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New knockout model confirms a role for androgen receptors in regulating anxiety-like behaviors and HPA response in mice

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Abstract

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

Keywords

Androgen receptor; Testosterone; Anxiety; Androgen insensitivity; CreLox technology; HPA axis; Corticosterone; Photoperiod

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

Clinical data indicate that gonadal hormones affect anxiety in humans. Women are more prone to episodes of anxiety during the premenstrual and postpartum periods, when estrogen (E) levels are low (Dean and Kendell, 1981; Douma et al., 2005; Freeman, 2002), and as they approach menopause, when E levels start to decline (Llaneza et al., 2012; Tangen and Mykletun, 2008). In addition, E treatment ameliorates anxiety in some postmenopausal women (Frye, 2009; Yazici et al., 2003), and in breast cancer survivors regardless of age (Decker et al., 2003). Like E, testosterone (T) has also been linked to anxiety levels in women, with low levels correlating with high anxiety levels (Giltay et al., 2012). Accordingly, treating women with T following ovariectomy decreases anxiety (Shifren et al., 2000). In aging men, anxiety levels increase with decreasing levels of T (Amore et al., 2009; Sternbach, 1998), and T treatment ameliorates anxiety in such men (Wang et al., 1996). Similarly, androgen blockade therapy for prostate cancer treatment has been reported to increase anxiety, which was alleviated when treatment ended (Almeida et al., 2004). Although the nature of the relationship between these steroid hormones and anxiety level is not always consistent (Demetrio et al., 2011; Kiesner, 2011; Thomson and Oswald, 1977), they appear to exert anxiolytic effects in a number of different circumstances.

Sex differences in anxiety-related behavior are also present in other mammals, including laboratory rodents. Although the direction of these differences varies by species and strain, gonadal hormones also have anxiolytic effects in rodents. Laboratory rodents are nocturnal prey species that are averse to open and brightly lit areas; therefore, behavioral tests like the open field (OF), novel object (NO), elevated plus maze (EPM) and light/dark (L/D) box, which subject animals to open or lit spaces, are used to infer anxiety levels. Results from these tests suggest that E acts as an anxiolytic. Specifically, when circulating E levels are high during proestrous, female rodents spend more time in the open arms of the EPM than when E levels are low during diestrous (Marcondes et al., 2001; Walf et al., 2009; Zuloaga et al., 2011a). Likewise, E-treated females enter the center area of an OF more often and spend more time in the open arms of an EPM than do untreated females (Walf et al., 2008), while females given inhibitors of estrogen biosynthesis show more anxiety-related behavior in the OF and EPM (Meng et al., 2011; however, Morgan and Pfaff, 2002). Similarly, aged females enter the center area of an OF more often and spend more time in the light side of the L/D box when treated with E than do oil treated females (Walf and Frye, 2010). These effects of E appear to be mediated through ER β (Hughes et al., 2008; Imwalle et al., 2005; Krezel et al., 2001; Rocha et al., 2005; Walf et al., 2009; Walf et al., 2008) and not ER α (Krezel et al., 2001; Lund et al., 2005; Walf and Frye, 2005).

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

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

Materials and Methods

Experimental Animals

We used the CreLox system in mice in an attempt to recapitulate the effect of a spontaneous mutation in the AR gene, namely the *sTfm*. Mice carrying a conserved lox sequence of 34 base pairs at the two ends of exon 2 of the AR gene ("floxed" AR, a generous gift from De Gendt et al., 2004) were crossed with transgenic animals carrying the cyclization recombination (Cre) transgene under the control of the universal adenovirus Ella promoter ("deleter" mice, Jax stock 003724). The presence of both genotypes in the same cell should result in the excision of the targeted sequence in AR exon 2, which encodes the first zinc finger of the DNA-binding domain, essential for the recognition of androgen response elements. This deletion should cause a frame-shift mutation resulting in the premature termination of AR transcription, rendering AR dysfunctional (De Gendt et al., 2004). Female offspring carrying such a recombined AR allele were identified by PCR, as described below, then bred to wildtype (WT) C57BL/6 (Jax) males; the recombined AR allele was transmitted to some female progeny without the cre transgene, as confirmed by PCR. These females were used to found a colony of mice perpetuating the recombined AR allele. We confirmed that breeding these females with WT males produces genetic male offspring that recapitulate the phenotype of XY mice carrying the *sTfm* mutant of AR:

