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# The Anxiolytic and Antidepressant-like Effects of Testosterone and Estrogen in Gonadectomized Male Rats

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## Abstract

**Background—**While the influence of testosterone levels on vulnerability to affective disorders is not straightforward, research suggests this hormone may confer some degree of resiliency in men. We recently demonstrated a role for the dentate gyrus in mediating testosterone's protective effects on depressive-like behavior in gonadectomized male rats. Here, testosterone may exert its effects through androgen receptor-mediated mechanisms or via local aromatization to estradiol.

**Methods—**Gonadectomized male rats were implanted with a placebo, testosterone, or estradiol pellet, and subsequent protective anxiolytic- and antidepressant-like effects of testosterone and its aromatized metabolite, estradiol, were then investigated in the open field and sucrose preference tests, respectively. Moreover, their influence on gene expression in the hippocampus was analyzed by genome-wide cDNA microarray analysis. Finally, the contribution of testosterone's aromatization within the dentate gyrus was assessed by local infusion of the aromatase inhibitor, fadrozole, whose efficacy was confirmed by LC-MS/MS.

**Results—**Both hormones had antidepressant-like effects associated with a substantial overlap in transcriptional regulation, particularly in synaptic plasticity- and mitogen-activated protein kinase pathway-related genes. Further, chronic aromatase inhibition within the dentate gyrus blocked the protective effects of testosterone.

**Conclusions—**Both testosterone and estradiol exhibit anxiolytic- and antidepressant-like effects in gonadectomized male rats, while similarly regulating critical mediators of these behaviors, suggesting common underlying mechanisms. Accordingly, we demonstrated that testosterone's protective effects are mediated, in part, by its aromatization in the dentate gyrus. These findings thus provide further insight into a role for estradiol in mediating the protective anxiolytic- and antidepressant-like effects of testosterone.

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**Keywords**

Depression; anxiety; hippocampus; testosterone; estradiol; aromatase

## Introduction

Androgens are suspected to serve a protective role in the development of affective disorders and can improve depressive symptoms in both men and women. However, the connection between testosterone levels and depression vulnerability is not readily apparent, as both low and high testosterone levels have been associated with depressive symptoms, along with equivocal efficacy reported in studies investigating testosterone as a standalone treatment or adjunct therapy in depressed individuals (1). Despite this, the incidence of depression in men increases with age, coinciding with a decline in testosterone levels (2,3,4), and testosterone replacement has some efficacy in improving depressive symptoms in this population, as well as in men with refractory depression as an adjunct treatment to antidepressant medication (5,6,7,8). Moreover, hypogonadism in young males can precipitate depressive symptomology, supporting a protective role of testosterone against the development of affective disorders. Accordingly, preclinical research has shown that testosterone has antidepressant-like effects in aged male mice (9), and protects against the development of depressive-like behavior in male rats following gonadectomy (10,11).

While it is clear that testosterone can exert modulatory effects on affective state, the underlying mechanisms remain poorly characterized. Within the brain, physiological actions of testosterone are primarily mediated by its 5α-reduced and aromatase-derived metabolites, dihydrotestosterone (DHT) and estradiol, respectively. Mounting evidence points to an active role of aromatized estradiol in the regulatory effects of testosterone on affective status (12,13). This is conceivably due in part to its effects on neuronal plasticity within limbic regions implicated in the pathophysiology of depression, including the hippocampus. Indeed, several reports have confirmed significant aromatase expression throughout the hippocampus of rodents and humans, in a steroid- and sex-independent manner (14,15,16). Given that the hippocampal formation is rich in both androgen and estrogen receptors (14,17), it is possible that testosterone may exert antidepressant and axiolytic effects via both androgen- and estrogen-dependent mechanisms within this brain region.

We recently demonstrated that testosterone protects against depressive-like behavior in gonadectomized male rats in the forced swim and sucrose preference tests, and that these effects directly involve activation of the mitogen-activated protein kinase (MAPK) signaling pathway within the dentate gyrus of the hippocampus (10,13). Importantly, these effects were likely due to testosterone's estrogenic metabolite, as supplementation of estradiol to gonadectomized rats, but not DHT, mimicked the efficacy of testosterone in the forced swim test (10). Although testosterone can activate MAPK signaling via both estrogen- (18) and androgen-dependent mechanisms, it is unclear whether similar mechanisms underlie the antidepressant-like effects of estradiol supplementation in gonadectomized male rats. Further, while both hormones profoundly regulate transcription and activation of depression-relevant signaling cascades within the hippocampus, these effects are complex and can be

different (even opposite) or similar in male and female subjects, owing to vast sexual dimorphisms in brain morphology, neurochemistry and function (19,20,21). As such, there is a critical need to delineate the contributions of testosterone and estradiol to mood-related symptoms in a sex-specific manner. Accordingly, our previous work demonstrated that chronic testosterone supplementation protected against depressive- and anxiogenic-like consequences of social isolation stress in gonadectomized male, but not female, rats (11).

Given that, in our hands, testosterone lacked protective efficacy in ovariectomized female rats, the present work focused on male rats to determine the nature of estradiol's contribution to the protective actions of testosterone at behavioral and molecular levels. In this work, we investigated the protective anxiolytic- and antidepressant-like effects of testosterone and its aromatized metabolite in gonadectomized male rats, and their influence on hippocampal gene expression. In addition, because systemically-administered estradiol has widespread effects on various brain regions involved in the display of affective behaviors, we directly examined the role of testosterone's conversion to estradiol in the dentate gyrus on anxiety- and depressive-like behaviors via local infusion of the aromatase inhibitor, fadrozole.

## Methods

### Animals

Adult male (250–270g) Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were pair-housed in 43x21.5x25.5cm plastic cages and kept on a 12h:12h light:dark cycle (lights on at 0700 hours). Food and water was available *ad libitum* except during testing. Behavioral experiments, except the sucrose preference test, were conducted during the first 4h of the light phase of the light:dark cycle and all animal protocols were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Florida State University.

### Surgery

Rats were anesthetized with a ketamine (70mg/kg) /xylazine (10mg/kg) mixture (i.p.). Bupivicaine (0.25% solution; 0.4mL/kg) was applied topically as analgesic and the non-steroidal anti-inflammatory drug meloxicam (1.0mg/mL) was injected subcutaneously.

### Gonadectomy and Hormone Supplementation

Gonadectomy and sham surgeries were performed as previously described (10,11). Following gonadectomy/sham surgery, 60-day slow release testosterone (25mg/pellet), β-estradiol 3-benzoate (0.1mg/pellet) or placebo pellets (Innovative Research of America, Sarasota, FL) were inserted subcutaneously into male rats 10cm from a small 2-cm incision below the shoulder blades.

## Osmotic Minipumps

Rats were implanted bilaterally with cannulae (Plastics One, Roanoke, VA, USA) aimed at the dentate gyrus area of the dorsal hippocampus (AP=−4.3; ML=±3.0; DV=−4.7mm) (10). Cannula placement was verified *a posteriori* by sectioning on a cryostat. All rats included in

analyses had correct placements. Behavioral data from 7 rats were excluded from analyses due to incorrect bilateral cannula placement. Two cannulae delivered 6μL/day of sterile saline containing or not 1.0μg fadrozole (Sigma-Aldrich, St. Louis, MO) via subscapular Alzet Osmotic Minipumps (Model 2004; Alza, Mountain View, CA). This dose was chosen based on reports that intracranial infusion of fadrozole within the 0.8–1.378μg dosing range effectively abolishes the local conversion of testosterone to estradiol (22,23). Prior to implantation, osmotic minipumps were incubated at 37°C for 48h in sterile saline to equilibrate and ensure accurate flow rate prior to implantation.

## Experimental Design

### Experiment 1: Depressive-like Behavior Following Gonadectomy and Hormone Replacements

Ten days following surgery, depressive-like behavior of sham-operated and gonadectomized male rats (n=6–8/group) receiving testosterone (GDX+T), estrogen (GDX+E), or placebo (GDX) pellet replacements were investigated using the sucrose preference test.

### Experiment 2: Effects of Testosterone and Estrogen Supplementation on Gene Expression in the Hippocampus of Gonadectomized Male Rats

Upon completion of behavioral testing, rats from Experiment 1 were sacrificed under non-stressful conditions, and their brains snap-frozen in 2-methylbutane and stored at −80°C until further processing for genome-wide cDNA microarray analysis. RNA was extracted from tissue punches of the dorsal hippocampus, and used to generate cDNA for microarray analysis and real-time quantitative polymerase chain reaction (qRT-PCR) for validation of selected targets.

