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THE ROLE OF THE ANDROGEN RECEPTOR IN ANXIETY-RELATED BEHAVIORS, THE HYPOTHALAMIC PITUITARY ADRENAL AXIS, AND SENSORIMOTOR GATING: STUDIES IN RODENTS WITH THE TESTICULAR FEMINIZATION MUTATION

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ABSTRACT

THE ROLE OF THE ANDROGEN RECEPTOR IN ANXIETY-RELATED BEHAVIORS, THE HYPOTHALAMIC PITUITARY ADRENAL AXIS, AND SENSORIMOTOR GATING: STUDIES IN RODENTS WITH THE TESTICULAR FEMINIZATION MUTATION

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Androgens such as testosterone play a role in the display of anxiety-related behaviors and sensorimotor gating. However, the role of a specific hormone receptor, the androgen receptor (AR) in the regulation of these behaviors is less clear, because testosterone can be converted to estrogens that act on estrogen receptors. In this series of experiments we investigated the role of the AR in anxiety and sensorimotor gating by comparing AR deficient male mice and rats with the testicular feminization mutation (Tfm) to their wild type siblings in rodent models of these behaviors. Since increased anxiety-related behaviors are often correlated with an elevation of the hypothalamic-pituitary-adrenal (HPA) axis we also measured the release of the adrenal stress hormone corticosterone at baseline and at time points after exposure to an anxiety-provoking situation. Results of these studies indicate increased indices of anxiety and increased activation of the HPA axis in both Tfm male rats and mice, while sensorimotor gating appeared normal in the absence of ARs. An investigation into the role of androgens and the AR prior to adulthood in the organization of anxiety-related behaviors and sensorimotor gating revealed that neonatally gonadectomized Tfm and wt male rats showed decreased anxiety-related behavior and increased sensorimotor gating compared to neonatally sham operated Tfm and wt males. These results suggest that androgens act

primarily via an AR-independent mechanism, perhaps through estrogen receptors, during development to influence these behaviors. Together our findings in Tfm rodents indicate a role of the AR and/or developmental androgens in the regulation of anxiety-related behavior, the HPA axis, and sensorimotor gating.

This dissertation is dedicated to my mom and Kristen without whom this would never have been accomplished

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Chapter 1: Introduction

In humans, there are startling gender differences in neuropsychiatric disorders such as dyslexia, autism, schizophrenia, and anxiety. For example, the onset of schizophrenia generally occurs earlier in men (Hambrect et al., 1992) and the symptoms tend to be more severe (Castle et al., 1995). Females, however, are twice as likely as men to suffer from generalized anxiety disorder (DSMIV, 1994). Gonadal hormones, testosterone (T) and estrogen (E) particularly through their activation of estrogen receptors (ERs), have been implicated in the development and maintenance of sex differences in neuropsychiatric disorders (Perlman et al., 2004: Ostlund et al., 2003). However, the role of the androgen receptor (AR), and particularly its influence on the development these disorders in males, is less understood.

An overwhelming body of evidence indicates that sex differences in mammalian behavior are the result of (a) differences in early hormone exposure that permanently "organize" the nervous system and/or (b) differences in exposure to hormones in adulthood that "activate" the neural circuitry that was previously organized. T is an important hormone in the organization and activation of male typical behavior. T can facilitate changes in behavior by acting on both ARs and ERs, which then alter gene expression and protein synthesis. In rodents, the AR is directly activated by T itself or a metabolite of T such as 5α-dihydrotestosterone (DHT). ERs are indirectly activated by T through the process of aromatization in which T is converted to estrogens such as estradiol (E2) by the enzyme aromatase, and subsequently E2 binds to ERs. Since T can act on both receptor types it can be difficult to discern whether AR, ER, or both receptors are involved in the masculinization of a particular behavior. As a result, genetic models

such as testicular feminization mutant (Tfm) rodents, which lack functional ARs, provide a unique approach for examining the role of sex hormone receptors in behavior.

Hormonal Influences on Anxiety

In humans, anxiety disorders are estimated to affect about 1 in 5 people (Kessler et al., 1994), and there are striking sex differences in the prevalence of these disorders. Women suffer more from social anxiety disorder than do men (Weinstock et al., 1999) and tend to receive the majority of diagnoses for specific phobias (i.e., arachnophobia; DSMIV, 1994). Differences in circulating sex hormones, E and T, are likely involved in the occurrence of gender differences in anxiety. E replacement has positive effects on mood and decreases anxiety in postmenopausal women (Yazici et al., 2003), while other studies indicate that T also plays a role in the regulation of anxiety in humans. Chemical castration, through androgen blockade therapy, increases anxiety and when treatment is ceased, anxiety levels decrease (Almeida et al., 2004).

Sex differences in anxiety-related behavior have been demonstrated in rats, as assessed through rodent models of anxiety, with females generally showing more activity and reduced indices of anxiety compared to males (Frye et al., 2000; Lucion et al., 1996). However, gender does not appear to play a major role in the display of these same behaviors in mice (Voikar et al., 2001). Sex hormones T and E are involved in regulating anxiety in both mice and rats, with increases in either hormone correlated with a decrease in anxiety-related behaviors (Frye and Walf, 2004; Bing et al., 1998). Recent reports indicate that anxiolytic actions of estrogens are mediated via activation of ERβ type receptors. ERβ knockout female mice exhibit greater anxiety in the elevated plus maze

anxiety test compared to their wild type (wt) siblings (Imwalle et al., 2002) and pharmacological activation of ERβ in male and female rats decreases anxiety-related behaviors in this same test (Lund et al., 2004). On the other hand, pharmacological activation of ERα increases indices of anxiety in some rodent tests of anxiety (Lund et al., 2004). These findings indicate that ERα and ERβ subtypes may play opposing roles in the regulation of anxiety-related behaviors. There is also evidence indicating a role of ARs in the regulation of anxiety-related behavior in rodents. In both mice and rats, DHT treatment reduces anxiety behaviors in the elevated plus maze (Aikey et al., 2002; Edinger and Frye, 2004; Frye and Edinger, 2004). However DHT can also interact with other hormone (ERβ) and non-hormone (GABA) receptors via further metabolism, making it difficult to decipher the specific receptors involved in reducing anxiety.

Hypothalamic-Pituitary-Adrenal (HPA) Axis

The hypothalamic-pituitary adrenal axis (HPA) plays a critical role in an animal's reaction to an anxiety-provoking situation and dysregulation of the HPA axis is related to the incidence of anxiety disorders. When an individual is exposed to a physical or psychological stressor, the paraventricular nucleus of the hypothalamus begins to secrete corticotropin releasing hormone (CRH) and vasopressin, and these hormones, in turn, activate the anterior lobe of the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH then activates the adrenal cortex, which results in the release of glucocorticoids (primarily cortisol in humans, and corticosterone in rodents). Glucocorticoids can then activate tissues throughout the body, including the brain which contains glucocorticoid receptors that when activated can play a role in the cessation of

CRH release through a negative feedback mechanism. The display of anxious behavior is often correlated with increased levels of blood ACTH and glucocorticoids with the elevation of these stress hormones likely increasing anxiety in stressful situations.

There are sex differences in rats in stress-induced blood corticosterone levels with females showing a greater elevation (Handa et al., 1994). These differences may, in part, result from the ability of testosterone to inhibit the HPA axis while estrogen can enhance its activation (Handa et al., 1994). There is also evidence that exposure to hormones around the time of birth can affect basal as well as stress-induced corticosterone levels (Seale et al., 2005a).

Hormonal Influences on Sensory motor gating

Prepulse Inhibition (PPI) is a common measure of sensorimotor gating in human and rodent models. PPI involves measuring a fast muscle twitch response to a loud acoustic stimulus that is preceded, by approximately 100ms, by a weaker acoustic stimulus or "prepulse". The prepulse should permit the subject to anticipate the loud pulse, and consequently startle less severely. Sensorimotor gating is necessary to filter sensory, motor, and cognitive information (Kodisi & Swerdlow, 1994) and is reduced in people with schizophrenia (Braff et al., 1978). These deficits in sensorimotor gating may be linked to symptoms of schizophrenia such as hallucinations, delusions, and disruptions in thought (Rigdon & Weatherspoon, 1992; Perry & Braff, 1994). Sex differences in PPI have also been reported in humans and rodents, in which males exhibit greater PPI than females (Swerdlow et al., 1993). Because reduced PPI has been associated with schizophrenia, it is curious that males, who are more severely affected by the disorder,

show *greater* PPI than females. This suggests that susceptible males may be those who do not exhibit a fully masculinized PPI response before onset of the disorder.

E has been suggested to play an important role in the regulation of PPI, and may contribute to the delay in onset and milder symptoms observed in women with schizophrenia. In female rats, administration of E causes an increase in PPI (Van den Buuse & Eikelis, 2001), and symptoms of schizophrenia in women are generally less severe when endogenous E levels are high (Hafner et al., 1993). Because sex hormones seem to affect both PPI and schizophrenia, investigating the influence of sex hormones and their receptors on PPI may shed light on the etiology of schizophrenia.

Organizational vs. Activational role of the AR

The role of the AR in sensorimotor gating, anxiety, and HPA axis regulation is not well understood, but is likely important. If ARs do contribute to these behaviors it is also essential to decipher whether ARs affect these behaviors during development and/or in adulthood. Once we know when androgen acts to regulate these functions, we can then look during these same time periods for morphological and/or functional changes in the brain that might critically mediate the change in behaviors.

Chapters

Chapter 1 (the present chapter): We introduce the role of sex hormones and their receptors in the regulation of anxiety-related behaviors, the HPA axis, and sensorimotor gating.

Chapter 2: We review evidence for the role of ARs in the masculinization of the brain and behavior with a focus on what has been learned from studies in rodents with the testicular feminization mutation.

Chapter 3: In this chapter we explore the role of the AR in anxiety-related behaviors, activation of the HPA axis, and sensorimotor gating by comparing Tfm and wt male mice in rodent models designed to measure these behaviors. Since Tfm male mice also have lower circulating T levels, behaviors were also compared in T-replaced Tfm and wt males.

Chapter 4: Here we explore the role of the AR in anxiety-related behaviors, activation of the HPA axis, and sensorimotor gating by comparing behavioral and physiological (corticosterone) responses in Tfm male, wt male, and wt female rats, and neural (c-fos) responses in Tfm and wild type males. Female rats were included in this experiment because, unlike in mice, sex differences in anxiety-related behaviors in rats are well documented, allowing us to observe whether Tfm male behavior better resembled that of wt males or females.

Chapter 5: In this chapter we investigate the role of androgens and the AR prior to adulthood in the organization of anxiety-related behaviors, sensorimotor gating, and HPA axis activity. In this experiment we compared behaviors in Tfm and wt male rats that were gonadectomized (Neo-Gdx) or sham-operated (Neo-Sham) on the day of birth. In adulthood, rats were either gonadectomized or sham-operated and implanted with T capsules to equilibrate adult circulating T and compared in tests of anxiety and sensorimotor gating. Corticosterone levels were also measured in these rats at baseline or following exposure to an anxiety-provoking situation.

Chapter 6: Here we present an overarching discussion of our findings and their implications.

Chapter 2: The Role of Androgen Receptors in the Masculinization of Brain and Behavior: What we've learned from the Testicular Feminization Mutation

Abstract

Many studies demonstrate that exposure to testicular steroids such as testosterone early in life masculinizes the developing brain, leading to permanent changes in behavior. Traditionally, masculinization of the rodent brain is believed to depend on estrogen receptors (ERs) and not androgen receptors (ARs). According to the aromatization hypothesis, circulating testosterone from the testes is converted locally in the brain by aromatase to estrogens, which then activate ERs to masculinize the brain. However, an emerging body of evidence indicates that the aromatization hypothesis cannot fully account for sex differences in brain morphology and behavior, and that androgens acting on ARs also play a role. The testicular feminization mutation (Tfm) in rodents, which produces a nonfunctional AR protein, provides an excellent model to probe the role of ARs in the development of brain and behavior. Tfm rodent models indicate that ARs are normally involved in the masculinization of many sexually dimorphic brain regions and a variety of behaviors, including sexual behaviors, stress response and cognitive processing. We review the role of ARs in the development of the brain and behavior, with an emphasis on what has been learned from Tfm rodents as well as from related mutations in humans causing complete androgen insensitivity.

Introduction

Exposure to testicular steroids such as testosterone (T) early in life masculinizes the developing brain, leading to permanent changes in behavior in a wide variety of animal models (Morris et al., 2004). According to the aromatization hypothesis, T is converted by aromatase into 17-β estradiol (E2), which then acts on estrogen receptors (ERs) to masculinize the brain (Naftolin et al., 1975). Traditionally, aromatization is believed to be the mechanism by which the rodent brain becomes masculinized and defeminized. Some sexually dimorphic regions within the hypothalamus adhere well to this hypothesis, including the sexually dimorphic nucleus of the preoptic area (SDN-POA) and anteroventral periventricular nucleus (AVPV). T spares neurons from death in the SDN-POA, while it promotes cell death in the AVPV, both through activation of ERs. The aromatization hypothesis also seems to hold for the development of some sexual and nonsexual rodent behaviors. Defeminization of rat sexual behavior, as measured by the proclivity to show lordosis (the female posture of sexual receptivity), and masculinization of aggressive behavior in rodents are largely controlled by estrogenic metabolites of T acting on ERs (Olsen, 1979; Vreeburg et al., 1977; Ogawa et al., 2000; Scordalakes and Rissman, 2004).

However, an emerging body of evidence suggests that the aromatization hypothesis cannot account for all sex differences in brain morphology and behavior. In primates, including humans, brain masculinization may be accomplished primarily via androgens acting directly on the androgen receptor (AR). For example, alpha-fetoprotein binds estrogen and prevents it from entering and masculinizing the brain in rodents (Bakker et al., 2006; Puts et al., 2006), but has very low affinity for estrogen in primates

(Swartz and Soloff, 1974). If the aromatization hypothesis were generally true in primates, it would seem that ovarian estrogens would cross the blood-brain barrier and masculinize the female brain. Moreover, people with complete androgen insensitivity syndrome (CAIS) exhibit feminine behavior (see below) and morphology. CAIS individuals have a 46,XY karyotype and develop testes that remain undescended in the abdominal cavity. Despite producing normal-to-high male levels of T, individuals with CAIS have completely nonfunctional ARs, and so are phenotypically female (Imperato-McGinley et al., 1982). Because individuals with CAIS regard themselves as females, we refer to them hereafter as women. Their feminine behavior suggests that functional ARs are required to masculinize the human brain. Conversely, human males with mutations rendering the aromatase enzyme dysfunctional present as normal males, despite the absence of aromatization in the brain or elsewhere (Grumbach and Auchus, 1999). Taken together, the feminine behavior of people with CAIS and the masculine behavior of men with dysfunctional aromatase suggest that ER stimulation has a very limited role, if any, in masculinizing the human brain.

In the meantime, there is growing evidence that even in rodents, androgens act on ARs to shape the brain and behavior. Traditionally, exploration into the role of ARs in brain morphology and behavior involved the administration of a nonaromatizable AR agonist (usually dihydrotestosterone: DHT) or antagonist (e.g., flutamide). Unfortunately, DHT administration does not guarantee exclusive activation of ARs. DHT can be metabolized to 3α-androstanediol (3α-diol) which has a low affinity for ARs but a high affinity for GABA receptors, and 3β-diol, an estrogenic compound which binds ERs (Kuiper et al., 1998). Even if administration of DHT did act solely upon ARs,

it might not reveal a contribution of the AR in cases where T acts synergistically upon both ERs and ARs. For example, T acts upon ARs to increase aromatase activity in many brain regions (Roselli et al., 1987; Rosenfeld et al., 1977), which would provide more estrogens to stimulate ERs. Therefore DHT treatment alone would not reveal such a normal role for ARs because while it might boost aromatase activity, there would be no aromatizable androgenic precursor to be converted by the enzyme to provide estrogens for ERs. Furthermore, neonatal DHT administration may not masculinize some areas of the brain because AR expression levels in those areas may depend on ERs (McAbee and DonCarlos, 1999a). Neonatal gonadectomy in male rats decreases AR mRNA, and replacement with T or E2, but not DHT, restores AR mRNA to levels similar to gonadally intact male rats (McAbee and DonCarlos, 1999a; McAbee and DonCarlos, 1999b). These findings suggest that T normally acts through ERs to upregulate AR. Because DHT treatment fails to restore AR mRNA, DHT may fail to masculinize brain areas simply because AR levels are too low. These data also suggest reciprocal interactions between ARs and ERs in the brain, a theme emphasized in the remainder of this review.

The AR antagonist flutamide is also an imperfect test of AR contributions since its administration, while blocking ARs, also alters T production from the testes, making it difficult to attribute changes in morphology and behavior solely to AR blockade (Clos et al., 1988; Ayub and Levell, 1987).

Testicular feminization mutant (Tfm) rodents provide a unique model for examining the role of the ARs in the brain and behavior, because this mutation in the AR gene renders the protein nonfunctional. As a result of this mutation (the rodent analog of

CAIS in humans), genetically male Tfm rodents appear phenotypically female: they possess nipples typical of female rodents, lack normal male genitalia, and are infertile. Since this trait is X-linked, only genetically male (XY) carriers are wholly androgen insensitive. Female Tfm carriers are heterozygous, carrying one *Tfm* and one wildtype (wt) allele of the *AR* gene, thus allowing the mutation to be passed on to future generations. Although Tfm rodents present a feminine exterior, we refer to them hereafter as Tfm males because they are genetically male, possess testes and, unlike women with CAIS, show signs of at least partial masculinization of many behaviors, as discussed below. We also refer to them as Tfm males to distinguish them from females carrying the *Tfm* allele.

Rat and mouse Tfm models differ in terms of the type of mutation in *AR*. In rats, androgen insensitivity results from a mutation involving a single base pair *replacement* in the *AR* gene (Yarbrough et al., 1990), while in the Tfm mouse the mutation results from a single base *deletion*, which causes a frameshift mutation (Charest et al., 1991). Thus there is only a single amino acid difference between wt and Tfm AR protein in rats resulting in expression of a normal sized but dysfunctional AR protein in Tfm rats. On the other hand, the mutation in Tfm mice introduces a premature stop codon, resulting in a shortened transcript and essentially no AR protein (He et al., 1991; Monks et al., 2007). Due to differences in the nature of these mutations, Tfm rats have some residual sensitivity to androgens through ARs, although it is greatly reduced (Yarbrough et al., 1990), while Tfm mice have virtually *no* sensitivity to androgen through ARs, as in CAIS women (Drews, 1998). In both models estrogen binding in the brain appears normal (Attardi et al., 1976; Olsen and Whalen, 1982).

Rat and mouse Tfm models also differ in terms of circulating T levels. Tfm male mice have significantly less endogenous T compared to their wt siblings (Jones et al., 2003), while Tfm male rats have circulating T levels in the high male range (Rosseli et al., 1987). In this regard, the Tfm rat resembles untreated humans with CAIS, who also have circulating T levels in the high-male range (Vague, 1983). In both rat and mouse Tfm models, indirect evidence suggests that T levels are near the normal male range in the perinatal period (Olsen, 1979; Goldstein and Wilson, 1972), but no one has actually measured circulating androgens in perinatal Tfm rodents.

Aromatase activity is also decreased in several areas of the adult Tfm brain compared to wt males (Roselli et al., 1987; Rosenfeld et al., 1977), so differences between wt males and Tfm males in brain morphology and behaviors associated with these regions may be caused by reduced ER activation. Therefore in the survey that follows, as we find evidence that the *Tfm* allele of the *AR* gene affects brain and behavior, we must keep in mind that such findings do not disprove a role for ER, since AR may be acting in part by affecting aromatase.

AR and Brain Morphology

Increasing evidence suggests that activation of AR also normally mediates masculinization of the nervous system and behavior. Some of the first evidence for this idea came from analysis of motoneurons of the spinal nucleus of the bulbocavernosus (SNB), which mediate penile reflexes during copulation (see Sengelaub and Forger, 2008, in this issue). There is a sex difference in the number of SNB motoneurons (males>females) that is dependent on AR activation, since the SNB system is feminized

in Tfm rats (Breedlove and Arnold, 1980; Breedlove and Arnold, 1981). Recent research utilizing Tfm rodent models suggests that the absence of functional ARs also alters discrete areas of the brain. This demasculinization of specific brain regions is not due to an effect on the overall size of the brain. In both rats and mice, males have greater brain weights than do females, which probably reflect the sex difference in overall body size. We now report for the first time that the brain weight of Tfm males is fully masculine in both species (Figure 1). Interestingly, body weight of Tfm males is intermediate between that of wt males and females in both species, indicating that sexual differentiation of overall brain weight and body weight are dissociable. These results suggest that this global masculinization of brain weight may occur via ER activation or via some other mechanism not dependent on ARs. However, they also set the stage for the following results where brain regions that are typically larger in males than in females are partially or wholly feminine in Tfm males, indicating that regional brain demasculinization in Tfm animals is not a generalized effect on overall brain size, but a specific decrease in some brain areas caused by lack of AR stimulation.

Posterodorsal medial amygdala (MePD)

Morphology of the posterodorsal medial amygdala (MePD), which receives olfactory and pheromonal information and is important for some aspects of male sexual behavior, is highly dependent on adult hormones. MePD volume is 1.5 times greater in male rats than in females, but this sex difference can be abolished by castration of adult males and/or administration of T to adult females (Cooke et al., 1999). Structural plasticity in the adult MePD appears to be mediated through activation of both ARs and ERs. Cooke et al.

(2003) found that treating castrated adult male rats with E2 and the nonaromatizable androgen DHT each masculinize aspects of MePD morphology. Treatment with E2 increases both volume and soma size of MePD cells compared to untreated castrates, while DHT treatment increased MePD soma size but did not affect volume. MePD volume and soma size have also been examined in Tfm male rats (Morris et al., 2005) and both were found to be partially demasculinized, significantly different from both wt males and females (Figure 2a-f). In contrast, the rostrocaudal extent of the MePD, which is greater in wt males than females, is fully masculinized in Tfm males. Because T levels are in the high male range and aromatase activity is normal in the medial amygdala of Tfm male rats (Rosseli et al., 1987), adequate amounts of estrogens should be available for ER activation. Given that both MePD soma size and volume are smaller in Tfm males than in wt males, T activity through ERs appears insufficient to fully masculinize these characteristics (Morris et al., 2005). On the other hand, masculinization of the rostrocaudal extent of the MePD appears to be independent of AR activation, with Tfm and wt males equivalent (Morris et al., 2005). Similarly, Tfm and wt male rats also show an asymmetry, with MePD volumes greater in the right hemisphere than the left. This laterality in MePD volume is not found in females, indicating that its presence may depend on some masculinizing factor unrelated to AR activation, the leading candidate being ER activation (Morris et al., 2005).

Suprachiasmatic Nucleus

Sexual dimorphisms have been found in the brain's biological clock, the suprachiasmatic nucleus (SCN), in both humans and rodents. The vasoactive intestinal polypeptide (VIP)

containing subnucleus of the SCN is twice as large in men as in women, and the vasopressin containing subnucleus of the SCN has a different shape in men than in women (Swaab et al., 1994; Swaab et al., 1985). Sexual orientation has also been correlated with the size of the human SCN. Swaab and Hofman (1990) found the vasopressin subnucleus of the SCN of homosexual men to be 1.7 times as large and contain twice as many cells as that of heterosexual men. Studies of sex differences in the SCN of rats have yielded somewhat conflicting findings in which males have a greater SCN volume than females (Robinson et al., 1986; Gorski et al., 1978) or there were no differences (Madeira et al., 1995; Bloch and Gorski, 1988). These discrepancies suggest that sex differences in the rat SCN may either be small and/or strain dependent. In gerbils, volume of the male SCN was twice that of the female SCN (Holman and Hutchison, 1991). Furthermore, castration of gerbils on the day of birth reduced SCN volume to female levels in adulthood, suggesting that the SCN is influenced by early androgen exposure. Morris et al. (2005) analyzed the SCN in Tfm male rats and found that volume and neuronal soma size was decreased compared to wt male rats, indicating that functional ARs are essential for the full masculinization of this brain area. However, given that aromatase activity is decreased in the SCN of Tfm male rats (Roselli et al., 1987), it is possible that the main role the AR plays in masculinization of the SCN is to regulate the level of aromatase and that ERs mediate downstream changes in SCN structure. This scenario would still indicate an important role for ARs in normal masculinization of the SCN, but it would also suggest that sufficient stimulation of ERs, such as with exogenous estrogen, can fully masculinize the nucleus.

Ventromedial Hypothalamus

The ventromedial hypothalamus (VMH) is another sexually differentiated region that is involved in sexual and parental behaviors. VMH volume and soma size are greater in males than in females, and males also have a higher concentration of ARs in the VMH than do females (Matsumoto and Arai, 1983; Madeira et al., 2001). The VMH contains four morphologically distinct regions, including the anterior (VMHa), dorsomedial (VMHdm), ventrolateral (VMHvl), and central (VMHc) subdivisions, which have distinct connectivity patterns to other brain regions and may affect a variety of functions (McClellan et al., 2006). In a recent study, volume and neuronal soma size of the entire VMH and its four subdivisions were compared between wt males, females, and Tfm male rats (Dugger et al., 2007). Confirming earlier findings, overall VMH volume was greater in males than in females. This difference was accounted for by a larger male VMHvl and a marginally greater male volume in the VMHdm. In Tfm males, overall VMH volume was intermediate between wt males and females, but did not significantly differ from either group. However, the VMHvl, which accounts for most of the sexual dimorphism in VMH volume as a whole, was significantly smaller in Tfm males than wt males and similar to that of females. Analysis of neuronal soma size revealed a sex difference in which males had a greater soma size than females in three of the four VMH subdivisions (VMHvl, VMHc, and VMHdm). Tfm males had significantly smaller somata than wt males in the VMHdm and marginally smaller somata in the VMHvl and VMHc. Together these data suggest that functional ARs normally play a role in the full masculinization of the VMH. In fact, ARs may be the predominant steroid receptor

mediating masculinization of the VMH, since Tfm males did not significantly differ from females on any measure. However, differences in AR-induced aromatase activity, as discussed for the SCN, may also influence VMH morphology. Finally, these results from the VMH, along with those discussed for the MePD and SCN, emphasize the fact that different morphological traits of a given brain region may or may not be affected by ARs, and that the effects of the AR on individual cells within a region may not affect overall volume.

Bed Nucleus of the Stria Terminalis

The bed nucleus of the stria terminalis (BST) is a sexually dimorphic region within the hypothalamus that plays a role in social and reproductive behaviors (De Vries and Panzica, 2006). A portion of this region, the posteromedial nucleus of the bed nucleus of the stria terminalis (BSTMPM) shows several sexual dimorphisms, including greater AR density and regional volume in males compared to females (Hines et al., 1992; Lisciotto and Morrell, 1994; Roselli, 1991). The sex difference in volume appears to result from hormone exposure both perinatally and in adulthood. Gonadectomy of newborn males decreases BSTMPM volume, and androgen treatment of newborn females increases it (Guillamon et al., 1988b), while gonadectomy of adult males also decreases regional volume (Malsbury and McKay, 1994). Based on neuroanatomical similarities of the BSTMPM and the MePD (proximity and high AR content), and similar hormone responsiveness between the two regions, it was hypothesized that ARs may also play a role in the sexual differentiation of this region. Durazzo et al. (2007) compared characteristics of the BSTMPM in wt male, wt female, and Tfm male rats and found group differences in regional volume, but not neuronal soma size. There was a sex

difference in BSTMPM volume in both the left and right hemisphere (males > females), while volume was increased in wt males compared to Tfm males only in the left hemisphere. These results suggest that, as in the MePD, ARs normally contribute to the full masculinization of the BSTMPM in males.

Arginine vasopressin (AVP) innervation of the septal area (including the BST), which plays a role in pair bonding, parental, and aggressive behavior (De Vries and Panzica, 2006), is also sexually differentiated. AVP innervation of this area is greater in males than in females and is dependent upon androgens in both early development and adulthood. Castration of male rats in adulthood decreases AVP innervation, although not as extensively as does castration neonatally (Wang et al., 1993). Androgens contribute to the sex difference in AVP via aromatized metabolites of T acting on ERs, although ARs also appear to contribute to the perinatal organization of this system to some degree. For example, gonadectomized rat pups treated in the early postnatal period with DHT show a partial masculinization of BST vasopressin, although this masculinization is less than that found in rat pups treated with E2 either alone or combined with DHT (Han and De Vries, 2003). On the other hand, Tfm male mice are not different from wt males after adult castration and treatment with E2 (Scordalakes and Rissman, 2004). These results suggest that the role of ARs in the masculinization of AVP innervation of the septal area in mice is minimal.

Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA)

Even the SDN-POA, in which abundant literature suggests that sex differences depend on the metabolism of T into E2 and activation of ERs, may normally depend on ARs for full masculinization. Regional volume and soma size of SDN-POA neurons are greater in male than in female rats (Gorski et al., 1980; Madeira et al., 1995), and perinatal hormone manipulations suggest that ERs, not ARs, are important in the masculinization of SDN-POA volume (Dohler et al., 1986). However, Tfm male rats show only a partial masculinization of the SDN-POA: volume is fully masculine but neuronal soma size is not, with Tfm males having smaller neurons in the SDN-POA than wt males (Morris et al., 2005). Again, as in several other brain regions, aromatase activity is decreased in the medial preoptic area of Tfm male rats (Roselli et al., 1987), so ARs may not be directly *necessary* for the masculinization of neuronal soma size in the SDN-POA, if sufficient estrogen is provided. Nevertheless, ARs appear to contribute normally to the full masculinization of the rat SDN-POA.

Locus Coeruleus

The locus coeruleus (LC), a brainstem nucleus implicated in the physiological response to stress and panic, is an example of a sexual dimorphism in which females show a larger volume and a greater number of neurons than do males (Guillamon et al., 1988a). This dimorphism appears to be under the control of both testicular and ovarian hormones, with testicular steroids decreasing and ovarian steroids increasing growth and survival of LC cells. Both neonatal and postpubertal gonadectomy of females decrease the number of neurons in the LC (De Blas et al., 1990; De Blas et al., 1995), while pre- and postnatal androgen administration also decreases LC neuron number (Guillamon et al., 1988a). In

males, ARs rather than ERs appear to play a critical role in the masculinization of the LC. Garcia-Falgueras et al. (2005) found that, compared to wt male littermates, Tfm males had a larger volume and greater number of neurons in the LC. Based on these results, Guillamon's group suggests that when a sexual dimorphism exists in which females show more neurons and/or greater volume it may be at least partially mediated via AR activation in males.

Hippocampus

The hippocampus is another sexually dimorphic area of the brain in which males show a greater overall volume than females (Madeira et al., 1995; Nunez et al., 2000). One area of the hippocampus, the dentate gyrus, is sexually dimorphic in some strains of mice in which males show a greater number, size, and density of cells within this region (Wimer and Wimer, 1984; Wimer et al., 1988; Wimer and Wimer, 1989). Study of Tfm male mice suggests that ARs may play some role in the development of the mouse dentate gyrus. Although no sex difference was reported in volume of the granule cell layer of the dentate gyrus (GCL) in C57BL6J mice, both wt males and females had a larger right than left GCL, a laterality that was absent in Tfm males and partially androgen insensitive Tfm carrier female mice (Tabibnia et al., 1999). These results suggest that ARs play a role in establishing laterality in the mouse GCL regardless of whether a sexual dimorphism exists. In the rat dentate gyrus, the presence of functional ARs also appears to affect morphology, despite a lack of sexual dimorphism. Jones and Watson (2005) found that Tfm male rats show a greater GCL volume than do females, although there was no sex difference in GCL volume. Why GCL volume is greatest in Tfm rats is

unclear, but the authors suggest it may result from increased ER activation within the GCL due to the higher levels of T in Tfm male rats.

