

Published in final edited form as:

Horm Behav. 2008 November ; 54(5): 726–734. doi:10.1016/j.yhbeh.2008.07.013.

Androgens with activity at estrogen receptor beta have anxiolytic and cognitive-enhancing effects in male rats and mice

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Abstract

Testosterone (T) and its metabolites may underlie some beneficial effects for anxiety and cognition, but the mechanisms for these effects are unclear. T is reduced to dihydrotestosterone (DHT), which can be converted to 5 α -androstane,3 α ,17 β -diol (3 α -diol) and/or 5 α -androstane-3 β ,17 β -diol (3 β -diol). Additionally, T can be converted to androstenedione, and then to androsterone. These metabolites bind with varying affinity to androgen receptors (ARs; T and DHT), estrogen receptors (ER β ; 3 α -diol, 3 β -diol), or GABA_A/benzodiazepine receptors (GBRs; 3 α -diol, androsterone). Three experiments were performed to investigate the hypothesis that reduced anxiety-like and enhanced cognitive performance may be due in part to actions of T metabolites at ER β . *Experiment 1:* Gonadectomized (GDX) wildtype and ER β knockout mice (β ERKO) were subcutaneously (SC) administered 3 α -diol, 3 β -diol, androsterone, or oil vehicle at weekly intervals, and tested in anxiety tasks (open field, elevated plus maze, light–dark transition) or for cognitive performance in the object recognition task. *Experiment 2:* GDX rats were administered SC 3 α -diol, 3 β -diol, androsterone, or oil vehicle, and tested in the same tasks. *Experiment 3:* GDX rats were androsterone- or vehicle-primed and administered an antagonist of ARs (flutamide), ERs (tamoxifen), or GBRs (flumazenil), or vehicle and then tested in the elevated plus maze. Both rats and wildtype mice, but not β ERKO mice, consistently had reduced anxiety and improved performance in the object recognition task. Androsterone was only effective at reducing anxiety-like behavior in the elevated plus maze and this effect was modestly reduced by flumazenil administration. Thus, actions at ER β may be required for T's anxiety-reducing and cognitive-enhancing effects.

Keywords

Anxiety; Cognition; 3 α -diol; 3 β -diol; Androsterone; Estrogen receptor beta

Introduction

Variations in the levels of testosterone (T) and of its metabolites may influence affect and / or cognition in men. Men who have higher endogenous levels of T have a lower incidence of depression (Earls, 1987). Conversely, young hypogonadal men, with low endogenous T and

dihydrotestosterone (DHT) levels, are more likely to be diagnosed with an anxiety or depressive disorder, and exhibit decreased performance in some cognitive tasks (Howell and Shalet, 2001; Kaminetsky, 2005). Treatment for these men includes T replacement, which can increase positive, and decrease negative, mood and improve cognition (Zitzmann, 2006). A similar pattern is observed in aging men, where T levels, along with age, negatively correlate with anxiety, and with decreased performance in visuospatial tasks (Janowsky, 2006; Janowsky et al., 1994; Li et al., 2002). Administration of T to these men reinstates their affective and cognitive performance (Alexander et al., 1998; Delhez et al., 2003; Janowsky, 2006). Together these data implicate androgens as having anxiolytic and cognitive-enhancing effects among men.

In animal models of androgen-deficiency, androgens can mediate both affective and cognitive behavior. Removing rats' primary source of endogenous androgens through androgen extirpation (i.e. castration or gonadectomy- GDX) results in increased anxiety-like behavior, and detriments in cognitive performance (Ceccarelli et al., 2001; Frye and Seliga, 2001; Edinger et al., 2004). Replacing their extirpated androgens decreases anxiety-like behavior in the open field, elevated plus maze, and defensive freezing tasks (Aikey et al., 2002; Edinger et al., 2004; Frye and Edinger, 2004) and enhances cognitive performance in the inhibitory avoidance (Edinger and Frye, 2004a) and conditioned contextual fear (Edinger et al., 2004) tasks. Administration of androgens to intact rodents can also decrease anxiety-like behavior and enhance learning and memory. When given T (Bitran et al., 1993) or anabolic steroids (Bitran et al., 1993; Rojas-Ortiz et al., 2006), intact male rats exhibited decreased anxiety-like behavior in the elevated plusmaze and Vogel paradigms. Together, these data from animal models demonstrate that androgens modulate affective and cognitive processes.

One possible mechanism by which T may influence hippocampally- mediated processes, such as affect and cognition, is through actions of its metabolites at estrogen receptors (ERs.; Frye et al., 1996; Pak et al., 2005). Estrogens and androgens have actions in the hippocampus to increase plasticity (Mukai et al., 2006). There are two isoforms of ER, estrogen receptor alpha and beta (referred to as ER α and ER β). Studies indicate that ER β , which is found in the hippocampus (Zhang et al., 2002), facilitates cell proliferation and learning and memory (Walf and Frye, 2008; Walf and Frye, 2007a; Rissman et al., 2002). Administration of selective ER modulators (SERMs) to female rats, which specifically target ER β , but not ER α , enhances cognition, and reduces anxiety-like, and depressive behaviors of ovx rats or wildtype mice, but not those that are ER β knockouts (β ERKO; Rhodes and Frye, 2006; Walf and Frye, 2005; Walf et al., 2008). Although T and its 5 α -reduced metabolite, DHT, do not bind to ERs in physiological concentrations, T can be aromatized to 17 β -estradiol (E₂; MacLusky et al., 1987), which binds to both ER α and ER β , and has anxiolytic-like and (reviewed in Walf and Frye, 2006), cognitive-enhancing effects in rodents (Frye and Rhodes, 2002; Frye et al., 2007; Rhodes and Frye, 2006; Walf et al., 2006; Luine, 2008). Alternatively, T is either metabolized by 5 α -reductase to dihydrotestosterone (DHT), which is then converted to 5 α -androstane-3 α ,17 β -diol (3 α -diol) and 5 α -androstane-3 β ,17 β -diol (3 β -diol), or T can also be converted to androstenedione and then further reduced to androsterone (Brown et al., 1994; Frye et al., 2007; Luttge, 1979). Administration of 3 α -diol alone decreases anxiety-like behavior in tasks, such as the open field and elevated plus maze, and increases cognitive performance in the inhibitory avoidance, conditioned fear, and place learning tasks, similar to that observed with T and DHT administration (Edinger and Frye, 2004a; 2005; 2007; Edinger et al., 2004; Frye and Lacey, 2001; Frye et al., 2001). Blocking formation of 3 α -diol through administration of indomethacin can reverse these beneficial effects of androgens on affect and cognition (Frye and Walf, 2004). 3 α -diol may bind to ER β and has a high affinity for GABA/ benzodiazepine receptors (GBRs), while 3 β -diol can bind with high affinity to ER β and is a

