Published in final edited form as:

Neurobiol Learn Mem. 2007 January ; 87(1): 78–85. doi:10.1016/j.nlm.2006.07.001.

Androgens' effects to enhance learning may be mediated in part through actions at estrogen receptor- β in the hippocampus

Kassandra L. Edinger^b and Cheryl A. Frye^{a,b,c,d,*}

^aDepartment of Psychology, The University at Albany-SUNY, Life Sciences Research Building Rm 1058, 1400 Washington Avenue, Albany, NY 12222, USA

^bDepartment of Biological Sciences, The University at Albany-SUNY, 1400 Washington Avenue, Albany, NY 12222, USA

^cThe Centers for Life Science, The University at Albany-SUNY, 1400 Washington Avenue, Albany, NY 12222, USA

^dNeuroscience Research, The University at Albany-SUNY, 1400 Washington Avenue, Albany, NY 12222, USA

Abstract

Testosterone (T) may enhance cognitive performance. However, its mechanisms are not well understood. First, we hypothesized that if T's effects are mediated in part through actions of its 5 α -reduced metabolites, dihydrotestosterone (DHT) and/or 3 α -androstenediol (3 α -diol) in the hippocampus, then T, DHT, and 3 α -diol-administration directly to the hippocampus should enhance learning and memory in the inhibitory avoidance task. In order to test this hypothesis, gonadectomized (GDX) male rats were administered T, DHT, or 3 α -diol via intrahippocampal inserts immediately following training in the inhibitory avoidance task. We found that T tended to increase, and DHT and 3 α -diol significantly increased, performance in the inhibitory avoidance task compared to vehicle-administered GDX rats. Second, we hypothesized that, if androgens' effects are due in part to actions of 3 α -diol in the hippocampus, then systemic or intrahippocampal administration of 3 α -diol should significantly enhance cognitive performance of GDX male rats. Third, we hypothesized that, if androgen metabolites can have actions at estrogen receptors (ERs) in the hippocampus, then administration of ER antisense oligonucleotides (AS-ODNs) directly to the hippocampus of GDX, 3 α -diol replaced, rats would decrease learning in the inhibitory avoidance task. We found that intrahippocampal administration of AS-ODNs for ER β , but not ER α , significantly decreased learning and memory of 3 α -diol replaced rats. Together, these findings suggest that T's effects to enhance learning and memory may take place, in part, through actions of its metabolite, 3 α -diol, at ER β in the dorsal hippocampus.

Keywords

Androgen; 3 α -Androstenediol; Estrogen receptors; Androgen receptors; Cognition; ER β

1. Introduction

Gender differences or developmental changes in androgen levels are correlated with differences in cognitive performance. Adult men with higher testosterone (T) levels show

better cognitive performance on spatial tasks than do women (Bannerman et al., 2002; Barrett-Connor & Goodman-Gruen, 1999; Christiansen & Knussman, 1987; Gouchie & Kimura, 1991; Moffat & Hampson, 1996; Moffat et al., 2003; Nass & Baker, 1991). Men with lower levels of T, due to hypogonadism or aging, demonstrate poorer cognitive functioning than their androgen-replete counterparts, and these deficits can be reduced by T-replacement therapy (Janowsky, Chavez, & Orwell, 1994; Lund, Bever-Stille, & Perry, 1999; Sternbach, 1998; Tan, 2001). These findings suggest that T-administration may alter cognitive behavior of people. In rodents, gonadectomy (GDX) decreases cognitive performance of male rats (Ceccarelli & Scaramuzzino, 2001; Frye & Seliga, 2001). Androgen replacement to GDX rats restores performance in the inhibitory avoidance (Edinger & Frye, 2004; Frye & Edinger, 2004; Frye, Edinger, Seliga, & Wawrzycki, 2004; Frye & Seliga, 2001), object recognition tasks (Ceccarelli & Scaramuzzino, 2001), and conditioned contextual fear tasks (Edinger, Lee, & Frye, 2004). These findings suggest that androgen-depletion causes deficits in cognitive performance which can be ameliorated through T-replacement.

T's effects to enhance cognitive performance may be influenced by its aromatization to estrogen (E_2), which can enhance cognition in people (Drake et al., 2000; Sherwin, 2002) and animals (Frye & Rhodes, 2002). T is also metabolized by 5 α -reductase to dihydrotestosterone (DHT), which is further metabolized by 3 α -hydroxysteroid dehydrogenase (3 α HSD) to 5 α -androstane, 3 α ,17 β -diol (3 α -diol). Systemic DHT and 3 α -diol administration are effective at enhancing cognition of GDX rats in a variety of learning tasks (Edinger & Frye, 2004; Edinger et al., 2004; Frye & Edinger, 2004; Frye et al., 2004; Frye & Lacey, 2001; Frye & McCormick, 2000; Frye, Park, Tanaka, Rosellini, & Svare, 2001; Frye, Rhodes, Rosellini, & Svare, 2002; Frye & Seliga, 2001). Further, blocking DHT's metabolism to 3 α -diol with indomethacin decreases DHT's cognitive-enhancing effects in male rats (Frye et al., 2004). In studies examining the effects of T, DHT, and 3 α -diol replacement on GDX rats, all androgen administrations significantly enhanced learning and memory in the inhibitory avoidance and conditioned contextual fear tasks (Edinger & Frye, 2004; Edinger et al., 2004). However, the only androgen that was consistently elevated in each of these groups was 3 α -diol (Edinger & Frye, 2004). These data suggest that some of T's effects to enhance cognitive performance may be mediated by actions of 3 α -diol in the hippocampus.

There are many substrates where androgens may exert their effects to enhance cognitive performance. For instance, T and DHT bind with high affinity to androgen receptors (ARs; Roselli, Horton, & Resko, 1987). Intrahippocampal administration of flutamide, an AR antagonist, can reverse the beneficial cognitive-enhancing effects of DHT-replacement to GDX male rats (Frye et al., 2004). However, these studies are not definitive, as flutamide has been shown to exert benzodiazepine-like effects in rodents (Ahmadiani, Mandgary, & Sayyah, 2003). In addition, 3 α -diol, a metabolite of T and DHT, does not typically bind to ARs (Roselli et al., 1987), but can be back-converted to DHT, which can act at ARs (Roselli et al., 1987). Together, these findings suggest that androgens can have effects at ARs in the hippocampus to enhance learning and memory.