Androgens also increase the pyramidal cell dendritic spine density (PSSD) in CA1 of the hippocampus in male and female rats (MacLusky et al., 2004; Leranth et al., 2004; Hajszan et al., in this issue). In females, estrogens can also increase CA1 PSSD, though they have no effect in males (Leranth et al., 2003; MacLusky et al., 2005). This result indicates that the androgen-induced increase in PSSD in males may be mediated through ARs. However, Tfm males with dysfunctional ARs showed a PSSD comparable to wt males (MacLuscky et al., 2006). Interestingly, DHT administration increased CA1 PSSD in both wt and Tfm males, suggesting that androgens may exert their influence through nonclassical pathways.

Our review of the literature indicates that, *for every known sexual dimorphism in the rat and mouse nervous system that has been examined in Tfm models so far*, results indicate that ARs normally contribute to masculinization of at least some aspects of morphology. In several cases, both regional volume and neuronal soma size are significantly demasculinized in Tfm males (SNB, MePD, VMH, SCN). Sometimes volume is demasculinized while neuronal soma size is not, sometimes the reverse is true. Our conclusion is that ARs are integral to masculine structural development throughout the rodent brain. These studies are summarized in Table 1.

AR and Behavior

In line with the emerging role of the AR in shaping brain morphology, evidence also suggests that the AR is important in shaping behavior, both during its early organization

and its later activation in adulthood. Although aromatization may account for many sex differences in rodent behavior, research using Tfm models suggests that the AR normally plays a critical role in a variety of behaviors ranging from sexual to cognitive.

Spatial Memory

Males outperform females in spatial memory performance in both rodents and humans (Rizk-Jackson et al., 2006; Roof, 1993; Astur et al., 1998; Jonasson, 2005). The sex differences in rodents have generally been attributed to the organizational effects of androgens, since neonatal castration of males or administration of T to newborn females eliminates such sex differences (Isgor and Sengelaub, 2003). The aromatization of androgens to estrogens may be particularly important in the development of the sex difference, since neonatal administration of E2 masculinizes spatial ability in female rats (Williams et al., 1990; Williams and Meck, 1991). However, other evidence suggests that perinatal AR activation may also play a role in enhancing male spatial memory performance. Isgor & Sengelaub (1998) found that prenatal E2 treatment did not masculinize performance in the Morris Water Maze (MWM) in female Sprague-Dawley rats, whereas treatment with either T or DHT did. In addition, males administered AR antagonists prenatally show poorer MWM performance than do control males, eliminating the sex difference in this behavior (Isgor and Sengelaub, 1998; Joseph et al., 1978).

Hormonal influence on such behaviors, however, may not be solely organizational. Adult circulating androgens and estrogens can also affect certain aspects of spatial memory performance and these effects may, at least partially, be mediated

through activation of ARs in adulthood (Gibbs, 2005; Sandstrom et al., 2006; Naghdi et al., 2001). To further explore the role of ARs in spatial memory, performance in the MWM has been examined in both the rat and mouse Tfm models. Jones and Watson (2005) compared MWM performance in wt male, female, and Tfm male rats and found that Tfm males showed an intermediate pattern of performance in which they took longer to reach male-typical performance levels. Tfm male mice also show impaired MWM performance compared to partially androgen-insensitive Tfm carrier females, although sex differences in wt mice were not found (Rizk et al., 2005). Together, these findings suggest that the AR is necessary for the full masculinization of spatial memory.

Other facets of memory also appear to be influenced by androgens, such as object memory in rodents, which is strongly influenced by circulating androgens and estrogens in adulthood (Frye and Lacey, 2001; Walf et al., 2006). Use of the Tfm models would further elucidate the role of T and its mechanism of action in this and other memory tasks.

Sex differences in human spatial ability also appear to be mediated by androgens (Puts et al., 2007; Puts et al., in press), and evidence supports a role of ARs in this sex difference. In the only published study of CAIS and spatial ability to date, CAIS females performed significantly worse on spatial tasks than did their male relatives (Imperato-McGinley et al., 1991). Although this finding suggests that androgens masculinize spatial ability via ARs, it is also possible that CAIS women exhibit less masculine spatial abilities because they were socialized in a manner concordant with their phenotypic gender. A more powerful comparison is that between CAIS women and their unaffected (46,XX) female relatives. If spatial ability is AR-mediated, then CAIS women should

perform significantly worse on tests of spatial ability than their unaffected female relatives, who are also socialized as females yet produce and receive some androgen stimulation. In fact, this is what was found (Imperato-McGinley et al., 1991). Even this comparison must be interpreted cautiously however, as it is possible that ovarian hormone production in unaffected females caused them to differ from their CAIS relatives.

Anxiety-Related Behavior

In humans, anxiety disorders are estimated to affect about 1 in 5 people (Kessler et al., 1994), and there are striking sex differences in the prevalence of these disorders. Women suffer more from social anxiety disorder than do men (Weinstock, 1999) and tend to receive the majority of diagnoses for specific phobias (e.g., arachnophobia; DSM-IV, 1994). Differences in circulating sex hormones, E2 and T, are likely involved in the occurrence of gender differences in anxiety. Estrogen replacement has repeatedly been shown to increase mood and decrease anxiety in postmenopausal women (Yazici et al., 2003). Other studies indicate that androgens also play a role in the regulation of anxiety in humans. Chemical castration through androgen blockade therapy in men with prostate cancer was correlated with an increase in anxiety. When treatment ceased, anxiety levels decreased (Almeida et al., 2004).

Gonadal hormones also play a role in the display of anxiety-related behavior in rodents as assessed by open field testing, exposure to a novel object, the elevated plus maze task, and light dark box (Edinger and Frye, 2004; Walf and Frye, 2005; Lund et al., 2005; Bridges and Starkey, 2004; Frye and Lacey, 2001). In general, increases in either

T or E2 in both male and female rodents are correlated with a decrease in anxiety-related behaviors (Frye and Walf, 2004; Bing et al., 1998; Frye et al. 2007). Recent reports have shed some light on the hormone receptors that are activated to provide anxiolysis. Imwalle et al. (2005) reported that estrogen receptor beta (ERβ) knockout female mice exhibit greater anxiety on an elevated plus maze anxiety test compared to their wt littermates. Furthermore, pharmacological activation of ERβ in rats decreased, while estrogen receptor alpha (ERα) activation increased, anxiety-related behaviors in an open field and when exposed to a novel object (Lund et al., 2005). Together these studies suggest that estrogen action via ERα and ERβ subtypes differentially regulates anxiety-related behaviors.

ARs also appear to be involved in anxiety in rodents. In rats, DHT treatment reduces anxiety behavior in the elevated plus maze (Edinger and Frye, 2004; Frye and Edinger, 2004). However, as previously mentioned, DHT can also interact with other hormone and non-hormone receptors via further metabolism. DHT can be metabolized to 3α-androstanediol (3α-diol) which has a low affinity for ARs but a high affinity for GABA receptors, and treatment with 3α-diol increases anxiolysis in gonadectomized rats (Frye and Edinger, 2004). 3β-diol, which can also be derived from DHT, binds ERs, with greater affinity for ERβ than ERα (Kuiper et al., 1998; Lund et al., 2004). Given what is known about the role of ERβ in the display of anxiety, it is likely that increases in 3β-diol levels following DHT administration can also alter this behavior (see Handa et al., in this issue). Consequently, Tfm male rodents with nonfunctional ARs provide a useful and novel method for exploring the role of the AR in anxiety-related behaviors, while avoiding some limitations of DHT treatment.

Studies using Tfm mice indeed suggest that ARs are involved in the anxiolytic actions of androgens. Rizk et al. (2005) found that, as a group, mice carrying the Tfm allele (Tfm males and Tfm carrier females) showed increased indices of anxiety on the elevated plus maze compared to wt male and female mice. Furthermore, in a novel object exposure test conducted in our laboratory, Tfm male mice tend to spend less time exploring the novel object in an open field compared to wt males, indicating that mice without functional ARs show greater anxiety-related behavior (Fig 3a). This difference does not appear to depend on inherent differences in circulating T levels between Tfm and wt males, as androgen treatment, which increases the time spent with a novel object in castrated wt males, does not alter the behavior of Tfm males in this test (Fig 3b).

Androgens play a critical role in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, with administration of T or DHT reducing the rise of stress hormones (adrenocorticotropic hormone (ACTH) and corticosterone) following exposure to a stressful situation (Handa et al., 1994; Lund et al., 2004; Lund et al., 2005; Lund et al., 2006). Increased HPA axis activation is often correlated with increased anxiety (Lund et al., 2005; Salome et al., 2004), which suggests that androgens may regulate the display of anxiety-related behavior by depressing HPA axis activation. In a recent study comparing activation of the HPA axis in Tfm and wt male mice, we found that Tfm males show an increased release of corticosterone following exposure to an open field with a novel object (Fig 4), indicating that the increased anxiety-related behavior in Tfm male mice described above may be related to hyperactivation of the HPA axis.

Play Fighting and Aggression

Play fighting (which includes two major components, playful attack and playful defense) is a common juvenile behavior in many mammalian species and appears to be regulated by gonadal hormones. In rats, play fighting, also called rough-and-tumble play, peaks between postnatal days 30-40 (Thor and Holloway, 1984), and occurs more frequently in males than females (Olioff and Stewart, 1978; Pellis and Pellis, 1990). Play fighting is decreased in male rats castrated at birth and its frequency begins to decline at the onset of puberty (Beatty et al., 1981; Meaney, 1988; Pellis and Pellis, 1990). Analysis of Tfm male rats suggests that ARs are involved in the development of play fighting behavior since, as juveniles, Tfm males show decreased play fighting behavior compared to wt males (Meaney et al., 1983; Meaney, 1988), although recent data suggest that this difference may depend on the testing paradigm (Field et al., 2006). Tfm males also fail to show the male-typical decline in playful attack with age, but like wt males they do show a decline in some aspects of playful defense, indicating that ARs are involved in some but not all aspects of play fighting (Field et al., 2006).

Unlike juvenile play fighting behavior, aggression in adulthood may rely more heavily on the activation of ERs. In male mice, castration decreases aggressive behavior while administration of T, E2, DHT, or a combination of E2 and DHT have been shown to increase male aggressive behavior (Gandelman, 1980; Matochik et al., 1994; Burge and Edwards, 1971; Finney and Erpino, 1976; Luttge and Hall, 1973). Gonadally intact Tfm mice also show decreased aggressive behavior compared to intact males, suggesting that ARs play a role in mediating this behavior (Ohno et al., 1974). However, when adult wt and Tfm male mice were castrated and supplemented with exogenous E2, they showed similar levels of aggression, indicating that low levels of circulating testicular hormones,

and not dysfunctional ARs, underlies decreased aggression in gonadally intact Tfm male mice (Scordalakes and Rissman, 2004). Studies using hormone receptor knockout mice also indicate that aggression may be mostly, if not entirely, mediated through activation of estrogen receptors, with enhancement of aggression via ERα activation and inhibition via ERβ activation (Scordalakes and Rissman, 2004; Nomura et al., 2002).

Sexual/Social Behavior

The masculinization and defeminization of male sexual behavior in rodents are traditionally believed to rely on the conversion of androgens to estrogens and the subsequent activation of ERs during the perinatal period, a hypothesis most recently supported by data from mice deficient in either aromatase or ERs (Ogawa et al., 2000; Matsumoto et al., 2003; Ogawa et al., 1997). However, newborn male rats administered an aromatase inhibitor retain masculine sexual behaviors (Dominguez-Salazar et al., 2002), while genetically produced AR knockout mice (ARKO) show less masculine sexual behavior (Sato et al., 2004), suggesting that ARs are also involved in male sexual behavior. Tfm male mice, similar to ARKO mice, also display impaired male sexual behavior (Ohno et al., 1974), but this impairment in coital behavior (mounts and thrusts), is not observed after E2 replacement in adulthood (Bodo and Rissman, 2007). This finding indicates that AR activation during development is of little importance for the expression of coital male behavior in mice, or that sufficient ER stimulation can overcome a lack of AR activation.

Masculinization of other sex-related behaviors, such as partner preference in mice, does appear to rely on the presence of a functional AR during development. For

example, while wt males prefer females, Tfm males act like wt females in showing no partner preference, even when E2 is equated across groups (Bodo and Rissman, 2007). Tfm males and females also prefer to explore male-soiled bedding, while males prefer exploring female-soiled bedding (Bodo and Rissman, 2007). Expression of the immediate early gene c-Fos in the MPOA and BST was also elevated in Tfm males and females but not in wt males after exposure to male-soiled bedding (Bodo and Rissman, 2007). Together these data suggest that ARs are necessary for the masculinization of at least some aspects of sexual and social behavior in mice. Similarly, people with CAIS do not differ from genetic (XX) females in sexual orientation, are just as likely to be married, and as children were involved in female typical play (Money et al., 1968; Hines et al., 2003).

In rats, defeminization of sexual behavior appears to be *AR-independent*. Olsen (1979) found that neonatally castrated Tfm male rats, compared to intact Tfm males, showed an increased display of lordosis, indicating that functional ARs are not necessary for the defeminization of this behavior, but rather androgens normally act through another mechanism (presumably ERs) to defeminize lordosis. The role of ARs in the development of masculine sexual behavior (i.e., mounting behavior), however, is not as clear. Tfm male rats have been reported to show variable, but consistently reduced sexual behavior compared to wt males (Beach and Buehler, 1977; Hamson et al., 2005; Olsen and Whalen, 1981; Olsen, 1992; Shapiro et al., 1976). Interestingly, c-Fos expression following a sexual encounter was similar in relevant brain areas (such as the medial preoptic area (MPOA) and medial amygdala (MeA)) of wt and Tfm males that showed comparable levels of sexual behavior (Hamson et al., 2005), suggesting that

activation of relevant neural circuitry may be normal in Tfm rats despite the lack of functional ARs.

Diminished sexual behavior in Tfm male rats may actually have little to do with their motivation, since partner preference is masculinized in Tfm male rats (Hamson et al., 2005; unlike in Tfm male mice), and more to do with a female's disinterest in *them*. Tfm male rats make fewer ultrasonic vocalizations during sexual encounters compared to wt males, a deficit that may reduce the receptivity of estrous females (Hamson et al., 2006). For Tfm animals in both species, we also cannot discount the influence of having a female genital phenotype on male sexual behavior because, as Frank Beach was fond of saying, "it's hard to be a good carpenter without a hammer".

In conclusion, just as Tfm models indicate that AR normally contributes to masculine development of every neural region examined so far, Tfm models also indicate that AR normally contributes to masculine development of a wide range of behaviors. We summarize these findings in Table 2.

Structure/function relationships in Tfm rodents

Thus far, little evidence has been generated to suggest a strong correlation between behavior and brain morphology in Tfm males. However, morphological differences have been found in brain areas that have been associated with particular behaviors. One such example includes differences in hippocampal morphology and spatial memory. Studies in Tfm mice and rats suggest that functional ARs are beneficial for optimal spatial memory performance (Jones and Watson, 2005; Rizk et al., 2005), and differences in GCL morphology have been reported in both species (Jones and Watson, 2005; Tabibnia

et al., 1999). However, morphological differences are not the same in the two Tfm models and it is difficult to interpret how an increased GCL volume in Tfm male rats, or a lack of GCL laterality in Tfm male mice, could result in spatial memory impairment. It is possible that morphological differences in unexamined hippocampal regions, such as CA1 of Tfm males, contribute to this deficit.

As reviewed above, several brain areas believed to regulate sexual behavior in rodents (MePD, VMH, SDN-POA, BST) are demasculinized in Tfm male rats, and thus may contribute to their reduced sexual behavior (Beach and Buehler, 1977; Shapiro et al., 1976; Hamson et al., 2005). In this instance, it is easier to infer how demasculinization of sex behavior-related circuitry could lead to a demasculinization of behavior. Ultrasonic vocalizations, which are disrupted in Tfm males, appear to rely heavily on androgens acting in the VMH (Harding and McGinnis, 2003; Harding and McGinnis, 2004), so the demasculinization of this nucleus in Tfm males (Dugger et al., 2007) may contribute to this deficit in masculine behavior. Furthermore, along with its role in sexual behavior, the BST is also an important component of the circuitry involved in the regulation of the HPA axis and anxiety-related behavior. Therefore, altered BST morphology in Tfm males could potentially underlie differences in HPA axis physiology and anxiety. Of course, it is also possible that structural changes in the brains of Tfm male rats and mice are unrelated to the differences observed in their behavior.

Conclusion

The Tfm models have provided overwhelming evidence that functional ARs are indeed necessary for the full masculinization of rodent brain and behavior, and studies of CAIS

strongly implicate a role of ARs in human behavior as well. Certain rodent brain sexual dimorphisms rely heavily on the presence of functional ARs (MePD, VMH, LC), whereas others do to a lesser extent (SDN-POA), and yet others appear largely unaffected by ARs (septal AVP innervation).

However, there is more to be learned, and there are many holes in the Tfm story. In terms of differences in brain morphology, very little has been examined in the Tfm mouse for at least two reasons: (1) sex differences in the rat brain are better documented, and (2) Tfm male mice also have much lower T levels than wt males in adulthood, making it more difficult to attribute morphological differences to the AR *per se*. Few studies of Tfm mouse behavior have provided exogenous T to equilibrate this factor as was done for Figure 3. It is also not known when during development T titers begin to decline in Tfm male mice, although, as mentioned earlier, indirect evidence suggests that T levels are normal during the pre- and early postnatal period (Goldstein and Wilson, 1972). One important benefit of the Tfm mouse model is that, unlike in the Tfm rat, the AR is completely nonfunctional, making it easier to detect contributions of ARs. Furthermore, genetic tools and approaches are already available in mice, offering more opportunities to dissect the differing contributions of the AR in various tissues.

For the behaviors discussed in this review, analysis has often been performed in only one of the two models or in some cases experiments were performed using Tfm mice that were not supplemented with hormones in adulthood to equate levels in wt and Tfm males. The strength and generalizabilty of previous findings would benefit from addressing these issues. There are undoubtedly other differences in the brain and behavior of Tfm and wt males that have yet to be discovered, some of which could

involve non-neuronal mechanisms. It is possible that AR deficiency could affect the morphology and number of glia such as astrocytes, and in turn influence behavior.

In many ways, the Tfm rodent model can serve as a starting point upon which the role of ARs in brain morphology and behavior can be explored, but it has its limitations. Standard use of these models does not resolve the issue of whether any differences are the result of organizational or activational influences of hormones, since the Tfm defect is present throughout ontogeny. We know that in the MePD, volume and soma size are dependent on adult androgens, so in other cases in which brain morphology differs in Tfm males, the notion that adult AR activation can alter morphology should not be discounted. Another question concerns cellular targets that mediate a particular androgen effect, and this is not readily answered with traditional Tfm models. For example, the cell type(s) (neurons, astroctyes, oligodendrocytes) that are the site of action for ARs to alter morphology or behavior are difficult to determine using this model. The development of conditional knockout models (in which the AR is deleted only in neurons, or only in astrocytes, or in both at particular times in the lifespan) could help answer such questions (see Juntti et al., 2008, in this issue). First, however, it would be beneficial to learn more about the role of global AR deficiency using the Tfm rodent models. Future research will undoubtedly add to what we have already learned from the Tfm models which, simply stated, is that the AR *does* play an important role in the masculinization of rodent brains and behaviors.

Chapter 3: Mice with the Testicular Feminization Mutation Demonstrate a Role for Androgen Receptors in the Regulation of Anxiety-Related Behaviors and the Hypothalamic-Pituitary-Adrenal Axis

Abstract

Testosterone (T) appears to play a role in anxiety and sensorimotor gating in rodents, but whether T acts through the androgen receptor (AR) to influence these behaviors is less clear. We compared adult genetic male mice with the testicular feminization mutation (Tfm), which lack functional ARs, to wild type male littermates (wt males) on an assay of sensorimotor gating (prepulse inhibition of the acoustic startle response; PPI) and several tests thought to reflect anxiety: open field exposure, novel object exposure, elevated plus maze (EPM), and light/dark (LD) box. PPI was similar between groups, but indices of anxiety in the novel object and LD box tests were increased in Tfm males with no significant differences found in the open field or EPM. Since Tfm male mice have decreased circulating T, the same tests were conducted in mice that were gonadectomized (wt males) or sham-operated (Tfm males) as adults and supplemented with T or nothing (B). Although no significant group differences were found in PPI, both B and T-treated Tfm males continued to show increased anxiogenesis compared to T-treated wt males in the novel object and LD box tests. Increased indices of anxiety in Tfm males appear to be related to hyper-activation of the hypothalamic-pituitary-adrenal axis since levels of the stress hormone corticosterone were elevated in Tfm males compared to wt males at

baseline and at several time points after exposure to a novel object. These findings demonstrate that ARs influence anxiety and stress response.

Introduction

In humans, gonadal hormones have been implicated in the development and maintenance of several mental disorders including anxiety, depression, and schizophrenia. Women suffer from anxiety disorders and depression more frequently than do men (Weinstock, 1999; Kornstein, 1997; Wilhelm et al., 1998), and variations in the ovarian hormone estrogen appear to contribute to the etiology of these disorders in women (Arpels, 1996; Yazici et al., 2003). Evidence in animal models supports a role of estrogen in mood disorders. In both male and female rodents, estrogens have been shown to decrease depressive and anxiety-related behavior (Frye and Lacey, 2001; Walf and Frye, 2005), particularly through activation of the estrogen receptor (ER) isoform ERβ (Lund et al., 2005; Imwalle et al., 2005).

Emerging evidence suggests that androgens also contribute to mood disorders. Boys and girls with low T levels show increased indices of depression and anxiety compared to those with high T (Granger et al., 2003). Furthermore, in hypogonadal and aging men who have low levels of circulating T, mood disorders are increased (Kaminetsky, 2005; Lund et al., 1999; Amore, 2005; Eskelinen et al., 2007; Cooper and Ritchie, 2000). T treatment of these individuals can decrease depressive symptoms and increase mood (Kumano, 2007; Kaminetsky, 2005; Cooper and Ritchie, 2000).

In rodents, androgens have also been shown to play a role in anxiety-related behavior, with an increase in T generally correlated with decreased indices of anxiety

(Frye et al., 2008; Bing et al., 1998; Bitran et al., 1993). Reducing androgen levels via gonadectomy in male rodents increases anxiety and fear-related behaviors (Adler et al., 1999; Bitran et al., 1993; Frye et al., 2008; Frye and Edinger, 2004). Supplementation with T also decreases indices of anxiety in several rodent models (Frye et al., 2008; Aikey et al., 2002; Edinger and Frye, 2005). As in hypogonadal men, diminished testicular production of T in mice is associated with indices of decreased mood (Umehara et al., 2006). In rodents, aging, which has also been correlated with increased anxiety-related behaviors (Koprowska et al., 2004; Boguszewski and Zagrodzka, 2002), is accompanied by a decline in T. A recent study suggests that anxiety-related behavior in aged male mice can be reduced by administration of androgens (Frye et al., 2008).

One mechanism through which androgens may influence anxiety-related behavior is through the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. While HPA axis activation is generally adaptive, hyper-activation may be maladaptive and has been associated with increased indices of anxiety and depression in both humans and rodents (Scott and Dinan, 1998; Landgraf et al., 1999; Lund et al., 2005). In rodents, administration of T, and its metabolites dihydrotestosterone (DHT) and 5alpha-androstane, 3beta,17beta-diol (3β-diol), can suppress the normal rise of stress hormones (adrenocorticotropic hormone (ACTH) from the pituitary and corticosterone from the adrenal cortex) following exposure to a stressful situation (Handa et al., 1994; Lund et al., 2004; Lund et al., 2005; Lund et al., 2006), suggesting androgens can influence HPA axis activity, and in turn, may potentially influence anxiety-related behaviors.

Gonadal hormones, particularly estrogens, have also been hypothesized to play a neuroprotective role in schizophrenia, which may contribute to the later onset and milder

symptoms of this disease in women compared to men (Hafner et al., 1998; Castle et al., 1995). One common characteristic in people with schizophrenia is a deficit in sensorimotor gating, the capacity to filter sensory, motor, and cognitive information (Kodisi and Swerdlow, 1994). This reduction in sensorimotor gating may be linked to symptoms of schizophrenia such as information processing deficits, disorders of thought and auditory hallucinations (Perry and Braff, 1994; Kumari et al., 2008). In an operational measure of sensorimotor gating, prepulse inhibition of the acoustic startle response (PPI; Swerdlow et al., 1996), people with schizophrenia show reduced PPI indicating a deficit in sensorimotor gating (Braff et al., 1992). This deficit is alleviated by treatment with atypical antipsychotic medications (Kumari et al., 2002).

Animal models suggest that gonadal hormones can also influence sensorimotor gating. In female rats, PPI varies depending on the phase of the estrus cycle (Koch, 1998) and administration of estrogen to ovariectomized females increases PPI (van den Buuse and Eikelis, 2001). T has also been shown to facilitate PPI in male and female rats (Gogos and Van den Buuse, 2003), however, another recent study suggests that in male rats PPI is unaffected by treatment with androgens or estrogens (Turvin et al., 2007). In mice the role of gonadal hormones in PPI has been less studied, and the roles of specific hormone receptors, particularly the AR, in modulating PPI are largely unknown, though studies in aromatase knockout (ArKO) males suggest that estrogenic metabolites of androgen acting through ERs can influence PPI (van den Buuse et al., 2003; Gogos et al., 2006).

One model for exploring the role of the AR in the brain and behavior is testicular feminization mutant (Tfm) mice (Zuloaga et al., 2008). Tfm mice have a mutation in the

AR gene that involves a single nucleotide deletion (Charest et al., 1991) which introduces a reading frame shift and premature stop codon, resulting in a shortened transcript and essentially no AR protein (He et al., 1991; Monks et al., 2007). Consequently these mice have virtually *no* sensitivity to androgen through the AR (Drews, 1998). Since this trait is X-linked, only genetic males (XY) are wholly androgen insensitive. To further investigate the role of the AR in anxiety-related behavior, regulation of the HPA axis, and sensorimotor gating in males, we compared behavioral and physiological responses in wt and Tfm male mice. Because Tfm male mice have significantly decreased circulating T levels (Jones et al., 2003) behavior was also assessed in mice provided with exogenous T in adulthood. These studies indicate that the AR regulates the HPA axis and plays a role in many behaviors associated with anxiety in mice, but plays little if any role in regulating PPI.

Method

Animals

C57BL6J mice bred in our Tfm colony at Michigan State University were group housed with a 12/12 L/D cycle, lights on at 0600. The mice in this colony have been sired exclusively by commercially purchased C57BL6J males for over 10 generations. Upon weaning at 21 days old, mice were ear punched and genotyped using a modified polymerase chain reaction (PCR) described elsewhere (Fernandez et al., 2003). Products of this reaction differentiated between the Tfm and the wt allele for the AR, and male versus female, based on the presence or absence of the Sry gene found only on the Y chromosome.

Procedure

Experiment 1: To address the role of the AR in sensorimotor gating and anxiety-related behavior, 90-120 day old wt male (N=9) and Tfm male mice (N=10) were tested for PPI and anxiety-related behaviors as described in detail below.

Experiment 2: Since endogenous T levels differ in Tfm and wt male mice (Tfm<< wt) and might explain differences in behavior, a second experiment was performed. Wt males were gonadectomized at 70-90 days of age and implanted with Silastic capsules containing T (N=10) or nothing (blank, B; N=9), while Tfms were sham-operated and also implanted with capsules containing T (N=10) or nothing (N=9). Tfm males were not gonadectomized because (1) their testes produce negligible amounts of T (Jones et al., 2003; present study), and (2) gonadectomy would be more intrusive in these animals because the testes reside in the abdominal cavity. To better mimic gonadectomy in wt males, Tfm males received an incision into the perineal cavity (procedure described in detail below). Three weeks later, mice were assessed for sensorimotor gating and anxiety-related behavior as described below.

Experiment 3: To assess whether differences in anxiety-related behavior might be related to differences in HPA axis activation, plasma corticosterone was collected from 90-120 day old wt and Tfm male mice at baseline (wt male: N=10; Tfm male: N=10) or at 20 min following the onset of exposure to an open field with a novel object (wt male: N=10; Tfm male: N=10).

Experiment 4: To assess whether the time course of HPA axis recovery differed in wt and Tfm males following exposure to an open field with a novel object, 90-120 day old mice were assayed for corticosterone at baseline (wt male: N=7; Tfm male: N=6) as well as at 20 (wt male: N=7; Tfm male: N=7), 40 (wt male: N=6; Tfm male: N=7), 60 (wt male: N=7; Tfm male: N=7), and 120 (wt male: N=6; Tfm male: N=6) min after exposure to a novel object in the open field testing arena.

Adult castration and hormone replacement

At 70-90 days, wt male mice were anesthetized with isoflurane and the testes externalized via bilateral incisions made in the scrotum. The vas deferens were tied off with silk suture prior to cutting. For Tfm males, bilateral incisions were made in the perineal cavity in a similar location as the scrotum in wt males. Animals also received a subcutaneous Silastic capsule (1.57 mm inner diameter, 3.18 mm outer diameter: 6 mm effective length) containing either free T (T) or nothing (blank (B)) via a 2 cm incision dorsally at the nape of the neck. These Silastic capsules were designed to deliver normal male physiological levels of T to adult mice. Incisions were closed with wound clips and the analgesic buprenorphine (0.1 mg/kg) was injected sc post operatively.

Behavior Testing

Animals in experiments 1 and 2 were tested for sensorimotor gating (PPI) and anxiety-related behaviors. Testing took place in the following order: PPI, open field/novel object test, elevated plus maze (EPM), and light/dark (LD) box, with each animal receiving a

minimum of 72 hours between tests. All tests were administered between 1000-1400 except for PPI, which was conducted during the dark cycle beginning at 1900. In order to address the possibility that exposure to the most fear provoking test (PPI) might alter behavior on the following tests, tests were also conducted in another cohort of intact wt (n=10) and Tfm (n=10) male mice in order from those judged to be the least to most anxiety-provoking (open field/novel object test, light dark box, elevated plus maze, PPI).

Prepulse Inhibition

Mice were tested for PPI 1 hour after lights off in a room illuminated by dim red light. PPI was measured in acoustic startle response chambers (SR Lab startle response system, San Diego Instruments, San Diego, CA). Animals were placed into the chamber for 18 minutes, the first 5 minutes of which is an acclimation period. For the remaining 13 minutes the fast twitch startle responses of mice were recorded via SR Lab software (San Diego Instruments) to a 100 decibel (dB) tone alone (acoustic startle response), or the same tone preceded by a 100 msec tone of 3, 8, 10, or 15 decibels. The prepulse should permit the subject to anticipate the loud pulse, and consequently startle less severely. PPI was calculated as the percentage decrease in startle response relative to responding to the 100 dB tone without a prepulse. Each of these trials was repeated 6 times at pseudo-random intervals. After the test, animals were removed and the chamber was cleaned with 70% ethanol.