negative modulator of GBRs (Handa et al., 2008; Pak et al., 2005; Edinger and Frye, 2007; Frye et al., 2007; Gee, 1988; Masonis and McCarthy, 1995). In particular, 3 β -diol not only has a high affinity for ER β , but, once bound, activates transcription with high potency (Lund et al., 2005). In addition, 3 α -diol-replaced GDX rats infused intracerebroventricularly with antisense oligonucleotides (AS-ODNS) targeted against ER β , but not ER α , had poorer inhibitory avoidance performance compared to rats infused with vehicle (Edinger and Frye, 2007). Together, these findings suggest that androgens' affective and cognitive effects may be mediated through actions at ER β .

In order to assess whether ER β is a potential target for androgens' anxiety-reducing and/or cognitive-enhancing effects, three experiments were conducted. In Experiment 1, GDX β ERKO and age-matched wildtype (WT) mice were administered sesame oil vehicle or T metabolites that bind to ER β (3 β -diol- Handa et al., 2008), GBRs (androsterone- McMahon and France, 2003) or both (3 α -diol- Edinger and Frye, 2007). Anxiety behavior was assessed using the open field, elevated plus maze, and light–dark transition task, and cognition was measured using the object recognition task. In Experiment 2, the effects of these androgen treatments were assessed in GDX rats in the same battery of tests as were the mice in Experiment 1. Finally, Experiment 3 was designed to elucidate whether some of the anxiolytic effects that androsterone exerts are via GBRs, ERs, and/or androgen receptors (ARs). As such, GDX rats were administered either a GBR (flumazenil; Hoffman and Warren, 1993), ER (tamoxifen; MacGregor and Jordan, 1998), or AR (flutamide; Simard et al., 1986) antagonist in conjunction with an injection of androsterone or sesame oil vehicle, and then tested in the elevated plus maze paradigm. We hypothesized that, if ER β is a substrate for androgens' beneficial effects on affective and/or cognitive behavior, then administration of 3 α -diol or 3 β -diol, but not androsterone, will reduce anxiety-like behavior, in the open field, elevated plus maze, and light–dark transition, and improve cognitive performance in the object recognition task of GDX rats and WT, but not β ERKO, mice. Furthermore, if androsterone does not have some anxiolytic effects, then it will have limited task-specific effects on reducing anxiety-like behavior.

Materials and methods

These methods were pre-approved by the Institutional Animal Care and Use Committee at the University at Albany-SUNY.

Animals and housing

Experiment 1—Mouse strain and genotyping—Male mice ($N=167$), approximately 55 days of age, were either WT ($n=51$) or β ERKO ($n=116$), derived from breeder pairs purchased from Jackson Laboratories (Bar Harbor, ME) and bred onto a C57BL/6 background. Mice were group-housed (3–5 per cage) in a temperature-controlled room (21 ± 1 °C) in the Laboratory Animal Care Facility. Mice were maintained on a 12/12-hour reversed light cycle (lights off at 8:00 am) with continuous access to Purina Rodent Chow and tap water in their home cages.

Genotype of mice cannot be determined based on phenotypic characteristics. As such, genomic DNA was isolated from tails and analyzed by PCR in our laboratory and/or the Molecular Core Facility at SUNY Albany to determine genotype of our experimental mice. A brief description of the PCR procedure utilized is as follows. DNA was denatured at 94 °C for 3 min, followed by 35 cycles of amplification: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final primer extension step at 72 °C for 2 min. Specific primers, obtained from Integrated DNA Technologies (Coralville, IL), used: ESR2-1, which lies upstream of insertion site in exon 2 (5'-GTTGTGCCAGCCCTGTTACT-3'), ESR2-1, which lies downstream of the insertion site in exon 2 (5'-TCACAGGACCAGACACCGTA-3'), and

ESR2-3, a neo gene-specific primer (5'-GCAGCCTCTGTTCCACATACAC-3'). Bands of approximately 106 and 160 base pairs were amplified for WT and β ERKO mice, respectively (Walf et al., 2008).

Experiments 2 and 3—Rats—Subjects ($N=103$; $N=75$) were male Long–Evans rats, approximately 55 days old, obtained from our in-house breeding colony (original stock from Taconic Farms, Germantown, NY). Rats were group-housed (3–4 per cage) in polycarbonate cages (45×24×21 cm) in the Laboratory Animal Care Facility of The Life Sciences Research Building at The University at Albany-SUNY in a controlled temperature room (21±1 °C) that was maintained on a 12:12 reverse light cycle (lights off at 8:00 am). Rats had continuous access to Purina Rodent Chow and tap water in their home cages.

Surgery

Young adult mice were GDX under sodium pentobarbital (80 mg/kg or to effect; Fort Dodge Animal Health, Fort Dodge, IA) at least 3–6 weeks before behavioral testing. Young adult rats were GDX under xylazine (12 mg/kg; Bayer Corp., Shawnee Mission, KS) and ketamine (60 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) anesthesia at least 3–6 weeks before behavioral testing.