### Experiment 3: Anxiety and Depressive-like Behaviors Following Aromatase Inhibition in the Dentate Gyrus

Ten days following surgery, anxiety and depressive- like behaviors of GDX male rats (n=8–14/group) receiving testosterone or placebo pellets and fadrozole or saline infusions into the dentate gyrus were investigated. Rats' behavior was first tested using the open field test, followed one day later by the sucrose preference test. Testosterone was extracted from tissue punches taken from the dorsal hippocampus surrounding the sites of fadrozole or saline infusions prior to mass spectrometric analysis to confirm effective local inhibition of aromatase.

## Behavioral Tests

### Open Field Test

Rats were placed in a large (1mx1m) open field under dim light and were allowed to freely explore the arena for 5min to provide measures of locomotor activity and general anxiety-like behavior, recorded by a digital camcorder placed above the open field. Locomotor activity and total duration spent in the center were analyzed in EthoVision XT version 8 (Noldus Information Technology, Leesburg, VA). The open field arena was cleaned with 70% ethanol between trials.

### Sucrose Preference Test

The sucrose preference test consisted of a two-bottle choice paradigm (11). After a 5-day habituation to two bottles of water, rats were given access to two pre-weighed bottles, one containing water and the other 1% sucrose, for 48 hours. The position of the sucrose solution was alternated with water at 24 hours to account for possible location preference. The bottles were weighed at 0800h and 1700h daily and the preference for sucrose over water was used as a measure of anhedonia.

## Statistical Analysis of Behavioral Data

All data were first subjected to the Anderson-Darling Normality test. Results from sucrose preference (Experiment 1) and open field tests followed a normal distribution and were analyzed using one-way or two-way analysis of variance (ANOVA), respectively, followed by post-hoc Fisher tests where appropriate. Non-normally distributed sucrose preference data (Experiment 3) were analyzed using the Kruskal-Wallis nonparametric test followed by Mann-Whitney post-hoc tests where appropriate.

## Microarray Analysis

### RNA Extraction and cDNA Synthesis

Tissue punches (1.0mm) from 200μm sections of the dorsal hippocampus were homogenized in 400μL TRIzol reagent (Life Technologies Inc., Grand Island, NY, USA) and total RNA was isolated in accordance with manufacturer's protocol. RNA purity and concentration were assessed by optical density spectroscopy using a ND–1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity (RQI) was determined by Experion RNA StdSens analysis (BioRad Laboratories Inc., Hercules, CA, USA) prior to cDNA synthesis. Only samples with RQI indices >8.0 were considered. Single-stranded cDNA was reversed transcribed from 10μg aliquots of each sample with the Superscript II First-Strand cDNA Synthesis kit (Life Technologies) following manufacturer's protocol.

### Gene Expression Profiling

Four micrograms of cDNA per sample (n=4–5/group) were labeled with Cy3 random nonamers and hybridized overnight at 42°C onto a NimbleGen 12x135K rat gene expression array (Roche NimbleGen Inc., Madison, WI, USA), representing 26,419 genes. All procedures from NimbleGen Array User's Guide for Gene Expression Arrays (Roche NimbleGen) were followed. To account for inter-array variability due to hybridization or procedural artifacts, biological replicates for each treatment condition were equally distributed across 2 arrays such that all groups were represented on each slide. Following hybridization, slides were scanned using the NimbleGen MS200 (2 m resolution; single-channel, 532 nm) to generate probe pixel intensity data. Resulting .tif image files containing probe intensities were processed with DEVA Software (v.1.6, NimbleGen) for alignment, summarization, background normalization and annotation. Probe-level intensities were summarized using Robust Multi-Array Analysis, followed by background subtraction and quantile normalization to generate probe-level gene expression for statistical analysis.

### Analysis of Differential Gene Expression

Files (.PAIR) were imported into ArrayStar 10.0 software (DNASTAR, Madison, WI), and normalized using global median probe expression. One-way ANOVA followed by moderated *t*-tests correcting for multiple comparisons determined significant differences in mRNA abundance between experimental conditions, with a minimum threshold fold-change of 1.5. Differences were considered statistically significant at $p<0.05$. Pathway analysis of differentially expressed genes was completed using the Database for Annotation, Visualization and Integrated Discovery (24,25).

### Semiquantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA used for microarray analysis (1μg) was reversed transcribed to generate cDNA as described above. Semiquantitative RT-PCR was used, as previously described (26), to validate selected genes of interest. Nicotinamide adenine dinucleotide dehydrogenase (NADH) was used as the reference gene for normalization of all target genes chosen for validation of microarray results. Primer sequences are listed in Supplementary Table S1. Normalized data are expressed as fold change relative to GDX control.

## Determination of Testosterone Levels in Brain Tissue

### Sample Preparation

Tissue (8–10mg) was added with 20uL internal standard (IS) mixture (testosterone-d3 in 90% MeOH; 1ng/g tissue) (Cerilliant, Round Rock, TX), followed by 200uL ice-cold MilliQ water. Tissue was sonicated, and 800ul MeOH was immediately added. Samples were vortexed, sonicated in an ice bath for 15min, and incubated at 4°C overnight. The next day, samples were vortexed and centrifuged for 10min, 3000*xg* (4°C). Supernatant was transferred to a clean 1.5mL tube and dried in a SpeedVac (45°C). Dried residue was resuspended in 1mL 10% MeOH and loaded onto Oasis HLB 1cc/30mg solid phase extraction cartridges pre-conditioned with 1mL MeOH and 1mL MilliQ water. Samples were passed through the column via gravity, and washed with 40% MeOH. Analytes were eluted from the column via gravity into 1.5mL tubes with 2x500uL 90% MeOH, and dried by SpeedVac (45°C). Dried extracts were stored at −20°C until LC-MS/MS analysis.

### Mass Spectrometry

Brain tissue extract was redissolved in 10% acetonitrile aqueous solution with 0.1% formic acid (20uL/10mg tissue). 3.75uL of the above solution was injected to be separated by liquid chromatography with a NanoAquity nanoLC system (Waters, Milford, MA). Eluents were ionized by nano-electrospray ionization (nESI) in positive mode and detected on-line with a Xevo TQ-S Triple Quadrupole Mass Spectrometer (Waters, Milford, MA). Samples and standards were run in triplicate. Chromatograms were acquired and the area under curve (AUC) was calculated for both testosterone and testosterone-d3. The concentration of testosterone in unknown samples was calculated by comparing the AUC to the standard curve of the calibration standards under the same condition. Data were analyzed by two-way ANOVA, followed by post-hoc Bonferroni's multiple comparisons tests. A detailed description of mass spectrometry conditions can be found in Supplemental Material.

## Results

### Experiment 1: Depressive-like Behaviors Following Gonadectomy and Hormone Replacements

GDX male rats exhibited reduced sucrose preference compared to sham-operated (Sham), GDX+T and GDX+E male rats (Figure 1; $F_{(3,28)} = 4.259$; all p's < 0.05 versus GDX). Sucrose preferences of GDX+T and GDX+E male rats were similar to those of Sham males (p's > 0.05 versus Sham).

### Experiment 2: Regulation of Hippocampal Gene Expression by Testosterone and Estradiol Supplementation Following Castration

Microarray analysis revealed substantial regulation of gene expression within the dorsal hippocampus of GDX male rats supplemented with either testosterone or estradiol. 4838 genes combined were found to be differentially expressed in GDX+T and GDX+E male rats compared to GDX controls. While 1327 genes were uniquely regulated by testosterone and 1209 by estradiol, a substantial set of genes, 2302, were significantly regulated by both hormones (Figure 2A). All genes in this pool were regulated in the same direction (either up- or down-regulated), suggesting that genomic actions of testosterone-derived estradiol may account for a significant portion of its transcriptional regulatory activity in the hippocampus of male rats (Figure 2B). Significantly enriched pathways for all treatments are presented in Table 1, in addition to a list of selected gene targets validated by qRT-PCR (Table 2). Differentially expressed genes for each experimental condition are listed in Supplementary Table S2.

