# THE EFFECTS OF ANDROGENS ON COGNITIVE, MORPHOLOGICAL, AND NEUROCHEMICAL FUNCTIONS IN FEMALE RATS

By

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Abstract

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Estrogen replacement therapy promotes memory function, but is associated with harmful side effects in women. Androgens minimize some of these side effects and enhance cognitive function in males and females, but results remain equivocal. The focus of this research is to determine whether androgens affect cognition of female rats.

Subchronic (two day injections) and acute (single injection) treatment with the androgens, dehydroepiandrosterone (DHEA), testosterone propionate (TP), dihydrotestosterone (DHT), and androstenedione (AD) were used in ovariectomized rats to assess effects on object placement (spatial) and object recognition (nonspatial) memory. Androgens differentially affected these tasks, with enhanced spatial memory by subchronic treatment with DHEA, DHT, and AD, and enhanced nonspatial memory with TP. Acute treatment with DHEA and AD, but not DHT enhanced spatial memory and acute TP enhanced nonspatial memory. DHEA and TP's enhancing effects on memory were not blocked by the aromatase inhibitor, letrozole, suggesting that these effects were mediated by androgens, and not via conversion of androgens to estrogen. DHEA, TP, DHT and AD did not influence anxiety levels on the elevated plus maze suggesting that memory enhancements were due to effects on mnemonic processes. Golgi impregnation

and analyses found that treatment with DHEA and TP increased apical and basal dendritic spine density of the prefrontal cortex, and only basal spine density of CA1 of the hippocampus, brain areas known to be involved in memory. Neurochemical analyses using high performance liquid chromatography (HPLC) found that treatment with DHEA, TP and estradiol benzoate (EB) altered norepinephrine, serotonergic, and dopaminergic activities in the prefrontal cortex, CA1, CA3, and dentate gyrus of the hippocampus, striatum, and vertical diagonal band. Subchronic treatment with DHT and AD altered mainly norepinephrine activity in the above mentioned brain areas.

In summary, the current findings provide novel behavioral, physiological, morphological, and neurochemical information about the cognitive role of androgens in female ovariectomized rats. Results show that androgens enhance memory through activational effects, and changes in dendritic spine density and brain monoaminergic activity may be important in mediating these effects. Furthermore, these androgens may have a potential role as hormone replacement therapy in postmenopausal women.

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# Introduction

## *Memory*

Memory plays a pivotal role in both human and nonhuman lives. The adaptive value of memory involves learning and being able to benefit from prior experiences in the environment, thus contributing to the survival of the organism throughout its life span. Memory includes acquisition, storage and retrieval of information. There are two different types of memory, short-term memory and long-term memory. We are mainly concerned with short-term memory, which holds onto information currently in use (Reisberg, 2001). Working memory is a form of short-term memory that stores information only for the period of time that it remains useful or while it is being worked on, and the retention period for working memory varies according to some researchers, lasting less than one minute to many hours (Reisberg, 2001; Dohanich, 2002).

Researchers have developed a variety of tasks to assess cognitive performance in nonhuman animals under different experimental conditions. Rats with lesions to the hippocampus have impaired spatial memory on the Morris water maze (Morris et al, 1982) and radial arm maze (Kesner et al, 1993). Rats have a natural tendency to spend more time exploring novel objects than familiar objects (nonspatial memory) and objects in locations where they have not previously encountered objects (place/spatial memory) (Ennaceur and Aggleton, 1994; Ennaceur et al, 1997). These novelty preferences have been developed into cognitive tasks with inter-trial delays, and rats with lesions to the fornix, a bundle of nerve fibers extending from the hippocampus to the hypothalamus, or hippocampus impaired memory on the object placement task, but not on the object recognition task (Ennaucer and Aggleton, 1994; Ennaucer et al, 1997; Mumby et al,

2002). Smaller lesions to the hippocampus have been shown to impair spatial memory tasks, while larger lesions to the hippocampus impair object recognition (Broadbent et al, 2004). Lesions to the medial prefrontal cortex primarily impaired recognition in monkeys (Bachevalier and Mishkin, 1986). Kritzer et al (2007) found that male rats performed a series of operant tasks that measured commonly accepted prefrontal cortex functions of spatial working memory, impulsivity and extra dimensional set-shifting of the medial area and reversal learning/preservation and motivation of the orbital area. Therefore, the hippocampus is not the only area that processes spatial information (Kritzer et al, 2007; Luine, 2007), while the prefrontal cortex is not the only area that processes recognition/nonspatial information (Broadbent et al, 2004).

The object recognition and object placement tasks adapted from Ennaceur and Aggleton (1994) and Ennaceur et al (1997) will be used with minor modifications to assess object recognition and place/spatial memory, respectively. Advantages of using these tasks are that subjects do not need to be deprived of food or water and thus there are no reinforcements which can interact with factors such as appetite, thirst, and anxiety levels; and subjects do not require the learning of a contingency rule, and thus, trials can be administered to subjects repeatedly (Ennaucer et al, 1997; Mumby et al, 2002).

There is general agreement in the literature that human aging leads to cognitive deficits, and increased risk of dementia and neurological diseases such as Alzheimer's disease (Grady and Craik, 2000; Graham et al, 1997). Aging in women is accompanied by menopause which leads to a decline in the production of the ovarian hormones, estrogen and progesterone. This decline in ovarian hormones may be linked to an increased risk in cognitive impairments (Sherwin, 1998). Rats may serve as an effective

model for investigating cognitive deficits in humans. The rat model can be used to enhance our understanding of the underlying substrates and mechanisms, that is the brain-behavior relation between the absence of and administration of steroids on cognition, and the neural substrates that support these actions (Vallee et al, 2001). Additionally, nonmnemonic processes such as sensory, motor, regulatory, and motivational components should also be investigated to separate them from genuine memory processes (Pellow and File, 1986; Dohanich, 2002).

***Synthesis and production of steroid hormones***

The term androgens, refers to a group of 19-carbon steroid hormones (Davis and Burger, 2003) (Figure 1). In both men and women, androgens comprise a considerable component of the total circulating pool of gonadal steroids. Testosterone, the principal androgen secreted by the testes in men, is present in women and secreted by the adrenal zona fasciculata, the outer layer of the adrenal gland (25%) and ovaries (25%), while the remaining 50% is produced from peripheral conversion of circulating androstenedione (Burger, 2002). Dehydroepiandrosterone sulfate (DHEAS) is only secreted from the adrenal zona reticularis, the inner layer of the adrenal, while dehydroepiandrosterone (DHEA) is produced by the adrenal zona reticularis (50%) and ovaries (20%), and 30% is derived from circulating DHEAS under the action of the steroid sulphatase (Burger, 2002). Androstenedione is produced approximately equally by the adrenal zona fasciculata (50%) and the ovaries (50%). Dihydrotestosterone (DHT) is a product of peripheral conversion from testosterone catalyzed by 5α-reductase, and a small quantity is directly secreted by the adrenal zona fasciculata (Burger, 2002). These are the major androgens in women and when listed in descending order of circulating serum

concentration, they are as follows: DHEAS (1-4 μg/ml), DHEA (1-10 ng/ml), androstenedione (0.50-0.20 ng/ml), testosterone (0.20-0.70 ng/ml), and DHT (0.02 ng/ml) (Burger, 2002). It should also be noted that these steroids of interest are produced *de novo* ('anew') in the brain, which may then be converted to another active steroid by cellular metabolism, and are capable of modifying neural activities (Beck and Handa, 2004; Baulieu, 1998).

In both sexes, androgen production from gonadal and adrenal sources decline with age. In women, circulating levels of DHEA are decreased by approximately 70% between the third and sixth decades of life (Labrie et al, 1997a). Between the second and fourth decades of life, not only DHEA and DHEAS, but also testosterone serum levels are decreased by 50% in women (Zumoff et al, 1995). Thus, most of the significant decline in circulating DHEA, DHEAS, androst-5-ene-3β, 17β-diol, an androgen metabolite, and androstenedione occurs between the age ranges of 20-30 and 50-60 years, whereas relatively smaller changes occur after the age of 60 years (Labrie et al, 1997a). These significant decreases in androgen levels with age are accompanied by central and peripheral changes that will be discussed in the following subsections.

Androgens have a broad spectrum of action, in part because they represent circulating substrates for the synthesis of a range of biologically active metabolites. As shown in Figure 1, biosynthesis of steroids begins with cholesterol being converted into pregnenolone by the enzyme P45O Scc which catalyzes side-chain cleavage (Burger, 2002; Davis and Burger, 2003). The P450 $C_{17}$ enzyme catalyzes both 17-hydroxylation and 17-20 bond cleavage required for the production of DHEA and androstenedione from pregnenolone and progesterone, respectively. DHEA can further be converted to its

sulfate ester, DHEAS. 3β-hydroxysteroid dehydrogenase (3β-HSD) catalyzes the conversion of pregnenolone to progesterone and DHEA to androstenedione, while 17β-hydroxysteroid dehydrogenase (17β-HSD) catalyzes conversion of androstenedione to testosterone (Burger, 2002; Davis and Burger, 2003; Beck and Handa, 2004). Testosterone can be converted into estradiol by the enzyme P450 aromatase, or into dihydrotestosterone by the enzyme, 5α-reductase (Beck and Handa, 2004).

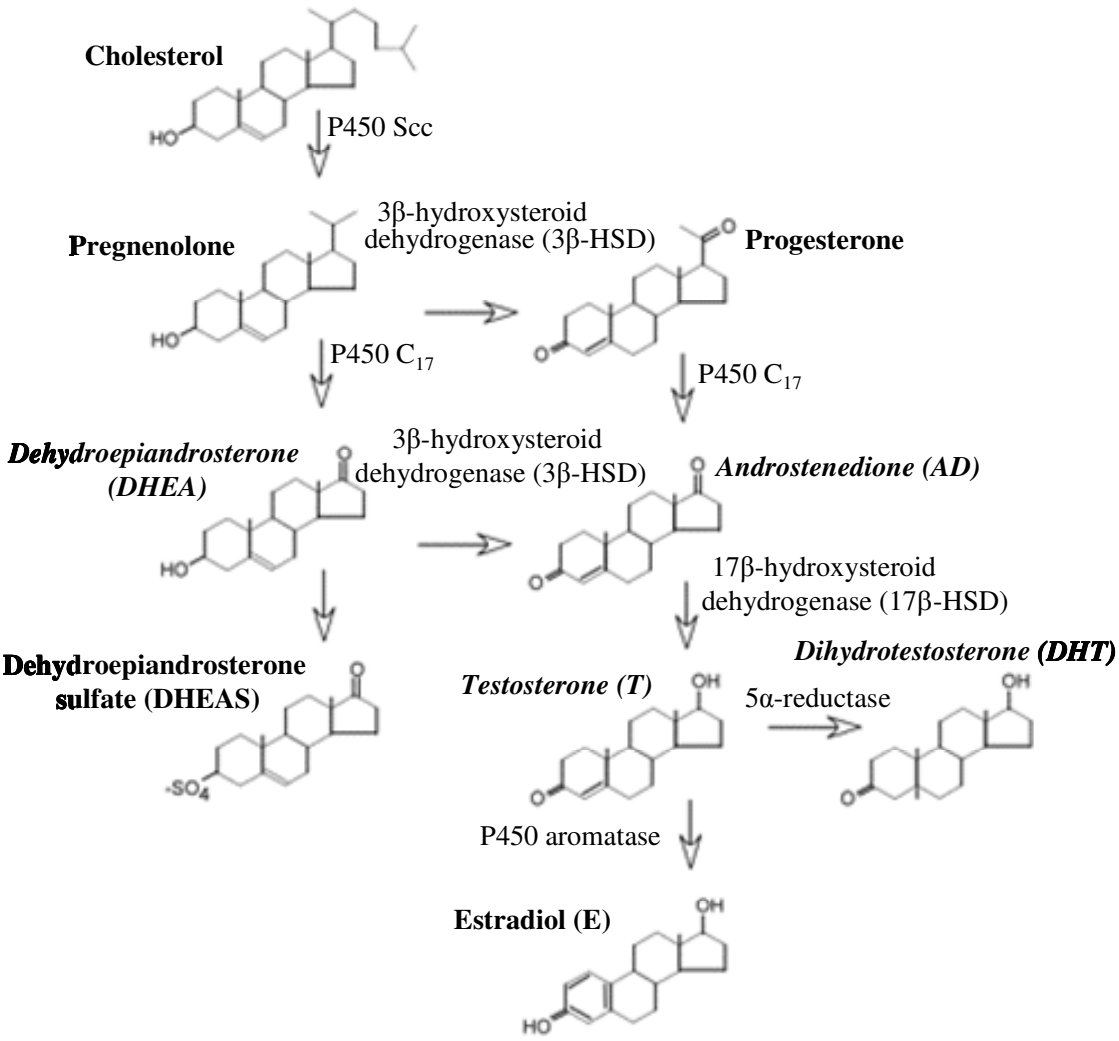



**Figure 1. The biosynthesis and metabolism of steroid hormones.** Steroid hormones and precursors are represented in bold, enzymes responsible for synthesis are represented in regular font, and androgens investigated in these experiments are represented in bold italics. (Adapted from Beck and Handa (2004) with some alterations).

***Hormone replacement therapy effects on memory in humans***

With a steady increase in life expectancy, human aging has become a concern of modern society. Aging can be characterized as a general process of alterations of biological functions, and includes a decline of neuronal abilities, particularly those involving impairments in cognitive and memory processes, and possibly dementia (Grady and Craik, 2000). Age-related functional decline may evolve into neurodegenerative diseases such as Alzheimer's disease. A study in an elderly population reported that the prevalence of cognitive impairment with and without dementia was 8% and 17%, respectively, and increased with age (Graham et al, 1997). Aging in women is accompanied by menopause, defined as the permanent cessation of menstrual cycles due to the loss of ovarian function, usually occurring between the ages of 45 to 55 years (Greendale et al, 1999). Due to the loss of ovarian function, production of the hormones, estrogen and progesterone decrease tremendously in the body. Common physiological symptoms of menopause include hot flashes, night sweats, vaginal dryness, and osteoporosis (Greendale et al, 1999). Women may take hormone replacement therapy (HRT) to alleviate these physical symptoms.

Studies have demonstrated that menopause is not only associated with negative physiological symptoms, but also with cognitive decline which may be ameliorated by estrogen replacement therapy. Postmenopausal women taking estrogen exhibited significantly better performance on a digit ordering task, that measured verbal working memory, and on a task involving matching colored pairs of dots, that measured spatial working memory, compared to nonusers (Duff and Hampson, 2000). When compared to HRT nonusers, postmenopausal women taking Premarin, conjugated equine estrogens,

had increased attention and concentration on a digit vigilance test, and delayed recall on a visual reproduction task (Smith et al, 2001). Similar treatment in postmenopausal women resulted in enhanced delayed recall of lists of words, and reasoning and concept formation by providing similarities or superordinate categories for paired items (Jacobs et al, 1998). Postmenopausal subjects administered transdermal patches of estrogen had enhanced visuospatial abilities measured by a mental rotation task (Duka et al, 2000), immediate recall of lists, short and long delay free recall (Maki et al, 2001), and delayed recall of paired associates (Wolf et al, 1999), compared to non-HRT users.

Women who experienced surgical menopause and had estrogen replacement injections, increased immediate paragraph recall compared to their own baseline before surgery and maintained immediate and delayed recall of paired associates compared to subjects taking placebo who decreased recall pre- to post-operatively (Phillips and Sherwin, 1992). Postmenopausal women with higher circulating estradiol levels compared to those with lower levels, were associated with better verbal memory for immediate and free recall of paired associates, less susceptibility to interference using the Stroop test (Wolf and Kirschbaum, 2002), better verbal memory using a serial learning task, and enhanced retrieval efficiency using delayed trials (Drake et al, 2000).

Some studies have found no cognitive effects of estrogen replacement therapy in postmenopausal women. Women taking Premarin or placebo performed similarly on the digit span and digit symbol tasks (Ditkoff et al, 1991). In another study, cognitive speed and accuracy, attention, and memory were not different in women treated with estrogen or placebo, and on visual detection tasks, recognition thresholds were longer in HRT users compared to non-HRT users (Polo-Kantola et al, 1998). In studies previously

mentioned, no hormonal effects were apparent on immediate and delayed recall of visual material, delayed recall of paragraphs, or digit span scores (Phillips and Sherwin, 1992), immediate and delayed recall of paragraphs and paired associates, performance on the Stroop test (Smith et al, 2001), and short-term figural memory (Maki et al, 2001).

However, women taking HRT may be at increased risk of breast, endometrial and ovarian cancer (Persson, 2000). Recent groundbreaking studies with postmenopausal women receiving Premarin, or Prempro, consisting of estrogen and progesterone replacement therapy ended prematurely because the increased health risks outweighed the benefits. Two Women's Health Initiative (WHI) trials ended in May 2002 and February 2004 because women taking HRT as opposed to placebo were at increased risk of coronary heart disease, stroke, pulmonary embolisms, and invasive breast cancer (Writing Group for the WHI Investigators, 2002; The Women's Health Initiative Steering Committee, 2004). A subset of postmenopausal women from these trials termed the Women's Health Initiative Memory Study (WHIMS) were tested on the Modified Mini-Mental State Examination, and HRT users were at increased risk for dementia compared to non-HRT users (Shumaker et al, 2003; 2004). Prescriptions from January to June 2003 declined by 66% for Prempro and 33% for Premarin, indicating that many postmenopausal women discontinued hormone replacement therapy because of the harmful side effects demonstrated by these studies (Hersh et al, 2004). These studies encouraged researchers, physicians, and postmenopausal women to seek alternative hormone replacement therapies which included androgens as evidenced below.

Similar to estrogen, androgen levels decrease with aging in both sexes and may play a cognitive role in aging humans. A recent study by the National Institute of Aging

found that older men with low levels of free circulating testosterone are at higher risk of developing Alzheimer's disease compared to men with higher levels of this hormone (Moffat et al, 2004). Similarly, Alzheimer's disease patients have a reduction in circulating concentrations of testosterone, DHEAS and DHEA, compared to normal control subjects (Hogervorst et al, 2001; Yanase et al, 1996; Sunderland et al, 1989). In aging men, higher levels of bioavailable testosterone have been associated with better performance on standardized tests of cognitive function (Barrett-Connor et al, 1999; Yaffe et al, 2002).

Older men with testosterone replacement, performed significantly better on a subject ordered pointing task of working memory compared to before when there was no hormone supplementation (Janowsky et al, 2000). In older men, intramuscular injections of testosterone enhanced spatial memory, measured by recall of a walking route, spatial ability by block construction, and verbal memory measured by recall of a short story, compared with baseline and those treated with placebo (Cherrier et al, 2001). A follow-up study found that older men treated with testosterone and the aromatase inhibitor, anastrozole, increased testosterone, decreased estrogen levels, and enhanced spatial memory, while the group treated with testosterone only, increased testosterone and estrogen levels, and enhanced verbal memory, compared to baseline (Cherrier et al, 2005). Hypogonadal men who have low levels of testosterone, receiving testosterone gel replacement, have increased circulating testosterone and estradiol levels and improved verbal memory by immediate and delayed recall of words, while those receiving DHT gel replacement, have increased DHT and decreased testosterone levels, and improved spatial memory on a route test and spatial array test, compared to baseline (Cherrier et al, 2003).

Both of these studies, Cherrier et al (2003; 2005), demonstrated that verbal memory induced by testosterone administration depends on aromatization of testosterone to estradiol, whereas improvement in spatial memory occurs in the absence of increases in estradiol. Another study found eugonadal and hypogonadal men who received testosterone replacement had significantly better verbal fluency by using a word naming task, compared to those in the placebo group (O'Connor et al, 2001). Thus, older men or men with lower levels of androgens, given androgen replacement therapy, ameliorate the effects of memory impairments and the risk of Alzheimer's disease.

Similar to men, androgens affect cognition in aging women, although the number of studies conducted is sparse. Subjects with Alzheimer's disease had significantly lower DHEAS, the sulfated form of DHEA, but not DHEA levels compared to healthy controls, thus these low levels may be associated with cognitive impairment in patients with Alzheimer's disease (Yanase et al, 1996; Hillen et al, 2000). Older women with higher levels of endogenous testosterone compared to those with lower levels were associated with better performance on standardized tests of cognitive function (Barrett-Connor and Goodman-Gruen, 1999). Similarly, postmenopausal women with higher circulating endogenous levels of testosterone had better immediate and delayed recall of paired associates, and verbal fluency using an animal naming task, compared to those with lower levels (Wolf and Kirschbaum, 2002; Drake et al, 2000).

Transdermal testosterone administered to postmenopausal women enhanced divergent thinking, which involved tasks that required fluency and flexibility during creative thought by forming new representations (Krug et al, 2003). Women experiencing natural or surgical menopause, administered oral treatments of estrogen and

methyltestosterone maintained a steady level of performance on a visual memory task that required accurate placement of buildings on a test map, compared to women with estrogen treatment only who had decreased performance (Wisniewski et al, 2002). Similarly, women who experienced surgical menopause, and were administered estrogen alone, a combination of estrogen and testosterone, or testosterone alone had similar scores preoperatively and after hormone replacement postoperatively, on tests of short and long term memory, and logical reasoning, compared to those taking placebo who had lower scores postoperatively (Sherwin, 1988). Oral administration of DHEA to postmenopausal women enhanced recognition memory discrimination for words presented briefly, and perceptual identification of words (Hirshman et al, 2003; 2004). Similar to older women, healthy young women administered a single dose of testosterone enhanced delayed recall of object-location memory on a task that involved reconstructing the location of different objects studied within a spatial display, and visual spatial ability on a mental rotation test (Postma et al, 2000; Aleman et al, 2004).

Although the review above demonstrates a positive role of androgens in cognition of both men and women, other studies have failed to find effects or have found negative effects. Testosterone levels were negatively correlated with verbal fluency, with lower levels of the hormone associated with increased verbal fluency in older men (Wolf and Kirschbaum, 2002). Testosterone administered to elderly men blocked the practice effect in verbal fluency found in controls subjects, and had no effects on spatial or verbal memory (Wolf et al, 2000). Testosterone administered to postmenopausal women had no effects on convergent thinking which involved tasks that required a logical operation of thought, verbal memory, or immediate and free recall of words (Krug et al, 2003).

Despite significant increases in DHEA, DHEAS, androstenedione, and testosterone levels, DHEA replacement in postmenopausal women had no effects on cognitive performance measured by standardized tests, and a negative effect on recognition memory (Wolf et al, 1997; Hirshman et al, 2004). The cognitive role of androgens in humans still remains to be defined, as the literature thus far has shown positive, negative, or no effects on memory.

***Neuroprotective roles of steroid hormones***

Steroid hormones have been shown to protect neurons from a variety of insults associated with stroke, trauma, disease, and age (Wise et al, 2001). Both *in vitro* and *in vivo* studies have documented that estradiol reduced cell damage from oxidative stress in primary neurons, hippocampal cells, (Behl et al, 1997) and female rats with ischemic insults (Yang et al, 2000). Amino acid excitoxicity induced by glutamate in cortical neurons (Zaulyanov et al, 1999), and kainic acid toxicity in hippocampal neurons of ovariectomized rats (Azcoitia et al, 1998) is ameliorated by estradiol treatment. Estradiol also reduced amyloid neurotoxicity induced by β-amyloid plaques, an Alzheimer's insult, in neuroblastoma cells, primary cultures of rat, mouse, and human embryonic cortical neurons (Xu et al, 1998).

Similar to estrogen, androgens also have neuroprotective effects. DHEA protected against the toxicity induced by oxidative stress in both rat hippocampal cells and human hippocampal tissue from Alzheimer's disease patients (Bastianetto et al, 1999). β-amyloid neurotoxicity was significantly reduced with decreased hippocampal neuronal cell loss by testosterone and DHT treatment, while an antiestrogen did not inhibit androgen protection (Pike, 2001). Thus, androgen protection involved an estrogen-

independent mechanism. Excitatory amino acid (NMDA, AMPA, and kainic acid)-induced neurotoxicity *in vitro*, in hippocampal neurons, and *in vivo*, via NMDA infusion into the hippocampus of animals was reduced by DHEA and DHEAS treatment (Kimonides et al, 1998). DHEA and DHEAS also enhanced neuronal and glial survival in cultures of embryo mouse brains (Bologa et al, 1987; Roberts et al, 1987).

*In vivo* models have shown that androgens have neuroprotective effects after brain and spinal cord injury. In acute models of spinal cord and cerebral ischemia in the rat, DHEA improved functional neuronal recovery by enhanced performance on the beam walk test, spatial memory on the Morris water maze, and neurological reflexes, compared to vehicle treated subjects (Malik et al, 2003). Similarly, mice with spinal cord injury, treated with DHEA enhanced recovery of locomotor activity on the open field, left-right coordination and fine motor control compared to control subjects (Fiore et al, 2004).

***Androgen effects on peripheral functions***

There is growing evidence that androgens may have protective effects on breast cancer. DHEA, testosterone, and DHT inhibited cell proliferation and counteracted estradiol-induced proliferative action in human breast cancer cell lines and mammary tumors of rhesus monkeys (Gil-ad et al, 2001; Ando et al, 2002; Poulin et al, 1988; Zhou et al, 2000), and decreased incidence of mammary tumors in rats (Lubet et al, 1995; Luo et al, 1997). Taken together, androgens diminished the incidence of breast cancer and also estrogen-induced breast proliferation, and thus may limit cancer-promoting effects of estrogen.

Androgens may also play a role in bone physiology. Hip, spine, and total body bone mineral density was increased more by estrogen plus testosterone compared to

estrogen alone, and DHEA in postmenopausal women (Davis et al, 1995: Labrie et al, 1997b). Therefore, androgens may be more effective than estrogen in preventing osteoporosis in postmenopausal women (Savvas et al, 1988).

Androgens have been shown to have protective effects on cardiovascular variables. Oral administration of DHEA to rabbits with high cholesterol showed an inhibition in coronary plaque formation and aortic fatty streak formation (Gordon et al, 1988; Arad et al, 1989). Non-obese postmenopausal women with higher endogenous testosterone had a lower prevalence of atherosclerosis (Montalcini et al, 2007). Androgens have also shown to decrease total cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides, (Sarrel, 1998).

Androgen replacement therapy show increased improvements in both psychological and psychosomatic symptoms. Postmenopausal women receiving replacements of testosterone, or a combination of testosterone and estrogen, or DHEA had improved sleep quality, more energy, less joint pain, overall well-being, sexual well-being, decreased distress, irritability, nervousness, anxiety, and depression, compared to non-HRT users (Morales et al, 1994; Arlt et al, 1999; Montgomery et al, 1987; Sherwin and Gelfland, 1985; Watts et al, 1995).

Androgens can also diminish postmenopausal symptoms such as loss of libido and/or sexual satisfaction and vasomotor symptoms. Testosterone alone or used in combination with estrogen resulted in improvements in sexual activity, satisfaction, pleasure, and orgasm (Goldstat et al, 2003; Davis et al, 1995). Menopausal women who received estrogen replacement alone or in combination with testosterone, or DHEA reported a significantly reduced frequency of hot flashes, night sweats, sweating, and

diminished vaginal dryness compared to the placebo group (Sherwin an Gelfand, 1985; Watts et al, 1995; Stomati et al, 2000; Overlie et al, 2001).

Side effects and risks for women using androgen therapy include hirsutism, abnormal growth of hair on a woman's face and body, acne, androgen-dependent neoplasia, that is presence or formation of new, abnormal growth of tissue, temporal balding, deepening of the voice, and situations where increased libido, sexual thoughts and fantasies may lead to undesirable consequences and distress (Davis and Burger, 2003). If circulating androgen concentrations are kept within the upper limit of the normal physiological range, or administered together with estrogen, these effects are highly unlikely. It seems that the health benefits offered by androgen replacement exceeds the potential risks, when treatment is properly managed.

***Steroid hormone effects on memory in animals***

The ovarian steroids, estrogen and progesterone, not only activate and maintain reproductive and sexual behaviors, but have also been shown to affect cognitive performance in nonhuman animals (Dohanich, 2002). Over the years, there has been accumulating evidence regarding estrogen's influences on learning and memory in females, particularly female rodents. Estrogen has been shown to enhance working memory on a variety of cognitive tasks. Ovariectomized rats chronically treated with estrogen for 30 days, enhanced spatial working memory on the eight-arm radial maze, compared to control females (Daniel et al, 1997). Similarly, in our lab, chronic administration of estradiol for 12 days enhanced performance on the radial arm maze (Luine et al, 1998). Administration of estradiol for 5 days to female OVX mice, enhanced spatial memory on the object placement task compared to oil treated rats (Li et al, 2004).

On the T-maze, ovariectomized rats treated with estradiol benzoate for up to 3 days or 3 weeks made more correct reinforced alternation responses during acquisition compared to vehicle treated rats (Dohanich et al, 1994; Fader et al, 1998). Chronic estradiol replacement in both young and aged female rats significantly enhanced performance on another version of the T-maze, using a delayed matching to position paradigm that assessed spatial memory (Gibbs, 1999; 2000). Additionally, chronically treated estradiol rats not only had enhanced delayed matching to position acquisition on the T-maze task compared to control females, but also had increased potassium-stimulated acetylcholine release in the hippocampus (Gibbs et al, 2004). Furthermore, 192 IgG-saporin (SAP), an immuontoxin that selectively destroys cholinergic neurons in the medial septum and vertical diagonal band, when administered with estrogen or estrogen and progesterone, did not enhance spatial memory on the delayed matching to position T-maze task, compared to non-SAP treated females (Gibbs, 2002). Therefore, basal forebrain cholinergic neurons are necessary for hormone-mediated enhancement of delayed matching to position acquisition.

Estradiol replacement in female rats is also associated with non-spatial memory. Estradiol given for 5 or 28 weeks increased the number of active avoidance responses displayed by rats conditioned to a mild electric shock (Singh et al, 1994). Estradiol benzoate given 24 hours before testing to ovariectomized mice, enhanced performance on object recognition and object placement, tasks that assess nonspatial and spatial memory (Li et al, 2004).

Similar to aged women, estrogen replacement to aged rodents enhances memory. Acute (2 days) and chronic (28 days) administration of estrogen to aged female rats

enhanced spatial memory on the Morris water maze (Markham et al, 2002). In addition, in aged female mice, 5 days of estradiol replacement enhanced Morris water maze performance (Frick et al, 2002).

A post-training estradiol administration paradigm also enhances memory. Packard and Teather (1997a; 1997b) showed that rats trained on a hidden platform water maze task, immediately followed by post-training peripheral or intrahippocampal injections of estradiol, followed by testing 24 hours later, enhanced spatial memory compared to vehicle treated rats. Estradiol administered 2 hours post-training had no effect on retention, indicating a time-dependent enhancing effect of estradiol on memory enhancing processes (Packard and Teather 1997a; 1997b). In another acute paradigm, ovariectomized rats treated with 17α-, 17β-estradiol or diethylstilbestrol, a synthetic estrogen, 30 minutes before sample trials enhanced both nonspatial and spatial memory on the objection recognition and object placement tasks, respectively (Luine et al, 2003). Hormones given immediately post sample trial but not 2 hours post sample trial, enhanced nonspatial and spatial memory, suggesting estrogen's effects on menmonic processes, consolidation or encoding, and not performance parameters (Luine et al, 2003).

Similar to the role of estrogen in females, the role of the androgens, particularly testosterone and DHT in activating reproductive, sexual and aggressive behaviors have been investigated. However, recently investigators have been exploring the role of androgens in nonreproductive behaviors such as cognition, anxiety, and other related behaviors, particularly in males.

In working memory tasks such as the T-maze, castration and estradiol replacement in castrated male rats impaired acquisition, compared to intact and testosterone-replaced males (Kritzer et al, 2001). Similar to females, silastics of estradiol given to males enhanced acquisition of a delayed matching-to-position spatial task (Gibbs, 2005). However, neither castration nor testosterone treatment had any significant effect on acquisition, but testosterone-treated males performed better than castrated and estrogen-replaced males on increasing inter-trial delays, thus affecting delay-dependent working memory (Gibbs, 2005).

On the radial arm maze, castrated male rats committed significantly more working memory errors than control male rats (Spritzer et al, 2008). Aged male rats (22 months) made significantly more errors than young rats on the radial arm maze (Bimonte-Nelson et al, 2003). Testosterone but not DHT improved working memory on the radial arm maze in aged rats (Bimonte-Nelson et al, 2003). Spatial working memory but not acquisition was impaired in castrated males on the Morris water maze compared to intact males (Sandstrom et al, 2006). This impairment was attenuated by testosterone replacement. Male rats administered testosterone enanthate into the basolateral nucleus of the amygdala, which has connections to the hippocampus, enhanced spatial working memory on the Morris water maze compared to control animals, but flutamide, an androgen antagonist had no effect on spatial working memory (Naghdi et al, 2003). On a one-trial passive avoidance conditioning task, male rats administered acute treatments of testosterone enanthate, estradiol valeriate or nor-andorstenolone enhanced both short (10 min interval) and long-term memory (24 hr interval) (Vazquez-Pereyra et al, 1995).

In a conditioned place preference paradigm, intact male rats treated with a testosterone-hydroxypropyl-β-cyclodextrin inclusion complex displayed a preference for an environment previously paired with testosterone administration compared to an environment paired with saline administration (Alexander et al, 1994). Similarly, intact male rats spent significantly more time in the compartment previously paired with injections of testosterone into the nucleus accumbens compared to the compartment paired with saline injections (Packard et al, 1997). These studies indicate that testosterone has rewarding properties.

One of the main research groups associated with investigating the cognitive role of androgens in male rats is Frye and colleagues. Castrated male rats administered testosterone, DHT (testosterone's 5α-reduced metabolite) or 5α-androstane-3α, 17β-diol ((3α-diol) - testosterone's 3α-hydroxysteroid dehydrogenase (HSD) reduced metabolite) via silastics or intrahippocampal injections had greater analgesia measured by longer tail flick and paw lick latencies and enhanced learning measured by increased crossover latencies on the inhibitory avoidance task, compared to castrated males treated with a vehicle solution (Edinger and Frye, 2004; 2005; 2007). Similarly, other studies have shown that gonadally intact male rats had longer crossover latencies in the inhibitory avoidance task relative to gonadectomized rats (Frye and Seliga, 2001; Edinger et al, 2004). Treatment with 3α-diol is more effective than testosterone or DHT in enhancing conditioned place preference, with intact male rats having an increased preference for the non-preferred side of the chamber compared to that seen in home cage controls (Frye et al, 2001).

The steroids pregnenolone, DHEA and others derived from it have also been shown to alter working memory in mice. A single dose of DHEA (20 mg/kg) reversed the impairment induced by the muscarinic receptor antagonist, scopolamine in male mice on the Morris water maze (Shi et al, 2000). DHEAS, the sulfate derivative of DHEA, when given 30 min before testing to male mice also prevented scopolamine-induced amnesia in both the Y-maze and Morris water maze (Urani et al, 1998). Additionally, DHEA and DHEAS when given 30 min before testing to male mice prevented amyloid induced amnesia on the Y-maze (Maurice et al, 1998). Both DHEA and DHEAS injected 30 min before testing to male mice enhanced memory on the T-maze (Melchior and Ritzmann, 1996). Pre-training administration of pregnenolone sulfate, the sulfate derivative of pregnenolone, injected intraperitoneally or directly into the dorsal hippocampus of aged male rats attenuated retention deficits in the Y-maze, an effect which lasted for 10 days (Vallee et al, 1997).

These steroids also alter memory on passive and active avoidance conditioning tasks. Male mice administered the steroid, pregnenolone, and steroids metabolically derived from it, pregnenolone sulfate, DHEA, DHEAS, androstenedione, testosterone, DHT, or aldosterone immediately post-training improved retention for footshock active avoidance training, while estrone, estradiol, and progesterone were ineffective (Flood et al, 1992). Middle aged and old male mice injected with DHEA (20 mg/kg) immediately post-training improved retention performance in the foot shock active avoidance task, similar to levels produced in young mice (Flood and Roberts, 1988). DHEAS (0.125 – 10 mg/kg) given to male mice 60 min before training enhanced memory on a passive

avoidance task by decreasing the number of passive avoidance step-down descents and the active escape latency to reach a shock-free zone (Reddy and Kulkarni, 1998).

It must be noted that, developmentally, neonatal females treated with testosterone, developed a more male-like hippocampus, specifically the dentate gyrus was larger in these females compared to control females (Roof and Havens, 1992). Additionally, these testosterone treated females performed better than controls as well as males on the Morris water maze, a spatial navigation task. In another study, control males performed better than control females, but testosterone propionate treatment during the neonatal period reversed this pattern, and produced better performance in females than males on both the radial arm maze and the Morris water maze (Roof, 1993). Although this thesis investigates the activating effects of androgens on memory and anxiety behaviors in adult female rats, it must be noted that these and other studies indicate that circulating testosterone during brain development may play a role in the development of memory, and both the sex difference and testosterone influence are present early in life.

Studies investigating the cognitive role of estrogens in females and androgens in males are much greater in number compared to the limited number exploring the role of androgens in females. Like males, Frye and colleagues have also investigated the cognitive role of androgens in female rats. DHEA (3.0 mg/kg) administered to ovariectomized rats immediately post-training trials, enhanced spatial memory on the Morris water maze, compared to control females, 24 hours later (Frye and Lacey, 1999). DHEAS (3.0 mg/kg and 7.50 mg/kg) replaced females had decreased latencies to the goal arm in the Y-maze compared to control females, 24 hours later (Frye and Lacey, 1999). Frye and Sturgis (1995) found that DHEAS (3.20 and 6.40 mg/kg) administered to

ovariectomized female rats had enhanced spatial memory on the Morris water maze, and greater percent correct in the Y-maze compared to control females. Post-training injections of 3.0 mg/kg and 7.50 mg/kg of testosterone, DHT, or 3α-diol increased the percentage of correct choices in the Y-maze, 1 hour later and percentage of time exploring the novel objects in an object recognition task, 24 hours later, compared to control females (Frye and Lacey, 2001). Both doses of DHT and 3α-diol, but not testosterone increased latencies to cross the shock-associated side of the inhibitory avoidance chamber, 1 hour later (Frye and Lacey, 2001). Administration of 7.50 mg/kg of 3α-diol or testosterone to ovariectomized rats produced anti-seizure effects induced by kainic acid, prevented decrements in the Morris water maze, and resulted in a greater number of identifiable neurons in the hilar region of the hippocampus compared to control animals (Frye and Reed, 1998). These findings suggest that administration of androgens enhance cognitive performance and produce anti-seizure effects in female rats.

Some studies have found negative effects or no effects when androgens are administered to rodents. DHEAS selectively impaired hippocampus dependent contextual fear conditioning in both male and female rats, but had no effects on auditory cue fear conditioning (Fleshner et al, 1997). Male rats treated with intrahippocampal injections of testosterone enanthate or anisomycin, a protein synthesis inhibitor, had increased latencies to find the invisible platform in the Morris water maze compared to controls (Naghdi et al, 2005). Only the group receiving both testosterone and anisomycin decreased this latency. In another study, male rats were administered testosterone or DHT and tested on a two-lever attention task that required discrimination of visual signals and non-signals (Johnson and Burk, 2006). Testosterone (0.50 mg/kg) decreased accuracy on

non-signal trials, and decreased latencies to retrieve a reward, while dihydrotestosterone (0.50 mg/kg) decreased accuracy on non-signal trials during visual distractor sessions, compared to control males (Johnson and Burk, 2006). Old male mice treated with DHEA (20 mg/kg) did not improve memory retention on the Morris water maze (Shi et al, 2000). These findings do not support the hypothesis that androgens mediate cognitive enhancements in rodents.

***Steroid hormone effects on anxiety in animals***

Nonmnemonic processes that involve sensory, motor, regulatory, and motivational components may have indirect effects on memory performance (Pellow and File, 1986; Dohanich, 2002). Two tasks commonly used to measure anxiety and locomotor activity, are the elevated plus maze and the open field, respectively. The elevated plus maze consists of two open arms and two closed arms. Rats prefer enclosed spaces, and exploring an open space is an indicator of low anxiety (Pellow et al, 1985; Pellow and File, 1986). A long duration of time in open arms of the elevated plus maze or a high frequency of entries into the open arms is an indicator of low anxiety (Pellow et al, 1985; Pellow and File, 1986; Lonstein, 2005). The open field is an enclosed chamber, divided into squares. Greater number of entries into central compared to outer squares and greater number of total entries into squares, are indicative of lower levels of anxiety (Frye and Lacey, 2001; Edinger and Frye, 2004; Bowman et al, 2004)).

Castrated male rats administered testosterone, dihydrotestosterone or 3α-diol via silastics or intrahippocampal injections had less anxiety measured by a greater percentage of time spent in the open arms of the elevated plus maze, more exploratory behaviors on the open field in central squares compared to outer squares, and less defensive freezing in

response to shock (Edinger and Frye, 2004; 2005). Intact male rats and gonadectomized rats replaced with DHT had similar low levels of anxiety behavior on the open field and elevated plus maze, and less fear behavior in the defensive freezing task compared to gonadectomized control rats (Frye and Edinger, 2004). Intact or DHT-replaced rats that received blank inserts to the dorsal hippocampus demonstrated less anxiety behavior than rats administered implants of indomethacin, 3α-HSD inhibitor (Frye and Edinger, 2004). This finding demonstrated that testosterone's metabolite, DHT reduced anxiety behavior, and blocking metabolism to 3α-diol in the hippocampus increased these anxiety effects. Administration of flutamide, an androgen receptor antagonist to the hippocampus in intact and DHT replaced male rats increased anxiety behavior on the open field, elevated plus maze and defensive freezing task compared to intact, DHT-replaced controls, and flutamide only male rats (Edinger and Frye, 2006).

Intact male rats treated with DHEA (0.70 mg/kg), a weak androgen decreased anxiety on the elevated plus maze, measured by increased time spent in the open arms and number of open arm entries (Fedotova and Sapronov, 2004). Male rats administered silastics of a stronger androgen, testosterone propionate for one week also increased exploration on the open arms of the elevated plus maze, but not on the open field (Bitran et al, 1993).

DHEA also reduces anxiety behavior in mice. Testosterone or 3α-diol administered to aged intact male mice increased central square entries and total square entries in the open field, and time spent in open arms of the elevated plus maze, compared to vehicle treated mice (Frye et al, 2007). DHEA administered to male mice with high but not low anxiety, had significantly diminished behavioral despair measured

by the Porsolt's test, by decreasing the duration of immobility in a tank (Prasad et al, 1997). Both DHEA (5 μg/kg – 1.0 mg/kg) and DHEAS (0.05 and 0.50 mg/kg) increased the number of open arm entries on the elevated plus maze compared to vehicle treated male mice (Melchior and Ritzmann, 1994).