Screening procedure in mice

Prior to inclusion in the study, each mouse was examined for general health and normative responses to external stimuli (as described in Crawley et al., 2007). Observers who were blind to the genotypes of mice performed the behavioral evaluations, which included: the general appearance of mice (clean fur, whiskers, posture, gait, muscle tone), normative behavior (i.e. fur grooming, nestbuilding with Nestlet cotton squares provided in home cage, huddling with cage mates, ability to cage climb, paw withdrawal when gently pulled), and reflexes (blinking to cotton swab placed close to eyes, ear twitch when cotton swab is gently placed on ear). All mice were included in the study as no differences were noted between the WT or β ERKO mice for these measures.

Handling procedure in mice

Mice underwent a 5-day habituation/handling protocol, as per published methods (Frye et al., 2006), to accustom mice to steroid injections, handling by the investigator, and behavioral testing. Briefly, in the following order, mice were exposed to: handling, cage transfer, placement in a scale, travel on a cart in while in their homecages, subcutaneous (SC) injections with 0.2 cm³ vegetable oil SC, and placement in novel environment for 5 min.

Androgen administration

For Experiments 1 and 2, mice and rats were randomly-assigned to androgens with high affinity for GBRs (3 α -diol or androsterone), androgens with high affinity for ER β (3 α -diol or 3 β -diol) or vehicle. Rats and mice were administer sesame oil vehicle, 3 α -diol, 3 β -diol or androsterone acutely through SC injections (1 mg/kg) 1 h before testing in the anxiety tasks, and immediately following training in object recognition. These regimens have been used previously to produce physiological androgen levels (Edinger and Frye, 2004a; Fernandez-Guasti and Martinez-Mota, 2007). Crystalline androgens were obtained from Sigma (St. Louis, MO) and dissolved in sesame oil vehicle. For Experiment 3, animals were randomly-assigned to receive either androsterone or vehicle SC, in conjunction with an injection of intraperitoneal flumazenil (GBR antagonist; 15 mg/kg in 0.2% methyl-cellulose), flutamide SC (AR antagonist; 50 mg/kg in oil and 5% ethanol), tamoxifen SC (ER antagonist; 15 mg/

kg; in oil), or vehicle SC. Antagonists were obtained from Sigma (St. Louis, MO). Antagonist injections were given prior to androsterone/vehicle injections, 1 h before testing.

Procedure

Mice were tested once per week to assess behavior in the tasks described below. The order in which mice were tested through behavioral tasks was the same for all animals and was as indicated below. Rats were tested once in a single behavioral task (described below). Behavioral data were collected by trained observers and simultaneously video-recorded with a video-camera and/or videotracking system (Any-maze — Stoelting, Inc., Wood Dale, IL).

Behavioral testing

Open field—mice—Motor behavior of mice was assessed in a 39×39×30 cm activity monitor (AccuScan Instruments, Inc., Columbus, OH), as per previous methods (Frye and Walf, 2004). The activity chamber had a grid floor with a total of 16 equal squares delineated. An observer recorded the number of entries made by the mouse into the 12 peripheral or 4 central squares for 5 min, the number of interruptions in light beams in a horizontal plane were automatically recorded. Increased central entries are indicative of reduced anxiety-like behavior (Frye and Walf, 2004).

Open field—rats—The open field (76×57×35 cm) has a 48-square grid floor and was situated in a brightly-lit room. As per previously published methods, the number of central and peripheral squares (which were summed for total) that each rat entered during the 5 min test were recorded (Frye et al., 2000). Total entries made are an index of general motor behavior and an increased number of central entries is an index of anxiety.

Elevated plus maze—mice—The matte black stainless steel elevated plus maze (Columbus Instruments, Inc., Columbus, OH) has 2 open arms, which are 30 cm in length and 5 cm in width, and 2 closed arms, which are the same size but enclosed by 14.5 cm high walls. The arms of the maze are elevated 47.5 cm off the ground. Mice were placed in the center of the maze and the duration of open and closed arm entries (max latency=300 s) was measured. The time spent in the 2 open (5×40 cm) or closed (5×40×20 cm) arms was recorded for 5 min. Increased time spent on the open arms indicates decreased anxiety and the total arm entries made is an index of general motor behavior (Frye and Edinger, 2004, 2006; Walf and Frye, 2007b).

Elevated plus maze—rats—The elevated plus maze was situated in a brightly-lit room and consisted of four arms (2 open without walls and 2 enclosed by 30 cm high walls) 49 cm long and 10 cm wide, elevated 50 cm off the ground. Rats were placed at the junction of the open and closed arms and the number of entries and time spent on the open and closed arms were recorded (as per Frye et al., 2000; Walf and Frye 2007b). Total arm entries made in the plus maze are an index of general motor behavior and an increase in time spent on the open arms indicates decreased anxiety.

Light-dark transition task—mice—Mice are placed in the dark side of the chamber (24.5 cm×23.5 cm×35 cm) and allowed to move between the two chambers. Time spent in the light side of the box was recorded for 5 min (Frye et al., 2006).

Light-dark transition task—rats—Rats were placed on the side of a two-chambered box (30×40×40 cm) with white walls and floor and illuminated by a 40-watt light from above; the other side of the box was painted black and had a lid so it was not illuminated. The time spent on the light side of this chamber during 5 min compared to the dark side was

recorded (Walf and Frye, 2005). Increased time in the light side is indicative of decreased anxiety.