In addition to ARs, there are other possible targets for androgens' actions. 3 α -diol, which may have effects at GABA_A receptors (GBRs) and estrogen receptors (ERs; Frye, Van Keuren, & Erskine, 1996; Gee, Bolger, Brinton, Coirini, & McEwen, 1988; Masonis & McCarthy, 1995; Pak et al., 2005), can enhance learning and memory independent of T and DHT (Edinger & Frye, 2004, 2005). There are also multiple possible substrates for 3 α -diol's actions. While 3 α -diol has a low affinity for ARs, it has been shown to bind to GBRs (Frye, Van Keuren, et al., 1996; Gee et al., 1988; Masonis & McCarthy, 1995). In addition, other studies have shown that 3 α -diol may bind to ERs (Brown, Adler, Sharma, Hochberg, &

Maclusky, 1994). Together, these findings suggest that some of androgens' effects to enhance learning and memory may take place through actions at ERs.

Notably, there are two major subtypes of ERs, ER α and ER β , where 3 α -diol could possibly exert its effects (Kuiper, Shughrue, Merchenthaler, & Gustafsson, 1998; Tremblay et al., 1997). The classic ER, ER α , and ER β share ~97% of their DNA and ~60% of their ligand binding domains but have distinct N-terminal regions (Tremblay et al., 1997). While many studies have indicated that ER α may be an important modulator of reproductive function (Basu & Rowan, 2005; Connor et al., 2005; Hewitt & Korach, 2003; Shimizu et al., 2005; Walf, Rhodes, & Frye, 2004), other studies have shown that ER β is an important modulator of cell proliferation and learning and memory (Rissman, Heck, Leonard, Shupnik, & Gustafsson, 2002; Zhang, Cai, De Zhou, & Su, 2002). ER β KO mice, which lack functional ER β , show impaired acquisition in the Morris Water Maze task compared to their wildtype counterparts (Rissman et al., 2002), and show increased anxiety in the open field and elevated plus maze tasks (Krezel, Dupont, Krust, Chambon, & Chapman, 2001). In addition, administration of selective ER modulators that target ER β , but not ER α , have been shown to enhance cognitive performance, anti-anxiety, and anti-depressive behavior in female rats (Rhodes & Frye, 2006; Walf et al., 2004). Both extranuclear ER α and ER β have been localized to the hippocampus and the amygdala, both brain regions which are important modulators of learning and memory (Kalita, Szymezak, & Kacsmarck, 2005; Mehra, Sharma, Nyakas, & Vij, 2005; Shughrue, Lane, & Merchenthaler, 1997). Notably, 3 β -diol has been shown to bind with high affinity to ER β , but not ER α (Pak et al., 2005). Thus, it is important to dissociate the mechanisms that ER α and ER β may modulate reproductive and cognitive and affective processes. If actions at ER β underlie estrogens' mnemonic actions, then it may be possible to develop hormone therapies that target this region to result in beneficial behavioral effects.

To address whether T's effects to enhance cognitive performance may involve metabolism to DHT and/or 3 α -diol in the dorsal hippocampus, T, DHT, and 3 α -diol were administered via intrahippocampal inserts to GDX male rats. We hypothesized that, if actions of T's 5 α -reduced metabolites in the dorsal hippocampus are important for androgens' cognitive effects, then administration of T, DHT, and 3 α -diol directly to the hippocampus should enhance cognitive performance in the inhibitory avoidance task. To address whether T's effects to enhance cognitive performance were due, in part, to actions of 3 α -diol in the hippocampus, 3 α -diol or vehicle control was administered systemically or intrahippocampally to GDX male rats. We hypothesized that, if 3 α -diol in the hippocampus is important for T's effects, then systemic and intrahippocampal administration of 3 α -diol to GDX rats should similarly enhance performance in the inhibitory avoidance task. To address where in the hippocampus 5 α -reduced androgens may exert their effects on learning and memory, 3 α -diol replaced GDX rats were infused with vehicle control or antisense oligonucleotides (AS-ODNs) that targeted ER α , ER β , both ER α and ER β (ER), or that had a scrambled codon (ER scrambled).

2. Methods

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

2.1. Animals and housing

All male Long-Evans rats ($N = 110$) approximately 55 days of age, were obtained from the breeding colony at the University at Albany (original stock from Taconic Farms, Germantown, NY). Rats were housed in groups of 2–4 in a temperature- ($21 \pm 1^\circ\text{C}$) and humidity-controlled (45–55%) room in the laboratory animal care facility. The rats were

maintained on a 12/12 hour reversed light cycle (lights off 8:00 a.m.) with unrestricted access to Purina Rat Chow and tap water in their home cages.

2.2. Surgery and hormone replacement

Rats were anesthetized with xylazine (12 mg/kg) and ketamine (60 mg/kg) prior to surgical procedures. Rats were GDX 4–6 weeks prior to behavioral testing to ensure the absence of endogenous androgens.

All rats were stereotactically implanted with bilateral guide cannulae aimed over the hippocampus (from bregma AP = −3.8, ML = ±2.0, DV = −2.0; Paxinos & Watson, 1985). Cannulae consisted of 23-gauge thin wall stainless steel guide tubing with 30-gauge removable inserts, made to extend 3 mm beyond the dorsal tip of the guide cannulae.