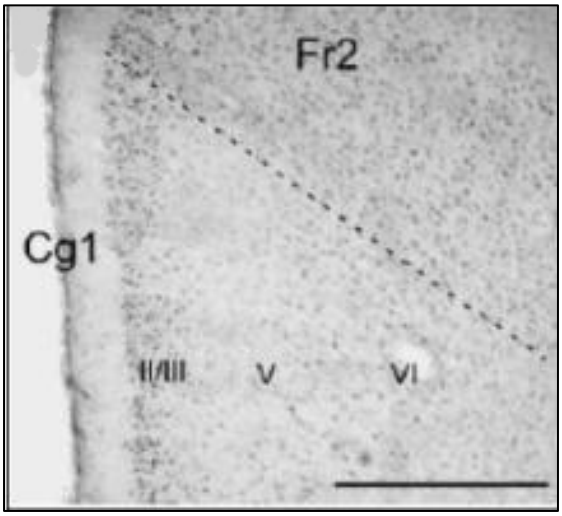
There are few studies investigating the effects of androgens administered to female rodents on anxiety, compared to male rodents. Frye and Lacey (2001) found that post-training injections of 3.0 mg/kg and 7.50 mg/kg of testosterone, DHT, or 3α-diol increased the number of entries into the center squares of a brightly lit open field and time spent in open arms on the elevated plus maze, 1 hour post administration. Interestingly, in another study, DHEA (3.0 mg/kg and 7.50 mg/kg) decreased total entries in the open field, 24 hours post injection, while DHEAS (7.50 mg/kg) decreased the ratio of central to total squares entered and total entries in the open field, 1 hour post injection (Frye and Lacey, 1999).

***Distribution of steroid hormone receptors and enzymes***

The actions of steroid hormones on the brain may be, but not necessarily mediated via nuclear steroid receptor proteins. Androgen receptors are widely distributed throughout the rat brain, particularly in the medial preoptic, arcuate, and ventromedial nuclei of the hypothalamus, the medial nucleus of the amygdala, the pyramidal cell layers of CA1 and CA3 of the hippocampus, the cingulate gyrus, lateral septum and bed nucleus of the stria terminalis (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992; Kritzer et al, 2004). (See Figure 2 for distribution of androgen receptors in the prefrontal cortex and CA1 of the hippocampus). Androgen receptors are also present in mammary tissue,

(Zhou et al, 2000; Dimitrakakis et al, 2002 31, 32), ovaries, uterus (Pelletier et al, 2000), and human osteoblasts, bone forming cells (Abu et al, 1997; Colvard et al, 1989).

A. Distribution of androgen receptors in the prefrontal cortex.




B. Distribution of androgen receptors in CA1 of the hippocampus.

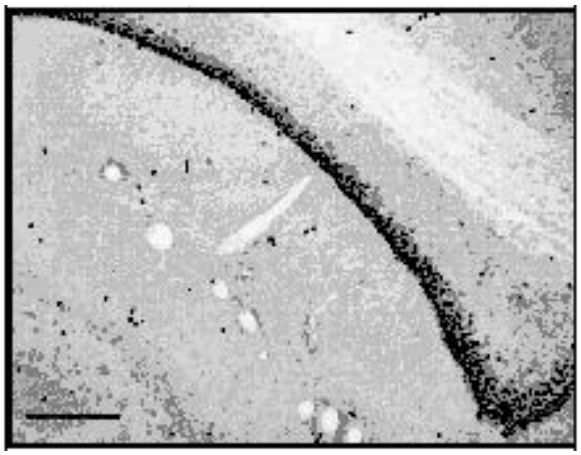


**Figure 2. Distribution of androgen receptors in the prefrontal cortex and CA1 of the hippocampus.** Labelled cells expressing androgen recetpors are depicted by small dots. A. Andorgen receptors are mainly labeled in the cingulate gyrus 1 (Cg1) of the prefrontal cortex (adapted from Kritzer, 2004). B. Androgen receptors are mainly labeled in CA1 of the hippocampus (adapted from Sarkey et al, 2008).

Estrogens act via two distinct nuclear estrogen receptors, ER-α and ER-β, which have different distributions within the brain. ER-α expression predominates in the ventromedial nucleus of the hypothalamus and subfornical organ, while ER-β is expressed to a greater extent than ER-α in the cerebral cortex, CA1 and CA3 of the hippocampus, the paraventricular nucleus of the hypothalamus, and the cerebellum of female rats (Shughrue et al, 1997). Both ER-α and ER-β are expressed in the bed nucleus of the stria terminalis, medial and cortical amygdaloid nuclei, preoptic area, lateral habenula, periaqueductal gray, parabrachial nucleus, locus ceruleus and nucleus of the solitary tract (Shughrue et al, 1997). ER-α is more abundantly expressed in the uterus, mammary gland, and pituitary, while ER-β is highly expressed in the brain and ovaries (Shughrue et al, 1998; Kupier et al, 1996; Hewitt and Korach, 2003; McEwen and Alves, 1999).

The enzymes involved in biosynthesis of steroid hormones have differential distributions in the brain, and thus the products may regulate different functions. Studies have shown that P450 Scc, P450 $C_{17}$, and P450 aromatase (testosterone), enzymes that catalyze pregnenolone, DHEA and androstenedione, and estradiol synthesis, respectively (Figure 1), are significantly localized in the pyramidal neurons of CA1 and CA3, and in granule cells of the dentate gyrus of the hippocampus (Kimoto et al, 2001; Furukawa et al, 1998). Furthermore, P450 aromatase is also distributed across the medial basal hypothalamus, amygdala, and frontal cortex (Lephart et al, 2001). The distribution of 5α-reductase, the enzyme that converts testosterone into dihydrotestosterone, is more widespread in the medial basal hypothalamus, amygdala, and frontal cortex and display

higher rates of enzymatic activity than P450 aromatase (Lephart et al, 2001; Jacobson et al, 1997).

When radioactive testosterone was infused into ovariectomized/adrenalectomized female rats, unchanged testosterone, and its metabolites DHT and estradiol were recovered, with highest levels of estradiol found in the amygdala, followed by the hypothalamus, preoptic areas and septum, while highest levels of DHT were found in the pituitary, followed by the hypothalamus and septum (Lieberburg and McEwen, 1977). When radioactive estradiol or DHT were infused, respectively, cell nuclear levels (pooled from the preoptic area, amygdala, hypothalamus, septum, hippocampus, midbrain-central grey, and parietal cerebral cortex) of estradiol as a testosterone metabolite did not correlate well with nuclear activity, but DHT as a testosterone metabolite correlated well (Lieberburg and McEwen, 1977). These findings emphasize the potential importance of local aromatization in the rat brain, suggest the existence of a DHT receptor site at the brain cell nuclear level, and confirm the existence of estradiol receptors. Brown et al (1994) has shown that ovariectomized/adrenalectomized female rats treated with estradiol benzoate followed by DHT, had decreased estrogen binding in the ventromedial nucleus, an effect that was inhibited by flutamide, confirming the effect was mediated through the androgen receptor system (Brown et al, 1994). DHT administered in the presence of estradiol decreased estrogen receptor content in the bed nucleus of the stria terminalis, arcuate nucleus, and ventromedial nucleus of ovariectomized/adrenalectomized rats (Brown et al, 1996). Both studies demonstrated that nonaromatizable androgens can act in the brain and downregulate estrogen receptors. The metabolite of dihydrotestosterone, 5α-androstane-3β, 17β-diol (3β-diol) has been shown to activate ERβ transcription in a

mouse hippocampal cell line, thus providing evidence for activation of estrogen receptor signaling pathways by an androgen metabolite (Pak et al, 2005).

*In vivo*, DHEA treatment in female ovariectomized mice upregulated androgen receptor levels in the medial prepotic area, lateral septum, and bed nucleus of the stria terminalis, while *in vitro*, DHEA augmented androgen receptor in hypothalamic cells, an effect that was not blocked by trilostane, an 3β-hydroxysteroid dehydrogenase inhibitor, and an effect that was blocked by the androgen antagonist, flutamide (Lu et al, 2003). DHEA also promoted androgen receptor expression and competed with DHT for binding to recombinant androgen receptor in a cell-free system (Lu et al, 2003). Male rats treated with DHEA, increased nuclear and cytoplasmic fractions of androgen receptors in the preoptic/anterior hypothalamic area (Bairamov and Sapronov, 2004). These results provide evidence that DHEA is capable of interacting with and regulating androgen receptor activity in rodents and *in vitro*.

***Role of aromatase inhibitors***

P450 aromatase is the rate limiting enzyme that catalyzes the conversion of testosterone to estrogen (Beck and Handa, 2004). Blockade of this step allows for treatment of diseases that are dependent on estrogen. Therefore, aromatase inhibitors have been approved for treatment options of hormone-dependent advanced breast cancer (Brodie et al, 2003). Competitive inhibitors such as anastrozole and letrozole bind to the active site of the enzyme, aromatase (Mokbel, 2002). Letrozole is classified as a third-generation type II inhibitor, as it is a nonsteroidal aromatase inhibitor and its action is reversible. (Mokbel, 2002). Third-generation aromatase inhibitors effectively block the production of estrogen without exerting effects on other steroidogenic pathways

(Bhatnagar, 2007). Letrozole has greater potency than other aromatase inhibitors, including anastrozole, exemestane, and formestane, and produces near complete inhibition in peripheral tissues and is associated with greater suppression of estrogen than is achieved with other aromatase inhibitors (Bhatnagar, 2007). Letrozole has been shown to protect against breast cancer in female mice (Brodie et al, 2003) and postmenopausal women (Trunet et al, 1997; Mouridsen et al, 2001; Mouridsen and Bhatnagar, 2005), and found to be more effective than other treatments (Mokbel, 2002). In addition to its primary use as a protective agent, letrozole can also be used to inhibit the conversion of androgens to estrogen in order to determine whether the mechanism involved is androgenic or estrogenic for enhancing cognitive function (Cherrier et al, 2005; Hajszan et al, 2004; Leranth et al, 2004). See subsection on "*Steroid hormone effects on brain morphology*" for details.

***Brain mechanisms involved in steroid hormone effects***

The actions of steroids on the brain were initially believed to be mediated entirely through control of gene transcription via nuclear steroid proteins. Over the last decade, the general model for steroid hormone action has been modified to include actions of the steroids via membrane receptor sites. In the classical, genomic pathway, steroid hormones, notably estrogen and androgens exit the circulatory system, pass though the lipid membrane of cells, and then combine with receptor proteins within the cells (McEwen and Alves, 1999; Heinlein and Chang, 2002a) (Figure 3). Following attachment to intracellular receptors, steroids interact directly with DNA in the cell nucleus and affect the rates at which proteins are synthesized. Genomic actions of

steroids develop very slowly, sometimes requiring many hours before peak effects are achieved (McEwen and Alves, 1999; Heinlein and Chang, 2002a).

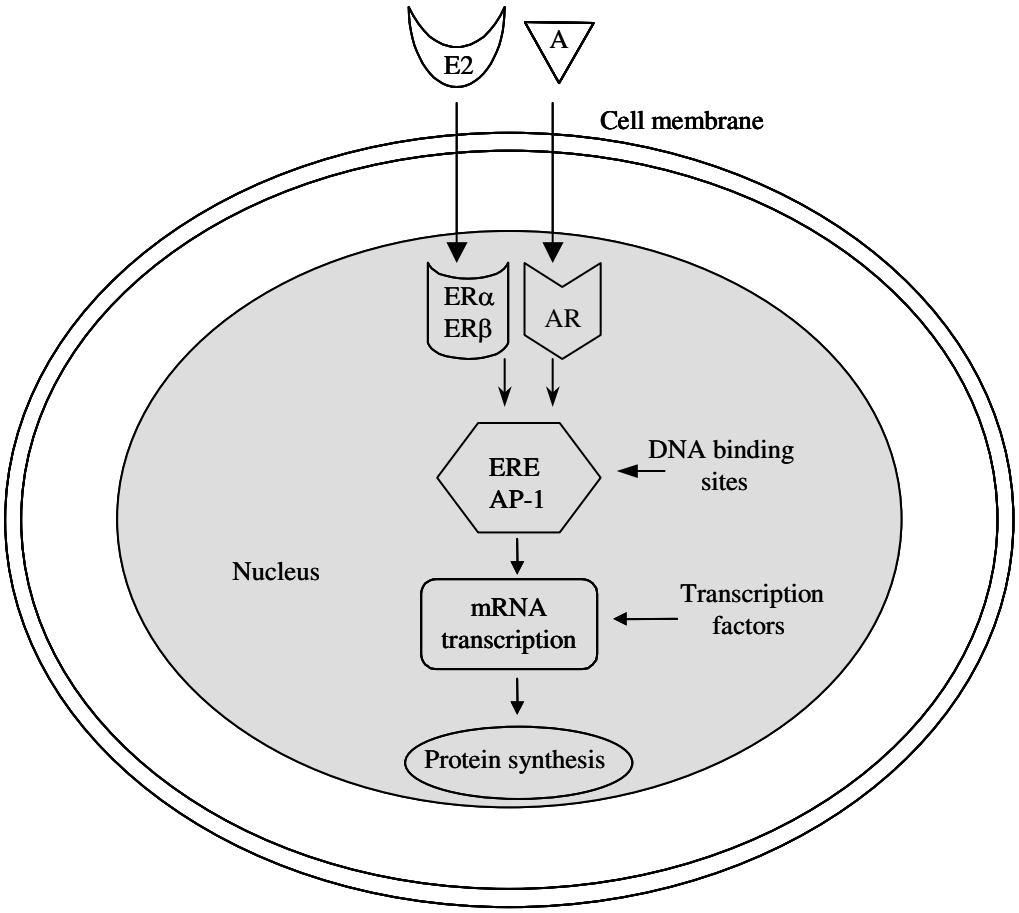



**Figure 3. Diagram of the genomic mechanism of action.** Estrogen and androgens can have slow and prolonged effects by binding to intracellular receptors. This complex can then interact with DNA binding sites such as estrogen response elements and AP-1 and mRNA transcription factors to alter production of protein synthesis. (E2: estrogen; A: androgen; ERE: estrogen response element). (Adapted from Dohanich, 2002).

The more recently described nongenomic pathway results in effects that are rapid in onset and short in duration, and may last seconds to minutes (Vasudevan and Pfaff, 2007; Heinlein and Chang, 2002b). Nongenomic actions of estrogen and androgens appear to be mediated though steroid membrane or G-protein coupled receptors that can activate two different signal transduction second messenger pathways that may involve calcium influx, when estradiol or androgens are administered. One pathway involves the mitogen activated protein kinase (MAPK) cascade which is propagated by Ras activation, followed by sequential phosphorylation and activation of MAP kinase and extracellular signal-regulated protein kinase (ERK) (Kelly and Levin, 2001) (Figure 4). Activated ERK is then translocated into the nucleus to interact directly with nuclear transcription factors such as CRE, SRE, AP-1 and immediate early genes such as c-fos and c-jun (Kelly and Levin, 2001). The other pathway involves activation of adenylyl cyclase, cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) or protein kinase C (PKC) which could act in parallel or by converging onto the MAP kinase pathway which also results in the activation of gene transcription via response elements (Vasudevan and Pfaff, 2007; Heinlein and Chang, 2002b). Physiological, cognitive, neurochemical, and morphological changes may be exerted through these genomic and nongenomic mechanisms by alterations in synthesis of proteins and transcription factors.

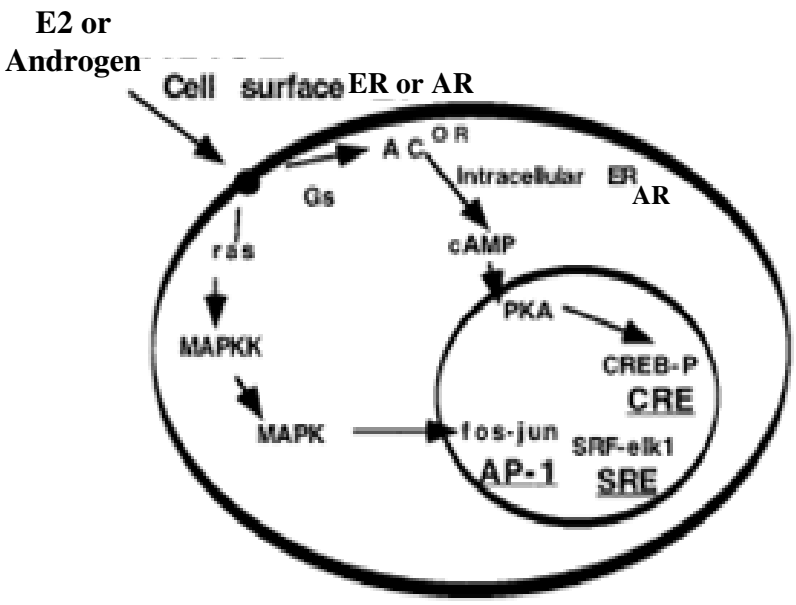



**Figure 4. Diagram of the nongenomic mechanism of action.** Estrogen (E2) and androgens can exert rapid effects by binding to cell membrane receptors or G-protein coupled receptors that can activate second messenger pathways. One pathway activates MAPK which stimulate or represses transcription through AP-1, SRE, and CRE. The other pathway involves activation of adenylyl cyclase, cAMP and PKA or PKC which could converge with the MAPK pathway that also results in activation of gene transcription via response elements. (E2: estrogen; ER: estrogen receptor; AR: androgen receptor; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; PKC: protein kinase C; MAPK: mitogen activated protein kinase). (Adapted from McEwen and Alves, 1999; Heinlein and Chang, 2002b).

A recent study has shown that acute administration of 17β-estradiol increased ERK2 phosphorylation in the diagonal band of Broca, nucleus accumbens, paraventricular and arcuate nucleus of female ovariectomized rats (Bryant et al, 2005). Similarly estradiol infused into the left ventricle increased extracellular ERK enzymatic activity as well as rapid (5 min) induction of ERK phosphorylation in the rat hippocampus (Kuroki et al, 2000). Female rats that are in proestrus and have elevated levels of estrogen, produced tonic phosphorylation and activation of ERK2/MAP kinase throughout the brain (Bi et al, 2001).

Similar to estrogens, the androgens, testosterone and DHT rapidly and transiently activated MAP kinase in cultured hippocampal neurons as evidenced by phosphorylation of ERK1 and ERK2 (Nguyen et al, 2005). Additionally, suppression of MAPK/ERK signaling blocked androgen-mediated neuroprotection against β-amyloid toxicity (Nguyen et al, 2005). Androgens also affect second messenger pathways in cancer cell lines. Androgen activation of MAPK/ERK and neuroprotection by decreasing β-amyloid toxicity was observed in PC12 cells, a cancer cell line derived from the adrenals, transfected with the androgen receptor (Nguyen et al, 2005). Peripherally, treatment of human prostate carcinoma derived cells with androgen or estradiol triggered simultaneous association of androgen receptor and estradiol receptor β with Src, activating the Src/Raf-1/ERK2 pathway (Migliaccio et al, 2000). In the breast cancer cell line, PMC42, the androgen, R1881 rapidly and transiently activated the ERK, peaking at 5 min, and has important implications treatment of breast cancer (Zhu et al, 1999).

Long-term potentiation (LTP) is a prolonged enhancement of synaptic transmission first described in the rabbit hippocampus, a structure important for the

establishment of memories and for temporary storage of long-term memories (Bliss and Lomo, 1973). *In vivo*, estradiol treatment in ovariectomized rats, facilitated the induction of LTP in CA1 of the hippocampus, compared to control, oil treatment in rats (Cordoba and Carrer, 1997). *In vitro*, 17β-estradiol administered to CA1, CA3 and dentate gyrus hippocampal slices potentiated excitatory postsynaptic potentials (Kim et al, 2006). Hippocampal slices administered 17β-estradiol exhibited a significant enhancement of LTP compared to control slices, via a MAPK dependent pathway (Kim et al, 2002). The majority of spine synapses on hippocampal CA1 pyramidal cells are glutamatergic and it is thought that both NMDA and non-NMDA glutamate receptors are colocalized at glutamatergic synapses on cells (Bekkers and Stevens, 1989). Woolley et al (1997) found that the estradiol treatment paradigm that increased the density of excitatory synaptic input to CA1 pyramidal cells, also increased these cells' sensitivity to NMDA receptor-mediated synaptic input.

Androgens also affect LTP. Studies have shown that hippocampal slices from ovariectomized female rats had decreased dendritic excitatory postsynaptic potential, while testosterone replacement increased excitatory postsynaptic potential amplitudes and the population of spike amplitude (Smith et al, 2002). DHEAS facilitated CA1 hippocampal long-term potentiation via sigma 1 (σ1) receptor and an amplification of Src-dependent NMDA receptor signaling (Chen et al, 2006a; 2006b). Thus, DHEAS may act as a sigma 1 receptor agonist and enhances NMDA-induced neuronal excitability. DHEA has been shown to potentiate the NMDA response in CA3 of the hippocampus, assessed by firing rates (Debonnel et al, 1996). Androstenedione also has been shown increase LTP in the dentate gyrus of rats (Schwartz et al, 2002).

***Steroid hormones effects on brain morphology***

Steroid hormones exert not only significant organizational effects but also activational effects on the nervous system (Praduz et al, 2006). It is now widely accepted that the adult mammalian brain retains considerable structural plasticity. Gonadal steroid hormones are a group of compounds that can activate cellular and morphological changes in the brain. Both estrogens and androgens have been shown to regulate synaptic plasticity (Gould et al, 1990; Hajszan et al, 2004; Leranth et al, 2004).

Dendritic spines are defined as small protrusions on the shaft of dendrites in the mammalian brain (Leuner et al, 2003). Dendritic spine structure reflects a dynamic structure that may undergo numerous changes in shape, size, and number, both slowly and rapidly. Dendritic spine shape varies over a continuum of morphologies, short to long, thin to thick-necked, headless to large bulbous heads (Sorra and Harris, 2000). In CA1 of the hippocampus, each head of a branched spine synapses with a different presynaptic axon, while others may have no presynaptic partner (Sorra et al, 1998). In contrast, different heads of spines on CA3 pyramidal cells can be innervated by the same or different presynaptic axons (Chicurel and Harris, 1992).

Dendritic spines serve several functions. Spines increase the packing density of synapses (Sorra and Harris, 2000). Dendrites with spines can reach beyond and connect with axons, thus increasing the density of possible connections. Spines are principal sites for excitatory synaptic transmission. Hippocampal spines differ from spines in other brain regions, because they rarely have inhibitory synapses on them (Harris and Stevens, 1989). Spines provide synapse specificity through molecular compartmentalization. Therefore, spines compartmentalize calcium such that localized changes in intracellular

calcium at an active synapse do not spread to neighboring inactive synapses (Muller and Connor, 1991).

The concept of synaptic and spine plasticity as a mechanism of learning and memory was first introduced over a century ago (Harris and Stevens, 1989). Dendritic spines represent a means by which new contacts between cells can be established and existing contacts strengthened, suggesting the formation of new memories (Sorra and Harris, 2000). Because most spines in the hippocampus form excitatory synapses, this leads to a significant increase in excitatory neurotransmission, which is often an important step in memory formation (Leuner et al, 2003). Therefore, hormone-induced structural changes in hippocampal circuitry provides an opportunity to link endocrine changes with cognitive functions.

Using Gogli impregnation, a landmark study has shown that removal of the circulating gonadal steroids, estrogen and progesterone, by ovariectomy of adult female rats, resulted in a significant decrease in dendritic spine density in CA1 pyramidal cells of the hippocampus, compared to intact rats (Gould et al, 1990). Ovariectomized (OVX) rats that were subcutaneously injected with 10 μg of estradiol benzoate (EB) 24 hours apart, prevented this decrease in spine density, 48 hours post-sacrifice (Gould et al, 1990). The effect of estradiol on spine density was augmented by 500 μg progesterone given 5 hours prior to sacrifice. Ovariectomy or gonadal steroid replacement did not affect spine density of other areas of the hippocampus, namely CA3 pyramidal cells or granule cells of the dentate gyrus (Gould et al, 1990). Female rats that were ovariectomized and implanted with silastic capsules of 17β-estradiol, had increased axospinous synapse

density in CA1 of the hippocampus, compared to those that were implanted with cholesterol silastics (Adams et al, 2001).

In the naturally occurring estrus cycle, Woolley et al (1990) found that rats in the estrus phase of the estrous cycle had a significantly decreased (30%) CA1 apical dendritic spine density compared to rats in the proestrus phase. Therefore, during the proestrus phase when estradiol and progesterone levels are highest, spine density is also highest. During estrus when steroid levels are at their lowest, spine density is also lowest. During diestrus when estradiol levels are intermediate, dendritic spines are also at an intermediate level (Woolley et al, 1990). Similar to Gould et al (1990), there were no significant changes in spine density in the CA3 and dentate gyrus of the hippocampus.

Dendritic changes occur in mice also. OVX female mice that were treated with 1 µg of 17β-estradiol for 5 days had increased spines with mushroom shapes, a more mature type of spine (Li et al, 2004). The postsynaptic markers, PSD95 and spinophilin, and the presynaptic marker, syntaxin were increased throughout all fields of the dorsal hippocampus, when treated with 17β-estradiol. However, total dendritic spine density in the CA1 was not enhanced with 17β-estradiol treatment, a finding that contrasts with that in the female rat. In the object placement task, a spatial memory task, 17β-estradiol enhanced memory (Li et al, 2004). Therefore, 17β-estradiol not only facilitated the spine maturation process but also enhanced hippocampal-dependent, spatial memory.

Steroid hormones also affect dendritic plasticity in nonhuman primates. Adult African green monkeys that were OVX had significantly lower CA1 spine density compared to animals that were OVX and received EB (100%) for one month (Leranth et al, 2002). Similarly, using electron microscopy, the volumetric density (number of spine

synapses/$\mu m^3$) of spine synapses was significantly lower in OVX animals compared to OVX and EB-replaced animals (Leranth et al, 2002). In young female OVX rhesus monkeys, estradiol cypionate, but not oil, increased spinophilin-immunoracrtive spine number in the prefrontal cortex (Tang et al, 2004). In aged female rhesus monkeys, 17β-estradiol but not oil, increased both apical and basal dendrtic spine density in the prefrontal cortex (Hao et al, 2006).

A previous study in our lab has shown that acute 17β- and 17α-estradiol administration enhance spatial memory in OVX female rats (Luine et al, 2003). The mechanism underlying these behavioral responses may be due to rapid changes in synaptic plasticity due to steroid hormones. OVX rats injected with 45μg/kg of 17β-estradiol had significantly greater CA1 spine synapse density after 4.5 hours, compared to OVX rats injected with oil or a lower dose of 17β-estradiol (15μg/kg) ( MacLusky et al, 2005). The higher dose of 17β-estradiol (45μg/kg) increased CA1 pyramidal spine synapse density at a shorter time interval, 30 minutes after injection, compared to oil treated or the lower dose of 17β-estradiol. An even higher dose of 17β-estradiol (60μg/kg) further augmented CA1 pyramidal spine synapse density. When the isomer 17α-estradiol was injected, CA1 pyramidal spine synapse density was significantly increased at 45 μg/kg and further increased by 15μg/kg, after 30 minutes (MacLusky et al, 2005).

*In vitro* studies have also explored the regulation of dendritic spine density by steroid hormones. Murphy and Segal (1996) have shown that 0.10 μg/ml of 17β-estradiol increased spine density of cultured hippocampal neurons compared to control, 17α-estradiol, progesterone, and estrogen and progesterone treated cells. 17β-estradiol

produced this significant effect on spine density within 48 hr of it application. An additional increase was seen for up to 4 days after exposure to 17β-estradiol (Murphy and Segal, 1996). Hippocampal slice cultures treated with 17β-estradiol and letrozole, a nonsteroidal aromatase inhibitor, showed significantly decreased spine synapse density and number of synaptic boutons compared to controls (Kretz et al, 2004). There was also a down regulation of spinophilin, a marker of dendritic spines, and synaptophysin, a protein of presynaptic vesicles (Kretz et al, 2004). These studies suggest that estrogen and estrogenic mechanisms are important in spine and synaptic plasticity.

More recent studies have shown that androgens enhance spine synapse density in first male and then female rats. CA1 spine synapse density decreased by almost 50% in castrated male rats compared to sham-operated controls (Leranth et al, 2003). Castrated rats that were treated with two injections of testosterone propionate (TP - 500 μg), 24 hours apart, and sacrificed 48 hours later, had increased spine syanpse density comparable to those of intact males. A similar increase was seen in males treated with 500 μg of dihydrotestosterone (DHT), but not 10 μg of estradiol (Leranth et al, 2003). CA1 spine synapse density was significantly lower in the hippocampi of gonadectomized rats replaced with TP ipsilateral to the fimbria/fornix transection and gonadectomized rats, compared to intact males, gonadectomized males replaced with TP, and gonadectomized males replaced with TP contralateral to the fimbria/fornix transection (Kovacs et al, 2003). This result suggested that the effects of TP on spine synapse density are partially dependent on subcortical mediation. In male rats with a *Tfm* mutation, which results in the synthesis of defective androgen receptors, and replaced with DHT and hydroxyflutamide increased spine synapse density of CA1, while EB and sesame oil had

no effect (MacLusky et al, 2006). These results were surprisingly similar to wild-type males. Thus, androgen effects on spine synapses may involve novel androgen response mechanisms. In the medial prefrontal cortex, another brain area that plays a crucial role in memory, castration reduced compared to intact male rats, while DHT (500 μg/rat/2 day) or estradiol benzoate (10 μg/rat/2 day) administered to castrated male rats increased spine synapse density, similar to intact male rats, although the DHT effect was significantly larger compared to EB (Hajszan et al, 2007). Unlike CA1, DHT or EB administered to castrated *Tfm* males, restored spine synapse density to intact *Tfm* male levels, but the EB effect was significantly larger compared to DHT (Hajszan et al, 2007).

DHEA, a weak androgen, administered with the same injection paradigm as Leranth et al (2003) used, increased CA1 spine syanpse density in female OVX rats compared to control OVX rats treated with sesame oil (Hajszan et al, 2004). A nonsteroidal aromatase inhibitor, letrozole, administered 1 hour before DHEA, blocked this induction of spines. Similar to males, TP and DHT increased spine syanpse density in CA1 in female OVX rats, although the effect was smaller in DHT treated females (Leranth et al, 2004). Letrozole blocked the increase in spines for TP treated but not DHT treated females. Flutamide (5mg), an androgen antagonist, increased CA1 spine syanpse density in female OVX rats (MacLusky et al, 2004). When DHEA and flutamide were given in combination, the effects on spine synapse density were additive (MacLusky et al, 2004). In OVX rats replaced with EB, CA1 spine synapse density increased significantly contralateral to the fimbria/fornix transection, compared to the ipsilateral hippocampus of estrogen replaced rats and ipsilateral and contralateral hippocampi of OVX rats (Leranth et al, 2000). This finding suggests that EB effects on spine synapse

density involves subcortical mediation as the fimbria/fornix contains the majority of subcortical efferents which have cholinergic, GABAergic, serotonergic systems to the hippocampus (Leranth et al, 2000).

Several studies have linked morphological changes with cognitive function. In male rats that were trained on the Morris water maze, a spatial memory task, using an invisible platform, there was an associated increase in frequency of shorter distances, that is, clustering between synaptic active zones in CA1, compared to control rats that had a visible platform, which requires no spatial memory (Rusakov et al, 1997). No training-associated changes in volume of CA1 pyramidal cells, density or sizes of synapses were found. Leuner et al (2003), found that male rats trained using the eyeblink trace conditioning paradigm, an associative learning task that requires the hippocampus for acquisition, had an associated increase in CA1 basal pyramidal spine density of the hippocampus, compared to animals exposed to unpaired stimuli. Thus far, only few studies have investigated the link between behavior responses and morphological changes in the hippocampus in female rats. OVX female rats were given two injections, 24 hours apart, of sesame oil, 10 µg of EB, 500 µg of TP, or 500 µg of DHT (Frick et al, 2004). Forty-eight hours later, EB but not TP or DHT significantly impaired spatial memory on the Morris water maze compared to controls. These rats were sacrificed after behavioral testing, but unlike previous findings, EB, TP or DHT did not increase spine synapse density in CA1 of the hippocampus. A separate experiment showed that EB significantly increased spine synapse density in behaviorally naïve female rats but not in behaviorally tested control and EB treated females (Frick et al, 2004). A recent study in our lab has shown that ovariectomy impairs nonspatial and spatial memory and also decreases spine

density in the prefrontal cortex and CA1, compared to intact rats (Wallace et al, 2006). In aged rats, nonspatial memory and spine density of the prefrontal cortex declined compared to young rats (Wallace et al, 2007). These results suggest that the morphological changes in the brain may be mediating memory.

***Steroid hormone effects on neurotransmitters***

Gonadal steroids alter sexual behavior, affective state, and learning and memory. One of the possible mechanisms mediating these actions is the regulation of synthesis and release of neurotransmitters in the central nervous system (McEwen and Parsons, 1982). Female rats that were ovariectomized had increased concentrations of dopamine (DA) in the striatum and hippocampus, while its metabolites, DOPAC and HVA increased in both the hypothalamus and striatum, and no changes were found in serotonin (5-HT) metabolism (Bitar et al, 1991). Testosterone propionate (TP) and estradiol treatment reversed DA changes in the striatum and hippocampus, respectively, while both TP and EB treatments reversed DOPAC and HVA changes in the hypothalamus and striatum (Bitar et al, 1991). Chronic estradiol treatment in ovariectomized female rats decreased norepinephrine (NE), 5-HT, and DA levels in the frontal cortex, while NE was increased in the vertical diagonal band, and DA and DOPAC were increased in the lateral septum, compared to control ovariectomized rats (Luine et al, 1998). The vertical diagonal band showed an increase in GABA in estradiol treated rats (Luine et al, 1998). Surprisingly no changes were found in the CA1, CA3, or dentate gyrus of the hippocampus. A possible reason is that steady state levels of neurotransmitters, not activity/turnover were measured, and neurons can alter activity or firing rate without affecting transmitter or metabolite levels (Luine et al, 1998). These changes in monoaminergic and amino acid

transmitters may contribute to estrogen's enhancing effects on the radial arm maze in ovariectomized female rats.

Serotonin receptors have also been shown to be altered by steroid hormones. Estradiol replacement in female rats increased 5-$HT_{2A}$ receptor density in the anterior frontal and anterior cingulate cortices, nucleus accumbens, and striatum (Sumner and Fink, 1995; Cyr et al, 2000). DHEA treatment increased 5-$HT_{2A}$ receptor expression in the striatum and cortical amygdala (Cyr et al, 2000). Serotonin transporter (SERT) mRNA and the density of SERT sites significantly increased in the basolateral amygdala, lateral septum and ventromedial hypothalamus of female rats replaced with estradiol (McQueen et al, 1997). Furthermore, 5-$HT_{2A}$ receptors in the female rat forebrain is significantly higher during proestrus, when the gonadal hormone, estrogen is high, compared to diestrus in intact rats (Sumner and Fink, 1997).

Many studies have explored levels of monamines in male rats after steroid hormone replacement. Castration in male rats significantly lowered levels of DA, DOPAC, and HVA in the septum and nucleus accumbens, while chronic administration of the steroid hormones, testosterone, estradiol, DHT, or a combination of estradiol and DHT reversed these effects (Alderson and Baum, 1981). Male gonadectomized rats replaced with dihydrotestosterone propionate prevented open field induced increases in DOPAC/DA, MHPG/NE, and 5HIAA/5-HT ratios, while estradiol replacement had the opposite effect with DOPAC/DA and MHPG/NE ratios increased to a greater level than in gonadectomized or intact animals in the medial prefrontal cortex (Handa et al, 1997). Similar to females, castration decreased while estrogen and also testosterone, but not DHT increased 5-$HT_{2A}$ receptor mRNA content in the dorsal raphe nucleus and density

of the 5-$HT_{2A}$ receptor binding sites in frontal and cingulate cortices, and nucleus accumbens of male rats (Sumner and Fink, 1998). The density of SERT sites increased in the arcuate, basolateral amygdala, and ventromedial hypothalamus with treatment of EB or TP, but not DHT, in castrated male rats (McQueen et al, 1999). These two studies suggest that the action of testosterone may depend upon its conversion to estrogen by aromatase.

Steroid hormones have also been shown to alter the dopaminergic system in male rats. Damage to monoamine terminals, specifically striatal dopamine transporter and hippocampal serotonin transporter sites with methamphetamine administration in rats may be related to severe impairment on the object recognition, a nonspatial task (Schroder et al, 2003). Estradiol and DHT replacement in castrated male rats attenuated acute and chronic decreases, respectively, of dopaminergic afferents detected via tyrosine hydroxylase immunoreactivity in the cerebral cortex, but did not attenuate noradrenergic afferents detected via dopamine-β-hydroxylase (Kritzer, 2000). Estradiol or DHEA administered to male rats or mice with MPTP-induced DA depletion, increased striatal DA, DOPAC and HVA compared to saline-MPTP-treated mice (D'Astous et al, 2003). Furthermore, DHEA and estradiol prevented MPTP-induced dopamine transporter tyrosine hydroxylase mRNA decreases. Similarly, female rats coinjected with $MPP^+$, MPTP's active metabolite and either estradiol or DHEA had significantly greater concentrations of dopamine in the striatum, compared to controls (Tomas-Camardiel et al, 2002). These studies suggest that DHEA and estradiol are involved in neuroprotection by preventing the depletion of dopamine.

In sum, there are few studies examining the role of androgens on monoaminergic

neurotransmitters and metabolites, and no studies examining monoaminergic turnover ratio levels in brain areas involved in memory in female rats.

### *Specific Research Aims*

Studies in the current and other labs have found chronic (Luine et al, 1998; Daniel et al, 1998; Markham et al, 2002) and acute estrogen dependent (Luine et al, 2003; Packard and Teather, 1997a; 1997b) enhancements of memory in female rats. Androgens enhance memory in both male (Bimonte-Nelson et al, 2003; Edinger and Frye, 2004) and female rats (Frye and Lacey, 1999; 2001), but studies are few and results are inconsistent, especially in female rats. Based on previously reported cognitive, physiological, neurochemical, and morphological effects of steroid hormones in rats, the role of androgens on these functions in female ovariectomized rats were examined. The overall goal was to gain information on whether androgen treatments to aged females might serve as an effective hormone replacement therapy. Experiments were designed to specifically address each of the following questions:

***1. To determine whether subchronic and acute androgen treatments enhance memory.***

It is hypothesized that androgens will enhance memory, when administered for a few days or acutely. Memory will be assessed using two behavioral tasks, object recognition and object placement. Subchronic and acute effects of the androgens, dehydroepiandrosterone (DHEA), testosterone propionate (TP), dihydrotestosterone (DHT), and androstenedione (AD) will be tested in OVX female rats.

***2. To determine whether androgens act as androgens or via conversion to estrogen to enhance memory.***

Letrozole, an aromatase inhibitor that prevents the conversion of androgens to estrogen, when given in conjunction with androgens, blocks the increase of spine synapse density in CA1 of the hippocampus in OVX female rats. Based on these previous results,

it is hypothesized that androgens act via conversion to estrogen and that letrozole will block the enhancement of spatial and nonspatial memory. Letrozole will be administered for a few days in conjunction with DHEA and TP to OVX female rats to determine whether object recognition and object placement will be affected.

***Mechanisms:* to determine whether neural effects of androgens underlie memory enhancements.**

***3. To determine whether androgen treatments alter dendritic morphology of brain areas involved in memory.***

Androgens increase hippocampal spine synapse density in OVX female rats. Based on these previous results, it is hypothesized that DHEA and TP will increase spine density in the prefrontal cortex and CA1 of the hippocampus. Golgi analysis will be used to assess whether treatment for a few days with the androgens, DHEA and TP, affect spine density in the prefrontal cortex and CA1 of the hippocampus.

***4. To determine whether androgen treatments alter neurotransmitter levels in brain areas involved in memory.***

Since monoamines modulate behaviors such as memory, it is hypothesized that androgens will modulate monoaminergic activity in discrete brain areas involved in memory. OVX female rats will be treated for a few days with the androgens, DHEA, TP, DHT, and AD, and estrogen, and monoaminergic neurotransmitters, metabolites, and turnover ratio levels will be measured in the prefrontal cortex, CA1, CA3, and dentate gyrus of the hippocampus, striatum, and vertical diagonal band using high performance liquid chromatography (HPLC).

Thus experiments will determine whether androgens enhance spatial and nonspatial memory in female rats and determine possible mechanisms responsible for the enhancements.

***Aim 1. Subchronic and acute treatment effects of androgens on memory, anxiety, and physiological parameters.***

Studies have revealed that exogenous estrogen and androgens enhance memory in both human and nonhuman females when circulating hormones are low (Duff and Hampson, 2000; Daniel et al, 1997; Hirshman et al, 2003; Frye and Lacey, 1999). However, studies with androgens are few, focus more on males than females, and results vary as different doses, treatment paradigms, and cognitive tasks have been used.

Subchronic treatment (2 days) with androgens has previously shown to increase CA1 spine synapse density in OVX female rats (Hajszan et al, 2004; Leranth et al, 2004). Acute treatment with estrogen, immediately post sample trial enhances memory in OVX female rats (Luine et al, 2003). In the current study, we are adapting the same treatment paradigms used by these researchers. Since studies are more abundant concerning cognitive enhancements with estrogen, experiments were initiated with the use of dehydroepiandrosterone (DHEA), a weak androgen, which has both androgenic and estrogenic properties, and can be aromatized to estrogen (Burger, 2002). DHEA has been found to have less masculinizing effects than other androgens, and is widely available as an over the counter hormone replacement therapy (Davis and Burger, 2003). We then investigated the more potent androgen, testosterone propionate (TP) which can also be aromatized to estrogen. Following this, we tested dihydrotestosterone (DHT), the only androgen that is nonaromatizable. The final steroid hormone to be investigated was androstenedione (AD), the aromatizable androgen that has been least researched regarding cognition.

We hypothesized that androgens will enhance spatial and nonspatial memory, both subchronically (Section A) and acutely (Section D) in ovariectomized female rats. Memory was assessed using the two behavioral tasks, object placement and object recognition, which have been previously been used in our lab (Luine et al, 2003; Luine et al, 2006). Using these cognitive tasks are advantageous as subjects do not need to be food or water deprived, they do not require learning of a contingency rule, and thus animals can be tested repeatedly over many trials (Ennaucer et al, 1997; Mumby et al, 2002).

To assess whether there are any potential indirect effects on memory performance, subjects subchronically treated with the androgens were tested on the elevated plus maze to assess anxiety levels (Section B). Furthermore, uterine weights and androgen levels in blood serum were measured to determine whether any peripheral effects exist and to verify effectiveness of these treatments (Section C).

## Materials and Methods

### *Subjects*

Two-month old OVX female Sprague Dawely rats were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN, and used for all experiments. Animals were double-housed in plastic tubs (42 x 24 x 20 cm) with wood chips for bedding, and kept on a 12-hour light (lights on at 7:00am), 12-hour dark cycle. Subjects had access to food and water *ad libitum*. A low diet of phytoestrogens (Harlan Teklad Global 16% protein rodent diet, Madison, WI) was used to eliminate possible phytoestrogen effects. A recent study in our lab has shown that long-term maintenance of OVX rats on a high phytoestrogen diet compared to those maintained on a low phytoestrogen diet was associated with better performance on object placement, increased dendritic spine density in CA1 and prefrontal

cortex, and increased uterine weights (Luine et al, 2006). All experiments were conducted in compliance with the Hunter College Institutional Animal Care and Use Committee and NIH Guide for Care and Use of Animals.