Object recognition—mice—The object recognition task, a test of non-spatial reference memory, was conducted in an open field box (46×57×30 cm), constructed of white laminate and located in a quiet room with bright lighting. Small, plastic, washable toys that have distinct circular (oranges, lemons, apples) or cone-like shapes (buoys, bottles) were utilized as target stimuli. A digital camera mounted on the ceiling above the testing chamber and connected to a computer with a videotracking system, objectively monitored and quantified animals' movements (Any-maze — Stoelting, Inc., Wood Dale, IL). Testing, which takes advantage of the natural affinity of rodents for novelty, was conducted as described previously (Walf et al., 2008; Frye and Walf, 2008). Briefly, during training, each mouse was placed in the open field box for 180 s and allowed to explore two identical objects (placed approximately 15 cm from the northeast and northwest walls). After 180 s, each mouse was removed from the box, immediately injected with vehicle or androgens and returned to its individual holding cage. One familiar object (identical to that which was used in training) and one novel object were placed in the same location of the box as was used during training. Whether the novel object was placed in the northeast or northwest location was counterbalanced within and between groups. The time spent exploring each object was recorded during both the training and testing phases using Any-maze video-tracking system. Object exploration was scored when the mouse was sniffing, or touching the object and was a body's length or less away from the object (i.e. less than 3 cm) while facing the object and/or oriented towards it. All mice investigated both objects during training for a similar duration. Four hours later, the time mice spent exploring the objects were recorded during testing. An increased percentage of time spent exploring the displaced object compared to the total amount of time spent exploring both objects during testing (duration spent with novel object / (duration spent with novel object+duration spent with familiar object)×100) was considered an index of enhanced performance in this task. Inclusion criteria are that animals had to investigate the objects. This was operationalized using a criteria setup in the Anymaze behavioral tracking program for when animals investigate both objects for greater than 0.5 s during the training period. Chance levels of performance are when mice spend 50% of the total exploration time investigating each object.

Object recognition—rats—The object recognition task was done as described above in mice and previously in rats (Walf et al., 2006). This task was conducted in an open field box (76×57×35 cm), constructed of white laminate and located in a quiet room with bright lighting. The same target stimuli and methods of data collection were utilized. Each rat was placed in the open field box for 180 s and allowed to explore two identical objects (placed approximately 15 cm from the northeast and northwest walls). After 180 s, each rat was removed from the box, immediately injected with vehicle or androgens and returned to its individual holding cage. One familiar object (identical to that which was used in training) and one novel object were placed in the same location of the box as was used during training. Whether the novel object was placed in the northeast or northwest location was counterbalanced within and between groups. The time spent exploring each object was recorded during both the training and testing phases using Any-maze videotracking system. Object exploration was scored when the rat was sniffing, or touching the object and was a body's length or less away from the object while facing the object and/or oriented towards it. All rats investigated both objects during training. Four hours later, the time rats spent exploring the objects were recorded during testing.

Statistical analyses

For Experiment 1, two-way analyses of variance (ANOVAs) tests, with Fisher's *post hoc* tests, as appropriate, were utilized to determine effects of genotype (WT vs. β ERKO) and androgen condition (vehicle, 3 α -diol, 3 β -diol, or androsterone) on dependent measures. For Experiment Two, one-way ANOVAs, with Fisher's *post hoc* tests, as appropriate, were used to evaluate the effects of androgen condition in a given task. Two-way ANOVAs, with Fisher's *post hoc* tests, were used to evaluate effects of androgen condition (vehicle, 3 α -diol, 3 β -diol, or androsterone) by antagonist (flumazenil, flutamide, tamoxifen, or vehicle; androsterone or vehicle) on behavioral measures. The α level for statistical significance was a p-value of <0.05 and a trend was considered when $p < 0.10$.

Results

Experiment 1

Open field—There was a main effect of genotype for central entries ($F_{3,159}=3.17$, $p < 0.05$) due to 3 α -diol or 3 β -diol increasing the number of central entries into the open field of WT, but not β ERKO, mice (Fig. 1). The main effects of genotype ($F_{1,159}=40.93$, $p < 0.01$) was due to WT mice making more total entries than β ERKO mice (Table 1).

Elevated plus maze—Androgen condition and genotype interacted ($F_{3,159}=4.09$, $p < 0.01$) insofar as 3 α -diol or 3 β -diol to WT, but not β ERKO, mice increased the duration of time (s) spent on the open arms compared to vehicle administration. However, androsterone increased the time spent on the open arms of the elevated plus maze among both WT and β ERKOs (Fig. 2) compared to vehicle.

There was a main effect of genotype ($F_{1,159}=6.49$, $p < 0.05$; Table 1), but not androgen condition, for the number of total entries in the elevated plus maze. WT mice made more total entries than did β ERKO mice. Additionally, 3 α -diol, 3 β -diol, or androsterone treatment, compared to vehicle, significantly increased the percent of open entries (Table 1) made by the WT, but not β ERKO animals ($F_{1,159}=5.560$, $p < 0.01$). A similar pattern was observed for percent open arm time (Table 1), where WT animals who received 3 β -diol or 3 α -diol spent a significantly higher percentage of their time on the open arm ($F_{1,159}=4.095$, $p < 0.01$).

Light dark transition-mice—Androgen condition and genotype interacted ($F_{3,159}=3.83$, $p = 0.01$), such that WT, but not β ERKO, mice administered 3 α -diol or 3 β -diol spent a significantly longer duration of time (in seconds) in the white chamber compared to mice administered vehicle (Fig. 3).

Object recognition—Androgen and genotype interacted ($F_{3,120}=3.07$, $p < 0.05$), where WT, but not β ERKO mice administered 3 α -diol or 3 β -diol compared to vehicle or androsterone, had an increased percentage of time spent with the novel object (Fig. 4).

Experiment 2

Open field—There was an increase in central entries in the open field (Fig. 1, bottom) for rats administered 3 α -diol or 3 β -diol ($F_{3,99}=5.82$, $p < 0.01$) compared to those administered vehicle or androsterone. There was no significant effect of androgen administration for the number of total entries in the open field.

Elevated plus maze—Administration of 3 α -diol, 3 β -diol, or androsterone significantly increased amount of time spent on the open arms of the elevated plus maze ($F_{3,94}=3.00$, $p < 0.05$; Fig. 2, bottom) compared to vehicle administration. There was no effect of androgen

treatment on total open arm entries compared to that of vehicle administration. Additionally, compared to rats administered vehicle, rats administered 3 β -diol, 3 α -diol, or androsterone made significantly greater percentage of entries (Table 1) onto the open arms ($F_{3,93}=3.501$, $p<0.05$), as well as spent a significantly greater percentage of time in the elevated plus maze (Table 1) out on the open arm ($F_{3,93}=3.243$, $p<0.05$).