small, abdominal testes, short anogenital distance, feminine external genitalia, and nipples (Figure 1). Hence, we refer to these mice as *induced* Tfm, or *i*Tfm mice, to distinguish them from the *s*Tfm mice. As with our *s*Tfm colony, these females also produced male offspring carrying the WT AR allele to serve as controls. AR immunocytochemistry (methods described below) confirmed full knock out of AR in experimental animals, as we found no AR immunoreactivity in the brains of *i*Tfm males (Figures 2B,D), while their WT brothers showed AR expression in typical AR positive regions (Figures 2A,C). Circulating T measures revealed that *i*Tfm males have lower adult circulating T levels than their WT counterparts (*i*Tfm: $M=4.2 \pm 0.23$ (SEM) nmol/l; WT: $M=29.8 \pm 4.9$ nmol/l; methods detailed below), further confirming the disruption of AR in these animals since *s*Tfm mice also have lower T levels than WTs (*s*Tfm: $M=3.26 \pm 0.52$ nmol/l; WT: $M=19.75 \pm 4.39$ nmol/l; Zuloaga et al., 2008).

Mice born in this *i*Tfm colony were housed in plastic cages (29×18×18cm) at approximately 27°C in a 12:12 LD cycle, and provided *ad libitum* tap water and rodent chow (Harlan Teklad 8640 Rodent Diet [Madison, WI]). Mice were ear punched for genotyping and weaned at postnatal (PN) day 23 and group housed with other phenotypically similar males or females. For experiments 1 and 3, WT and *i*Tfm males were castrated at postnatal (PN) day 60 and subcutaneously implanted either with testosterone-filled (T) or blank (B) capsules (1.6 mm-inner and 3.2 mm-outer diameter, 1.6 cm effective length). Behavior testing and blood collection took place on PN90–120. All housing conditions and experiments were performed in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the MSU Institutional Animal Care and Use Committee.

Polymerase chain reaction (PCR) identification of genotype

PCR was run to detect the recombined AR allele and the *Sry* gene. To discriminate the WT and recombined AR alleles, primers targeted and amplified the sequence that includes the lox sites and exon 2 of the AR gene. The primers used were AGC CTG TAT ACT CAG TTG GGG and AAT GCA TCA CAT TAA GTT GAT ACC. The resulting products were 860bp for the wild type AR, and 400bp for the recombined AR allele. Animals that were positive for both *Sry* and WT AR were classified as WT males whereas mice positive for both *Sry* and a recombined AR were classified as *i*Tfm males. The genotype determined by PCR was also verified based on the phenotype of each mouse as described below (experiments 1 and 3), and/or at sacrifice (experiment 2).

AR Immunocytochemistry (ICC)

To facilitate detecting AR, all mice in these studies were injected with 1mg of testosterone propionate in 0.1ml sesame oil sc two hours before being sacrificed. Animals were then injected with an overdose of sodium pentobarbital i.p. and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered Saline (PBS, pH 7.4). Brains were collected and post-fixed for 2 hours in 4% paraformaldehyde, at 4°C. Tissues were then transferred into a 20% sucrose solution and kept at 4°C until they sunk to the bottom (approx. 2 days). Once sunk, brains were microtome sectioned in the coronal plane at 35µm. All reactions for the ICC were performed at room temperature and on a rotomixer

unless otherwise indicated. Tissues were rinsed in four 5 minute washes in a phosphate-buffered gelatin Triton solution (PBS-GT; 0.1% gelatin, 0.3% Triton X-100, in PBS, pH 7.4), followed by 0.5% sodium borohydride in PBS-GT for 15 minutes, and three 5 minute washes in PBS-GT. Sections were then incubated in 10% NGS in PBS-GT for 1 hour to block non-specific binding of the secondary antibody. Two 5 minute washes in PBS-GT, and later a 10 minute incubation in avidin block followed. This was succeeded by two 5 minute PBS-GT washes and then incubation for 10 minutes in biotin block (Avidin/Biotin Blocking Kit, Vector, cat#SP-2001). Two 5 minute PBS-GT washes took place before the tissue was incubated for 36 hours at 4°C in 1% NGS in PBS-GT with AR primary antisera at 1:5000 concentration (rabbit monoclonal - Abcam, code#ab52615, clone ID EP670Y). Following incubation in the primary antibody, the tissue was rinsed in PBS-GT, and incubated 1 hour in 1% NGS in PBS-GT with biotinylated goat antirabbit secondary antisera at 1:1000 concentration (Jackson Immunoresearch, lot # 88762, code # 711-065-152). Brain sections were rinsed again in PBS-GT, followed by a 1 hour incubation in PBS-GT with an Avidin-Biotin complex solution made 30 minutes before use (1 drop of each solution A and B per 10ml of PBS-GT; ABC Elite Kit [standard], Vector Laboratories, catalogue # PK6100). The tissue was again washed in PBS-GT before being stained with NiCl-enhanced diaminobenzidine (DAB, Sigma, St. Louis, MO) in a 0.05 M Tris Buffer, pH 7.2. Following staining, the tissue was first washed in PBS-GT and then in mounting solution before being mounted and coverslipped after dehydration through graded alcohols and xylene.