***Behavioral measures***

***Open field***

Acclimation began approximately two weeks after ovariectomy and one week after arrival in the animal colony. On day one of acclimation, subjects were placed in an open field chamber made of black plexi glass. The field consists of 3 x 5 grid with each square measuring 9 squared inches. The overall measurements of the chamber were 27 x 45 x 12 inches. Subjects were allowed to explore the field for 6 minutes. No behavioral measures were recorded, as this served the purpose of habituating the animals to the large field. Any extreme behaviors such as excessive defecation and remaining in one corner of the field for an extended period of time were noted.

***Object recognition***

The object recognition memory task was adapted from Ennaceur and Aggleton (1994) and Ennaceur et al (1997), with minor modifications. The object recognition chamber was a smaller version of the open field chamber, a 3 x 3 grid with each square measuring 9 squared inches. The overall measurements of the chamber were 27 x 27 x 12 inches. On day two of habituation, trials of object recognition began, with one inter-trial delay interval per day. During acclimation, trials were separated by 1 min, 10 min, 1 hr and 2 hr inter-trial delay intervals (Bisagno et al, 2003; Luine et al, 2003; Luine et al, 2006). During the sample trial (T1) of the object recognition memory task, subjects were individually placed in the center of the chamber and introduced to two identical objects

such as various bottles, cans, containers, placed equidistant from the two corners on the left side of the chamber. Subjects were allowed to explore the objects in the chamber for 3 minutes, and the time spent exploring the two identical objects was recorded with one timer. Exploration was defined as when the subject sniffed, whisked or looked at the objects no more than 2 cm away. Subjects that did not explore the objects in T1 and/or T2 were omitted from the analysis. After T1, subjects were removed and placed in their home cages for their inter trial delay. For the recognition/retention trial (T2), one of the old or familiar objects from T1 was replaced with a new or novel object. Subjects were again individually placed in the center of the chamber, allowed to explore the objects for 3 minutes, and the time spent exploring the old and novel objects was recorded with two timers. Objects and the positioning of objects were fully counterbalanced between groups. At the end of each individual trial, the objects and chamber were cleaned with coverage spray. For the object recognition memory test, when hormones were administered, the inter-trial delay was 4 hr.

***Object placement***

The object placement memory task was adapted from Ennaceur et al (1997) with minor modifications. Object placement acclimation trials began two days after object recognition acclimation trials were completed. The procedures for object placement were similar to those of object recognition. However, during acclimation, trials were separated by 10 min, 40 min, and 1 hr inter-trial delays. Unlike object recognition, instead of replacing one of the old objects with a new object in T2, one of the identical objects was moved to a new location (horizontally to the right end of the chamber) in the object placement task. The location of objects was fully counterbalanced between groups. The

time spent exploring the old and new locations was recorded with two timers. The objects used in the object placement task were various candleholders and statues. Object location in T2 was fully counterbalanced between groups. For the object placement memory test, when hormones were administered, the inter-trial delay was 2 hr for subchronic treatment and 4 hr for acute treatment. Bisagno et al (2003) has shown that a 4 hr inter-trial delay of the object placement task is more difficult for females to perform compared to 4 hr object recognition. Previous studies in our lab use the same paradigm (Luine et al, 2003; Wallace et al, 2006).

***Elevated plus maze***

The elevated plus maze is designed to assess anxiety-related behaviors (Pellow and File, 1986). The elevated plus maze was made of wood, painted in gray. The maze is adapted from Pellow and File (1986), with four arms extending (50 x 10 cm), two alternate arms that are completely open and two alternate arms that are enclosed by 40 cm high walls. The maze was 50 cm above the floor and arms extended from a 10 x 10 cm central open square, a neutral area. Individual subjects were placed in the central open square facing an open arm and allowed to explore the arms for 5 min. An arm was counted as being entered when a subject placed its head and two forepaws completely within that space (Lonstein, 2005). The number of entries and time spent in open and closed arms were recorded. The time in the central, neutral area was not recorded. A long duration of time spent in open arms of the elevated plus maze or a high frequency of entries into the open arms is an indicator of low anxiety (Pellow et al, 1985; Pellow and File, 1986; Lonstein, 2005). Subjects were administered hormones subchronically (see details below), tested on a 4 or 2 hr inter-trial delay of object recognition or object

placement, respectively, followed by two or four hours of rest, and then tested on the elevated plus maze. Therefore, subjects were tested on the elevated plus maze, 54 hours after the second injection.

***Hormone treatments***

***Subchronic hormonal treatment***

Several cohorts of OVX female rats were used (Table 1). Five days after acclimation, half of the animals received single subcutaneous injections of the vehicle, sesame oil (SO) and the other half received injections of the hormones dissolved in SO. Twenty-four hours later, subjects were injected again with the same treatments. Forty-eight hours later, subjects were tested on 4 hr or 2 inter-trial intervals of object recognition or object placement, respectively. This subchronic treatment paradigm has been used in several studies and has shown to increase CA1 spine density and spine synapse density in OVX female rats, both of which have been implicated in memory (Gould et al, 1990; Hajszan et al, 2004; Leranth et al, 2004). These subjects were allowed a wash out period of the hormones for one week to two weeks, and then injected with the same or another hormone and tested on object recognition or object placement (see details in Table 1). Treatments were reversed when the same cohort was used for several memory tests. All steroid hormones were obtained from Sigma-Aldrich Corp. (St. Louis, MO). See details of the subchronic injection and testing paradigm in Figure 5A.

***Acute hormonal treatment***

Several cohorts of OVX female rats were used (Table 5). After a wash out period of one to two weeks of the subchronic treatment hormones, subjects were tested on a sample trial of object recognition or object placement. Immediately post sample trial

(T1), half of the subjects received single subcutaneous injections of the vehicle, propylene glycol (PG) and the other half received injections of the hormone dissolved in PG. Four hours after T1, subjects were tested on the recognition/retention trial (T2) of object recognition or object placement. Another acute treatment involved injecting subjects 2 hours post sample trial (delayed injections) of object placement. Two hours later, subjects were tested on T2 of object placement. This post sample trial injection schedule is adapted from McGaugh (1989) and Packard et al (1994). Posttraining injection of hormones allows for determining whether a treatment influences task performance via an effect on memory processes or by psychological/performance parameters (Packard et al, 1994). A previous study in our lab has shown that 17β-estradiol and diethylstilbestrol immediately post sample trial but not delayed post sample trial, enhanced object recognition and object placement in OVX female rats (Luine et al, 2003). See details of the acute injection and testing paradigm in Figure 5B.

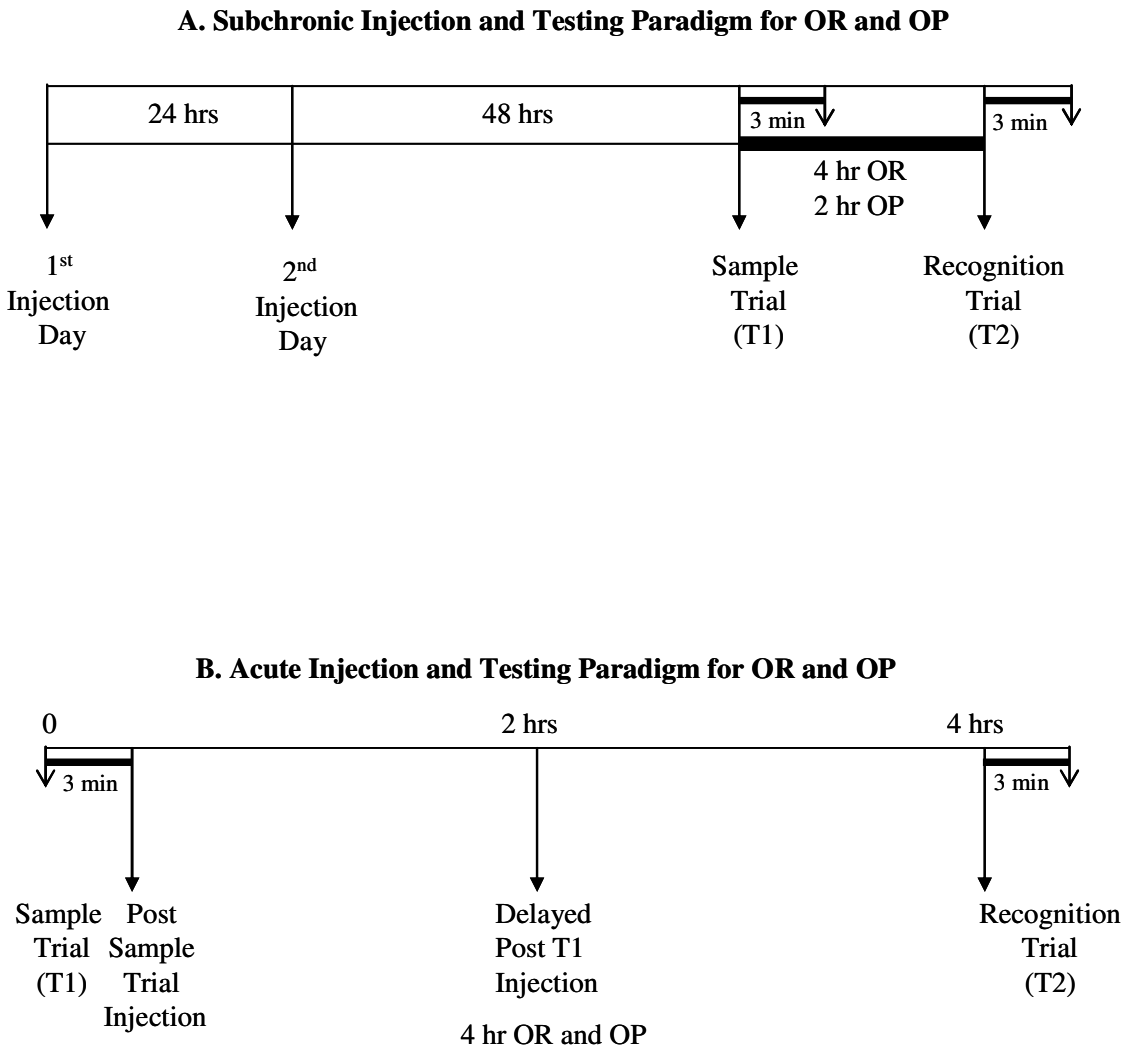



**Figure 5. Subchronic and acute paradigms used for object recognition and object placement memory tasks.** A. In the subchronic treatment paradigm, subjects received two subcutaneous injections 24 hrs apart. Testing on a 4 hr inter-trial delay of object recognition or a 2-hour inter-trial delay of object placement occurred 48 hrs later. B. In the acute treatment paradigm, subjects received a single subcutaneous injection immediately after or 2 hrs after the sample trial. The inter-trial delay for both OR and OP was 4 hrs.

***Physiological measures***

***Uterine weight***

To assess whether androgens have any effect on uterine weight, subjects were subchronically treated with androgens and sacrificed 48 hrs later. Uteri were removed, stripped of fat and connective tissue, and wet weights were recorded in mg. See Section C for specific details on hormone treatments.

***Radioimmunoassay***

Blood samples were collected from trunk blood of subjects that were subchronically treated androgens and sacrificed 48 hrs later. See Section C for specific details on hormone treatments. Testosterone, DHEAS, the sulfated form of DHEA, and androstenedione levels were measured by radioimmunoassay (RIA) using the Coat-A-Count® assay kits available from Diagnostic Products Corporation, Los Angeles, CA. Blood samples were allowed to sit in the refrigerator overnight, centrifuged the following day, and serum was removed. The Coat-A-Count procedure is a solid-phase RIA based on testosterone, DHEAS, or androstenedione-specific antibody immobilized to the wall of a polypropylene tube. Samples were analyzed in duplicates. The calibrators used for the testosterone assay were (0, 20, 100, 400, 800, and 1,600 ng/dL), for the DHEAS assay were (0, 5, 20, 200, 500, and 1000 μg/dL) and for the androstenedione assay were (0, 0.15, 0.40, 1.5, and 4.0, and 10 ng/ml), which were also in duplicates. Briefly, 50 μl of each calibrator and 50 μl of sample for testosterone and DHEAS assays, and 100 μl of each calibrator and 100 μl of sample for the androstenedione assay were pipetted into two tubes each. One ml of the isotope $^{125}$I-testosterone, $^{125}$I-DHEAS, and $^{125}$I-androsenedione was added to every tube and vortexed. $^{125}$I-labeled testosterone, DHEAS, and

androstenedione compete with testosterone, DHEAS, and androstenedione, respectively for antibody sites. Samples for the testosterone and androstenedione assays were incubated overnight in a refrigerator at 2-8°C. Samples for the DHEAS assay were incubated for 30 mins at 37 °C in a water bath. All tubes were then decanted thoroughly, to separate bound from free testosterone, and counted on a 1470 Automatic Gamma Counter (Perkin Elmer Life Sciences). Testosterone and androstenedione levels were expressed in pg/ml, and DHEAS levels were expressed in ng/ml. See section C for specific details on hormone treatments.

S*tatistical analysis*

Data were analyzed using NCSS (NCSS Statistical Software, Kaysville, UT). Two-sample t-tests were used to test for group differences in exploration time in the sample trial and Mann-Whitney U tests were used to test for group differences in ratios in T2 (time spent with new object or location/ time spent with old object or location + time spent with new object or location) for object recognition and object placement. Two-sample t-tests were used to test for group differences on the elevated plus maze for percentage of open arm entries (number of open arm entries/(number of open arm entries + number of closed arm entries) x 100) and percentage of time spent in open arms (time spent in open arms/(time spent in open arms + time spent in closed arms) x 100). One-way ANOVAs were used to test for differences among groups in uterine weights (group x weight (mg), and androgen serum levels (group x pg/ml). *Post hoc* Fisher's LSD tests were used to test for differences between groups if significant F values were found ($p < 0.05$).

***A. Subchronic androgen treatments: object placement and object recognition***

See specific details above in general methods (Fig. 5A) and different cohorts used for experiments in Table 1. For all experiments, subjects that did not explore objects in T1 and/or T2 were removed from analysis.

***DHEA***

OVX female rats were subchronically administered a vehicle solution of SO (0.20 ml; n = 13) or DHEA (1 mg; n = 13) dissolved in SO and tested on a 2 hr inter-trial delay of object placement. This dose of DHEA and treatment paradigm has been shown to induce CA1 spine synapse density in OVX female rats (Lernath et al, 2004).

A new cohort of OVX female rats were subchronically treated with SO (0.20 ml; n = 8) or DHEA (1 mg; n = 8) dissolved in SO and tested on a 4 hr inter-trial delay of object recognition.

***Testosterone propionate***

A new cohort of OVX female rats were subchronically treated with SO (0.10 ml; n = 8) or TP (500 μg; n = 8) and tested on a 2 hr inter-trial delay of object placement.

A new cohort of OVX female rats were subchronically treated with SO (0.10 ml; n = 8) or TP (500 μg; n = 8) and tested on a 4 hr inter-trial delay of object recognition.

Because 500 μg of TP did not enhance object placement, the same cohort of OVX female rats used to test object recognition above, were subchronically administered SO (0.20 ml; n = 9) or a higher dose of TP (1 mg; n = 9) and tested on a 2 hr inter-trial delay of object placement.

***Dihydrotestosterone***

A new cohort of OVX female rats were subchronically administered SO (0.10 ml;

n = 8) or DHT (500 μg; n = 9) and tested on a 2 hr inter-trial delay of object placement.

A new cohort of OVX female rats were subchronically treated with SO (0.10 ml; n = 7) or DHT (500 μg; n = 8) and tested on a 4 hr inter-trial delay of object recognition.

Because 500 μg of DHT did not enhance object placement or object recognition, the same cohort of OVX female rats used to test object recognition above were subchronically treated with SO (0.20 ml; n = 9) or a higher dose of DHT (1 mg; n = 8) and tested on a 4 hr inter-trial delay of object recognition.

The same cohort of OVX female rats used in the above task were subchronically administered SO (0.20 ml; n = 9) or DHT (1mg; n = 9) and tested on a 2 hr inter-trial delay of object placement.

***Androstenedione***

A new cohort of OVX female rats were subchronically administered SO (0.20 ml; n = 9) or AD (1mg; n = 9) and tested on a 2 hr inter-trial delay of object placement.

The same cohort of OVX female rats were subchronically treated with SO (0.20 ml; n = 9) or AD (1mg; n = 9) and tested on a 4 hr inter-trial delay of object recognition.

## Results

***Subchronic treatment effects of androgens: object placement and object recognition***

***DHEA***

For object placement, the spatial memory task, DHEA treated subjects had significantly greater exploration times in T1 compared to SO treated subjects ($p < 0.05$) (Fig 6A). Similarly, DHEA treated subjects spent a significantly greater proportion of time with the new location in the recognition trial (T2) of object placement compared to the SO treated subjects ($p < 0.05$) (Fig. 6B). Unlike object placement, for object

recognition, the nonspatial memory task, SO (8 sec) and DHEA (12 sec) treated subjects spent similar amounts of time exploring the objects during the sample trial (Fig. 6C). In the recognition trial of object recognition, SO and DHEA treated subjects spent a similar proportion of time exploring the new object, with neither showing a significant preference for the novel object (Fig. 6D). Therefore, subchronic treatment with DHEA enhances memory in the object placement task but not in the object recognition task. This enhancement in spatial memory may be a result of increased exploration time in T1 by DHEA treated rats.

***Testosterone propionate***

SO and TP treated subjects had similar exploration times in the sample trial (Fig. 7A) and exploration ratios in T2 for object placement (Fig 7B). For object recognition, TP treated subjects had similar exploration times in the sample trial (Fig. 7C). Unlike object placement, TP treated subjects spent a significantly greater proportion of time with the new object in the recognition trial compared to SO treated subjects ($p < 0.01$) (Fig. 7D). Since 500 μg of TP did not enhance spatial memory, we assessed whether the higher dose, 1 mg of TP had any effects on memory. Similar to the lower dose of TP, SO and TP treated subjects had similar exploration times in T1 (Fig. 8A) and exploration ratios in the recognition trial of object placement (Fig. 8B). Therefore, TP enhances only nonspatial memoy at the lower dose but not spatial memory.

***Dihydrotestosterone***

SO and DHT (500 μg) treated subjects had similar exploration times in the sample trial (Fig. 9A) and proportion of time spent with the new location in T2 for object placement (Fig 9B). Similarly, SO and DHT (500 μg) treated subjects had similar

exploration times in T1 (Fig. 9C) and proportion of time spent with the new object in T2 of object recognition (Fig. 9D).

Subjects treated with SO and the higher dose of DHT (1 mg) had similar exploration ratios in T1 for object placement (Fig. 10A). In contrast to the lower dose of DHT (500 μg), subjects treated with the higher dose of DHT (1 mg) spent a significantly greater proportion of time with the new location compared to SO treated subjects ($p < 0.05$) (Fig. 10B). Similar to the low dose of DHT (500 μg), subjects treated with SO and the higher dose of DHT (1mg) had similar exploration times in T1 (Fig. 10C) and proportion of time spent with the new object for object recognition (Fig. 10D). Therefore, DHT does not enhance spatial or nonspatial memory at the lower dose, but enhances spatial and not nonspatial memory at the higher dose.

***Androstenedione***

SO and AD treated subjects had similar exploration times for T1 of object placement (Fig. 11A). AD treated subjects spent a significantly greater proportion of time with the novel location compared to SO treated subjects for object placement ($p < 0.01$) (Fig. 11B). SO and AD treated subjects had similar exploration times in the sample trial (Fig. 11C) and proportion of time spent with the new object for object recognition (Fig. 11D), unlike object placement. Therefore, AD enhances spatial but not nonspatial memory. (See summary Table 2 for subchronic treatment effects of all androgens tested on object placement and object recognition memory tasks).

**Table 1. Summary of subchronic androgen treatments and cohorts used for behavioral, physiological, morphological, and neurochemical analyses.**

| Cohort | Treatment | Dose | N | Task |
|---|---|---|---|---|
| 1 | SO; DHEA | 0.20 ml; 1 mg | 13; 13 | 2 hr OP |
| 2 | SO; DHEA | 0.20 ml; 1 mg | 8; 8 | 4 hr OR; EPM |
| 3 | SO; Let + DHEA | 0.20 ml; 1 mg + 1 mg | 8; 8 | 2 hr OP |
| 3 | SO; TP | 0.10 ml; 500 μg | 8; 8 | 2 hr OP |
| 4 | SO; TP | 0.10 ml; 500 μg | 8; 8 | 4 hr OR |
| 4 | SO; TP | 0.20 ml; 1 mg | 9; 9 | 2 hr OP; EPM |
| 5 | SO; Let + TP | 0.10 ml; 1 mg + 1 mg | 6; 6 | 4 hr OR |
| 5 | SO; DHT | 0.10 ml; 500 μg | 7; 8 | 4 hr OR |
| 6 | SO; DHT | 0.10 ml; 500 μg | 8; 9 | 2 hr OP |
| 6 | SO; DHT | 0.20 ml; 1 mg | 9; 8 | 4 hr OR; EPM |
| 6 | SO; DHT | 0.20 ml; 1 mg | 9; 9 | 2 hr OP |
| 6 | SO; DHEA; TP | 0.20 ml; 1 mg; 500 μg | 6; 6; 6 | Golgi, uterine weights; T & DHEAS RIAs |
| 7 & 8 | SO; DHEA;<br>TP; EB | 0.15 ml; 1 mg;<br>500 μg; 50 μg/kg | 9; 10<br>9; 9 | HPLC;<br>uterine Weights |
| 9 | SO; AD | 0.20 ml; 1 mg | 9; 9 | 4 hr OR |
| 9 | SO; AD | 0.20 ml; 1 mg | 9; 9 | 2 hr OP |
| 9 | SO; AD; DHT | 0.20 ml; 1 mg; 1 mg | 6; 6; 6 | HPLC; uterine weights; AD RIA |
| 10 | CMC; Let | 0.20 ml; 1 mg | 8; 10 | 2 hr OP |
| 10 | CMC; Let | 0.20 ml; 1 mg | 6; 6 | 4 hr OR |
| 11 | SO; AD | 0.20 ml; 1 mg | 8; 8 | 4 hr OR; EPM |

Subjects were subchronically treated with androgens and tested on object placement, object recognition, or EPM. Subjects in other cohorts were eventually sacrificed and brains were used for golgi and HPLC analysis, blood serum was analyzed for androgen levels, and uteri were weighed to determine whether any peripheral effects existed. When the same cohort of subjects was injected with different hormones and tested on different tasks, one to two weeks was allowed between tasks and for wash out of the hormones. Subjects that did not explore objects in T1 and/or T2 were removed from analysis. (EPM: elevated plus maze; OR: object recognition; OP: object placement; RIA: radioimmunoassay).

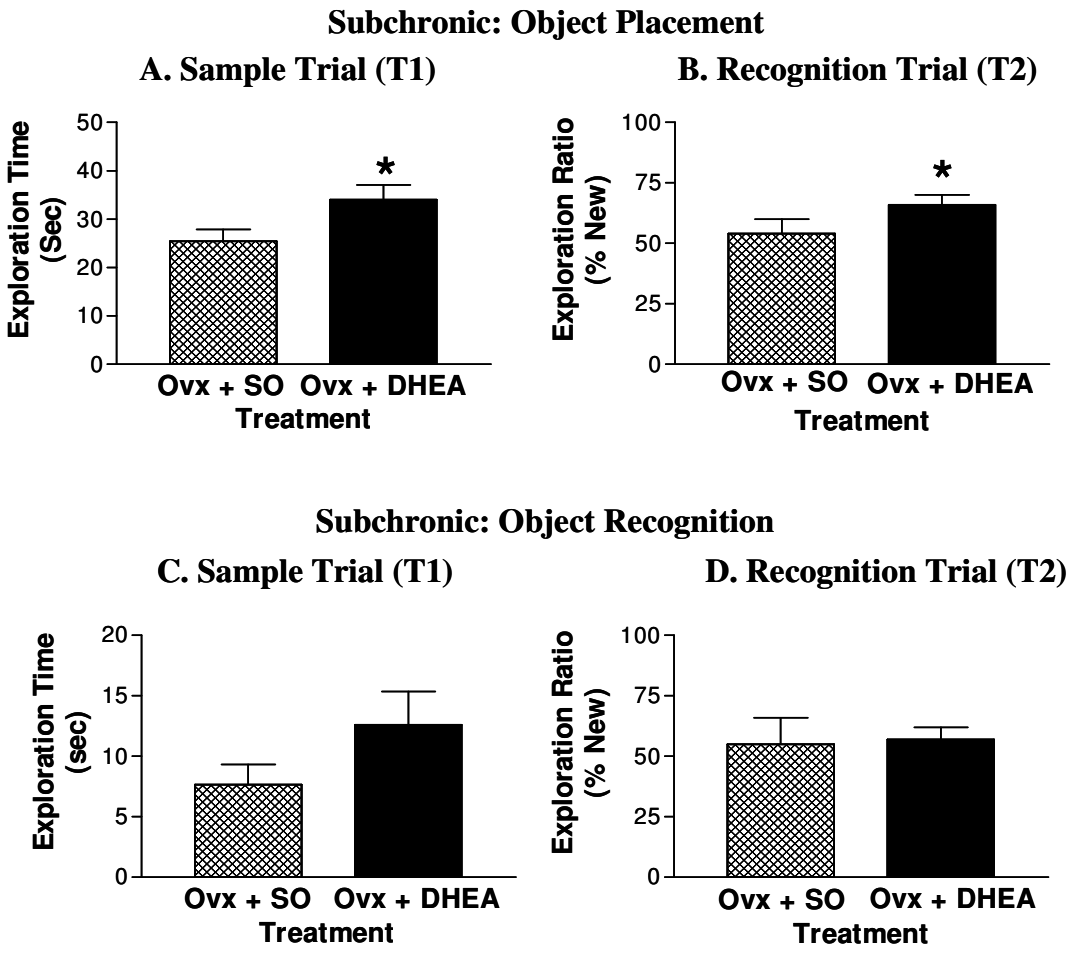



**Figure 6. Effect of subchronic DHEA treatment on object placement and object recognition.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 2 hr inter-trial delay of object placement is shown for SO (n = 13) and DHEA (n = 13; 1 mg) treated subjects. A two-sample t-test showed significant differences in T1 exploration times, where (* $p < 0.05$). (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (* $p < 0.05$). (C) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for SO (n = 8) and DHEA (n = 8; 1 mg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (D) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

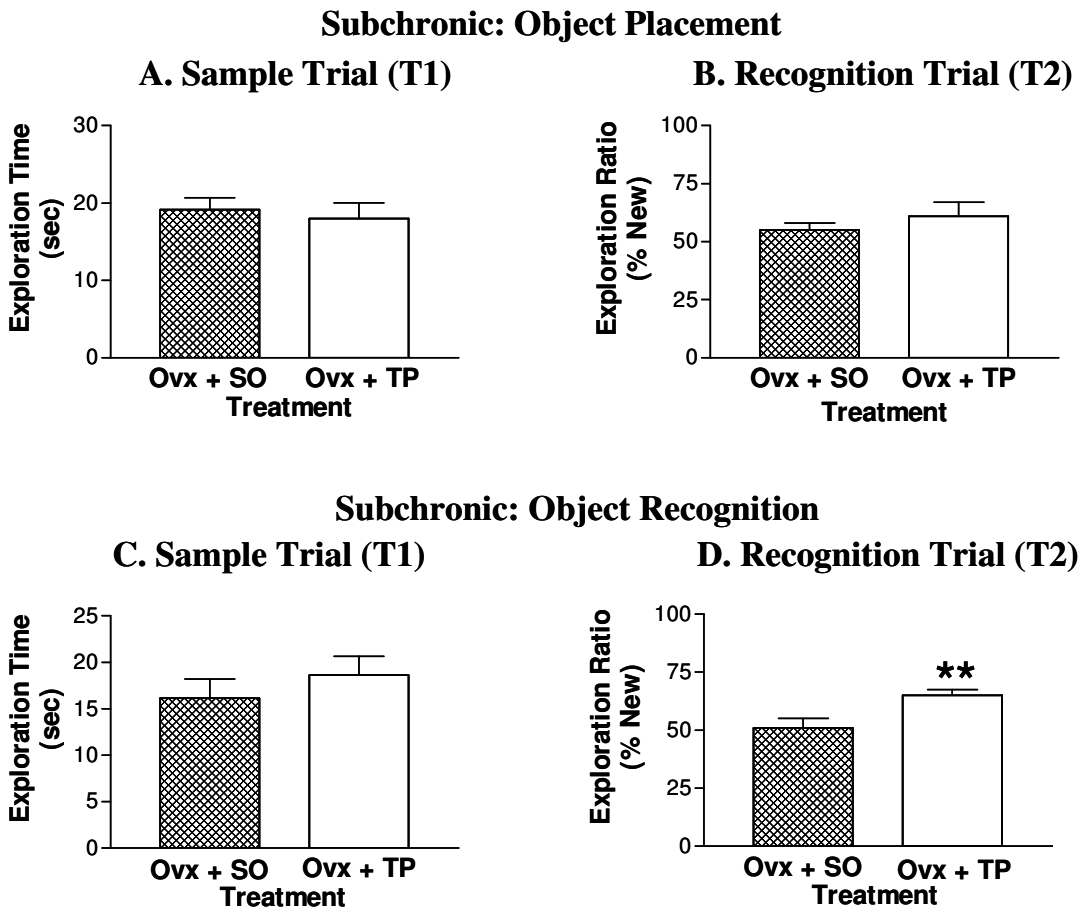



**Figure 7. Effect of subchronic TP treatment on object placement and object recognition.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 2 hr inter-trial delay of object placement is shown for SO (n = 8) and TP (n = 8; 500 μg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups. (C) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for SO (n = 8) and TP (n = 8; 500 μg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (D) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (** $p < 0.01$).

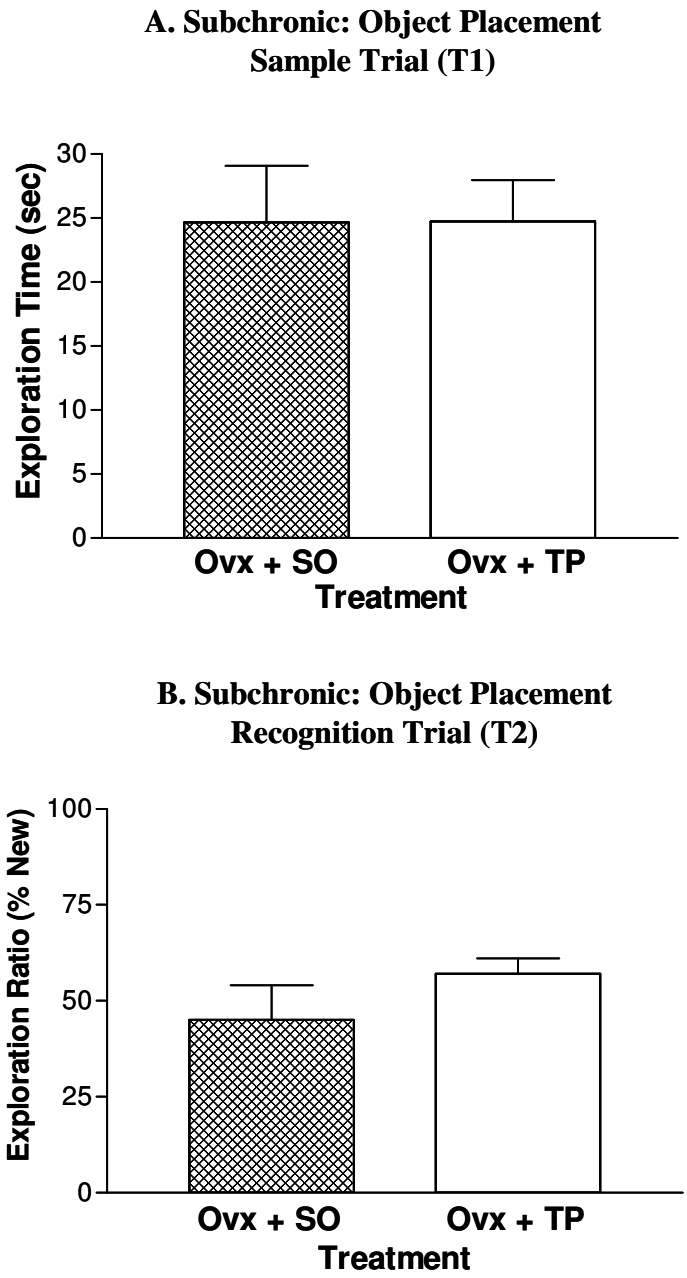



**Figure 8. Effect of a higher dose of subchronic TP treatment on object placement.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 2 hr inter-trial delay of object placement is shown for SO (n = 9) and TP (n = 9; 1 mg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

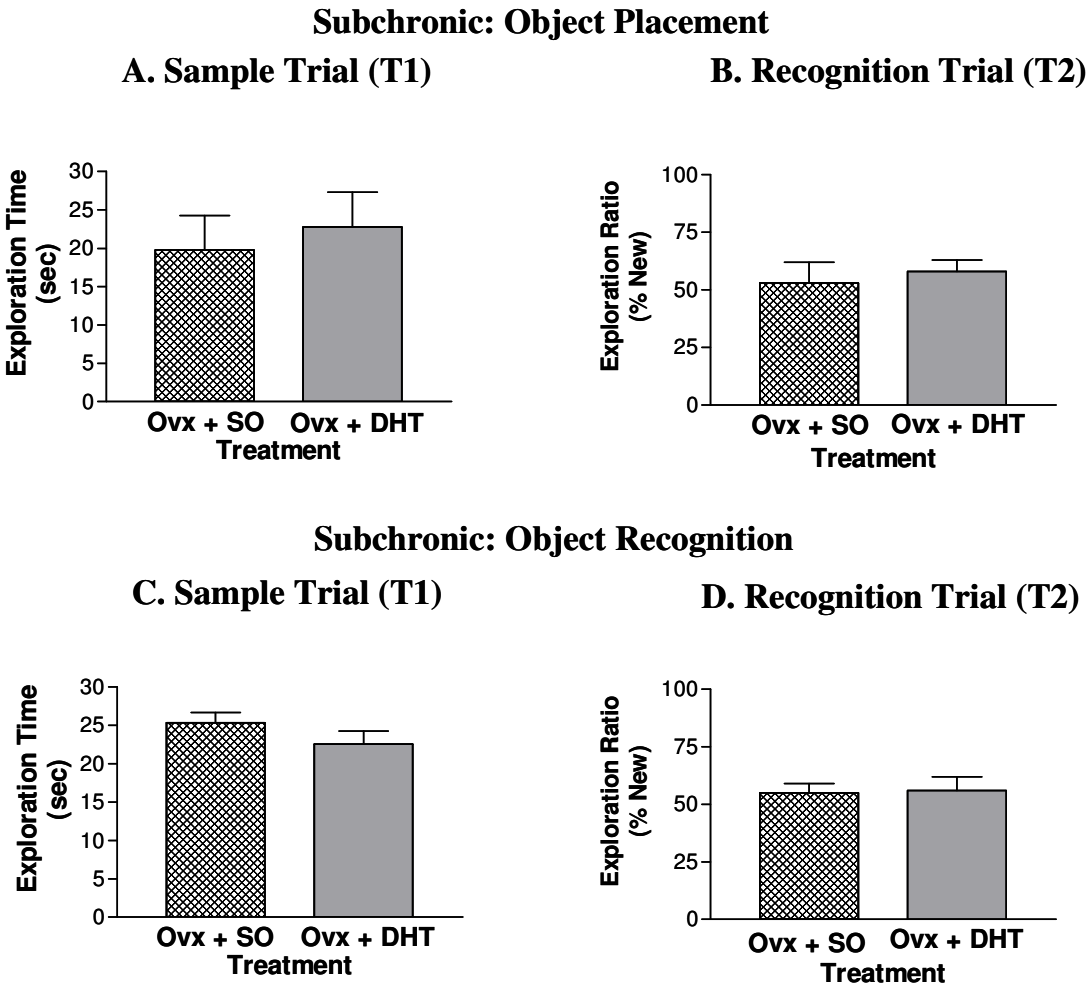



**Figure 9. Effect of subchronic DHT treatment on object placement and object recognition.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 2 hr inter-trial delay of object placement is shown for SO (n = 8) and DHT (n = 9; 500 μg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups. (C) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for SO (n = 7) and DHT (n = 8; 500 μg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (D) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

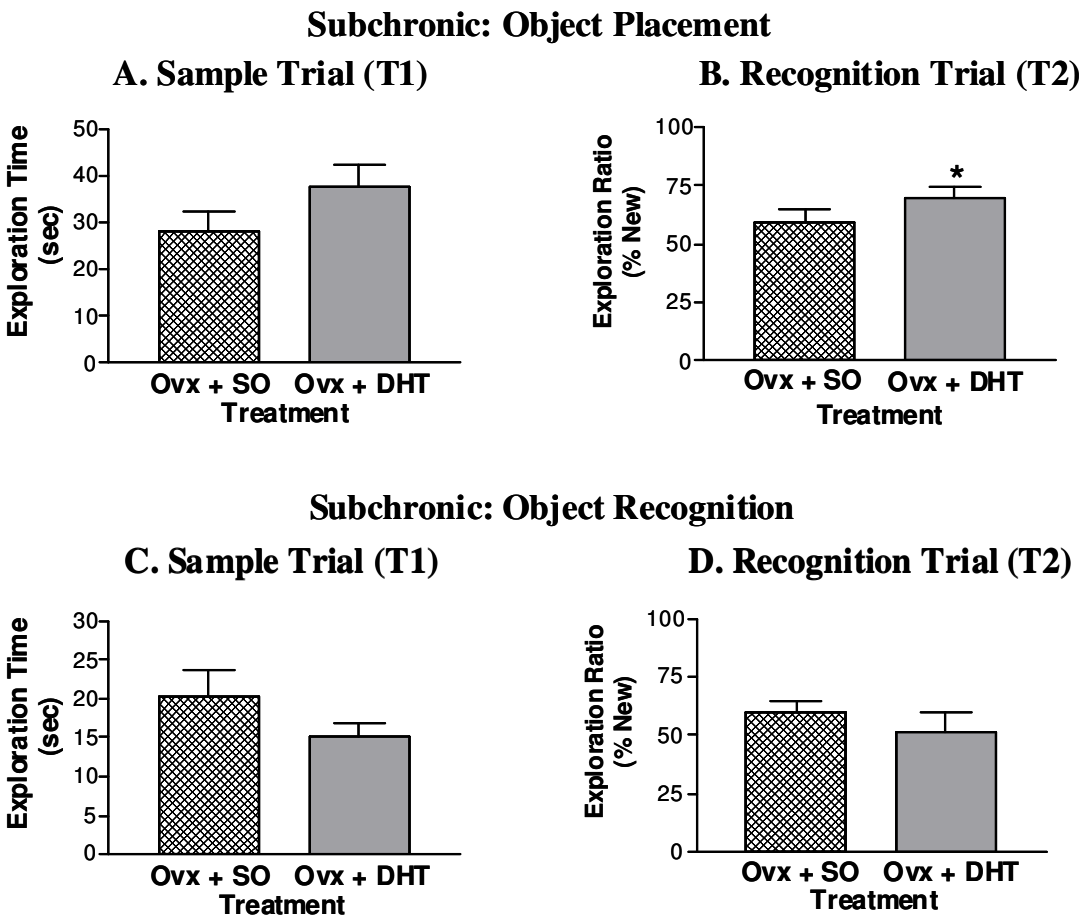



**Figure 10. Effect of a higher dose of subchronic DHT treatment on object placement and object recognition.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 2 hr inter-trial delay of object placement is shown for SO (n = 9) and DHT (n = 9; 1 mg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (* $p < 0.05$). (C) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for SO (n = 9) and DHT (n = 8; 1 mg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (D) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

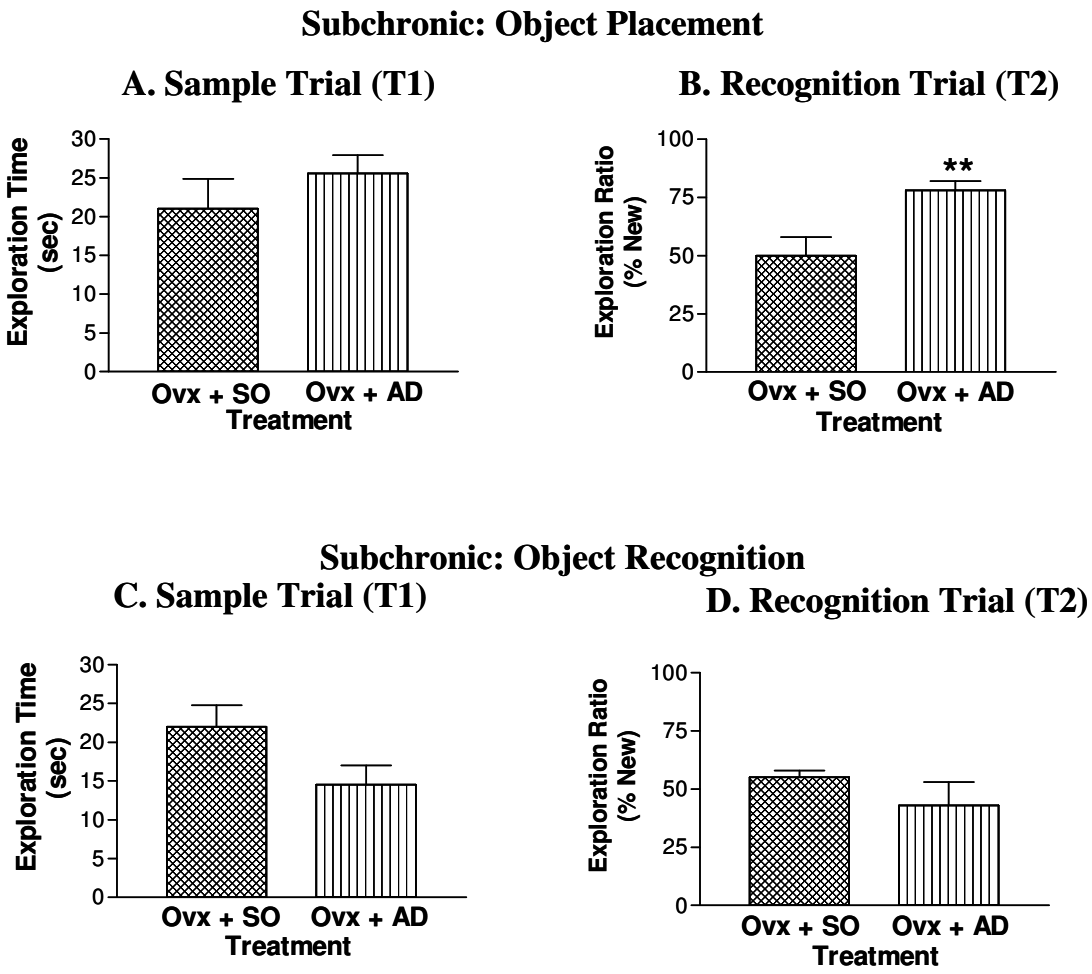



**Figure 11. Effect of subchronic AD on object placement and object recognition.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 2 hr inter-trial delay of object placement is shown for SO (n = 9) and AD (n = 9; 1 mg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (** $p < 0.01$). (C) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for SO (n = 9) and AD (n = 9; 1 mg) treated subjects. (D) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

***B. Subchronic androgen treatments: elevated plus maze***

Studies have shown that androgens decrease anxiety related behaviors in both male and female gonadectomized rats (Edinger and Frye, 2004; 2005; Frye and Lacey, 2001). Since androgens may have potential indirect effects on memory performance via sensory, motor, regulatory, and affective processes, subjects were subchronically treated with androgens and tested on the elevated plus maze to assess anxiety levels.