Light dark transition-rats—A significant increase in time spent in the light chamber of the light dark transition box was observed in rats administered 3 α -diol or 3 β -diol compared to androsterone or vehicle ($F_{3,100}=2.58$, $p=0.05$; Fig. 3, bottom).

Object recognition—Administration of 3 α -diol or 3 β -diol to rats increased percentage of time spent investigating the novel object in the object recognition task ($F_{3,105}=3.3$, $p<0.05$; Fig. 4, bottom), compared to androsterone or vehicle administration.

Experiment 3

Elevated plus maze—There was a main effect for androsterone treatment, such that rats administered androsterone, compared to vehicle, had increased open arm time ($F_{1,59}=7.005$, $p<0.01$; Fig. 5), number of open arm entries ($F_{1,59}=8.332$, $p<0.01$), percentage of time in the plus maze on the open arms ($F_{1,59}=6.834$, $p<0.05$; Fig. 5), but not for percentage of open arm entries (Fig. 5). Additionally, in open arm entries, there was a main effect for number of open arm entries ($F_{3,59}=3.183$, $p<0.05$) where flumazenil administration resulted in significantly fewer open arm entries (1.0+0.3) compared to flutamide administration (1.9+0.5).

Discussion

The findings supported the hypothesis that actions of androgens at ER β may underlie their cognitive-enhancing and anxiolytic effects. Mice deficient in ER β did not demonstrate the reductions in anxietylike behavior in the elevated plus maze, open field, or light–dark transition; nor did they benefit from the cognitive enhancements observed in the WT animals that were administered 3 α -diol or 3 β -diol. Among GDX rats, 3 α -diol and 3 β -diol, which can bind to ER β with high affinity, significantly decreased anxiety in the elevated plus maze, open field, and light–dark transition tasks compared to vehicle administration. 3 α -diol and 3 β -diol significantly increased performance in the object recognition task over administration of vehicle and androsterone, which does not bind to ER β . Experiment 3 demonstrated that androsterone can have anxiolytic effects in the elevated plus maze. Despite the addition of antagonists for ARs, ERs, and GBRs, there was a main effect for androsterone administration in decreasing anxiety-like behavior. However, flumazenil did significantly block this effect for open arm entries. Thus, these data suggest that one potential target of androgen metabolites may be ER β for improved performance in the open field, elevated plus maze, light–dark transition task, and object recognition task.

Our findings are congruent with past research showing that androgens have cognitive-enhancing effects. GDX can produce cognitive deficits in rodents, which can be reversed with T-replacement (Frye and Seliga, 2001), or replacement of its metabolites (Edinger and Frye, 2004a). Given that different metabolites bind with varying affinity to different substrates to produce these effects, it is possible that androgens' cognitive-enhancing effects work through a combination of actions of its metabolites at ARs, GBRs, and/or ER β (Edinger and Frye, 2007). However, the cumulative results obtained from experiments one and two implicate actions at ER β as having more robust effects on cognition, which is supported by additional research that utilized ER AS-ODNs for both ER β and ER α . When administered directly into the hippocampus, along with 3 α -diol, ASODNs for ER β and not

ER α decreased cognitive performance in GDX rats (Edinger and Frye, 2007). The present findings extend this previous data by illustrating that administration of 3 β -diol alone, which cannot be back-converted to T or aromatized to E $_2$, is effective at enhancing cognitive performance. In addition, administration of androgens to WT, but not β ERKO mice, was effective at enhancing cognitive performance. Together, these findings show that actions at ER β are, in part, responsible for mediating androgen's cognitive-enhancing effects.

The present findings are consistent with past research illustrating the importance of T and its metabolites in mediating anxiolysis in rodents. GDX increases anxiety of male rats, an effect that can be reversed through administration of T (Fernandez-Guasti and Martinez-Mota, 2003; Frye and Seliga, 2001), and of T's metabolites (Bitran et al., 1993; Edinger and Frye, 2004b). Additionally, studies have found DHT and 3 α -diol to be just as effective, if not more so, than sole administration of T, at decreasing anxiety in GDX rats (Edinger and Frye, 2004a; Edinger and Frye, 2004b). Additionally, work by Fernández-Guasti and Martínez-Mota, (2005) demonstrated, that although androsterone does bind to GBRs, it does not always produce strong anxiolytic effects, either chronically or acutely. Experiment 3 demonstrated that androsterone can have anxiolytic effects. Our results are congruent with previous findings from other laboratories demonstrating that androsterone does have modest effects on reducing anxiety-like behavior (Aikey et al., 2002). Despite the addition of antagonists for ARs, ERs, and GBRs, there was a main effect for androsterone to decrease anxiety-like behavior in the elevated plus maze, as defined by increasing open armtime, percent open arm time, and open arm entries. However, flumazenil did block this effect for open arm entries. Similarly, flumazenil did not significantly alter defensive burying time when testosterone propionate, which is capable of being metabolized into androsterone and 3 α -diol, was concurrently administered (Fernández-Guasti and Martínez-Mota, 2005). This suggests that, although androsterone can modestly reduce anxiety-like behavior in a task-specific manner, likely through its activation of GBRs (Fernandez-Guasti and Martinez-Mota, 2005), which are a common target for benzodiazepines prescribed for anxiety (reviewed in Nemeroff, 2003), these effects may not be as robust as those produced via ER β 's activation, and may require more chronic administration to produce a consistent significant anxiolytic effect (Aikey et al., 2002). The current data extend these previous findings by implicating ER β as a potential site for androgens' anxiolytic effects. Administration of 3 β -diol, which cannot be backconverted to T, or be aromatized into E $_2$, resulted in decreased anxiety-like effects similar to those seen when T is administered alone. In addition, administration of androgens to β ERKO mice, which lack functional ER β , was not effective at reducing anxiety-like behavior. Together, this evidence suggests that the 5 α -reduced metabolites of T mediate anxiety-like behavior via ER β .