For experiment 1, GDX rats were implanted with inserts tamped in crystalline T, DHT, 3 α -diol, or left empty (control; n = 15 per group) aimed at the dorsal hippocampus immediately following training in the inhibitory avoidance task. For experiment 2, GDX rats were either administered vehicle control (n = 7), implanted with an insert tamped in crystalline 3 α -diol (n = 7), or injected sub-cutaneously with a 1 mg/kg injection of 3 α -diol (n = 7). For experiment 3, 3 α -diol replaced rats (n = 30) received a 1 mg/kg subcutaneous injection of 3 α -diol immediately following training. This paradigm has been shown to increase 3 α -diol in plasma and in the hippocampus to physiological levels, and to enhance learning and memory in the inhibitory avoidance tasks (Edinger & Frye, 2004, 2005). 3 α -diol replaced rats were administered 2 μ g of AS-ODNs (ER, ER α , or ER β), scrambled ODNs (ER scrambled), or saline control through bilateral cannulae aimed directly to the CA1 region of the dorsal hippocampus 2 h prior to training in the inhibitory avoidance tasks, in conjunction with 3 α -diol immediately following training in the inhibitory avoidance task, and 30 min prior to testing. Rats were infused with antisense ODNs (ER with the sequence 5'-CAT-GCT-CAT-GCT-CAG-3', ER α with the sequence 5'-CAT-GGT-CAT-GGT-CAG-3', and ER β with the sequence 5'-GAA-TGT-CAT-AGC-TGA-3'), scrambled control ODNs (ERscrambled; ER α and β scrambled ODNs that have the same base pairs and GC ratio, but the order randomized, and little or no homology to any mRNA sequences posted at GenBank; 5'-ATC-GTG-GAT-CGT-GAC-3' and 5'-AAG-GTT-ATC-GCA-AGT-3'), respectively (Liang et al., 2002). This regimen was adapted from previously published regimens that have been used successfully in our lab (Walf, Ciriza, Garcia-Segura, & Frye, in press; Walf, Rhodes, Meade, Harney, & Frye, in press). Immunocytochemistry on tissues from similar experiments have shown that this same ER β AS-ODN regimen, when infused into the ventricles knocks down expression of ER β immunoreactivity in the CA1 region of the hippocampus, which we directly targeted with AS-ODN in this study (Walf, Ciriza, et al., in press). GDX control rats with no 3 α -diol replacement (n = 6) were administered vehicle control to the hippocampus.

2.3. Site analyses and tissue dissection

Some brains were used to examine location of cannulae placement with light microscopy, while others were used to conduct Western Blot Analyses. After testing in Experiments 1 and 2, rats were perfused with 0.9% saline followed by 10% formalin. Brains were removed from the skull, fixed in formalin, and then sliced on a cryostat at 40 μ m. Slices were then stained with cresyl violet and infusion location was determined with light microscopy. Rats that did not receive implants to the CA1 region of the dorsal hippocampus (n = 3) and instead received implants that were too ventral and located within the posterior nucleus of the hypothalamus were excluded from data analyses (see Table 1).

In Experiment 3, rats were re-administered their 3 α -diol and AS-ODN regimen and rapidly decapitated. Brains were quickly removed from the skull, and placed on dry ice. Whole brains were stored at -80°C until the hippocampus and pituitary (as a positive control) were dissected out. Cannulae placement was verified during dissection. For dissections, brains were first gently thawed on ice. The hippocampus and pituitary were each placed in eppendorf tubes containing protease inhibitor cocktail dissolved in distilled water (Roche, 11836145001). Samples were transferred to test tubes on ice and were homogenized. Homogenized samples were then stored in the original eppendorf tubes containing the protease inhibitor overnight at 4°C .

2.4. Western blot analysis

To ensure effective knockdown of ERs with the AS-ODN regimen utilized, western blot analyses, as used in previous experiments from our laboratory, were used to measure differences in ER protein expression in the hippocampus (Walf, Rhodes, et al., in press). To measure the extent to which AS-ODNs bound to the translation sites of receptor genes and inhibited translation, standard western blotting techniques were employed (Schreihöfer et al., 2002). Polyclonal anti-rabbit antibodies against ER α (C1355; Upstate Biotechnology, Lake Placid, NY; Tanabe, Miyasaka, Kubota, & Aso, 2004; Tanaka, Ohtani-Kaneko, Yokosuka, & Watanabe, 2004) or ER β (PA1-311; Affinity Bioreagents; Mehra et al., 2005) were used. As a positive control, western blot analyses using a monoclonal antimouse β -actin antibody (A1978; Sigma) were also performed. Samples were homogenized and centrifuged for 20 min at $11 \times 1000g$. Following centrifugation, supernatant was separated from pellet and stored at -20°C . Protein concentration in each sample was then assessed using a Bradford Assay (Bradford, 1976). Based on the assay information, $10 \mu\text{g}$ of protein and sample buffer (50 mM TrisCl – pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), yielding a final volume of $10 \mu\text{l}$, were loaded onto 1.0 mm gels. Samples were then resolved on 10% SDS polyacrylamide gel with 25 mM Tris-glycine buffer, pH 8.3, which contained 20% methanol, at 100 V for 1.5–2 h at room temperature. They were then electrophoretically transferred to nitrocellulose membrane (Pierce; with pore size of $0.45 \mu\text{m}$) using a 25 mM Tris-glycine buffer, pH 8.3, containing 20% methanol, at 100 V for 1.5 h at 4°C . Following transfer, Pierce's Blocking Buffer (Pierce, Rockford, IL) was used to block nitrocellulose membranes with 0.05% Tween-20 overnight at 4°C . The membranes were washed in buffer (PBS with 0.05% Tween-20) on a rocking platform before incubation with the primary antibodies for ER α (1:1000 dilution), ER β (1:1000 dilution), or β -actin (1:1000 dilution) in blocking buffer with 0.05% Tween-20 overnight at 4°C . Following incubation with the primary antibody, membranes were washed in buffer on a rocking platform and were incubated with the appropriate horseradish peroxidase-labeled goat antirabbit (1:3000 dilution for ER α , 1:2000 dilution for ER β), or goat antimouse secondary antibody (1:5000 dilution for β -actin,) supplied in the chemiluminescence kit (Pierce, Supersignal West FEMTO Max Sensitivity kit; 34095), in blocking buffer with 0.05% Tween-20, for 1 h with agitation. Finally, membranes were washed in buffer, soaked in the chemiluminescence medium for 5 min, and exposed to X-ray film, and film was developed.

2.5. Inhibitory avoidance task

Rats were tested in the inhibitory avoidance task, a measure of emotional memory (O'Connell, Early, & Leonard, 1994; Venault et al., 1986). The hippocampus mediates performance in the inhibitory avoidance task (Edinger & Frye, 2004; Martinez, Quirarte, Diaz-Cintra, Quiroz, & Prado-Alcala, 2002). Hippocampal integrity is important for performance in this task (Martinez et al., 2002). In addition, the inhibitory avoidance task is sensitive to androgen administration (Edinger & Frye, 2004; Edinger et al., 2004). Administration of androgens systemically (Edinger et al., 2004) or directly to the

hippocampus (Edinger & Frye, 2004) increases performance in task. Thus, this task allowed us to examine the effects and mechanisms by which androgens hippocampally mediated learning and memory.