**Methods**

***DHEA***

Subjects subchronically treated with SO (0.20 ml; n = 8) or DHEA (1 mg; n = 8), that were previously tested on a 4 hr inter-trial delay of object recognition (see section A), were also tested on the elevated plus maze. Two hours after T2 of the 4 hr object recognition or 54 hours after the second injection, subjects were tested on the elevated plus maze. See specific details of the elevated plus maze in the general methods and cohorts used in Table 1.

***Testosterone propionate***

Subjects subchronically administered with SO (0.20 ml; n = 9) or the higher dose of TP (1 mg; n = 9) that were previously tested on a 2 hr inter-trial delay of object placement (see section A), were also tested on the elevated plus maze. Four hours after T2 or 54 hours after the second injection, subjects were tested on the elevated plus maze.

***Dihydrotestosterone***

Subjects subchronically treated with SO (0.20 ml; n = 9) or the higher dose of DHT (1 mg; n = 8) that were previously tested on a 4 hr inter-trial delay of object

recognition (see section A), were also tested on the elevated plus maze. Two hours after T2 or 54 hours after the second injection, subjects were tested on the elevated plus maze.

***Androstenedione***

A new cohort of subjects was subchronically treated with SO (0.20 ml; n = 8) or AD (1 mg; n = 8) and tested on a 4 hour inter-trial delay of object recognition, followed by testing on the elevated plus maze. Two hours after T2 or 54 hours after the second injection, subjects were tested on the elevated plus maze.

## Results

***Subchronic treatment effects of androgens: elevated plus maze***

For the elevated plus maze (EPM), DHEA treated subjects made a greater percentage of entries in open arms (19%) compared to SO treated subjects (11%), although this finding was not significant (Fig. 12A). SO and DHEA treated subjects spent a similar percentage of time spent in open arms (Fig. 12B). Therefore, this dose of DHEA (1 mg) does not influence anxiety on the elevated plus maze.

SO and TP treated subjects had a similar percentage of open arms entries (Fig. 13A) and percentage of time spent on open arms (Fig. 13B) on elevated plus maze. Therefore, this dose of TP (1 mg) does not influence anxiety on the elevated plus maze.

SO and DHT treated subjects had similar percentage of open arm entries (Fig. 14A) and percentage of time spent in the open arms (Fig. 14B) of the elevated plus maze. Therefore, this dose of DHT (1 mg) does not influence anxiety on the elevated plus maze.

For, SO and AD treated subjects, there were no significant differences in percentage of open arm entries (Fig. 15A) and percentage of time spent in open arms (Fig. 15B) on the elevated plus maze. Therefore, this dose of AD (1 mg) does not

influence anxiety on the elevated plus maze. (See summary Table 2 for subchronic treatment effects of all androgens tested on elevated plus maze).

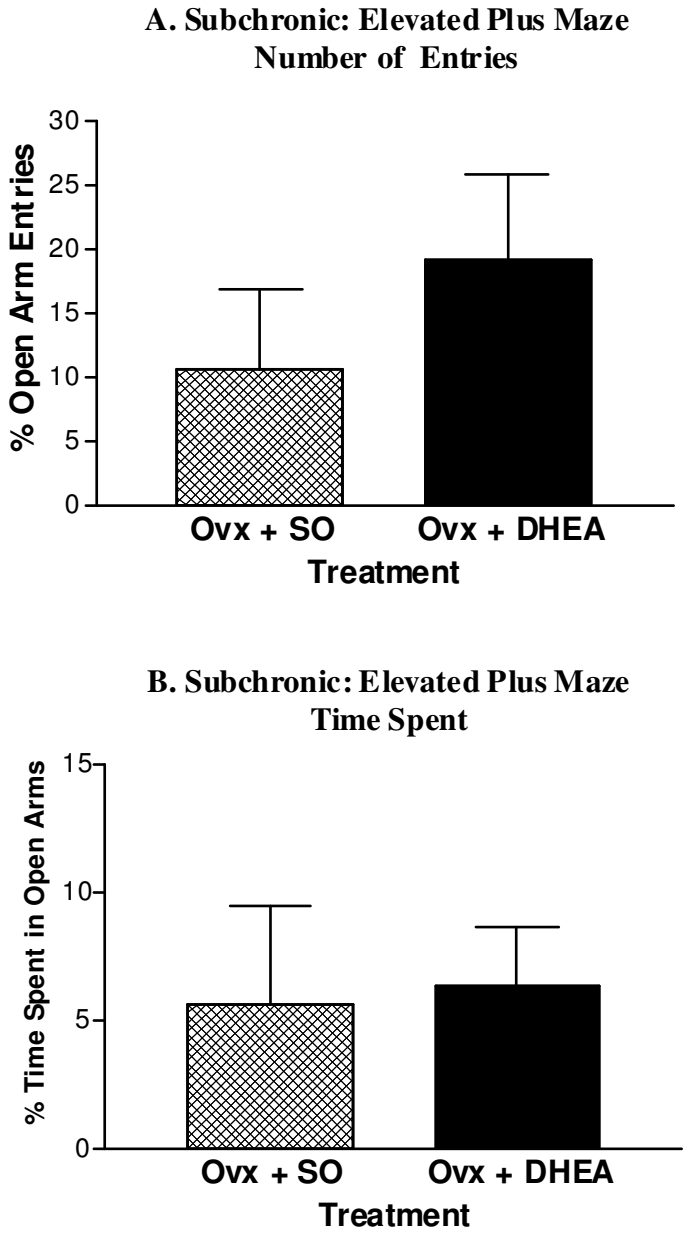



**Figure 12. Effect of subchronic DHEA on elevated plus maze.** (A) Percentage of open arm entries ± SEM (number of open arm entries/(number of open arm entries + number of closed arm entries) x 100) is shown for SO (n = 8) and DHEA (n = 8; 1 mg) treated subjects. A two-sample Mann-Whitney U test showed that there were no significant differences between groups. (B) Percentage of time spent in open arms ± SEM (time spent in open arms/(time spent in open arms + time spent in closed arms) x 100) is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

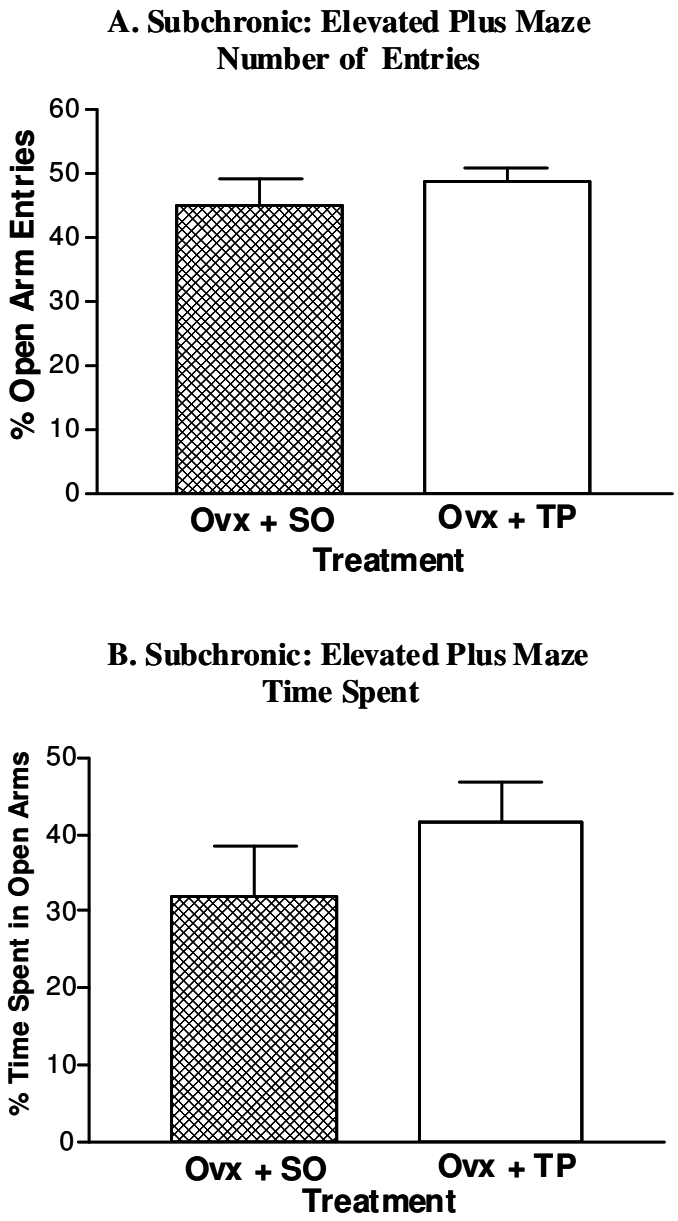



**Figure 13. Effect of subchronic TP on elevated plus maze.** (A) Percentage of open arm entries ± SEM (number of open arm entries/(number of open arm entries + number of closed arm entries) x 100) is shown for SO (n = 9) and TP (n = 9; 1 mg) treated subjects. A two-sample Mann-Whitney U test showed that there were no significant differences between groups. (B) Percentage of time spent in open arms ± SEM (time spent in open arms/(time spent in open arms + time spent in closed arms) x 100) is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

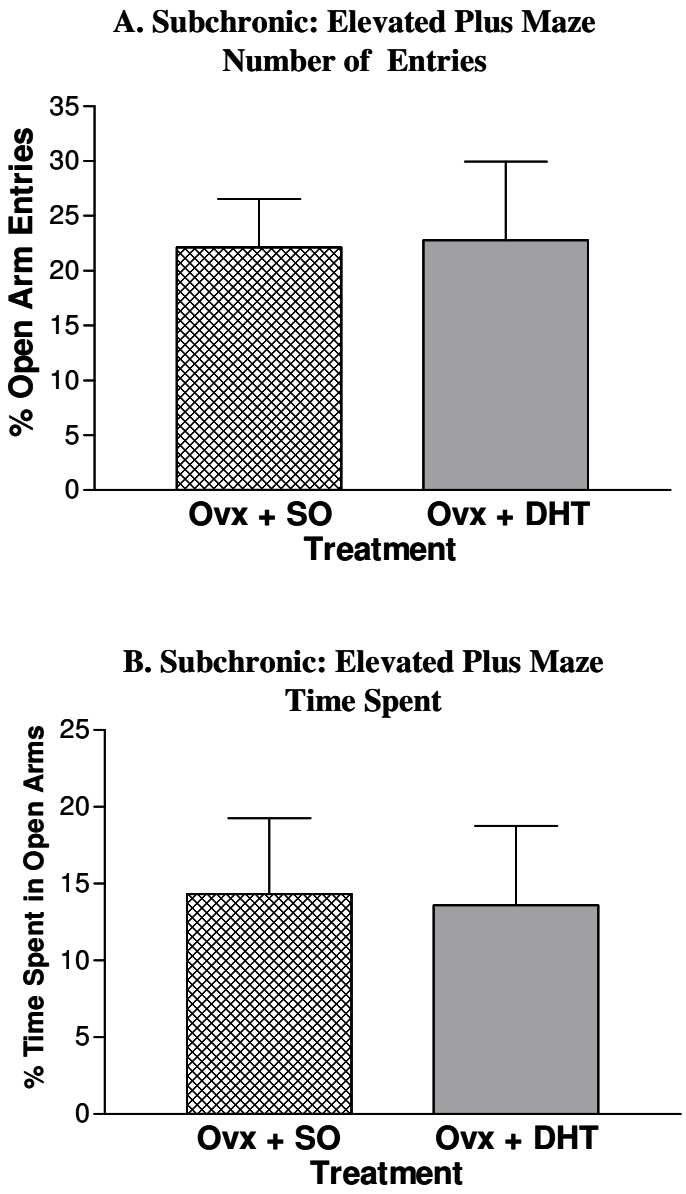



**Figure 14. Effect of subchronic DHT on elevated plus maze.** (A) Percentage of open arm entries ± SEM (number of open arm entries/(number of open arm entries + number of closed arm entries) x 100) is shown for SO (n = 8) and DHT (n = 8; 1 mg) treated subjects. A two-sample Mann-Whitney U test showed that there were no significant differences between groups. (B) Percentage of time spent in open arms ± SEM (time spent in open arms/(time spent in open arms + time spent in closed arms) x 100) is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

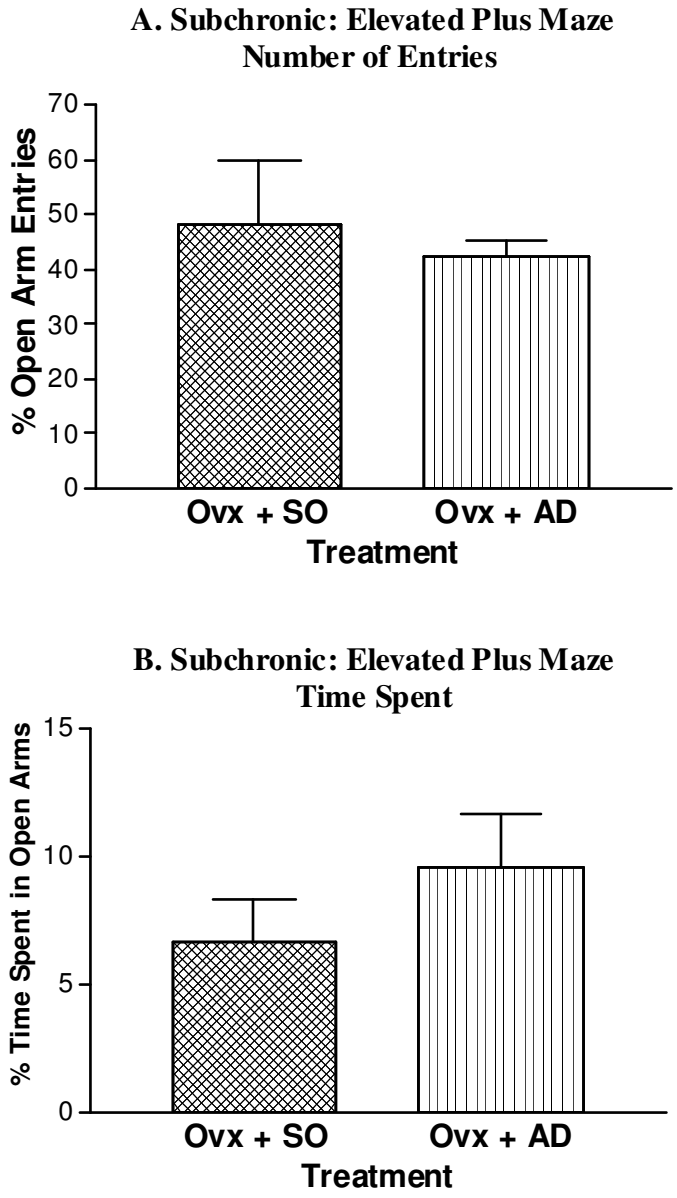



**Figure 15. Effect of subchronic AD on elevated plus maze.** (A) Percentage of open arm entries ± SEM (number of open arm entries/(number of open arm entries + number of closed arm entries) x 100) is shown for SO (n = 8) and AD (n = 8; 1 mg) treated subjects. A two-sample Mann-Whitney U test showed that there were no significant differences between groups. (B) Percentage of time spent in open arms ± SEM (time spent in open arms/(time spent in open arms + time spent in closed arms) x 100) is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

**Table 2. Summary of subchronic androgen treatment effects on behavioral and physiological measures.**

| Subchronic Treatment | Dose | OP | OR | EPM | Uterine Weight | DHEAS RIA | T RIA | AD RIA |
|---|---|---|---|---|---|---|---|---|
| DHEA | 1 mg | ✓ | ----- | ----- | ✓ | ✓ | ----- | NT |
| TP | 500 µg | ------ | ✓ | ----- | ✓ | ✓ | ✓ | NT |
| TP | 1 mg | **-----** | NT | NT | NT | NT | NT | NT |
| DHT | 500 µg | **-----** | ----- | NT | NT | NT | NT | NT |
| DHT | 1 mg | ✓ | ----- | ----- | ----- | NT | NT | ----- |
| AD | 1 mg | ✓ | ----- | ----- | ----- | NT | NT | ✓ |

(OP: object placement; OR: object recognition; EPM: elevated plus maze;
NT: not tested; ✓: enhancement; -----: no difference).

### *C. Subchronic androgen treatments: physiological measures*

Estradiol has been shown to stimulate proliferation of uterine tissue (Gibbs et al, 2004), while Hajszan et al (2004) demonstrated that OVX female rats treated subchronically with DHEA, a weak androgen did not increase uterine wet weights. Uterine weights and androgen levels in serum blood were measured in subjects treated subchronically with androgens and estrogen to compare effects of androgens to determine whether peripheral effects exist and to verify effectiveness of the treatments.

## Methods

### *Uterine Weight*

Two separate cohorts of subjects (Table 1) were treated subchronically and sacrificed 48 hours after the second subchronic injection of SO (n = 6; 0.15 ml), DHEA (n = 6; 1 mg), TP (n = 6; 500 μg) and SO (n = 6; 0.20 ml), DHT (n = 6; 1 mg) and AD (n = 6; 1 mg) and EB (n = 4; 50 μg/kg). (EB treated uteri were obtained from a different cohort). Uteri were removed and weighed. When cohorts were combined, there were 12 SO treated subjects. The cohorts were combined to determine whether there were any peripheral effects as a result of the hormones administered. See general methods for details on uterine weight analysis.

### *Radioimmunoassay*

Blood samples were collected from the trunk blood of subjects from the same cohorts as above that were subchronically treated with SO (0.15 ml), DHEA (1 mg), or TP (500 μg) for the testosterone and DHEAS RIAs, and SO (0.20 ml), DHT (1 mg), or AD (1 mg) for the androstenedione RIA (Table 1), and sacrificed 48 hrs later. For the testosterone RIA, two subjects were removed from the analysis, as levels of testosterone

were not detected (SO (n = 5); DHEA (n = 5); TP (n = 6)). For the DHEAS RIA, one subject was removed from the analysis as levels of DHEAS were not detected (SO (n = 6); DHEA (n = 5); TP (n = 6)). For the androstenedione RIA, two subjects were removed from the control group, as androstenedione levels were not detected (n = 4), DHEA (n = 6), or TP (n = 6). See general methods for details on RIA anaylsis.

## Results

### *Subchronic treatment effects of androgens: physiological measures*

#### *Uterine weights*

A one-way ANOVA showed that there was a significant treatment effect for uterine wet weights ($F(5,34) = 110.56$, $p < 0.00001$) (Fig. 16). *Post hoc* with Fisher's LSD revealed that uterine weights for DHEA, TP, and EB treated subjects were significantly different from each other and significantly greater than SO, DHT and AD treated subjects. Uterine weights for EB treated subjects were significantly greater than all other groups. SO, DHT and AD treated subjects had uterine weights that were very similar to each other and not significantly different from each other. This indicates that EB, DHEA and TP act peripherally in the uterus, while DHT and AD have no effects peripherally in the uterus.

#### *Serum hormone levels*

Blood serum levels of testosterone, DHEAS, and androstenedione were measured by RIA in OVX female rats treated subchronically with androgens. A one-way ANOVA revealed that serum testosterone levels significantly differed between groups ($F(2,13) = 20.64$, $p < 0.0001$) (Table 3). *Post hoc* analysis with Fisher's LSD showed that TP treated subjects had significantly higher levels of testosterone compared to SO and DHEA

treated subjects (Table 3). Testosterone levels were very low, less than one ng/ml in SO and DHEA treated subjects compared to the high level, over 1600 ng/ml in TP treated subjects. This indicates that injected TP significantly elevated testosterone in blood serum levels and DHEA was not converted to testosterone levels that were detectable.

A one-way ANOVA revealed that serum DHEAS levels differed between groups ($F(2,14) = 5.40$, $p < 0.05$) (Table 3). *Post hoc* analysis revealed that DHEA and TP treated subjects had significantly higher levels of DHEAS compared to SO treatment (Table 3). Serum DHEAS levels were similar in both DHEA and TP treated subjects, around 147 pg/ml.

A one-way ANOVA showed that serum levels of androstenedione significantly differed between groups ($F(2,13) = 19.56$, $p < 0.001$) (Table 4). *Post hoc* analysis revealed that AD treated subjects had significantly higher AD levels compared to SO and DHT (Table 4). This suggests that androstenedione was not converted to DHT as it was not detectable by RIA.

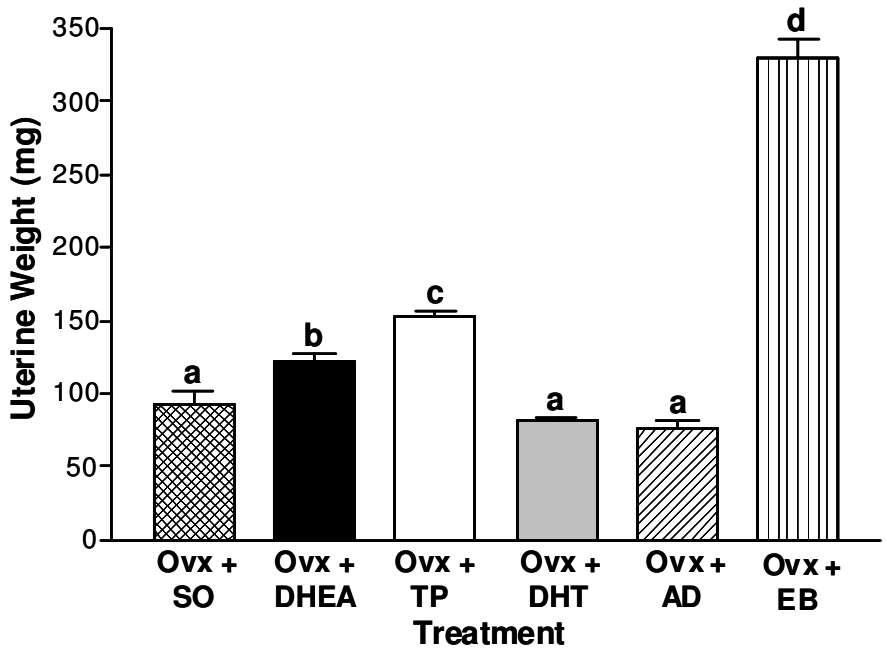



**Figure 16. Effect of subchronic DHEA, TP, DHT, AD and EB on uterine weight.** Uterine wet weights ± SEM are shown for SO (n = 12), DHEA (n = 6; 1 mg), TP (n= 6; 500 μg), DHT (n = 6; 1 mg), AD (n = 6; 1 mg), and EB (n = 4; 50 μg/kg) treated subjects. A one-way ANOVA showed that there was a significant treatment effect for uterine wet weights ($F(5,34) = 110.56$, $p < 0.00001$). *Post hoc* with Fisher's LSD revealed that uterine weights for DHEA, TP and EB treated subjects were significantly greater than SO, DHT, and AD treated subjects. Groups labeled with different letters are significantly different from each other.

**Table 3. Serum testosterone and DHEAS concentrations measured by RIA.**

| Treatment | Serum testosterone (pg/ml) | Serum DHEAS (ng/ml) |
|---|---|---|
| SO | 0.25 ± 0.25 | 83.96 ± 15.18 [a] |
| DHEA | 0.91 ± 0.91 | 147.08 ± 16.54 [b] |
| TP | 1664.09 ± 330.02 * | 147.22 ± 16.02 [b] |

Serum testosterone levels (pg/ml) ± SEM are shown for SO (n = 5), DHEA (n = 5) and TP (n = 6). A one-way ANOVA showed that there was a significant treatment effect ($F(2,13) = 20.64$, $p < 0.0001$). *Post hoc* with Fisher's LSD revealed that treatment with TP significantly elevated testosterone levels compared to SO and DHEA treatments. A group labeled with an asterisk (*) represents one that is significantly different from all other groups. Serum DHEAS levels (ng/ml) ± SEM are shown for SO (n = 6), DHEA (n = 5) and TP (n = 6). A one-way ANOVA showed that there was a significant treatment effect ($F(2,14) = 5.40$, $p < 0.05$). *Post hoc* with Fisher's LSD revealed that treatment with DHEA and TP significantly elevated DHEAS levels compared to SO treatment. Groups labeled with different letters are significantly different from all other groups.

**Table 4. Serum androstenedione concentrations measured by RIA.**

| Treatment | **Serum androstenedione (pg/ml)** |
|---|---|
| SO | 1.75 ± 1.18 |
| DHT | 9.00 ± 6.89 |
| AD | 848.83 ± 171.61 *** |

Serum androstenedione levels (pg/ml) ± SEM are shown for SO (n = 4), DHT (n = 6) and AD (n = 6). A one-way ANOVA showed that there was a significant treatment effect ((F (2,13) = 19.56, $p < 0.001$). *Post hoc* with Fisher's LSD revealed that treatment with AD significantly elevated AD levels compared to SO and DHT treatments. A group labeled with an asterisk (*) represents one that is significantly different from all other groups.

***D. Acute androgen treatments: object placement and object recognition***

Acute treatment with estrogen and androgens in female OVX rats, immediately post-training, have shown to enhance memory (Packard and Teather, 1997a; 1997b; Frye and Lacey, 1999; 2001). Based on these findings and the cognitive enhancing effects of spatial and nonspatial memory with subchronic treatment with androgens (Section A), it is hypothesized that acute administration of the androgens, DHEA, TP, DHT, and AD, immediately post sample trial will enhance both spatial and nonspatial memory in female OVX rats.

**Methods**

See specific details above in general methods (Fig. 5B) and different cohorts used for experiments in Table 5. Subjects that did not explore objects in T1 and/or T2 were removed from analysis.

***DHEA***

Since subchronic DHEA treatment enhanced 2 hr object placement (Fig. 6B), we investigated whether acute treatment with DHEA also enhanced object placement on a 4 hr inter-trial delay. The cohorts used for subchronic DHEA treatment experiments were also used for all acute DHEA treatment experiments (Table 5). Immediately following the sample trial of object placement, OVX female rats were acutely treated with propylene glycol (PG) (0.20 ml; n = 14) or DHEA (1 mg; n = 15) dissolved in PG. Four hours after the sample trial, subjects were tested on T2 of object placement.

OVX female rats were tested on a delayed post sample trial injection paradigm, using 4hr object placement. Subjects were first tested on a sample trial of 4 hr object placement. Two hours after the sample trial, subjects were injected with PG (0.20 ml; n =

8) or DHEA (1 mg; n = 8). Four hours after the sample trial, subjects were tested on T2 of a 4 hr inter-trial delay of object placement.

OVX female rats were treated with an immediate post sample trial injection of PG (0.20 ml; n = 8) or DHEA (1 mg; n = 8). Four hours after the sample trial, subjects were tested on T2 of object recognition.

***Testosterone propionate***

Since subchronic TP treatment enhanced object recognition (Fig. 7D), we investigated whether acute treatment with TP also enhanced object recognition. One of the cohorts used for subchronic TP treatment experiments was also used for the current acute TP treatment experiment (Table 5). Immediately following the sample trial of object recognition, OVX female rats were acutely treated with PG (0.10 ml; n = 8) or TP (500 μg; n = 8). Four hours after the sample trial, subjects were tested on T2 of object recognition.

***Dihydrotestosterone***

Since subchronic DHT treatment enhanced object placement (Fig. 10B), we investigated whether acute treatment with DHT also enhanced object placement. One of the cohorts used for suchronic DHT treatment experiments was used for this acute DHT treatment experiment (Table 5). Immediately following the sample trial of object placement, OVX female rats were acutely treated with PG (0.20 ml; n = 9) or DHT (1 mg; n = 9). Four hours after the sample trial, subjects were tested on T2 of object placement.

***Androstenedione***

Since subchronic treatment with AD enhanced object placement (Fig. 11B), we

investigated whether acute treatment with AD also enhance object placement. A different cohort of OVX female rats were acutely treated with PG (0.20 ml; n = 8) or AD (1 mg; n = 9), immediately following the sample trial of object placement. Four hours after the sample trial, subjects were tested on T2 of object placement.

## Results

### *Acute treatment effects of androgens: object placement and object recognition*

#### *DHEA*

Both PG and DHEA treated subjects had similar exploration ratios in T1 (Fig. 17A). Similar to subchronic treatment with DHEA, subjects administered DHEA immediately post sample trial spent a significantly greater proportion of time at the novel location compared to PG treated subjects for object placement ($p < 0.05$) (Fig. 17B). When injections were delayed 2 hours post sample trial, PG and DHEA treated subjects had similar exploration times in T1 (Fig. 17C) and spent a similar proportion of time at the novel location (Fig. 17D). This finding suggests that DHEA is acting via an effect on memory processes and not performance parameters. For the 4 hr inter-trial delay of object recognition, subjects treated with PG and DHEA immediately post T1 had similar exploration times (Fig. 18A) and spent a similar proportion of time with the new object (Fig. 18B). Therefore, acute treatment with DHEA immediately post T1 enhances spatial but not nonspatial memory.

#### *Testosterone propionate*

Subjects treated with PG and TP immediately following the sample trial had similar exploration ratios (Fig. 19A). Similar to subjects treated subchronically with TP, subjects treated acutely with TP spent a significantly greater proportion of time with the

new object compared to PG treated subjects ($p < 0.01$) (Fig. 19B). Therefore, acute treatment with TP enhances nonspatial memory.

***Dihydrotestosterone***

Subjects treated with PG and the higher dose of DHT (1mg), immediately following the sample trial had similar exploration ratios (Fig. 20A). Unlike subjects treated subchronically with this dose of DHT, subjects treated acutely with PG and DHT spent a similar proportion of time at the new location (Fig. 20B). Therefore, DHT immediately post T1 does not enhance spatial memory.

***Androstenedione***

Subjects treated with PG and AD immediately following the sample trial had similar exploration ratios (Fig. 21A). Similar to subjects treated with subchronic AD, subjects treated acutely with TP spent as significantly greater proportion of time with the new location compared to PG treated subjects ($p < 0.01$) (Fig. 21B). Therefore, acute treatment with AD enhances spatial memory. (See summary Table 6 for all androgens tested on object placement and object recognition memory tasks).

**Table 5. Summary of acute androgen treatments and cohorts used for behavioral analysis.**

| Cohort | Treatment | Dose | N | Task |
|---|---|---|---|---|
| 1 & 2 | PG; DHEA | 0.20 ml; 1 mg | 14; 15 | 2 hr OP |
| 2 | PG; DHEA | 0.20 ml; 1 mg | 8; 8 | 4hr OP (delayed T1 injection) |
| 2 | PG; DHEA | 0.20 ml; 1 mg | 8; 8 | 4 hr OR |
| 4 | PG; TP | 0.10 ml; 500 μg | 8; 8 | 4 hr OR |
| 6 | PG; DHT | 0.20 ml; 1 mg | 9; 9 | 4 hr OP |
| 12 | PG; AD | 0.20 ml; 1 mg | 8; 9 | 4 hr OP |

Subjects were treated acutely with androgens and tested on object placement or object recognition. When the same cohort of subjects was injected with different androgens and tested on different behavioral tasks, one to two weeks was allowed between tests and for wash out of hormones. Subjects that did not explore objects in T1 and/or T2 were removed from analysis. (OP: object placement; OR: object recognition).

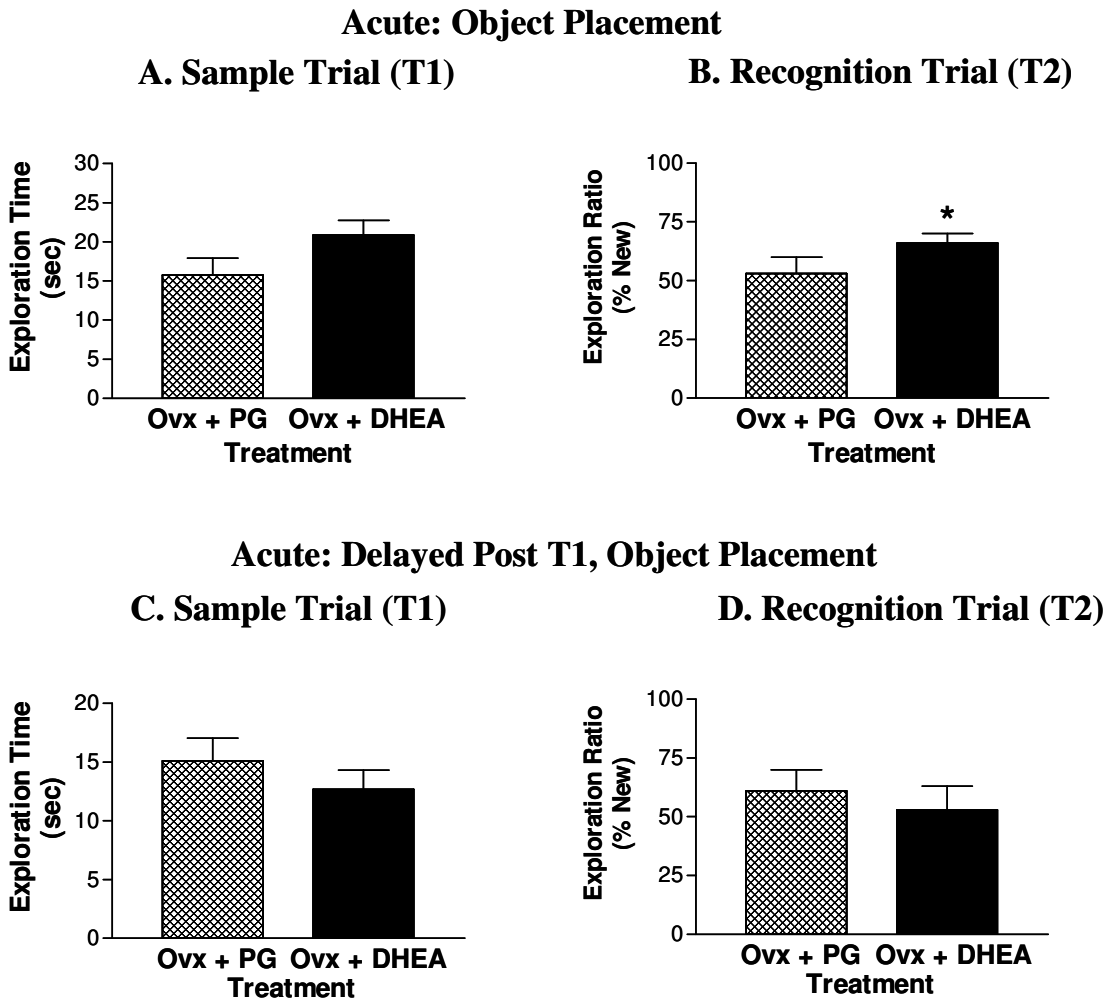



**Figure 17. Effect of acute DHEA on object placement.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object placement is shown for PG (n = 14) and DHEA (n = 15; 1 mg) treated subjects, injected immediately post sample trial (T1). A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (* $p < 0.05$). (C) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object placement is shown for PG (n = 8) and DHEA (n = 8; 1 mg) treated subjects, injected two hrs post sample trial (T1). A two-sample t-test showed that there were no significant differences between groups. (D) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

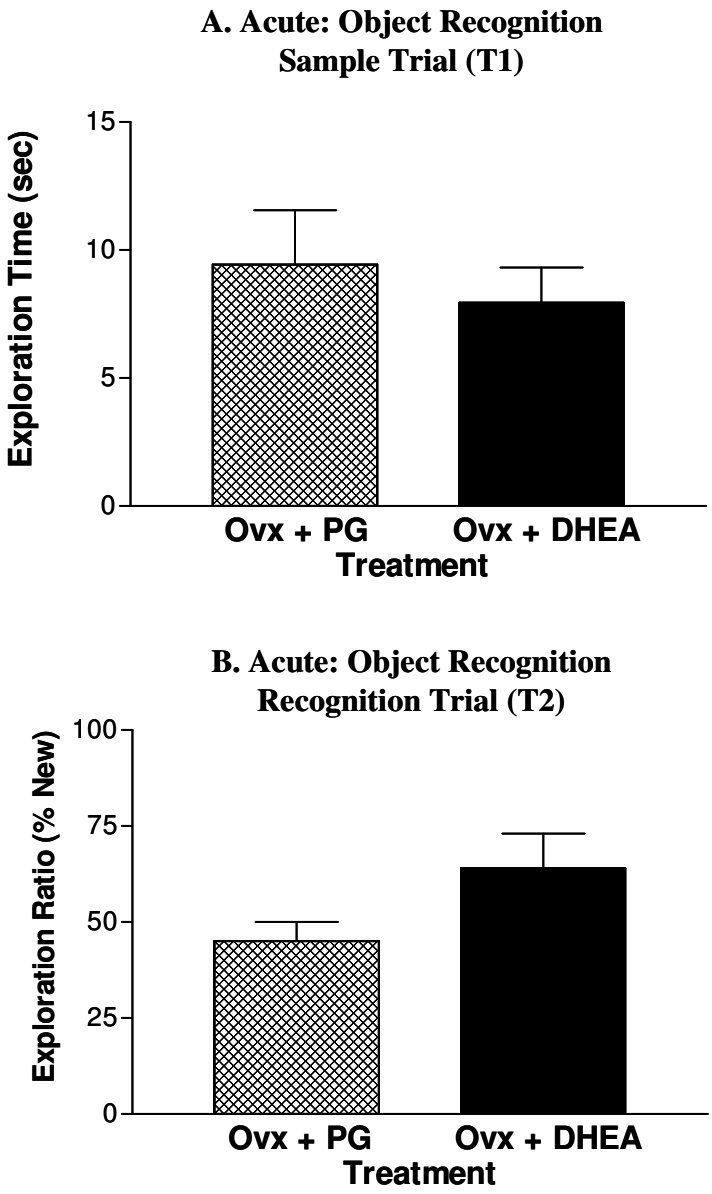



**Figure 18. Effect of acute DHEA on object recognition.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for PG (n = 8) and DHEA (n = 8; 1 mg) treated subjects, injected immediately post sample trial (T1). A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

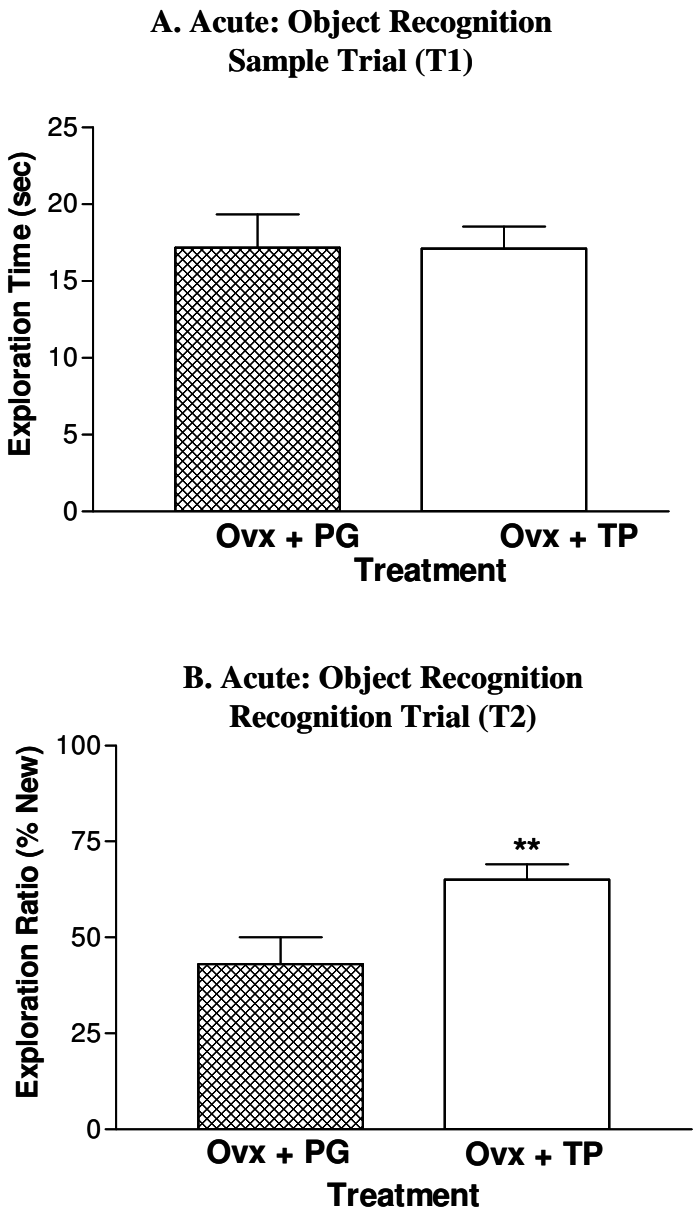



**Figure 19. Effect of acute TP on object recognition.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for PG (n = 8) and TP (n = 8; 500 µg) treated subjects, injected immediately post sample trial (T1). A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (** $p < 0.01$).

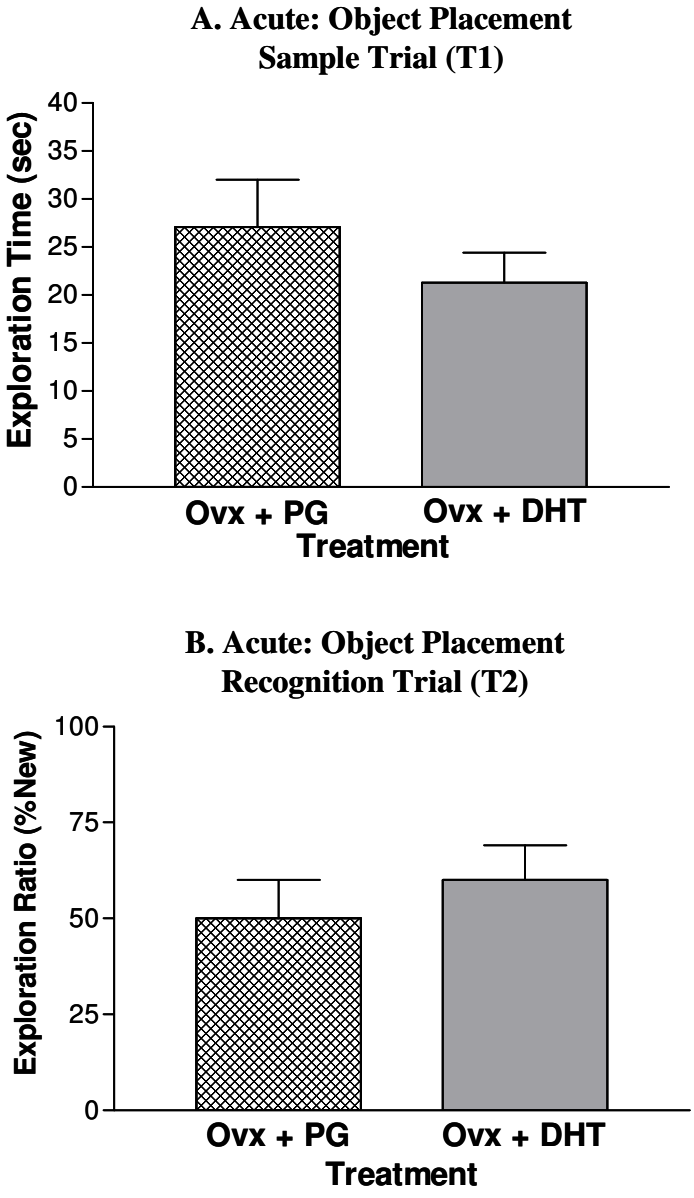



**Figure 20. Effect of acute DHT on object placement.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object placement is shown for PG (n = 9) and DHT (n = 9; 1 mg) treated subjects, injected immediately post sample trial (T1). A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups.