### Experiment 3: Anxiety and Depressive-like Behaviors Following Aromatase Inhibition in the Dentate Gyrus

Whereas low to undetectable levels of testosterone were observed in the dentate of GDX animals, physiological levels were detected in testosterone-supplemented rats (Fig. 4B; $p < 0.001$). Moreover, testosterone-supplemented animals infused with fadrozole exhibited higher levels than their saline-injected counterparts ($p < 0.001$), confirming effective local inhibition of testosterone's aromatization to estradiol by fadrozole infusion (Figure 4B; Hormone: $F_{(1,12)} = 441.50$, $p < 0.0001$; Infusion: $F_{(1,12)} = 30.33$, $p = 0.0001$; Interaction: $F_{(1,12)} = 27.59$, $p = 0.0002$). Compared to GDX animals receiving saline or fadrozole, testosterone-supplemented GDX animals spent more time in the center of the open field (Figure 3A,B; Hormone: $F_{(1,40)} = 5.95$, $p = 0.019$; Infusion: $F_{(1,40)} = 1.79$, $p = 0.19$; Interaction: $F_{(1,40)} = 4.19$, $p = 0.047$). Delivery of fadrozole directly into the dentate gyrus (Figure 4A; −3.60mm to −4.52mm from Bregma) blocked this effect ($p < 0.05$). Neither testosterone replacement nor fadrozole infusion affected locomotor activity in GDX male rats (Figure 3C; Hormone: $F_{(1,40)} = 2.74$, $p = 0.11$; Infusion: $F_{(1,40)} = 1.08$, $p = 0.30$; Interaction: $F_{(1,40)} = 2.26$, $p = 0.14$). GDX animals receiving testosterone replacement and saline infusion exhibited increased sucrose preference compared to placebo-treated GDX animals (Figure 3D; $H = 9.04$, $p = 0.029$). Interestingly, this effect was blocked by the infusion of fadrozole, indicating that the conversion of testosterone to estrogen within the dentate gyrus, in part, mediates hedonic behavior (Mann-Whitney post-hoc $p < 0.02$). However, the variability in sucrose preference scores following chronic aromatase inhibition

(Figure 3E) suggests a possible role of DHT and/or other androgenic metabolites and brain regions in mediating the protective effects of testosterone on anhedonia induced by gonadal hormone depletion in male rats.

## Discussion

Our findings showed that depressive- and anxiety-like behaviors induced by gonadectomy (GDX) in male rats could be effectively reversed by supplementation with either testosterone or estradiol, confirming previous findings that the protective effects of testosterone on depressive-like behavior are likely mediated via aromatization to estradiol. Consistent with these data, there was substantial overlap in changes in hippocampal gene expression regulated by both chronic testosterone and estradiol treatment. Further, by chronically blocking aromatase activity within the dentate gyrus, we have demonstrated a modulatory role of locally-synthesized estradiol in this brain region in organizing aspects of antidepressant- and anxiolytic-like behavioral actions of testosterone.

The contributing role of gonadal hormones in pronounced sex differences in depressive disorders is both organizational and activational in origin. Sexual differentiation of the brain is a hormone-dependent process, whereby testes-derived testosterone irreversibly masculinizes the brain, relying principally on its local conversion to estradiol via p450 aromatase. In adulthood, gonadal testosterone produces activational, or reversible, effects which contribute to sex differences at molecular, cellular and functional levels (27,28,29). As such, investigation of sex differences relevant to depressive disorders is inherently complex. In this study, a focus on activational effects of testosterone and estradiol on affective behaviors were permitted by depletion of the peripheral source of testosterone via gonadectomy of adult male rats.

Many of testosterone's effects in the brain are due to actions of its more physiologically active metabolites, DHT and estradiol. These may affect transcription through directly binding to their nuclear receptors (AR, ERα/β) or by rapidly modulating signaling pathways via membrane-bound receptors. In addition, non-genomic actions of estradiol and testosterone may rapidly activate signaling pathways to regulate subsequent transcriptional processes. Both may be required for behavioral outcomes related to actions of circulating or brain-derived sex steroids. In support of previous findings, estradiol supplementation in GDX male rats mimicked the antidepressant-like effects of testosterone in the sucrose preference test, suggesting that metabolism to estradiol may mediate several antidepressant-like effects of testosterone. This is further supported by the striking similarity in hippocampal transcriptional regulation by chronically-administered testosterone and estradiol in GDX male rats. Transcriptional changes from testosterone-derived estradiol most likely come from this pool of genes, whereas those unique to estradiol supplementation may be consequent to exogenous administration.

A large body of evidence exists debating underlying mechanisms contributing to the different efficacy profiles of antidepressants in men and women. Among these, signaling pathways related to synaptic plasticity emerged as critical mediators of current antidepressant efficacy. Specifically, the mitogen-activated protein kinase (MAPK)/

extracellular signal-regulated kinase (ERK) pathway has been identified as an integral component of the antidepressant response profile (30,31,32,33). For example, stress-mediated alterations in ERK signaling convey depressive-like behavioral responses in rodents that are reversible by chronic fluoxetine treatment (33). Interestingly, this pathway represents a possible convergence point for actions of testosterone and estradiol within the hippocampus. MAPK/ERK signaling can be activated directly by testosterone via androgen receptor-mediated mechanisms, by aromatized estradiol through binding to ERβ, or through interaction of both sex hormones via AR-ERβ complexes (18,34). This pathway is therefore an excellent target for the actions of locally-synthesized estradiol in the hippocampus.

We have recently demonstrated testosterone-dependent regulation of ERK2 mRNA and protein within the dentate gyrus, where compromised ERK activity abrogated the antidepressant-like effects of testosterone. In addition, ERK2 overexpression within this region attenuated gonadectomy-induced anhedonic behaviors, mimicking the effects of testosterone supplementation (10). In the present experiments, estradiol protected against anhedonia induced by gonadal testosterone depletion to an extent comparable to that observed following testosterone supplementation. Given the similarity in behavioral responses of GDX male rats receiving either hormone in the sucrose preference test, it is conceivable that testosterone's antidepressant-like actions are mediated through local aromatization, involving activation of MAPK/ERK in the dentate gyrus. In agreement with this hypothesis, we found significant overlapping transcriptional regulation of genes in the classical MAPK/ERK pathway by testosterone and estradiol when compared to placebo-treated GDX male rats (Table 1).

Within the classical MAPK pathway, specifically, we observed numerous differentially expressed genes upstream of MAPK/ERK. Positive regulators of MAPK/ERK signaling, *RasGRP3* and *Raf1*, were significantly upregulated by both testosterone and estradiol supplementation. In addition, both hormone treatments significantly upregulated various growth factors (such as *Fgf1* and *Fgf*5) known to stimulate this intracellular signaling cascade. Interestingly, chronic administration of either hormone significantly downregulated ERβ mRNA (*Esr2*) in GDX male rats, possibly due to prolonged activation of this receptor. This suggests that activation of MAPK/ERK signaling following chronic treatment with either testosterone or estradiol may not result directly from genomic effects of hormone supplementation, but rather from non-genomic activation of intracellular signaling cascades by aromatase-derived estradiol via ERβ, which in turn may positively regulate MAPK/ERK activity via transcriptional regulation of upstream targets.

Such synaptocrine mechanisms of estradiol locally synthesized from testosterone within the hippocampus could inevitably lead to sustained modulation of synaptic plasticity via regulation of dendritic spine stability or formation. In support of this theory, both hormone treatments upregulated *Arhgef6 and Arhgef7* expressions. Guanine exchange factors (GEFs) are relays of signals from synaptic receptors to the cytoskeleton and regulate small GTPase effectors, which stimulate or repress actin-binding protein activity (35). Downstream, we observed significant upregulation of LIM-domain protein kinase 1 (*Limk1*), an important regulator of actin cytoskeleton stabilization (35). Atrophy of apical dendrites and decreased neurogenesis within the dentate gyrus in response to stress have been demonstrated in

preclinical studies (36,37,38). Moreover, reduction in hippocampal volume and neuronal atrophy within the hippocampus have been observed in individuals with MDD (39,40,41). Such structural and functional alterations in the hippocampus may contribute to the depressive-like behaviors observed in the present study.

While these results support a major role for aromatase-derived estradiol in mediating the protective effects of testosterone against depressive- and anxiety-like behavior in GDX male rats, we acknowledge that exogenous administration of estradiol is a limitation of this study, as it produces an unnatural peripheral source of estradiol in male rats. It is therefore plausible that the behavioral effects of estradiol were due to widespread effects on multiple limbic regions where estradiol is known to influence affective behavior, such as the nucleus accumbens and amygdala. Consequently, we infused the aromatase inhibitor, fadrozole, directly into the dentate gyrus of the hippocampus to determine the extent to which locally-synthesized estradiol may influence anxiety- and depressive-like behavior. While local inhibition of aromatase activity clearly blocked the anxiolytic-like effects of testosterone replacement in GDX male rats, significant variability was observed in fadrozole-treated rats in the sucrose preference test, suggesting additional contributions of extra-hippocampal and androgen-dependent mechanisms in mediating the protective effects of testosterone on hedonic behavior in male rats.