Rats were trained on day one. On training day, rats were placed in a two-compartment ($24 \times 18 \times 19$ cm each), stainless steel box separated by a guillotine door. One chamber was white and brightly lit from above while the other chamber was black and covered so as to block all light. Rats were allowed to explore the brightly lit chamber (2 min). Following a 30-min latency, rats were again allowed to explore the white chamber (1 min). The door was lifted and the crossover latency was recorded (max latency 20 min). The door was closed behind them and a mild shock administered (0.25 mA, 2-s duration). Twenty-four hours later, rats were habituated in the white chamber (1 min). The door was lifted and the crossover latency was measured (a maximum latency of 5 min was allowed). Longer latencies to cross to the shock-associated side indicate better performance.

3. Results

3.1. Experiment 1

Intrahippocampal androgen administration increased cognitive performance in the inhibitory avoidance task ($F_{(3,56)} = 6.138$, $p = 0.001$). Intrahippocampal DHT ($p = 0.007$) and 3 α -diol ($p = 0.007$) significantly increased, and T ($p = 0.06$) tended to increase, crossover latencies compared to GDX controls (see Fig. 1).

3.2. Experiment 2

Systemic ($p = 0.001$) and intrahippocampal ($p = 0.001$) administration of 3 α -diol significantly increased crossover latencies compared to GDX rats in the inhibitory avoidance task ($F_{(2,18)} = 17.28$, $p = 0.0001$). There were no differences between systemic and intrahippocampal administration of 3 α -diol ($p = 0.92$; see Fig. 2).

3.3. Experiment 3

3 α -Diol replacement significantly enhanced cognitive performance in the inhibitory avoidance task compared to GDX rats administered vehicle control ($F_{(5,30)} = 5.37$, $p = 0.001$). Intrahippocampal administration of the AS-ODNs for ER ($p = 0.002$), and ER β (0.008), but not ER α ($p = 0.94$) or ER scrambled ($p = 0.92$), significantly decreased cognitive performance in the inhibitory avoidance task ($F_{(5,30)} = 5.37$, $p = 0.001$; see Fig. 3).

The efficacy of our AS-ODN infusion regimen is revealed by western blot analyses. Western blots using the β -actin antibody revealed presence of protein in samples. Bands for ER α were seen in rats administered ER β AS-ODN, but not in rats administered ER α AS-ODN. Similarly, bands for ER β were seen in rats administered ER α AS-ODN, but not in rats administered ER β AS-ODN (see Fig. 3, inset), demonstrating that ERs were knocked down. This replicates findings from previous experiments, where immunocytochemistry on tissue from animals treated with these AS-ODNs to the hippocampus illustrated decreased representation of the corresponding ER (Walf, Ciriza, et al., in press).

4. Discussion

The results of this experiment are consistent with our hypothesis. First, we hypothesized that intrahippocampal administration of T and its 5 α -reduced metabolites, DHT and 3 α -diol, would enhance learning in the inhibitory avoidance task compared to GDX controls. In support, T tended to increase, and DHT and 3 α -diol significantly increased, crossover

latencies compared to that of vehicle-administered GDX rats. Notably, DHT and 3 α -diol administration was more effective than T at enhancing cognitive performance. These findings suggest that androgens' effects to enhance learning and memory may be mediated in part by actions of T's 5 α -reduced metabolites in the dorsal hippocampus. Second, we hypothesized that, if T's effects are due in part to actions of 3 α -diol in the hippocampus, then systemic and intrahippocampal administration of 3 α -diol to GDX male rats should similarly enhance cognitive performance in the inhibitory avoidance task. In support, systemic and intrahippocampal administration of 3 α -diol both significantly increased crossover latencies compared to GDX male rats. Because there were no differences between intrahippocampal and systemic groups, systemic 3 α -diol replacement was used in the third experiment. Third, we hypothesized that, if some of T's effects to enhance learning and memory are due to its metabolism to 3 α -diol and its subsequent actions at ER β in the hippocampus, then infusions of AS-ODNs for ER β directly to the hippocampus of 3 α -diol replaced GDX rats should decrease learning and memory in the inhibitory avoidance task. In support, we found that systemic administration of 3 α -diol to GDX rats significantly increases learning and memory compared to GDX control rats. As verified by western blots, ER α AS-ODNs were effective at knocking down expression of ER α , but not ER β . Similarly, ER β AS-ODNs to the hippocampus were effective at knocking down expressions of ER β , but not ER α . We found that infusions of ER or ER β -specific AS-ODNs, but not ER α or scrambled AS-ODNs, were effective at decreasing learning and memory in 3 α -diol replaced rats. However, it is important to note that, although there are several variants of ER β expressed in the brain, our Western Blot technique did not discern between these different types. Together, these findings suggest that T's effects to enhance learning and memory may take place, in part, through its metabolism to 3 α -diol, and its subsequent actions at ER β in the hippocampus.

The present findings, as in previous inhibitory avoidance (Frye & Edinger, 2004; Frye et al., 2004), object recognition (Ceccarelli & Scaramuzzino, 2001), and conditioned contextual fear (Edinger et al., 2004) experiments, indicate that GDX associated deficits in affective and cognitive performance of male rats can be reduced through T-replacement. However, T's effects are poorly understood due to its multiple routes of metabolism. One possible route for T's effects to decrease anxiety and enhance cognitive performance is through its metabolism to E₂, which may be responsible for some of T's effects. Studies have found that E₂ to male rodents enhances learning (Luine & Rodriguez, 1994; Packard, Kohlmaier, & Alexander, 1996). However, previous studies have found that T, DHT, and 3 α -diol were similarly effective at enhancing cognitive performance (Edinger et al., 2004; Frye & Edinger, 2004; Frye et al., 2004). In addition, in the second and third studies, we found that systemic administration of 3 α -diol alone was effective at enhancing learning and memory of GDX male rats. Previous findings have indicated that blocking DHT's metabolism to 3 α -diol through intrahippocampal administration of indomethacin attenuates androgens' cognitive behavioral effects in the inhibitory avoidance task (Frye et al., 2004). This indicates that some of androgens' effects to enhance cognitive performance may be mediated in part by its metabolism to 3 α -diol.