It is important to consider actions at intra-cellular receptors (ARs and ERs) versus rapid actions at membrane receptors (GBRs) when considering these results. T has been known to act via both classical and non-classical signaling mechanisms (Walker and Cheng, 2005). Both T and DHT have a high affinity for ARs (Roselli et al., 1987). When ARs are blocked by administration of an AR antagonist, flutamide, the beneficial effects of DHT are not seen (Edinger and Frye, 2006). However, unlike T and DHT, 3 α -diol and 3 β -diol do not bind readily to ARs (Roselli et al., 1987). Although it has long been thought that androgens non-classical membrane actions occurred through aromatization to E $_2$, current research suggests that T and/or its metabolites may act directly at membrane receptors. For example, 3 α -diol has a high affinity for GBRs (Handa et al., 2008; Pak et al., 2005), and actions at GBRs can decrease anxiety-like behavior (reviewed in Nemeroff, 2003). However, in the current experiment, administration of 3 β -diol, which does not bind to GBRs, and instead binds with high affinity to ER β , resulted in decreased anxiety-like behavior, at levels similar to that of animals administered 3 α -diol alone. In future studies, it will be important to examine these effects at genomic and non-genomic targets. Until recently, much of the research done to

determine that 3β -diol works through ER β has been done on cell cultures. Little behavioral research using animal models exists that can confirm these results. However, our research findings are analogous to those found using the animal and human derived cell cultures. Using mouse hippocampus-derived neuronal cell lines (HT-22), it has been determined that 3β -diol can bind to ER β , and not to AR, and that it is this binding that is responsible for direct regulation by androgens in the neuronal cell types that lack ARs (Pak et al., 2005). Similar findings utilizing human neuroblastoma-derived cell line SK-N-SH demonstrated that DHT and 3β -diol can stimulate arginine vasopressin (AVP) through ER β . AVP is a neuropeptide that can assist in the regulation of stress and anxiety (Pak et al., 2007). Evidence still exists that supports our findings on the importance of 3α -diol, and 3β -diol binding to, and exerting their effects through ER β , and not an AR. Despite these caveats, past findings support our current results demonstrating that 3β -diol can reduce anxiety-like behavior and increase cognitive performance.

In conclusion, these results suggest that T's anxiolytic and cognitive-enhancing effects may be mediated largely through its metabolites, 3α -diol and 3β -diol, and may require actions at ER β . This is supported by findings that GDX rats or WT mice administered 3α -diol and 3β -diol exhibited improved anxiety and cognition. We also found that, in mice lacking functional ER β , there were no affective or cognitive improvements with administration of 3α -diol or 3β -diol. Determining the precise route and mechanisms in which testosterone and/or its metabolites exert their beneficial effects have potential implications in regards to the ever increasing incidence of prostate cancer, proportion of aging individuals, and the continued use of enhancing steroids within the young and aging population.

Acknowledgments

This research was supported, in part, by grants from the Karo Bio Research Foundation, National Science Foundation (IBN03-16083), and National Institute of Mental Health (MH0676980), as well as an intramural faculty research award grant to CAF. Technical assistance provided by Fabiola Estrada, Mary Beth Gillepsie, Mary Unger, and Jari Willing is greatly appreciated.

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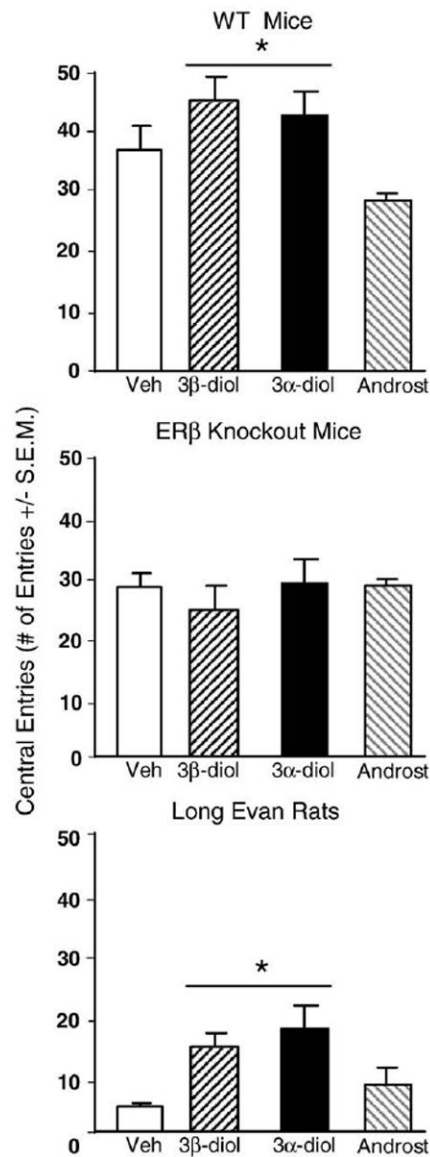


Fig. 1.

Represents mean central entries in the open field for: wildtype (WT) mice (top) administered acute vehicle control (white bar; $n=15$), 3β-diol (horizontally-striped bar; $n=11$), 3α-diol (black bar; $n=13$), or androsterone (androst; gray-stripe bar; $n=12$); to ERβ receptor knockout (βERKO) mice (middle) administered acute vehicle control (white bar; $n=21$), 3β-diol (horizontally-striped bar; $n=34$), 3α-diol (black bar; $n=34$), or androsterone (androst; gray-stripe bar; $n=29$); and to rats (bottom) administered acute vehicle control (white bar; $n=22$), 3β-diol (horizontally-striped bar; $n=26$), 3α-diol (black bar; $n=25$), or androsterone (androst; gray-stripe bar; $n=25$). Rats and WT mice administered 3α-diol or 3β-diol had significantly more central entries than animals administered androsterone or vehicle. * Denotes significant difference ($p<.05$).