Experiment 1: Validation of the *ΔTfm* model

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

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

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

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

Based on findings in experiments 1 and 2, and the relation between anxiety-related behavior and HPA axis activity, experiment 3 was conducted to determine whether disabling AR through recombination alters HPA response to mild stress. Terminal blood samples for corticosterone (CORT) levels were collected on PN90 from behaviorally naïve T-treated WT and *ΔTfm* mice at baseline, and at 20, 40 60, and 120 min after a 10-min exposure to the

L/D box. Castrated WT males implanted with blank capsules provided an additional control group. Exposure to the L/D box and blood collection took place during the animals' active phase, 2 hours after lights off, parameters chosen based on the robust group differences seen in Experiment 2 (detailed below).

Castration and Silastic capsule implant for androgen treatment

At PN60 before any testing began, mice in experiments 1 and 3 were anesthetized with isoflurane and their backs shaved for capsule implants. Castrations in WT mice were performed through a 5-mm long incision along the midline of the scrotal sac. Testes were visualized and silk suture was used to tie the vas deferens, blood vessels and associated fat pad before removing the testes. The incision was closed using surgical staples. In *iTfm* mice, the testes were undescended and small as expected (De Gendt et al., 2004; Figure 1D); thus, an abdominal incision was made through which the testes were visualized on each side of the bladder, tied and removed as described above. The abdominal muscle wall was closed with suture and the overlying skin closed with clips.

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

Anxiety-related behavior testing

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

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

Elevated plus maze test—The EPM consisted of four arms, 25.4 cm in length and 5.7 cm in width, stretched out from a center square at 90 degree intervals. Two of the opposing arms had walls of 13.3 cm in height. The maze rested 43.8 cm from the floor. Testing took place with an overhead lamp that provided 50 lux lighting in the open arms and 25 lux in the

closed arms. Animals were placed into the maze facing a closed arm and allowed 10 minutes to move freely. An overhead camera recorded their activity. Measures taken from video recordings included the number of open arm entries (4 paws in an open arm), the number of stretch attends into open arms (2 paws in an open arm), the total percentage of time spent in open arms (time in open arm/[time spent in closed arms + time spent in open arms]), and the number of head dippings while in an open arm.

Light/dark box test—The L/D box consisted of a rectangular Plexiglas arena divided at the center by a wall to yield two areas measuring 24.1×18.4 cm each. The “light” part of the box was open-topped and had 3 transparent walls, while the “dark” part was a fully-enclosed black box. The dividing black wall between the light and dark sides of the box had a 10.2×5.1cm opening, which allowed mice to move freely between the sides. Testing took place with an overhead lamp providing 500 lux in the light area and 2 lux in the dark area. Animals were placed on the light side of the apparatus facing the connecting doorway and allowed 10 minutes to move freely. Behavior coding started after animals first enter the dark side. Measures made from video recordings included number of stretch attends towards the light chamber (2 paws in light side), number of entries into the light chamber (4 paws in light side), total time spent in the light chamber, and number of rearings made during time spent in the light chamber (number of rears/time in light side).

Blood collection for CORT sampling

To study CORT hormone response, we exposed mice to the L/D box two hours into their active phase. Mice were anesthetized with isoflurane and decapitated for blood collection. Blood was collected into heparinized tubes, which were centrifuged at 8°C for 20 minutes at 3000 rcf. Plasma samples were stored at –80°C until analyses. Samples were assayed for corticosterone using Coat-a-Count Corticosterone kits (Diagnostics Products Corporation, Los Angeles, CA. USA) at the Diagnostic Center for Population and Animals Health at MSU. All samples were run in duplicate and the average of the two samples from each mouse was used for analysis. Blood samples for basal T level analysis were taken from intact adult WT and *iTfm* animals. All procedures were the same as mentioned above, and Coat-a-Count Total Testosterone kits were used for T assays (Diagnostics Products Corporation, Los Angeles, CA. USA).