Open Field/Novel object test

Open field/novel object testing for mice took place in a 16"x 16" Plexiglas chamber (Versamax animal activity monitor, Accuscan Instruments Inc, Columbus, OH) that was illuminated from above. The chamber was equipped with a grid of invisible infrared beams, and breaks in beams were used to measure movement characteristics such as rearings (vertical movements that involve removal of the forelimbs from the floor) and anxiety-related indices, including the number of entries into and time spent in the center area of the field or visiting the novel object at the field center. Animals were first placed into a corner of the empty open field and data were collected for 5 minutes. After 5 minutes mice were removed, the chamber was cleaned with 70% ethanol, and a novel object (a 2" d x 0.75" h Petri dish with red and blue tape) was placed in the center of the chamber. Three minutes after first removal from the open field, the mouse was then replaced into the chamber and data were collected for another 5 minutes. The chamber was cleaned with 70% ethanol between each test.

Elevated Plus Maze (EPM)

The EPM (Phenome Technologies Inc.; Lincolnshire, IL) consisted of two open and two closed arms (30 x 5 cm) that extended from a center platform and was elevated 50 cm above the floor. Testing took place in a dimly lit room with a video camera suspended above the maze to record behavior for assessment at a later time by an observer blind to the experimental condition. During testing, animals were placed in the center area of the EPM facing an open arm and allowed to freely explore for the 10 minute duration of the test. The numbers of entries into and the amount of time spent on the open and closed

arms were assessed as indices of anxiety-related behavior. The EPM was cleaned with 70% ethanol between each test.

Light/Dark (LD) Box

The LD box (Phenome Technologies Inc.; Lincolnshire, IL) consisted of a rectangular box divided into two regions, a dark (21 cm length x 24 cm width x 19 cm height) and a light (21 cm length x 24 cm width x 19 cm height) area connected by a (10 cm height x 5 cm width) opening between the two. The dark region was constructed of black plastic and was covered by a black plastic lid, while the light region was constructed of clear Plexiglas and was illuminated by a 60 watt light 3 feet directly above it. The small opening connecting the two chambers allowed the animals to freely enter either area. Animals were placed in the light side of the chamber facing the opening to the dark chamber and were allowed to move freely between the two compartments for 10 minutes with behavior recorded via an overhead video camera. The number of entries into, time spent in the two compartments, and the number of rearings in the light compartment were scored at a later time as indices of anxiety-related behavior by an observer blind to the experimental condition. The chamber was cleaned with 70% ethanol between each test.

Plasma Collection, Corticosterone and Testosterone Assay

In experiment 3, blood was collected from adult mice between 0900-1100 at baseline or 20 min after exposure to an open field with a novel object (a 2" d x 0.75" h Petri dish with red and blue tape). In experiment 4, blood was collected from adult mice between 0900-1100 at 20, 40, 60, and 120 min after exposure to an open field with a novel object

or from control mice that remained in their home cage. Exposure to a novel object in an open field was for 10 minutes, after which they were returned to their home cage where they remained until sacrifice. Control mice remained in their home cage until sacrifice. Mice were deeply anesthetized with isoflurane and decapitated, with trunk blood collected within 2 minutes of cage disturbance. Blood was also collected from mice in experiment 2 to verify T levels after supplementation. These mice were deeply anesthetized with sodium pentobarbital and blood was collected through cardiac puncture. All blood was collected in 1.5 ml tubes containing 250µl of heparin and held on crushed blue ice until centrifugation. Samples were centrifuged at 8° C for 20 minutes and plasma was collected and stored frozen at –20° C. Plasma was assayed for corticosterone and T at the Diagnostic Center for Population and Animal Health at Michigan State University using Coat a Count Corticosterone and Coat a Count Total Testosterone kits (Diagnostics Products Corporation, Los Angeles, CA, USA). All plasma samples were run in duplicate and the results averaged.

Statistical Analysis

For PPI, a mixed design ANOVA was used to analyze data for all treatment groups and/or genotypes as between group factors and the different prepulse trials as within group (or repeated measures) factors. Anxiety-related behaviors were analyzed in experiment 1 using Student's t-tests, or in experiment 2 using a 2x2 analysis of variance (ANOVA) with hormone treatment (T, no T) and genotype (wt male and Tfm male) as independent factors. To compare behavioral differences in mice exposed to the PPI test prior to anxiety tests (test order 1) and mice tested in order from least to most anxiety

provoking (test order 2) a 2-way ANOVA was used with genotype and test order (test order 1, test order 2) as independent factors. In experiment 3, T and corticosterone were analyzed using a 2x2 analysis of variance (ANOVA) using testing condition (open field/novel object exposure, no manipulation) and genotype (wt male and Tfm male) as independent factors. In experiment 4 differences in corticosterone were analyzed using a two-way ANOVA (genotype x time). All significant main effects or interactions were further analyzed using Newman-Keuls multiple comparisons post hoc tests. Differences were considered significant when $p<0.05$ and all data are reported as means ± standard error of the mean (SEM).

Results

Experiment 1.

For PPI, a mixed design ANOVA (genotype as a between groups factors and prepulse intensity as a within groups factor) revealed an expected effect of prepulse intensity ($F(3,42)= 87.679$, $p<.001$) in which PPI increased as intensity of the prepulse increased. There was also a significant effect of genotype in which wt males showed an overall greater PPI than did Tfm males ($F(1,45)= 4.869$, $p<.05$; Figure 1a). This difference appeared to be independent of prepulse intensity as there was only an overall effect of genotype and no significant interaction. No significant difference in startle response to the loud acoustic pulse alone was found in this test (wt males: 63.9 ± 6.2; Tfm males: 50.3 ± 8.4; in arbitrary units).

In the open field test, no significant differences were found between wt and Tfm males in the number of entries into or time spent in the center area of the chamber (Figure

2a). There was also no significant difference in the total number of rearings, although there was a trend in which wt males tended to rear more frequently than did Tfms ($t(17)= 1.971$, $p=.065$; Figure 2e, left-most bars). When mice were exposed to a novel object, independent t-tests revealed that wt males entered the center area of the open field containing the object more frequently ($t(17)= 2.225$, $p<.05$; Figure 2b) and spent more time visiting the novel object ($t(17)= 2.177$, $p<.05$; Figure 2b) than did Tfm males. The number of rearings was also greater in wt than Tfm males ($t(17)= 3.279$, $p<.01$; Figure 2e, middle bars).

In the EPM there were no group differences in the number of entries into the open arm of the maze, though wt males did show a trend toward spending more time in the open arms of the maze than did Tfm males ($t(17)= 1.781$, $p=.09$; Figure 2c). In the LD box, independent t-tests revealed that wt males showed a greater number of entries into the light area of the box ($t(17)= 2.148$, $p<.05$; Figure 2d), spent more time in the light area ($t(17)= 2.178$, $p<.05$; Figure 2d), and reared more frequently in the light area ($t(17)= 2.372$, $p<.05$; Figure 2e, right-most bars) than did Tfm males.

When behavior tests were conducted in another cohort of wt and Tfm male mice, in order from least to most anxiety-provoking (test order 2), similar group differences and/or similarities were found for all tests except PPI. Time spent visiting the novel object, in the light area of the LD box, and in the open arm of the EPM were greater (or marginally greater) in wt than in Tfm males (($t(18)= 3.376$, $p<.01$; ($t(18)= 2.290$, $p<.05$); ($t(18)= 2.067$, $p=.057$) respectively; data not shown), and again there were no group differences in the open field test. In mice in test order 2 there was no significant main effect of genotype for PPI (Figure 1b) and again ASRs did not differ. As a result of the

discrepancy in group differences in PPI mice from test order 1 and 2 were pooled to determine if there was an overall effect of genotype. This analysis did not indicate a significant main effect of genotype for PPI. No significant main effect of test order was found for behaviors in the open field, novel object test, LD box, EPM, or PPI test between mice in test order 1 and test order 2, indicating behaviors in Tfm and wt males were not significantly affected by test order.

Experiment 2.

There was a significant main effect of T treatment on body weight with no main effect of genotype or interaction. Specifically, body weight was greater in T-treated wt males than all other groups (all p's<.05; Table 1). Blood concentrations of T also revealed a significant main effect of treatment with no main effect of genotype or interaction. Animals provided T capsules had circulating T levels that were in the physiological range, slightly lower than in gonadally intact wt males (Table 1). Castrated wt males given B capsules had circulating levels of T that approximated those of untreated Tfm mice in both studies (Table 1).

A mixed design ANOVA using genotype and hormone treatment as between groups factors and prepulse intensity as a within groups factor again revealed the expected effect of prepulse intensity ($F(3,96)= 48.293$, $p<.001$; Figure 3b) in which PPI increased as the intensity of the prepulse increased. However, no significant main effects or interactions were found between groups for PPI in this cohort. As in experiment 1, there were no significant group differences for startle response to the 100 dB pulse alone (Figure 3a).

In the open field test, a 2x2 ANOVA revealed no significant main effects or interactions between groups in the number of entries into and time spent in the center area of the chamber (Figure 4a), or in the number of rearings (Figure 4e, left-most bars). In the novel object test there was a significant main effect of genotype ($F(1,34)= 9.35$, $p<.05$), with no main effect of treatment or interaction, indicating that as a group Tfm males spent less time visiting the novel object than did wt males, replicating the results of experiment 1 in gonadally intact mice. Post hoc comparisons revealed significant differences between T-treated wt males and all other groups (p's<.05; Figure 4b, left), indicating that T increases this measure, but only in wt males and not in Tfm males. A significant main effect of genotype was also revealed for the number of rearings in the novel object test ($F(1,34)= 6.714$, $p<.05$), with no main effect of treatment or interaction, indicating that wt males reared more than did Tfms. Post hoc comparisons revealed significant differences between T-treated wt males and both Tfm groups (T or no T, p's<.05; Figure 4e, middle bars). No significant main effects or interactions were found for the number of novel object visits, though there was a trend toward a main effect of treatment ($F(1,34)= 3.323$, $p=.076$; Figure 4b, right).

In the EPM no significant main effects or interactions were found for time spent on or number of visits to the open arms (Figure 4c). In the LD box a significant main effect of treatment was found for time spent in the light area ($F(1,34)= 4.209$, $p<.05$), with no significant main effect for genotype or interaction. Post hoc tests revealed that T-treated wt males spent more time in the light area than did either B-treated wt males or T-treated Tfm males (p's<.05; Figure 4d, left), though T-treated wt males did not significantly differ from B-treated Tfm males. A significant main effect of genotype was

found for the number of rearings in the light area of the LD box ($F(1,34)= 6.356$, $p<.05$), with no significant main effect of treatment or interaction. Post hoc comparisons revealed significant differences between T-treated wt males and all other groups ($p's<.05$; Figure 4e, right bars), indicating that T-treated wt males reared more than any other group in the light area of the LD box. No significant main effects or interactions were found for the number of light area visits, although there was a trend toward a main effect of treatment ($F(1,34)= 3.966$, $p=.054$; Figure 4d, right).

Experiment 3.

Two-way ANOVA revealed a significant main effect of exposure ($F(1,38)= 908$, $p<.001$) in which mice exposed to an open field with a novel object showed an increased corticosterone response 20 min after exposure compared to mice in the basal condition (left in their cage). There was also a significant main effect of genotype ($F(1,38)= 177.4$, $p<.001$) with Tfm males showing an increased corticosterone response compared to wt males. Post hoc comparisons revealed that Tfm males had greater blood corticosterone levels at baseline ($p<.01$) and after exposure to an open field with a novel object ($p<.001$). A significant interaction was also found between exposure and genotype on corticosterone concentrations ($F(3,36)= 69.1$, $p<.001$), which indicated that the increase in corticosterone from baseline to that induced by exposure to a novel object was greater in Tfm males than in wt males (Fig 5a). Two-way ANOVA revealed a significant main effect of genotype on T levels ($F(1,38)= 134$, $p<.001$), with no main effect of exposure or interaction between genotype and exposure. Specifically, compared to wt males, Tfm

males had lower levels of T (Table 1). As expected, body weight was also significantly greater in wt than in Tfm males ($t(17)= 3.382$, $p<.01$; Table 1).

Experiment 4.

A 2-way ANOVA confirmed the expected main effect of time on corticosterone levels ($F(4,52)= 55.58$, $p<.001$) indicating that compared to basal corticosterone levels, Tfm and wt males had greater corticosterone levels overall after exposure to a novel object. A main effect of genotype was also revealed in which Tfm males showed overall greater blood corticosterone concentrations compared to wt males ($F(1,55)= 33.67$, $p<.001$). Post hoc comparisons revealed that Tfms had higher corticosterone concentrations compared to wt males at 20, 40, and 60 min ($p's<.05$; Fig 5b).

Discussion

Tfm male mice showed increased indices of anxiety in several behavioral tests (novel object and LD box tests), but not others (open field and EPM), indicating a task-specific increase in anxiety-related behavior in Tfm males compared to wt males. In the novel object and LD box tests, Tfm males showed decreased exploration of a novel object and of the light area of the LD box as well as a generalized decrease in exploratory behavior in these tests, as assessed by the number of rearings. T-treatment in gonadectomized wt males decreased indices of anxiety in the novel object and LD box tests suggesting that T-treatment in adult male mice can produce anxiolysis. However, these behaviors were unaffected by T treatment in Tfm males, which indicates that T's anxiolytic action in wt males is likely mediated through the AR. These findings support previous research

indicating that the AR is involved in the regulation of anxiety-related behavior in rodents (Edinger and Frye, 2006; Fernandez-Guasti and Martinez-Mota, 2005; Rizk et al, 2005).

Since the Tfm mutation is present from ontogeny, it is unclear whether differences between wt and Tfm males in anxiety-related behavior are the result of "organizational" or "activational" influences of ARs (Arnold and Breedlove, 1985). Other findings in rodents indicate that activation of ARs in adulthood plays a prominent role in regulating anxiety-related behavior (Edinger and Frye, 2006; Fernandez-Guasti and Martinez-Mota, 2005). In our study, increased anxiety-related behavior in wt males given T capsules versus B capsules confirms that T can act in adulthood to affect this behavior, although it does not directly implicate the AR. It is possible that decreased AR activation during development in Tfm males resulted in their increased indices of anxiety. Little is known about the organizational role of ARs in anxiety, although a study showed that neonatal treatment of rats with the AR antagonist flutamide did not affect indices of anxiety in the elevated plus maze (Zimmerberg and Farley, 1993).

Corticosterone levels were also elevated in Tfm males compared to wt males at both baseline and 20 min after initial exposure to an open field with a novel object (experiment 3). During recovery, the corticosterone response remains consistently elevated in Tfm males compared to wt males at 40 minutes and 60 minutes (experiment 4). These findings indicate that the increased anxiety-related behavior in Tfm male mice may be related to a hyper-activation of the HPA axis. Previous studies suggested a relationship between trait anxiety and increased HPA axis activity after exposure to rodent tests of anxiety. Rats bred for high compared to low anxiety behavior show

elevations in stress hormones ACTH and corticosterone that correlate with increased indices of anxiety in the EPM and open field (Landgraf et al., 1999; Salome et al., 2004).

Androgens have been shown to play a role in the regulation of the HPA axis response to stress in other models. Gonadectomy of adult wt male rats increased the release of corticosterone and ACTH following footshock stress or exposure to an open field (Handa et al., 1994). Furthermore, T or DHT replacement in gonadectomized rats decreased the rise in stress hormones to levels similar to those of intact rats (Handa et al., 1994). Androgen's influence on the HPA axis does not appear to be solely activational, however. Neonatal gonadectomy in male rats increased the corticosterone response to restraint stress, an increase that is not reduced by adult testosterone propionate (TP) replacement as it is in rats gonadectomized as adults (McCormick et al., 1998). Neonatal androgen exposure can also alter the female corticosterone response: female rats administered TP on the day of birth showed decreased basal and stress induced corticosterone levels (Seale et al., 2005a). Other evidence supports the notion that the AR is involved in the organization of the HPA axis (McCormick and Mahoney, 1999; Seale et al., 2005b). Seale et al. (2005b) demonstrated that perinatal AR blockade, by administration of the AR antagonist flutamide, increased both basal and stress induced corticosterone levels in adulthood compared to rats given vehicle perinatally. Furthermore, serum corticosterone and ACTH levels are elevated in AR knockout male mice, in which AR deficiency is present from ontogeny (Miyamoto et al., 2007), as in Tfm males.

Previous research therefore indicates that increased corticosterone levels found in Tfm male mice could be related to organizational influences, activational influences, or

both, resulting from a lack of functional ARs. However, since Tfm males have decreased levels of T compared to wt males, it is also possible that when provided ample T, Tfm males would show corticosterone levels similar to wt males. Evidence suggests that T, after metabolism to DHT, can be converted to 3β-diol which reduces HPA axis activity via activation of ERβ (Lund et al., 2004; Lund et al., 2005; Lund et al., 2006). Therefore, without additional experiments, we cannot conclude that the increased corticosterone levels seen in Tfm male mice result directly from a lack of functional ARs. However, Tfm male rats, which have elevated T levels compared to wt males, also show an increased corticosterone response compared to wt males 20 min following exposure to an open field with a novel object (Zuloaga et al., in preparation). Therefore, even when provided similar T, Tfm male mice may still continue to show increases in corticosterone compared to wt males. This outcome would be consistent with our behavioral measures of anxiety suggesting that Tfm male mice show increased anxiogenesis regardless of endogenous T levels.

Indirect evidence suggests that in Tfm male mice, T levels are near the normal male range during the perinatal period (Goldstein and Wilson, 1972) and decrease during development largely due to a reduction in the testicular enzyme 17α-hydroxylase (Murphy and O'Shaughnessy, 1991). Thus, Tfm male mice may have ample T substrate to activate ERs perinatally, leading us to suspect that the increased HPA axis activity seen in Tfm male mice may indeed reflect a lack of functional ARs, although when AR is critical remains unclear.

Since plasma corticosterone binding globulin (CBG) levels were not measured in this study, it is possible that CBG concentrations are elevated in Tfm males and could

contribute to differences in corticosterone levels. An increase in CBG levels would indicate that more corticosterone would need to be released in order for similar corticosterone receptor binding to occur, because more circulating corticosterone is being bound by CBGs and is therefore rendered biologically inactive. Increased T has been shown to decrease plasma CBG concentrations (Viau and Meaney, 2004). Therefore, if indeed a decrease in circulating T (or a decrease in functional ARs independent of circulating T) in Tfm males increases plasma CBGs, elevated plasma corticosterone levels in Tfm males may reflect an attempt to achieve normal levels of arousal rather than a hyper-arousal.

Intact Tfm male mice also showed an overall decrease in PPI compared to wt male mice in test order 1, suggesting that the AR may be involved in sensorimotor gating. However, in test order 2 there were no group differences in PPI and in experiment 2 neither T treatment nor genotype affected PPI. These findings suggest that the original differences found between wt and Tfm male mice may be subtle and somewhat unreliable. Alternatively, group differences in PPI may have been reduced by prior exposure to tests of anxiety in intact animals. However, hormone manipulated mice in experiment 2 were tested for PPI without prior exposure to tests of anxiety and did not show differences in PPI, indicating that test order did not contribute to group differences. Together these data suggest that in mice the role of T and ARs in the regulation of PPI is minimal. This corresponds with a report of normal PPI in mice with low circulating T levels (Umehara et al., 2006).

Tfm males provide a useful model for examining the role of ARs in behavioral and physiological responses, while avoiding some of the limitations of pharamacological

AR agonists (e.g., DHT) and antagonists (e.g., flutamide). DHT, along with activating ARs, can be metabolized to 3α-androstanediol (3α-diol) which has a low affinity for ARs but a high affinity for GABA receptors. 3α-diol also appears to play a role in anxiolysis in rodents, as its administration decreases anxiety-related behavior (Frye and Edinger, 2004; Edinger and Frye, 2004; Edinger and Frye, 2005). An estrogenic metabolite, 3β-diol, which binds ERs with greater affinity for ERβ than ERα, can also be derived from DHT (Kuiper et al., 1998; Lund et al., 2004). 3β-diol administration has also been demonstrated to reduce anxiety-related behavior and activation of the HPA axis through its actions on ERβ (Lund et al., 2004; Lund et al., 2005; Lund et al., 2006). Thus effects of DHT treatment on anxiety-related behaviors and HPA axis activity could be mediated either through AR, ERβ, or GABA receptors.

A limitation of the Tfm model is that Tfm male rats have been reported to show decreased aromatase activity in several areas of the brain (Rosseli et al., 1987). Therefore it is possible that T-treatment in Tfm males failed to affect anxiety-related behavior because there was insufficient ER activation due to a decrease in the conversion of T to estrogens. However, Tfm male mice have been reported to show both similar and slightly decreased levels of aromatase activity in the hypothalamus compared to wt males (Naftolin et al., 1975; Rosenfeld et al., 1977). In the study that indicated a decrease in Tfm aromatase activity, it was the conversion of T to the less potent estrogen, estrone, which contributed to an overall decrease in aromatization, while the conversion of T to E2 was actually elevated in Tfm male mice (Rosenfeld et al., 1977). Estrone, unlike E2, has a much higher affinity for ERα than ERβ, and evidence suggests that a decrease in ERα activation would not contribute to increased anxiogenesis (Lund et al., 2005; Krezel

et al., 2001). Furthermore, aromatase knockout (ArKO) male mice have been shown to exhibit normal levels of anxiety behavior (Dala et al., 2005), suggesting that aromatization of T to estrogens plays a minimal role in the display of these behaviors in male mice.

In conclusion, the present findings in Tfm mice indicate that the AR is involved in the regulation of anxiety-related behaviors in males, as demonstrated by differences between wt and Tfm males in some tests of anxiety. Furthermore, these differences in anxiety-related behavior may be related to an increased activation of the HPA axis in Tfm males since they show increased levels of blood corticosterone at baseline as well as at several time points following exposure to an open field with a novel object. Further work will be needed to explore whether ARs act during development and/or adulthood to affect anxiety-related behaviors. On the other hand, the role of the AR in sensorimotor gating in mice, as indicated by PPI, appears minimal, suggesting that any role of gonadal steroids on sensorimotor gating may be mediated via estrogen receptors rather than ARs.

Chapter 4: Elevated Anxiety-Related Behavior and Corticosterone Response in Rats with the Testicular Feminization Mutation

Abstract

Testosterone has been shown to influence anxiety and sensorimotor gating in rodents but the role of a target receptor, the androgen receptor (AR), in mediating these influences is unclear. In this study male rats with the testicular feminization mutation (Tfm), which lack functional ARs, were compared to wild type (wt) male and female littermates on an assay of sensorimotor gating (prepulse inhibition (PPI) of the acoustic startle response (ASR)) and tests of anxiety (open field, novel object exposure, elevated plus maze (EPM), and light/dark (LD) box). No differences were found for PPI, although Tfm males had an increased ASR compared to wt males and females. Tfm males also showed increased indices of anxiety compared to wt males and females in the open field and when exposed to a novel object, with no significant differences found in the EPM or LD box. When exposed to a novel object, Tfm males showed a peak corticosterone response similar to wt females and greater than wt males, although baseline and recovery levels did not differ from wt males. Analysis of the immediate early gene c-Fos revealed activation of several brain areas after exposure to an open field with a novel object, with greater activation in Tfm males compared to wt males in some regions (medial preoptic area) and lesser activation in others (dentate gyrus, posterodorsal medial amygdala). These findings demonstrate that ARs play a role in the regulation of some anxiety-related behaviors in rats, but have little or no role in sensorimotor gating.

Introduction

In humans, gonadal hormones play an important role in the regulation of anxiety and sensorimotor gating (the capacity to filter sensory, motor, and cognitive information). Women are diagnosed with disorders of anxiety and unipolar depression more than men, and decreases in mood often coincide with a decline in levels of the ovarian hormone estrogen during menopause (Arpels, 1996). Furthermore, estrogen replacement therapy has been reported to increase mood and decrease anxiety in postmenopausal women (Yazici et al., 2003). In men a similar but less abrupt decline in androgen levels is also often accompanied by increased symptoms of anxiety and depression (Kaminetsky, 2005; Lund et al., 1999; Eskelinen et al., 2007; Cooper and Richie, 2000). Androgen treatment of these men, or of younger men with decreased testicular production of testosterone (T) alleviates some of these symptoms (Kaminetsky, 2005; Cooper and Richie, 2000; Kumano, 2007). Similarly, boys and girls with low T levels show greater indices of depression and anxiety than those with high T (Granger et al., 2003).

There are also sex differences in sensorimotor gating (men > women; Swerdlow et al., 1993) as assessed by an experimental model of sensorimotor gating, prepulse inhibition of the acoustic startle response (PPI; Swerdlow et al., 1996). Furthermore, PPI varies in women according to stage of the menstrual cycle suggesting that variations in sex hormones can influence PPI (Swerdlow et al., 1997; Jovanovic et al., 2004).

As in humans, hormones also appear to play a role in the display of anxiety-related behaviors and sensorimotor gating in rodents. In rats there are sex differences in tests of anxiety with females often showing decreased anxiety-related behaviors and greater activity compared to males (Archer, 1975; Masur et al., 1980; Slob et al., 1981;

Seliger, 1977; Lucion et al., 1996). Furthermore, administration of both estrogens and androgens generally result in decreased indices of anxiety in rodents (Frye and Lacey, 2001; Walf and Frye, 2005; Lund et al., 2005; Frye et al., 2008; Bing et al., 1998; Bitran et al., 1993). Evidence suggests that the anxiolytic actions of estrogens are largely mediated through activation of the estrogen receptor (ER) isoform ERβ (Lund et al., 2005; Imwalle et al., 2005). On the other hand, the actions of androgens may be mediated through several mechanisms including androgen receptors (ARs), ERs, and/or GABA receptors (Edinger and Frye, 2006; Lund et al., 2005; Edinger and Frye, 2005).

Along with influencing anxiety, sex hormones have also been shown to mediate hypothalamic-pituitary-adrenal (HPA) axis activity (Handa et al., 1994; Lund et al., 2005), which in turn may affect behavior in rodents exposed to an aversive situation. In particular, T decreases while estrogen increases the release of stress hormones adrenocorticotropic hormone (ACTH) from the pituitary gland, and corticosterone from the adrenal cortex. Specific activation of sex hormone receptors ERα and ERβ increase and decrease HPA axis activity respectively, while AR activation also appears to decrease activity, although its role is less clear (Handa et al., 1994; Lund et al., 2004; Lund et al., 2005; Lund et al., 2006).

Sensorimotor gating in rodents, like humans, is influenced by sex hormones. Sex differences in PPI have been reported in some strains of rats and mice (Lehman et al., 1999; Ralph et al., 2001; Ison and Allen, 2007) and PPI is influenced by estrus cycle in rats (Koch, 1998). Administration of estrogens and androgens has also been demonstrated to affect PPI (van den Buuse and Eikelis, 2001; Gogos and Van den Buuse,

2003), though little is known about the role of specific hormone receptors, including the AR, that may be mediating these changes.

To investigate the role of ARs in anxiety-related behaviors, regulation of the HPA axis, and PPI, we compared behavior and hormonal (corticosterone) responses in wild type (wt) male, wt female, and testicular feminization mutant (Tfm) male rats with dysfunctional ARs. To further probe the role of ARs in males, corticosterone recovery and neural activation were also measured in Tfm and wt male rats at baseline and after exposure to an anxiety-provoking situation. Tfm rats are a genetic model for exploring the role of the AR in the brain and behavior (Zuloaga et al., 2008a). In rats this mutation results from a single base pair replacement in the AR gene (Yarbrough et al., 1990), and thus there is only a single amino acid difference between wt and Tfm AR protein that results in expression of a normal sized but largely dysfunctional AR protein in Tfm rats (Yarbrough et al., 1990). Since this trait is X-linked, only genetic males (XY) are androgen insensitive.

Materials and Methods

Animals

Long Evans rats, which were bred with commercially purchased Long Evans (Charles River) sires for over 10 generations, were group housed in our Tfm colony at Michigan State University with a 12/12 L/D cycle, lights on at 0600. Upon weaning at 21 days old, ear punches were performed to obtain DNA for determining whether rats were wt male,

wt female, Tfm male, or female Tfm carriers. Rats were genotyped using a modified polymerase chain reaction (PCR) to detect the Tfm versus the wt allele for the AR, and to detect the presence or absence of the Sry gene found only on the Y chromosome (Fernandez et al, 2003). Wt males, wt females, and Tfm males were used in the following experiments. All animals received care that meets standards of the National Institutes of Health and all experiments were approved by the MSU IACUC.

Experiment 1: The role of ARs in sensorimotor gating and anxiety-related behavior. One hundred twenty-150 day old wt male (N=9), wt female (N=11), and Tfm male rats (N=10) were tested for PPI and anxiety-related behaviors as described below.

Experiment 2: The role of ARs in HPA axis regulation. Plasma corticosterone was collected from 120-180 day old rats at baseline (wt male: N=10; wt female: N=10; Tfm male: N=10) or at 20 minutes after initial exposure to an open field with a novel object (wt male: N=10; wt female: N=10; Tfm male: N=10).

Experiment 3: The role of ARs in HPA axis recovery. One hundred twenty-180 day old rats were assayed for corticosterone at baseline (wt male: N=7; Tfm male: N=6), 20 (wt male: N=7; Tfm male: N=7), 40 (wt male: N=6; Tfm male: N=6), 60 (wt male: N=6; Tfm male: N=6), and 120 (wt male: N=6; Tfm male: N=6) minutes after exposure to an open field with a novel object. For this experiment we chose to focus only on wt and Tfm males in order to further probe the role of ARs in the male corticosterone response to stress. Furthermore, sex differences in the rodent corticosterone response to stress are already well documented (Handa et al., 1994b; reviewed in Kudielka and Kirschbaum, 1995).

Experiment 4: The role of ARs in immediate early gene activation. To investigate how specific brain areas may be activated differently due to the presence or absence of functional ARs in males, we examined activation of the immediate early gene c-Fos in 120-150 day old Tfm and wt male rats at baseline (wt male: N=7; Tfm male: N=7) and after exposure to an open field with a novel object (wt male: N=7; Tfm male: N=7).

Behavior Testing

Animals in Experiment 1 were tested for sensorimotor gating (PPI) and anxiety-related behaviors. Testing took place in the following order: PPI, open field/novel object test, elevated plus maze (EPM), and light dark (LD) box, with a minimum of 72 hours between tests. All tests were administered between 1000-1400 except for PPI, which was conducted during the dark cycle beginning at 1900. To address the possibility that exposure to the most fear provoking test (PPI) can alter behavior in tests that follow, anxiety tests were also conducted on another cohort of intact wt male (n=11), wt female (n=6) and Tfm rats (n=9) in an order that we deemed was least to most anxiety-provoking (open field/novel object test, LD box, EPM) without testing for PPI.

Prepulse Inhibition

Rats were tested for PPI 1 hour after lights off in a room illuminated by dim red light. PPI was measured in acoustic startle response chambers (SR Lab startle response system, San Diego Instruments, San Diego, CA). Animals were placed into the chamber for 18 minutes, the first 5 minutes of which is an acclimation period. For the remaining 13 minutes the fast muscle twitch startle responses of animals were recorded to a 100 decibel

tone alone (acoustic startle response; ASR), or to that same tone preceded by tones of 3, 8, 10, and 15 decibels via SR Lab software (San Diego Instruments). The prepulse should permit the subject to anticipate the loud pulse, and consequently startle less severely. Each of these trials was repeated 6 times at pseudo-random intervals. After the test, animals were removed and the chamber was cleaned with 70% ethanol.