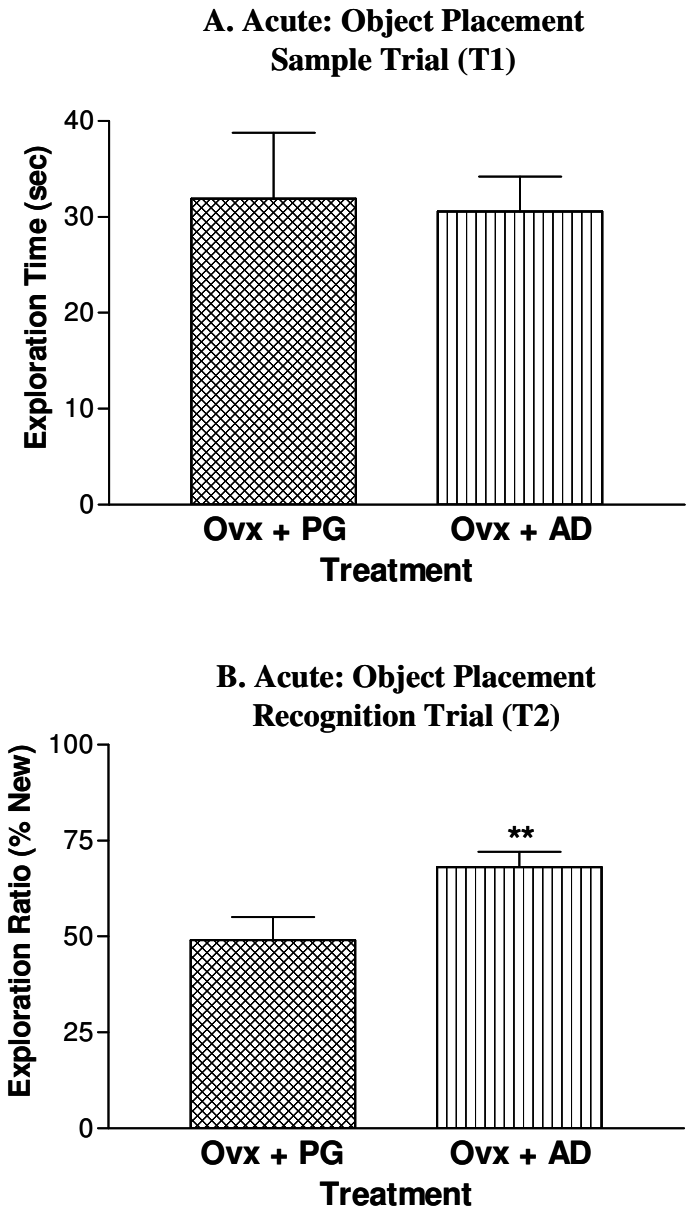



**Figure 21. Effect of acute AD on object placement.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object placement is shown for PG (n = 8) and AD (n = 9; 1 mg) treated subjects, injected immediately post sample trial (T1). A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (** $p < 0.01$).

**Table 6. Summary of acute androgen treatment effects on behavioral measures.**

| Acute Treatment | Dose | OP | OR |
|---|---|---|---|
| DHEA (Immediate post T1) | 1 mg | ✓ | ----- |
| DHEA (Delayed post T1) | 1 mg | ----- | NT |
| TP (Immediate post T1) | 500 µg | NT | ✓ |
| DHT (Immediate post T1) | 1 mg | ----- | NT |
| AD (Immediate post T1) | 1 mg | ✓ | NT |

(OP: object placement; OR: object recognition; EPM: elevated plus maze; NT: not tested; ✓: enhancement; -----: no difference).

## *Discussion*

### *Behavioral measures*

#### *Subchronic treatment effects of androgens: object placement and object recognition*

The current data show that subchronic treatment (two injections 24 hrs apart followed by testing 48 hrs later) with androgens enhanced both object recognition and object placement memory in ovariectomized (OVX) female rats. DHEA, DHT, and AD enhanced object placement, spatial memory, while TP enhanced object recognition, nonspatial memory (see summary in Table 2). These treatments, estradiol, DHEA, TP, or DHT have been previously shown to increase CA1 spine density and spine synapse density in OVX female rats (Gould et al, 1990; Hajszan et al, 2004; Leranth et al, 2004). These morphological changes have been implicated in memory enhancements. Similar to the current findings, behavioral studies in our lab have shown that estradiol benzoate (50mg/kg) administered to OVX female rats, enhanced both nonspatial and spatial memory (Jacome et al, in prep.). Additionally, previous findings have reported that chronic, longer term treatment (5-28 days) with estradiol benzoate administered to OVX female rats, enhanced cognitive performance on the radial arm maze, Morris water maze, T-maze, a delayed matching to position version of the T-maze task, and the active avoidance task (Daniel et al, 1997; Luine et al, 1998; Dohanich et al, 1994; Fader et al, 1998; Gibbs et al, 1999; 2000; Singh et al, 1994). However, subchronic and chronic behavioral studies with androgens in female rats are lacking, and thus the current work fills in the gap.

DHEA treated subjects had significantly greater exploration times during the sample trial (T1) and exploration ratios in the recognition/retention trial (T2) of object

placement, compared to control subjects. Greater exploration ratios for DHEA treated subjects during the recognition trial may be due to increased exploration times during the sample trial, and thus better consolidation leading to enhanced spatial memory. Unlike spatial memory, DHEA did not enhance nonspatial memory.

The literature focuses on acute effects of DHEA. DHEA and/or DHEAS, the sulfate derivative of DHEA, administered pre- and post-training, enhanced performance on the Morris water maze, Y-maze, T-maze, and active and passive avoidance tasks in male mice, and on the Morris water maze and Y-maze in OVX female rats (Shi et al, 2000; Urani et al, 1998; Flood et al, 1992; Reddy and Kulkarni, 1998; Frye and Lacey, 1999). Therefore, to our best knowledge, these are the first experiments to show subchronic enhancements of DHEA on spatial memory and not nonspatial memory in female rats.

TP enhanced nonspatial memory, but not spatial memory. The lower dose of TP (500 μg) enhanced object recognition, while neither the lower nor the higher dose of TP (1 mg) enhanced object placement. Because there were no significant differences between SO and TP treated subjects for exploration times during the sample trial, these data indicate that any significant differences between the groups in terms of discrimination during the recognition trial does not result from differences in total object exploration, which may enhance consolidation. In aged male rats chronically administered testosterone (60 days) enhanced spatial memory on the Morris water maze, and T-maze acquisition in castrated male rats (Bimonte-Nelson et al, 2003; Kritzer et al, 2001). Studies have found that acute doses of testosterone administered to male rats enhanced performance on the Morris water maze, passive avoidance tasks, and

conditioned place preference (Naghdi et al, 2003; Vazquez-Pereyra et al, 1995; Alexander et al, 1994; Packard et al, 1997). In female OVX rats, post-training injections of testosterone enhanced performance on the Y-maze and object recognition task, and prevented kainic acid decrements on the Morris water maze (Frye and Lacey, 2001; Frye and Reed, 1998). Thus, our results provide novel findings that few days of treatment with TP enhances nonspatial memory.

Similar to DHEA, DHT enhanced spatial memory, but not nonspatial memory. The higher dose of DHT (1 mg) but not the lower dose (500 μg) enhanced spatial memory, while neither the lower nor the higher dose of DHT enhanced nonspatial memory. Differences in exploration ratios during the recognition trial are not due to exploration times in the sample trial, as they were similar for both the SO and DHT treated groups. Previous findings have shown that chronic or acute DHT or 3α-diol, DHT's metabolite administered to castrated male rats enhanced cognition on the inhibitory avoidance task (Edinger and Frye, 2004; 2007). In female rats, post-training injections of DHT or 3α-diol enhanced performance on the Y-maze and object recognition task in female OVX rats (Frye and Lacey, 2001). This is the first time the present data show that subchronic DHT enhanced spatial memory on the object placement task.

Similar to both DHEA and DHT, AD (1 mg) enhanced spatial memory, but not nonspatial memory. There are no studies exploring few to many days treatment effects of AD on memory. Thus, these are novel findings on the role of AD on spatial memory of female rats.

Studies have shown that the hippocampus is involved in spatial memory, as lesions to the hippocampus and/or fornix impaired spatial memory (Morris et al, 1982; Kesner et al, 1993; Ennaceur and Aggleton, 1994; Ennaceur et al, 1997; Mumby et al, 2002). Lesions to the medial prefrontal cortex impaired recognition memory (Bachevallier and Mishkin, 1986). Object recognition is less dependent on the hippocampus and requires prefrontal cortical input (Broadbent et al, 2004; Ennaceur and Aggleton, 1994). Lesions of at least 80% of the hippocampus are necessary to impair object recognition while smaller lesions of the hippocampus (30%) impair spatial memory tasks.

Furthermore, androgen receptors in the rat brain are located in the CA1 and CA3 regions of the hippocampus, cerebral cortex, amygdala, and lateral septum, all of which have been implicated in memory (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992). Estrogen receptors, particularly ER-β are also located in CA1 and CA3 of the hippocampus, and the cerebral cortex (Shughrue et al, 1997). The enzymes P450 aromatase and 3β-hydroxysteroid dehydrogenase which catalyze the conversion of testosterone to estradiol, and DHEA to AD, respectively are significantly localized in CA1, CA3, and the dentate gyrus of the hippocampus (Kimoto et al, 2001; Furukawa et al, 1998). Furthermore, 5α-reductase, the enzyme that converts testosterone into DHT, is located in the frontal cortex (Lephart et al, 2001). Therefore, DHEA, TP, DHT, and AD may be binding on to intracellular androgen receptors in the hippocampus and cortex to mediate memory enhancements. Furthermore, since enzymes responsible for conversion of androgens are present in the hippocampus and frontal cortex, androgens may be converted into other androgens and bind on to androgen receptors, or converted to

estrogen and bind on to estrogen receptors in these brain areas known to mediate enhancements in memory.

***Subchronic treatment effects of androgens: elevated plus maze***

The same hormones and treatments that enhanced memory were used to assess anxiety on the elevated plus maze. Although DHEA had a greater percentage of open arm entries compared to SO treated rats, this finding was not significant. SO and DHEA treated subjects also spent a similar percentage of time in the open arms also. Similar to DHEA, subchronic treatment with TP, DHT, and AD did not affect anxiety levels on the elevated plus maze (see summary in Table 2). Subjects were administered subchronic doses of the androgens, tested on a 4 or 2 hr inter-trial delay of object recognition or object placement, respectively, followed by two hours rest, and then tested on the elevated plus maze. Because animals were tested on a memory task several hours before elevated plus maze testing, and the long duration between hormone administration and testing, may be factors involved in the androgens having no effects on anxiety.

Previous studies have shown that castrated male rats replaced with chronic (silastics) or acute (1hr before testing) testosterone, DHT, or 3α-diol had decreased anxiety on the elevated plus maze with a greater percentage of time spent in the open arms compared to control animals (Edinger and Frye, 2004; 2005; 2006; Frye and Seliga, 2001; Frye and Edinger 2004) . In male rats and mice, DHEA also decreased anxiety on the elevated plus maze (Fedotova and Sapronov, 2004; Melchior and Ritzmann, 1994). Other anxiety behaviors such as more exploratory behaviors on the open field and less defensive freezing in response to shock were also reduced by these androgens (Edinger and Frye, 2004; 2005; Frye and Edinger, 2004) . OVX female rats administered DHEA or

DHEAS and tested on the elevated plus maze, 1 and 24 hrs post injection, were unaffected (Frye and Lacey, 1999), while the same treatment paradigm of testosterone, DHT, and 3α-diol replacement decreased anxiety levels on the elevated plus maze (Frye and Lacey, 2001). Thus, the current study used a longer duration between androgens administered and time of testing on the elevated plus maze, as opposed to previous studies that have used a shorter duration between hormone administration and testing, or chronic administration of hormones in silastics. These factors may account for the apparent discrepancy between the current study compared to those in the literature. Because the androgens, DHEA, TP, DHT, and AD did not affect anxiety on the elevated plus maze, enhanced effects on object recognition and object placement were not due to decreased anxiety, but effects on mnemonic processes.

***Physiological measures***

***Subchronic treatment effects of androgens: uterine weights***

Since proliferative effects of estradiol may be responsible for untoward effects of breast and uterine cancer in postmenopausal women taking estrogen replacement therapy (Persson, 2000), uterine weights were measured to assess whether such effects exist in androgen treated subjects. DHEA, TP, and EB treated subjects had significantly greater uterine weights than SO, DHT, and AD treated subjects (see summary in Table 2). Uterine weights for EB treated subjects were at least twice the weights of all other groups. SO, DHT, and AD treated subjects had uterine weights that were very similar to each other and not significantly different from each other. Since ER-α receptors are more abundantly expressed in the uterus, EB may have activated these receptors to cause proliferative effects (Shughrue et al, 1998; Hewitt and Korach, 2003). DHEA and TP

may also be aromatized to estrogen to bind on to ER-α receptors in the uterus and thus having significantly heavier weights than the other two androgens, DHT and AD. Androgen receptors are also present in the uterus (Pelletier et al, 2000), suggesting that binding of the androgens occurs at the androgen receptors in the uterus.

***Subchronic treatment effects of androgens: androgen serum levels***

To assess the physiological impact of treatments, androgen levels were measured in subjects subchronically treated. TP but not DHEA significantly increased testosterone levels in the blood serum 48 hrs later (see summary in Table 2). This suggests that DHEA was not converted into testosterone levels that were detectable in the blood. The physiological level of circulating testosterone is approximately 4000 pg/ml in male rats (Bowman et al, 2006). Thus, these female rats have approximately a quarter of the circulating testosterone compared to male rats, and may account for enhanced nonspatial memory.

Subchronic treatment with DHEA and TP significantly increased DHEAS serum levels. Subchronic treatment with AD significantly elevated AD levels compared to SO and DHT treatments. This finding suggests that AD was not converted to DHT to the point of being undetectable in blood serum. Together, these increased hormone levels of DHEAS and AD may be responsible for enhanced spatial memory (see summary in Table 2).

***Acute treatment effects of androgens: object placement and object recognition***

The current data show that not only few days of treatment with androgens enhanced memory but also acute, immediate post sample trial injections of androgens, adapted from McGaugh (1989) and Packard et al (1994), enhanced spatial and nonspatial

memory. Similar to subchronic treatments, the same doses of DHEA and AD, and TP enhanced spatial and nonspatial memory, respectively (see summary Table 6). Unlike few days of treatment, acute treatment with DHT did not enhance spatial memory.

DHEA administered immediately post sample trial enhanced spatial memory, 4 hours later on the recognition trial, compared to control treated subjects. A delayed post sample trial injection paradigm, whereby DHEA was administered two hours after the sample trial, did not enhance object placement. As expected, exploration times in the sample trial were similar for both groups, because subjects were not injected as yet. Packard and Teather (1997a; 1997b) have shown that a single post-training injection of estradiol, followed by testing 24 hrs later, was effective at enhancing spatial memory on the Morris water maze, whereas the same treatment delayed 2 hours post-training had no effect when subjects were tested 24 hrs later on the retention trial (Packard and Teather, 1997). Similarly, our lab has shown that estradiol or diethylstilbestrol given 30 minutes before the sample trial or immediately post sample trial enhanced both nonspatial and spatial memory, but not when hormones were administered 2 hours post sample trial (Luine et al, 2003). Frye et al (2007) found that administration of estradiol immediately after training, but not 1.5 hrs or 1 hr later enhanced performance on a 4 hr inter-trial delay of object placement and object recognition, respectively (Frye et al, 2007; Walf et al, 2006). Administration of hormones immediately post sample trial or delayed post sample trial highlights the temporal effectiveness of treatment on memory. Therefore, the androgens, DHEA, TP and AD are effective in memory consolidation during the period post T1 and before delayed post T1, 1, 1.5 and2 hrs later. Furthermore, these androgens are effective at activating the mnemonic processes necessary for encoding, consolidation,

storage and retrieval of information that occur during and between the recognition trial (Packard et al, 1994). This delayed post T1 treatment paradigm rules out the possibility of hormonal influences on nonmnemonic factors such as sensory, motoric, motivational, or affective processes which may have an effect on memory performance (Packard et al, 1994; Luine et al, 2003).

Similar to our data, acute studies in rodents have demonstrated that DHEA enhances memory. Immediate post-training injections of a single dose of DHEA (20 mg/kg), followed by testing six days later, reversed scopolamine impairment in male mice on the Morris water maze (Shi et al, 2000) and improved performance on an active avoidance training task (Flood and Roberts, 1988; Flood et al, 1992). DHEA or DHEAS administered to male mice 30 min before testing prevented scopolamine-induced impairment on the Y-maze, and Morris water maze (Urani et al, 1998) amyloid-induced amnesia on the Y-maze (Maurice et al, 1998), and enhanced memory performance on the T-maze (Maurice et al, 1998), while DHEAS given to male mice 60 min before training enhanced performance on a passive avoidance task (Reddy and Kulkarni, 1998). In female OVX rats, immediate post-training DHEA (3 mg/kg), followed by testing on the Morris water maze 24 hrs later, enhanced spatial memory, while the same treatment paradigm using DHEAS (3.20 or 6.40 mg/kg) enhanced performance on the Y-maze task (Frye and Lacey, 1999). In female OVX rats, DHEAS given 30 min prior to testing, enhanced spatial memory on the Morris water maze and performance on the Y-maze (Frye and Sturgis, 1995). Similar to our findings, object recognition was unaffected by DHEA and also DHEAS (Frye and Lacey, 1999). Interestingly, our treatment paradigm used a lower dose of DHEA (1mg) to enhance spatial memory.

Similar to few days treatment with TP, acute TP enhanced nonspatial memory. Studies have found that acute doses of testosterone administered directly into the amygdala of male rats 30 min before testing enhanced performance on the Morris water maze (Naghdi et al, 2003). Male rats injected with testosterone peripherally, 45 min before testing, enhanced performance on a passive avoidance task (Vazquez-Pereyra et al, 1995), while male mice receiving post-training testosterone enhanced performance on an active avoidance task (Flood et al, 1992). Subcutaneous injections of testosterone or injections directly into the nucleus accumbens 30 min before testing, enhanced conditioned place preference in male rats (Alexander et al, 1994; Packard et al, 1997). In female OVX rats, post-training injections of testosterone enhanced performance on the Y-maze, 1 hr and object recognition task, 24 hrs later (Frye and Lacey, 2001).

Surprisingly, acute DHT did not enhance object placement, as opposed to few days treatment with this hormone. Previously, castrated male rats that received DHT or its metabolite 3α-diol via post-training injections directly into the hippocampus, enhanced cognitive learning on the inhibitory avoidance task (Edinger and Frye, 2007). In female rats, post-training injections of 3.0 mg/ kg and 7.5 mg/kg of DHT or 3α-diol enhanced performance on the Y-maze and passive avoidance task, 1 hour later and on the object recognition task, 24 hrs later (Frye and Lacey, 2001). However, chronic administration of DHT did not improve spatial memory on the Morris water maze (Bimonte-Nelson, 2003) Based on the literature, possibly a higher dose of acute DHT may enhance object placement memory in female OVX rats.

Acute treatment with AD enhanced spatial memory. Only one study in male mice has found that post-training AD enhanced performance on an active avoidance task

(Flood et al, 1992). Thus, the current finding provides novel information that acute AD enhances spatial memory.

Since the acute paradigm, involves injections immediately after the sample trial, acute enhancements of the androgens, DHEA, AD and TP on object placement and object recognition memory may be mediated via the nongenomic pathway which is rapid in onset and short in duration (Vasudevan and Pfaff, 2007). Androgens may bind on to extracellular or G-protein couple receptors that may activate the MAPK cascade with phosphorylation and activation of MAP kinase and ERK, or the activation of adenylyl cyclase, cAMP, PKA or PKC that could act in parallel or by converging onto the MAP kinase pathway (Kelly and Levin, 2001; Heinlein and Chang, 2002b). Treatment with acute estradiol or androgens have shown to activate the MAP kinase pathway via ERK phosphorylation in the hippocampus of female OVX rats, hippocampal neurons and cancer cell lines (Nguyen et al, 2005; Migliaccio et al, 2000). Thus, acute treatment with DHEA, TP, and AD may be mediating its rapid enhancements via these nongenomic pathways.

***Aim 2. To determine whether androgens act as androgens or via conversion to estrogen to enhance memory.***

P450 aromatase is the rate limiting enzyme that catalyzes the conversion of testosterone to estrogen (Beck and Handa, 2004). Aromatase inhibitors such as anastrozole and letrozole, bind on to the active site of the enzyme aromatase, and suppress estrogen production (Mokbel, 2002). Thus, these aromatase inhibitors are used as a protective agent in the treatment of estrogen-dependent advanced breast cancer (Brodie et al, 2003; Mouridsen et al, 2001).

Letrozole has greater potency than other aromatase inhibitors, and it can also be used to inhibit the conversion of androgens to estrogen in order to test whether effects of aromatizable androgens on cognitive function shown here, are due to action as androgens or estrogen. Exogenous gonadal steroids, both androgens and estrogen have been positively associated with cognitive performance in both human and nonhumans (Duff and Hampson, 2000; Daniel et al, 1997; Hirshman et al, 2003; 2004; Frye and Lacey, 1999). This response has been linked with the effects of these steroids on synaptic and spine plasticity, especially in the hippocampus, a brain region vital for the processing of mnemonic information (Harris and Stevens, 1989; Broadbent et al, 2004; Ennacuer et al, 1997; Gould et al, 1990; Wallace et al, 2006). For example, subchronic treatment with the androgens, DHEA or TP increase spine synapse density in CA1 of the hippocampus in OVX female rats, while letrozole when given in conjunction with these androgens, blocks this increase, supporting the idea that estrogen is the active hormone (Hajszan et al, 2004; Leranth et al, 2004). In Aim 1, suchronic treatment with DHEA or TP enhanced spatial (Fig. 6B) and nonspatial memory (Fig. 7D), respectively. Letrozole will be

administered subchronically (2 days) in conjunction with DHEA or TP to OVX female rats to determine whether spatial and nonspatial memory will be affected. Based on these previous morphological and behavioral results, it is hypothesized that androgens will act via conversion to estrogen and letrozole will block the enhancement of spatial and nonspatial memory using object placement and object recognition tasks, respectively.

## Methods

### *Letrozole and DHEA: object recognition*

Subjects were subchronically treated with carboxymethylcellulose (CMC) (0.20 ml; n = 8) or letrozole ((Novartis AG, Basel, Switzerland; 1 mg dissolved in 2.5% CMC; n = 10) for two days. Subjects were then tested on a 2 hr inter-trial delay of object placement, 48 hrs later. This experiment was done to verify that letrozole only does not have any enhancements in spatial memory. See specific details for cohorts used in Table 1 and methods for object placement and statistical analysis in Aim 1.

In the second experiment, subjects were subchroincally treated with SO (0.20 ml; n = 8) or letrozole (1 mg dissolved in 2.5% CMC) and DHEA (1mg; n = 8) and tested on a 2 hr inter-trial delay of object placement. Letrozole injections were administered 1 hour before DHEA to allow for complete aromatase blockade before administration of the androgen (Hajszan et al, 2004).

### *Letrozole and testosterone propionate: object placement*

Subjects were subchronically treated with CMC (0.20 ml; n = 6) or letrozole (1 mg dissolved in 2.5% CMC; n = 6) for two days. Subjects were then tested on a 4 hr inter-trial of object recognition, 48 hrs later. This experiment was done to verify that letrozole does not have any enhancements in nonspatial memory. See specific details for

cohorts used in Table 1 and methods for object recognition and statistical analysis in Aim 1.

In the second experiment, subjects were subchronically treated with SO (0.20 ml; n = 6) or letrozole (1 mg dissolved in 2.5% CMC) and TP (500 μg; n =6) and tested on a 4 hr inter-trial delay of object recognition. Letrozole injections were administered 1 hour before TP to allow for complete aromatase blockade before administration of the androgen (Leranth et al, 2004).

## Results

### *Effects of letrozole and DHEA on object placement*

Both SO and letrozole treated groups had similar exploration times in T1 (Fig. 22A) and exploration ratios in T2 of object placement (Fig. 22B). This indicates that similar to our control animals, letrozole only does not enhance spatial memory.

Subjects that were subchronically treated with SO or letrozole and DHEA had similar exploration ratios in T1 (Fig. 22C). Letrozole and DHEA treated subjects spent a significantly greater proportion of time with the new location compared to SO treated subjects ($p < 0.01$) (Fig. 22D), an effect that is greater than DHEA only treated subjects (Fig. 6B). Therefore, letrozole does not block DHEA enhanced spatial memory. Based on the above experiment, the effect of letrozole and DHEA enhancing spatial memory is due to DHEA and not letrozole. (See summary in Table 7 for effects of letrozole and DHEA on memory tasks).

***Effects of letrozole and testosterone propionate on object recognition***

Both SO and letrozole treated groups had similar exploration times in T1 (Fig. 23A) and exploration ratios in T2 of object recognition (Fig. 23B). This indicates that similar to our control subjects, letrozole only does not enhance nonspatial memory.

Subjects that were subchroincally treated with SO or letrozole and TP had similar exploration times (Fig. 23C). Letrozole and TP treated subjects spent a significantly greater proportion of time with the new object compared to SO treated subjects ($p < 0.05$) (Fig. 23D). Therefore, letrozole does not block TP enhanced nonspatial memory. Based on the above experiment, the effect of letrozole and TP enhancing nonspatial memory is due to TP and not letrozole. (See summary in Table 7 for effects of letrozole and TP on memory tasks).

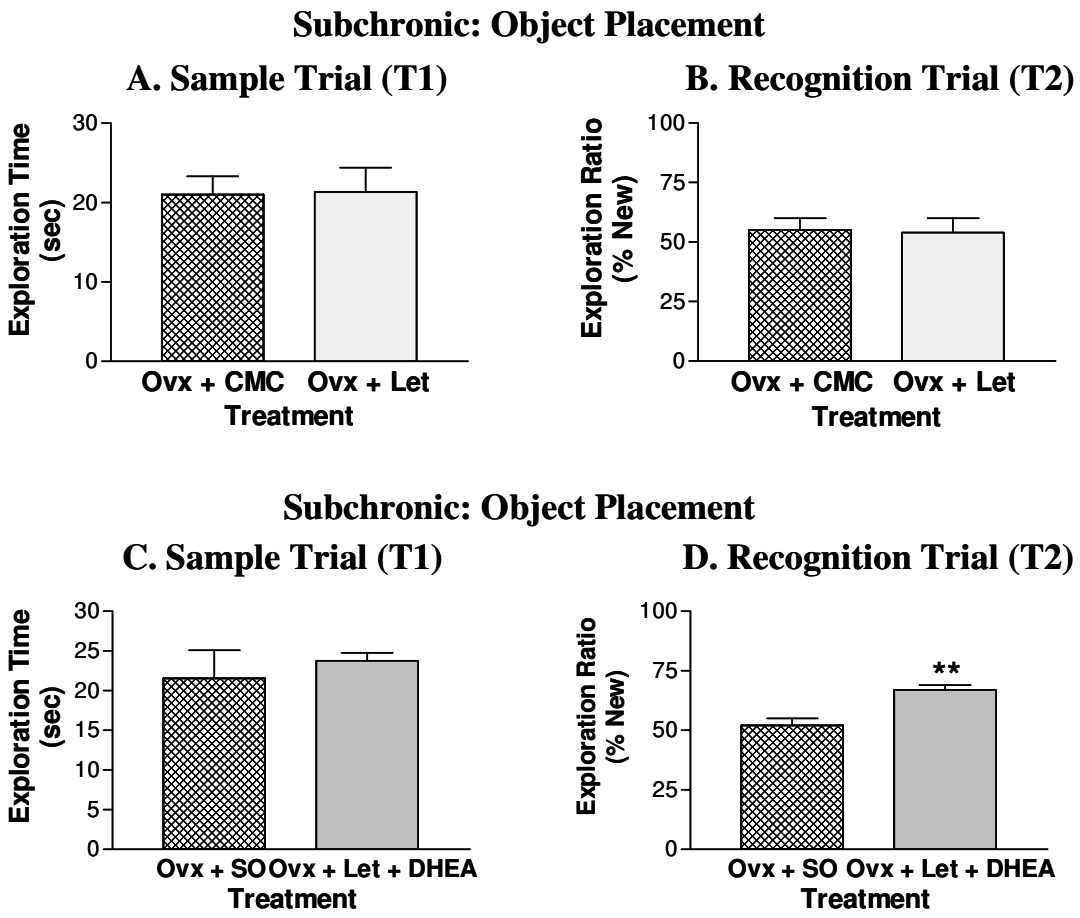



**Figure 22. Effect of subchronic letrozole alone, and letrozole and DHEA on object placement.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 2 hr inter-trial delay of object placement is shown for CMC (n = 8) and letrozole (n = 10; 1 mg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups. (C) Exploration time ± SEM with objects in the sample trial (T1) of a 2 hr inter-trial delay of object placement is shown for SO (n = 8), and letrozole (1 mg) and DHEA (n = 8; 1 mg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (D) Exploration ratio ± SEM (time with new location/time with old location + time with new location) in the recognition trial (T2) of object placement is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (** $p < 0.01$).

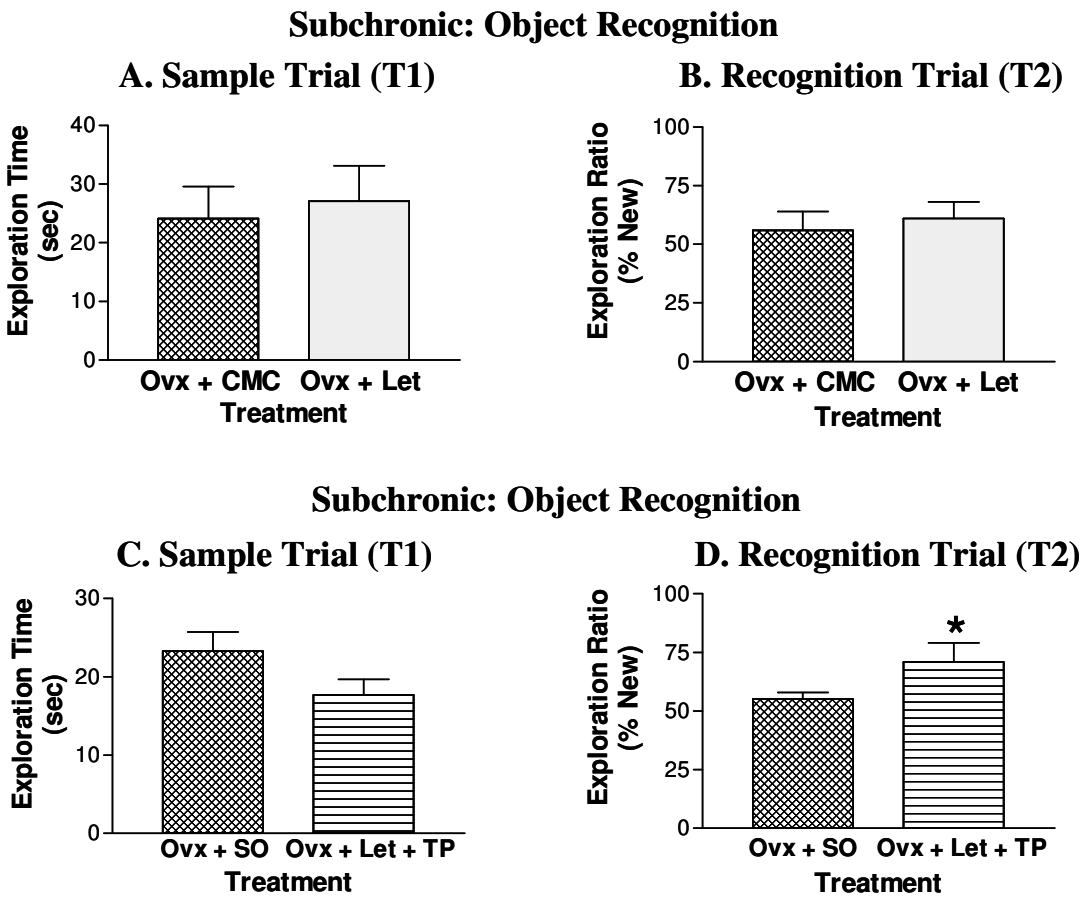



**Figure 23. Effect of subchronic letrozole alone, and letrozole and TP on object recognition.** (A) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for CMC (n = 6) and letrozole (n = 6; 1 mg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (B) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed that there were no significant differences between groups. (C) Exploration time ± SEM with objects in the sample trial (T1) of a 4 hr inter-trial delay of object recognition is shown for SO (n = 6), and letrozole (1 mg) and TP (n = 6; 500 μg) treated subjects. A two-sample t-test showed that there were no significant differences between groups. (D) Exploration ratio ± SEM (time with new object/time with old object + time with new object) in the recognition trial (T2) of object recognition is shown. A two-sample Mann-Whitney U test showed significant differences in exploration ratio times, where (* $p < 0.05$).

**Table 7. Summary of letrozole and androgen treatment effects on behavioral measures.**

| Treatment | Dose | OP | OR |
|---|---|---|---|
| DHEA (aim 1) | 1 mg | ✓ | ----- |
| Letrozole | 1 mg | ----- | ----- |
| Letrozole + DHEA | 1 mg + 1 mg | ✓ | NT |
| TP (aim 1) | 500 µg | ----- | ✓ |
| Letrozole + TP | 1 mg + 500 µg | NT | ✓ |

(OP: object placement; OR: object recognition; EPM: elevated plus maze; NT: not tested; ✓: enhancement; -----: no difference).

## Discussion

Subchronic letrozole, administered with DHEA did not block DHEA's enhancing effects on spatial memory in female OVX rats (see summary in Table 7). These memory enhancing effects were not due to differences in exploration time in the sample trial, as both groups had similar times. It was confirmed that letrozole alone did not enhance spatial memory in OVX rats. Administration of letrozole along with TP did not block TP's enhancing effects on nonspatial memory. Furthermore, it was confirmed that letrozole alone did not enhance nonspatial memory. The finding that, letrozole, the competitive inhibitor that binds to the active site of the enzyme, P450 aromatase, and inhibits the conversion of androgens to estrogen, particularly for our purposes, DHEA and TP to estrogen, was unable to block these androgens' enhancing effects on memory was surprising. The current experiments used the same treatment doses and paradigm as Hajszan et al, 2004 and Leranth et al, 2004, whereby letrozole was administered with DHEA or TP, for 2 days, followed by sacrifice and golgi analysis 48 hrs later. Unlike our behavioral findings, letrozole administered along with DHEA and TP, but not DHT blocked the induction of CA1 spine synapse density in female OVX rats, an effect that is present when DHEA or TP was administered alone (Hajszan et al, 2004; Leranth et al, 2004). These experiments are the first to show that androgens act as androgens and not via conversion to estrogen to enhance spatial and nonspatial memory.

The question that has been debated for a long time is whether steroid gonadal hormones express their effects via estrogenic or androgenic actions. Aromatization is an essential step in sexual behavior of male rats. Treatment with the aromatase inhibitors, 1, 4, 6-androstatrien-3,17-dione (ATD) and aminogluthethimide (AG) suppressed

ejaculation, and reduced the number of mounts and intromissions, but concurrent administration with EB prevented this inhibitory effect (Larsson and Beyer, 1977). Morphologically, two days of administration of EB to female OVX rats increased spine density and spine synapse density in CA1 of the hippocampus, while the same treatment in castrated male rats had no effect (Gould et al, 1990; Leranth et al, 2003). Thus, morphologically, testosterone action in the female is highly dependent on local estrogen synthesis in the brain, but not so for males. In hippocampal slice cultures, 17β-estradiol administered along with letrozole significantly decreased spine synapse density and number of synaptic boutons, and downregulated spinophilin, a marker of dendritic spines, and synaptophysin, a protein of presyanptic vesicles (Kretz et al, 2004). Furthermore, the release of estradiol is dose-dependently down-regulated after administration of letrozole to the medium, from 120 pg/5 ml for controls compared to 40 pg/5 ml for letrozole ($10^{-7}$ M) treated hippocampal neurons(Kretz et al, 2004). Administration of pregnenolone and DHEA protected hippocampal neurons against excitatory kainic acid neurotoxicity while addition of the aromatase inhibitor, fadrozole, blocked these neruoprotective effects (Veiga et al, 2003). All of these studies support estrogenic actions as being functionally important, as opposed to androgenic actions. In contrast, letrozole did not block DHEA induction of CA1 spine synapses in male castrated rats (MacLusky et al, 2004). Our findings indicate that similar to male rats, aromatization of the androgens, DHEA and TP to estrogen does not appear to play a major role in the enhancement of spatial and nonspatial memory.

More recent studies support the androgenic mechanism hypothesis. In male rats, testosterone enanthate injected into the CA1 of the hippocampus impaired acquisition of

spatial learning memory on the Morris water maze, while addition of anastrozole, an aromatase inhibitor improved spatial learning memory (Moradpour et al, 2006). In another study, intact male rats administered letrozole committed less errors than control animals on the T-maze (Alejandre-Gomez et al, 2007). Similar to this, our study revealed that when letrozole was administered together with the androgens, DHEA or TP, spatial and nonspatial memory was enhanced, respectively. Human fetal tissue neurons treated with 17β-estradiol, testosterone, or the nonaromatizable androgen, milbolerone protected against serum deprivation-mediated apoptosis (Hammond et al, 2001). The aromatase inhibitor, ATD does not prevent testosterone-mediated neuroprotection while the anti-androgen, flutamide eliminated this effect (Hammond et al, 2001). Therefore, this neuroprotective effect of testosterone may be mediated through androgen receptors. Administration of letrozole to intact female rats reduced latency and increased probe trial performance which was used to assess memory consolidation on the Morris water maze (Aydin et al, 2008). Letrozole reduced norepinephrine and dopamine in the hippocampus, while it increased norepinehprine, dopamine, and DOPAC in the prefrontal cortex by in a dose-dependent manner (Aydin et al, 2008). Thus, inhibition of estrogen synthesis in the brain may have beneficial effects on spatial memory consolidation and increased catecholamines in the prefrontal cortex. In another study, letrozole down-regulated estradiol release, glutamate acid decarboxylase (GAD) expression, the enzyme that catalyzes GABA synthesis, and GABA synthesis, and the number of GAD positive cells in hippocamal cell cultures (Zhou et al, 2007). Inhibition of GAD expression and GABA synthesis can lead to disinhibition of CA1 neurons which can result in increased excitation of neurons that can promote structural and electrical changes in the

hippocampus (Murphy et al, 1998). Enhanced memory after letrozole administration in intact rats, support our findings of enhanced memory with letrozole and androgen treatment in OVX female rats, while increased catecholamines in the prefrontal cortex, a brain area known to be involved in memory, and decreased GABA synthesis may be mechanisms by which androgens are enhancing memory in our current findings.

In humans, androgenic actions have been associated with enhanced cognition. Postmenopausal women receiving testosterone replacement therapy had significant improvements in immediate and delayed visual and verbal memory, all of which were unaffected by letrozole, compared to those receiving no letrozole treatment (Shah et al, 2006). In older men treated with testosterone, and the aromatase inhibitor, anastrozole, increased testosterone, decreased estrogen levels, and enhanced spatial memory (Cherrier et al, 2005). This study demonstrated improvement in spatial memory occurs in the absence of increases in estradiol. In postmenopausal women, circulating plasma steroid concentrations of testosterone (0.60 nmol/L), DHEA (15 nmol/L) and DHEAS (2500 nmol/L) are greater than estrone (0.10 nmol/L) and estradiol (0.04 nmol/L) levels (Simpson, 2002). Consequently, these androgens form a reservoir of precursor, which is available for conversion to testosterone and thus estrogens in numerous peripheral tissue sites. These circulating levels of estrogens in postmenopausal women are not drivers of estrogen action, they are reactive rather than proactive (Simpson, 2002).

In the same study where letrozole administered with DHEA, blocked the increase of CA1 spine synapse density in ovariectomized female rats, no increases in uterine weight was found, compared to EB treated subjects (Hajszan et al, 2004). DHEA alone did not increase uterine weight, which suggests that the conversion of DHEA to estrogen

occurred within the brain itself and not in the uterus (Hajszan et al, 2004). Intact female rats treated with letrozole, had decreased uterine weights in a dose-dependent manner (Aydin et al, 2008). Therefore, peripherally letrozole does not have proliferative effects in the uterus.

A possible mechanism by which these androgens are exerting their androgenic effects may be by DHEA and TP binding on to androgen receptors in the brain, particularly in CA1 and CA3 of the hippocampus and cortex, areas known to be involved in spatial and nonspatial memory (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992; Broadbent et al, 2004; Ennaceur and Aggleton, 1994, Ennaceur et al, 1997). P450 aromatase is also distributed in pyramidal neurons of CA1 and CA3, in granule cells of the dentate gyrus of the hippocampus, and in the frontal cortex (Kimonto et al, 2001; Furukawa et al, 1998; Lephart et al, 2001). Other areas of the brain that may be involved in memory are subcortical regions that project via the fimbria/fornix to and from the hippocampus, and include the medial septum/diagonal band of Broca, the median raphe, and the supramammillary area (Leranth et al, 2000). When the fimbria/fornix was transected unilaterally in OVX rats replaced with estradiol, hippocampal spine synapse density increased on the side where the fimbria/fornix pathway was intact (Leranth et al, 2000). However, in castrated males, in the hippocampi ipsilateral to the fimbria/fornix lesion, testosterone replacement is still capable of finding a significant increase in CA1 spine synapse density (Kovacs et al, 2003). Thus, the effects of testosterone on CA1 synaptogenesis in the male may include components of both local androgen action and distal effects on neurons projecting to the hippocampus via the fimbria/fornix.

Local aromatization in the rat brain takes place, as radioactive testosterone when infused into OVX/adrenalectomized female rats, unchanged testosterone and its metabolites DHT and estradiol were recovered with highest levels of estradiol found in the amygdala, preoptic area and septum (Lieberburg and McEwen, 1977). DHEA has been shown to upregulate androgen receptor levels in the medial preoptic area, lateral septum, and bed nucleus of the stria terminalis (Lu et al, 2003; Bairamov and Sapronov, 2004). Therefore, DHEA is capable of interacting with and regulating androgen receptor activity.