While the present study indicates that some of androgens' effects to enhance learning and memory may be due to metabolism to DHT and/or 3 α -diol, the mechanism for this action is still unclear. It is possible that the effects of androgens to enhance performance in the inhibitory avoidance task are due to a combination of effects mediated via ARs, GBRs, and/or ER β . T and DHT can act at ARs (Roselli et al., 1987). However, 3 α -diol does not typically bind with high affinity to ARs (Roselli et al., 1987), and has been shown to have actions at GBRs (Frye, McCormick, Coopersmith, & Erskine, 1996; Frye, Van Keuren, et al., 1996), and at ER β in the hippocampus (Brown et al., 1994; Pak et al., 2005). Studies in KO mice have revealed that lacking the functional receptor, ER β , results in decreased

performance in learning and memory tasks (Rissman et al., 2002), while KO mice lacking the functional receptor ER α showed no deficits in learning tasks, but instead showed deficits in reproductive measures (Hewitt & Korach, 2003). In addition, activity at ER β has been shown to enhance learning and memory, as well as affective processes, of female rats (Rhodes & Frye, 2006; Walf et al., 2004).

The present study extends these findings to suggest that 3 α -diol, which has been shown to have effects on learning and memory (Edinger & Frye, 2004, 2005; Edinger et al., 2004; Frye et al., 2004; Rosellini, Svare, Rhodes, & Frye, 2001), may have actions at ER β to produce these effects. In the present study, administration of AS-ODNs for ER β directly to the hippocampus of 3 α -diol replaced rats significantly decreased emotional learning and memory compared to 3 α -diol replaced rats administered either vehicle control or AS-ODNs for ER α . It is important to note that T can be aromatized to E $_2$ in the hippocampus (MacLusky, Hajszan, Prange-Kiel, & Leranth, 2006), which could mediate androgens' actions at ER β . Given that T can be aromatized to E $_2$, which has a much higher affinity for ER β than does 3 α -diol, it may be expected that T would be more effective at enhancing cognitive performance than its 5 α -reduced metabolites. However, in the present experiment, administration of DHT and 3 α -diol, which cannot be aromatized to E $_2$, were more effective than T at increasing performance in the inhibitory avoidance task, and administration of AS-ODNs for ER β to rats that were 3 α -diol-replaced significantly decreased cognitive performance in the inhibitory avoidance task. It is possible that these disparate effects are related to T being aromatized to E $_2$ and reduced to DHT and/or 3 α -diol occurring in different regions of the hippocampus. It is also possible that blocking ER β alters signaling through other pathways. While there is as yet no definitive evidence that downregulating ER β via AS-ODN administration could impair other signaling mechanisms that are important for androgen action, it remains entirely possible that blocking this mechanism could alter androgen action, even if 3 α -diol does not interact directly with ER β . For example, local E $_2$ and/or androgen biosynthesis may be necessary to maintain synaptic integrity in the hippocampus (MacLusky et al., 2006). Blocking ER β signaling could thus alter the local regulation of synaptic plasticity, interfering with the mechanisms that might be required to mediate the effects of 3 α -diol action on cognitive function, even if these actions do not involve direct 3 α -diol mediated signaling through ER β . Together, these findings suggest that T's effects to enhance learning and memory may take place, in part, through its metabolism to DHT and/or 3 α -diol and their subsequent actions on hippocampal function via AR, GBR, and ER β -mediated mechanisms, which may complement effects mediated via local steroidogenesis of estrogens and/or androgens.

The present findings also extend previous research indicating that androgens may have actions in the hippocampus to enhance cognitive performance. The hippocampus is an important brain region for cognitive behaviors and may be a target for androgens' actions (Kaut & Bunsey, 2001; Martinez et al., 2002). Further, ER β , a possible substrate for androgens' actions, has been localized to the hippocampus (Kalita et al., 2005; Mehra et al., 2005; Shughrue et al., 1997). T-administration to GDX rats increases hippocampal neuronal excitability (Smith, Jones, & Wilson, 2002), and decreases stress-induced cell death in the hippocampus (Frye & McCormick, 2000). Previous studies have found that systemic androgen administration enhances learning in hippocampally mediated, but not amygdala-mediated, portions of the conditioned contextual fear paradigm (Edinger et al., 2004). In this study, direct administration of T and its 5 α -reduced metabolites to the hippocampus enhanced learning. Systemic and intrahippocampal administration of 3 α -diol similarly enhanced cognitive performance of GDX male rats, indicating that some of androgens' actions may take place in the hippocampus. In addition, intrahippocampal and systemic administration of 3 α -diol similarly enhanced cognitive performance of GDX male rats. Blocking actions at ER β through intrahippocampal administration of AS-ODNs resulted in

decreased cognitive performance in 3 α -diol replaced rats. Together, these findings suggest that androgens can have actions in the hippocampus to enhance learning and memory.

However, it is also possible that androgens are acting in other brain regions to produce cognitive-enhancing effects. Androgen administration can increase c-Fos and Fos-related antigens in the central nucleus of the amygdala, the nucleus accumbens, and the frontal cortex (Johannsson-Steensland, Nyberg, & Chahl, 2002). T to the amygdala or nucleus accumbens has been shown to enhance learning (Frye et al., 2002; Naghdi, Oryan, & Estemadi, 2003; Rosellini et al., 2001). ER β has been localized to the amygdala, as well (Kalita et al., 2005; Mehra et al., 2005; Shughrue et al., 1997). However, as previously noted, administration of T, DHT, and 3 α -diol to GDX rats can enhance learning and memory in the contextual, hippocampally mediated portion of the conditioned contextual fear task, but not in the cued, amygdala-mediated portion of the task (Edinger et al., 2004).

In summary, the present findings suggest that T's effects to enhance learning and memory may take place through actions of its 5 α -reduced metabolites in the hippocampus. In support, administration of T tended to increase, and DHT or 3 α -diol directly to the hippocampus of GDX male rats significantly increased, learning and memory in the inhibitory avoidance task. We also found that systemic and intrahippocampal administration of 3 α -diol similarly enhanced cognitive performance in the inhibitory avoidance task. In addition, the present findings suggest that some of T's effects to enhance learning and memory may be due to actions of 3 α -diol at ER β in the hippocampus. We found that systemic or intrahippocampal administration of 3 α -diol alone was enough to significantly enhance learning and memory of GDX male rats. In addition, we found that administration of AS-ODNs that targeted ER or ER β , but not ER α , were effective at blocking 3 α -diol's cognitive-enhancing effects. Together, these findings suggest that some of androgens' effects to enhance learning and memory may be due, in part, to metabolism to 3 α -diol and its subsequent actions at ER β in the hippocampus. These data on the specific actions of steroid hormones are particularly relevant, given the increased use of hormone replacement therapy in the aging population (Witek, 2000).