Open Field/Novel object test

Open field/novel object testing took place in a 122cm x 122cm white plastic box illuminated from directly above by a 60 watt light. A grid was drawn in the box to demarcate entries into the center area and activity (grid crossings). Rats were first placed into a corner of the empty open field and behavior was recorded via an overhead video camera for 5 minutes. After 5 minutes rats were removed, the box was cleaned with 70% ethanol, and a novel object (a 4" diameter x 8" high cylindrical metal oxygen tank cap) was placed in the center of the chamber. Three minutes after removal from the open field, the rat was replaced into the chamber which now contained the novel object and behavior was recorded for another 5 minutes. The box was again cleaned with 70% ethanol following the novel object test. The number of entries into the center area, time spent in the center area, visits to the novel object, and time spent visiting the novel object were assessed as indices of anxiety-related behavior. The number of grid crossings and rearings were assessed as measures of activity and all behaviors were coded at a later time by a "blind" observer.

Elevated Plus Maze (EPM)

The EPM consisted of two open and two closed arms (50 x 10 cm) that extended from a center platform and was elevated 50 cm above the floor. Testing took place in a dimly lit room in which animals were placed in the center area of the EPM facing an open arm and allowed to move freely between the arms for the 10 minute duration of the test. Behavior was recorded using an overhead video camera and was coded at a later time by a blind observer. The number of entries into and the amount of time spent on the open arms were assessed as indices of anxiety-related behavior. The maze was cleaned with 70% ethanol between each test.

Light/Dark (LD) Box

The LD box consisted of a rectangular box divided into two regions, one dark (40 cm length x 38 cm width x 29 cm height) and one light (31 cm length x 28 cm width x 29 cm height). The dark region was constructed of black plastic and was covered by a black lid, while the light region was constructed of transparent Plexiglas and was illuminated by a 60 watt light that was 3 feet directly above it. The two chambers were connected by a small opening (7.5 height x 7.5 cm width) that allowed the animals to freely enter either area. Animals were placed in the light side of the chamber facing the opening to the dark chamber and were allowed to move freely between the two compartments for 10 minutes. Behavior was recorded via an overhead video camera for coding at a later time by a blind observer. The number of entries into and time spent in the two compartments, as well as the number of rearings in the light compartment, were assessed as indices of anxiety and activity.

Plasma Collection and Hormone Assays

In Experiment 2 blood was collected from adult rats between 0900-1100 at baseline or 20 min after initial exposure to the open field with a novel object. In Experiment 3 blood was collected from adult rats between 0900-1100 at 20, 40, 60, and 120 minutes after exposure to an open field with a novel object or at baseline. Novel object exposed rats were placed in an open field with a novel object for 10 minutes after which they were returned to their home cage where they remained until sacrifice. Baseline rats remained in their home cage until sacrifice. Rats were deeply anaesthetized with isoflurane and decapitated, with trunk blood collected within 2 minutes of cage disturbance. All blood was collected in 1.5 ml tubes containing 250µl of heparin and held on crushed blue ice until centrifugation. After centrifugation plasma was collected and frozen at –20° C until the assay was performed. Plasma was assayed for corticosterone and T at the Diagnostic Center for Population and Animal Health at Michigan State University using Coat- A-Count Corticosterone and Coat-A-Count Total Testosterone kits according to the manufacturer's instructions (Diagnostics Products Corporation, Los Angeles, CA, USA). All plasma samples were run in duplicate and results were averaged. Intraassay and interassay coefficients of variation were < 5 and < 7%, respectively for corticosterone and < 12 and < 7%, respectively for testosterone.

Tissue Processing and c-Fos Immunocytochemistry

One hour after a 10 minute exposure to an open field with a novel object, or at baseline, rats were administered an intraperitoneal overdose of pentobarbital, and perfused through the heart with 100 ml of 0.9% saline followed by 200 ml of phosphate-buffered 4%

paraformaldehyde. Brains were removed, placed in 4% paraformaldehyde, and refrigerated overnight. The following morning brains were transferred into a 20% sucrose solution where they remained until sectioning. No later than one week after collection, brains were sectioned at 40 μm on a freezing microtome into three alternative series and stored at -20° C in cryoprotectant. For c-Fos labeling, one series of sections was rinsed in several changes of 0.05 M tris buffered saline (TBS) to clear cryoprotectant and then incubated as free-floating sections in 0.1% sodium borohydride in TBS for 15 minutes to clear residual fixative. Sections were then rinsed in TBS, incubated in 1% hydrogen peroxide and 0.3% Triton-X in TBS (TBS-TX) for 10 minutes, again rinsed in TBS, then incubated in 20% normal goat serum (NGS) in TBS-TX for 25 minutes. After rinsing in TBS, tissue was incubated in primary antisera (c-Fos rabbit polyclonal: 1:10,000, Santa Cruz Biotechnology) in 2% normal goat serum and TBS-TX for 48 hours at room temperature. Tissue was then rinsed in TBS and incubated for 1 hour in biotinylated goat-anti rabbit antibody in TBS-TX (1:500, Vector Laboratories, Burlingame, CA) followed by rinses in TBS and a 1 hour incubation in avidin-biotin complex (ABC Elite kit, Vector Laboratories, Burlingame, CA). Following rinses in TBS, tissue was developed for visualization of c-Fos positive cells in a hydrogen peroxide, diaminobenzidine, and nickel solution for 5 minutes, after which sections were rinsed in TBS and immediately mounted on slides. The following day sections were counterstained with neutral red, dehydrated, defatted, and coverslipped with Permount.

Microscope analysis

Analysis of c-Fos positive cells was conducted on a Nikon light microscope equipped with a video camera and Bioquant stereological software (Bioquant, Nashville, TN, USA). Briefly, anatomical position was determined using a stereotaxic atlas (Paxinos and Watson, 1998). Discrete brain regions were outlined using a 20x objective and cells considered c-Fos positive (containing black nuclear label) within this region were counted. C-Fos positive cells were quantified in androgen and/or stress responsive brain areas including the basolateral amygdala (BLA), medial amygdala (MEA), posterodorsal medial amygdala (MePD), ventral lateral septum (VLS), posteromedial nucleus of the bed nucleus of the stria terminalis (BSTMPM), paraventricular nucleus of the hypothalamus (PVN), ventromedial nucleus of the hypothalamus (VMH), medial preoptic area (MPOA), dentate gyrus (DG), and CA1. The observer was blind to the experimental condition of the tissue.

Statistical Analysis

For PPI, a mixed design repeated measures analysis of variance (ANOVA) was used to analyze data with genotype as a between groups factor and prepulse intensity as within (or repeated) groups factor. ASR and anxiety-related behaviors were analyzed using one-way ANOVAs. Acoustic startle response by trial was analyzed using a repeated measures 2-way ANOVA with ASR trial order (1-6) and genotype as factors. To compare differences in anxiety-related behaviors in rats exposed to either the PPI test prior to anxiety tests (test order 1) and not exposed to the PPI test (test order 2), 2-way ANOVAs using genotype and test order as factors were performed. In experiment 2, testosterone and corticosterone concentrations were analyzed using a 2-way ANOVA

with testing condition (novel object exposure, no manipulation) and genotype (wt male, wt female, and Tfm male) as factors. In experiment 3 differences in corticosterone were analyzed using a two-way ANOVA (genotype x time). Separate 2-way ANOVAs were run for each brain region analyzed for C-Fos expression with testing condition and genotype as factors. All significant main effects or interactions were further analyzed using Tukey's post hoc tests. Differences were considered significant when $p<0.05$ and all data are reported as means ± standard error of the mean (SEM) with N= the number of animals.

Results

Experiment 1: The role of ARs in sensorimotor gating and anxiety-related behavior

A mixed design repeated measures ANOVA for PPI revealed the expected effect of prepulse intensity ($F(3,108)= 3.793$, $p<.01$; Figure 1a) in which PPI increased along with an increase in prepulse decibel intensity, with no main effect of genotype or interaction between genotype and prepulse intensity. Analysis of ASR revealed a significant main effect of genotype ($F(2,27)= 4.668$, $p<.01$). Post hoc comparisons revealed that Tfm males showed an increased ASR compared to both wt males and females ($ps<.05$; Figure 1b). An analysis of ASR by trial revealed a significant main effect of trial ($F(5,162)= 4.789$, $p<.01$) indicating that, as a whole, rats showed a decrease in ASR, particularly after the initial ASR trial. There was also a significant main effect of genotype ($F(2,162)= 14.72$, $p<.01$) and a genotype x trial interaction ($F(10,162)= 4.789$, $p<.01$), again because Tfm males showed an elevated ASR. Post hoc comparisons revealed that ASR was elevated in Tfm males compared to wt males and females only in the initial trial

(ps<.001; Figure 1c), and this difference largely accounted for the overall increase in ASR in Tfm males.

In the open field test there was a significant effect of genotype for time spent in the center area of the chamber ($F(2,27)= 9.738$, $p<.01$), and the number of entries into the center area of the chamber ($F(2,27)= 4.111$, $p<.05$). Post hoc comparisons revealed that Tfm males spent less time in the center area of the open field compared to both wt males and females (ps<.05 and .01 respectively; Figure 2a) and visited the center area less than did wt females ($p<.05$; Figure 2a). There was also a significant sex difference in the number of rearings in the open field ($F(2,27)= 4.790$, $p<.05$), as wt females reared more frequently than did wt males (ps<.05; Table 1, test order 1). No significant effect of genotype was found for the total number of grid crossings or time spent grooming (Table 1, test order 1).

In the novel object test there was a significant effect of genotype for time spent visiting the novel object ($F(2,27)= 5.233$, $p<.05$) and the number of novel object visits ($F(2,27)= 4.729$, $p<.05$). Post hoc comparisons revealed that Tfm males spent less time visiting the novel object compared to both wt males ($p<.05$) and females ($p<.01$; Figure 2b) and visited the object less frequently than did wt females ($p<.05$; Figure 2b). There were no significant differences in the number of grid crossings, or time spent grooming (Table 1, test order 1), although there was a marginal effect of genotype on rearings ($F(2,27)= 3.292$, $p=.053$) suggesting that wt females rear more than wt or Tfm males (Table 1, test order 1).

In the EPM there were no group differences in the number of entries into the open arms or in time spent in the open arms, although there was a significant difference

in total arm entries ($F(2,27)= 4.627$, $p<.05$), indicating that Tfm males made fewer total arm entries than did females ($p<.05$; Table 1, test order 1). In the LD box there were no significant differences in the number of entries into the light area of the box, time spent in the light area, or rearings in the light area (Table 1, test order 1). Body weight significantly differed between groups of rats used in these tests ($F(2,27)= 44.54$, $p<.001$) with Tfm males (401.46 ± 14.05g) having body weights intermediate between wt males (539.43 ± 22.12g) and wt females (319.38 ± 13.56g).

When anxiety-related behavior tests (open field/novel object tests, LD box, and EPM) were conducted in rats from least to most anxiety provoking and not preceded by the PPI test (test order 2), no significant differences were found for measures of anxiety in the open field test (time spent in the center area of the open field or number of visits to the center area) or grid crossings. However, Tfm males again tended to show fewer visits and decreased time in the center area of the open field compared to wt males and females (See Table 1, test order 2). There was a significant effect of genotype for the number of rearings ($F(2,21)= 3.458$, $p<.05$) indicating that wt females reared more than did Tfm males ($p<.05$; Table 1, test order 2). A significant main effect was also found for time spent grooming in the open field ($F(2,21)= 3.747$, $p<.05$) with Tfm males grooming more than wt males ($p<.05$; Table 1, test order 2).

As in the novel object test in test order 1, there was a significant effect for time spent visiting the novel object ($F(2,21)= 17.55$, $p<.001$) and the number of novel object visits ($F(2,21)= 12.12$, $p<.001$) in the test order 2 cohort. Post hoc comparisons revealed that Tfm males spent less time visiting the novel object compared to both wt males ($p<.05$) and females ($p<.01$) and visited the object less frequently than did wt females

($p<.01$), exhibiting the same pattern of differences as seen in the earlier cohort of animals in test order 1 (Table 1). Wt males also spent less time visiting the novel object and had a fewer number of visits to the object than did wt females ($ps<.05$). In this test, there was a significant effect for the number of grid crossings ($F(2,21)= 5.833$, $p<.01$) with females showing a greater number of grid crossings than wt males ($p<.05$) and Tfm males ($p<.01$) and a significant difference in the number of rearings $F(2,21)= 8.420$, $p<.01$) with females showing a greater number of rearings compared to wt and Tfm males ($ps<.01$). No differences were found for time spent grooming in the novel object test. There were also no significant differences in indices of anxiety or activity in the EPM or LD box in the test order 2 cohort (Table 1).

Significant differences in anxiety-related behavior and activity were not found in the open field and the LD box between rats in test order 1 and test order 2 (Table 1). However, in the novel object test there was a main effect of test order in the number of rearings ($F(1,49)= 15.91$, $p<.001$), with no other significant differences in anxiety-related or activity behaviors. Specifically, females showed a greater number of rearings in test order 1 than in test order 2 ($p<.05$). In the EPM there was also a main effect of test order in which animals in test order 2 spent more time in the open arms ($F(1,49)= 4.868$, $p<.05$), had more visits to the open arms ($F(1,49)= 12.49$, $p<.001$), and more total arm entries ($F(1,49)= 35.61$, $p<.001$) than did animals in test order 1. Post hoc comparisons revealed that wt females from the test order 2 cohort spent more time in the open arms and visited the open arms more frequently than females from the test order 1 cohort ($ps<.05$). Furthermore, all groups showed an increase in total arm entries in test order 1

compared to test order 2 (ps<.05). See Table 1 for a comparison of behavior in anxiety-related tests in rats tested in the two orders.

Experiment 2: The role of ARs in HPA axis regulation

Two-way ANOVA revealed a significant main effect of exposure ($F(1,54)= 585.8$, $p<.001$) in which rats exposed to an open field with a novel object showed an increased corticosterone response 20 minutes after exposure compared to rats in the basal condition. There was also a significant main effect of genotype ($F(2,54)= 8.484$, $p<.001$) and a significant interaction between exposure and genotype on corticosterone concentrations ($F(2,54)= 8.50$, $p<.001$). Post hoc comparisons revealed that all groups had similar blood corticosterone levels at baseline, but after exposure to an open field with a novel object Tfm males and wt females showed an increased corticosterone response compared to wt males (ps<.001; Fig 3a). Two-way ANOVA revealed a significant main effect of genotype on T levels ($F(2,54)= 11.40$, $p<.001$), with no main effect of exposure or genotype x exposure interaction. Specifically, T levels were greater in Tfm males (16.3 ± 4.0 nmol/L) than in wt males (5.9 ± 1.3 nmol/L; $p<.05$), and both groups had higher T levels than wt females (0.1 ± 0.03; ps<.01).

Experiment 3: The role of ARs in HPA axis recovery

Two-way ANOVA revealed a significant main effect of time on corticosterone levels ($F(4,50)= 95.22$, $p<.001$). Compared to basal corticosterone levels, corticosterone levels were greater in wt and Tfm males at 20 and 40 minutes after the onset of novel object exposure (ps< .01). A significant genotype x time interaction was also revealed

($F(4,50)= 3.874$, $p<.01$). Tfm males showed increased corticosterone levels compared to wt males at 20 and 120 ($ps<.05$) minutes after novel object exposure, but similar corticosterone levels compared to wt males at baseline, 40, and 60 minutes (Figure 3b). A significant main effect of genotype was not found in this test.

Experiment 4: The role of ARs in immediate early gene activation

Replicating our earlier findings (35), body weight was significantly greater in wt males (526.4 ± 24.4g) than Tfm males (379.5 ± 18.6g), though brain weight did not significantly differ (wt male: 1.68 ± 0.03g; Tfm male: 1.62 ± 0.04g) in rats used in this experiment. A significant main effect of exposure on the number of c-Fos immunopositive cells was found in every brain region examined (Table 2). These results indicate that compared to the baseline condition, c-Fos immunoreactivity (ir) was increased in these areas after exposure to an open field with a novel object.

In the DG a main effect of genotype was revealed ($F(1,24)= 14.85$, $p<.001$) and reflected that fact that wt males exposed to a novel object had greater c-Fos-ir compared to novel object exposed Tfm males ($p<.001$; Table 2; Figure 4a). There was also a main effect of genotype in the MPOA ($F(1,24)= 12.35$, $p<.01$). Post hoc comparisons revealed that c-Fos-ir in rats exposed to a novel object but not controls was increased in Tfm compared to wt males ($p<.001$; Table 2; Figure 4b). A significant interaction was also found for c-Fos-ir in the MPOA ($F(1,24)= 10.08$, $p<.01$), indicating that the increase in c-Fos expression from baseline to one hour after novel object exposure was also greater in Tfm males. Only a significant interaction was found in the MePD ($F(1,24)= 5.630$, $p<.05$), due to a greater increase in c-Fos expression from baseline to one hour after novel

object exposure in wt compared to Tfm males (Table 2; Figure 4c). No other significant main effects of genotype or interactions were found for the brain regions examined.

Discussion

Tfm male rats showed increased indices of anxiety in two tests (open field and novel object test), but not two others (LD box and EPM), indicating a task-specific or moderate increase in anxiety-related behavior compared to wt males and females. In the open field and novel object tests, Tfm males showed decreased exploration of the center area of the open field and novel object compared to both wt males and females. The current findings support previous research indicating involvement of the AR in the regulation of anxiety-related behavior in rodents (Edinger and Frye, 2006; Fernandez-Guasti and Martinez-Mota, 2005), including our previous findings in Tfm mice (Zuloaga et al., 2008b).

Tfm males, like wt males, were also consistently less active in tests of anxiety compared to wt females as assessed by the number of rearings and grid crossings in the open field and novel object tests, and the total number of arm entries in the EPM. Differences, however, did not reach significance in several cases (Table 1). These findings are consistent with reports of sex differences in ambulation and rearing in the open field and activity in the EPM, with females more active (Archer, 1975; Masur et al., 1980; Slob et al., 1981; Seliger, 1977; Lucion et al., 1996). Activity measures did not differ between Tfm and wt males suggesting that sex differences in activity in rats in our study and others may be mediated via differential activation of ERs.

Test order significantly influenced anxiety-related and activity measures in the EPM and rearings in the novel object test, with wt female rats most affected. In the EPM,

wt females showed an increase in the amount of time spent on and entries into the open arms when tested in order from least to most anxiety-provoking (test order 2) compared to animals first tested for PPI (test order 1), while all groups showed greater activity in the EPM in test order 2. In the novel object test, wt female rats also showed a greater number of rearings in test order 2 compared to test order 1. These findings are consistent with other studies showing that that prior exposure to a stressful or anxiety producing situation can influence behavior in tests of anxiety (MacNeil et al., 1997; File, 1993; Ballaz et al., 2007). Since estrus status was not controlled for, it is also possible that the phase of the estrus cycle may have differed in females in test order 1 and 2 cohorts when exposed to the different tests of anxiety, and this difference may have contributed to changes in behavior between the two groups. Phase of estrus cycle has previously been shown to influence behavior in tests of anxiety, most notably in the EPM (Zuluaga et al., 2005; Marcondes et al., 2001; Mora et al., 1996; Vinogradova, 1999).

Consistent with other studies comparing sex differences in the corticosterone response to a stressful situation (Handa et al., 1994a; Seale at al., 2004), wt female rats showed an increased corticosterone response compared to wt male rats after exposure to an open field with a novel object. A novel finding in our study is that Tfm males also showed an increased peak corticosterone response (20 min) compared to wt males, though recovery levels at 40 and 60 minutes after novel object exposure did not significantly differ from wt males. Since Tfm males had elevated T levels compared to wt males, and T generally has an inhibitory effect on corticosterone release (Handa et al., 1994b; Seale et al., 2004; Mitsushima et al., 2008), this finding suggests that ARs play a role in decreasing the corticosterone response to stress. There was also a difference in

corticosterone levels between wt and Tfm males at 120 minutes after exposure to a novel object. Since corticosterone recovery levels were normal at 40 and 60 minutes this difference does not appear to reflect a prolonged difference in recovery, rather it may reflect a difference in basal corticosterone levels at this time of day. We recently found that Tfm mice also show a heightened corticosterone response to a novel object compared to wt males (Zuloaga et al., 2008a), supporting the notion that AR may play a role in adrenal steroid regulation across mammals.

Although an increased corticosterone response to stress in females compared to males is not associated with increased indices of anxiety, increased corticosterone responses within a gender have been associated with elevated anxiety. In female rats administration of the ERβ agonist diarylpropionitrile (DPN) reduces anxiety as well as corticosterone levels in the EPM (Lund et al., 2005). Similarly, in male rats pharmacological activation of ERβ decreases the corticosterone response to stress (Lund et al., 2004; Lund et al., 2005; Lund et al., 2006) and decreases anxiety-related behaviors in the EPM (Lund et al., 2005). Furthermore, male rats bred for high anxiety also show increases in corticosterone compared to rats showing normal anxiety behaviors (Landgraf et al., 1999). Therefore, elevated anxiety-related behaviors and corticosterone responses in Tfm males compared to wt males are likely correlated. However, since plasma corticosterone binding globulins (CBGs) were not quantified, it remains possible that differences in CBGs may have contributed to differences corticosterone levels found between wt and Tfm males.

PPI did not differ between groups, indicating that the AR plays a minimal role in this measure of sensorimotor gating in rats. These findings are in line with other studies

showing that administration of DHT does not affect PPI in male and female rats gonadectomized in adulthood (Turvin et al., 2007; van den Buuse and Eikelis, 2001), though the role of developmental AR activation on PPI had not previously been studied. Since AR deficiency is present from ontogeny in Tfm male rats, these findings further suggest that activation of ARs during development have a negligible effect on PPI. In rodents, PPI enhancing effects of T and estrogen are more likely mediated by the activation of ERs (van den Buuse and Eikelis, 2001).

Although PPI did not differ across groups, ASR was enhanced in Tfm males compared to wt males and females in this test suggesting a role for ARs in this behavior. This difference was most notable in the first ASR trial, with only a moderate elevation in the following trials. This result suggests that emotional responses in Tfm males are increased during the first exposure to a loud acoustic stimulus, but are greatly diminished after the initial exposure to the pulse. Elevated ASR in Tfm males is in line with studies showing administration of T (Turvin et al., 2007; Toufexis et al., 2005) and DHT (Turvin et al., 2007) can attenuate ASR. As for PPI, the influence of AR stimulation in development on ASR has not been previously examined, therefore increased ASR in Tfm rats may result from reduced AR activation in adulthood, development, or both. Increased ASR in Tfm male rats also appears to relate to our findings of increased anxiety-related behaviors in Tfm males since elevations in ASR have been suggested to reflect anxiety in mice and rats (Walker and Davis, 1997; Crawley et al., 1997; Belzung et al., 2000; Hode et al., 2000). Therefore, increased ASR in Tfm male rats likely also reflects increased anxiety-related behavior.

As might be expected, there was an increase in c-Fos activation in all brain regions examined in rats exposed to the novel object compared to rats sacrificed at baseline. However, c-Fos-ir differed between wt and Tfm males in some brain areas (DG, MPOA, MePD) but not others (LSV, PVN, BSTMPM, VMH, BLA, MEA, CA1) following exposure to an open field with a novel object. In brain regions in which c-Fos activation differed between wt and Tfm males there was both increased (MPOA) and decreased (DG, MePD) immunoreactivity in Tfm males, suggesting that the AR seems to act specifically, modulating neural reactivity differently in different brain regions.

Decreased activation of the DG was found after novel object exposure in Tfm compared to wt males. This is similar to findings in another study in which decreased c-Fos activation in the DG was observed in rats bred for high versus low anxiety (58). The hippocampus, including the DG, is an androgen responsive brain region, with abundant ARs particularly in CA1 (Xiao and Jordan, 2002) and lower levels in the DG (Brannvall et al., 2005; Tabori et al., 2005), which plays a role in anxiety-related behavior (Edinger and Frye, 2004, Edinger and Frye, 2005, Edinger and Frye, 2006). The hippocampus also regulates HPA axis feedback via inhibitory projections to the PVN, where increased c-Fos-ir has been correlated with increased HPA axis activity and anxiety (Lun et al., 2006; Salome et al., 2004). While this could be a pathway through which androgens inhibit anxiety and HPA axis activity we found no differences in c-Fos activation of the PVN, although it remains possible that there was a greater excitation of neurosecretory neurons in the PVN of Tfm males not revealed in our analysis of c-Fos.

C-Fos-ir was increased in the MPOA of novel object-exposed Tfm males compared to wt males. This increase is consistent with previous findings demonstrating a

correlation between elevated c-Fos activation of the MPOA and increased anxiety-related behavior in an open field and the open arm of the EPM (Salome et al., 2004). The MPOA contains abundant ARs (Handa et al., 1987; Handa et al., 1996; Simerly et al., 1990) and has been identified as an androgen responsive brain region involved in sexual behaviors as well as the regulation of the HPA axis (McCormick et al., 2002; Viau and Meaney, 1996). Therefore, these data may reflect an alteration in HPA axis activity of Tfm males.

A decrease in c-Fos-ir was found in the MePD of Tfm male rats, an area that has also been found to have decreased volume in Tfm rats compared to wt males (Morris et al., 2005). The MePD is particularly involved in receiving olfactory and pheromonal information and is important for some aspects of male sexual behavior (Lehman et al., 1983; Swann et al., 2001). However, the MePD projects to two other hormone and stress responsive regions (BNST, MPOA) and through these projections may regulate anxiety-related behaviors (Coolen and Wood, 1998; Gomez and Neuman, 1992). In areas where we detected no change in c-Fos activation between Tfm and wt males, it remains possible that there were actual differences in the activation of specific cell types within these regions, yet net cell activation was the same. Altered activity in areas outside of the brain may have also contributed to differences in behavioral and physiological responses. Differences between Tfm and wt males, particularly in HPA axis activation, may result from AR mediated changes in pituitary activity. AR knockout male mice show elevations in ACTH and corticosterone that appear to be caused by an impairment of negative feedback resulting from a decrease in pituitary glucocorticoid receptors (Miyamoto et al., 2007).

Aromatase activity is also decreased in several areas of the brain in adult Tfm compared to wt males (Rosseli et al., 1987), so we cannot discount the possibility that differences between these groups result from decreased activation of ERs in Tfm males. However, anxiety-related behaviors are also increased in Tfm male mice compared to wt males where it is not clear whether aromatase levels are significantly altered (Naftolin et al., 1975; Rosenfeld et al., 1977), and other studies have also implicated an involvement of ARs in the regulation of the HPA axis and anxiety-related behaviors (Miyamoto et al., 2007; Edinger and Frye, 2006; Fernandez-Guasti and Martinez-Mota, 2005).

In conclusion, Tfm male rats showed increased anxiety-related behavior compared to wt males and females in tests thought to reflect anxiety (open field, novel object test, ASR), with no differences in PPI. Compared to wt males, Tfm males also showed increased peak corticosterone responses and differences in immediate-early gene activation in select brain regions (DG, MPOA, and MePD) following exposure to an open field with a novel object that are consistent with greater anxiety in Tfm males than wt males. Together these data indicate a role of the AR in the regulation of anxiety, the HPA axis, and activation of discrete brain areas in rats that may underlie anxiogenesis.

Chapter 5: The Organizational Role of Testicular Hormones and the Androgen Receptor in Anxiety-Related Behaviors and Sensorimotor Gating in rats

Abstract

Exposure to testosterone (T) early in life, which can act upon both androgen receptors (ARs) and, via aromatization of T into estrogens, upon estrogen receptors, organizes adult behaviors in rodents. We compared behaviors in wild type (wt) male rats and AR-deficient rats with the testicular feminization mutation (Tfm), that were either gonadectomized (Neo-Gdx) or sham-operated (Neo-Sham) on the day of birth. In adulthood, all rats were either gonadectomized or sham-operated and implanted with T capsules to equilibrate adult circulating T. In each of four tests of anxiety (open field, novel object exposure, light dark box, elevated plus maze), Neo-Gdx rats showed decreased indices of anxiety compared to Neo-Sham rats, with no differences between wt or Tfm males within treatment groups. These results indicate that testicular hormones act perinatally to increase adult indices of anxiety, and functional ARs are not required for this effect. Acoustic startle response (ASR) was also reduced by Neo-Gdx, but only in Tfm males, suggesting that perinatal stimulation of ERs can increase ASR, but this effect is counteracted in normal males by stimulation of ARs. Corticosterone levels were elevated in Neo-Gdx rats at baseline with no differences in hormone levels after exposure to a novel object in an open field. Sensorimotor gating, as measured by prepulse inhibition (PPI) of the ASR, was increased by neonatal castration, in both wt and Tfm rats. These findings indicate a role of T prior to adulthood in the organization of anxiety-

related behaviors and sensorimotor gating in rats, which appears to be primarily AR-independent.

Introduction

In rodents, exposure to hormones around the time of birth can "organize" adult sex differences in both the central nervous system and behavior (Phoenix et al., 1959; Jacobson et al., 1981; Breedlove et al., 1982; Arnold and Breedlove; 1985). Traditionally, sex differences in behavior and brain morphology are believed to result from testicular production of testosterone (T) in males in early development. According to the aromatization hypothesis, T is converted locally in the brain by the enzyme aromatase to estrogens, which then activate estrogen receptors (ERs) to masculinize the rodent brain (MacLusky and Naftolin, 1981). However, emerging evidence suggests that activation of androgen receptors (ARs) also contributes to the masculinization of brain and behavior in rodents (Zuloaga et al., 2008; Durazzo et al., 2007; Dugger et al., 2007; Morris et al., 2005), so the issue of the relative contribution of ER and AR to masculinization of rodent behavior remains open.

Sex differences exist in many different rodent behaviors and the most apparent are related to sexual behavior. Neonatal administration of T to guinea pigs masculinizes sexual behaviors in both males gonadectomized on the day of birth and females (Phoenix et al., 1959) indicating a role of T in the organization of this sex difference. Early hormone exposure also contributes to sex differences in rodent spatial memory performance in the Morris water maze (males perform better than females). Neonatal castration of males or administration of T to newborn females eliminates such sex

differences (Isgor and Sengelaub, 2003), an effect that appears to be mediated through activation of both ERs and ARs (Williams et al., 1990; Jones and Watson, 2005; Isgor and Sengelaub, 1998).