Statistics

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

Experiment 2—Analysis for the OF and NO tests consisted of one-way ANOVAs with genotype (WT versus *iTfm*) as the independent factor. For the EPM and LD box tests, an

initial two-way ANOVA was run with genotype (WT versus *iTfm*) and test order (EPM/LD versus LD/EPM) as independent factors. As these did not reveal a test order effect, one-way ANOVAs were conducted with genotype as the independent factor.

Experiment 3—Analysis consisted of a two-way ANOVA with group (WT+T, *iTfm*+T, WT+B) and blood collection time (baseline, 20, 40, 60, 120 minutes after exposure to the L/D box) as independent factors, followed by a post-hoc LSD test. Differences were considered significant when $p < 0.05$. For two-way ANOVAs and pairwise comparisons that showed significant differences, eta squared and Cohen's d effect sizes were calculated, respectively.

Results

Experiment 1: Validation of the *iTfm* model in the resting phase

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

For the NO test, there was a significant main effect of genotype on the number of rears ($\eta^2 = .015$) because WT males reared more than *iTfm* mice ($d = .72$). There was no main effect of hormone or interaction, paralleling what we found in the OF. Post-hoc comparisons confirmed that WT+T mice reared more than T-treated *iTfm* males ($d = .755$; Figure 3B). T treatment also reduced the latency to approach the novel object, but only in WT animals; a post-hoc LSD test showed that WT+T animals approached the object faster than WT+B mice ($d = .887$; Figure 3C). *iTfm* males in both groups were intermediate on this measure, not differing from either WT group. No main effect of genotype or hormone, and no interactions were found for number of object visits and total time spent with the object.

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

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

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

As in the previous tests during the resting phase, we found a difference between WT and *iTfm* animals in the number of rears in the OF test in the active phase ($d=.941$; Figure 4A), but no significant differences for time spent in the center, latency to enter the center, or total time spent in the center.

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

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

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

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

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

Discussion

ARs appear to play a role in mediating the anxiolytic effects of testosterone in mice, as measured by anxiety-related behaviors and HPA activity in both the resting and active

phases of the circadian cycle. Using cre/lox technology to recapitulate the spontaneous Tfm model (Zuloaga et al., 2008a), we replicated the role of ARs in the anxiolytic effects of T in a so-called “induced” Tfm (*i*Tfm) model and also expanded on these findings. Specifically, T treatment reduced some indices of anxiety only in WT males, not *i*Tfm males. Compared to *i*Tfm+T male mice, WT+T males entered the open arms of the EPM more times, took less time to approach a novel object in the NO test, and showed less exploration in the OF and NO tests, as indicated by the number of rearings. Testing conditions in this first experiment replicated the phenomenon found earlier (Zuloaga et al., 2008a) where *s*Tfm and WT male mice were tested for anxiety in their resting phase (lights on). Both studies indicated an anxiolytic role of AR, although there were some differences between the two Tfm models in terms of which parameters displayed statistically significant differences (Zuloaga et al., 2008a). This may be due to minor differences in how the tests were conducted or scored, as the testing staff was different in the two studies. In *s*Tfms, the natural mutation is caused by a single base deletion in the coding region of the n-terminus, which creates a change in the reading frame, resulting in a truncated AR that lacks both DNA and steroid binding domains (Charest et al., 1991). In *i*Tfms, exon 2 of the AR gene is excised (De Gendt et al., 2004). Exon 2 codes for the 1st zinc finger of the AR DNA-binding domain, and, though this also creates a premature termination of transcription, the resulting transcripts would be different, but as no functional protein is produced in either model, it is difficult to see how the different transcripts would affect behavior.