Acknowledgments

We thank Alicia Walf, Jonathan Meade, and Dr. Harney for their assistance with the Western Blot Analyses in this study. This research was supported by grants from the National Institute of Mental Health (06769801) and the National Science Foundation (03-16083 and 98-96263).

References

- Ahmadiani A, Mandgary A, Sayyah M. Anticonvulsant effect of flutamide on seizures induced by pentylenetetrazole: involvement of benzodiazepine receptors. *Epilepsia*. 2003; 44(5):629–635. [PubMed: 12752460]
- Bannerman DM, Deacon RM, Offen S, Friswell J, Grubb M, Rawlins JN. Double dissociation of function with the hippocampus: spatial memory and hyponeophagia. *Behavioral Neuroscience*. 2002; 5:884–901. [PubMed: 12369808]
- Barrett-Connor E, Goodman-Gruen D. Cognitive function and endogenous sex hormones in older women. *Journal of the American Geriatric Society*. 1999; 47:1289–1293.
- Basu A, Rowan BG. Genes related to estrogen action in reproduction and breast cancer. *Frontiers in Bioscience*. 2005; 1:2346–2372. [PubMed: 15970500]
- Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye *binding. *Analytical Biochemistry*. 1976; 72:248–252. [PubMed: 942051]

- Brown TJ, Adler GH, Sharma M, Hochberg RB, Maclusky NJ. Androgen treatment decreases estrogen receptor binding in the ventromedial nucleus of the rat brain: a quantitative in vitro autoradiographic analysis. *Molecular Cellular Neuroscience*. 1994; 5:549–555.
- Ceccarelli A, Scaramuzzino AM. Effects of gonadal hormones and persistent pain on non-spatial working memory in male and female rats. *Behavioral Brain Research*. 2001; 123:65–76.
- Christiansen K, Knussman R. Sex hormones and cognitive function in men. *Neuropsychobiology*. 1987; 18:27–36. [PubMed: 3444523]
- Connor EE, Wood DL, Sonstegard TS, da Mota AF, Bennett GL, Williams JL, Capuco AV. Chromosomal mapping and quantitative analysis of estrogen-related receptor alpha-1, estrogen receptors alpha and beta and progesterone receptor in the bovine mammary gland. *Journal of Endocrinology*. 2005; 185:593–603. [PubMed: 15930184]
- Drake EB, Henderson VW, Stanczyk FZ, McCleray CA, Brown WS, Smith CA, Rizzo AA, Murdock GA, Buckwalter JG. Associations between circulating sex steroid hormones and cognition in normal elderly women. *Neurology*. 2000; 8:599–603. [PubMed: 10680789]
- Edinger KL, Frye CA. Testosterone's analgesic, anxiolytic, and cognitive-enhancing effects may be due in part to actions of its 5 α -reduced metabolites in the hippocampus. *Behavioral Neuroscience*. 2004; 118:1352–1364. [PubMed: 15598144]
- Edinger KL, Frye CA. Testosterone's anti-anxiety and analgesic effects may be due in part to actions of its 5 α -reduced metabolites in the hippocampus. *Psychoneuroendocrinology*. 2005; 30:418–430. [PubMed: 15721054]
- Edinger KL, Lee B, Frye CA. Mnemonic effects of testosterone and its 5 α -reduced metabolites in the conditioned fear and inhibitory avoidance tasks. *Pharmacology Biochemistry and Behavior*. 2004; 78:559–568.
- Frye CA, Edinger KL. Testosterone's metabolism in the hippocampus may mediate its anti-anxiety effects in male rats. *Pharmacology Biochemistry and Behavior*. 2004; 78:473–481.
- Frye CA, Edinger KL, Seliga AM, Wawrzycki JM. 5 α -Reduced androgens may have actions in the hippocampus to enhance cognitive performance of male rats. *Psychoneuroendocrinology*. 2004; 29:1019–1027. [PubMed: 15219653]
- Frye CA, Lacey EH. Posttraining androgens' enhancement of cognitive performance is temporally distinct from androgens' increases in affective behavior. *Cognitive Affective Behavioral Neuroscience*. 2001; 2:172–182.
- Frye CA, McCormick CM. The neurosteroid, 3 α -androstenediol, prevents inhibitory avoidance deficits and pyknotic cells in the granule layer of the dentate gyrus induced by adrenalectomy in rats. *Brain Research*. 2000; 855:166–170. [PubMed: 10650145]
- Frye CA, McCormick CM, Coopersmith C, Erskine MS. Effects of paced and non-paced mating stimulation on plasma progesterone, 3 α -diol and corticosterone. *Psychoneuroendocrinology*. 1996; 21:431–439. [PubMed: 8844881]
- Frye CA, Park D, Tanaka M, Rosellini R, Svare B. The testosterone metabolite and neurosteroid 3 α -androstenediol may mediate the effects of testosterone on conditioned place preference. *Psychoneuroendocrinology*. 2001; 26:731–750. [PubMed: 11500254]
- Frye CA, Rhodes ME. Enhancing effects of estrogen on inhibitory avoidance performance may be in part independent of intracellular estrogen receptors in the hippocampus. *Brain Research*. 2002; 956:285–293. [PubMed: 12445697]
- Frye CA, Rhodes ME, Rosellini RA, Svare B. The nucleus accumbens as a site of action for rewarding properties of testosterone and its 5 α -reduced metabolites. *Pharmacology Biochemistry Behavior*. 2002; 74:119–127.
- Frye CA, Seliga AM. Testosterone increases analgesia, anxiolysis, and cognitive performance of male rats. *Cognitive Affective Behavioral Neuroscience*. 2001; 1:371–381.
- Frye CA, Van Keuren KR, Erskine MS. Behavioral effects of 3 α -androstenediol. I. modulation of sexual receptivity and promotion of GABA_A/benzodiazepine receptors-stimulated chloride flux. *Behavioral Brain Research*. 1996; 79:109–118.
- Gee KW, Bolger MB, Brinton RE, Coirini H, McEwen BS. Steroid modulation of the chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of