In summary, DHEA and TP enhance spatial and nonspatial memory via androgenic mechanisms. These studies are the first to show enhancement of these memory tasks with subchronic treatment of androgens, not via aromatization to estrogen. A few studies using letrozole support our findings, and the proposed mechanism of action may be through binding of androgens to androgen receptors present in the hippocampus, prefrontal cortex, and subcortical areas.

***Aim 3. Effects of androgens on dendritic morphology in brain areas mediating cognition.***

It is now known that steroid hormones not only play a critical role in physiology and behavior during development, but also in adulthood. The neural mechanisms underlying these hormonal effects on brain function have been the subject of numerous studies. One such approach involves investigating influences of steroid hormones on spine and synaptic plasticity, a concept that has been introduced over a century ago as a mechanism of learning and memory (Harris and Stevens, 1989; Praduz et al, 2006).

Behaviorally, both estrogen and androgens have positive effects on cognitive functions in both human and nonhuman females (Duff and Hampson, 2000; Hirshman et al, 2003; Daniel et al, 1997; Frye and Lacey, 1999; 2001). The hippocampus and more recently, the prefrontal cortex, are brain areas known to be involved in memory (Kesner et al, 1993; Mumby et al, 2002; Ennaceur et al, 1997; Kritzer et al). Morphologically, the hippocampus retains the potential for considerable plasticity in response to changes in circulating levels of gonadal steroids. This was first recognized as a result of studies underlying cyclical alterations in hippocampal function that occurred during the female reproductive cycle. In female rats, dendritic spine density in CA1 pyramidal neurons of the hippocampus, rapidly changed during the estrous cycle, with a peak immediately following the proestrus estrogen surge (Woolley et al, 1990). Exogenous subchronic estradiol (2 days) administered to female OVX rats to mimic the estrous cycle, also increased spine density of CA1 (Gould et al, 1990). More recently, acute treatment with estradiol (Maclusky et al, 2005) and subchronic treatment with the androgens, DHEA

(Hajszan et al, 2004), TP and DHT (Leranth et al, 2004) have also shown to increase CA1 hippocampal spine synapse density in OVX female rats.

Subchronic doses of DHEA and TP used in Hajszan et al, 2004 and Leranth et al, 2004 enhanced spatial memory (Fig. 6B) and nonspatial memory (Fig. 7D), respectively, in Aim 1. In the current study we are adapting the same doses and treatment paradigm and using golgi analysis to determine whether subchronic treatment with the androgens, DHEA and TP affect dendritic morphology of brain areas known to be involved in memory. It is hypothesized that subchronic treatment with DHEA and TP will increase spine density in both the prefrontal cortex and CA1 of the hippocampus, and these increases is one mechanism which underlies enhancements in memory.

## Methods

### *Golgi impregnation and analysis*

Ovariectomized rats were subchronically treated with vehicle or hormones. Forty-eight hours later, animals were overdosed with carbon dioxide, followed by rapid decapitation. Brains were removed and blocks were made of the prefrontal cortex and the hippocampus. The staining procedure was carried out using FD Rapid GolgiStain™ kit (FD NeuroTechnologies Consulting and Services, Inc.). This technique has been previously used in our lab (Luine et al, 2006; Wallace et al, 2006; 2007). Brains were first rinsed in 0.1 M phosphate buffer (pH 7.4) and then immersed in Golgi solution, a combination of potassium dichromate and mercuric chloride, for 14 days in the dark at room temperature. This solution was changed once after 12 hours of immersion. Brains were then transferred into a sucrose-based solution and stored for 2-7 days at 4°C in the dark. This solution was changed once after 12 hours of immersion. Brains were removed

and sectioned at 100 μm on a cryostat at -22°C. To prevent brain tissue from ice crystal damage and preserve the cell morphology while in the cryostat, brain tissue was flash frozen before sectioning using Super Friendly Freeze'it™ (Fischer Scientific). Sections were mounted on gelatin coated slides, a drop of sucrose solution was placed on each of the sections and excess solution was absorbed with filter paper. Slides were allowed to air dry at room temperature. After sections were sufficiently dry, they were rinsed in distilled water and immersed in a silver nitrate solution for 10 minutes. Slides were dehydrated in 70% (5 minutes), 95% (two times, 5 minutes each), 100% (2 times, 5 minutes each) ethanol, respectively. Sections were then cleared in Protocol® Safeclear™ (3 times, 3 minutes each) and coverslipped using Permount® (Fisher Scientific).

Dendritic spine density of pyramidal neurons in layer II/III of the medial prefrontal cortex and CA1 of the hippocampus was analyzed using the Spot Advanced program, version 3.5 for Windows (©Diagnostic Instruments, Inc., 1997-2002) and a Nikon Eclipse E400 microscope. Neurons were located and spines were counted according to the general methods of Wellman (2001) for the prefrontal cortex and Gould et al (1990) for the hippocampus. Dendrites counted had to meet several criteria. First, the cell body had to be located within the area of interest. Second, the length of the dendritic branch had to be unbroken. Third, the length of the dendritic branch had to be isolated well enough for an unobstructed view. These criteria were applied to ensure that the same population of dendrites and spines within and between animals were being analyzed. Spines on 6 tertiary apical dendrites and 6 secondary basal dendrites in the medial prefrontal cortex and CA1 meeting these criteria were counted under oil immersion (100x), for each animal by two investigators and averaged (see Figure 24).

The length of the dendrite was measured using the Spot Advanced software. The number of spines was divided by the length of the spine (μm) to calculate spine density. Spine density values were multiplied by 10 and expressed as spines/10 μm. The spine density for the six counts was averaged for each subject.

One-way ANOVAs were used to test for differences among groups in spine density (group x spine density). *Post hoc* Fisher's LSD tests were used to test for differences between groups if significant F values were found ($p < 0.05$).

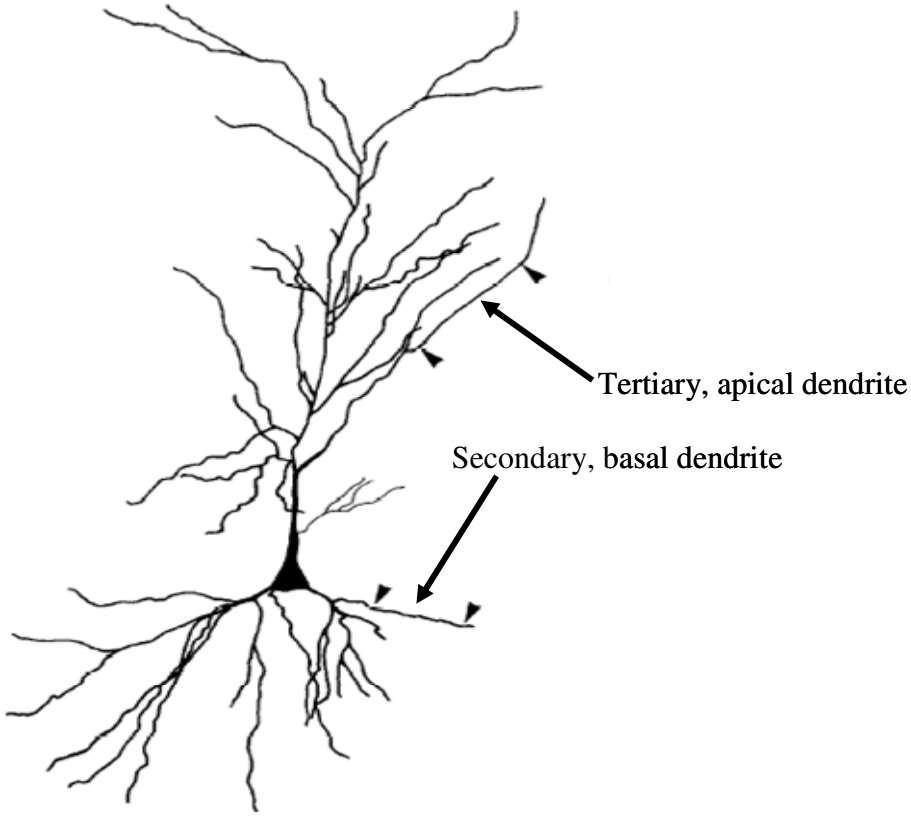



**Figure 24. Diagram of a CA1 pyramidal neuron.** The regions located between the arrow heads represent tertiary, apical dendrite and secondary basal dendrite segments that were used in analysis. (Adapted from Gould et al, 1990).

***DHEA and TP: prefrontal cortex and CA1 of the hippocampus***

OVX rats were subchronically treated with SO (0.15 ml; n = 6), DHEA (1 mg; n = 6) or TP (500 μg; n = 6). See Table 1 for subjects used. Forty-eight hours later, subjects were sacrificed, brains were removed and processed as above. See above for details on methods. The spine counts in CA1 of the hippocampus for a control, SO treated subject was removed because the slide was not stained sufficiently and the values were very low, thus leaving an n = 5.

## Results

***Effects of DHEA and TP: prefrontal cortex***

A one-way ANOVA showed that there was a significant treatment effect ($F(2,15) = 7.83$, $p < 0.01$) in the prefrontal cortex for tertiary apical dendrites of pyramidal neurons (Fig. 25A). Fisher's LSD *post hoc* test revealed that spine density of apical dendrites was significantly greater for both DHEA (19%) and TP (24%) treated subjects compared to control, SO treated subjects. See photomicrographs of golgi impregnated tertiary apical dendrites from pyramidal neurons of layers II/III of the prefrontal cortex and notice increases in spine density for both DHEA and TP treated groups compared to control (Fig. 25A). Similarly, a one-way ANOVA showed that there was a significant treatment effect ($F(2,15) = 9.96$, $p < 0.01$) in the prefrontal cortex for secondary basal dendrites of pyramidal neurons (Fig. 25B). *Post hoc* with Fisher's LSD revealed that spine density for basal dendrites was significantly greater for both DHEA (32%) and TP (37%) treated subjects compared to control, SO treated subjects. (See summary in Table 8 for percentage increases in dendritic spine density of the prefrontal cortex for androgen treated groups).

***Effects of DHEA and TP: CA1 of the hippocampus***

There were no significant differences between groups for the spine density of tertiary apical dendrites of pyramidal neurons in CA1 of the hippocampus (Fig. 26A). However, there was a trend with the means being higher for the treated groups, DHEA and TP. On the other hand, a one-way ANOVA revealed that there was a significant treatment effect ($F(2,14) = 8.14$, $p < 0.01$) in the CA1 for secondary basal dendrites of pyramidal neurons (Fig. 26B). *Post hoc* with Fisher's LSD revealed that spine density for basal dendrites was significantly greater by the same amount for both DHEA (28%) and TP (28%) treated subjects compared to control, SO treated subjects. (See summary in Table 8 for increases in dendritic spine density of CA1 for androgen treated groups).

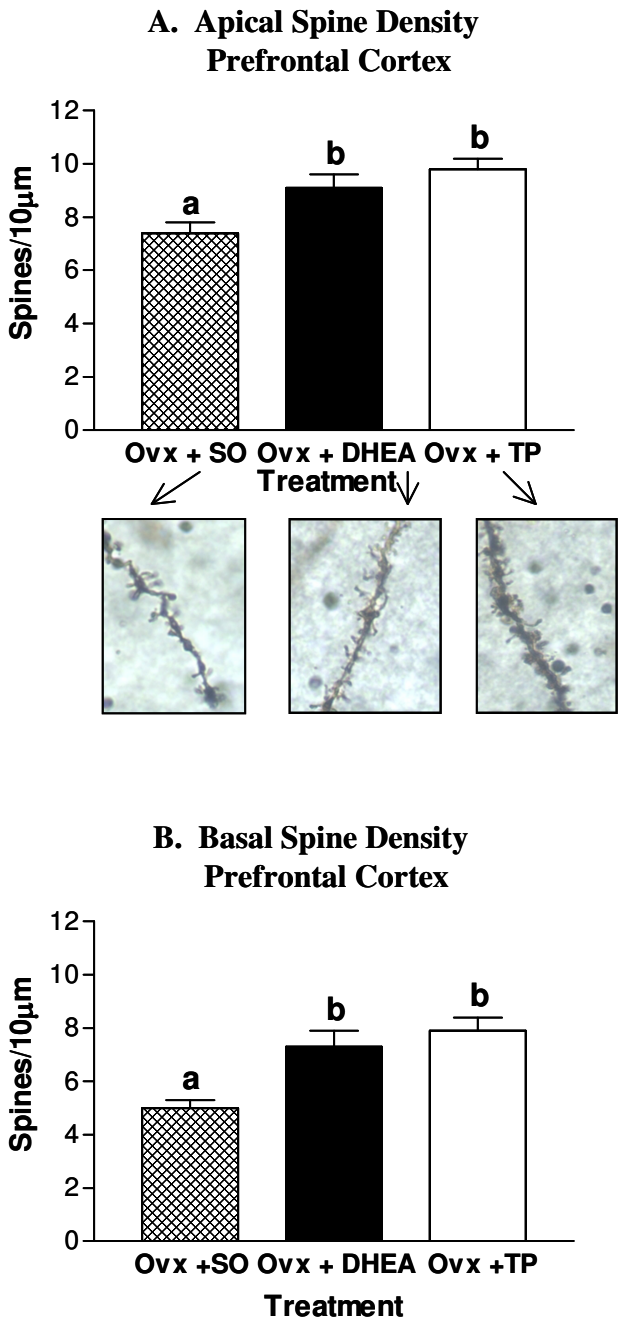



**Figure 25. Effect of subchronic DHEA and TP on spine density in the prefrontal cortex.** (A) All data was analyzed using one-way ANOVAs and *post hoc* with Fisher's LSD. Dendritic spine densities (spines/10 µm) ± SEM are shown for SO (n = 6), DHEA (n = 6; 1 mg) and TP (n = 6; 500 µg). Apical dendritic spine density of the prefrontal cortex: ($F(2,15) = 7.83$, $p < 0.01$). Photomicrographs (100x) of tertiary apical dendrites from pyramidal neurons (layer II/III) of the prefrontal cortex are shown. (B) Dendritic spine densities (spines/10 µm) ± SEM are shown. Basal dendritic spine density of the prefrontal cortex: ($F(2,15) = 9.96$, $p < 0.01$). Groups labeled with different letters are significantly different from each other.

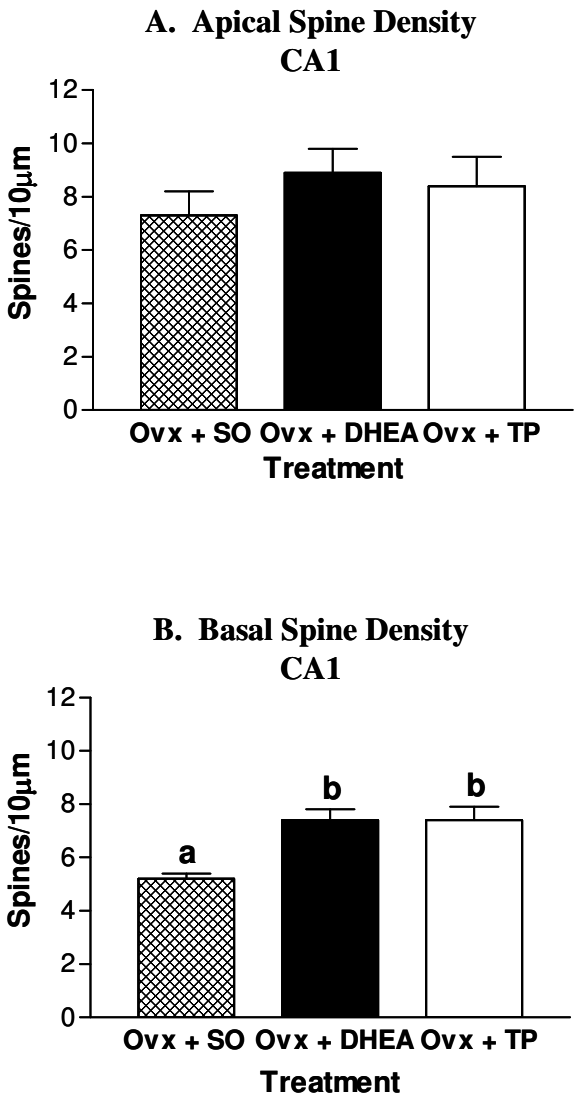



**Figure 26. Effect of subchronic DHEA and TP on spine density in CA1 of the hippocampus.** (A) All data was analyzed using one-way ANOVAs and *post hoc* with Fisher's LSD. Dendritic spine densities (spines/10 μm) ± SEM are shown for SO (n = 5), DHEA (n = 6; 1 mg) and TP (n = 6; 500 μg). Apical dendritic spine density of CA1: there were no significant differences between groups. (B) Dendritic spine densities (spines/10 μm) ± SEM are shown. Basal dendritic spine density of CA1: ($F(2,14) = 8.14$, $p < 0.01$). Groups labeled with different letters are significantly different from each other.

**Table 8. Summary of androgen treatment effects on dendritic spine density of the prefrontal cortex and CA1 of the hippocampus.**

| Treatment | Dose | PFC Apical | PFC Basal | CA1 Apical | CA1 Basal |
|---|---|---|---|---|---|
| SO | 0.10 – 0.20 ml | ----- | ----- | ----- | ------ |
| DHEA | 1 mg | ↑ 19% | ↑ 32% | ----- | ↑ 28% |
| TP | 500 μg | ↑ 24% | ↑ 37% | ----- | ↑ 28% |

Percentage increases for androgen treated groups are compared to control, SO treated group. (PFC: prefrontal cortex).

## Discussion

Spine densities of apical tertiary dendrites and basal secondary dendrites of pyramidal neurons in the prefrontal cortex were significantly increased with subchronic treatment of DHEA and TP compared to control subjects. Spine density for both apical and basal dendrites increased slightly more for TP (24% and 37%) than DHEA treated subjects (19% and 32%), compared to control subjects (see Table 8). Surprisingly, the spine density of tertiary apical dendrites of pyramidal neurons in CA1 of the hippocampus was not significantly different for the treated groups, DHEA and TP compared to controls, although there was a trend for spine density to be slightly higher in the treated groups. In contrast, subchronic treatment with both androgens, DHEA (18%) and TP (18%) increased spine density of secondary basal dendrites by the same amount in CA1 of the hippocampus, compared to controls (see Table 8).

Studies have documented changes in hippocampal and prefrontal cortex morphology when rodents are treated with steroid hormones. The current technique for golgi impregnation and counting has previously been used in our lab (Luine et al, 2006; Wallace et al, 2006). Luine et al (2006) found increased apical spine density in both CA1 and the prefrontal cortex of OVX female rats treated with phytoestrogens, compared to controls. One of the first landmark studies showed that ovariectomy resulted in decreased apical and basal dendritic spine density in CA1 pyramidal neurons, while subchronic treatment with EB, and a combination of EB and progesterone resulted in increased dendritic spine density of apical and basal branches, respectively (Gould et al, 1990). Wallace et al (2006) subsequently confirmed the effect of ovariectomy on spines and also showed that these subjects had poorer object recognition and object placement

performance compared to intact females. Although these researchers found changes mainly in apical spine density of CA1, all of these previous studies used estrogen replacement, while in the current study androgen replacement was used. Thus it appears that estrogen may act on apical dendrites while androgens act on basal dendrites. Gould et al (1990) concluded that there were more dramatic changes in response to estrogen in the density of spines on apical than on basal dendrites. The current data suggest that basal dendrites are more sensitive to and respond slightly more to androgens, compared to apical dendrites in both the prefrontal cortex and CA1 of the hippocampus.

In both male and female rats, gonadectomy reduced, while hormone replacements with the androgens DHEA, TP, and DHT increased CA1 apical spine synapse density (Leranth et al, 2003; MacLusky et al, 2004; Hajszan et al, 2004; Leranth et al, 2004). The same doses of DHEA (1 mg) and TP (500 μg) and subchronic treatment paradigm used in these experiments were also used in the current study. Acute doses of EB have also been shown to enhance CA1 apical spine synapse density, 30 minutes and 4.5 hours after injection (MacLusky et al, 2005). These studies all investigated steroid hormone influences on spine synapse density. The current study is the first to investigate androgens' influences on spine density in OVX female rats and presents novel information that both DHEA and TP increase spine density.

Some other studies have examined the role of steroid hormones on spine plasticity in the prefrontal cortex. A recent study in our lab has shown that apical but not basal dendritic spine density of the prefrontal cortex declined in aged rats, when estrogen levels are lower, compared to young rats (Wallace et al, 2007). In OVX female rhesus monkeys, estradiol increased spinophilin-immunoreactive spine number in the prefrontal cortex

(Tang et al, 2004), while in aged female monkeys, estradiol increased apical and basal dendritic spine density in the prefrontal cortex (Hao et al, 2006). In male rats, castration reduced, while administration of DHT or EB increased spine synapse density in the medial prefrontal cortex (Hajszan et al, 2007). Gonadally intact *Tfm* male rats and castrated *Tfm* rats replaced with DHT had considerably reduced spine synapse density compared to intact wild-type males (Hajszan et al, 2007). Since the *Tfm* mutation results in the synthesis of defective androgen receptors, this study indicated that androgen receptors may mediate a large part of the synaptogenic action of androgens in the prefrontal cortex of male rats. Thus far, there are no studies that have examined the influences of androgens on spine density in the prefrontal cortex of female rats. The current results show that both androgens, DHEA and TP increase spine density in the prefrontal cortex. Based on the finding that androgen receptors may mediate synaptic plasticity of androgens in the prefrontal cortex (Hajszan et al, 2007), and androgen receptors are found in both the prefrontal cortex and CA1 of the hippocampus (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992; Kritzer et al, 2004), DHEA and TP's increases in dendritic spine density may be mediated by androgen receptors.

The hippocampal area, CA1 projects directly to the medial prefrontal cortex. In rats, injections of retrograde tracers centered in the infralimbic cortex but extending into the cingulate gyrus 1 (Cg1) and Cg3 areas label CA1 pyramidal cells (Swanson, 1981). Similarly, Hoover and Vertes (2007) found that one of the main sources of afferent projections to the prelimbic and infralimbic cortex of the prefrontal cortex is from the hippocampus. Paired-pulse stimulation of the hippocampus produced short-term facilitation of the monosynaptic EPSP in pyramidal cells of the prefrontal cortex

(Degenetais et al, 2003). Behaviorally, object placement is dependent on an intact hippocampus and/or fornix (Ennaceur et al, 1997), and may also rely on prefrontal cortical input (Ennaceur and Aggleton, 1994). Object recognition is less dependent on the hippocampus and requires prefrontal cortical input (Broadbent et al, 2004; Ennaceur et al, 1997). Other areas of the brain that may be involved in memory are subcortical regions which project via the fimbria/fornix to the hippocampus, and include the medial septum/diagonal band of Broca, the median raphe, and the supramammillary area (Leranth et al, 2000). When the fimbria/fornix was transected unilaterally in OVX rats replaced with estradiol, hippocampal spine synapse density increased only on the side where the fimbria/fornix pathway was intact (Leranth et al, 2000). In castrated males, in the hippocampi ipsilateral to the fimbria/fornix lesion, testosterone replacement is still capable of a significant increase in CA1 spine synapse density (Kovacs et al, 2003). Thus, DHEA and TP's increases in spine density of the prefrontal cortex and hippocampus may be working cooperatively in these brain areas to enhance spatial and nonspatial memory.

Several studies have linked morphological changes with cognitive function. In male rats, that were trained on the Morris water maze, using an invisible platform that required spatial memory, there were associated changes in synaptic spatial distribution (Rusakov et al, 1997). Thus, there was an associated increase in frequency of shorter distances, that is clustering between synaptic active zones in CA1 compared to control rats that had a visible platform and required no spatial memory (Rusakov et al, 1997). Also, male rats trained using the eyeblink trace conditioning paradigm, an associative task that requires the hippocampus for acquisition, had an associated increase in CA1

pyramidal spine density (Leuner et al, 2003). In female rats, ovariectomy impaired nonspatial and spatial memory on the object recognition and object placement tasks, and also decreased spine density in the prefrontal cortex and CA1, compared to intact rats (Wallace et al, 2006). Female aged rats had significantly lower spine density in the prefrontal cortex and decreased performance on the object recognition task, compared to young rats (Wallace et al, 2007). In our lab, spatial memory on the object placement task was enhanced with acute doses of both 17α- and 17β-estradiol in OVX female rats (Luine et al, 2003). Similarly, a separate study showed that both isomers of this hormone increased CA1 spine synapse density (MacLusky et al, 2005). We found that subchronic treatment with DHEA and TP enhanced spatial (Fig. 6B) and nonspatial memory (Fig 7D), respectively. These same doses of DHEA (1 mg) and TP (500 μg) that enhanced spatial and nonspatial memory increased spine density in both the prefrontal cortex and CA1 of the hippocampus. Thus increases in spine density may underlie enhancements in memory.

Dendritic spines represent a means by which new contacts between cells can be established and existing contacts strengthened, suggesting the formation of new memories (Sorra and Harris, 2000). Studies have shown that treatment with estradiol in OVX rats increased NMDA receptor binding levels in CA1 (Weiland et al, 1992) and increased NMDA mediated excitatory neurotransmission (Wong and Moss, 1992). Furthermore, estradiol induced NMDA receptor-mediated synaptic input is correlated well with estradiol increased dendritic spine density (Woolley et al, 1997). The androgen, DHT has also been found to increase NMDA receptor binding in CA1 of male rats (Romeo et al, 2005). Presynaptic boutons afferent to CA1 dendritic spines can be divided

into two types, single synapse boutons (SSBs), which are synaptically connected to one dendritic spine and multiple synapse boutons (MSBs), which are synpatically connected to more than one dendritic spine (Woolley et al, 1996). Ovariectomized rats treated with estrogen had increased relative frequency of MSBs compared to SSBs, and also the average number of synapses each MSB forms, compared to controls (Woolley et al, 1996). A follow-up study found that the majority of multiple synapse boutons in OVX rats treated with estradiol form synapses with more than one postsynaptic cell (Yankova et al, 2001). Thus, in addition to increasing the density of excitatory synaptic input to individual CA1 pyramidal cells, estradiol also increases the divergence of input from individual presynaptic boutons to multiple postsynaptic CA1 pyramidal cells (Yankova et al, 2001). This finding suggests the formation of new synaptic connections between previously unconnected hippocampal neurons. Therefore, increased spine density in CA1 of the hippocampus by DHEA and TP may lead to increased synaptic connections that may in turn lead to increased excitatory neurotransmission, which is often an important step in memory formation, and thus enhancements in spatial and nonspatial memory.

Long-term potentiation (LTP) is a phenomenon in which brief repetitive stimulation of synapses results in a long-lasting increase in synaptic strength (Bliss and Lomo, 1973). LTP serves as a model for cellular changes that may be involved in learning and memory. Hippocampal slices treated with 17β-estradiol exhibited a significant enhancement of LTP compared to control slices, via a MAP kinase dependent pathway (Kim et al, 2002). 17β-estradiol has also been shown to potentiate EPSPs in CA1 hippocampal slices (Kim et al, 2006). Exposure to estradiol increased spine density in hippocampal neurons, an effect that was mimicked by an activator of protein kinase C,

PMA, and blocked by the NMDA antagonist, APV (Murphy and Segal, 1996). Estradiol also caused a rise in calcium reactivity with application of glutamate (Murphy and Segal, 1996). A follow-up study found that estradiol not only increased spine density in CA1 but also caused a large increase in phosphorylated CREB and in CREB binding protein (Murphy and Segal, 1997). Estradiol increased both apical and basal spine density of CA1 pyramidal neurons, while this effect was blocked by Erk MAP kinase inhibitor and an estrogen inhibitor (Mruakami et al, 2006). These changes may be mediating DHEA and TP's effects on spine density.

Androgens have also been shown to alter hippocampal excitability. Testosterone significantly increased EPSPs in CA1 hippocampal slices in both male and female gonadectomized rats (Smith et al, 2002). DHEAS facilitated CA1 hippocampal LTP via an amplification of Src-dependent NMDA receptor signaling (Chen et al, 2006b). In the CA1 of freely behaving rats, it is more difficult to evoke an apical than a basal LTP, and thus the threshold for evoking the apical LTP was higher than the basal LTP (Leung and Shen, 1995). Differential sensitivity to LTPs may be a reason why DHEA and TP increased basal spine density but not apical spine density in CA1.

Taken together, since the prefrontal cortex and hippocampus are brain regions involved in memory, and CA1 of the hippocampus sends projections to the prefrontal cortex, increases in spine density in pyramidal neurons of these brain areas by treatment with DHEA and TP may be mediating enhanced memory performance in object placement and object recognition, respectively. Increased spine density with the androgens may lead to increased excitatory neurotransmission by forming new synaptic

contacts or LTP via genomic mechanisms by binding on to androgen receptors or via the nongenomic, MAP kinase pathway, and may mediate enhanced memory.

***Aim 4. Effects of androgens on monoaminergic activity in brain areas mediating cognition.***

Another possible mechanism mediating learning and memory enhancements is the regulation of synthesis and release of neurotransmitters in the brain. Studies have shown that estradiol modulates monoaminergic activity, with changes in norepinephrine (NE) in both the frontal cortex and vertical diagonal band (VDB), and changes in serotonin (5-HT), and dopamine (DA) in the frontal cortex (Luine et al, 1998) of OVX female rats. These alterations in monoamines may contribute in estradiol's enhancing effects on the radial arm maze (Luine et al, 1998). There are few studies examining the role of androgens in monoaminergic activity in female rats. In female rats, estradiol and DHEA replacement altered serotonin receptors in the cingulate cortex, striatum and lateral septum (Sumner and Fink, 1995; Cyr et al, 2000). Gonadectomy in male rats and replacement with EB and DHT has been shown to alter NE, DA, and serotonin (5-HT) activity in the medial prefrontal cortex (Handa et al, 1997).

In Aim 1, it was found that subchronic treatment with the androgens, DHEA (Fig. 6B), DHT (Fig. 10B) and AD (Fig. 11B) enhanced spatial memory, while TP (Fig. 7D) enhanced nonspatial memory OVX female rats. In Aim 3, subchronic treatment with the androgens, DHEA and TP increased spine density in both the prefrontal cortex and CA1 of the hippocampus (Fig. 25A, 25B 26B). A previous study in our lab has shown that subhcronic treatment with EB (50 μg/kg) enhanced both spatial and nonspatial memory (Jacome et al, in prep.). These results suggest that enhancements in memory with subchronic androgens may be mediated by increases in dendritic spine density in the prefrontal cortex and CA1.

The identity of neurotransmitters present in spines regulated by androgens is unknown. Moreover, few studies have examined whether androgens affect monoaminergic activity in female rats. Our previous findings show memory enhancements (Aim 1; Jacome et al (in prep)) and increases in spine density (Aim 3) with androgens. In the current study, we are adapting the same doses and treatment paradigm to determine whether subchronic treatment with the androgens, DHEA, TP, DHT, and AD, and estrogen affect monoaminergic activity in brain areas important for memory function. It is hypothesized that subchronic treatment with the androgens and estrogen will alter monoaminergic neurotransmitters and metabolites in the prefrontal cortex, CA1, CA3 and dentate gyrus of the hippocampus, striatum, and VDB, and these changes may contribute to memory enhancements.

## Methods

### *Neurochemical analyses*

Subjects were subchronically treated with vehicle or hormones, and 48 hours later were overdosed with carbon dioxide and sacrificed via rapid decapitation. Brains were removed and rapidly frozen and stored at –70˚C. Brains were then sliced into thick sections using a razor blade. Using a 500 μm-diameter cannula, tissue samples from various brain regions were obtained from the frozen sections under a dissecting microscope with the stage maintained at approximately –11 ˚C and placed in 1.5 ml Eppendorf tubes. Between two to eight punches were taken, depending upon the area and neurotransmitter being measured. The brain areas punched bilaterally and the number of punches obtained from each area were, prefrontal cortex (6-8 punches), CA1 (4 punches),

CA3 (4 punches), and dentate gyrus of the hippocampus (4 punches), vertical diagonal band (3 punches), and striatum (2 punches).

Monoamine and metabolite neurotransmitter levels were measured by dissolving the punches in 60µl of sodium acetate buffer, pH 5.0, followed by freezing and thawing to disrupt cellular structures and release cellular components, including the neurotransmitter of interest. α-A-Methyl-dopamine was added as an internal standard and samples were centrifuged at 12000 rpm for 12 minutes. The supernatant was removed and the pellet was re-suspended in 100 or 200 µl of 2.0N NaOH, depending on the amount of punches, for protein analysis using Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA).

High-performance liquid chromatography (HPLC) with electrochemical analysis was used to quantify neurotransmitter levels. The 40 µl supernatant was used in the detection of monoamines, including dopamine and its metabolites, 3,4-dihydroxyphenylalanine (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG); and serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA). Monoamines were measured in a Waters Associates chromatographic system (Waters 2690) consisting of an alliance module containing an automated refrigerated, injector pump, Symmetry $C_{18}$ 5µm 4.6 X 150 mm reverse-phase column (Novapak 3 micron), and an ESA Coulochem III detector (0.45V potential). The mobile phase, described elsewhere (Alves et al, 2002.) contained 3% acetonitrile and peak sharpness was increased by the addition of 100% methanol (99.5% mobile phase: 0.5% methanol).

Millennium software (Waters Associates) was used to run the chromatography system and concentrations of transmitters and metabolites were calculated by reference to standards using peak area integration. Monoamine levels were measured in the above mentioned brain areas.

One-way ANOVAs were used to test for differences among groups in level of monoamines, metabolites and turnover ratios (group x pg/μg protein). *Post hoc* Fisher's LSD tests were used to test for differences between groups if significant F values were found ($p < 0.05$).

***A. Treatment with DHEA, TP, and EB***

Ovariectomized rats were treated subchronically with SO (0.15 ml; n = 9), DHEA (1mg; n = 10), TP (500 μg; n = 9) or EB (50 μg/kg; n = 9). Forty-eight hours after the second injection, subjects were sacrificed, brains were sectioned and HPLC analysis was performed on different brain areas. See above methods for details and cohort used in Table 1.

## Results

Monoamine levels differed in several brain areas of subjects that were subchroincally treated with SO, DHEA, TP, or EB and sacrificed 48 hrs later. For the neurochemical analyses, two comparisons were made. All treated groups, DHEA, TP and EB were compared to the control, SO group, and the androgen treated groups, DHEA and TP were compared to the EB treated group.

***Prefrontal cortex***

In the prefrontal cortex, a one-way ANOVA ($F(3,30) = 3.70$, $p < 0.05$) with *post hoc* Fisher's LSD showed that norepinephrine (NE) levels significantly decreased in

DHEA compared to SO treated subjects (Fig. 27A). NE's metabolite, MHPG significantly decreased in EB compared to SO treated subjects and significantly increased in the androgen treated subjects, DHEA and TP compared to EB treated subjects ($F(3,30) = 11.48$, $p < 0.0001$). The turnover ratio, MHPG/NE significantly increased in DHEA compared to EB treated subjects ($F(3,30) = 4.09$, $p < 0.05$). EB treatment led to significantly elevated levels of serotonin (5-HT) compared to SO treated subjects, while androgen treatment with DHEA and TP led to significantly lowered levels of 5-HT compared to the EB treatment ($F(3,30) = 9.14$, $p < 0.001$) (Fig. 27B). No significant differences were observed for 5-HT's metabolite, 5-HIAA. The turnover ratio, 5-HIAA/5-HT significantly increased in DHEA compared to EB treated subjects ($F(3,30) = 3.14$, $p < 0.05$). There were no significant changes in dopamine (DA) and its metabolite, HVA nor for its metabolites and DA turnover ratios (Fig. 27C, 27D). Thus, DHEA generally increased, while EB increased monoamine activity in the prefrontal cortex.

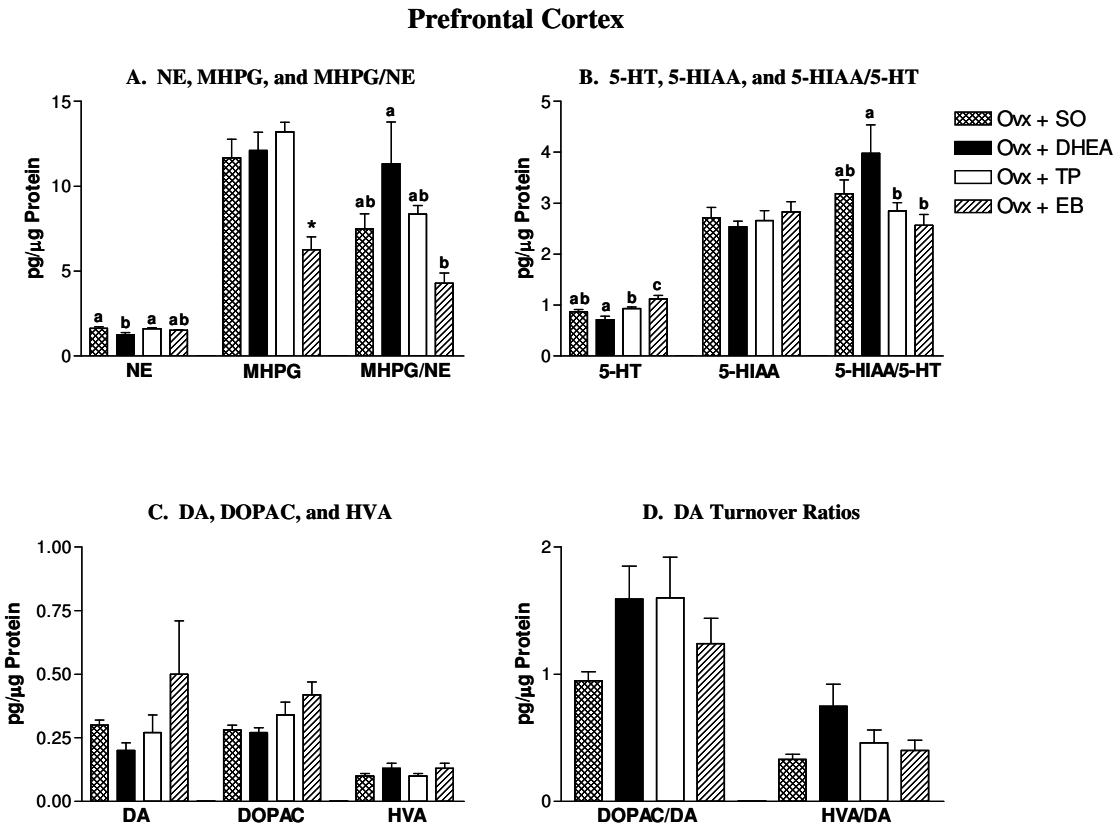



**Figure 27. Effect of subchronic DHEA, TP and EB on monoamine, metabolite and turnover levels in the prefrontal cortex.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein) and *post hoc* with Fisher's LSD. Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for NE activity. NE: $F(3,30) = 3.70$, $p < 0.05$. MHPG: $F(3,30) = 11.48$, $p < 0.0001$. MHPG/NE: $F(3,30) = 4.09$, $p < 0.05$. (B) Monoamine and metabolite levels are pg/µg protein ± SEM shown for 5-HT activity. 5-HT: $F(3,30) = 9.14$, $p < 0.001$. 5-HIAA/5-HT: $F(3,30) = 3.14$, $p < 0.05$). (C) Monoamine and metabolite levels are pg/µg protein ± SEM shown for DA activity. There were no significant differences for DA activity. (D) Turnover ratio levels are pg/µg protein ± SEM shown for DA and its metabolites. There were no significant differences for DA turnover ratio levels. Groups labeled with different letters are significantly different from each other. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

***CA1 of the hippocampus***

In CA1 of the hippocampus, TP treatment resulted in significantly lowered levels of NE, compared to SO and EB treatments ($F(3,33) = 4.74$), $p < 0.01$) (Fig 28A). MHPG ($F(3,33) = 12.47$, $p < 0.0001$) and MHPG/NE ($F(3,33) = 4.08$, $p < 0.05$) levels significantly increased in EB compared to SO treated subjects, and significantly decreased in the androgen treated subjects, DHEA and TP compared to EB treated subjects. There were no significant differences for 5-HT (Fig. 28B). DHEA and TP treatments led to decreased levels of 5-HIAA, compared to SO and EB treatments ($F(3,33) = 25.37$, $p < 0.00001$). Similarly, the turnover ratio, 5-HIAA/5-HT significantly decreased in the androgen treated groups, DHEA and TP compared to the EB treated group ($F(3,33) = 3.89$, $p < 0.05$). No significant changes were observed for DA, its metabolite, HVA (Fig. 28C), and its turnover ratio, HVA/DA (Fig. 28D). DOPAC was significantly lowered in TP compared to SO treated subjects, and in DHEA and TP compared to EB treated subjects ($F(3,33) = 6.38$, $p < 0.01$). EB treatment resulted in significantly increased turnover levels of DOPAC/DA, compared to SO treatment, while treatment with the androgens, DHEA and TP resulted in significantly decreased levels compared to EB treatment ($F(3,33) = 5.00$, $p < 0.01$) (Fig. 28D). Therefore, DHEA generally decreased, while EB increased monoaminergic activity in CA1 of the hippocampus.