In rats there are also sex differences in tests of anxiety with females typically showing decreased indices of anxiety and greater activity compared to males (Powell et al., 2002; Archer, 1975; Masur et al., 1980; Slob et al., 1981; Seliger, 1977; Lucion et al., 1996; Zimmerberg and Farley, 1993). These differences appear to depend on both organizational and activational effects of hormones as well as both ERs and ARs. Neonatal gonadectomy in male rats eliminates sex differences in anxiety-related behaviors in the elevated plus maze (Lucion et al., 1996). Furthermore, female rats receiving either neonatal treatment with an ER antagonist (tamoxifen) or prepubertal ovariectomy show greater indices of anxiety in the EPM compared to control females (Zimmerberg and Farley, 1993). In adulthood, administration of both estrogens and androgens generally result in decreased anxiety-related behavior in rodents (Frye and Lacey, 2001; Walf and Frye, 2005; Lund et al., 2005; Frye et al., 2008; Bing et al., 1998; Bitran et al., 1993) apparently by acting on both ERs and ARs (Lund et al., 2005; Imwalle et al., 2005; Edinger and Frye, 2006; Fernandez-Guasti and Martinez-Mota, 2005; Zuloaga et al., 2008). Sex differences in anxiety-related behaviors are also associated with sex differences in HPA axis activation in which female rats show a greater release of stress hormones from the pituitary (adrenocorticotropin hormone) and adrenal glands (corticosterone) following exposure to an anxiety-provoking or stressful situation (Handa et al., 1994a; Kudielka and Kirshbaum, 1999). Androgens and

estrogens acting in development and adulthood appear to contribute to these differences (Handa et al., 1994b; Seale et al., 2004; Seale et al., 2005; Bingham and Viau, 2008).

Sex differences in sensorimotor gating have also been reported in humans and some strains of mice and rats (Swerdlow et al., 1993; Lehman et al., 1999; Ralph et al., 2001; Ison and Allen, 2007) as assessed by an experimental model of sensorimotor gating, prepulse inhibition (PPI) of the acoustic startle response (ASR; Geyer et al., 1990). In adult rodents, circulating levels of both androgens and estrogens can influence aspects of PPI (Jovanovic et al., 2004; van den Buuse and Eikelis, 2001; Gogos and Van den Buuse, 2003), and these effects appear to be largely mediated through activation of ERs rather than ARs (van den Buuse and Eikelis, 2001; Zuloaga et al., submitted).

In order to further investigate the role of developmental androgens and ARs in anxiety-related behaviors, sensorimotor gating, and activation of the HPA axis we compared behavioral and hormonal responses in wild type (wt) male rats and AR deficient rats with the testicular feminization mutation (Tfm). Tfm rats represent a genetic model for exploring the role of the AR in brain and behavior (Zuloaga et al., 2008). In rats this mutation results from a single base pair replacement in the AR gene resulting in the expression of a normal sized but dysfunctional AR protein and consequently sensitivity to androgens through ARs is greatly reduced (Yarbrough et al., 1990; Naess et al., 1976). Wt and Tfm male rats were either gonadectomized (Neo-Gdx) or sham-operated (Neo-Sham) on the day of birth, then treated in adulthood to provide equivalent T before assessing anxiety, ASR, PPI and adrenal responses. We confirm reports that neonatal testicular hormones lead to greater indices of anxiety in adulthood, and because we find almost all effects of neonatal castration are equivalent in wt and Tfm

males, conclude that most of these neonatal effects of T increasing adult anxiety are primarily due to stimulation of ERs. However, we also find evidence that neonatal stimulation of AR normally results in *reduced* ASR in adulthood.

Materials and Methods

Animals

Long Evans rats were bred at Michigan State University and group housed in a vivarium with a 12/12 L/D cycle, lights on at 0600, with food and water ad lib. On the day of birth, Tfm and wt male pups were either gonadectomized (Neo-Gdx Tfms; N=11: Neo-Gdx males; N=9) or sham-operated (Neo-Sham Tfms; N=10: Neo-Sham males; N=9: procedure described in detail below). All rats were weaned at 21 days old, at which point ear punches were taken and rats were genotyped using a modified polymerase chain reaction (PCR) described elsewhere (Fernandez et al., 2003). Products of this reaction differentiated between the Tfm and the wt allele for the AR, and male versus female, based on the presence or absence of the Sry gene found only on the Y chromosome. In adulthood, animals were either gonadectomized (for those that were sham-operated at birth) or sham-operated and implanted with T capsules to equilibrate adult circulating T (procedure described in detail below).

Neonatal castration: On the day of birth, pups were removed from their home cage and anesthetized by hypothermia. After pups were deeply anesthetized, incisions were made on either side of the lower abdominal cavity and the gonads were visualized and removed or left intact (Sham-operated). Incisions were closed with surgical glue and pups were

placed under a lamp until they were warm and mobile before returning to their home cage.

Adult castration and hormone replacement: At 120 days, rats were anesthetized with isoflurane and either gonadectomized (for those that were sham-operated at birth) or sham-operated (for those that were gonadectomized at birth). In rats receiving gonadectomy, testes were externalized via bilateral incisions made in the scrotum (wt males) or in the lower abdominal area (Tfm males) and testes were tied off with silk suture and removed. In sham-operated rats bilateral incisions were also made into either the scrotum (wt males) or lower abdominal area (Tfm males). Incisions in the abdominal muscles were closed with silk suture and the overlying skin was closed with wound clips, while scrotal incisions were closed with wound clips. All rats also received two subcutaneous Silastic capsules (1.57 mm inner diameter, 3.18 mm outer diameter: 20 mm effective release length) containing either free T (T) or nothing (blank (B)) via a 2 cm incision dorsally at the nape of the neck. We recently demonstrated that these Silastic capsules deliver normal male physiological levels of T to adult rats (Morris et al., 2008). The analgesic buprenorphine (0.05 mg/kg) was injected sc post operatively.

Behavior Testing

Two weeks after adult gonadectomy or sham-operation, animals were tested for anxiety related behavior, sensorimotor gating, and blood corticosterone levels. Testing took place in an order we judged was least to most anxiety-provoking: open field/novel object test, light dark (LD) box, elevated plus maze (EPM), PPI, and blood corticosterone

collection (all described in detail below), with a minimum of 72 hrs between tests. All tests were administered between 1000-1400 except for PPI, which was conducted during the dark cycle beginning at 1900.

Open Field/Novel object test

Open field/novel object testing took place in a 48"x 48" white plastic box illuminated from directly above by a 60 watt light. A grid was drawn in the box to demarcate entries into the center area and activity (grid crossings). Rats were first placed into a corner of the empty open field and behavior was recorded via an overhead video camera for 5 min. After 5 min, rats were removed, the box was cleaned with 70% ethanol, and a novel object (a 4" diameter x 8" high cylindrical metal oxygen tank cap) was placed in the center of the chamber. Three minutes after removal from the open field, the rat was replaced into the chamber which now contained the novel object and behavior was recorded for another 5 min. The number of entries into the center area, time spent in the center area, visits to the novel object, and time spent visiting the novel object were assessed as indices of anxiety-related behavior. The number of grid crossings and rearings were assessed as measures of activity and all behaviors were coded at a later time by a blind observer. The box was again cleaned with 70% ethanol before testing the next subject.

Light/Dark (LD) Box

The LD box (Phenome Technologies Inc.; Lincolnshire, IL, USA) consisted of a rectangular box that was divided into two regions, one dark (40 cm length x 38 cm width

x 29 cm height) and one light (31 cm length x 28 cm width x 29 cm height). The dark region was constructed of black plastic and was covered by a black lid, while the light region was constructed of transparent Plexiglas and was illuminated by a 60 watt light that was 3 feet directly above it. The two chambers were connected by a small opening (7.5 cm height x 7.5 cm width) that allowed the animals to freely enter either area. Animals were placed in the light side of the chamber facing the opening to the dark chamber and were allowed to move freely between the two compartments for 10 min. Behavior was recorded via an overhead video camera for coding at a later time by a blind observer. The number of entries into and time spent in the two compartments, as well as the number of rearings in the light compartment were assessed as indices of anxiety and activity. The box was cleaned with 70% ethanol between subjects.

Elevated Plus Maze (EPM)

The EPM consisted of two open and two closed arms (50 x 10 cm) that extended from a center platform and was elevated 50 cm above the floor. Testing took place in a dimly lit room in which rats were placed in the center area of the EPM facing an open arm and allowed to move freely between the arms for 10 min. Behavior was recorded using an overhead video camera and was coded at a later time by a blind observer. The number of entries into and the amount of time spent on the open arms were assessed as indices of anxiety-related behavior. The maze was cleaned with 70% ethanol between each test. Total arm entries (open and closed) were assessed as a measure of activity.

Prepulse Inhibition (PPI) and Acoustic Startle Response (ASR)

Rats were tested for PPI 1 hour after lights off in a room illuminated by dim red light. PPI was measured in acoustic startle response chambers (SR Lab startle response system, San Diego Instruments, San Diego, CA). Animals were placed into the chamber for 18 min, the first 5 min of which is an acclimation period. For the remaining 13 min the fast muscle twitch startle responses of animals to a 100 decibel tone alone (acoustic startle response; ASR), or preceded by 100 msec by tones of 3, 8, 10, or 15 decibels were recorded via SR Lab software (San Diego Instruments). The prepulse should permit the subject to anticipate the loud pulse, and consequently startle less severely. Each of these trials was repeated 6 times at pseudo-random intervals. After the test, animals were removed and the chamber was cleaned with 70% ethanol.

Corticosterone Collection and Assay: In the final test, blood was collected from rats between 0900-1100 at baseline or 20 min after initial exposure to an open field with a different novel object (black tape dispenser; 7" length x 3" width x 3" height) located in the center. Novel object-exposed rats were placed in the open field with a novel object for 10 min, after which they were returned to their home cage where they remained until sacrifice. Baseline rats remained in their home cage until sacrifice. Rats were deeply anaesthetized with isoflurane and decapitated, with trunk blood collected within 2 minutes of cage disturbance. All blood was collected in 1.5 ml tubes containing 250µl of heparin and held on crushed blue ice until centrifugation. After centrifugation, plasma was collected and frozen at –20° C until the assay was performed. Plasma was assayed for corticosterone at the Diagnostic Center for Population and Animal Health at Michigan State University using a Coat-A-Count Corticosterone kit (Diagnostics Products

Corporation, Los Angeles, CA, USA). All plasma samples were run in duplicate and results were averaged. Brain weights were also measured in these animals immediately following sacrifice.

Statistical Analysis: For PPI, a mixed design repeated measures analysis of variance (ANOVA) was used to analyze data with genotype and neonatal treatment as a between groups factors and prepulse intensity as within (or repeated) groups factor. Anxiety-related behaviors and ASR were each analyzed separately using 2-way ANOVA. ASR by trial was analyzed using a mixed design repeated measures ANOVA with genotype and neonatal treatment as a between groups factors ASR trial order (1-6) as a within groups factor. Corticosterone levels were analyzed using 3 and 2-way ANOVA. All significant main effects or interactions were further analyzed using Tukey's multiple comparisons tests. Differences were considered significant when $p<0.05$ and all data are reported as means ± standard error of the mean (SEM) with N = number of animals in each group.

Results

In the open field test, a 2x2 ANOVA revealed a significant main effect of neonatal treatment for time spent in the center area of the chamber ($F(1,35)= 6.191$, $p<.05$; Figure 1a) and the number of entries to the center area ($F(1,35)= 4.763$, $p<.05$; Figure 1b) indicating that Neo-Gdx rats spent more time in and visited the center area more than Neo-Sham rats, with no main effects of genotype nor any interactions. There was also a significant main effect of neonatal treatment for the number of grid crossings in the open

field test ($F(1,35)= 12.79$, $p<.01$), indicating a greater number of crossings in Neo-Gdx compared to Neo-Sham males (Figure 1c, left), but no significant effect on number of rearings (Figure 1c, right). Post hoc comparisons revealed that Neo-Sham males had significantly fewer grid crossings than did either Neo-Gdx male or Neo-Gdx Tfm groups ($ps<.05$ and .01 respectively). No significant main effects of genotype or interactions were found for any measures in the open field test.

In the novel object test there was a significant main effect of neonatal treatment for the number of novel object visits ($F(1,35)= 7.872$, $p<.01$; Figure 2b) and the number of grid crossings ($F(1,35)= 4.660$, $p<.05$; Figure 2c, left) with no main effects of genotype or interactions. These results represent an elevation in both measures in Neo-Gdx rats compared to Neo-Sham rats. Post hoc comparisons did not reveal any other significant group differences, indicating only an overall effect of neonatal treatment in these measures. No significant main effects or interaction was found for time spent visiting the novel object (Figure 2a) or the number of rearings (Figure 2c, right).

In the LD box a significant main effect of neonatal treatment was found for time spent in the light area ($F(1,34)= 9.663$, $p<.01$), the number of entries into the light area ($F(1,34)= 9.663$, $p<.01$), and the number of rearings in the light area ($F(1,34)= 9.894$, $p<.01$), with no significant main effect of genotype or interaction (Figure 3a-c). Specifically, Neo-Gdx rats showed an elevation in all measures compared to Neo-Sham rats. There were no significant main effects of genotype or any interactions.

Again a significant main effect of neonatal treatment was found in the EPM for the number of entries into the open arms ($F(1,34)= 9.894$, $p<.01$), indicating that Neo-Gdx rats had more open arm entries than Neo-Sham rats (Figure 4b). Significant effects

of neonatal treatment were not found for time spent on the open arms ($F(1,34)= 3.682$, $p=.063$; Figure 4a) or total number of arm entries ($F(1,34)= 3.707$, $p=.062$; Figure 4c), although there was a trend for neonatally gonadectomized rats to show elevations in both measures. No significant main effects of genotype or interactions were found for any measures in the EPM.

A mixed design ANOVA using genotype and neonatal treatment as between groups factors and prepulse intensity as a within groups factor revealed the expected effect of prepulse intensity ($F(3,140)= 22.86$, $p<.001$) in which PPI increased as intensity of the prepulse increased. There was also a significant main effect of neonatal treatment ($F(1,35)= 9.970$, $p<.01$) in which Neo-Gdx rats showed overall increased PPI compared to Neo-Sham rats (Figure 5a). There was no significant effect of genotype and no interaction.

A significant main effect of neonatal treatment was also found for ASR ($F(1,35)= 9.760$, $p<.01$), indicating that Neo-Gdx rats showed a decreased ASR compared to Neo-Sham rats (Figure 5b), which is consistent with the above findings that Neo-Gdx reduces measures of adult anxiety. Specifically, Neo-Sham Tfms showed an increased ASR compared to Neo-Gdx Tfm and Neo-Gdx male groups (ps<.05 and .01 respectively). No significant main effect of genotype or interaction was found for average ASR. An analysis of ASR by trial revealed the expected significant effect of trial ($F(5,175)= 7.24$, $p<.01$) indicating that, as a whole, rats showed a decrease in ASR particularly after the initial ASR trial. However, there was also a significant main effect of neonatal treatment ($F(1,35)= 9.760$, $p<.01$), again indicating that Neo-Sham rats show greater ASR than Neo-Gdx rats. This difference was largely accounted for by an increased ASR in Neo-

Sham Tfm males compared to all other groups in trials 1 (ps<.05) and 2 (ps<.05), and compared to Neo-Gdx males in trial 5 (p<.05; Figure 5c).

A 3-way ANOVA (exposure x neonatal treatment x genotype) revealed a significant main effect of exposure ($F(1,54)= 585.8$, $p<.001$) in which rats exposed to an open field with a novel object showed an increased corticosterone response compared to rats in the basal condition (Figure 6a and 6b). Separate 2-way ANOVAs (neonatal treatment x genotype) were also performed to compare corticosterone levels at either baseline or at 20 minutes after exposure to an open field with a novel object. At baseline there was a significant main effect of treatment ($F(1,34)= 8.546$, $p<.05$), with no main effect of genotype or interaction, indicating that compared to Neo-Sham rats, Neo-Gdx rats had higher corticosterone levels (Figure 6a). Post hoc comparisons did not reveal any further significant group differences, indicating only an overall effect of neonatal treatment in this measure. Analysis of corticosterone levels following novel object exposure revealed no significant main effects or interaction (Figure 6b).

There was a significant main effect of genotype ($F(1,35)= 30.53$, $p<.001$), neonatal treatment ($F(1,35)= 25.66$, $p<.001$), and an interaction between genotype and neonatal treatment ($F(1,35)= 7.60$, $p<.01$) on body weight. Specifically, body weight was greater in Neo-Sham males compared to all other groups (all ps<.001; Figure 7b). A 2-way ANOVA for brain weight also revealed a significant main effect of neonatal treatment ($F(1,34)= 8.546$, $p<.05$), with no main effect of genotype or interaction. Post hoc comparisons revealed reduced brain weights in Neo-Gdx Tfm and Neo-Gdx male groups compared to Neo-Sham males (ps <.01; Figure 7a).

Discussion

Compared to Neo-Sham rats, Neo-Gdx rats showed decreased anxiety-related behavior in all our tests of anxiety, displaying a greater number of visits to the center area of the open field, to a novel object in the open field, to the light area of the LD box, and to the open arms of the EPM, as well as a decreased ASR. Neo-Gdx rats also spent more time in the center area of the open field and light area of the LD box, and tended to spend more time in the open arms of the EPM. These findings are in accord with a previous report of decreased anxiety-related behavior in the EPM in male rats that were gonadectomized at birth compared to sham-operated rats (Lucion et al., 1996). Unlike that previous study, in our study, T levels were equilibrated in adulthood, strengthening the idea that differences in T during development, rather than differences in circulating levels of T in adulthood, increase adult expression of anxiety. These findings also indicate that sex differences in anxiety behaviors that have been reported in rats (females < males: Lucion et al., 1996; Zimmerberg and Farley, 1993, Imhof et al., 1993) may, at least partially, be related to organizational effects of T. However, female gonadal hormones, acting both in development and adulthood also appear to contribute to these sex differences (Zimmerberg and Farley, 1993; Frye and Walf, 2004).

For all of these effects of neonatal castration on anxiety-related behaviors, wt and Tfm males were equivalent, indicating that the organizational influence of T on anxiety-related behaviors is primarily AR-independent, and rather, may be mediated through activation of ERs. We previously found that gonadally intact Tfm male rats showed increased indices of anxiety in the open field and novel object tests compared to wt male rats (Zuloaga et al., 2008), but in the present study we did not see this difference. It is

possible that exposure to a surgical procedure (gonadectomy) two weeks prior to behavior testing or neonatal anesthesia may have affected anxiety-related behaviors in such a way as to eliminate differences between these groups. Neonatal anesthesia has previously been demonstrated to affect hippocampal morphology and cognitive behavior (Rothstein et al., 2008; Nunez et al., 2000) though its effects on anxiety-related behavior have not been studied. Adult gonadectomy and T replacement also eliminated normal differences between Tfm males and wt males in circulating T (Tfm males > wt males: Roselli et al., 1987; Zuloaga et al., 2008) introducing the possibility that increased T levels in Tfm males, or differences in other gonadal secretions may have contributed to differences we previously found between these groups.

Several activity measures (grid crossings in the open field and novel object tests, transitions between compartments and rearings in the light area of the light dark box, and total arm entries in the EPM) were elevated in Neo-Gdx compared to Neo-Sham rats. These data are also in accord with other reports in which neonatally gonadectomized rats have shown increased ambulation in the open field and more total arm entries in the EPM (Slob et al., 1986; Lucion et al., 1996). Therefore, data from this study and others indicate that the presence of T during development contributes to sex differences in activity in adult rodents (females generally more active than males). Since there were no differences in activity measures between Neo-Sham Tfm and Neo-Sham wt males, our data also indicate that T does not act through the AR to alter this behavior. Presumably T's organizational influence on activity is ER-mediated.

There was only a single exception to the above pattern that AR is irrelevant to neonatal T effects on adult anxiety, and that was in the ASR, which has also been

suggested to reflect anxiety in mice and rats (Walker and Davis, 1997; Crawley et al., 1997; Belzung et al., 2000; Hode et al., 2000). Again, neo-Sham rats showed a greater ASR, but this difference was largely due to effects in Tfm rats. We previously found an increased ASR in gonadally intact Tfm males compared to wt males and females (Zuloaga et al., 2008), and in this study Neo-Sham Tfm males showed an increased startle response compared to Neo-Sham males as well as Neo-Gdx males of either group. While in this study the increased mean ASR in Tfm males compared to wt males among Neo-Sham animals did not reach significance (Figure 5b, left), the trend was clearly consistent with our previous finding, and analysis of ASR by trial indicates Neo-Sham Tfms display greater ASR than their wt counterparts (Figure 5c). Thus depriving Tfm males of testicular secretions during development eliminates the effect of the mutation on ASR in adulthood. This result suggests an interaction of AR and ER stimulation for this behavior. One possible explanation is that neonatal ER stimulation increases startle (as it appears to do for all other measures of anxiety we tested), but neonatal stimulation of ARs can counteract that effect for this particular behavior. This mechanism would explain why Neo-Sham Tfms show a greater startle than Neo-Sham males: they receive ER stimulation that increases startle, but do not receive the AR stimulation that would ameliorate this effect of ER. In this scenario, wt males fail to show a significant reduction in ASR following neonatal castration because the loss of ER stimulation is counterbalanced by the loss of AR stimulation.

Our finding of elevated corticosterone levels in Neo-Gdx rats is in accord with a recent report of increased basal corticosterone in neonatally gonadectomized rats (Bingham and Viau, 1998). Furthermore, prenatal administration of flutamide (an AR

antagonist) or 1,4,6-androstatriene-3,17-dione (ATD: an aromatase inhibitor) also results in increased basal corticosterone levels in rats, indicating that androgens act through both ARs and ERs around the time of birth to organize basal corticosterone levels (Seale et al., 2005). However, supporting our previous finding in gonadally intact Tfm and wt males, there were no significant differences in basal corticosterone levels between Neo-Sham Tfm and wt males in the current study, which suggests that AR deficiency during ontogeny does not affect this measure (Zuloaga et al., 2008).

As opposed to the basal condition, levels of corticosterone following exposure to a novel object did not differ between groups. We have previously found increased corticosterone levels in gonadally intact Tfm compared to wt males after novel object exposure (Zuloaga et al., 2008) and others have demonstrated that neonatal gonadectomy increases the corticosterone responses to stress in adult rats (Bingham and Viau, 2008). However rats in the current study had been subjected to repeated stress prior to corticosterone analysis, while rats in other studies, including our previous study, were not. Exposure to repeated stress alters the corticosterone response to stress (Stamp and Herbert, 1999; Melia et al., 1994; Retana-Marquez, 2003), and as a result, may have reduced differences between groups. Basal levels of corticosterone can also be altered by prior exposure to stress (Ottenweller et al., 1994; Servatius et al., 1994) suggesting that basal corticosterone levels may have been elevated in rats in the current study.

PPI was also increased in Neo-Gdx rats compared to Neo-Sham rats, indicating an organizational effect of T in sensorimotor gating. Gonadal hormone administration in adulthood was previously shown to influence PPI in rats (Eikelis et al., 2001, Gogos and van den Buuse et al., 2003), but to our knowledge this is the first evidence of an

organizational effect of hormones on PPI. Again this effect appears to be primarily dependent on ER rather than AR activation since there were no differences between Neo-Sham Tfm and Neo-Sham wt males.

Brain weight analysis also revealed an effect of neonatal treatment on brain weight with Neo-Sham rats having heavier brains than Neo-Gdx rats. This difference was largely accounted for by heavier brain weights in Neo-Sham wt males compared to both Neo-Gdx groups, though Neo-Sham Tfm males also tended to have heavier brains than did Neo-Gdx rats. Decreased brain weight in Neo-Gdx rats compared to Neo-Sham wt males resembles generalized sex differences in brain weight (males > females; Peiffer et al., 2003; Zuloaga et al., 2008a), and indicate that brain weight is increased by testicular secretions during development in male rats. Confirming previous findings in intact rats (Zuloaga et al., 2008a, Zuloaga et al., 2008b) brain weight did not significantly differ between Tfm and wt males indicating that T increases brain weight via an AR-independent mechanism. Because the Tfm mutation decreases body weight, but has no effect on brain weight, masculinization of these two parameters are at least partially independent in rodents (Zuloaga et al., 2008).

In conclusion, neonatally gonadectomized rats showed reduced anxiety-related behavior compared to sham-operated rats in tests thought to reflect anxiety (open field, novel object test, LD box, EPM, ASR), indicating that testicular secretions during the neonatal period normally result in increased anxiety in adult male rats. Virtually all of these effects of neonatal testicular secretions are equivalent in wt and Tfm males, indicating that AR does not mediate these effects. Only in ASR did we find evidence that neonatal AR stimulation affects adult anxiety, and even this effect suggests a contributing

role of ERs as well. Compared to Neo-Sham rats, Neo-Gdx rats also showed increased PPI and basal corticosterone levels as well as decreased brain weights, and these effects are also independent of AR. Together, these findings indicate a role of T prior to adulthood in the organization of anxiety-related behaviors, the HPA axis, sensorimotor gating, and brain weight, and suggest that ERs mediate these masculinizing effects of neonatal T.

Chapter 6: Discussion

Together our studies in Tfm rodents indicate a role of the AR and/or developmental androgens in the regulation of anxiety-related behavior, the HPA axis, and sensorimotor gating. The contribution of ARs and developmental androgens to these behavioral and physiological responses are discussed in this chapter.

Anxiety-Related Behaviors and Activity

In Chapters 3 and 4 we demonstrated that Tfm males in both mice and rats show increased indices of anxiety compared to wt males in several behavioral tests. These findings indicate that in male rodents ARs normally act to reduce anxiety-related behaviors. Tfm male rats were unlike wt males or females, displaying elevated anxiety compared to both groups, suggesting that AR normally plays this role in females as well. In this study there were not robust sex differences in tests of anxiety that have previously been reported (females less anxious than males; Lucion et al., 1996; Zimmerberg and Farley, 1993). However, females tended to show decreased indices of anxiety compared to males, particularly when tests were performed from least to most anxiety-provoking. Together these results indicate that Tfm male rats were neither masculinized nor feminized in this behavior—rather it suggests that functional ARs are needed for the display of "normal" emotional responses in both sexes.

Differences in anxiety-related behaviors appear to be task specific and to vary between the two rodent species. In both Tfm mice and rats, anxiety-related behaviors are increased upon exposure to a novel object. However, both species showed increased indices of anxiety in one other test: mice in the LD box and rats in the open field. Why

Tfm male mice and rats differ in terms of the specific tests in which they display elevated anxiety is not readily apparent but it suggests that the absence of ARs results in a moderate rather than robust increase in anxiety-related behaviors. If the role of the AR was more robust we would likely see increased anxiety-related behavior in Tfm males in all tests of anxiety.

Alternatively, rodents may have differed in anxiety-related behaviors in only specific tests because neural mechanisms underlying the behaviors may differ between tasks. Compared to other traditional tests of anxiety, exposure to an open field in rats has been demonstrated to involve specifically the basloateral amygdala (BLA) and inputs from other areas (particularly the hippocampus) into this region (Hale et al., 2008). In contrast, a single exposure to the elevated plus maze in rats has been shown to activate areas of the hypothalamus including the paraventricular nucleus and dorsomedial hypothalamus as well as the cingulate cortex, inferior colliculus, and median raphe nucleus (Albrecht-Souza et al., 2008; Andrade and Graeff, 2001). A role of the hippocampus and median raphe nucleus has also been demonstrated in the regulation of behavior in the light/dark box (Andrade and Graeff, 2001; Bannerman et al., 2003). While other studies indicate that brain activation patterns are grossly similar between traditional tests of anxiety (Salome et al., 2004) as are the neurotransmitters involved (Ludwig and Schwarting, 2007; Steiner et al., 1997; Schmitt and Hiemke, 1999), it is always possible that there are subtle variations in the relative contributions of different brain regions to each test, and that AR has differing levels of involvement in the different brain regions. As a result, further studies are needed to clarify differences underlying behaviors in these tests of anxiety.

Our investigation into the role of androgens and the AR prior to adulthood in the organization of anxiety-related behaviors (Chapter 5) did not replicate some of the differences we had previously seen between gonadally intact Tfm and wt males. Specifically, neonatally sham-operated Tfm and wt male rats did not show behavioral differences in the open field and novel object test, although ASR was again increased in Tfm rats. This discrepancy (discussed in chapter 5) may have resulted from effects of neonatal anesthesia or adult gonadectomy in rats used in the study in chapter 5. Since we found increased anxiety-related behavior in Tfm male rats in the open field and novel object tests in two different cohorts of gonadally intact rats these differences in chapter 3 do not appear to be transient.

Ambulation did not differ between wt and Tfm male mice (data not reported) or rats (see chapter 4) indicating that ambulatory behavior is not AR-dependent. However, another measure of activity, rearing, was greater in wt than in Tfm male mice with no differences found between wt and Tfm male rats. Differences between these Tfm models could result from differences in (a) the role of the AR in the regulation of rearing in mice and rats, (b) T during development between the rat and mouse models, or (c) the nature of the mutation between the two models that results in residual AR functionality in rats compared to virtually none in mice (Yarbrough et al., 1990; Charest et al., 1991). Whatever the nature of the species difference, this outcome suggests that in mice the absence of functional ARs decreases the display of rearing. In male rats, activity measures are more likely mediated through activation of the ER during development based on our evidence from neonatally gonadectomized and sham-operated wt and Tfm males in Chapter 5. In this study, neonatal gonadectomy significantly increased

ambulation and moderately increased rearings in tests of anxiety while Tfm and wt male groups did not differ in these measures, indicating an organizational effect of androgens that is AR-independent.

In humans there is abundant evidence that androgens are involved in anxiety and depression. A decrease in T with aging and low levels of T in younger men is often associated with elevated anxious and depressive symptomology (Kaminetsky, 2005; Lund et al., 1999; Amore, 2005; Eskelinen et al., 2007; Cooper and Ritchie, 2000). There is also emerging evidence that the AR is related to the etiology of mood disorders, particularly from comparisons of AR polymorphisms in which the number of CAG repeats in the AR gene is negatively correlated with levels of AR mediated transcription (Mhatre et al., 1993; Kazemi-Esfarjani et al., 1995). A greater number of CAG repeats in the AR gene is associated with symptoms of anxiety and depression in adolescents (Su et al., 2007) as well as aging men (Harkonen et al., 2003). Another study suggests that a 5-alpha reductase inhibitor (finasteride) is also linked to decreases in mood. Individuals taking finasteride to treat male pattern hair loss reported increased symptoms of depression and anxiety after 2 months of treatment with the drug (Rahimi-Ardabili, 2007). Since 5-alpha reductase inhibition causes a decrease in DHT, which subsequently leads to decreased AR activation, this result again suggests a role of the AR in mood disorders. Recent evidence also indicates that the AR may mediate mood via regulation of the HPA axis. The brains of depressed individuals, compared to non-depressed controls, contained lower levels of AR mRNA in the paraventricular nucleus of the hypothalamus (PVN), a key brain region involved in the regulation of the HPA axis (Lund et al., 2006). Since AR activation in the PVN is believed to inhibit HPA axis

activation, a decrease in AR mRNA levels suggests a mechanism through which the HPA axis could become hyper-activated and consequently exacerbate symptoms of depression. This mechanism may contribute to the greater adrenal steroid response of Tfm male rodents in our studies.

Together, our evidence and that of others suggests a potential therapeutic role of AR stimulation in the treatment of anxiety and depression. AR agonists have been shown to be beneficial in disorders involving muscle wasting, osteoporosis, and sexual behavior. However side effects, including enlargement of the prostate in males and virilization in females, can out-weight the benefits (Gao and Dalton, 2007). The development of selective androgen receptor modulators (SARMs) may offer a solution to this problem (Gao and Dalton, 2007). SARMs can act on specific tissues in the body, thus avoiding the ill effects of AR stimulation in non-target tissues. As a result, it appears feasible to generate a SARM that could activate AR in the brain and muscle (producing positive effects) but not in the prostate (reducing negative effects). To date, only a few SARMs have reached clinical trials.