Both the aromatized and 5α-reduced metabolites of testosterone have demonstrated robust anxiolytic- and antidepressant-like efficacy in male rats. The primary androgenic metabolite of testosterone, DHT, is further converted to 5α-androstane, 17β-diol-3α-diol (3α-diol) and 5α-androstane, 17β-diol-3β-diol (3β-diol). In contrast to the high AR-affinity of DHT, 3α-diol and 3β-diol are strong ERβ agonists (42). Systemic administration of 3α-diol, 3β-diol and selective ERβ agonists (e.g. diarylpropionitrile) are known to reduce restraint-stress induced CORT and ACTH elevation and decrease anxiety-like behavior in gonadectomized male rats and wild-type—but not ERβ knockout—mice to a similar extent as testosterone (43,44,45,46,47). Importantly, a direct role of androgenic metabolites in the hippocampus on anxiety-like behavior has been demonstrated in intact and DHT-replaced male rats, where infusion of the androgen receptor antagonist, flutamide, into the CA1 blocked the anxiolytic-like effects of hormone replacement (48). It is therefore possible that both androgen and estrogen actions on ERβ in the hippocampus may have elicited the behavioral benefits of chronic hormone supplementation in this study. Indeed, the downregulation of ERβ mRNA by both hormones observed herein support their action at this receptor in the hippocampus.

We have demonstrated that the protective anxiolytic- and antidepressant-like effects of testosterone in GDX male rats are, in part, mediated by conversion to its active metabolite, estradiol. These effects were associated with changes in hippocampal gene expression related to MAPK/ERK signaling and synaptic plasticity, along with alterations in numerous pathways of potential relevance. Functional studies within the dentate gyrus further suggest that this brain region is critically involved in aspects of anxiety- and depressive-like behavior that are modulated by aromatase-derived estradiol in the hippocampus of GDX male rats. Through use of microarray technology to profile gene expression patterns associated with hormone-dependent behavioral outcomes, the present findings not only

support past discoveries, but also provide novel avenues worth exploring in greater detail using complementary deep sequencing and behavioral techniques.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

1. Johnson JM, Nachtigall LB, Stern TA. The effect of testosterone levels on mood in men: a review. Psychosomatics. 2013; 54:509–514. [PubMed: 24016385]
2. Shores MM, Moceri VM, Gruenewald DA, Brodkin KI, Matsumoto AM, Kivlahan DR. Low testosterone is associated with decreased function and increased mortality risk: a preliminary study of men in a geriatric rehabilitation unit. J Am Geriatr Soc. 2004; 52:2077–2081. [PubMed: 15571546]
3. Shores MM, Sloan KL, Matsumoto AM, Moceri VM, Felker B, Kivlahan DR. Increased incidence of diagnosed depressive illness in hypogonadal older men. Arch Gen Psychiatry. 2004; 61:162–167. [PubMed: 14757592]
4. McIntyre RS, Mancini D, Eisfeld BS, Soczynska JK, Grupp L, Konarski JZ, et al. Calculated bioavailable testosterone levels and depression in middle-aged men. Psychoneuroendocrinology. 2006; 31:1029–1035. [PubMed: 16908107]
5. Pope HG Jr, Cohane GH, Kanayama G, Siegel AJ, Hudson JI. Testosterone gel supplementation for men with refractory depression: a randomized, placebo-controlled trial. Am J Psychiatry. 2003; 160:105–111. [PubMed: 12505808]
6. Pope HG Jr, Amiaz R, Brennan BP, Orr G, Weiser M, Kelly JF, et al. Parallel-group placebo-controlled trial of testosterone gel in men with major depressive disorder displaying an incomplete response to standard antidepressant treatment. J Clin Psychopharmacol. 2010; 30:126–134. [PubMed: 20520285]
7. Seidman SN, Rabkin JG. Testosterone replacement therapy for hypogonadal men with SSRI-refractory depression. J Affect Disord. 1998; 48:157–161. [PubMed: 9543205]
8. Zarrouf FA, Artz S, Griffith J, Sirbu C, Kommor M. Testosterone and depression: systematic review and meta-analysis. J Psychiatr Pract. 2009; 15:289–305. [PubMed: 19625884]
9. Frye CA, Walf AA. Depression-like behavior of aged male and female mice is ameliorated with administration of testosterone or its metabolites. Physiol Behav. 2009; 97:266–269. [PubMed: 19254734]
10. Carrier N, Kabbaj M. Extracellular signal-regulated kinase 2 signaling in the hippocampal dentate gyrus mediates the antidepressant effects of testosterone. Biol Psychiatry. 2012a; 71:642–651. [PubMed: 22265242]
11. Carrier N, Kabbaj M. Testosterone and imipramine have antidepressant effects in socially isolated male but not female rats. Horm Behav. 2012b; 61:678–685. [PubMed: 22426002]
12. Fink G, Sumner B, Rosie R, Wilson H, McQueen J. Androgen actions on central serotonin neurotransmission: relevance for mood, mental state and memory. Behav Brain Res. 1999; 105:53–68. [PubMed: 10553690]
13. McHenry J, Carrier N, Hull E, Kabbaj M. Sex differences in anxiety and depression: Role of testosterone. Front Neuroendocrinol. 2013; 35:42–57. [PubMed: 24076484]
14. Hojo Y, Hattori TA, Enami T, Furukawa A, Suzuki K, Ishii HT, et al. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. PNAS. 2004; 101:865–870. [PubMed: 14694190]

15. Roselli CE, Liu M, Hurn PD. Brain aromatization: classic roles and new perspectives. Semin Reprod Med. 2009; 27:207–217. [PubMed: 19401952]
16. Yague JG, Wang AC, Janssen WG, Hof PR, Garcia-Segura LM, Azcoitia I, et al. Aromatase distribution in the monkey temporal neocortex and hippocampus. Brain Res. 2008; 1209:115–127. [PubMed: 18402929]
17. Tabori NE, Stewart LS, Znamensky V, Romeo RD, Alves SE, McEwen BS, et al. Ultrastructural evidence that androgen receptors are located at extranuclear sites in the rat hippocampal formation. Neuroscience. 2005; 130:151–163. [PubMed: 15561432]
18. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, et al. Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. EMBO J. 2000; 19:5406–5417. [PubMed: 11032808]
19. Cahill L. Why sex matters for neuroscience. Nat Rev Neurosci. 2006; 7:477–484. [PubMed: 16688123]
20. Cosgrove KP, Mazure CM, Staley JK. Evolving knowledge of sex differences in brain structure, function, and chemistry. Biol Psychiatry. 2007; 62:847–855. [PubMed: 17544382]
21. Simerly RB. Wired on hormones: endocrine regulation of hypothalamic development. Curr Opin Neurobiol. 2005; 15:81–85. [PubMed: 15721748]
22. Clancy AN, Michael RP. Effects of testosterone and aromatase inhibition on estrogen receptor-like immunoreactivity in male rat brain. Neuroendocrinology. 1994; 59:552–560. [PubMed: 8084379]
23. Huddleston GG, Paisley JC, Clancy AN. Effects of estrogen in the male rat medial amygdala: infusion of an aromatase inhibitor lowers mating and bovine serum albumin-conjugated estradiol implants do not promote mating. Neuroendocrinology. 2006; 83:106–116. [PubMed: 16825796]
24. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nat Protoc. 2009; 4:44–57. [PubMed: 19131956]
25. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009; 37:1–13. [PubMed: 19033363]
26. Hollis F, Duclot F, Gunjan A, Kabbaj M. Individual differences in the effect of social defeat on anhedonia and histone acetylation in the rat hippocampus. Horm Behav. 2011; 59:331–337. [PubMed: 20851702]
27. Arnold AP, Breedlove SM. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm Behav. 1985; 19:469–498. [PubMed: 3910535]
28. Gillies GE, McArthur S. Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. Pharmacol Rev. 2010; 62:155–198. [PubMed: 20392807]
29. McCarthy MM, Konkle AT. When is a sex difference not a sex difference? Front Neuroendocrinol. 2005; 26:85–102. [PubMed: 16083951]
30. Einat H, Yuan P, Gould TD, Li J, Du J, Zhang L, et al. The role of the extracellular signal-regulated kinase signaling pathway in mood modulation. J Neurosci. 2003; 23:7311–7316. [PubMed: 12917364]
31. Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci. 2000; 20:9104–9110. [PubMed: 11124987]
32. Manji HK, Drevets WC, Charney DS. The cellular neurobiology of depression. Nat Med. 2001; 7:541–547. [PubMed: 11329053]
33. Qi X, Lin W, Li J, Li H, Wang W, Wang D, et al. Fluoxetine increases the activity of the ERK-CREB signal system and alleviates the depressive-like behavior in rats exposed to chronic forced swim stress. Neurobiol Dis. 2008; 31:278–285. [PubMed: 18586506]
34. Zadran S, Qin Q, Bi X, Zadran H, Kim Y, Foy MR, et al. 17-Beta-estradiol increases neuronal excitability through MAP kinase-induced calpain activation. PNAS. 2009; 106:21936–21941. [PubMed: 19995977]
35. Penzes P, Cahill ME. Deconstructing signal transduction pathways that regulate the actin cytoskeleton in dendritic spines. Cytoskeleton (Hoboken). 2012; 69:426–441. [PubMed: 22307832]