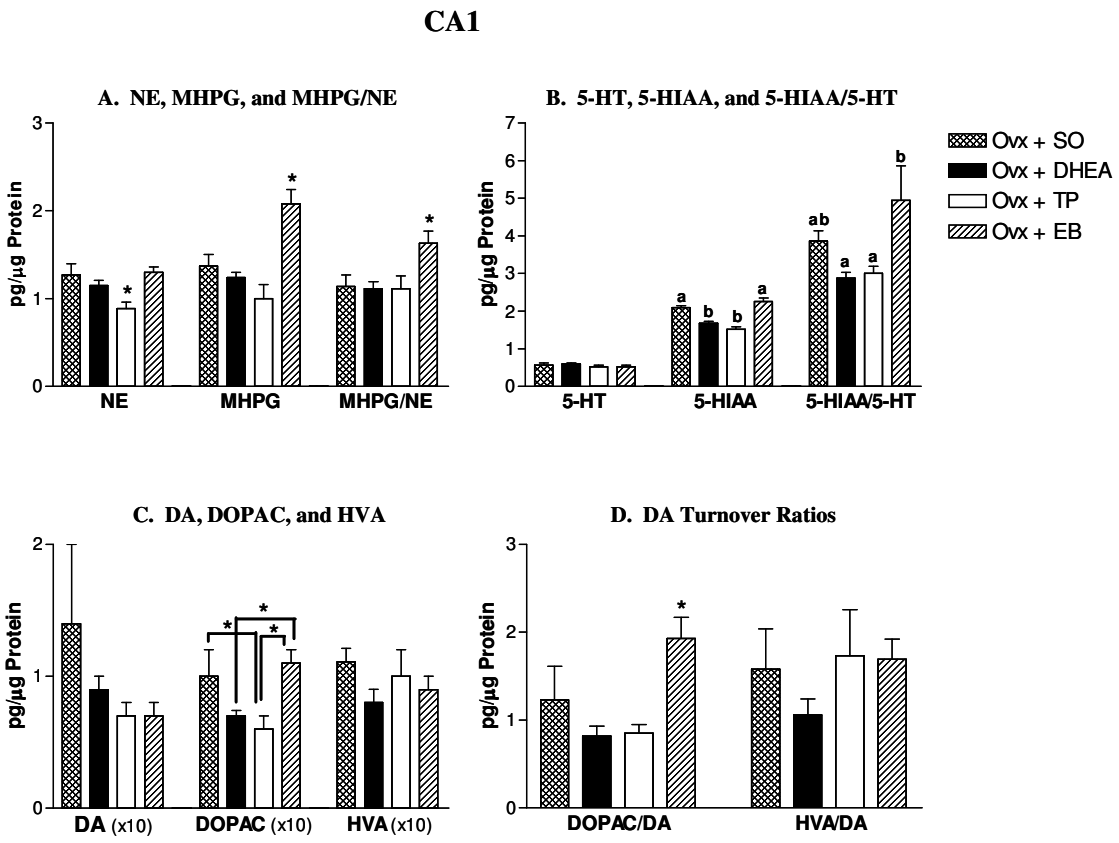



**Figure 28. Effect of subchronic DHEA, TP and EB on monoamine, metabolite and turnover levels in CA1 of the hippocampus.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein), and *post hoc* with Fisher's LSD. Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for NE activity. NE: $F(3,33) = 4.74$, $p < 0.01$. MHPG: $F(3,33) = 12.47$, $p < 0.0001$. MHPG/NE: $F(3,33) = 4.08$, $p < 0.05$. (B) Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for 5-HT activity. 5-HIAA: $F(3,33) = 25.37$, $p < 0.00001$. 5-HIAA/5-HT: $F(3,33) = 3.89$, $p < 0.05$. (C) Monoamine and metabolite levels are pg/µg protein ± SEM shown for DA activity. DOPAC: $F(3,33) = 6.38$, $p < 0.01$. (D) Turnover ratio levels are pg/µg protein ± SEM shown for DA and its metabolites. DOPAC/DA: $F(3,33) = 5.00$, $p < 0.01$. Groups labeled with different letters are significantly different from each other. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

***CA3 of the hippocampus***

All treatments, DHEA, TP and EB significantly lowered NE levels in CA3 of the hippocampus compared to SO treatment ($F(3,32) = 10.36$, $p < 0.0001$) (Fig. 29A). No changes were observed for MHPG. The turnover ratio, MHPG/NE significantly increased in TP compared to SO and EB treated subjects ($F(3,32) = 7.05$, $p < 0.001$). There were no significant differences for 5-HT (Fig. 29B). TP treatment led to significantly lowered levels of 5-HIAA compared to EB treatment ($F(3,32) = 3.51$), $p < 0.05$). The turnover ratio, 5-HIAA/5-HT significantly increased in DHEA and EB compared to SO treated subjects, and significantly decreased in TP compared to EB treated subjects ($F(3,32) = 5.98$, $p < 0.01$). DA ($F(3,32) = 34.70$, $p < 0.00001$) significantly increased in TP compared to SO and EB treated subjects, while HVA ($F(3,32) = 3.51$, $p < 0.05$) significantly increased in TP compared to EB treated subjects (Fig. 29C). Treatment with TP and EB resulted in significantly elevated levels of DOPAC compared to SO, while treatment with DHEA resulted in significantly lowered levels of DOPAC compared to EB ($F(3,32) = 5.38$, $p < 0.01$). The turnover ratio, DOPAC/DA significantly increased in EB compared to SO treatment and significantly decreased in the androgen treatments, DHEA and TP compared to EB treatment ($F(3,32) = 8.95$, $p < 0.001$) (Fig. 29D). Treatment with TP resulted in decreased turnover levels of HVA/DA, compared to SO and EB treatments ($F(3,32) = 5.38$, $p < 0.01$). Thus, TP generally decreased while EB increased monoaminergic activity in CA3 of the hippocampus.

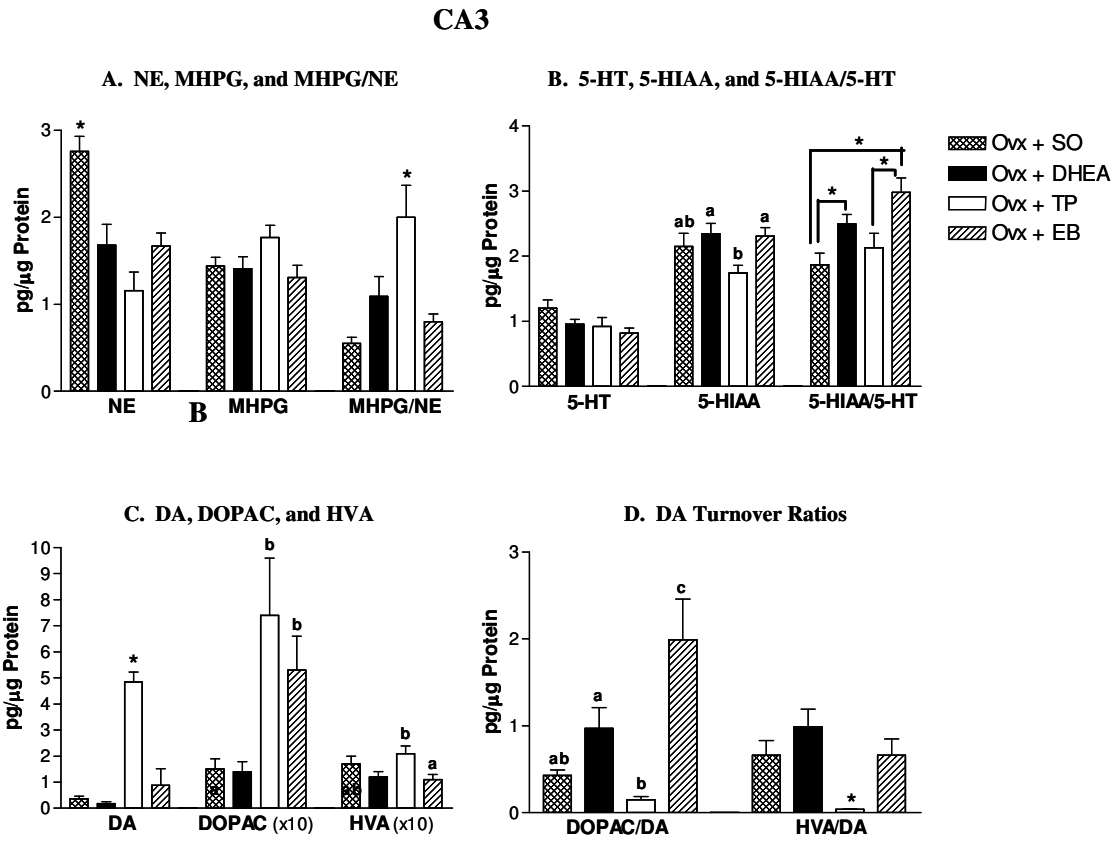



**Figure 29. Effect of subchronic DHEA, TP and EB on monoamine, metabolite and turnover levels in CA3 of the hippocampus.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein) and *post hoc* with Fisher's LSD. Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for NE activity. NE: $F(3,32) = 10.36$, $p < 0.0001$. MHPG/NE: $F(3,32) = 7.05$), $p < 0.001$. (B) Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for 5-HT activity. 5-HIAA: $F(3,32) = 3.51$, $p < 0.05$. 5-HIAA/5-HT: $F(3,32) = 5.98$, $p < 0.01$. (C) Monoamine and metabolite levels are pg/µg protein ± SEM shown for DA activity. DA: $F(3,32) = 34.70$, $p < 0.00001$. HVA: $F(3,32) = 3.51$, $p < 0.05$. DOPAC: $F(3,32) = 5.38$, $p < 0.01$. (D) Turnover ratio levels are pg/µg protein ± SEM shown for DA and its metabolites. DOPAC/DA: $F(3,32) = 8.95$, $p < 0.001$. HVA/DA: $F(3,32) = 5.38$, $p < 0.01$. Groups labeled with different letters are significantly different from each other. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

***Dentate gyrus of the hippocampus***

No changes were observed for NE in the dentate gyrus of the hippocampus (Fig. 30A). TP and EB treatments significantly decreased and increased, respectively, both MHPG ($F(3,29) = 46.81$, $p < 0.00001$) and MHPG/NE ($F(3,29) = 22.97$, $p < 0.00001$) levels, compared to SO treated subjects, and significantly decreased in DHEA and TP treatments compared EB treatment. TP treatment resulted in significantly increased 5-HT levels compared to SO and EB treatments ($F(3,29) = 3.80$, $p < 0.05$) (Fig. 30B). No significant differences were observed for 5-HIAA or the turnover ratio, 5-HIAA/5-HT. TP treatment also resulted in significantly elevated levels of DA compared to SO and EB treatments ($F(3,29) = 17.46$, $p < 0.00001$) (Fig. 30C). DOPAC levels significantly decreased in both TP and EB compared to SO treated subjects and significantly increased in DHEA compared to EB treated subjects ($F(3,28) = 4.81$, $p < 0.01$). DHEA and TP treatments significantly decreased levels of HVA compared to SO treated subjects ($F(3,28) = 3.11$, $p < 0.05$). The turnover ratio, DOPAC/DA significantly decreased in TP treated subjects compared to SO and EB treated subjects ($F(3,28) = 9.11$, $p < 0.001$) (Fig. 30D). HVA/DA levels significantly decreased in both androgen treated groups, DHEA and TP compared to EB ($F(3,28) = 4.80$, $p < 0.01$). Therefore, DHEA and TP generally decreased, while EB increased monoaminergic activity in the dentate gyrus.

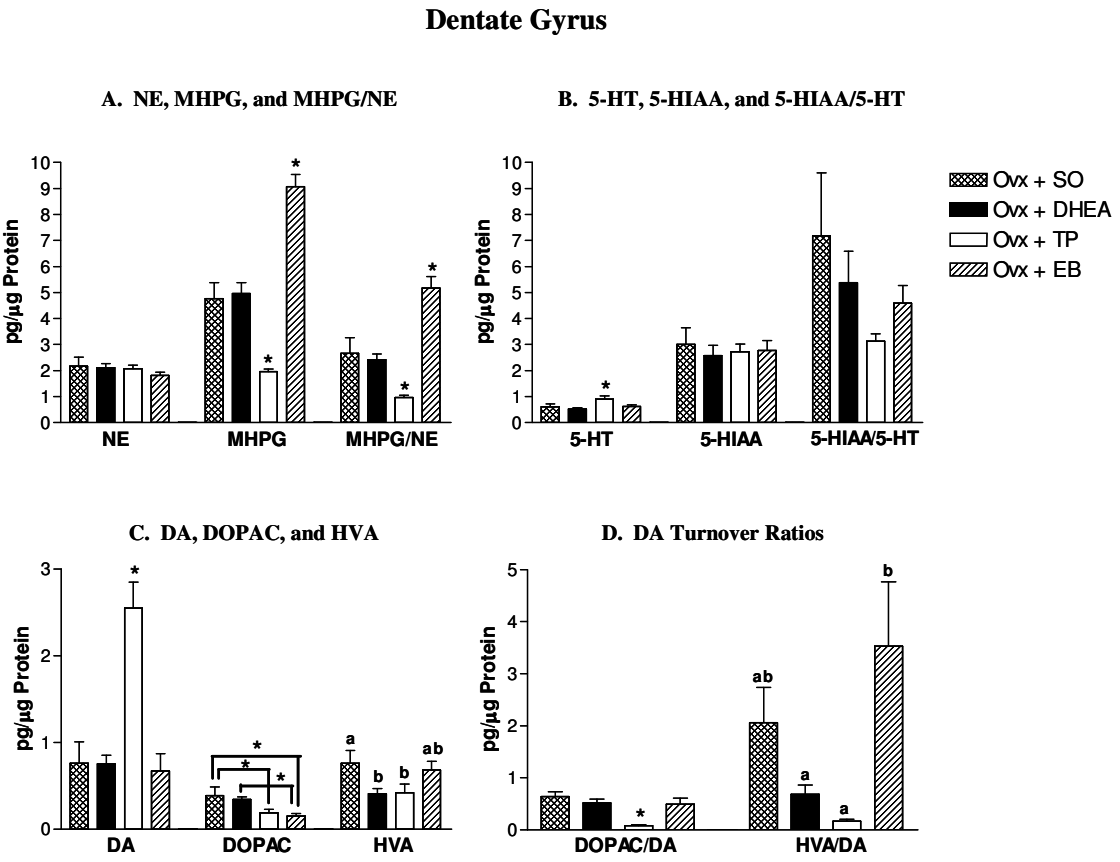



**Figure 30. Effect of subchronic DHEA, TP and EB on monoamine, metabolite and turnover levels in the dentate gyrus of the hippocampus.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein) and *post hoc* with Fisher's LSD. Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for NE activity. MHPG: ($F(3,29) = 46.81$, $p < 0.00001$). MHPG/NE: $F(3,29) = 22.97$, $p < 0.00001$). (B) Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for 5-HT activity. 5-HT: ($F(3,29) = 3.80$, $p < 0.05$). (C) Monoamine and metabolite levels are pg/µg protein ± SEM shown for DA activity. DA: $F(3,29) = 17.46$, $p < 0.00001$. DOPAC: $F(3,28) = 4.81$, $p < 0.01$. HVA: $F(3,28) = 3.11$, $p < 0.05$. (D) Turnover ratio levels are pg/µg protein ± SEM shown for DA and its metabolites. DOPAC/DA: $F(3,28) = 9.11$, $p < 0.001$. HVA/DA: $F(3,28) = 4.80$, $p < 0.01$. Groups labeled with different letters are significantly different from each other. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

***Striatum***

In the striatum, all treated groups, DHEA, TP and EB significantly increased NE levels compared to the SO group, while the androgen groups, DHEA and TP significantly decreased NE levels, compared to the EB group ($F(3,33) = 175.88$, $p < 0.00001$) (Fig. 31A). MHPG levels significantly decreased in DHEA and increased in TP and EB compared to SO treatment, while both DHEA and TP significantly decreased MHPG levels compared to EB treatment ($F(3,33) = 107.49$, $p < 0.00001$). The turnover ratio, MHPG/NE was significantly decreased in all treated groups, DHEA, TP, and EB compared to the SO treated group ($F(3,33) = 25.56$, $p < 0.00001$). 5-HT levels significantly increased with DHEA and TP treatment and decreased with EB treatment compared to SO treatment, while it significantly increased with the androgen treatments, DHEA and TP compared to EB treatment ($F(3,33) = 56.95$, $p < 0.00001$) (Fig. 31B). No changes in 5-HIAA were observed. TP and EB treatments significantly decreased and increased the turnover ratio, 5-HIAA/5-HT, respectively, compared to SO treatment, while the androgen treatments, DHEA and TP significantly decreased it compared to EB treatment ($F(3,33) = 60.73$, $p < 0.00001$). DA levels significantly increased with DHEA and EB compared to SO treatment, while DHEA and TP treatment significantly increased and decreased DA levels, respectively, compared to EB treatment ($F(3,33) = 27.75$, $p < 0.00001$) (Fig. 31C). DA's metabolite, DOPAC significantly decreased and increased with DHEA and TP treatment, respectively, compared to both SO and EB treatments ($F(3,33) = 28.67$, $p < 0.00001$). DHEA and EB treatments significantly decreased the turnover ratio, DOPAC/DA, compared to SO treatment, while DHEA and TP treatments significantly decreased and increased it, respectively, compared to EB treatment ($F(3,33)$

= 32.62, $p < 0.00001$) (Fig. 31D). There were no significant changes in HVA or HVA/DA turnover levels. Therefore, DHEA and TP generally decreased, while EB increased monoaminergic activity in the striatum.

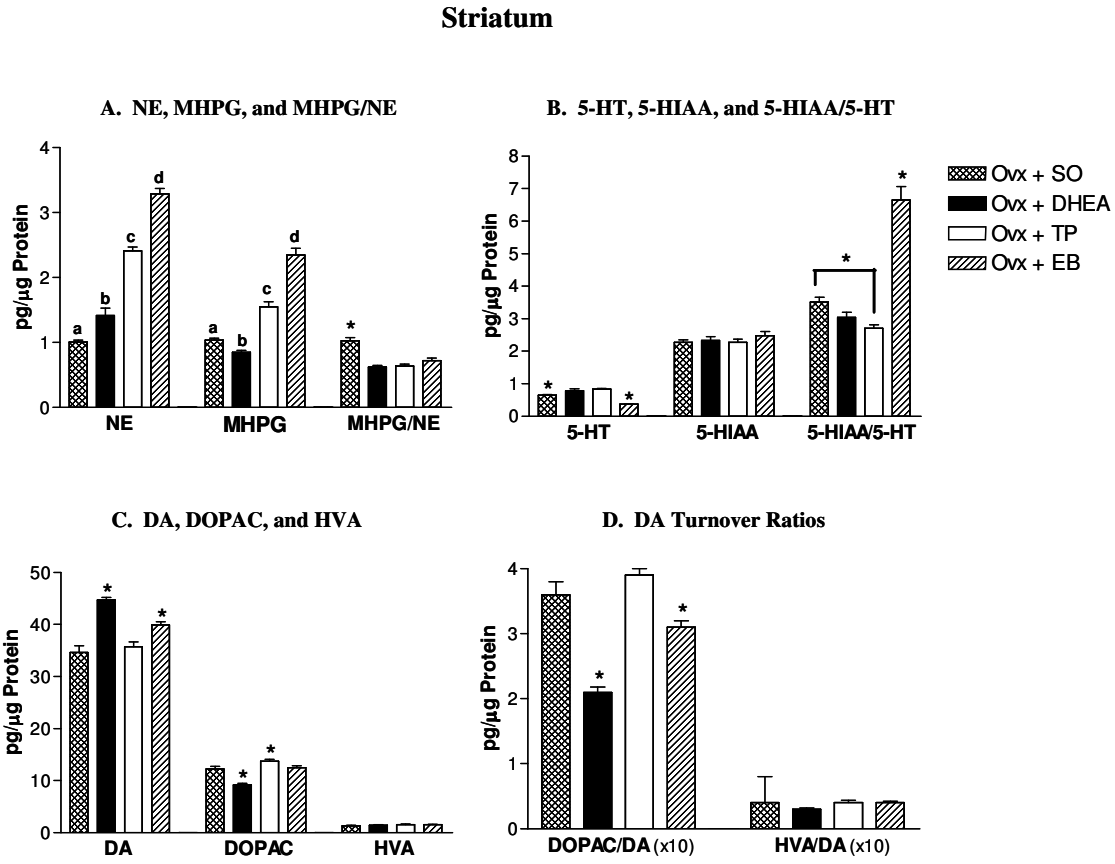



**Figure 31. Effect of subchronic DHEA, TP and EB on monoamine, metabolite and turnover levels in the striatum.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein) and *post hoc* with Fisher's LSD. Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for NE activity. NE: $F(3,33) = 175.88$, $p < 0.00001$. MHPG: $F = (3,33) = 107.49$, $p < 0.00001$. MHPG/NE: $F(3,33) = 25.56$, $p < 0.00001$. (B) Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for 5-HT activity. 5-HT: $F(3,33) = 56.95$, $p < 0.00001$. 5-HIAA/5-HT: $F(3,33) = 60.73$, $p < 0.00001$. (C) Monoamine and metabolite levels are pg/µg protein ± SEM shown for DA activity. DA: $F(3,33) = 28.67$, $p < 0.00001$. DOPAC: $F(3,33) = 28.67$, $p < 0.00001$. (D) Turnover ratio levels are pg/µg protein ± SEM shown for DA and its metabolites. DOPAC/DA: $F(3,33) = 32.26$, $p < 0.00001$. Groups labeled with different letters are significantly different from each other. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

***Vertical diagonal band***

In the vertical diagonal band (VDB), NE levels significantly decreased and increased with TP and EB treatments, respectively, compared to SO treatment, while NE levels significantly decreased with DHEA and TP compared to EB treatment ($F(3,33) = 41.28$, $p < 0.00001$) (Fig. 32A). TP and EB treatments resulted in significantly decreased and increased MHPG levels, respectively, compared to SO treatment, and both androgen treatments, DHEA and TP resulted in decreased MHPG levels compared to EB treatment ($F(3,33) = 31.98$, $p < 0.00001$). The turnover ratio, MHPG/NE significantly decreased with DHEA compared to SO and EB treatments ($F(3,33) = 6.17$, $p < 0.01$). DHEA and EB treatments led to significantly elevated 5-HT levels compared to SO treatment, while DHEA and TP treatments led to significantly lowered 5-HT levels compared to EB treatment ($F(3,33) = 128.37$, $p < 0.00001$) (Fig. 32B). TP treatment significantly decreased 5-HIAA levels compared to both SO and EB treatments ($F(3,33) = 17.08$, $p < 0.00001$). The turnover ratio, 5-HIAA/5-HT significantly decreased with all treatments, DHEA, TP and EB compared to SO treatment, and significantly increased with the androgen treatments, DHEA and TP compared to EB treatment ($F(3,33) = 33.70$, $p < 0.00001$). All treated groups, DHEA, TP and EB significantly decreased DA levels compared to SO, while TP decreased it compared to the EB group ($F(3,33) = 184.48$, $p < 0.00001$) (Fig. 32C). DOPAC levels were significantly elevated by DHEA and lowered by TP and EB compared to SO treatment, while DHEA and TP significantly elevated and lowered DOPAC levels, respectively, compared to the EB treatment ($F(3,33) = 363.71$, $p < 0.00001$). DA's metabolite, HVA significantly decreased and increased with DHEA and EB treatments, respectively, compared to SO treatment, and DHEA and TP

treatments significantly decreased HVA levels compared to EB treatment ($F(3,33) = 71.87$, $p < 0.00001$). The turnover ratio, DOPAC/DA significantly increased and decreased with DHEA and TP treatments, respectively compared to both SO and EB treatments ($F(3,33) = 244.12$, $p < 0.00001$) (Fig. 32D). HVA/DA turnover levels were significantly increased with TP and EB compared to SO treatment, and significantly decreased with DHEA and TP compared to EB treatment ($F(3,33) = 90.06$, $p < 0.00001$). Thus, EB generally increased monoaminergic activity in the VDB. See summary Table 9 for increases and decreases in turnover ratio levels by treated groups in all brain areas.

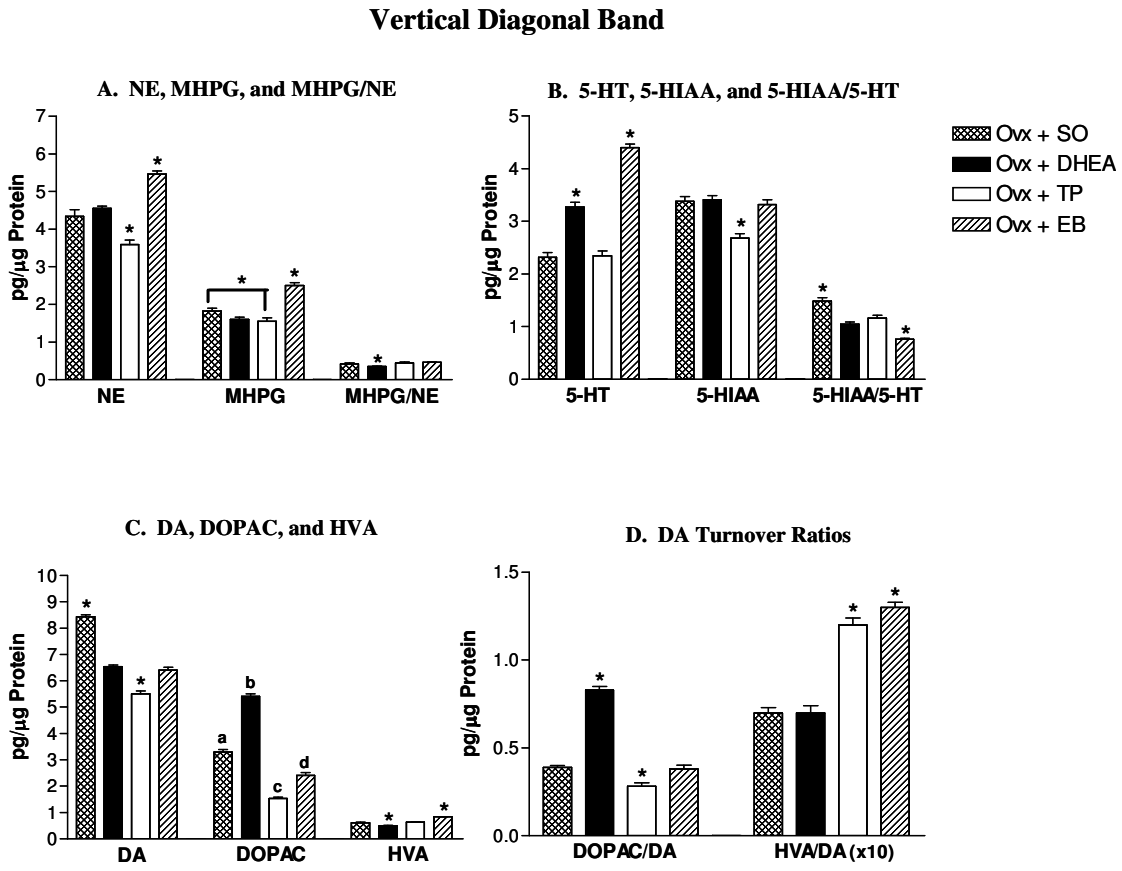



**Figure 32. Effect of subchronic DHEA, TP and EB on monoamine, metabolite and turnover levels in the vertical diagonal band.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein) and *post hoc* with Fisher's LSD. Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for NE activity. NE: $F(3,33) = 41.28$, $p < 0.00001$. MHPG: $F(3,33) = 31.98$, $p < 0.00001$. MHPG/NE: $F(3,33) = 6.17$, $p < 0.01$. (B) Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for 5-HT activity. 5-HT: $F(3,33) = 128.37$, $p < 0.00001$. 5-HIAA: $F(3,33) = 17.08$, $p < 0.00001$. 5-HIAA/5-HT: $F(3,33) = 33.70$, $p < 0.00001$. (C) Monoamine and metabolite levels are pg/µg protein ± SEM shown for DA activity. DA: $F(3,33) = 184.48$, $p < 0.00001$. DOPAC: $F(3,33) = 363.71$, $p < 0.00001$. HVA: $F(3,33) = 71.87$, $p < 0.00001$. (D) Turnover ratio levels are pg/µg protein ± SEM shown for DA and its metabolites. DOPAC/DA: $F(3,33) = 244.12$, $p < 0.00001$. HVA/DA: $F(3,33) = 90.06$, $p < 0.00001$. Groups labeled with different letters are significantly different from each other. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

**Table 9. Summary of DHEA, TP, and EB treatment effects on monoamine turnover levels in brain areas mediating cognition.**

| Area | Treatment | MHPG/NE | 5-HIAA/5HT | DOPAC/DA | HVA/DA |
|---|---|---|---|---|---|
| **PFC** | SO | ----- | ----- | ----- | ----- |
| | DHEA | ↑EB | ↑ EB | ----- | ----- |
| | TP | ----- | ----- | ----- | ----- |
| | EB | ↓ DHEA | ↓ DHEA | ----- | ----- |
| | | | | | |
| **CA1** | SO | ↓ EB | ----- | ↓ EB | ----- |
| | DHEA | ↓ EB | ↓ EB | ↓ EB | ----- |
| | TP | ↓ EB | ↓ EB | ↓ EB | ----- |
| | EB | ↑ SO, DHEA, TP | ↑ DHEA, TP | ↑ SO, DHEA, EB | ----- |
| | | | | | |
| **CA3** | SO | ↓ TP | ↓ DHEA, EB | ----- | ↑ TP |
| | DHEA | ----- | ↑ SO | ↓ EB | ----- |
| | TP | ↑ SO, EB | ↓ EB | ↓ EB | ↓ SO, EB |
| | EB | ↓ TP | ↑ SO, TP | ↑ DHEA, TP | ↑ TP |
| | | | | | |
| **DG** | SO | ↑ TP, ↓ EB | ----- | ↑ TP | ------ |
| | DHEA | ↓ EB | ----- | ----- | ↓ EB |
| | TP | ↓ SO, EB | ----- | ↓ SO, EB | ↓ EB |
| | EB | ↑ SO, DHEA, TP | ----- | ↑ TP | ↑ DHEA, TP |
| | | | | | |
| **ST** | SO | ↑ DHEA, TP, SO | ↑ TP, ↓ EB | ↑ DHEA, EB | ----- |
| | DHEA | ↓ SO | ↓ EB | ↓ SO, EB | ----- |
| | TP | ↓ SO | ↓ SO, EB | ↑ EB | ----- |
| | EB | ↓ SO | ↑ SO, DHEA TP | ↓ SO, TP ↑DHEA | ----- |
| | | | | | |
| **VDB** | SO | ↑ DHEA | ↑ DHEA, TP, EB | ↓ DHEA, ↑ TP | ↓ TP, EB |
| | DHEA | ↓ SO, TP, EB | ↓ SO, ↑ EB | ↑ SO, EB | ↓ EB |
| | TP | ----- | ↓ SO, ↑ EB | ↓ SO, EB | ↑ SO, ↓ EB |
| | EB | ↑ DHEA | ↓ SO, DHEA, EB | ↓ DHEA, ↑ TP | ↑ SO, DHEA, EB |

All treated groups were compared to SO, control group or androgen treated groups, DHEA and TP were compared to EB treated group.
(PFC: prefrontal cortex; DG: dentate gyrus; ST: striatum, VDB: vertical diagonal band; ↑↓: increased or decreased; -----: no difference).

***B. Treatment with AD and DHT***

Studies have shown that DHT enhances memory in both male and female rats (Edinger and Frye, 2004; 2007; Fyre and Lacey, 2001) and regulate monoaminergic activity in the brain (Handa et al, 1997). There are no studies in the literature exploring AD effects on memory and monoaminergic activity in brain areas involved in memory. In Aim 1, subchronic treatment with DHT and AD enhanced spatial memory. It is hypothesized that subchronic treatment with these androgens will alter monoaminergic activity in the same brain areas analyzed in Section A of the current aim, and these changes may contribute to memory enhancements. Furthermore, DHT the nonaromatizable androgen may have a different pattern of brain monoaminergic activity, compared to AD, an aromatizable androgen.

**Methods**

Ovariectomized rats were treated subchronically with SO (0.20 ml; n = 6), DHT (1 mg; n = 6) or AD (1 mg; n = 6). These subchronic doses of DHT (Fig. 10B) and AD (Fig. 11B) enhance spatial memory. Forty-eight hours after the second injection, subjects were sacrificed, brains were sectioned and HPLC analysis was performed on the same brain areas used in the previous experiment. See above methods for details and cohort used in Table 1.

**Results**

Subchronic treatment with the hormones, DHT and AD altered monoamine levels in several brain areas. All significant data are represented by graphs and values of both significant and non-significant data are shown in Table 10.

***Prefrontal cortex***

In the prefrontal cortex, no significant differences were observed for NE. However, treatment with DHT significantly increased levels of NE's metabolite, MHPG compared to SO and AD treatments ($F(2,15) = 9.46$, $p < 0.01$) (Fig. 33A). There were no significant changes for the turnover ratio, MHPG/NE. There were no significant changes for 5-HT, its metabolite 5-HIAA and its turnover ratio, 5-HIAA/5-HT, DA and its metabolites, DOPAC and HVA (Table 9). However, turnover levels for DOPAC/DA significantly increased in AD compared to SO and DHT treated subjects ($F(2,15) = 6.14$, $p < 0.05$) (Fig. 33B).

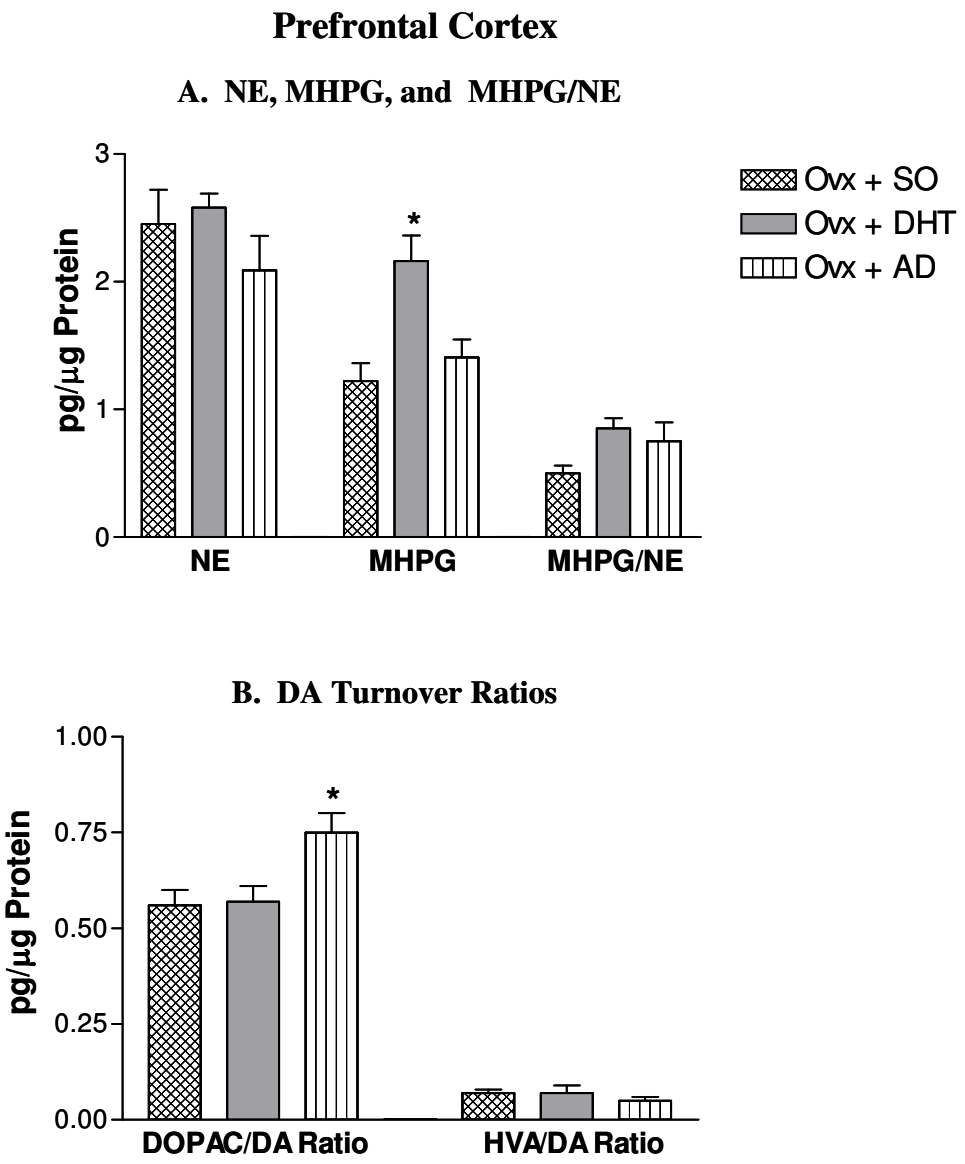



**Figure 33. Effect of subchronic DHT and AD on monoamine, metabolite and turnover levels in the prefrontal cortex.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein) and *post hoc* with Fisher's LSD. Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for NE activity. MHPG: $F(2,15) = 9.46$, $p < 0.01$. (B) Turnover ratio levels are pg/µg protein ± SEM shown for DA and its metabolites. DOPAC/DA: $F(2,15) = 6.14$, $p < 0.05$. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

***CA1 and dentate gyrus of the hippocampus, and striatum***

Changes were observed for NE activity in CA1 and the dentate gyrus of the hippocampus, and the striatum, while no changes were observed for 5-HT and DA activity (Table 9). No changes occurred in CA3 of the hippocampus (Table 9). In CA1, AD significantly increased MHPG ($F(2,15) = 5.34$, $p < 0.05$), but there were no changes in MHPG/NE levels (Fig. 34A).

DHT treatment significantly elevated NE levels in the dentate gyrus compared to SO and AD treatments ($F(2,15) = 6.93$, $p < 0.01$) (Fig. 34B). DHT and AD treatments significantly increased MHPG ($F(2,15) = 15.06$, $p < 0.001$) and MHPG/NE ($F(2,15) = 9.41$, $p < 0.01$) levels compared to SO treatment.

In the striatum, DHT treatment led to significantly decreased levels of NE compared to SO and AD treatments ($F(2,15) = 18.30$, $p < 0.0001$) (Fig. 34C). MHPG ($F(2,15) = 55.30$, $p < 0.00001$) and MHPG/NE ($F(2,15) = 26.40$, $p < 0.0001$) levels significantly increased with AD treatment compared to SO and DHT treatments.

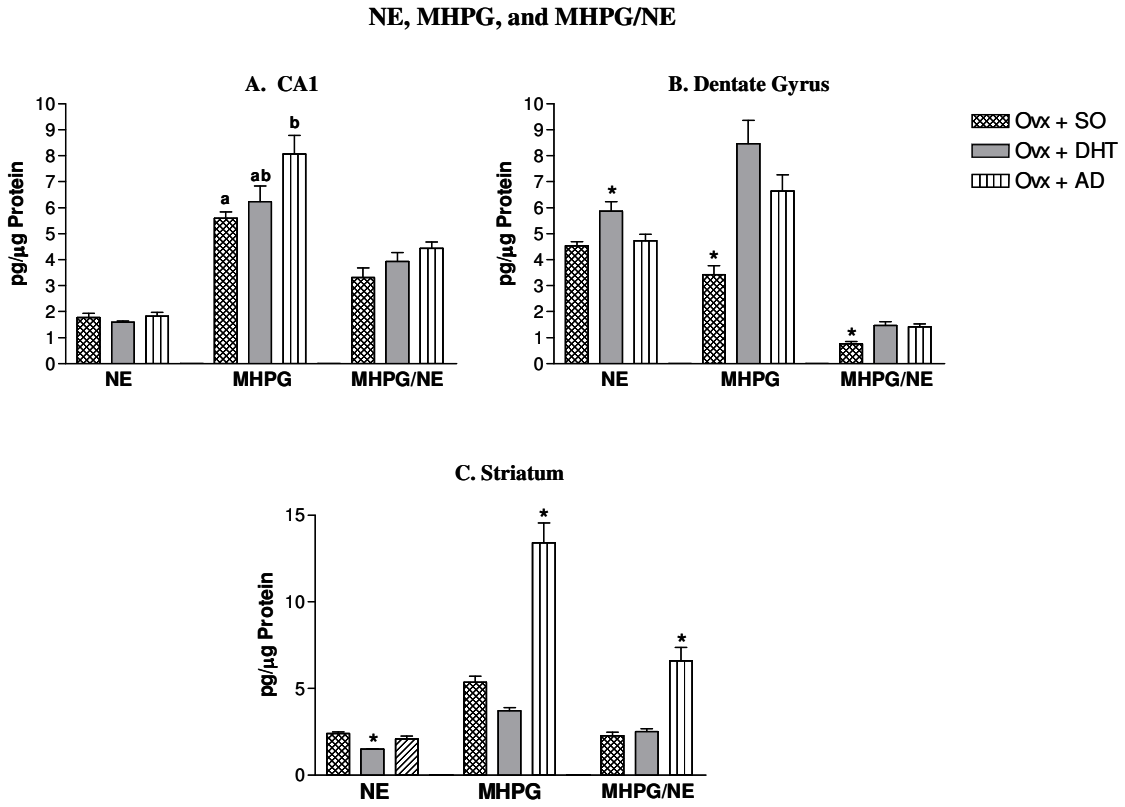



**Figure 34. Effect of subchronic DHT and AD on monoamine, metabolite and turnover levels in the CA1, dentate gyrus and striatum.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein) and *post hoc* with Fisher's LSD. Monoamine, metabolite, and turnover ratio levels are pg/µg protein ± SEM shown for NE activity in CA1 of the hippocampus. MHPG: $F(2,15) = 5.34$, $p < 0.05$.
(B) Monoamine, metabolite, and turnover ratio levels are pg/µg protein ± SEM shown for NE activity in the dentate gyrus of the hippocampus. NE: $F(2,15) = 6.93$, $p < 0.001$. MHPG ($F(2,15) = 15.06$, $p < 0.001$) and MHPG/NE: $F(2,15) = 9.41$, $p < 0.01$. (C) Monoamine, metabolite, and turnover ratio levels are pg/µg protein ± SEM shown for NE activity in the striatum. NE decreased in DHT compared to SO and AD treated groups: $F(2,15) = 18.30$, $p < 0.0001$. MHPG: $F(2,15) = 55.30$, $p < 0.00001$. MHPG/NE: $F(2,15) = 26.40$, $p < 0.0001$. Groups labeled with different letters are significantly different from each other. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

***Vertical diagonal band***

Treatment with DHT increased MHPG ($F(2,15) = 7.22$, $p < 0.01$) levels compared to AD treatment, and increased MHPG/NE ($F(2,15) = 20.06$, $p < 0.0001$) levels compared to SO and AD treatments in the vertical diagonal band (Fig. 35A). DHT treatment led to significantly elevated 5-HIAA levels compared to AD treatment ($F(2,15) = 4.04$, $p < 0.05$) (Fig. 35B). No significant changes were observed for 5-HT or its turnover ratio, 5-HIAA/5-HT. AD treatment significantly increased DA ($F(2,15) = 6.11$, $p < 0.05$) and DOPAC ($F(2,15) = 3.66$, $p = 0.05$) levels compared to SO treatment (Fig. 35C). There were significant changes for HVA levels. The turnover ratio, HVA/DA significantly decreased with DHT and AD treatments compared to SO treatment ($F(2,15) = 11.14$, $p < 0.01$) (Fig. 35D). See summary Table 11 for increases and decreases of turnover ratio levels by treated groups in all brain areas.