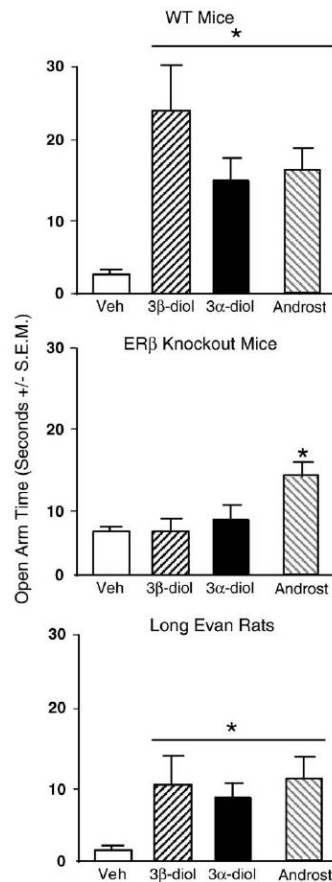


Fig. 2. Represents mean time spent on the open arm in the elevated plus maze for: wildtype (WT) mice (top) administered acute vehicle control (white bar; $n=15$), 3β-diol (horizontally-striped bar; $n=11$), 3α-diol (black bar; $n=13$), or androsterone (androst; gray-stripe bar; $n=12$); to ERβ receptor knockout (βERKO) mice (middle) administered acute vehicle control (white bar; $n=21$), 3β-diol (horizontally-striped bar; $n=34$), 3α-diol (black bar; $n=34$), or androsterone (androst; gray-stripe bar; $n=29$); and to rats (bottom) administered acute vehicle control (white bar; $n=22$), 3β-diol (horizontally-striped bar; $n=26$), 3α-diol (black bar; $n=25$), or androsterone (androst; gray-stripe bar; $n=25$). Rats administered 3α-diol, 3β-diol, or androsterone spent significantly more time on the open arm than those given vehicle. * Denotes significant difference ($p<0.05$).

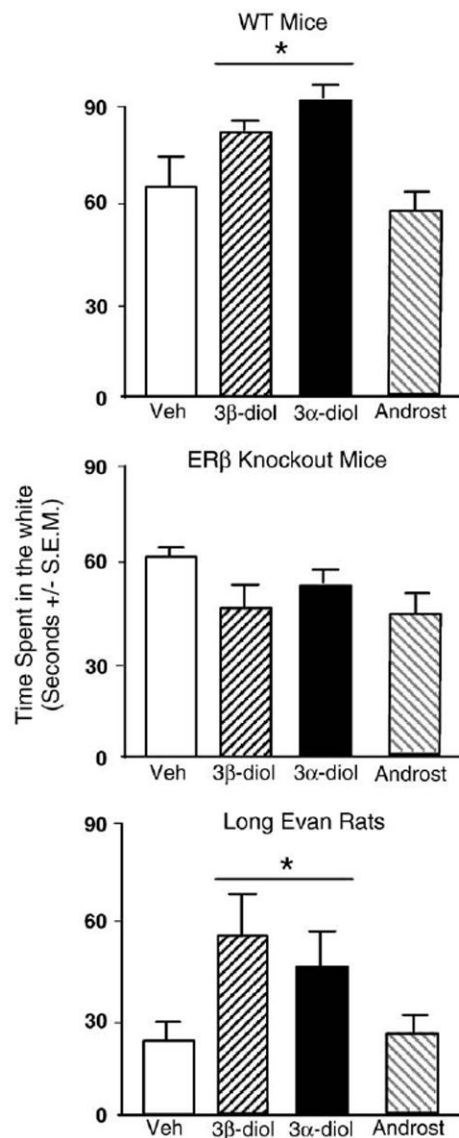


Fig. 3.

Represents mean time spent on the light side in the light–dark task for: wildtype (WT) mice (top) administered acute vehicle control (white bar; $n=15$), 3β-diol (horizontally-striped bar; $n=11$), 3α-diol (black bar; $n=13$), or androsterone (androst; gray-stripe bar; $n=12$); and to ERβ receptor knockout (βERKO) mice (middle) administered acute vehicle control (white bar; $n=21$), 3β-diol (horizontally-striped bar; $n=34$), 3α-diol (black bar; $n=34$), or androsterone (androst; gray-stripe bar; $n=29$); and to rats (bottom) administered vehicle control (white bar; $n=22$), 3β-diol (horizontally-striped bar; $n=26$), 3α-diol (black bar; $n=25$), or androsterone (androst; gray-stripe bar; $n=25$). Rats and WT, but not βERKO, mice administered 3α-diol or 3β-diol displayed significantly less anxiety-like behavior than those that received vehicle or androsterone. * denotes significant difference ($p < 0.05$).

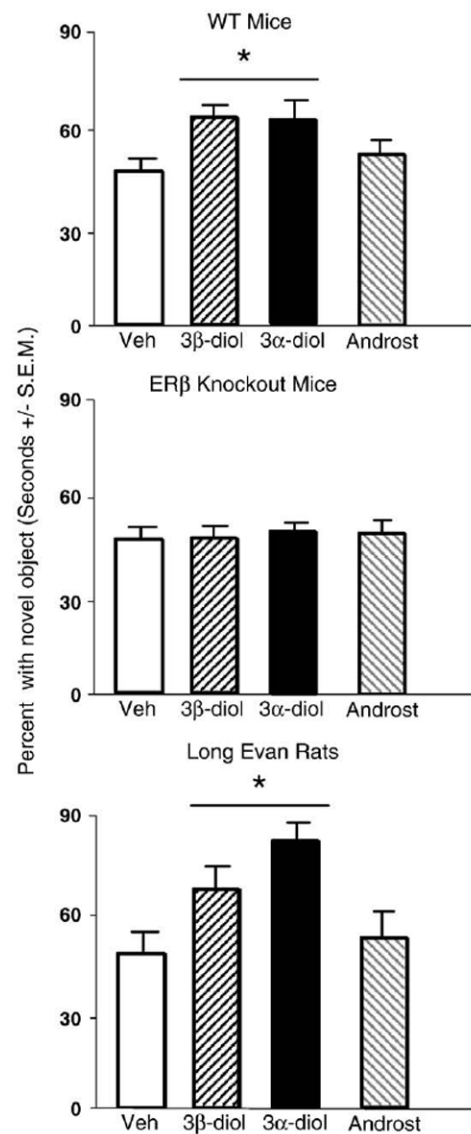


Fig. 4.