- action. *Journal of Pharmacology and Experimental Therapeutics*. 1988; 246:803–812. [PubMed: 2841455]
- Gouchie C, Kimura D. The relationship between testosterone levels and cognitive ability patterns. *Psychoneuroendocrinology*. 1991; 16:323–334. [PubMed: 1745699]
- Hewitt SC, Korach KS. Oestrogen receptor knockout mice: role for oestrogen receptors alpha and beta in reproductive tissues. *Reproduction*. 2003; 125:143–149. [PubMed: 12578528]
- Janowsky JS, Chavez B, Orwell E. Testosterone influences spatial cognition in older men. *Behavioral Neuroscience*. 1994; 108:324–332.
- Johannsson-Steensland P, Nyberg F, Chahl L. The anabolic-androgenic steroid, nandrolone decanoate, increases the density of Fos-like immunoreactive neurons in limbic regions of the guinea-pig brain. *European Journal of Neuroscience*. 2002; 15:539–544. [PubMed: 11876781]
- Kalita K, Szymezak S, Kacsmarck L. Non-nuclear estrogen receptor beta and alpha in the hippocampus of male and female rats. *Hippocampus*. 2005; 15:404–412. [PubMed: 15669092]
- Kaut KP, Bunsey MD. The effects of lesions to the rat hippocampus or rhinal cortex on olfactory and spatial memory: retrograde and anterograde findings. *Cognitive Affective Behavioral Neuroscience*. 2001; 3:270–286.
- Krezel W, Dupont S, Krust A, Chambon P, Chapman PF. Increased anxiety and synaptic plasticity in estrogen receptor beta-deficient mice. *Proceeds of National Academy of Sciences*. 2001; 9:11278–11282.
- Kuiper GG, Shughrue PJ, Merchenthaler I, Gustafsson JA. The estrogen receptor beta subtype: a novel mediator of estrogen action in neuroendocrine systems. *Frontiers in Neuroendocrinology*. 1998; 19:253–286. [PubMed: 9799586]
- Liang YG, Akishita M, Kim S, Ako J, Hashimoto M, Iijima K, Ohike Y, Watanabe T, Sudoh N, Toba K, Yoshizumi M, Ouchi Y. Estrogen receptor beta is involve in the anorectic active of estrogen. *International Journal of Obesity and Related Metabolic Disorders*. 2002; 26:1103–1109. [PubMed: 12119576]
- Luine V, Rodriguez M. Effects of estradiol on radial arm maze performance of young and aged rats. *Behavioral Neural Biology*. 1994; 62:230–236.
- Lund BC, Bever-Stille KA, Perry PJ. Testosterone and andropause: the feasibility of testosterone replacement therapy in older men. *Pharmacotherapy*. 1999; 8:951–956. [PubMed: 10453966]
- MacLusky NJ, Hajszan T, Prange-Kiel J, Leranth C. Androgen modulation of hippocampal synaptic plasticity. *Neuroscience*. 2006; 138(3):957–965. [PubMed: 16488544]
- Martinez GL, Quirarte S, Diaz-Cintra C, Quiroz ML, Prado-Alcala RA. Effects of lesions of hippocampal fields CA1 and CA3 on acquisition of inhibitory avoidance. *Neuropsychobiology*. 2002; 46:97–103. [PubMed: 12378127]
- Masonis AE, McCarthy MP. Direct effects of the anabolic/androgenic steroids, stanozolol and 17 alpha-methyltestosterone, on benzodiazepine binding to the gamma-aminobutyric acid receptor. *Neuroscience Letters*. 1995; 7:35–38. [PubMed: 7603620]
- Mehra RD, Sharma K, Nyakas C, Vij U. Estrogen receptor alpha and beta immunoreactive neurons in normal adult and aged female rat hippocampus: a qualitative and quantitative study. *Brain Research*. 2005; 14:22–35. [PubMed: 16122717]
- Moffat SD, Hampson E. A curvilinear relationship between testosterone and spatial cognition in humans: possible influence of hand preference. *Psychoneuroendocrinology*. 1996; 21:323–337. [PubMed: 8817730]
- Moffat SD, Zonderman AB, Metter EJ, Blackman MR, Harman SM, Resnick SM. Longitudinal assessment of serum free testosterone concentration predicts memory performance and cognitive status in elderly men. *Journal of Clinical Endocrinology Metabolism*. 2003; 87:5001–5007. [PubMed: 12414864]
- Nass R, Baker S. Androgen effects on cognition: Congenital adrenal hyperplasia. *Psychoneuroendocrinology*. 1991; 16(1–3):189–201. [PubMed: 1961839]
- Naghdi N, Oryan S, Estemadi R. The study of spatial memory in adult male rats with injection of testosterone ethanate and flutamide into the basolateral nucleus of the amygdala in Morris Water maze. *Brain Research*. 2003; 16:1–8. [PubMed: 12589883]