36. Magarinos AM, Deslandes A, McEwen BS. Effects of antidepressants and benzodiazepine treatments on the dendritic structure of CA3 pyramidal neurons after chronic stress. Eur J Pharmacol. 1999; 371:113–122. [PubMed: 10357248]
37. Schmidt HD, Duman RS. The role of neurotrophic factors in adult hippocampal neurogenesis, antidepressant treatments and animal models of depressive-like behavior. Behav Pharmacol. 2007; 18:391–418. [PubMed: 17762509]
38. Watanabe Y, Gould E, McEwen BS. Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. Brain Res. 1992; 588:341–345. [PubMed: 1393587]
39. Neumeister A, Wood S, Bonne O, Nugent AC, Luckenbaugh DA, Young T, et al. Reduced hippocampal volume in unmedicated, remitted patients with major depression versus control subjects. Biol Psychiatry. 2005; 57:935–937. [PubMed: 15820716]
40. Sheline YI, Gado MH, Kraemer HC. Untreated depression and hippocampal volume loss. Am J Psychiatry. 2003; 160:1516–1518. [PubMed: 12900317]
41. Stockmeier CA, Mahajan GJ, Konick LC, Overholser JC, Jurjus GJ, Meltzer HY, et al. Cellular changes in the postmortem hippocampus in major depression. Biol Psychiatry. 2004; 56:640–650. [PubMed: 15522247]
42. Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. 1997; 138:863–870. [PubMed: 9048584]
43. Handa RJ, Sharma D, Uht R. A role for the androgen metabolite, 5alpha androstane 3beta, 17beta diol (3β-Diol) in the regulation of the hypothalamo-pituitary–adrenal axis. Front Endocrinol. 2011; 2:65.
44. Edinger KL, Frye CA. Testosterone's anti-anxiety and analgesic effects may be due in part to actions of its 5α-reduced metabolites in the hippocampus. Psychoneuroendocrinology. 2005; 30:418–430. [PubMed: 15721054]
45. Frye CA, Koonce CJ, Edinger KL, Osborne DM, Walf AA. Androgens with activity at estrogen receptor beta have anxiolytic and cognitive-enhancing effects in male rats and mice. Horm Behav. 2008; 54:726–734. [PubMed: 18775724]
46. Kudwa AE, McGivern RF, Handa RJ. Estrogen receptor β and oxytocin interact to modulate anxiety-like behavior and neuroendocrine stress reactivity in adult male and female rats. Physiol Behav. 2014; 129:287–296. [PubMed: 24631553]
47. Osborne DM, Edinger KL, Frye CA. Chronic administration of androgens with actions at estrogen receptor beta have anti-anxiety and cognitive-enhancing effects in male rats. Age (Dordr). 2009; 31:191–198. [PubMed: 19685169]
48. Edinger KL, Frye CA. Intrahippocampal administration of an androgen receptor antagonist, flutamide, can increase anxiety-like behavior in intact and DHT-replaced male rats. Horm Behav. 2006; 50:216–222. [PubMed: 16631174]

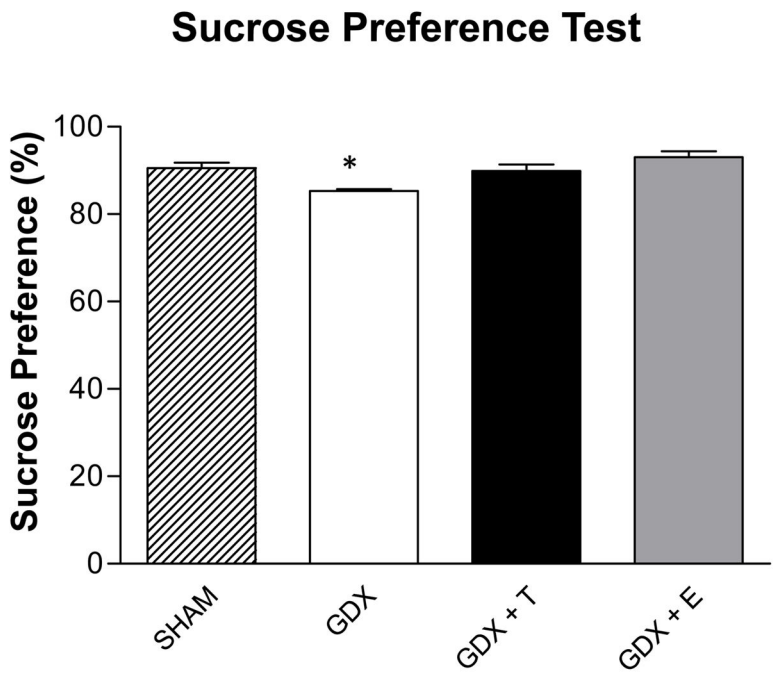


**Figure 1.**

Testosterone or estrogen replacement prevents gonadectomy-induced anhedonia. While gonadectomized rats receiving a placebo pellet (GDX) have a sucrose preference lower than sham-operated animals, gonadectomized rats with testosterone (GDX+T) or estrogen (GDX +E) replacement exhibit similar levels compared to sham-operated animals (Sham). * $p < 0.05$ *vs.* SHAM, GDX+T and GDX+E, Fisher's PLSD post-hoc test.

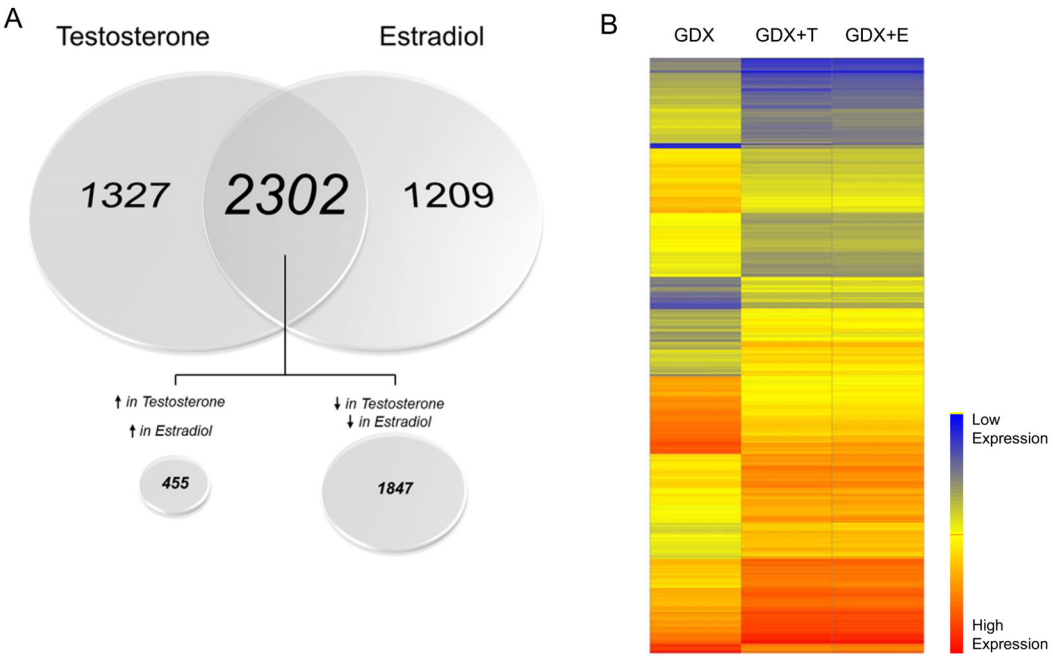


**Figure 2.**

Testosterone or estrogen replacements induce a similar profile of gene expression in the dorsal hippocampus of gonadectomized rats. (2A) The Venn diagram represents the genes uniquely regulated by testosterone replacement (left section, 1 327 genes), estradiol replacement (right section, 1 209 genes), or commonly regulated by both treatments (intersection, 2 302 genes). Only the genes with a minimum fold-change of 1.5 were considered. (2B) Heatmap representation of hierarchical clustering of gene expression levels. Rats receiving testosterone (GDX+T) or estradiol (GDX+E) replacements exhibit a similar profile of regulation compared to gonadectomized animals treated with a placebo pellet (GDX).