In Chapter 5 we also present evidence that the removal of androgens during development can lead to a decrease in anxiety-related behavior; essentially resulting in male rats that act like females (less anxious than control males). However, in humans there is an opposite gender difference in anxiety (females > males; DSM-IV, 1994) and decreased T during development is more likely to contribute to *increased* anxiety (Granger et al., 2003; Evardone and Alexander, 2008). For example, Evardone and Alexander (2008) recently demonstrated that larger 2^{nd} to 4^{th} finger length ratios (indicative of lesser prenatal androgen exposure) were correlated with greater levels of

anxiety in men. Given the conflicting findings between rats and humans in anxiety, it isn't realistic to extend our findings in neonatally gonadectomized rats to humans. It has also been suggested that sex differences reported in rat models of anxiety may not actually reflect differences in anxiety *per se*. A factor analysis of behavior in male and female rats in the EPM revealed that anxiety primarily contributed to behavior in males while activity was the primary contributor to female behavior (Fernandes et al., 1999). This interpretation indicates that there are sex differences in motivating factors that underlie behavior in tests of anxiety and raises the question of whether male rats are *truly* more anxious than female rats.

Hypothalamic-Pituitary-Adrenal Axis

Compared to wt males, Tfm mice and rats showed an increased peak corticosterone response following exposure to an open field with a novel object, indicating an involvement of the AR in HPA axis regulation. Tfm male mice demonstrate a particularly hyperactive corticosterone response with elevations at baseline, peak, and during recovery compared to wt males, whereas only peak corticosterone levels are elevated in Tfm male rats. This species difference likely reflects differences in T levels between the two Tfm models in which Tfm mice show decreased T, while Tfm rats show increased T compared to wt male siblings. Therefore, although T's actions through the AR are greatly reduced in Tfm male rats, there is ample T to be converted to estrogens and activate ERβ to decrease the corticosterone response to stress. Since T is reduced in Tfm male mice, activation of both AR and ERβ is probably also reduced, resulting in persistently increased corticosterone levels. It remains possible that residual AR function

in Tfm male rats (Yarbrough et al., 1990), compared to virtually none in Tfm male mice (He et al., 1991; Monks et al., 2007), may also have accounted for their lesser elevation in corticosterone levels. However, recent demonstrations of the powerful inhibitory role of ERβ on HPA axis activity (Lund et al., 2005; Lund et al., 2006) suggests that differences in ERβ activation may represent the greatest contribution to this species difference.

In both Tfm models, increased HPA axis activation is reflected by increased anxiety-related behavior compared to wt males. Similar elevations in corticosterone levels have been demonstrated in male mice and rats that display trait anxiety behaviors (Jankoveski et al., 2008; Landgraph et al., 1999). This finding indicates that the absence of functional ARs in Tfm males predisposes them to HPA axis hyperactivation, which contributes to increased anxiety-related behavior in these rodents. In humans, hyperactivity of the HPA axis is correlated with disorders of anxiety and depression (Swaab et al., 2005). Common symptoms of Cushing's syndrome, a disorder characterized by high levels of cortisol in the blood, are anxiety and depression. Furthermore, the combined dexamethasone/CRH test of HPA axis activity can identify, with near 80% sensitivity, individuals that are depressed (Heuser et al., 1994). In non-depressed individuals, pretreatment with dexamethasone (a corticosteroid) suppresses pituitary–adrenal responses to corticotropin releasing hormone (CRH); however, in depressed individuals the same procedure *enhances* hormonal responses to CRH indicating a hyperactivation of the HPA axis. As mentioned above, depressed individuals also show decreased AR mRNA in the PVN, suggesting a mechanism through which

ARs can alter activation of the HPA axis, and in turn contribute to the occurrence of mood disorders.

However, unlike depressed individuals, people with disorders of anxiety do not show increases in HPA axis hyperactivity, indicating that in humans the link between anxiety and hyper-activation of the HPA axis is less clear. Furthermore, in rodents, subjects that show increased indices of anxiety also commonly show increased depressive-like behavior, therefore it is difficult to decipher whether increased release of stress hormones in these rodents is specifically associated with anxiety-related behaviors. In our experiments, we did not test Tfm males in rodent models of depression, such as the forced swim test, so along with being more anxious it remains possible that these rodents may also show increased indices of depression, and it may be that increased depressive-like behavior (not anxiety-related behavior) is particularly related to HPA axis hyper-activation. As a result, further research is needed to explore the specific link between HPA axis hyper-activation and anxiety.

Our investigation into the role of androgen and ARs in the development of adult HPA axis activation yielded inconclusive results given that these rats had been exposed to a form of chronic stress prior to testing for corticosterone. As discussed in chapter 4, prior exposure to stress can alter both basal (Ottenweller et al., 1994; Servatius et al., 1994) and stress-induced increases in stress hormone levels (Stamp and Herbert, 1999; Melia et al., 1994). Therefore analysis of corticosterone levels in new cohorts of rats, that were not previously exposed to stress, would help to elucidate the role of androgens and the AR in the development of adult HPA axis function.

It remains possible that differences in anxiety-related behaviors and the HPA axis in all our experiments may, at least partially, be attributed to epigenetic influences. Maternal licking and grooming behaviors have been shown to alter glucocorticoid receptor expression in the hippocampus of offspring, a modification that can influence the HPA axis as well as stress responses (Weaver et al., 2004; Weaver, 2007). In neonatally gonadectomized and sham-operated rats, exposure to either cryo-anesthesia or neonatal surgery may have resulted in differences in maternal care. Furthermore, the removal of androgens through neonatal gonadectomy may also decrease maternal licking and grooming since the presence of androgens in neonates elicits greater maternal care (Moore, 1982). Likewise, maternal care may have also differed for neonatal Tfm males compared to wt males because sex hormone secretions likely differ between these groups during this time period. Some or all of these epigenetic influences may have contributed to differences in anxiety-related behaviors and corticosterone responses presented in this dissertation.

Immediate Early Gene (c-Fos) Activation

In chapter 4 we reported differences in c-Fos immunoreactivity between wt and Tfm male rats in 3 brain areas (MPOA, DG, and MePD) after exposure to an open field with a novel object. However, we do not know whether differences in activation of these brain areas are specifically related to either differences in anxiety-related behaviors, corticosterone responses, both, or neither. It is quite possible that elevated anxiety and HPA axis activation in Tfm males involve independent mechanisms and distinct pathways. Although the dentate gyrus and the MPOA have been indicated in playing a

role in regulating both responses, it may be that alterations in activity within these regions reflect only differences in anxiety-related behaviors. Differences in corticosterone responses may not be reflect differences in brain activation between Tfm and wt males, rather alterations in pituitary activity may primarily contribute to differing corticosterone responses. Alternatively, group differences in HPA axis activity may reflect alterations in brain activity reported in Chapter 4 and differences in anxiety-related behaviors may be entirely unrelated. In either case, we do not know whether behavioral or physiological differences contribute to, or are a consequence of, alterations in neural activity.

In any case we did find significant differences in immediate early gene activation in 3 brain regions between wt and Tfm males. As previously mentioned, differential activation of the DG and MPOA may reflect differences in either anxiety-related behavior or HPA axis activity. The MPOA has been identified as an area within which androgens suppress activation of the HPA axis (Viau and Meaney, 1996). Therefore increased activation within this area in Tfm males may reflect an absence of androgen suppression, due to a lack of functional ARs, which results in elevated excitatory input to other regions involved in the HPA axis, such as the PVN. Alternatively, increased activation of the MPOA could reflect elevated anxiety-related behavior. C-Fos immunoreactivity is increased in the MPOA of rats bred for high anxiety (HA) compared to low anxiety rats (LA) after exposure to an open field (Salome et al., 2004). However, HA rats also show HPA axis hyper-activation, so whether elevated c-Fos in the MPOA is related specifically to anxiety-related related behavior, HPA axis activity, or both mechanisms, remains unclear.

C-Fos-ir within the DG was decreased in Tfm males compared to wt males after exposure to an open field with a novel object. The dentate gyrus plays a role in the regulation of anxiety-related behaviors in rodents (Chen at al., 2007) and androgens have been shown to act in the hippocampus to decrease anxiety, though it is unclear whether androgens act on cells within the dentate gyrus or other regions of the hippocampus to regulate this behavior. The hippocampus, including the dentate gyrus, contains abundant adrenal hormone receptors and is involved in the regulation of the HPA axis. Therefore differences in activation of the dentate gyrus could represent differences in anxiety-related behavior, the HPA axis, or both. It is also possible that a decrease in c-Fos-ir in the dentate gyrus could be a consequence of increased cell death within this region resulting from chronically elevated corticosterone levels, although I did not quantify cell number within this region.

Differences I found in c-Fos activation within the MePD (Tfm males < wt males) are the most difficult to relate to differences in anxiety-related behavior and the HPA axis because although the MePD has abundant AR in wt males, this region is not generally believed to directly influence anxiety behaviors. The MePD is particularly involved in receiving olfactory and pheromonal information and is important for some aspects of male sexual behavior (Lehman et al., 1983; Swann et al., 2001). Nevertheless, the MePD projects to two other hormone and stress responsive regions (BNST, MPOA) and through these projections may regulate anxiety-related behaviors and the HPA axis (Coolen and Wood; 1998; Gomez and Newman, 1992). It may be that androgens act through ARs in the MePD to inhibit activation of cells in these other brain areas, resulting in decreases in anxiety and/or HPA activation. Alternatively, decreased c-Fos-ir in Tfm males may

reflect a more traditional role of the MePD; such as a disruption in the processing of olfactory and pheromonal cues.

Even in areas where no change in c-Fos activation was detected between Tfm and wt males, it remains possible that there were actual differences in the activation of specific cell types within these regions, yet net cell activation was the same. Such are the limitations of c-Fos. Dual immunocytochemistry of c-Fos combined with glutamate decarboxylase (GAD' a marker for GABAergic cells), corticotropic releasing factor (CRF), or arginine vasopressin (AVP), would be useful to better characterize the cell types that are activated following exposure to a novel object.

Sensorimotor Gating

The role of the AR in sensorimotor gating appears minimal based on our experiments in Tfm rodents. In mice our initial test for PPI indicated a moderate, but significant decrease in PPI in Tfm male mice compared to wt males. However, in our follow-up studies in intact and T-replaced mice we did not find any differences between Tfm and wt males, suggesting that differences we had seen initially were transient and did not represent group norms in this behavior. In comparisons of PPI between wt and Tfm male rats (chapters 4 and 5), we did not find any differences, again indicating little or no role for the AR in the regulation of sensorimotor gating. Furthermore, PPI did not differ between gonadectomized wt male mice treated with T or nothing. This result suggests that in mice, adult circulating T levels also appear to have little or no influence on sensorimotor gating.

Interestingly, in chapter 5, we demonstrated that neonatal gonadectomy significantly increased PPI, but that this is unrelated to AR since the results were identical in wt and Tfm male rats. These results suggest that the T during postnatal development in males normally increases sensorimotor gating by acting upon ERs. As we are aware of no other studies that have explored the organizational role of androgens in sensorimotor gating, this appears to be the first demonstration of this kind. In humans, sex differences have been reported in PPI, but this difference appears to be largely the result of differences in circulating estrogen, not T levels, that result in fluctuations in PPI in women (Swerdlow et al., 1997). Since deficits in sensorimotor gating are associated with schizophrenia, this could mean that exposure to androgens during development in males predisposes them to schizophrenia. There is not a sex difference in overall incidence of schizophrenia, but the development and severity of this disease do show a marked gender difference. In men the onset of schizophrenia typically occurs during development (early adulthood and adolescence; Hambrect et al., 1992), a period during which T levels are elevated. Furthermore, in adolescent male rats, but not female rats, there is an overproduction of dopamine receptors, which are subsequently eliminated in early adulthood (Andersen et al., 1997), and T replacement in gonadectomized male and female rats increases D1 receptor binding (Andersen et al., 2002). Overproduction of dopamine has been linked to schizophrenia (Lieberman et al., 1987) therefore this could be a mechanism through which developmental T could predispose an individual to this disorder.

Future Directions

Our findings in Tfm rodents indicate an increase in anxiety-related behavior and HPA axis activation that appears to be AR dependent. However, further experiments would be useful in verifying the role of ARs in these responses. AR deficiency in Tfm males might also alter the expression of estrogen receptor subtypes ERα and ERβ and through this mechanism may influence the display of anxiety-related behaviors and the HPA axis. An immunocytochemical comparison of ERα and ERβ in the brain of Tfm and wt male rodents would help to answer this question. In order to differentiate the respective contributions of AR and ERβ, one could generate double-knockout AR/ERβko mice and compare their anxiety-related behaviors and corticosterone responses with ARko, ERβko, and wild type mice. This experiment would be helpful in clarifying the individual roles of these steroid hormone receptors in the regulation of these responses and could also be used to compare relative contributions of these hormone receptors in both males and females.

Our findings in chapter 4 also suggest that ARs may also play a role in decreasing anxiety-related behaviors in female rats. Administration of DHT to female rats decreases anxiety-related behaviors (Frye and Lacey, 2001), further indicating a role of the AR in regulating this behavior in female rats. However, as mentioned before, DHT can act on other receptor types in the brain to decrease anxiety-related behaviors (Lund et al., 2004), so the role of ARs in the regulation of anxiety-related behaviors in female rats remains somewhat inconclusive. In order to further explore the ARs role in females one could compare groups of female rats that were (1) administered DHT, (2) administered both DHT and flutamide, or (3) treated with nothing. If ARs are important in regulating anxiety-related behaviors we would expect DHT treated females to show decreased

anxiety compared to both DHT/flutamide treated and untreated female rats. It is also possible that DHT/ flutamide treated rats would show decreased anxiety-related behaviors compared to untreated females if metabolites of DHT, acting through other receptor types (GABA/ERβ), play a role in regulating this behavior.

Another issue that remains largely unanswered is the role of the AR at different developmental time-points in the regulation of anxiety-related behaviors and the HPA axis, particularly in mice. One way to investigate the developmental role of ARs is through the generation of conditional AR knockout mice in which we could eliminate AR function at several different points in the lifespan. This approach would help decipher whether it is the lack of prenatal, early postnatal, pubertal, or adult AR stimulation that contributes to the elevated anxiety-related behaviors and corticosterone responses we observed in Tfm male mice. It is also possible that activation of ARs at several developmental time-points contribute to differences we report in Tfm males.

Conclusion

The findings presented in this dissertation add to emerging evidence that ARs contribute to the masculinization of the rodent brain and behavior (see Chapter 2). Specifically, these data support a role of the AR in the regulation of anxiety-related behaviors and the HPA axis. Furthermore, supporting the organizational hypothesis (Phoenix et al. 1959), we present evidence of an organizational role of androgens in behavior. Extending previous findings, we demonstrated that anxiety-related behaviors were affected by the absence of androgens in development, and present a novel role of organizational androgens, probably acting though ERs, in the development of sensorimotor gating.

Together, research presented in this dissertation represents a contribution to the field of behavioral neuroendocrinology and may potentially contribute to the development of novel therapies for the treatment of psychiatric disorders in humans.

APPENDIX

Table 1. Key morphological findings in the Tfm rodent brain. MePD: posterodorsal medial amygdala; SCN: suprachiasmatic nucleus; VMHdm: dorsomedial aspect of the ventromedial hypothalamus; VMHvl: ventrolateral aspect of the ventromedial hypothalamus; BSTMPM: posteromedial nucleus of the bed nucleus of the stria terminalis; SDN-POA: sexually dimorphic nucleus of the preoptic area; LC: locus coeruleus; DG: dentate gyrus; SNB: spinal nucleus of the bulbocavernosus; LS: lateral septum; AVP: arginine vasopressin.

Region	Species	Morphological Characteristics	Tfm ♂'s are	Refs
MePD	Rat	Volume: wt ♂ > Tfm ♂ > wt ♀. Soma Size: wt ♂ > Tfm ♂ > wt ♀.	Int Int	Morris et al, 2005
SCN		Volume: wt ♂ > Tfm ♂ ~ wt ♀. Soma Size: wt ♂ > Tfm ♂ ~ wt ♀, but wt ♂ ~ wt ♀.	Fem Fem?	Morris et al, 2005
VMHdm		Volume: No significant group differences. Soma Size: wt ♂ > Tfm ♂ ~ wt ♀.	--- Fem	Dugger et al, 2007
VMHvl		Volume: wt ♂ > Tfm ♂ ~ wt ♀. Soma Size: wt ♂ > wt ♀, but Tfm ♂ not significantly different from either sex.	Fem Int?	Dugger et al, 2007
BSTMPM (Left Hemisphere)		Volume: wt ♂ > Tfm ♂ ~ wt ♀. Soma Size: No significant group differences.	Fem ---	Durazzo et al, 2007
SDN-POA		Volume: wt ♂ ~ Tfm ♂ > wt ♀. Soma Size: wt ♂ > Tfm ♂ ~ wt ♀, but wt ♂ ~ wt ♀.	Masc Fem?	Morris et al, 2005
LC		Volume: wt ♂ < Tfm ♂ ~ wt ♀. Neuron Number: wt ♂ < Tfm ♂ ~ wt ♀.	Fem Fem	Garcia-Falgueras et al, 2005
DG		Volume: wt ♂ ~ Tfm ♂ > wt ♀, but wt ♂ not significantly different from either group.	Masc?	Jones and Watson, 2005
SNB		Neuron Number: wt ♂ > Tfm ♂ ~ wt ♀. Soma Size: wt ♂ > Tfm ♂	Fem Fem	Breedlove and Arnold, 1981
LS	Mouse	AVP Density: wt ♂ ~ Tfm ♂ > wt ♀.	Masc	Scordalakes and Rissman, 2004

Table 2. Key Behavioral Findings in Tfm Rodents

Spatial Memory			
Morris Water Maze	Rat	Tfm ♂'s show an intermediate spatial memory performance between wt ♂'s and ♀'s, with wt ♂'s displaying superior performance.	Jones and Watson, 2002
	Mouse	Tfm ♂'s show decreased spatial memory performance compared to Tfm carrier ♀'s, though there was no sex difference in wt mice.	Rizk et al., 2005
Anxiety-related behavior			
Novel Object Exposure	Mouse	Tfm ♂'s show greater anxiety-related behavior than wt ♂'s.	Present Study
Elevated Plus Maze	Mouse	Tfm mice (Tfm ♂'s and carrier ♀'s) show increased indices of anxiety compared to wt mice (♂'s and ♀'s).	Rizk et al., 2005
Play Fighting			
Juvenile play fighting	Rat	Tfm ♂'s show decreased play fighting compared to wt ♂'s.	Meaney et al, 1983
	Rat	Tfm ♂'s fail to show wt ♂ typical decline in playful attack and one form of playful defense (complete rotations).	Field et al, 2006
Aggression			
Resident-intruder test	Mouse	Similar indices of aggression in Tfm and wt ♂'s; both more aggressive than ♀'s.	Scordalakes and Rissman, 2004
Social/Sexual Behavior			
Feminine mating behavior	Rat	Tfm ♂'s show wt ♂ typical defeminization of sexual behavior.	Olsen, 1979
Masculine mating behavior	Rat	Tfm ♂'s show reduced masculine sexual behavior compared to wt ♂'s.	Beach and Buehler, 1977; Olsen and Whalen, 1981
	Mouse	Tfm ♂'s show reduced masculine sexual behavior compared to wt ♂'s, a difference that is eliminated in E2 treated mice.	Ono et al, 1974; Bodo and Rissman, 2007
Partner Preference	Rat	Tfm ♂'s show a masculinized partner preference.	Hamson et al, 2005
	Mouse	Tfm ♂'s show a demasculinized partner preference.	Bodo and Rissman, 2007
Olfactory preference test	Mouse	Tfm ♂'s show a demasculinized soiled bedding preference.	Bodo and Rissman, 2007

Table 3. Body weight and testosterone levels in wt and Tfm male mice. In T and B treated mice, body weight was greater in T-treated wt males than all other groups (p's<.05), and T levels were elevated in T compared to B-treated mice (p<.001). In intact untreated mice, wt males had elevated body weights and T levels compared to Tfm males (p<.01). T: testosterone, B: blank.

Mice	Weight	T (nmol/L)
T or B Treated		
WT Male (T)	29.38 ± .60	14.22 ± 2.95
WT Male (B)	25.93 ± .96	2.63 ± 1.52
TFM Male (T)	26.49 ± .56	16.81 ± 2.17
TFM Male (B)	25.78 ± 1.38	4.43 ± 2.28
Intact		
WT Male	28.38 ± .70	19.75 ± 4.39
TFM Male	24.92 ± .74	3.26 ± .52

Table 4. Behavior in tests of anxiety in wt male, Tfm male, and wt female rats in test order 1 (Open Field/Novel Object Test, EPM, and LD box, preceded by the PPI test) and test order 2 (Open Field/Novel Object Test, LD box, and EPM, *not* preceded by the PPI test). * indicates $p<.05$ compared to wt males, ^ indicates $p<.05$ compared to wt females, ∞ indicates $p<.01$ compared to wt females, # indicates $p<.05$ compared to the same genotype in test order 1.

Open Field	Center Time (s)	Center Entries	Rearings	Grid Crossings	Grooming (s)
Test order 1					
Wt Males	16.1 ± 2.2	6.0 ± 1.3	23.6 ± 3.6^	113.1 ± 14.7	6.3 ± 2.5
Tfm Males	8.5 ± 2.1* ∞	3.7 ± 0.9^	26.1 ± 4.3	96.6 ± 16.3	6.5 ± 2.7
Wt Females	21.4 ± 2.0	7.6 ± 0.7	37.2 ± 2.0	134.7 ± 7.2	2.8 ± 0.8
Test order 2					
Wt Males	18.5 ± 4.3	5.2 ± 1.0	32.4 ± 3.4	108.0 ± 7.5	2.4 ± 0.1
Tfm Males	10.9 ± 3.2	3.3 ± 0.9	28.4 ± 2.6^	99.3 ± 9.6	5.7 ± 1.6*
Wt Females	24.2 ± 4.9	5.8 ± 0.9	41 ± 2.0	126.4 ± 2.1	3.3 ± 0.7
Novel Object Test	**Object Time (s)**	**Object Visits**	**Rearings**	**Grid Crossings**	**Grooming (s)**
Test order 1					
Wt Males	38.1 ± 5.5	5.9 ± 0.8	12.2 ± 2.5	68.1 ± 9.7	9.8 ± 2.7
Tfm Males	15.2 ± 4.4*^	3.3 ± 0.9^	15.6 ± 3.2	63.2 ± 12.0	27.1 ± 6.7
Wt Females	42.3 ± 8.0	7.6 ± 1.3	24.4 ± 4.2	86.2 ± 11.5	14.1 ± 6.3
Test order 2					
Wt Males	43.8 ± 9.5^	4.7 ± 0.9^	23.4 ± 2.7∞	79.8 ± 12.0^	8.3 ± 2.0
Tfm Males	11.7 ± 4.5* ∞	1.9 ± 0.7 ∞	20.5 ± 4.1∞	66.5 ± 11.0∞	18.7 ± 6.4
Wt Females	80.8 ± 12.0	9.0 ± 1.1	41.6 ± 4.3#	125.8 ± 7.8	3.8 ± 1.2

Elevated Plus Maze	Open arm Time (s)	Open Arm Entries	Total Arm Entries
Test order 1			
Wt Males	41.1 ± 5.8	2.8 ± 0.4	13.6 ± 1.6
Tfm Males	23.2 ± 6.4	1.6 ± 0.4	11.1 ± 1.3^
Wt Females	45.4 ± 11.0	3.1 ± 0.6	17.2 ± 1.5
Test order 2			
Wt Males	41.6 ± 10.0	3.8 ± 0.9	20.7 ± 1.8#
Tfm Males	46.2 ± 15.2	3.5 ± 0.9	20.5 ± 2.7#
Wt Females	79.3 ± 13.6#	6.8 ± 1.5#	27.2 ± 1.7#

Light Dark Box	Light Area Time (s)	Light Area Entries	Light Area Rearings
Test order 1			
Wt Males	206.7 ± 46.8	6.9 ± 1.0	21.5 ± 4.9
Tfm Males	84.5 ± 28.4	6.3 ± 0.8	7.9 ± 2.9
Wt Females	134.0 ± 39.9	5.4 ± 0.7	13.3 ± 5.9
Test order 2			
Wt Males	114.5 ± 31.2	5.4 ± 1.3	10.8 ± 3.1
Tfm Males	86.8 ± 46.0	4.6 ± 1.7	6.5 ± 3.2
Wt Females	109.3 ± 30.4	6.6 ± 1.5	15.4 ± 4.9

Table 5. The number of c-Fos positive cells per mm^2 in wt and Tfm male rats at baseline or after exposure to an open field with a novel object. ≈, similar c-Fos immunoreactivity in Tfm compared to wt males; +, increased c-Fos immunoreactivity in Tfm compared to wt males; -, decreased c-Fos immunoreactivity in Tfm compared to wt males. * indicates $p<.01$ compared to same group baseline, # indicates $p<.05$ compared to same group baseline,^ indicates $p<.01$ compared to novel object exposed wt males, ∞ indicates $p<.05$ compared to novel object exposed wt males.

		Baseline		Open Field w/Novel Object		
Brain Region	Bregma	wt male	Tfm male	wt male	Tfm male	Responses in Tfm vs wt males
Forebrain						
LSV	0.7 mm	74.88 ± 17.28	58.45 ± 7.72	461.50 ± 32.23*	429.45 ± 35.78*	≈
BSTMPM	-0.8 mm	24.78 ± 4.46	25.03 ± 2.69	148.99 ± 15.01*	127.07 ± 10.17*	≈
Hypothalamus						
PVN	-1.8 mm	94.45 ± 15.78	102.51 ± 11.94	396.27 ± 45.89*	400.32 ± 59.40*	≈
MPOA	-0.9 mm	61 ± 9.82	68.73 ± 10.32	176.75 ± 11.98*	312.44 ± 36.04*^	+
VMH	-3.14 mm	12.23 ± 2.60	14.78 ± 2.214	93.52 ± 18.09*	83.12 ± 7.45*	≈
Amygdala						
BLA	-3.14 mm	26.54 ± 4.61	34.58 ± 5.62	72.11 ± 9.20*	60.83 ± 17.28#	≈
MEA	-2.56 mm	13.99 ± 2.69	17.356 ± 4.46	154.26 ± 8.41*	151.22 ± 24.80*	≈
MePD	-3.6 mm	5.07 ± 1.36	8.41 ± 0.91	48.46 ± 6.10*	30.06 ± 6.65*∞	-
Hippocampus						
CA1	-3.6 mm	51.22 ± 7.73	63.57 ± 6.96	111.39 ± 9.43*	105.29 ± 11.42*	≈
DG	-3.6 mm	98.32 ± 3.67	77.07 ± 4.99	254.92 ± 13.68*	185.32 ± 18.17*^	-

Figure 1. (A) Brain and (B) body weights of adult wildtype (wt) male, Tfm male, and wt female mice and rats. All animals were between 120-150 days old at the time of sacrifice. Brain weight measurements did not include the olfactory bulb and were taken after prolonged post-fixation in 10% formalin. Wt males show an increased brain weight compared to females, and Tfm males resemble wt males, in both mice and rats. Tfm males show a body weight intermediate between wt males and females. * indicates $p < .05$ compared to wt males.

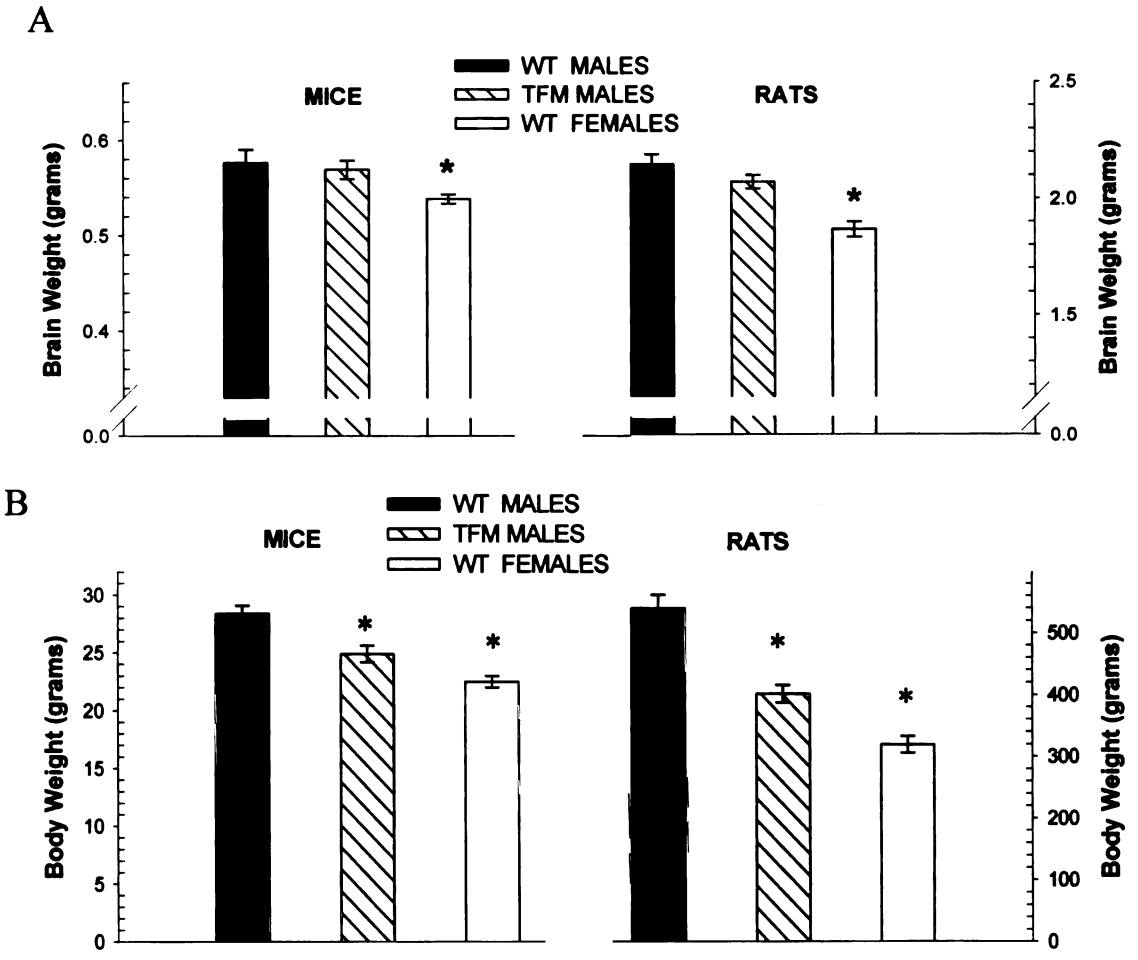


Figure 2. The posterodorsal medial amygdala (MePD) in Nissl-stained coronal sections from a wildtype (wt) male (top), a Tfm male with a dysfunctional androgen receptor (middle), and a female rat (bottom). The panels on the left are from the caudalmost appearance of the MePD, which served as an anchor point to assess changes in the nucleus across the rostrocaudal dimension. The appearance of the MePD, as well as the optic tract (ot), the stria terminalis (st), the anterolateral part of the amygdalohippocampal transition area (AHiAL), and the lateral ventricle (v) are equivalent in wt males (**a**), Tfm males (**b**), and females (**c**), indicating that the caudal termination of the MePD occurs in the homologous region of the brain across groups. The panels on the right are from the approximate middle of the rostrocaudal extent of the MePD where the nucleus is larger in wt males (**d**) than in females (**f**) and is intermediate in size in Tfm males (**e**). The MePD also extends farther rostrally in wt males and Tfm males than in females (Morris et al., 2005). Scale bar = 250 μm in a (applies to a-c), d (applies to d-f).