- O'Connell B, Early B, Leonard BE. Phencyclidine prevents spatial navigation and passive avoidance deficits in ibotenate lesioned rats. *Neuropharmacology*. 1994; 33:1094–1101.
- Packard MG, Kohlmaier JR, Alexander GM. Posttraining intrahippocampal estradiol injections enhance spatial memory in male rats: interaction with cholinergic systems. *Behavioral Neuroscience*. 1996; 110:626–632. [PubMed: 8889009]
- Pak TR, Chung WC, Lund TD, Hinds LR, Clay CM, Handa RJ. The androgen metabolite, 5 α -androstane-3 β , 17 β -diol, is a potent modulator of estrogen receptor- β -mediated gene transcription in neuronal cells. *Endocrinology*. 2005; 146:147–155. [PubMed: 15471969]
- Paxinos, G.; Watson, C. The rat brain. New York: Academic Press; 1985.
- Rhodes ME, Frye CA. ER β -selective SERMS produce mnemonic-enhancing effects in the inhibitory avoidance and water maze tasks. *Neurobiology of Learning and Memory*. 2006; 85:183–191. [PubMed: 16326119]
- Rissman EF, Heck AL, Leonard JE, Shupnik MA, Gustafsson JA. Disruption of estrogen receptor β gene impairs spatial learning in female mice. *Proceeds of the National Academy of Sciences*. 2002; 99:3996–4001.
- Roselli CE, Horton LE, Resko JA. Time-course and steroid specificity of aromatase induction in rat hypothalamus-preoptic area. *Biology of Reproduction*. 1987; 37:628–633. [PubMed: 3676409]
- Rosellini RA, Svare BB, Rhodes ME, Frye CA. The testosterone metabolite and neurosteroid 3 α -androstane-20-one may mediate the effects of testosterone on conditioned place preference. *Brain Research*. 2001; 27:162–171. [PubMed: 11744084]
- Schreihofer DA, Rowe DF, Rissman EF, Scordalakes EM, Gustafsson JA, Shupnik MA. Estrogen receptor- α (ER α), but not ER β , modulates estrogen stimulation of the ER α -truncated variant, TERP-1. *Endocrinology*. 2002; 143:4196–4202. [PubMed: 12399412]
- Sherwin BB. Estrogen and cognitive functioning in men with mild cognitive impairment. *Journal of Molecular Neuroscience*. 2002; 19:219–223. [PubMed: 12212785]
- Shimizu T, Kamegai J, Tamura H, Ishii S, Sugihara H, Oikawa S. The estrogen receptor (ER) α , but not ER β , gene is expressed in hypothalamic growth hormone-releasing hormone neurons of the adult female rat. *Neuroscience Research*. 2005; 52:121–125. [PubMed: 15811559]
- Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor α and β mRNA in the rat central nervous system. *Journal of Computational Neurology*. 1997; 388:507–525.
- Smith MD, Jones LS, Wilson MA. Sex differences in hippocampal slice excitability: role of testosterone. *Neuroscience*. 2002; 3:517–530. [PubMed: 12094208]
- Sternbach H. Age-associated testosterone decline in men: clinical issues for psychiatry. *American Journal of Psychiatry*. 1998; 155:1310–1318. [PubMed: 9766760]
- Tan RS. Memory loss as a reported symptom of andropause. *Archives of Andrology*. 2001; 47:185–189. [PubMed: 11695841]
- Tanabe F, Miyasaka N, Kubota T, Aso T. Estrogen and progesterone improve scopolamine-induced impair spatial memory. *Journal of Medical and Dental Science*. 2004; 51:89–98.
- Tanaka M, Ohtani-Kaneko R, Yokosuka M, Watanabe C. Low-dose perinatal diethylstilbestrol exposure affected behaviors and hypothalamic estrogen receptor- α -positive cells in the mouse. *Neurotoxicology Teratol*. 2004; 26:261–269.
- Tremblay GB, Tremblay A, Copeland NG, Gillbert DJ, Jenkins NA, Labrie F, Giguere V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β . *Molecular Endocrinology*. 1997; 11:353–365. [PubMed: 9058381]
- Venault P, Chapouthier G, Carvalho LP, Simian J, Morre M, Dodd RH, Rossier J. Benzodiazepine impairs and beta-carboline enhances performance in learning and memory tasks. *Nature*. 1986; 321:864–866. [PubMed: 3724846]
- Walf AA, Ciriza I, Garcia-Segura LM, Frye CA. Antisense oligonucleotides for estrogen receptor α and β attenuate estrogen's modulation of affective and sexual behavior. (in press).
- Walf AA, Rhodes ME, Frye CA. Antidepressant effects of ER β -selective estrogen receptor modulators in the forced swim test. *Pharmacology Biochemistry Behavior*. 2004; 78:523–529.

- Walf AA, Rhodes ME, Meade JR, Harney JP, Frye CA. Estradiol-induced conditioned place preference may require actions at estrogen receptors in the nucleus accumbens. *Neuropsychopharmacology*. 2005 [Epub ahead of print].
- Witek A. Novel estrogen receptor: a chance for more specific and safe endocrinological treatment? *Ginekol Pol*. 2000; 71:98–106. [PubMed: 10765606]
- Zhang JQ, Cai WQ, De Zhou S, Su BY. Distribution and differences of estrogen receptor beta immunoreactivity in the brain of adult male and female rats. *Brain Research*. 2002:73–80. [PubMed: 12062475]

Fig. 1. Mean ($\pm$ SEM) crossover latencies (s) on testing day for gonadectomized rats administered control implants (vehicle; open bar), testosterone (Test; striped bar), dihydrotestosterone (DHT; closed bar), or 3 α -androstane-20-one (3 α -diol; stippled bar) implants to the dorsal hippocampus. # denotes tendency ($p < 0.10$) to be different from vehicle * denotes a significant difference ($p < 0.05$) from control ($n = 15$ /group).

Fig. 2. Mean ($\pm$ SEM) crossover latencies (s) on testing day for gonadectomized rats administered vehicle control (vehicle; open bar), or gonadectomized rats administered 3 α -diol through subcutaneous injection (SC Diol; closed bar) or intrahippocampal implant (Hippo Diol; horizontal striped bar).

Fig. 3. Mean ($\pm$ SEM) crossover latencies (s) on testing day for gonadectomized rats administered vehicle control (vehicle; open bar), or 3 α -diol-replaced gonadectomized rats administered saline to the hippocampus (3 α -diol, closed bar), scrambled ODN (ER scrambled; horizontally striped bar), ER AS-ODN (ER α and ER β ; vertically striped bar), ER α AS-ODN (ER α ; stippled bar), or ER β AS-ODN (ER β ; diagonally striped bar). * denotes a significant difference ($p < 0.05$) from control. ($n = 6$ /group). Inset depicts representative images of ER α , ER β , and actin protein levels in the hippocampus of rats administered ER α and ER β AS-ODN, and in the pituitary of vehicle control rats.

Table 1

Data from individual rats administered rats receiving implants containing DHT or 3 α -diol to brain regions other than the CA1 region of the hippocampus compared to the average crossover latency of rats receiving DHT or 3 α -diol to the CA1 region of the hippocampus

Condition	Crossover latency
DHT missed site ($n = 1$)	126.0
3 α -Diol missed site ($n = 2$)	270.0
DHT to CA1 region ($n = 15$)	215.2
3 α -Diol to CA1 region ($n = 15$)	186.1