The role of functional AR was also examined in a different cohort of mice during their active phase (lights off) to assess the possibility that anxiety-related behavior and the apparent role of AR might be affected by circadian phase. To a large extent, we found the same pattern of differences in both phases: WT males reared more in the OF and NO tests, and performed more stretch attends in the EPM, a measure of higher anxiety (McLean et al., 2011). However, differences in anxiety-like behavior between WT and *i*Tfm males in the LD box were revealed only in the active phase, with *i*Tfm males rearing significantly less and spending significantly less time on the light side of the box than WT controls, suggesting an effect of photoperiodicity on the anxiolytic effects of activated AR in this particular test. Two potential mechanisms may underlie this interaction. First, T might work through functional AR in WT mice to dampen the effects of rising CORT levels that occur during the active phase (Halberg et al., 1959; Zuloaga et al., 2011b), which in turn may reduce anxiety-like behavior in the LD box. The effect that we saw of T on CORT levels in WT males in the active phase (Figure 5) is consistent with this view. Second, T and AR may reduce the anxiogenic effects that bright light has on animals during their active phase through a mechanism that is independent of CORT. Since the core of the suprachiasmatic nucleus of the hypothalamus (SCN) responds to photic inputs to the retina, and core cells contain AR (Karatsoreos and Silver, 2007), T may act directly on SCN neurons to regulate their response to light.

Results based on the OF, NO, EPM and LD box tests during the resting and active phases leave questions still unanswered. First, it is not apparent why the anxiolytic effects of AR affect some measures of anxiety-related behavior in these tests and not others, considering that they are all exploration-based anxiety tests. Other studies (Juntti et al., 2010; Raskin et

al., 2009) also suggest that T signaling through AR regulates some, but not all, behaviors, including anxiety-related behaviors. Second, since *iTfm* mice lack AR throughout development and AR is crucial for differentiation of several brain regions and behaviors (Bodo and Rissman, 2007; Dugger et al., 2007; Durazzo et al., 2007; Garcia-Falgueras et al., 2005; Jones and Watson, 2005; Meaney et al., 1983; Morris et al., 2005; Olsen and Whalen, 1981; Rizk et al., 2005; Zuloaga et al., 2008b), it is unclear whether the effects seen are solely due to the adult activational effects of T.

Here, we also demonstrate for the first time that the capacity for T to reduce HPA activity requires a functional AR. WT mice treated with T show lower basal CORT levels than WT +B or *iTfm*+T males. When exposed to the LD box, all animals showed a CORT increase from their respective baseline levels, which tapers down with time; however, only WT+T males return to baseline levels by 60 minutes after exposure, while WT+B and *iTfm*+T take longer, indicating a capacity for T to curtail HPA activity through functional AR. Neither the presence of T alone nor functional AR alone was sufficient to curtail HPA activity, as CORT levels in *iTfm*+T and WT+B mice remained above baseline after 60 minutes. That intact WT male mice have lower *baseline* CORT compared to mice that lack functional AR has previously been shown in our lab based on *sTfm* mice (Zuloaga et al., 2008a), but here we additionally demonstrate that T acts through functional AR to lower HPA response and recovery. Since previous work was conducted in the resting phase (Zuloaga et al., 2008a), the present finding also extends the importance of T and AR in regulating HPA activity to the active phase. Our results also agree with Evuarherhe and colleagues' (2009) finding that T-treated castrated adult rats show lower basal corticosterone levels than castrated controls. In addition, castration increases corticotropin releasing hormone and parvocellular arginine vasopressin mRNA in the paraventricular nucleus of the hypothalamus, a phenomenon that is abolished with T restoration (Evuarherhe et al., 2009; Zhou et al., 1994). It is possible that AR mediates these modulatory effects of T on mRNA levels.

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

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

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Highlights

- Testosterone (T) acts through androgen receptors (AR) to produce anxiolytic effects
- Photoperiod interacts with AR's capacity to modulate some anxiety-related behavior
- T acting through AR lowers baseline corticosterone (CORT) levels
- T acts through AR to hasten HPA return to baseline levels after mild stress
- Cre-lox recombination of AR allele disrupts testosterone's anxiolytic effects

Figure 1.

The external phenotype of *iTfm* males is feminine. AGD: anogenital distance. Compared to WT males, *iTfm* males have a visibly shorter anogenital distance (A vs. B) and have much smaller and undescended testis (arrow in C vs. D). *iTfm* males also have external nipples (arrows in A) typical of WT females but not WT males (B).

Figure 2. Brain AR immunoreactivity confirms full AR KO in *iTfm* animals. Robust nuclear AR immunoreactivity is present in the amygdala (A) and hippocampus (C) of WT males, while absent in the amygdala and hippocampus (B and D, respectively) of *iTfm* males. Black scale bar: 200 μ m; white scale bar: 40 μ m.

Figure 3.

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

Figure 4.

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

Figure 5.

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