Figure 2.

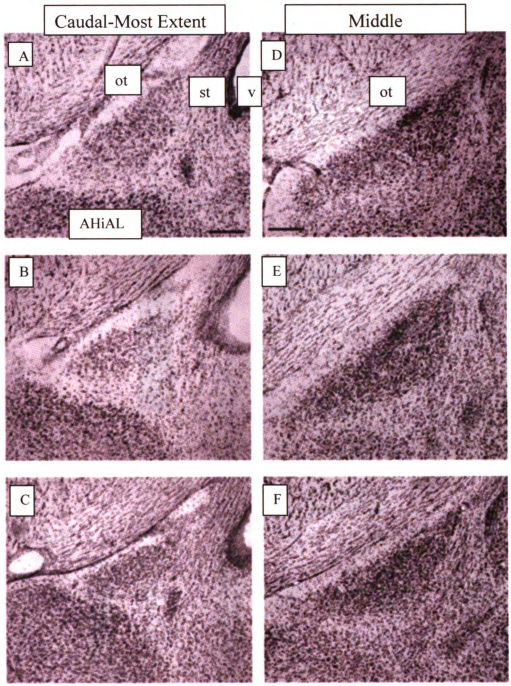


Figure 3. Time spent visiting a novel object in adult male wt and Tfm mice that were placed first in an empty open field and after five minutes were briefly removed and placed back into the open field that contained a small object (a 2"x 2" Petri dish with red and blue tape) in the center. (a) Upon exposure to a novel object, wt males spent more time exploring the novel object than did Tfm males.
(b) Wt male mice castrated as adults spent less time visiting the object, an effect that was averted if they were treated with testosterone (T) rather than blank capsules. T had no effect in Tfm males, indicating that androgen receptors mediate this effect. * indicates $p < .05$ compared to (a) Tfm males or (b) T-treated Tfm males.

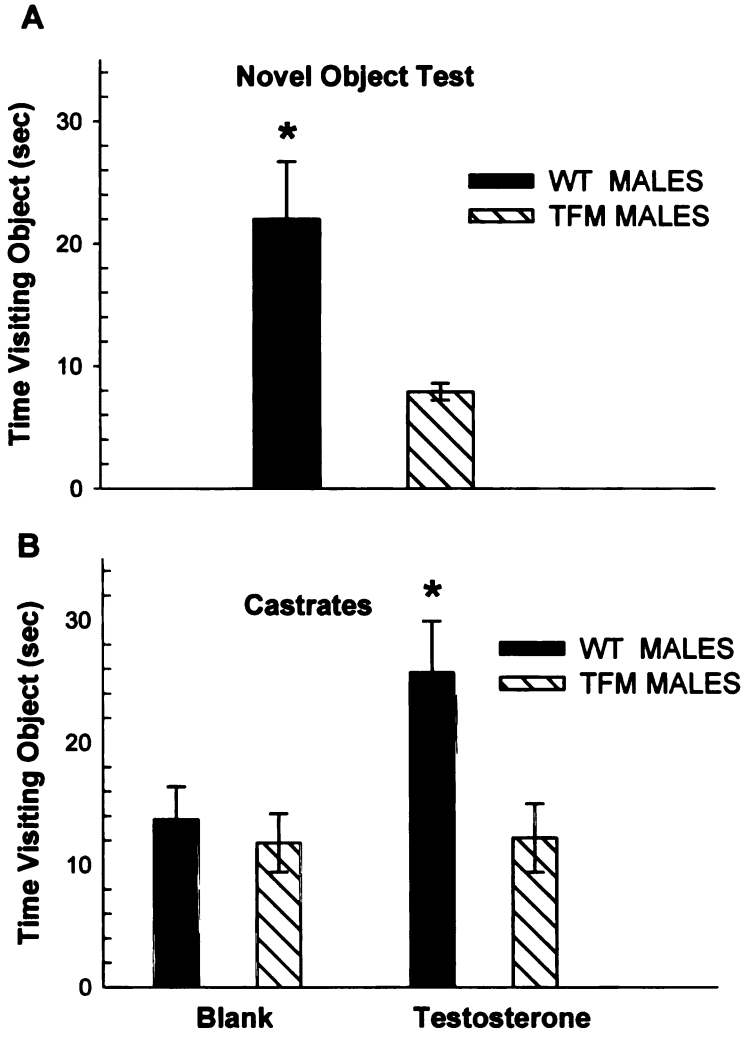


Figure 4. Plasma corticosterone levels 20 minutes after initial exposure to the 10 minute open field/novel object test were significantly greater in Tfm male mice compared to wt male mice. * indicates p< .01 compared to Tfm males.

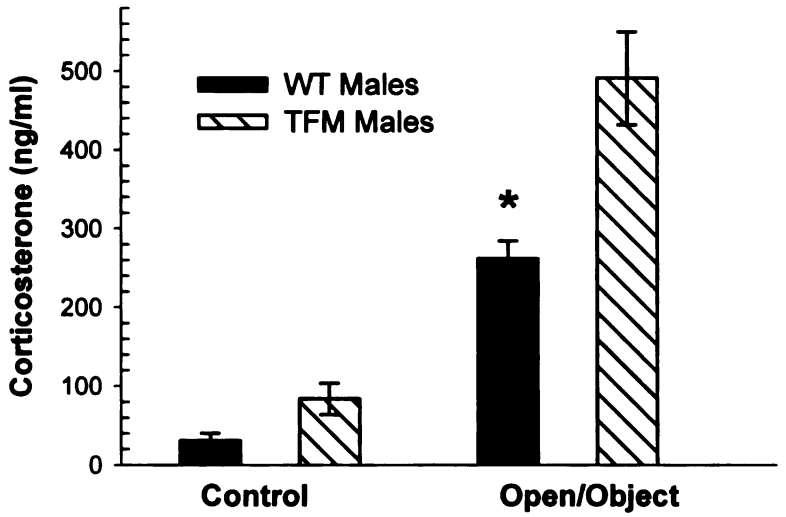


Figure 5. Prepulse inhibition in gonadally intact wt and Tfm male mice in (A) test order 1 (PPI, open field/novel object test, EPM, LD box) and (B) test order 2 (open field/novel object test, LD box, EPM, PPI). There is an overall decrease in prepulse inhibition in Tfm compared to wt males ($p < .05$) in test order 1, but no significant difference in test order 2. When test order 1 and 2 are pooled together, no significant difference between Tfm and wt males in PPI is found.

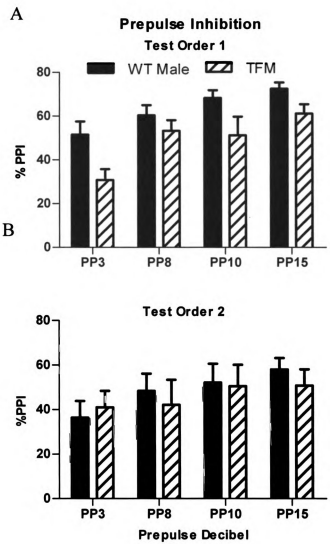


Figure 6. Anxiety-related behavior in gonadally intact wt and Tfm male mice. The amount of time spent in/with and number of visits to: (A) the center area of the open field, (B) a novel object in the open field arena, (C) the open arms of the elevated plus maze, and (D) the light area of the light dark box. (E) depicts the number of rearings in the open field, novel object, and LD box tests. These tests indicate greater anxiety-related behaviors in Tfm mice compared to wt mice in the novel object test and light dark box, but not the other two tests. * indicates $p<.05$ compared to wt males.

Figure 6.

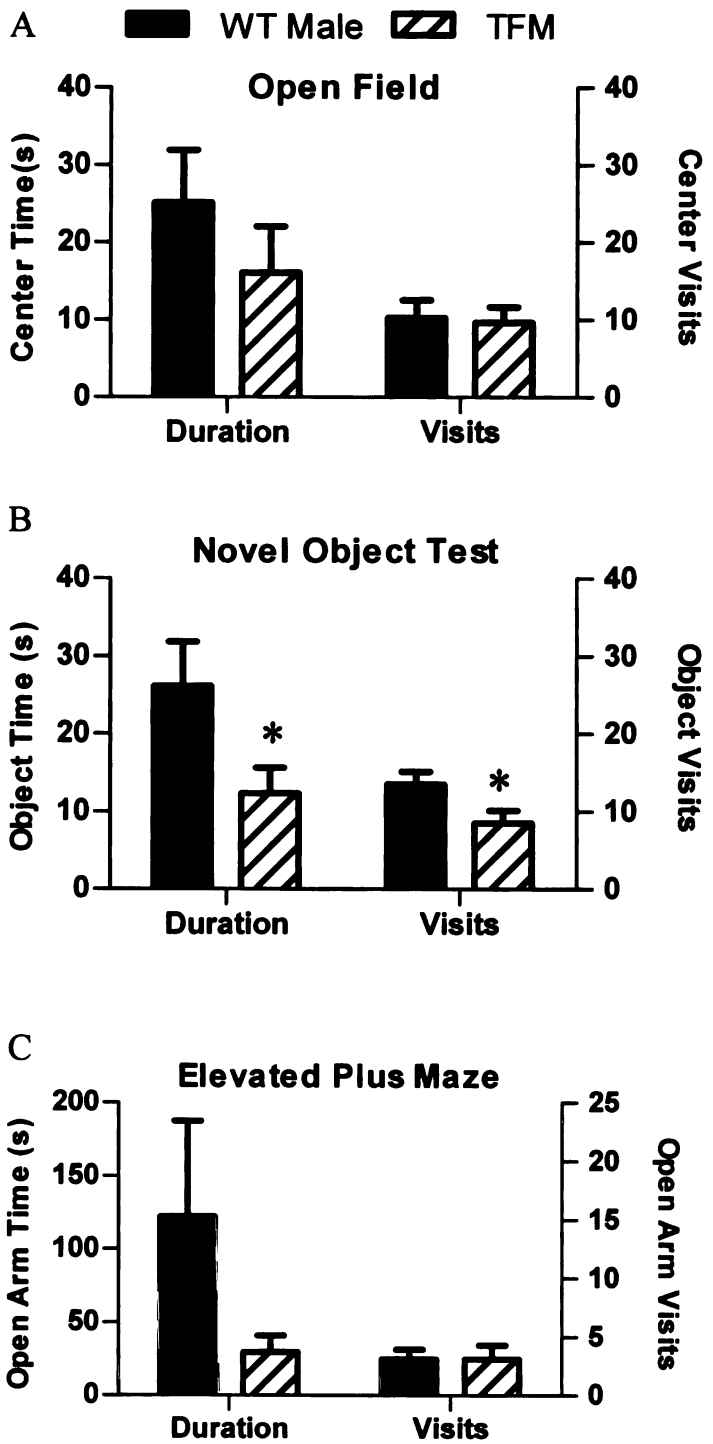

A
WT Male
TFM
Open Field
Center Time(s)
Center Visits
40
30
20
10
0
Duration
Visits
B
Novel Object Test
Object Time (s)
Object Visits
*
*
C
Elevated Plus Maze
Open Arm Time (s)
Open Arm Visits
200
150
100
50
25
20
15
5

Figure 6 cont.

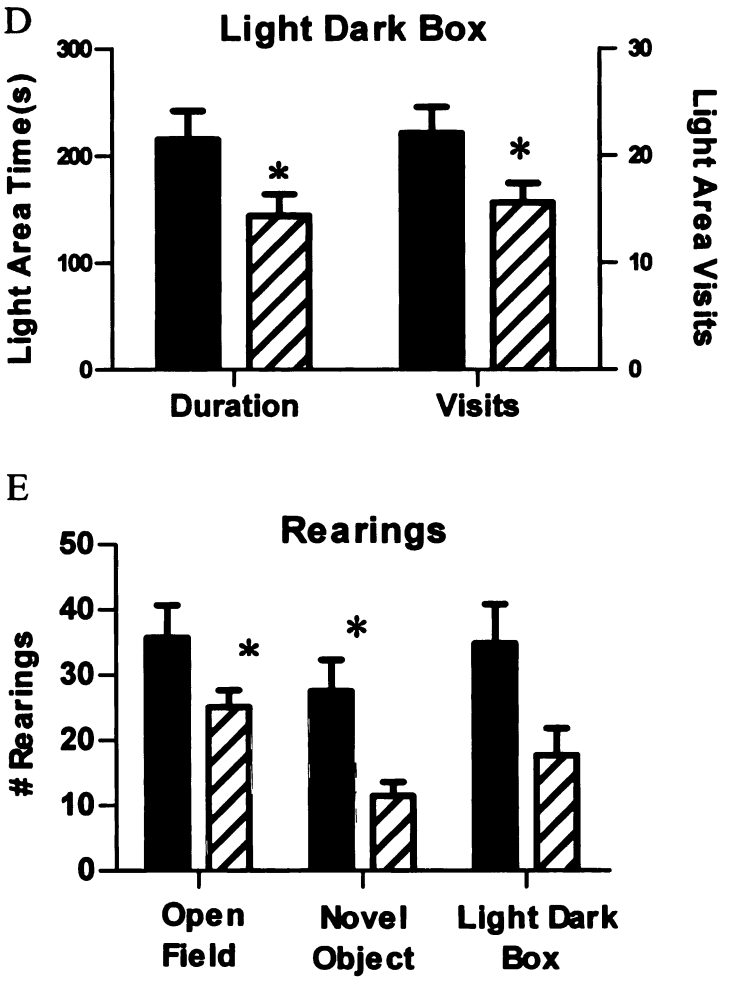
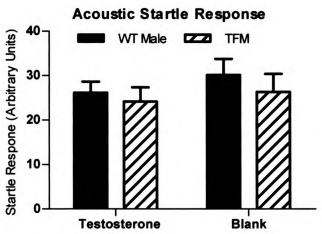


Figure 7. (A) Acoustic startle response and (B) Prepulse inhibition in T and B-treated wt and Tfm male mice. No significant group differences were found in acoustic startle response or prepulse inhibition. T: testosterone, B: blank.

A



B

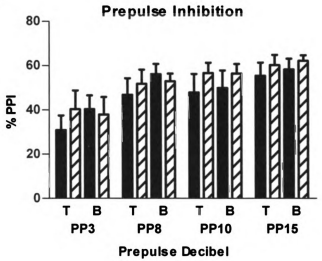


Figure 8. Anxiety-related behavior in T and B-treated wt and Tfm male mice. The amount of time spent in/with and number of visits to: (A) the center area of the open field, (B) a novel object, (C) the open arms of the elevated plus maze, and (D) the light area of the light dark box. (E) depicts the number of rearings in the open field, novel object, and LD box tests. These results confirmed the previously found differences between wt males and Tfm males for the novel object and LD box tests (see Figure 2) and suggest that T amelioration of anxiety-related behaviors are mediated by AR, since Tfm animals showed no behavioral response to T treatment in any test. * indicates $p<.05$ compared to T-treated wt males. T: testosterone, B: blank.

Figure 8.

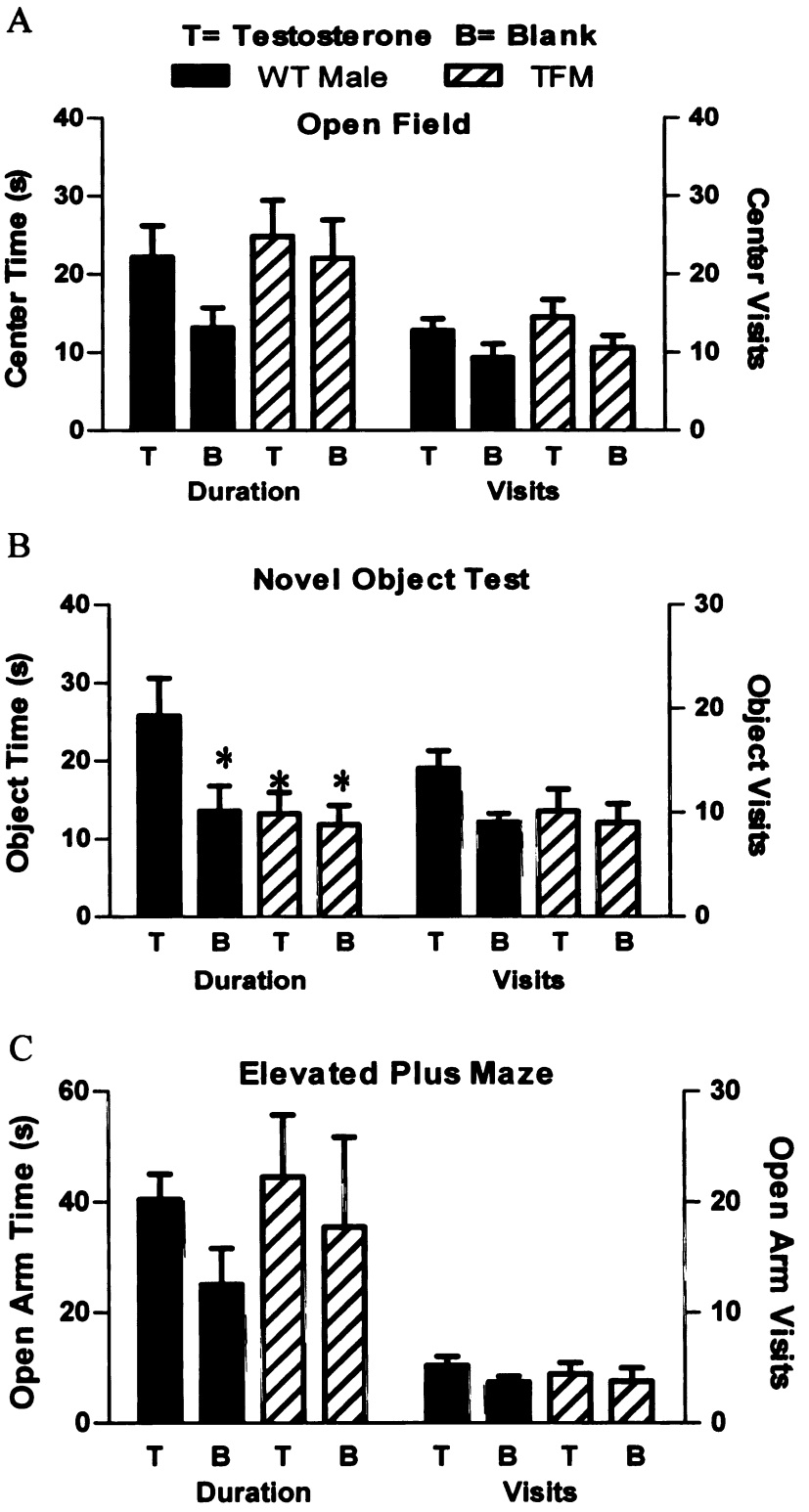

A
T= Testosterone B= Blank
WT Male
TFM
Open Field
Center Time (s)
Center Visits
40
30
20
10
0
T B T B T B T B
Duration
Visits
B
Novel Object Test
Object Time (s)
Object Visits
*
*
*
C
Elevated Plus Maze
Open Arm Time (s)
Open Arm Visits
60

Figure 8 cont.

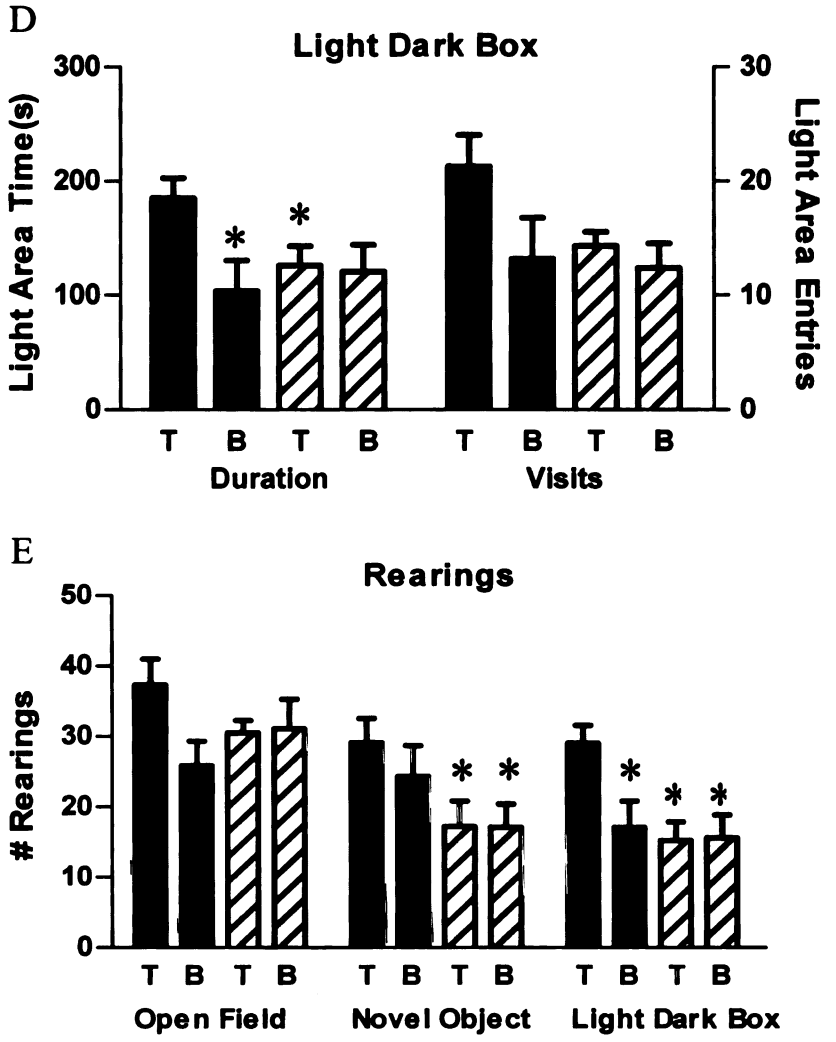


Figure 9. (A) Plasma corticosterone levels at baseline and 20 min after initial exposure to an open field with a novel object were elevated in Tfm males compared to wt males. *p< .01 compared to wt males. (B) Plasma corticosterone levels at baseline and 20, 40, 60, and 120 min after initial exposure to an open field with a novel object in Tfm and wt males. *indicates p< .05 compared to the same time point in wt males.

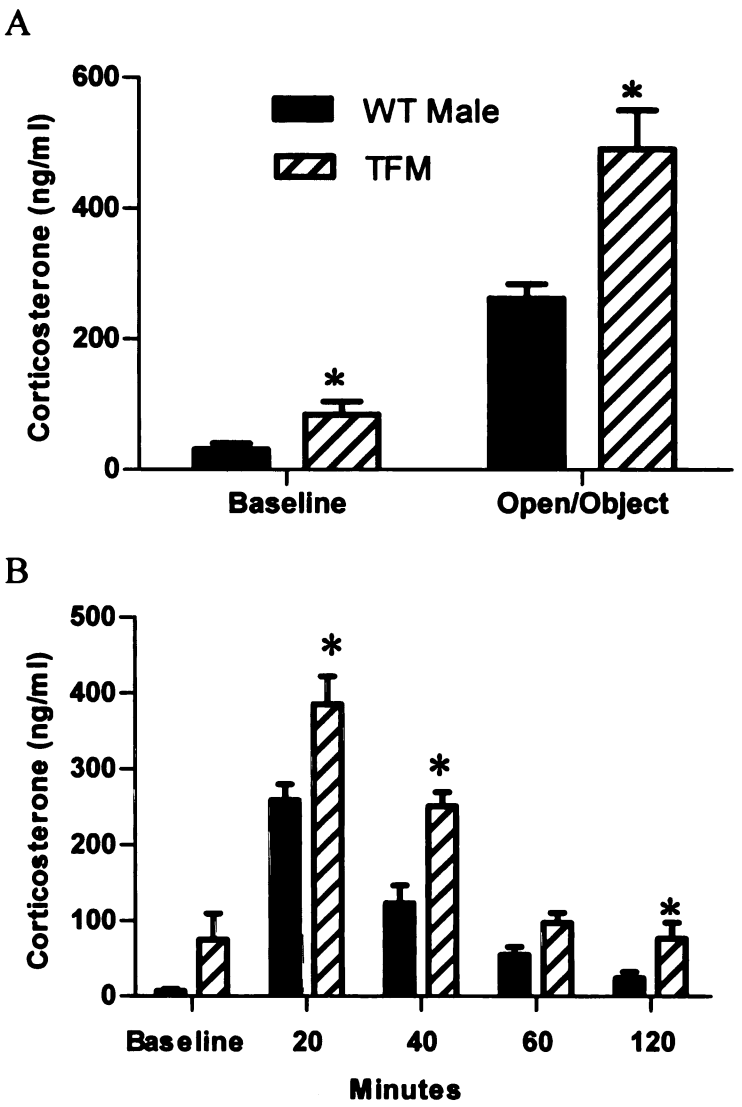


Figure 10. Prepulse inhibition (PPI; A), acoustic startle response (B), and acoustic startle response by trial in gonadally intact wt male, Tfm male, and wt female rats. There were no group differences in PPI, but ASR was greater in Tfm males than either wt males or females, primarily due to differences in startle response to the first pulse. * indicates $p<.05$ compared to wt males and females. # indicates $p<.001$ compared to same trial wt males and females.

Figure 10.

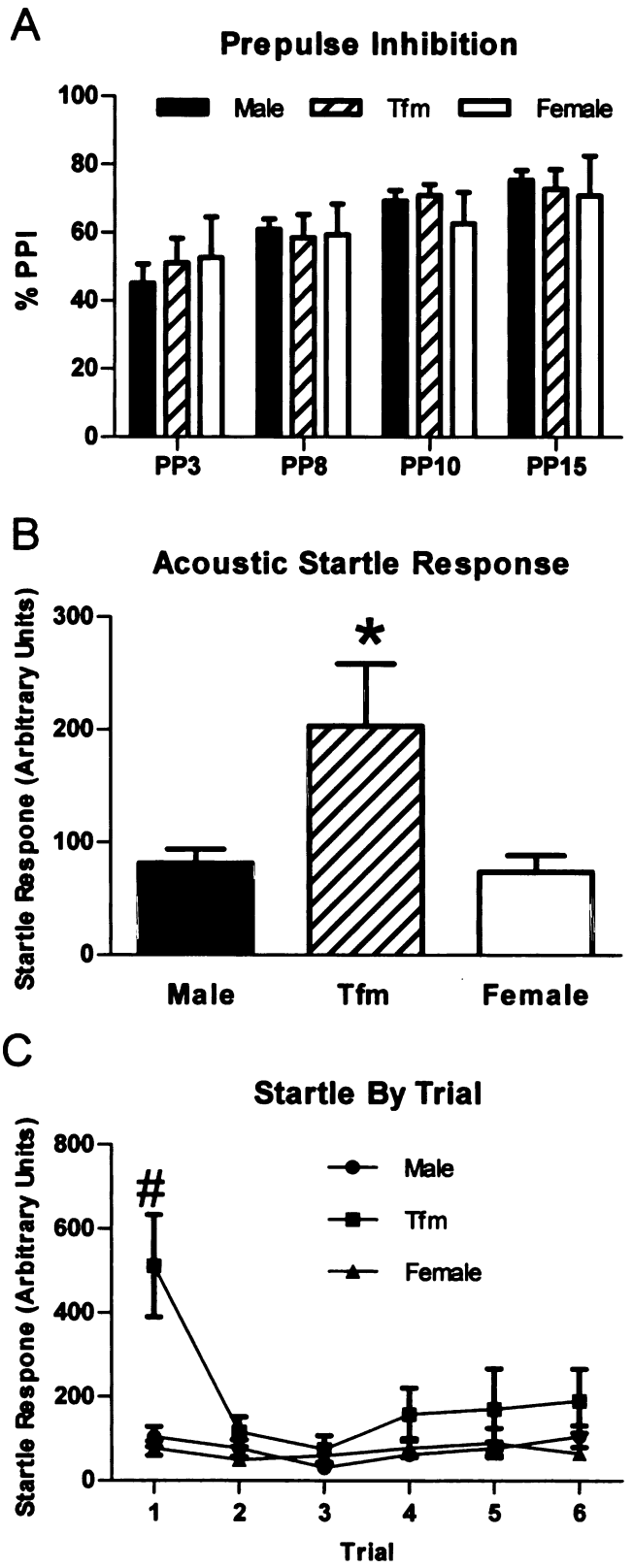

A
Prepulse Inhibition
Male
Tfm
Female
% PPI
100
80
60
40
20
0
PP3
PP8
PP10
PP15
B
Acoustic Startle Response
Startle Respone (Arbitrary Units)
300
200
100
0
*
Male
Tfm
Female
C
Startle By Trial
Startle Respone (Arbitrary Units)
800
600
400
200
0
#
Male
Tfm
Female
1
2
3
4
5
6
Trial

Figure 11. The amount of time spent in/with and number of visits to: (A) the center area of the open field or (B) a novel object in the open field arena in wt male, Tfm male, and wt female rats. We found no significant differences between wt males and females in these measures, but Tfm males displayed signs of greater anxiety than other groups. * indicates $p<.05$ compared to Tfm males. # indicates $p<.01$ compared to Tfm males.

Figure 11.

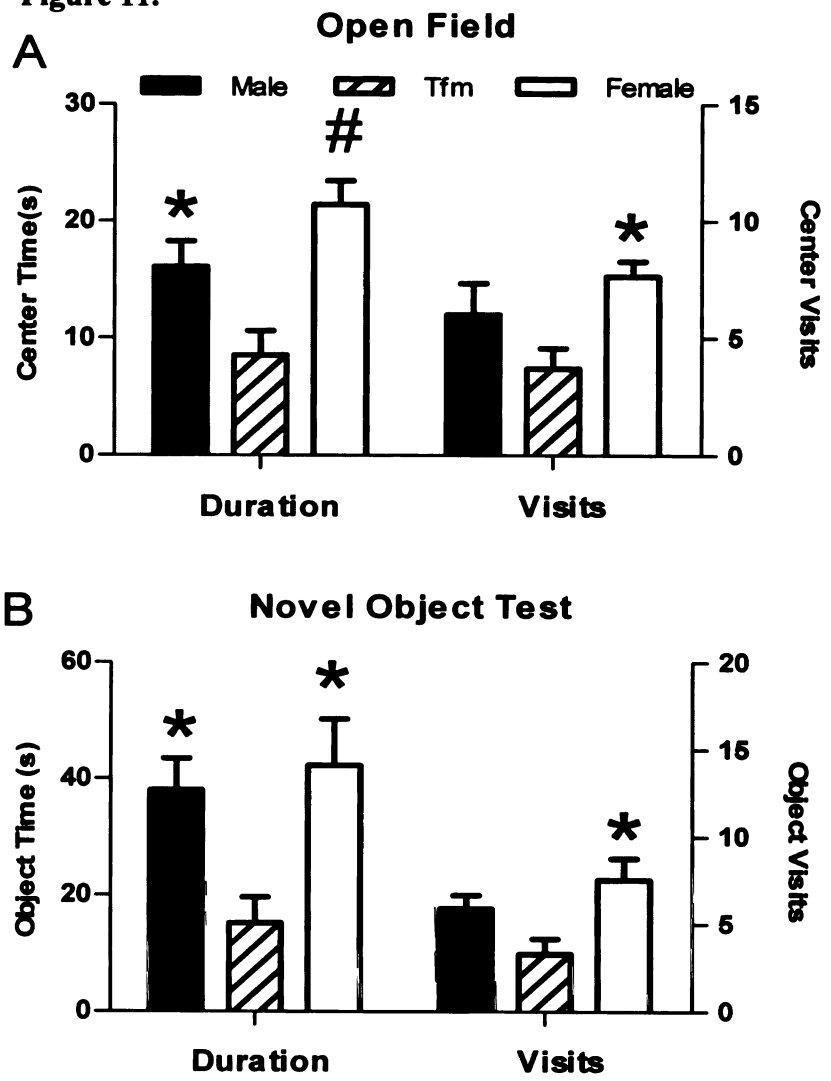

A
Open Field
Male
Tfm
Female
Center Time(s)
Center Visits
30
20
10
0
15
10
5
0
#
*
*
Duration
Visits
B
Novel Object Test
Object Time (s)
Object Visits
60
40
20
0
20
15
10
5
0
*
*
*
Duration
Visits

Figure 12. (A) Plasma corticosterone levels at baseline and 20 minutes after initial exposure to an open field with a novel object were elevated in Tfm males and wt females compared to wt males. * indicates $p < .001$ compared to the same treatment Tfm male and wt female groups. (B) Plasma corticosterone levels from a second cohort of rats at baseline and 20, 40, 60, and 120 minutes after initial exposure to an open field with a novel object in Tfm and wt males. * indicates $p < .05$ compared to the same time point Tfm male group.