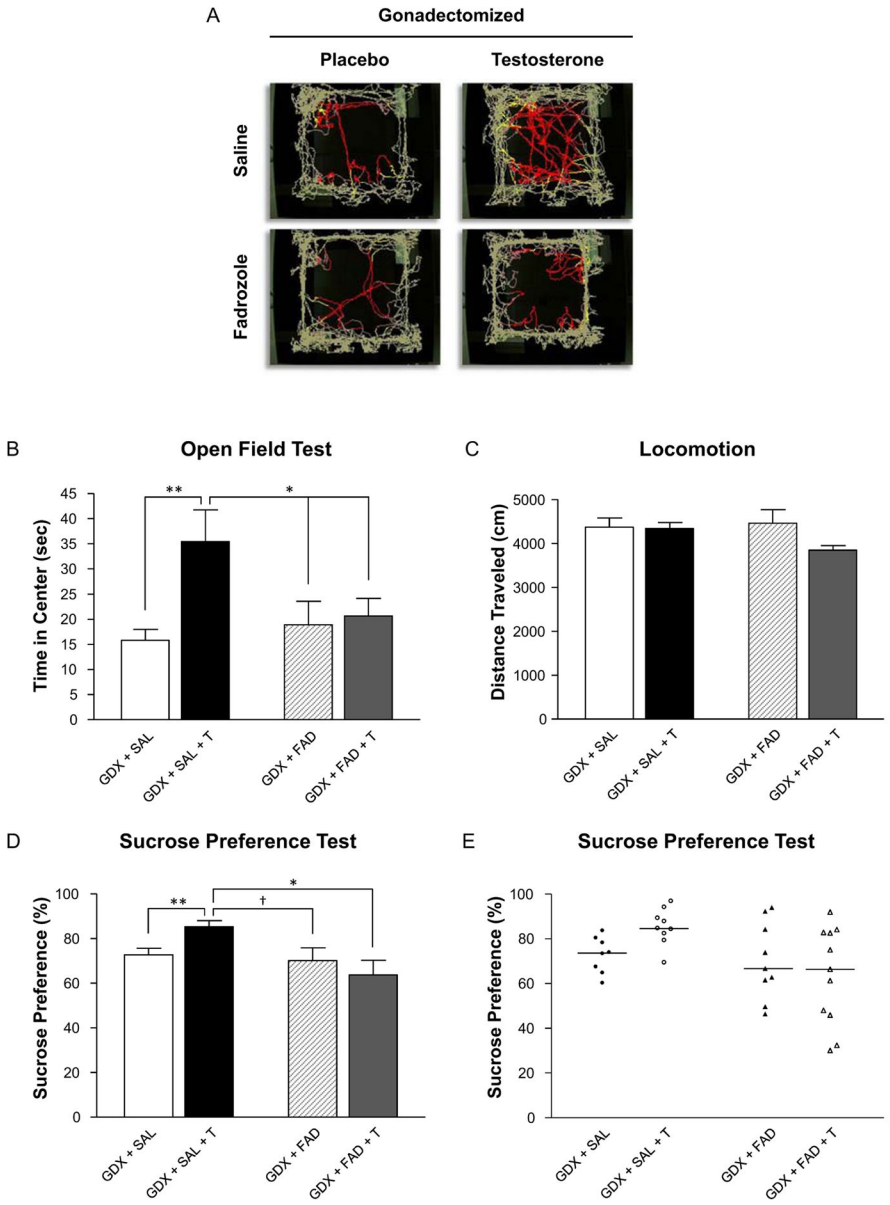


**Figure 3.**

Aromatase inhibition in the dorsal dentate gyrus blocks the anxiolytic- and antidepressant-like effects of testosterone replacement. (3A,B) When infused with saline (GDX+T+SAL), gonadectomized rats receiving testosterone replacement spent more time in the center of an open field than gonadectomized rats receiving a placebo pellet and infused with saline (GDX+SAL), but not when infused with the aromatase inhibitor fadrozole (GDX+FAD+T). ** $p < 0.01$ *vs.* GDX+SAL, * $p < 0.05$ *vs.* GDX+FAD and GDX+FAD+T, Fisher's PLSD post-hoc test. (3C) None of the treatments affected rats' locomotion in the open field. (3D) Testosterone replacement increased sucrose preference of gonadectomized rats infused with saline, but not fadrozole. (3E) Representation of individual values depicted in panel 3C, highlighting the greater variability observed following treatment with the aromatase

inhibitor fadrozole, compared to animals infused with saline. ** $p < 0.01$ *vs.* GDX+SAL, * $p < 0.05$ *vs.* GDX+FAD+T, † p = 0.0503 *vs.* GDX + FAD, Mann-Whitney post-hoc test.

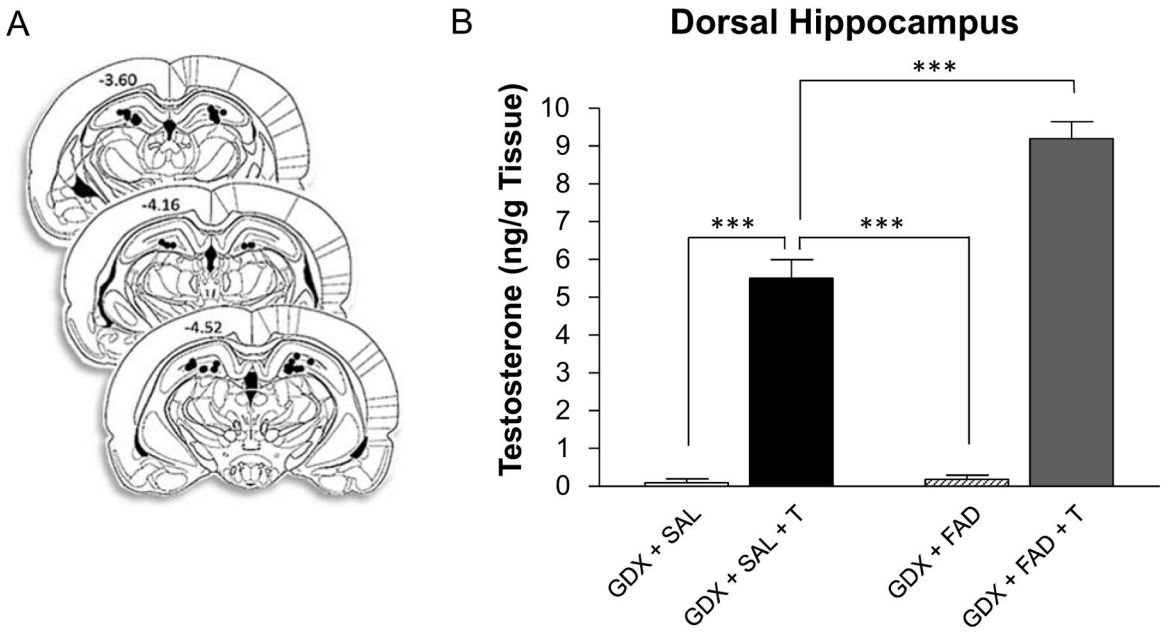


**Figure 4.**

Fadrozole effectively inhibits local conversion of testosterone to estradiol in the dorsal hippocampus. (4A) Representation of the infusion sites (black circles) on The Rat Brain Atlas (Paxinos and Watson), ranging from −3.60 mm to −4.52 mm from Bregma. (4B) Significantly elevated levels of testosterone were measured in the dorsal hippocampus of testosterone-supplemented male rats receiving fadrozole infusions compared to those receiving saline infusions. Conversely, low to undetectable levels of testosterone were observed in placebo-treated rats, regardless of local aromatase inhibition.

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**Table 1**

Significantly enriched KEGG pathways for genes uniquely regulated by testosterone replacement, estradiol replacement, or commonly regulated by both treatments.