Represents mean percentage of time with novel object for: wildtype (WT) mice (top) administered acute vehicle control (white bar; $n=15$), 3β-diol (horizontally-striped bar; $n=11$), 3α-diol (black bar; $n=13$), or androsterone (androst; gray-stripe bar; $n=12$); to ERβ receptor knockout (βERKO) mice (middle) administered acute vehicle control (white bar; $n=21$), 3β-diol (horizontally-striped bar; $n=34$), 3α-diol (black bar; $n=34$), or androsterone (androst; gray-stripe bar; $n=29$); and to rats (bottom) administered vehicle control (white bar; $n=22$), 3β-diol (horizontally-striped bar; $n=26$), 3α-diol (black bar; $n=25$), or androsterone (androst; gray-stripe bar; $n=25$). 3α-diol and 3β-diol, but not vehicle or androsterone, significantly increased the percentage of time rats and WT, but not βERKO, mice spent exploring the novel object. * Denotes significant difference ($p<0.05$).

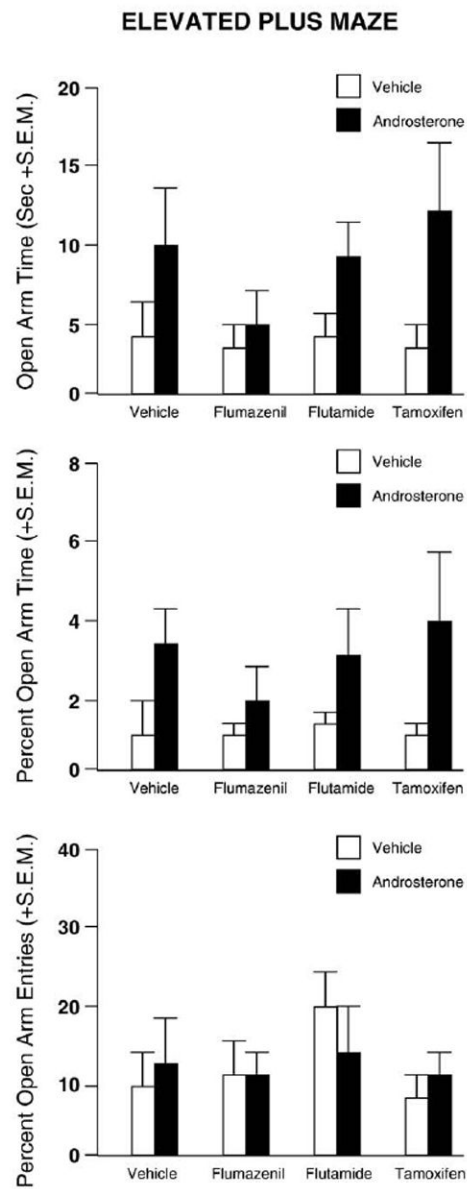


Fig. 5. Represents mean time spent out on the open arm, percentage of time on the open arm, and percentage of open arm entries for the elevated plus maze for acute coadministration of vehicle (white bars) or androsterone (black bars), with flumazenil (vehicle, $n=10$; androsterone $n=7$), flutamide (vehicle, $n=10$; androsterone, $n=10$), tamoxifen (vehicle, $n=10$; androsterone, $n=9$), or vehicle (vehicle, $n=9$; androsterone, $n=10$) in the elevated plus maze task. There was a significant main effect for animals administered androsterone spending significantly more time (top) and a greater percentage of time (middle) on the open arm than vehicle treated animals. There was no effect for percentage of open arm entries (bottom). * Denotes significant difference ($p<0.05$).

Table 1

Mice: Behavior (mean±sem) of vehicle-administered WT (*n*=15) or estrogen receptor β knockout (β ERKO; *n*=21) mice, 3β -diol-administered WT (*n*=11) or β ERKO (*n*=34) mice, 3α -diol-administered WT (*n*=13) or β ERKO (*n*=34) mice or androstereone (Androst) administered WT (*n*=12) or β ERKO (*n*=29)

Condition	OF-total entries	EPM-total open arm entries	EPM-% open arm time	EPM-% open arm entries
Wildtype mice*				
Vehicle	94.7±6.8	3.2±0.5	1.0±0.4	6.9±2.5
3β -diol	112.3±11.2	3.9±0.4	8.0±2.5**	26.6±4.1**
3α -diol	107.4±6.4	5.0±0.5	5.1±1.2**	21.3±3.6**
Androstereone	102.8±8.4	4.2±0.6	5.4±1.5**	20.1±3.3**
β ERKO mice				
Vehicle	146.9±8.8	7.7±1.2	2.1±0.4	13.5±2.4
3β -diol	162.4±4.6	9.6±1.4	2.3±0.6	11.1±1.9
3α -diol	164.5±10.5	10±1.3	2.5±0.8	10.1±2.4
Androstereone	131.6±4.1	10.8±1.2	4.8±0.8	15.9±2.1
Rats				
Vehicle	119.9±4.9	7.7±0.8	0.4±0.2	5.1±2.7
3β -diol	118.1±3.9	9.6±1.4	4.2±1.4**	17.4±4.1**
3α -diol	121.9±5.9	8.1±0.6	3.2±0.8**	16.9±3.3**
Androstereone	126.6±4.9	7.8±0.5	4.5±1.1**	20.1±3.3**

Rats: behavior (mean±sem) of vehicle administered (*n*=22), 3β -diol-administered (*n*=26), 3α -diol-administered (*n*=25) or Androst administered (*n*=25).

* Denotes significance <0.05 compared to β ERKO knockout mice.

** Denotes significance <0.05 compared to vehicle administration.