Figure 12.

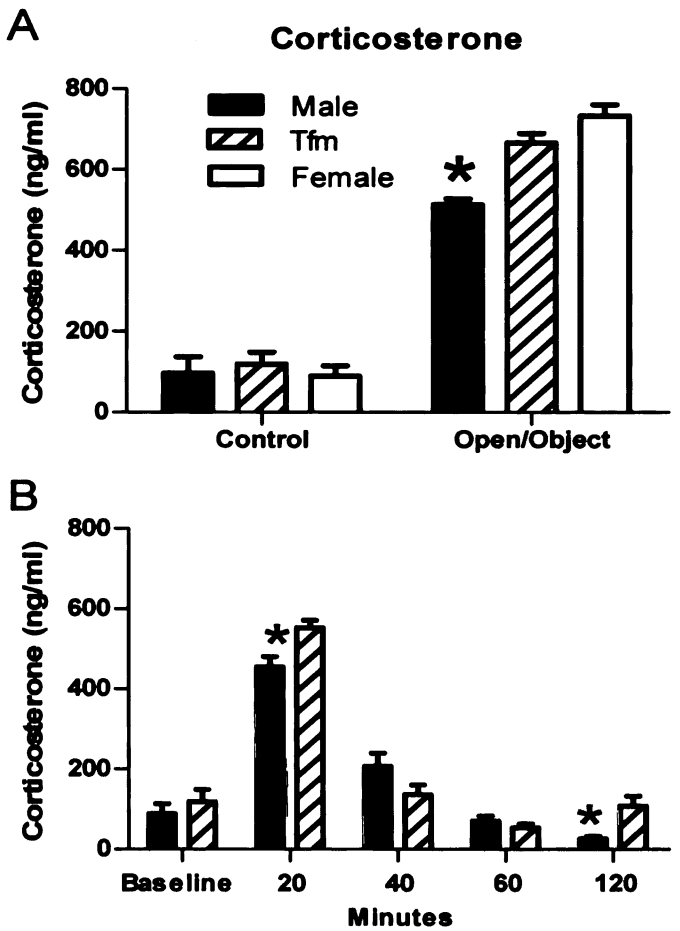

A
Corticosterone
Male
Tfm
Female
Corticosterone (ng/ml)
800
600
400
200
0
*
Control
Open/Object
B
Corticosterone (ng/ml)
800
600
400
200
0
*
*
Baseline
20
40
60
120
Minutes

Figure 13. The number of c-Fos positive cells per mm^2 in wt and Tfm male rats at baseline or after exposure to an open field with a novel object. There were no differences between wt and Tfm males at baseline, but following exposure to a novel object Tfm males displayed fewer c-Fos positive cells in the dentate gyrus and MePD, and more c-Fos positive cells in the MPOA, than did wt males. * indicates $p < .05$ compared to the same treatment Tfm male group. # indicates a significantly greater increase from control treatment compared to Tfm males, $p < .05$.

Figure 13.

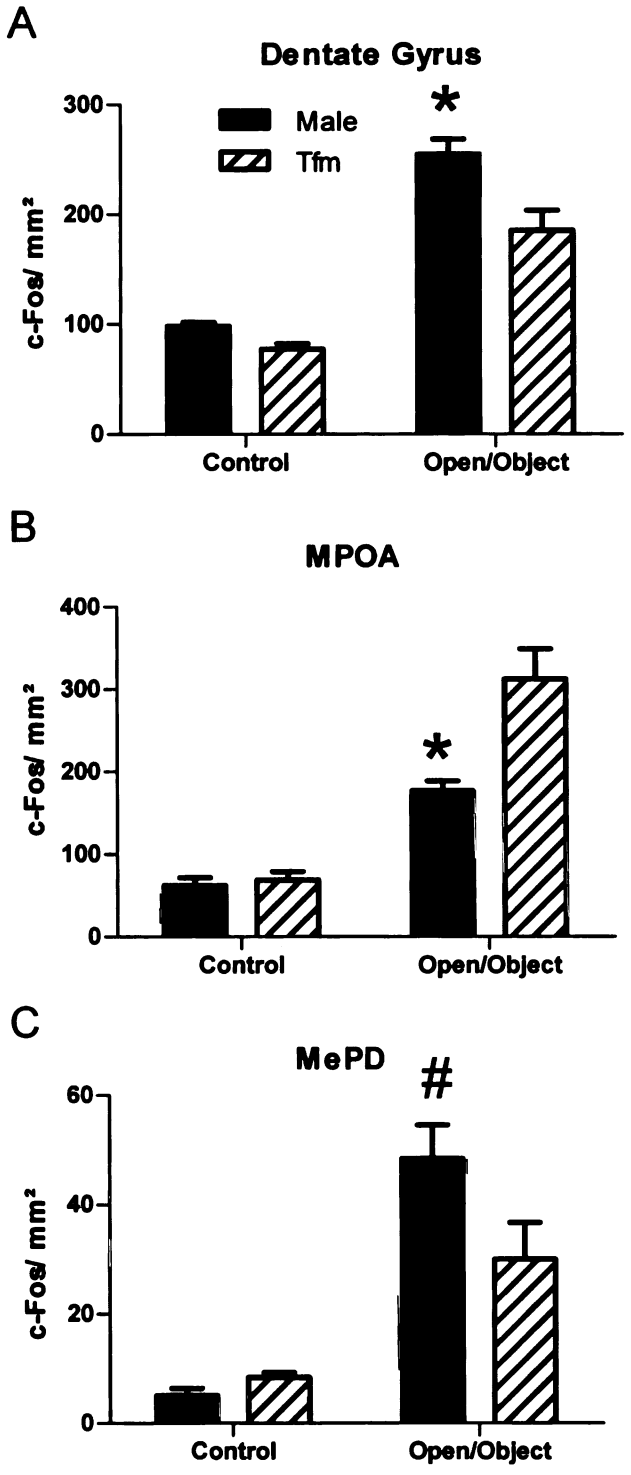

A
Dentate Gyrus
Male
Tfm
*
300
200
100
0
c-Fos/ mm²
Control
Open/Object
B
MPOA
400
300
200
100
0
*
c-Fos/ mm²
Control
Open/Object
C
MePD
#
60
40
20
0
c-Fos/ mm²
Control
Open/Object

Figure 14. Neonatal castration increased (A) the amount of time spent in and (B) the number of visits to the center area of the open field in both Tfm and wt male rats (ps<.05). (C) Neo-Gdx rats also showed a greater number of grid crossings (p<.01) than did Neo-Sham rats (left), but the number of rearings did not differ between groups (right). These data indicate decreased anxiety-related behavior and greater open field activity in Neo-Gdx rats compared to Neo-Sham controls. Mean ± SEM.

Figure 14.

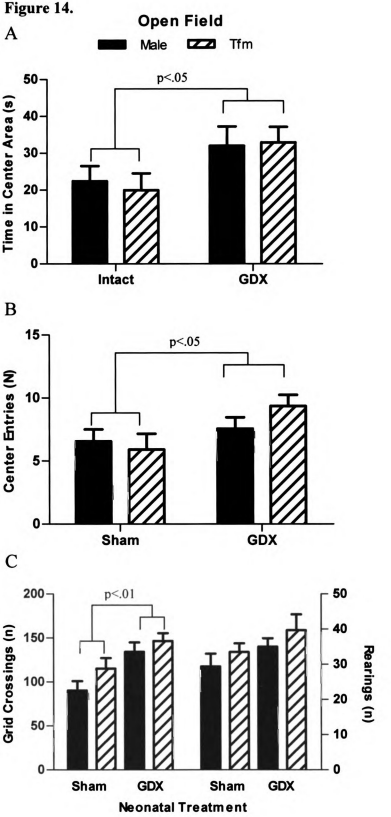

A
Open Field
Male
Tfm
p<.05
Time in Center Area (s)
50
40
30
20
10
0
Intact
GDX
B
p<.05
Center Entries (N)
15
10
5
0
Sham
GDX
C
p<.01
Grid Crossings (n)
200
150
100
50
0
Rearings (n)
50
40
30
20
10
0
Sham
GDX
Sham
GDX
Neonatal Treatment

Figure 15. (A) The amount of time spent with and (B) number of visits to a novel object in the open field arena in Neo-Gdx or Neo-Sham Tfm and wt males. (C) The number of grid crossing (left) and rearings (right) in the novel object test. Neo-Gdx rats visited the novel object more frequently than Neo-Gdx rats ($p<.05$) and showed a greater number of grid crossings than did Neo-Sham rats ($p<.05$). Time spent visiting the object and the number of rearings did not significantly differ between groups. These data indicate that neonatal castration decreases some indices of anxiety and increases activity in male rats, and that an intact AR is not required for these effects. Mean ± SEM.

Figure 15.

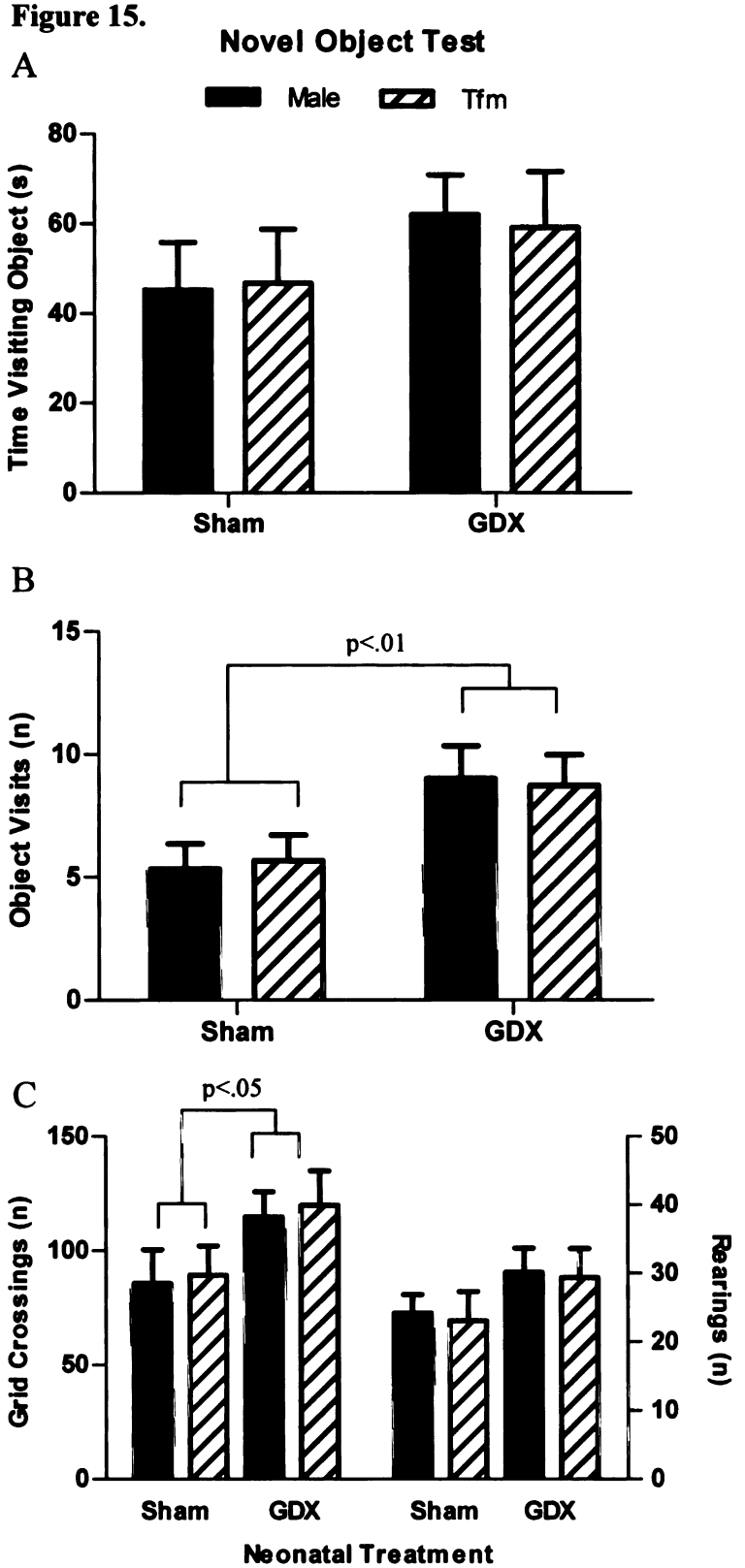

A
Novel Object Test
Male
Tfm
Time Visiting Object (s)
80
60
40
20
0
Sham
GDX
B
p<.01
Object Visits (n)
15
10
5
0
Sham
GDX
C
p<.05
Grid Crossings (n)
150
100
50
0
Rearings (n)
50
40
30
20
10
0
Sham
GDX
Sham
GDX
Neonatal Treatment

Figure 16. (A) The amount of time spent in the light area, (B) number of visits to the light area, and (C) rearings in the light area of the LD Box. As a group, Neo-Gdx rats spent more time in ($p<.01$), showed more visits to ($p<.01$), and reared more frequently in the light area ($p<.01$) than did Neo-Sham rats. These data indicate decreased anxiety-related behavior and greater activity following neonatal castration in rats. Mean ± SEM.

Figure 16.

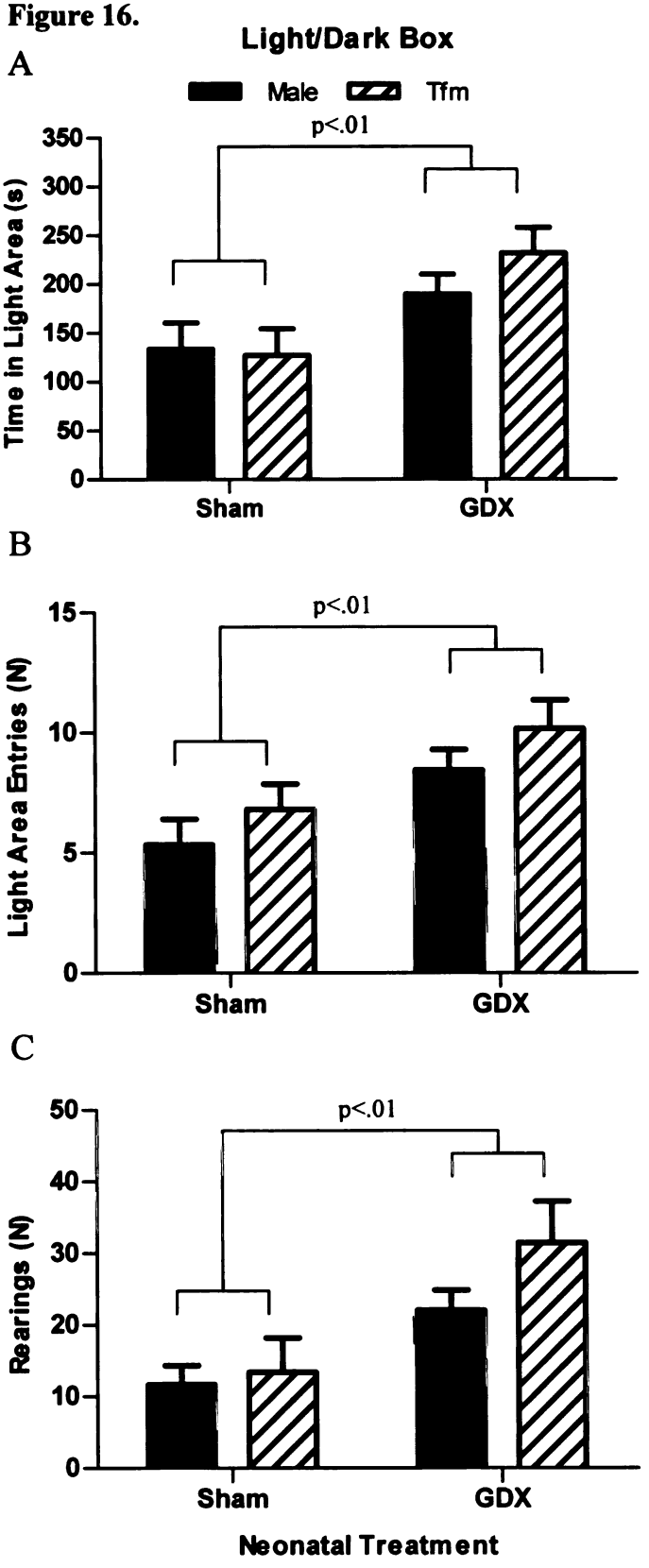

A
Light/Dark Box
Male
Tfm
p<.01
Time in Light Area (s)
350
300
250
200
150
100
50
0
Sham
GDX
B
p<.01
Light Area Entries (N)
15
10
5
0
Sham
GDX
C
p<.01
Rearings (N)
50
40
30
20
10
0
Sham
GDX
Neonatal Treatment

Figure 17. (A) The amount of time spent on and (B) number of entries into the open arms of the EPM in Neo-Gdx or Neo-Sham Tfm and wt males. (C) The number of total (open and closed) arm entries. Neo-Gdx rats had greater number of open arm visits than Neo-Sham rats ($p<.01$). They also tended to spend more time on the open arms ($p=.063$) and show a greater number of total arm entries ($p=.062$) than Neo-Sham males. These data indicate that neonatal castration decreases some indices of anxiety and may increase activity in adult rats in the EPM. Mean ± SEM.

Figure 17.

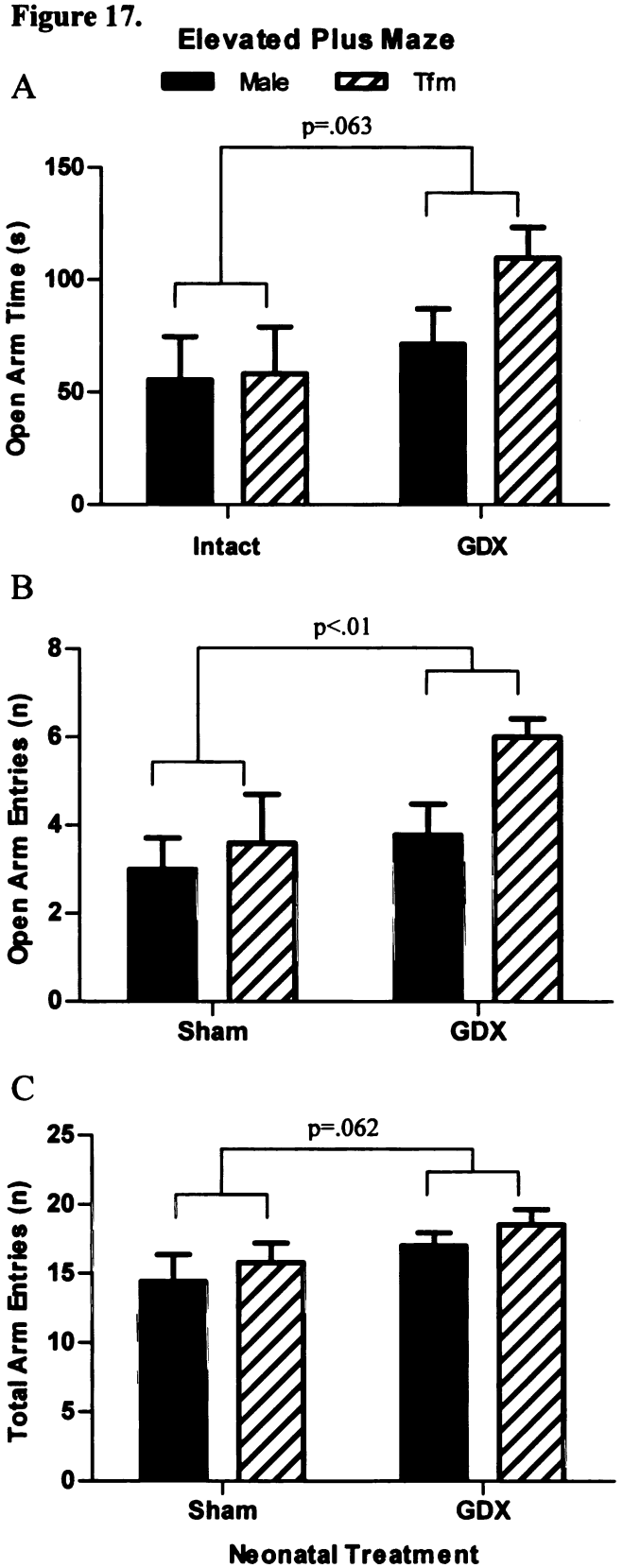

Elevated Plus Maze
A
Male
Tfm
p=.063
150
100
50
0
Open Arm Time (s)
Intact
GDX
B
p<.01
8
6
4
2
0
Open Arm Entries (n)
Sham
GDX
C
p=.062
25
20
15
10
5
0
Total Arm Entries (n)
Sham
GDX
Neonatal Treatment

Figure 18. Prepulse inhibition (PPI; A), acoustic startle response (B), and acoustic startle response by trial (C) in Neo-Gdx and Neo-Sham wt and Tfm male rats. PPI was increased in Neo-Gdx compared to Neo-Sham rats ($p<.01$), an effect that was equivalent in wt and Tfm males. ASR was decreased in Neo-Gdx compared to Neo-Sham rats ($p<.01$), a difference largely accounted for by an increased ASR in Neo-Sham Tfm males. Because neonatal testicular secretions increase adult ASR only in Tfm males, there seem to be both AR- and ER-mediated developmental influences on this behavior. * indicates $p<.05$ compared to all other groups in the same trial. # indicates $p<.05$ compared to same trial Neo-Gdx males. Mean ± SEM.

Figure 18.

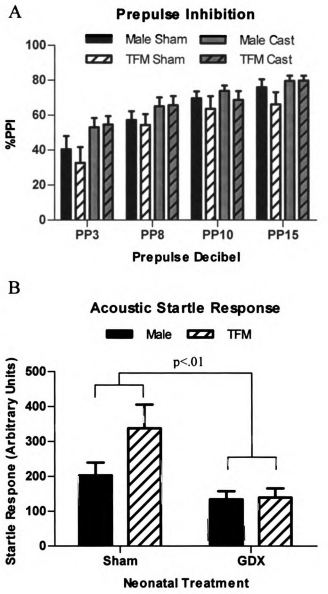

A
Prepulse Inhibition
Male Sham
Male Cast
TFM Sham
TFM Cast
%PPI
100
80
60
40
20
0
PP3
PP8
PP10
PP15
Prepulse Decibel
B
Acoustic Startle Response
Male
TFM
p<.01
Startle Respone (Arbitrary Units)
500
400
300
200
100
0
Sham
GDX
Neonatal Treatment

Figure 18 cont.

C

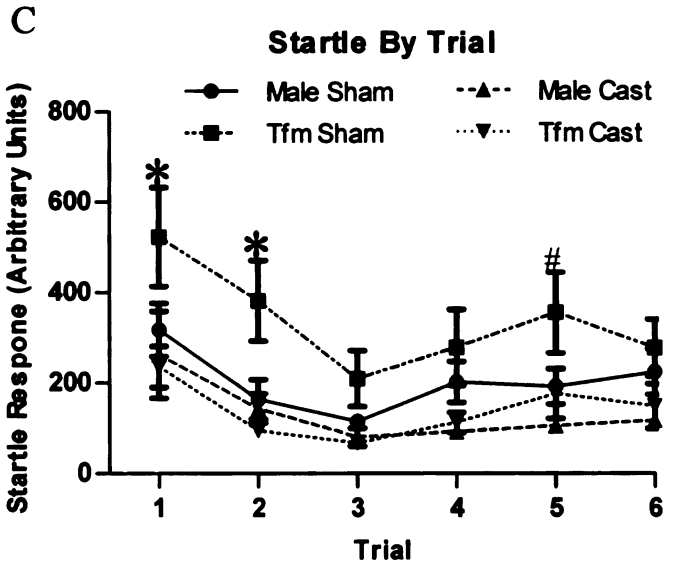


Figure 19. Plasma corticosterone levels at (A) baseline and (B) 20 minutes after initial exposure to an open field with a novel object. Baseline corticosterone levels were elevated in Neo-Gdx rats ($p<.05$) with no differences after exposure to a novel object. Mean ± SEM.

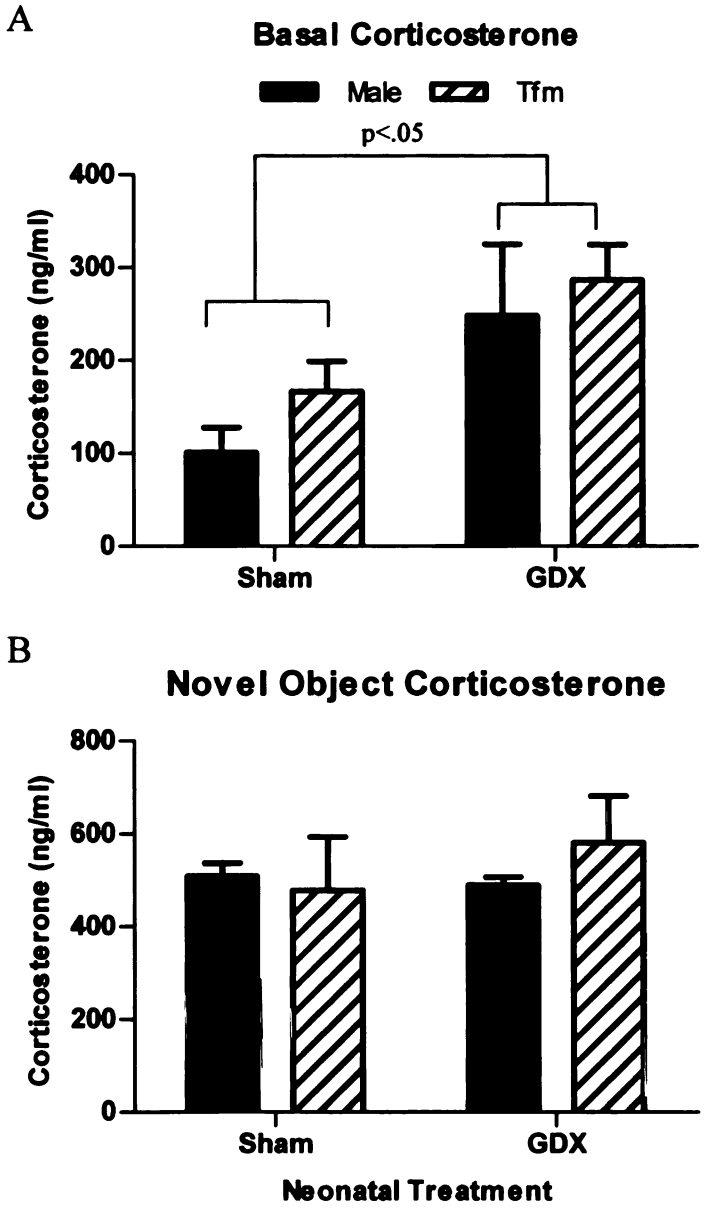
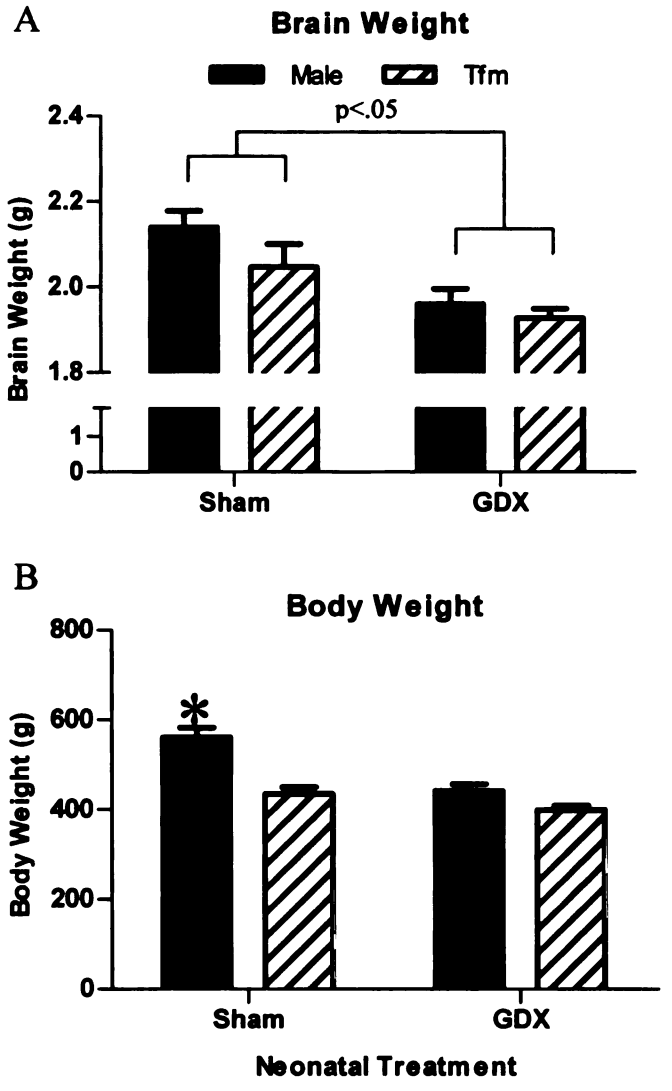


Figure 20. (A) Brain and (B) body weight in Neo-Gdx and Neo-Sham wt and Tfm male rats. Brain weight was greater in Neo-Sham compared to Neo-Gdx rats ($p<.05$), and was equivalent in wt and Tfm males. Thus neonatal stimulation of ER may be responsible for the larger brain weight in male versus female rats. In contrast, body weight was greater in Neo-Sham wt males compared to all other groups, suggesting that neonatal AR stimulation may normally contribute to masculinization of adult body weight. * indicates $p< .001$ compared to all other groups. Mean ± SEM.



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