| | Term | Fold Enrichment | Gene Count | Genes | *Fisher Exact p-Value* |
|---|---|---|---|---|---|
| *Commonly Regulated* | | | | | |
| | Cytokine-cytokine receptor interaction | 2.5322 | 34 | *Loc678883, Csf2, Rgd1561292, Rgd1565911, Il9r, Il6st, Ctf1, Tnfsf14, Kitlg, Il13, Il11, Lif, Rgd1565225, Il12rb1, Cxcr5, Loc691874, Csf2rb, Epo, Il4, Il3, Flt3, Rgd1559932, Tnfrsf13c, Tnfrsf14, Edar, Osm, Ccr8, Loc500598, Ccr6, Tnfsf13b, Il20ra, Cd40lg, Cxcl13, Ccr4, Ifna11, Loc678867, Il12b, Ngfr* | *<0.00001* |
| | Neuroactive ligand-receptor interaction | 1.9040 | 34 | *Cckar, Taar7e, Grik2, Drd4, F2rl1, Ppyr1, Bdkrb1, Adrb3, Ptgir, Hrh1, P2ry4, Ltb4r, S1pr4, Galr2, Mcpt8l2, Mc5r, Mc3r, Taar4, Taar3, Gpr156, Ptger4, Gabra3, Ptgdrl, Npbwr1, Htr4, Mcpt8, Ntsr1, Sstr5, Gpr35, Rgd1565970, Ltb4r2, Ptgdr, Gpr50, Uts2r, Tshr, Glp1r, Ctsg* | *0.0002* |
| | MAPK signaling pathway | 1.5444 | 28 | *Fgfr2, Fgf6, Fgf5, Fgf8, Fgf17, Rgd1561394, Map4k1, Hspa1a, Hspa1b, Casp3, Rasgrp3, Map3k2, Pla2g12b, Dusp16, Sos2, Pla2g1b, Fgf1, Traf6, Fgf3, Ntf4, Pla2g10, Chp2, Raf1, Cacng1, Cacna2d2, Cacna2d4, Hspb1, Pla2g2d, Pla2g2f* | *0.0130* |
| | Jak-STAT signaling pathway | 2.5333 | 24 | *Csf2, Rgd1561292, Rgd1565911, Il9r, Loc685840, Il6st, Loc687396, Ctf1, Il13, Il11, Lif, Il12rb1, Rgd1565225, Sos2, Csf2rb, Epo, Il4, Il3, Socs2, Rgd1559932, Il24, Osm, Cblc, Il20ra, Ifna11, Il12b* | *0.0000* |
| | Regulation of actin cytoskeleton | 1.6848 | 24 | *Fgfr2, Fgf6, Myl7, Fgf5, Fgf8, Arhgef7, Limk1, Arhgef6, Diaph2, Fgf17, Abi2, Itga10, Raf1, Bdkrb1, Iqgap1, Vcl, Itgb7, Sos2, Itgad, Mylc2pl, Ins2, Fgf1, Pip4k2c, Fgf3* | *0.0077* |
| | Cell adhesion molecules (CAMs) | 1.9827 | 20 | *Cldn7, Selp, Cldn18, Mpz, Cldn4, Cldn6, Rt1-M2, Cdh1, Rt1-Ec2, Rt1-Da, Pdcd1, Pdcd1lg2, Cldn23, Sdc3, Cd40lg, Itgb7, Rt1-T18, Cd2, Cd22, Spn* | *0.0023* |
| | Vascular smooth muscle contraction | 2.0774 | 16 | *Pla2g10, Prkch, Raf1, Prkcd, Kcnmb1, Itpr1, Ptgir, Plcb4, Gnaq, Calml3, Pla2g12b, Pla2g1b, Calm4, Plcb1, Pla2g2d, Pla2g2f* | *0.0039* |
| | Tight junction | 1.8058 | 16 | *Myl7, Cldn7, Cldn18, Cldn4, Cldn6, Myh3, Rgd1561394, Prkci, Prkch, Prkcd, Cldn23, Tjp1, Ash1l, Exoc4, Mylc2pl, Myh7b* | *0.0150* |
| | GnRH signaling pathway | 2.3413 | 15 | *Pla2g10, Raf1, Prkcd, Itpr1, Plcb4, Gnaq, Map3k2, Calml3, Pla2g12b, Sos2, Pla2g1b, Calm4, Plcb1, Pla2g2d, Pla2g2f* | *0.0016* |
| | Hematopoietic cell lineage | 2.6334 | 14 | *Il4, Il3, Csf2, Il9r, Flt3, Fcer2, Kitlg, Rt1-Da, Il11, Cd19, Tfrc, Cd2, Cd22, Epo* | *0.0007* |
| | Melanogenesis | 2.2327 | 14 | *Kitlg, Lef1, Raf1, Fzd7, Loc679869, Plcb4, Gnaq, Loc683733, Calml3, Calm4, Creb3l4, Wnt9a, Wnt6, Plcb1, Wnt8a* | *0.0035* |
| | Long-term depression | 2.6278 | 12 | *Plcb4, Gnaq, Pla2g10, Pla2g12b, Pla2g1b, Raf1, Prkg2, Plcb1, Gsbs, Pla2g2d, Itpr1, Pla2g2f* | *0.0016* |

| | Term | Fold Enrichment | Gene Count | Genes | *Fisher Exact p-Value* |
|---|---|---|---|---|---|
| | Fc epsilon RI signaling pathway | 2.3166 | 12 | *Il4, Il3, Csf2, Pla2g10, Sos2, Pla2g12b, Pla2g1b, Raf1, Il13, Prkcd, Pla2g2d, Pla2g2f* | *0.0049* |
| | Autoimmune thyroid disease | 2.6458 | 11 | *Il4, Prf1, Rgd1565911, Rgd1565225, Cd40lg, Rgd1559932, Rt1-T18, Rt1-M2, Ifna11, Rt1-Ec2, Tshr, Rt1-Da* | *0.0024* |
| | Glycerophospholipid metabolism | 2.3664 | 10 | *Pla2g10, Dgkg, Pla2g12b, Pla2g1b, Pemt, Lycat, Pcyt1b, Pla2g2d, Chat, Pla2g2f* | *0.0085* |
| | VEGF signaling pathway | 1.9827 | 10 | *Sh2d2a, Ptgs2, Pla2g10, Pla2g12b, Pla2g1b, Chp2, Raf1, Hspb1, Pla2g2d, Pla2g2f* | *0.0270* |
| | B cell receptor signaling pathway | 1.9563 | 10 | *Loc688112, Bcl10, Loc690948, Chp2, Raf1, Loc687079, Loc683446, Pira2, Lilrb3l, Cd19, Rasgrp3, Lilrb3, Sos2, Cd22, Cd79b, Cd79a, Ddah1* | *0.0300* |
| | Gap junction | 1.8113 | 10 | *Tjp1, Plcb4, Loc679312, Gnaq, Map3k2, Sos2, Raf1, Prkg2, Plcb1, Itpr1* | *0.0470* |
| | Melanoma | 1.9137 | 9 | *Fgf6, Fgf5, Fgf8, Cdkn2a, Fgf17, Raf1, Cdh1, Fgf1, Fgf3* | *0.0430* |
| | Maturity onset diabetes of the young | 4.5144 | 8 | *Onecut1, Gck, Hnf4a, Foxa2, Bhlha15, Pdx1, Neurog3, Ins2* | *0.0002* |
| | Steroid hormone biosynthesis | 2.7297 | 8 | *Hsd17b2, Cyp11a1, Cyp11b1, Cyp11b2, Cyp21a1, Ugt1a1, Ugt1a6, Ugt1a9, Cyp17a1, Ugt1a8, Ugt1a7c, Ugt1a3, Ugt1a2, Ugt1a5, Ugt2a1* | *0.0076* |
| | Allograft rejection | 2.2146 | 8 | *Il4, Prf1, Cd40lg, Rt1-T18, Rt1-M2, Il12b, Rt1-Ec2, Rt1-Da* | *0.0260* |
| | Arginine and proline metabolism | 2.2146 | 8 | *Pycr1, Abp1, Ckm, Prodh2, Dao, Aldh1a7, Agmat, Aldh3a2* | *0.0260* |
| | Glycerolipid metabolism | 2.3342 | 7 | *Cel, Dgkg, Lipg, Lycat, Aldh1a7, Lipc, Aldh3a2* | *0.0280* |
| | Asthma | 3.8275 | 6 | *Il4, Il3, Cd40lg, Prg2, Il13, Rt1-Da* | *0.0036* |
| | Primary immunodeficiency | 2.5892 | 6 | *Cd19, Cd40lg, Tnfrsf13c, Aire, Rag1, Cd79a, Loc687079* | *0.0250* |
| | Ether lipid metabolism | 2.5892 | 6 | *Pla2g10, Pla2g12b, Pla2g1b, Lycat, Pla2g2d, Pla2g2f* | *0.0250* |
| | alpha-Linolenic acid metabolism | 4.3153 | 5 | *Pla2g10, Pla2g12b, Pla2g1b, Pla2g2d, Pla2g2f* | *0.0045* |
| | Histidine metabolism | 3.0566 | 5 | *Abp1, Ftcd, Aldh1a7, Uroc1, Aldh3a2* | *0.0210* |
| *Testosterone Only* | Pathways in cancer | 1.4751 | 22 | *Wnt5a, Fgf14, Wnt1, Max, Ptk2, Stat4, Lamb2, Sos1, Pik3ca, Egf, Pik3r1, Loc685653, Loc685590, Bmp4, Loc499735, Crebbp, Rxrg, Hgf, Stat1, Rad51, Fzd6, Wnt2b, Cdkn1a, Ncoa4, Loc685605, Crk, Loc685626* | *0.0420* |
| | Neuroactive ligand-receptor interaction | 1.7036 | 21 | *Rgd1308470, Tspo, Drd3, Tacr2, Gabrb2, Npy2r, Bdkrb2, Gabbr2, Gcgr, Grid2, Mcpt8l2, Mcpt8l3, C5ar1, Pth2r, Gabra4, Gzma, Gabra6, Mcpt8, Mcpt10, Crhr2, Prlr, Chrm3, Rgd1565970, Drd1a, Glp1r* | *0.0110* |
| | Focal adhesion | 1.6350 | 15 | *Rgd1563410, Loc295810, Itga1, Mylk2, Hgf, Itga4, Vav2, Actg1, Ptk2, Lamb2, Sos1, Pik3ca, Loc680688, Egf, Loc685605, Crk, Rapgef1, Loc685653, Loc685626, Pik3r1, Loc685590, Thbs4* | *0.0400* |

| | Term | Fold Enrichment | Gene Count | Genes | *Fisher Exact p-Value* |
|---|---|---|---|---|---|
| | Insulin signaling pathway | 2.0933 | 13 | *Socs3, Phkb, Phka1, Fbp1, Mknk2, Eif4ebp1, Hk3, Sos1, Pik3ca, Prkaa1, Loc685605, Crk, Rapgef1, Pik3r1, Loc685626, Loc685653, Loc685590* | *0.0088* |
| | ErbB signaling pathway | 2.7506 | 11 | *Nrg3, Erbb4, Ptk2, Cdkn1a, Eif4ebp1, Sos1, Camk2d, Pik3ca, Loc685605, Egf, Crk, Pik3r1, Loc685626, Loc685653, Loc685590* | *0.0019* |
| | TGF-beta signaling pathway | 2.2243 | 9 | *Bmp4, Rgd1309707, Loc684620, Loc679119, Loc690504, Gdf6, Rbl1, Smad5, Crebbp, Bmp7, Thbs4* | *0.0190* |
| | Progesterone-mediated oocyte maturation | 2.1988 | 9 | *Anapc1, Loc499735, Cpeb1, Cdc25c, Rps6ka6, Mapk13, Pik3ca, Anapc7, Loc685605, Pik3r1, Loc685626, Loc685653, Loc685590* | *0.0200* |
| | Inositol phosphate metabolism | 3.2700 | 8 | *Pip5k3, Aldh6a1, Plce1, Pik3c2a, Synj1, Pik3c3, Pik3ca, Ocrl, Loc685605, Loc685653, Loc685626, Loc685590* | *0.0027* |
| | Phosphatidylinositol signaling system | 2.4291 | 8 | *Pip5k3, Plce1, Pik3c2a, Synj1, Pik3c3, Pik3ca, Ocrl, Loc685605, Loc685653, Loc685626, Pik3r1, Loc685590* | *0.0160* |
| | Hematopoietic cell lineage | 2.1800 | 8 | *Il1r1, Dntt, Cd3e, Rt1-Db1, Fcer2, Itga1, Mme, Itga4* | *0.0290* |
| | Retinol metabolism | 2.5218 | 7 | *Cyp1a2, Ugt1a1, Rdh5, Rdh12, Ugt1a6, Cyp4x1, Ugt1a9, Rdh7, Ugt1a8, Ugt1a7c, Ugt1a3, Ugt1a2, Ugt1a5, Cyp4a8, Retsat* | *0.0200* |
| | Sphingolipid metabolism | 3.0364 | 6 | *Sgpl1, Pomt2, Sptlc2, Acer1, Galc, Sgms1, Asah2* | *0.0130* |
| | Intestinal immune network for IgA production | 2.8340 | 6 | *Rt1-Db1, Madcam1, Itga4, Pigr, Il10, Loc688090, Rt1-Bb* | *0.0180* |
| | Hedgehog signaling pathway | 2.4525 | 6 | *Bmp4, Wnt5a, Wnt1, Loc679119, Bmp7, Wnt2b* | *0.0340* |
| | Renin-angiotensin system | 5.3137 | 5 | *Ren, Rgd1565970, Mcpt8l2, Mme, Mcpt8l3, Cma1, Mcpt8, Mcpt10* | *0.0019* |
| | Taurine and hypotaurine metabolism | 6.3764 | 3 | *Gad2, Baat, Gad1* | *0.0097* |
| *Estradiol Only* | Neuroactive ligand-receptor interaction | 1.8553 | 16 | *Taar7a, Taar8c, Prss1, Oxtr, Lpar2, Nr3c1, Ptgfr, Gh1, Taar9, Nmur1, Galr3, Avpr1b, F2, Glp2r, Htr2c, Htr1f* | *0.0120* |
| | Endocytosis | 1.9649 | 13 | *Rgd1309707, Loc684620, Rt1-M4, Dnm3, Il2rb, Ret, Loc690504, Cbl, Rufy1, Rgd1561176, Zfyve20, Chmp2b, Rab22a, Rab11b, Smurf2, Pdcd6ip* | *0.0150* |
| | Jak-STAT signaling pathway | 2.6228 | 12 | *Il2rb, Il23r, Il5, Cbl, Stat1, Ptpn11, Il20, Gh1, Ifna2, Stat4, Ifna1, Rgd1565225, Il20rb, Ccnd2, Ifna11* | *0.0020* |
| | Calcium signaling pathway | 1.8362 | 11 | *Loc497963, Slc8a2, Gna11, Ppp3r2, Oxtr, Sae1, Ptgfr, Avpr1b, Gnas, Cacna1e, Cacna1f, Nos2, Loc678722, Htr2c* | *0.0370* |
| | Ubiquitin mediated proteolysis | 2.1700 | 9 | *Loc691436, Rgd1309707, Loc684620, Loc690504, Ube2z, Slc8a2, Loc499959, Cbl, Sae1, Ube2c, Cul5, Wwp2, Smurf2, Loc678722, Ube2s* | *0.0220* |
| | Insulin signaling pathway | 2.0714 | 9 | *Ppp1r3c, G6pc, Ppp1r3b, Sorbs1, Pklr, Cbl, Acaca, Pde3b, Ins1* | *0.0290* |
| | Retinol metabolism | 4.1194 | 8 | *Rdh8, Cyp4a2, Cyp4a3, Adh1, Cyp2c7, Cyp2c23, Dhrs9, Adh7, Cyp2a1* | *0.0006* |

| Term | Fold Enrichment | Gene Count | Genes | *Fisher Exact p-Value* |
|---|---|---|---|---|
| Natural killer cell mediated cytotoxicity | 2.4304 | 8 | *Ifna2, Ifna1, Rgd1565225, Tnfrsf10b, Ifna11, Ppp3r2, Gzmb, Nfatc3, Hcst, Ptpn11* | *0.0170* |
| Autoimmune thyroid disease | 3.4863 | 7 | *Tg, Rt1-M4, Ifna2, Ifna1, Rgd1565225, Il5, Rt1-Ha, Ifna11, Gzmb* | *0.0036* |
| Drug metabolism | 2.9537 | 7 | *Adh1, Aldh1a3, Cyp2c7, Cyp2c23, Adh7, Cyp2d3, Cyp2a1* | *0.0090* |
| Antigen processing and presentation | 2.4166 | 7 | *Rt1-M4, Ifna2, Ifna1, Rgd1565225, Cd8a, Rfx5, Rt1-Ha, Ifna11, Nfyc* | *0.0250* |
| PPAR signaling pathway | 2.5674 | 6 | *Plin, Cyp4a2, Sorbs1, Cyp4a3, Scd, Loc301444, Cyp8b1, Dbi* | *0.0290* |
| Notch signaling pathway | 3.1000 | 5 | *Dtx2-Ps1, Psen1, Rbpsuh, Dtx2, Skiip, Rbpj, Ptcra* | *0.0220* |
| Tyrosine metabolism | 3.5742 | 4 | *Adh1, Aldh1a3, Adh7, Aoc3* | *0.0240* |

**Table 2**

qRT-PCR validation of selected genes uniquely regulated by testosterone replacement, estradiol replacement, or commonly regulated by both treatments.

| | | ***TESTOSTERONE (vs. PLACEBO)*** | | ***ESTRADIOL (vs. PLACEBO)*** | |
|---|---|---|---|---|---|
| | **Gene Symbol** | **Fold Change** | ***p-Value*** | **Fold Change** | ***p-Value2*** |
| *Commonly Regulated* | *Ngfr* | −2.749 | 0.039 | −2.889 | 0.046 |
| | *Gabra3* | 2.811 | 0.041 | 1.725 | 0.039 |
| | *Raf1* | 1.810 | 0.034 | 2.190 | 0.007 |
| | *Nt4* | −6.228 | 0.016 | −3.894 | 0.022 |
| *Testosterone Only* | *Pik3r1* | 3.351 | 0.001 | — | — |
| | *Egf* | 1.749 | 0.050 | — | — |
| | *Hgf* | 1.603 | 0.035 | — | — |
| | *Eif4ebp1* | −1.330 | 0.013 | — | — |
| *Estradiol Only* | *Vegf* | — | — | −2.157 | 0.028 |
| | *Gr* | — | — | 1.604 | 0.032 |
| | *5ht2c* | — | — | 1.474 | 0.041 |

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