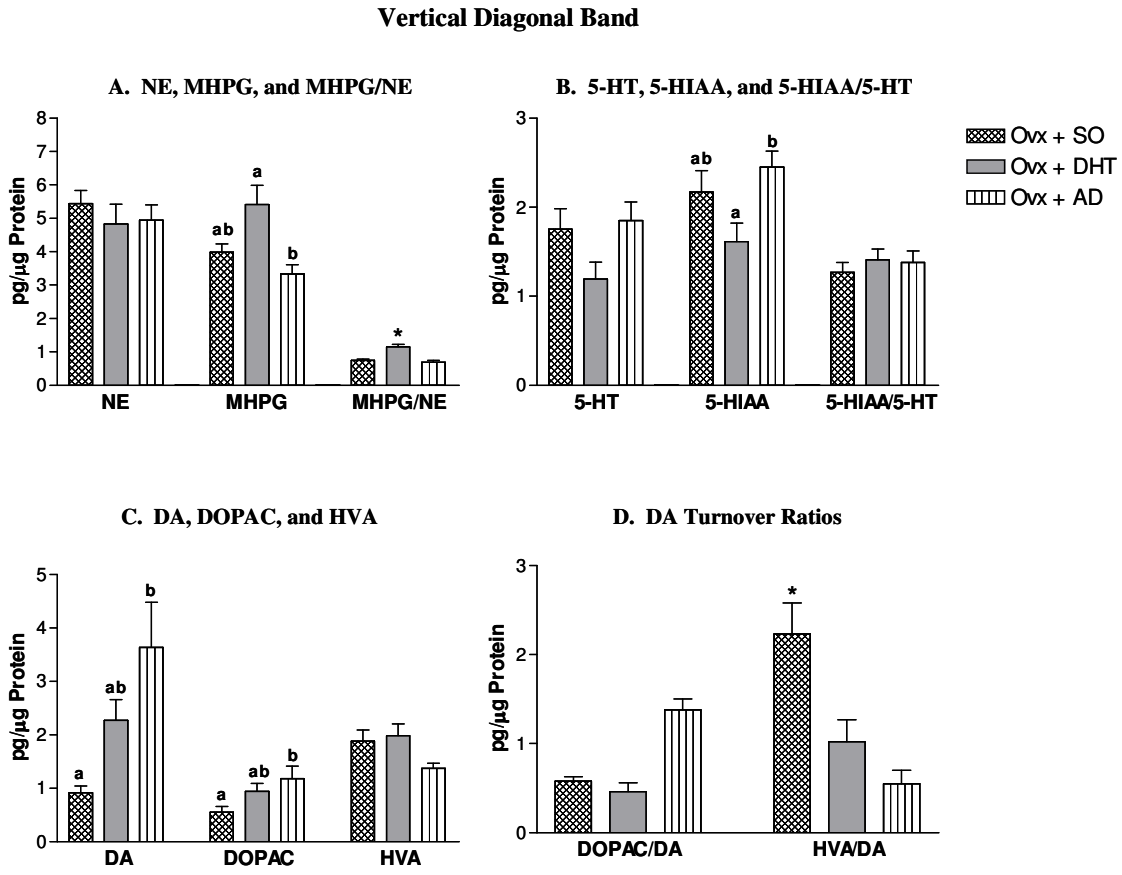



**Figure 35. Effect of subchronic DHT and AD on monoamine, metabolite and turnover levels in the vertical diagonal band.** (A) All data was analyzed using one-way ANOVAs (group x pg/µg protein) and *post hoc* with Fisher's LSD. Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for NE activity. MHPG: $F(2,15) = 7.22$, $p < 0.01$. MHPG/NE: $F(2,15) = 20.06$, $p < 0.0001$. (B) Monoamine, metabolite and turnover ratio levels are pg/µg protein ± SEM shown for 5-HT activity. 5-HIAA: $F(2,15) = 4.40$, $p < 0.05$. (C) Monoamine and metabolite levels are pg/µg protein ± SEM shown for DA activity. DA: $F(2,15) = 6.11$, $p < 0.05$). DOPAC: $F(2,15) = 3.66$, $p = 0.05$. HVA: $F(2,15) = 3.07$, $p = 0.07$. (D) Turnover ratio levels are pg/µg protein ± SEM shown for DA and its metabolites. HVA/DA: $F(2,15) = 11.14$, $p < 0.01$. Groups labeled with different letters are significantly different from each other. A group labeled with only an asterisk (*) represents one that is significantly different from all other groups.

**Table 10. Summary of monoamine, metabolite, and turnover ratio levels in AD and DHT groups.**

| Area | Treatment | NE | MHPG | MHPG/NE | 5-HT | 5-HIAA | 5-HIAA/5-HT |
|---|---|---|---|---|---|---|---|
| **PFC** | SO | 2.45 ± 0.27 | **1.22 ±** 0.14** | 0.50 ± 0.06 | 1.62 ± 0.06 | 2.91 ± 0.13 | 1.80 ± 0.09 |
| | DHT | 2.58 ± 0.11 | **2.16 ± 0.20** | 0.85 ± 0.08 | 1.65 ± 0.14 | 3.18 ± 0.28 | 1.95 ± 0.12 |
| | AD | 2.09 ± 0.27 | **1.41 ± 0.14** | 0.75 ± 0.15 | 1.41 ± 0.13 | 2.83 ± 0.22 | 2.03 ± 0.12 |
| **CA1** | SO | 1.78 ± 0.16 | **5.60 ±* 0.24** | 3.31 ± 0.37 | 0.50 ± 0.08 | 1.57 ± 0.22 | 3.23 ± 0.32 |
| | DHT | 1.59 ± 0.05 | **6.24 ± 0.60** | 3.93 ± 0.34 | 0.51 ± 0.10 | 1.52 ± 0.10 | 3.40 ± 0.50 |
| | AD | 1.83 ± 0.14 | **8.07 ± 0.71** | 4.43 ± 0.25 | 0.51 ± 0.03 | 1.87 ± 0.27 | 3.64 ± 0.37 |
| **CA3** | SO | 2.21 ± 0.18 | 2.84 ± 0.22 | 1.33 ± 0.12 | 0.50 ± 0.06 | 1.20 ± 0.20 | 2.48 ± 0.21 |
| | DHT | 1.88 ± 0.09 | 2.84 ± 0.12 | 1.52 ± 0.07 | 0.50 ± 0.04 | 1.12 ± 0.08 | 2.27 ± 0.18 |
| | AD | 2.20 ± 0.24 | 2.81 ± 0.16 | 1.48 ± 0.07 | 0.50 ± 0.05 | 1.17 ± 0.05 | 2.46 ± 0.31 |
| **DG** | SO | **4.54 ±** 0.16** | **3.42 ±*** 0.35** | **0.76 ±** 0.09** | 0.91 ± 0.02 | 2.90 ± 0.22 | 3.23 ± 0.30 |
| | DHT | **5.86 ± 0.37** | **8.64 ± 0.89** | **1.46 ± 0.16** | 1.16 ± 0.24 | 3.64 ± 0.52 | 3.63 ± 0.66 |
| | AD | **4.73 ± 0.24** | **6.64 ± 0.62** | **1.41 ± 0.12** | 1.01 ± 0.11 | 3.21 ± 0.33 | 3.29 ± 0.35 |
| **ST** | SO | **2.40 ±**** 0.09** | **5.36 ±***** 0.34** | **2.27 ±**** 0.20** | 0.81 ± 0.08 | 1.60 ± 0.10 | 2.05 ± 0.12 |
| | DHT | **1.49 ± 0.03** | **3.71 ± 0.20** | **2.51 ± 0.17** | 0.78 ± 0.08 | 1.46 ± 0.12 | 1.93 ± 0.14 |
| | AD | **2.10 ± 0.16** | **13.42 ± 1.14** | **6.59 ± 0.78** | 0.82 ± 0.06 | 1.78 ± 0.13 | 2.21 ± 0.16 |
| **VDB** | SO | 5.44 ± 0.39 | **3.98 ±** 0.26** | **0.74 ±**** 0.04** | 1.75 ± 0.23 | **2.17 ±* 0.24** | 1.27 ± 0.11 |
| | DHT | 4.82 ± 0.60 | **5.41 ± 0.58** | **1.15 ± 0.07** | 1.19 ± 0.19 | **1.61 ± 0.21** | 1.41 ± 0.12 |
| | AD | 4.94 ± 0.46 | **3.34 ± 0.27** | **0.69 ± 0.06** | 1.85 ± 0.21 | **2.45 ± 0.18** | 1.38 ± 0.13 |

Levels are pg/ug protein ± SEM shown for SO, DHT and AD treated subjects. Data were analyzed by one-way ANOVAs and *post hoc* with Fisher's LSD. Significant differences are in bold and indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and *****p < 0.00001. Significant data are represented by graphs in Figures 33, 34, and 35. (PFC: prefrontal crotex; DG: dentate gyrus; ST: striatum, VDB : vertical diagonal band).

**Table 10 cont'd.**

| Area | Treatment | DA | DOPAC | HVA | DOPAC/DA | HVA/DA |
|---|---|---|---|---|---|---|
| **PFC** | SO | 0.64 ± 0.06 | 0.36 ± 0.05 | 0.04 ± 0.01 | **0.56 ±* 0.04** | 0.07 ± 0.01 |
| | DHT | 0.72 ± 0.07 | 0.40 ± 0.02 | 0.04 ± 0.01 | **0.57 ± 0.04** | 0.07 ± 0.02 |
| | AD | 0.60 ± 0.09 | 0.47 ± 0.09 | 0.04 ± 0.01 | **0.75 ± 0.05** | 0.05 ± 0.01 |
| **CA1** | SO | 0.53 ± 0.06 | 0.30 ± 0.05 | 0.06 ± 0.01 | 0.57 ± 0.07 | 0.11 ± 0.01 |
| | DHT | 0.55 ± 0.04 | 0.23 ± 0.03 | 0.05 ± 0.01 | 0.44 ± 0.04 | 0.08 ± 0.01 |
| | AD | 0.59 ± 0.07 | 0.33 ± 0.07 | 0.05 ± 0.01 | 0.54 ± 0.05 | 0.09 ± 0.01 |
| **CA3** | SO | 0.56 ± 0.05 | 0.25 ± 0.03 | 0.03 ± 0.004 | 0.44 ± 0.02 | 0.05 ± 0.01 |
| | DHT | 0.57 ± 0.09 | 0.25 ± 0.04 | 0.03 ± 0.004 | 0.45 ± 0.07 | 0.06 ± 0.01 |
| | AD | 0.65 ± 0.06 | 0.25 ± 0.02 | 0.03 ± 0.004 | 0.39 ± 0.04 | 0.05 ± 0.01 |
| **DG** | SO | 0.83 ± 0.06 | 0.57 ± 0.09 | 0.11 ± 0.02 | 0.06 ± 0.07 | 0.10 ± 0.002 |
| | DHT | 1.12 ± 0.11 | 0.75 ± 0.08 | 0.12 ± 0.01 | 0.67 ± 0.02 | 0.11 ± 0.01 |
| | AD | 0.94 ± 0.11 | 0.61 ± 0.08 | 0.12 ± 0.02 | 0.65 ± 0.04 | 0.13 ± 0.01 |
| **ST** | SO | 49.67 ± 5.25 | 8.21 ± 0.48 | 4.56 ± 0.13 | 0.17 ± 0.02 | 0.10 ± 0.01 |
| | DHT | 50.20 ± 3.69 | 7.20 ± 0.41 | 4.16 ± 0.40 | 0.14 ± 0.01 | 0.09 ± 0.01 |
| | AD | 49.97 ± 1.71 | 7.88 ± 0.82 | 3.64 ± 0.50 | 0.16 ± 0.01 | 0.07 ± 0.01 |
| **VDB** | SO | **0.92 ±* 0.12** | **0.55 ±* 0.11** | 1.88 ± 0.21 | 0.58 ± 0.05 | **2.23 ±** 0.35** |
| | DHT | **2.27 ± 0.39** | **0.94 ± 0.15** | 1.98 ± 0.22 | 0.46 ± 0.1 | **1.02 ± 0.25** |
| | AD | **3.63 ± 0.85** | **1.18 ± 0.23** | 1.37 ± 0.10 | 1.38 ± 0.12 | **0.55 ± 0.15** |

**Table 11. Summary of DHT and AD treatment effects on monoamine turnover levels in brain areas mediating cognition.**

| Area | Treatment | MHPG/NE | 5-HIAA/5-HT | DOPAC/DA | HVA/DA |
|---|---|---|---|---|---|
| **PFC** | SO | ----- | ----- | ↓ AD | ----- |
| | DHT | ----- | ----- | ↓ AD | ----- |
| | AD | ----- | ----- | ↑ SO, DHT | ----- |
| | | | | | |
| **CA1** | SO | ----- | ----- | ----- | ----- |
| | DHT | ----- | ----- | ----- | ----- |
| | AD | ----- | ----- | ----- | ----- |
| | | | | | |
| **CA3** | SO | ----- | ----- | ----- | ----- |
| | DHT | ----- | ----- | ----- | ----- |
| | AD | ----- | ----- | ----- | ----- |
| | | | | | |
| **DG** | SO | ↓ DHT, AD | ---- | ----- | ----- |
| | DHT | ↑ SO | ----- | ----- | ----- |
| | AD | ↑ SO | ----- | ----- | ----- |
| | | | | | |
| **ST** | SO | ↓ AD | ----- | ----- | ----- |
| | DHT | ↓ AD | ----- | ----- | ----- |
| | AD | ↑ SO, DHT | ----- | ----- | ----- |
| | | | | | |
| **VDB** | SO | ↓ DHT | ---- | ---- | ↑ DHT, AD |
| | DHT | ↑ SO, AD | ---- | ---- | ↓ SO |
| | AD | ↓ DHT | ---- | ---- | ↓ SO |

Treated groups were compared to each other. (PFC: prefrontal cortex; DG: dentate gyrus; ST: striatum; VDB: vertical diagonal band; ↑↓: increased or decreased; -----: no difference).

## Discussion

### *DHEA, TP, and EB treatment effects*

For discussion purposes, only turnover ratios will be presented to determine levels of activity in the brain as previous researchers have shown that alterations in monoaminergic turnover ratios in brain areas involved in memory may contribute to cognitive changes (Bisagono et al, 2002; 2003; Bowman et al, 2003; Macbeth et al, 2008). All treated groups, DHEA, TP and EB will be compared to SO and the androgens, DHEA and TP will be compared to the estrogen, EB to determine whether the pattern of monoaminergic activity is different between androgens and estrogen (Table 9). In the prefrontal cortex of OVX female rats, subchronic treatment with DHEA increased both MHPG/NE and 5-HIAA/5-HT turnover levels compared to EB treatment. Increased turnover ratios correspond to increased neurotransmission and activity in noradrenergic and serotonergic systems and may be associated with enhancements in memory. Collier et al (2004) found that decreases in MHPG/NE activity in the cingulate cortex were associated with impaired spatial memory on the morris water maze. Furthermore, activation of noradrenergic transmission in the prefrontal cortex is increased when rats perform correctly on the T-maze, a spatial working memory task (Rossetti and Carbon, 2005). There is increasing evidence that 5-HT is associated with memory and its receptor subtypes are expressed in the frontal cortex (Manuel-Apolinar et al, 2005; Mitchell et al, 2006; Lamirault and Simon, 2001; Meneses, 1999). The 5-HT receptor agonist, 8-OH-DPAT administered into the prefrontal cortex of rats has been shown to reverse short term memory impairments (Liy-Salmeron and Meneses, 2008). The prefrontal cortex is more involved in recognition memory, but may also be involved in spatial memory, as

projections are sent from the hippocampus to the prefrontal cortex (Wallace et al, 2007; Swanson, 1981; Hoover and Vertes, 2007). The hippocampus and prefrontal cortex may be working cooperatively as both regions demonstrated enhanced correlated neuron firing in a forced-choice task that tests spatial memory (Jones and Wilson, 2005). The increased level of turnover in both the norepinephrine and serotonergic systems may be mediating enhancements in spatial memory, especially since there are projections between the hippocampus and the prefrontal cortex. In the prefrontal cortex, DHEA appears to be acting differently than EB, as EB treatment decreased levels of norepinehprine and serotonergic activity, while DHEA increased activity. Possibly, DHEA is acting as an androgen (evidenced in Aim 2), in the prefrontal cortex, and not being aromatized to estrogen, because androgen receptors are present in the cortex (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992).

In CA1 of the hippocampus, EB subjects had increased noradrenergic, serotonergic, and dopaminergic activity, while androgen treated subjects, DHEA and TP had decreased activity. On the other hand, DPN, the ER-β agonist, decreased MHPG/NE levels compared to controls in the prefrontal cortex (Jacome, 2007). The hippocampus has been shown to be involved in spatial memory and to a lesser extent, nonspatial memory (Morris et al, 1982; Kesner et al, 1993; Ennaucer et al, 1997; Ennaucer and Aggleton, 1994; Broadbent et al, 2004). Androgen receptors are present in CA1 of the hippocampus (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992). Similar to the prefrontal cortex, the androgens are having opposite effects on monoaminergic turnover levels compared to EB. It seems that DHEA and TP may be acting as androgens in CA1 with decreased noradrenergic, serotonergic, and dopaminergic activity compared to

estradiol. Decreased serotonin levels may be associated with enhanced memory. 5-HT agonists have been shown to impair radial arm maze performance, while 5-HT antagonists enhance memory (Winter and Petti, 1987; Meneses et al, 2007; Liy-Salmeron and Menses, 2008). Furthermore, concurrent administration of fluoxetine, a serotonin reuptake inhibitor and estrogen eliminated any enhancements on spatial memory performance obtained from chronic estrogen administration (Taylor et al, 2004). Activation of norepinephrine receptors may lead to inhibitory responses in the hippocampus. Application of norepinephrine or an agonist of this monoamine to CA1 hippocampal neurons, activated GABAergic neurons which then reduced electrical synaptic transmission via the cAMP/PKA cascade (Zsiros and Maccaferri, 2008). Therefore, decreased activity of the noradrenergic and serotonergic systems in CA1 of the hippocampus may mediate enhancements in spatial and nonspatial memory.

In CA3 of the hippocampus, TP increased noradrenergic activity and decreased serotonergic and dopaminergic activity while EB had opposite effects. Similar to EB, DHEA also increased serotonergic activity. CA3 is involved in spatial memory as cell loss in this area is associated with learning deficits on the morris water maze (Briones and Therrien, 2000). Androgen receptors are also located in CA3 of the hippocampus (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992). A trisynaptic circuit exists in the hippocampus, where neural activity passes through the dentate gyrus which in turn projects through mossy fibers to the CA3, and which then projects via Schaffer collaterals to CA1 (Becker, 2005). Since the androgens, DHEA and TP generally decreased turnover ratio levels in these hippocampal areas, monoaminergic alterations in this trisynaptic circuit may be mediating enhancements in memory.

In the dentate gyrus, the androgens generally decreased noradrenergic and dopaminergic activity, while EB increased activity. The dentate gyrus is the hippocampal region characterized by new neuron production during adulthood and this neurogenesis can be influenced by circulating estrogen levels (Tanapat et al, 1999). Specifically, elevated estrogen levels associated with proestrus produce a transient increase in dentate gyrus neurogenesis (Tanapat et al, 1999). The formation of new neurons in the hippocampus may be involved in enhancements of memory (Prickaerts et al, 2004). The number of adult generated neurons in the dentate gyrus of the rat doubled in response to training on associative learning tasks that require the hippocampus (Gould et al, 1999). Furthermore, LTP induction promoted survival of 1-2 week old dentate granule cells (Bruel-Jungerman et al, 2006). Testosterone replacement in gonadectomized male rats has shown to increase neurogenesis in the dentate gyrus (Spritzer and Galea, 2007). In female OVX and adrenalectomized rats, testosterone replacement reduced the number of apoptotic neurons in the dentate gyrus (Frye and Mcormick, 2000). Since lower levels of DA in the frontal cortex is associated enhanced performance on the radial arm maze (Luine et al, 1998) and the hippocampus sends projections to the prefrontal cortex (Swanson, 1981; Hoover and Vertes, 2007), DHEA and TP's decreased dopaminergic activity in the dentate gyrus may be enhancing memory via these projections. TP decreased HVA/DA and DOPAC/DA in both CA3 and dentate gyrus of the hippocampus. New granule cells that are formed in the dentate gyrus during adulthood may extend axons from the dentate into CA3 of the hippocampus (Hastings and Gould, 1999). Thus, monoaminergic alterations in dopaminergic activity in both CA3 and the dentate may be interacting to mediate enhancements in memory.

In the striatum, MHPG/NE levels were decreased in all treated groups, DHEA, TP, and EB compared to controls. TP decreased while EB increased 5-HIAA/5-HT ratio, compared to SO treatment, while both androgen treatments, DHEA and TP decreased it compared to EB treatment. DHEA and EB decreased DOPAC/DA levels compared to SO treatment, while DHEA and TP decreased and increased it, respectively, compared to EB treatment. Although this is a more complex pattern of monoaminergic changes, taken together, both androgens, DHEA and TP decreased norepinephrine and serotonergic activity, while DHEA decreased dopaminergic activity in the striatum. Decreased levels of NE were associated with enhanced spatial memory, while high levels of NE have been shown to impair working memory (Luine et al, 1998; Ramos and Arnsten, 2007). Thus, reduced activity in all three monoaminergic systems may be mediating DHEA and TP's enhancements in memory.

In the vertical diagonal band, MHPG/NE decreased with DHEA compared to SO and EB treatments. 5-HIAA/5HT decreased with all treatments, DHEA, TP and EB compared to SO treatment, and increased with the androgen treatments, DHEA and TP compared to EB treatment. DHEA increased and TP decreased DOPAC/DA levels, compared to both SO and EB treatments. In contrast, MHPG/NE levels decreased with subchronic DPN treatment (Jacome, 2007). HVA/DA turnover levels were increased with TP and EB treatments compared to SO, and decreased with DHEA and TP compared to EB treatment. The pattern of monoaminergic activity by the androgens in the vertical diagonal band is also complicated. Lesions to the medial septum and vertical diagonal band led to impaired spatial memory (Olton et al, 1978; Walsh et al, 1996; Riekkinen et al, 1990. Medial septum-vertical diagonal band lesions also depleted hippocampal

cholinergic innervation (Riekkinen et al, 1990). Furthermore, the vertical diagonal band is rich in cholinergic cell bodies and receives dense monoaminergic and GABAergic innervation and contributes to memory function (Zaborszsky et al, 1993). Therefore, alterations in monoaminergic activity in the vertical diagonal band via connections with other brain areas, such as the hippocampus may contribute to cognitive enhancements of both DHEA and TP.

***DHT and AD treatment effects***

Since DHT is a nonaromatizable androgen, and AD is an aromatizable androgen that can be converted to estrogen, it was expected that there would be a different pattern of brain monoaminergic activity between the two androgens. In the prefrontal cortex, turnover levels for DOPAC/DA increased in AD compared to SO and DHT treated subjects. Rats treated with a D1 receptor agonist, SKF 81297 had improvements in spatial and nonspatial memory and increased phosphorylation in CREB and DARPP-32 in the prefrontal cortex, while there were significant impairments of spatial memory with the selective D1 antagonist SCH 23390 and associated decreased phosphorylation of CREB and DARPP-32 (Hotte et al, 2005; 2006). A D1 agonist infused into the medial prefrontal cortex of rats improved memory at long delays on the radial arm maze (Floresco and Phillips, 2001). Furthermore, DA depletion in the prefrontal cortex impaired spatial delayed response performance, which was attenuated by treatment with DA agonists (Brozoski et al, 1979). DHT replacement in castrated male rats has been shown to attenuate chronic decreases of dopaminergic afferents via tyrosine hydroxylase immunoreactivity in the cerebral cortex (Kritzer, 2000). In contrast, in male gonadectomized rats replaced with DHT propionate, MHPG/NE, DOPAC/DA, and 5-

HIAA/5-HT levels in the medial prefrontal cortex decreased compared to intact, gonadectomized, and estradiol replaced rats introduced to a novel environment on an open field (Handa et al, 2007). Thus, AD enhancements in spatial memory may be mediated by increased dopaminergic activity.

In CA1 of the hippocampus, surprisingly NE's metabolite, MHPG but not MHPG/NE levels increased in AD compared to SO treatment. When NE was directly infused into CA1, memory was enhanced on a passive avoidance task (Bevilaqua et al, 1997a; 1997b), thus NE appears to aid in memory. There were no changes in CA3 or the dentate gyrus of the hippocampus. In the striatum, MHPG/NE was increased with AD compared to SO and DHT treatments. Direct hippocampal connections have been found between the hippocampus and the striatum (Rossato et al, 2006; Voorn et al, 2004). As mentioned previously, the hippocampus which primarily subserves spatial memory (Morris et al, 1984; Broadbent, 2004). Therefore, the hippocampus and striatum may be interacting to increase noradrenergic turnover ratios by the androgens AD and DHT to enhance spatial memory. In the vertical diagonal band, DHT increased MHPG/NE, while both treatment groups, DHT and AD decreased HVA/DA compared to SO treatment. In the vertical diagonal band, OVX female rats replaced with EB had increased NE levels that were associated with enhanced spatial memory on the radial arm maze (Luine et al, 1996). As mentioned previously, lesions to the medial-septal VDB impaired spatial memory, an area that has cholinergic innervation (Riekkinen et al, 1990; Walsh et al, 1996; Zaborszsky et al, 1993). Thus, these alterations in monoaminergic activity may underlie enhancements in spatial memory.

Taken together, the pattern seen here is that either AD and/or DHT treatments increased norepinephrine activity in the CA1, striatum, and vertical diagonal band. Since both hormones enhanced spatial memory, increased norepinehprine activity may be mediating enhancements in memory as decreases in NE activity were associated with impaired spatial memory (Collier et al, 2004). In contrast, the treatments, DHEA and TP decreased norepinehprine activity in most brain areas, and enhanced spatial and nonspatial memory, respectively. It must also be noted that uterine weights were similar in DHT and AD treated subjects, but significantly different than DHEA and TP treated subjects. Therefore, DHEA and TP may be mediating changes centrally, in the brain and peripherally, in the uterus differently than DHT and AD.

## General Discussion

The current data in Aim 1 demonstrated that two day treatment with the aromatizable androgens, DHEA and AD, and the nonaromatizable androgen, DHT enhanced spatial memory, while the more potent, aromatizable androgen, TP enhanced nonspatial memory. Previous studies have focused mostly on longer term, chronic administration of estrogen in female rats (Daniel et al, 1997; Luine et al, 1998; Gibbs et al, 1999; 2000), or androgen administration in male rats (Bimonte-Nelson et al, 2003; Sandstrom et al, 2006; Naghdi et al, 2003), and effects on memory. The current study is the first to demonstrate that two day treatment with androgens in female rats enhance both spatial and nonspatial memory performance, using object placement and object recognition tasks, respectively. Furthermore, results revealed that the androgens, DHEA, TP, DHT, and AD did not affect anxiety levels on the elevated plus maze. Thus, unlike other studies (Frye and Lacey, 2001; Edinger and Frye, 2004; 2005; 2006; Frye and Seliga, 2001), the memory enhancing effects of these androgens do not appear to be influenced via effects on anxiety, but effects on mnemonic processes. (See summary of all data from current work in Figure 36).

Physiological measures revealed that DHEA, TP and EB significantly increased uterine weights, compared to control, OVX rats, while there were no increases in DHT and AD treated rats. However, EB treated subjects had uterine weights that were at least twice the weights of all other groups. These results suggest that peripherally, DHEA and TP may be aromatized to estrogen and bind to estrogen receptors, specifically ER-α which is abundantly present in the uterus (Hewitt and Korach, 2003; Shughrue et al, 1998) to produce this uterotropic effect, but a much smaller effect than EB. The

proliferative effects of EB are consistent with other studies (Gibbs et al, 2004; Hajszan et al, 2004). DHT and AD seem to have protective effects on the uterus with weights being much less heavier than other treated groups. This finding suggests that the androgens, DHT and AD may be potential agents for hormone replacement therapy in postmenopausal, without the uterotropic effect that estrogen possesses. The physiological impact of treatment was also assessed by measuring androgen serum levels in subjects. Treatment with TP but not DHEA significantly increased testosterone levels, which is approximately a quarter of the circulating testosterone in male rats (Bowman et al, 2006). Thus, this elevated testosterone level, although much lower than male rats, may account for enhanced nonspatial memory. Furthermore, increased serum levels of DHEAS, the sulfated form of DHEA, with DHEA and TP treatment, and elevated serum levels of AD, but not DHT with AD treatment may be responsible for enhancement in spatial memory.

Results also revealed that similar to two day treatment, acute treatment with DHEA and AD, and TP, immediately post sample trial enhanced spatial memory, and nonspatial memory, respectively. The lack of effect by treatment with androgens delayed 2 hours post sample trial highlights the temporal effectiveness of androgens to facilitate memory. Furthermore, these androgens are effective at activating mnemonic processes necessary for encoding, consolidation, storage, and retrieval of information that occur during and between the recognition trial, and not acting indirectly on psychological performance parameters (Packard et al, 1994; Luine et al, 2003). The current results are consistent with other studies that have shown that estradiol treatment immediately post-training but not delayed post-training enhances spatial and nonspatial memory (Packard and Teather, 1997; Luine et al, 2003; Frye et al, 2004; Walf et al, 2006). Additionally,

treatment with androgens immediately post-training have shown to enhance spatial memory (Frye and Lacey, 1999). It was surprising that unlike subchronic treatment with DHT, and previous findings (Edinger and Frye, 2007; Frye and Lacey, 2001), acute treatment did not enhance spatial memory. If DHT is contrasted with the other androgens investigated, DHT is the only nonaromatizable androgen and this factor may play a role in why two day treatment and not one day, acute DHT enhanced memory. Furthermore, it is possible that a higher dose of acute administered DHT may enhance spatial memory.

Both two day and acute treatment with the androgens, DHEA, TP, DHT, and AD provide novel findings of enhancements in spatial and nonspatial memory. The neural mechanisms involved in these enhancements are yet to be elucidated. Subchronic treatment (2 days of injections, followed by testing 48 hrs later) effects may be mediated via the classical, genomic pathway as these actions develop slowly and take hours before effects are achieved (McEwen and Alves, 1999). Both androgen and estrogen receptors, are present in brain areas involved in spatial and nonspatial memory, CA1, CA3 of the hippocampus, and the frontal cortex (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992; Shughrue et al, 1997; Morris et al, 1982; Broadbent et al, 2004; Ennaceur and Aggleton, 1994; Mumby et al, 2002). Thus androgens may bind on to intracellular, androgen receptors in the hippocampus and frontal cortex to mediate enhancements in memory. Acute enhancements in memory may be mediated by the nongenomic pathway which is rapid in onset and short in duration (Vasudevan and Pfaff, 2007). Thus, it has been evidenced that androgens may bind on to membrane androgen receptors that may activate the MAPK cascade with phosphorylation and activation of MAP kinase and ERK or activation of adenylyl cyclase, cAMP, PKA, or PKC that could act in parallel or by

converging onto the MAP kinase pathway (Kelly and Levin, 2001; Heinlein and Chang, 2002b). Thus, acute treatment with the androgens may be mediating rapid cognitive enhancements via these nongenomic mechanisms. Recently, it has been proposed that there may be crosstalk between the genomic and nongenomic pathways (Luconi et al, 2002). Therefore, androgens may be exerting its memory enhancements not only separately, but via interactions between the two pathways.

Results in Aim 2 are the first to show that DHEA and TP's enhancing effects on spatial and nonspatial memory were due to the action of androgens and not via conversion to estrogen, as effects were not blocked by the aromatase blocker, letrozole. This finding contrasts previous studies that have shown that letrozole, when given in conjunction with DHEA or TP, blocked the increase in spine synapse density in CA1 of the hippocampus, an area involved in memory (Hajszan et al, 2004; Leranth et al, 2004). Consistent with the current data, recent findings have shown that when androgens act as androgens and not via conversion to estrogen, they have protective effects on memory in both humans and nonhumans (Cherrier et al, 2005; Moradpour et al, 2006; Alejamdre-Gomez et al, 2007; Aydin et al, 2008). Specifically, letrozole administered to intact female rats, enhanced spatial memory on the Morris water maze, and also increased NE, DA, and DOPAC in the prefrontal cortex and increased NE and DA in the hippocampus (Aydin et al, 2008). Our neurochemical data show that noradrenergic activity was increased by DHEA in the prefrontal cortex and noradrenergic and dopaminergic activity was decreased by both DHEA and TP in CA1 of the hippocampus. This previous finding (Aydin et al, 2008) supports our data and suggest that DHEA and TP are acting as androgens to alter monoaminergic activity in brain areas mediating cognition.

Taken together, findings in Aims 1 and 2 suggest that the exogenous androgens, DHEA, TP, DHT and AD administered to OVX female rats have restorative effects on memory via an androgenic mechanism. These results suggest an important role for these androgens as potential hormone replacement therapies (HRTs) in postmenopausal women. In postmenopausal women, not only estrogen, but also androgens significantly decline (Labrie et al, 1997a). The decrease in both estrogen and androgens is accompanied by physiological symptoms such as hot flashes, night sweats, vaginal dryness, osteoporosis (Greendale et al, 1999) and decline in cognitive function that can be attenuated by exogenous administration of these steroid hormones (Duff and Hampson, 2000; Smith et al; Krug et al, 2003; Hirshman et al, 2003; 2004). Since the premature discontinuation of the Women's Health Initiative trials because of increased risk of heart disease, stroke, and breast cancer due to estrogen and progesterone replacement in postmenopausal women (Writing Group for the WHI Investigators, 2002; The Women's Health Initiative Steering Committee, 2004), androgen replacement has shown to be a promising agent to be used in postmenopausal women. Androgens have shown to have protective effects centrally in hippocampal neurons (Bastianetto et al, 1999; Pike, 2001; Kimonides) and peripherally in human breast cancer cell lines (Gil-ad et al, 2001; Ando et al, 2002; Poulin et al, 1998; Zhou et al, 2000; Lubet al, 1997), bone density (Davis et al, 1995; Labrie et al, 1997b), cardiovascular variables (Gordon et al, 1988; Arad et al, 1989; Montalcini et al, 2007), and in psychological and psychomatic symptoms, and previous menopausal symptoms mentioned (Morales et al, 1994; Arlt et al, 1999; Montgomery et al, 1987; Sherwin and Gelfland, 1985; Watts et al, 1995; Stomati et al, 2000; Overlie et al, 2001). Together with these findings and the current

findings of memory enhancements with androgens via androgenic mechanisms, these androgens, DHEA, TP, DHT and AD may be potential HRTs in postmenopausal women. Since DHT and AD treatments did not increase uterine weights compared to DHEA and TP, these are more plausible agents for hormone replacement therapy (Figure 36). These are the first studies to investigate and show that the nonaromatizable androgen, DHT and the aromatizable androgen, AD do not have uterotropic effects. In female rats, letrozole given together with DHEA did not increase uterine weight compared to EB treatment (Hajszan et al, 2004). Furthermore, postmenopausal women given testosterone replacement had significant improvements in memory and this finding was unaffected by letrozole (Shah et al, 2006). If DHEA and TP are given as HRT, it is proposed that they are administered with letrozole to protect from uterotropic effects via aromatization to estrogen. However, risks for women using androgen therapy include masculinizing effects such as increase hair growth, deepening of voice, and temporal balding, just to name a few (Davis and Burger, 2003). Although findings from rats cannot be fully extrapolated on to humans, it is proposed that if circulating androgens are kept within the upper limit of the normal physiological range, given in short-term or low doses, or administered together with estrogen, these masculinizing effects are highly unlikely. When treatment is properly managed, the health benefits offered by androgen replacement exceed the potential risks.

The mechanisms involved in subchronic enhancements of memory by androgens were explored in Aims 3 and 4. Results in Aim 3 indicated that DHEA and TP increased both apical and basal spine density in the prefrontal cortex, and only basal spine density in CA1 of the hippocampus. Our findings are consistent with others who found increased

spine density or spine synapse density in CA1 of the hippocampus with two day treatment of estrogen or the androgens, DHEA and TP, respectively in female OVX rats (Gould et al, 1990; Hajszan et al, 2004; Leranth et al, 2004). In the prefrontal cortex of aged female rats, when estrogen levels are lower, spine density decreased compared to young rats (Wallace et al, 2007), while in aged female monkeys, estradiol increased spine density (Hao et al, 2006). In male rats, castration reduced, while DHT or EB increased spine synapse density in the medial prefrontal cortex (Hajszan et al, 2007). Thus, these are the first studies to examine the influences of androgens on spine density in the prefrontal cortex and CA1 of female OVX rats.

The same doses of DHEA and TP that enhanced spatial and nonspatial memory, increased spine density in the prefrontal cortex and CA1 of the hippocampus. Although object placement is dependent on the hippocampus and/or fornix (Ennaceur et al, 1997), it may also rely on prefrontal cortical input (Ennaceur and Aggleton, 1994), while object recognition is less dependent on the hippocampus and requires prefrontal cortcial input (Broadbent et al, 2004; Ennaceur et al, 1997). Furthermore, anatomically, CA1 projects to the medial prefrontal cortex (Swanson, 1981; Hoover and Vertes, 2007), and correlated neuronal firing takes place in the hippocampus and frontal cortex (Degenetais et al, 2003; Jones and Wilson). Therefore, DHEA and TP's increases in spine density of the prefrontal cortex and CA1 of the hippocampus may be working separately and cooperatively in these brain areas to enhance spatial and nonspatial memory. Increase in dendritic spines leads to increased synaptic contacts (Sorra and Harris, 2000; Woolley et al, 1996; Yankova et al, 2001), increased NMDA binding and NMDA mediated excitatory neurotransmission (Weiland et al, 1992; Wong and Moss, 1992; Romeo et al,

2005; Woolley et al, 1997) and increased LTP (Kim et al, 2002; Chen et al, 2006b; Smith et al, 2002) with estrogen and androgen administration, and may be possible mechanisms by which the current androgens, DHEA and TP enhance memory via increases in dendritic spine density.

Studies have shown that spatial learning induced increases in clustering of synaptic active zones and spine density in CA1 (Rusakov et al, 1997; Leuner et al, 2003). Our behavioral experiments in Aim 1 and morphological experiments in Aim 3 were done separately. A recent study in our lab has shown that subhchronic treatment with EB enhanced spatial memory in OVX rats, while there were no increases in spine density of the prefrontal cortex and CA1 for control and EB behaviorally naïve subjects or control and EB treated subjects tested on the behavioral task (Monde et al, 2008). Conversely, EB significantly increased CA1 spine synapse density among behaviorally naïve females, while spine densities did not differ among control and EB females tested on the Morris water maze (Frick et al, 2004). Another study in our lab showed decreased spine density in the prefrontal cortex and CA1 and impaired spatial memory in OVX compared to intact rats (Wallace et al, 2006). These differences in findings between behaviorally tested and behaviorally naïve subjects highlight the importance of investigating the effects of steroid hormones on behavior and morphology separately, as in the current study.

In our last aim, Aim 4, we investigated whether treatment with androgens and estrogen altered monoamine turnover levels in brain areas involved in memory. Generally, in the prefrontal cortex, CA1, CA3, and dentate gyrus of the hippocampus, striatum, and vertical diagonal band, there was a different pattern of noradrenergic,

serotonergic, and dopaminergic turnover levels for the androgens, DHEA and TP compared to EB. For many of these brain areas known to be involved in memory, either DHEA and/or TP increased or mainly decreased monoaminergic turnover ratio levels, while EB had opposite effects. In most of the brain areas investigated, EB increased at least one of the monoaminergic turnover levels. Thus, DHEA and TP may be acting as androgens and not being aromatized in these various brain areas (similar to memory enhancements in Aim 2), and binding on to androgen receptors compared to EB which is most likely binding on to estrogen receptors (Sar et al, 1990; Simerly et al, 1990; Clancy et al, 1992; Shughrue et al, 1997). It must be noted that in the prefrontal cortex, DHEA increased MHPG/NE and 5-HIAA/5HT levels. DHEA also enhanced spatial memory on the object placement task, with not only significantly increased exploration ratios, but also increased exploration time in the sample trial. DHEA subjects had increased entries and time in the open arms of the elevated plus maze compared to controls, although this finding was not significant. Therefore, increased noradrenergic and serotonergic activity in the prefrontal cortex may be a factor for these increased behavioral responses. No studies thus far have explored the effects of DHEA and other androgens on cognitive, motor, and anxiety levels and associated changes in monoaminergic activity in brain areas such as the prefrontal cortex and hippocampus. However, rats introduced to a novel environment, increased turnover ratio levels for both norepinephrine and serotonin in the medial prefrontal cortex (Handa et al, 1997). AD and/or DHT treatments increased mainly noradrenergic activity in the prefrontal cortex, CA1 and dentate gyrus of the hippocampus, striatum, and vertical diagonal band, while no differences in turnover ratio levels were observed in CA3 of the hippocampus. NE, 5-HT, and DA have all been

associated with learning and memory and receptors for these monoamines are present in the brain areas investigated (Collier et al, 2004; Manuel-Apolinar et al, 2005; Mitchell et al, 2005; Lamirault and Simon, 2001; Hotte et al, 2006). There are multiple connections between many of the investigated brain areas, between the hippocampus and prefrontal cortex (Swanson, 1981; Hoover and Vertes, 2007; Degenetais et al, 2003; Jones and Wilson, 2005), and CA1, CA3, and dentate gyrus of the hippocampus (Becker, 2005). Thus, it is plausible that androgen mediated alterations in the monoaminergic systems may interact among these brain areas to play a role in associated enhancements in spatial and nonspatial memory.

A question to be addressed is whether morphological and neurochemical findings may relate to each other. Norepinephrine receptors are found in dendritic spines of pyramidal neurons in the prefrontal cortex (Aoki et al, 1998). Serotonin receptors are also found in spines of the prefrontal cortex, the majority of which are postsynaptic and are predicted to be glutamatergic (Miner et al, 2003). Consequently, reuptake inhibitors which increase levels of neurotransmitters, such as the serotonin reuptake inhibitor, fluvoxamine, increased dendritic spine density, while the norepinephrine reuptake inhibitor increased total length of dendrites and the number of dendrites in CA1 of the hippocampus (Norrholm and Ouimet, 2000). Dopamine receptors in the prefrontal cortex, hippocampus, and striatum are also found in dendritic spines postsynaptically, where most glutamatergic synapses are established (Yao et al, 2008). The current work did not directly investigate androgens' effects on monoaminergic neurotransmitters and how this relates to dendritic spine density in brain areas mediating cognition. However, speculation from previous studies may predict that androgens, particularly DHEA and

TP's alterations in noradrenergic, dopaminergic, and serotonergic turnover levels, predominantly in the prefrontal cortex and CA1 may be occurring in dendritic spines, which in turn may increase spine density, a mechanism which may enhance spatial and nonspatial memory.

The current studies provide a background for which future experiments can be carried out. It would be interesting to investigate whether two day treatment with letrozole, the aromatase inhibitor, given in conjunction with DHEA and TP will cause increases in spine density in both the prefrontal cortex and CA1 of the hippocampus. This experiment may elucidate whether increases in dendritic spine density are due to actions of androgens or via conversion to estrogen. Since two day treatment with DHT and AD enhanced spatial memory, golgi analysis should be performed to determine whether these androgens also increase dendritic spine density in the prefrontal cortex and CA1. In the current findings, DHEA and TP increased spine density in these two brain areas. Furthermore, DHT increased CA1 spine synapse density in female OVX rats (Leranth et al, 2004). Taken together, it is predicted that the androgens, DHT and AD will increase dendritic spine density in both the prefrontal cortex and CA1 of the hippocampus. Both morphological and neurochemical experiments should be done in two day androgen treated subjects that are both naïve and behaviorally tested to determine whether there are differences between the two, since findings in the literature do not show morphological changes in naïve and behaviorally tested subjects or increases in spine density of naïve compared to behaviorally tested subjects (Monde et al, 2008; Frick et al, 2004). Androgen and estrogen receptor binding studies, particularly in the prefrontal cortex and hippocampus, brain areas known to be involved in memory, with two treatment of the

androgens, DHEA, TP, DHT, and AD should be done. These experiments will determine whether androgens are acting as androgens and binding on androgen receptors or being aromatized to estrogen and binding on to estrogen receptors. These future experiments will add to and clarify the current experiments.

In conclusion, the current studies provide novel behavioral, physiological, morphological, and neurochemical information about the cognitive role of androgens. The androgens, DHEA, TP, DHT and AD act activationally to produce these effects in adult female, ovariectomized rats. Both subchronic and acute treatment paradigms enhanced spatial and nonspatial memory. DHEA and TP's action on memory were via androgens and not conversion to estrogen. Androgen increases in dendritic spines and alterations in monoaminergic activity may mediate these enhancements in memory. These findings suggest a potential role for androgen replacement therapy in postmenopausal women, without many of the harmful side effects estrogens possess.

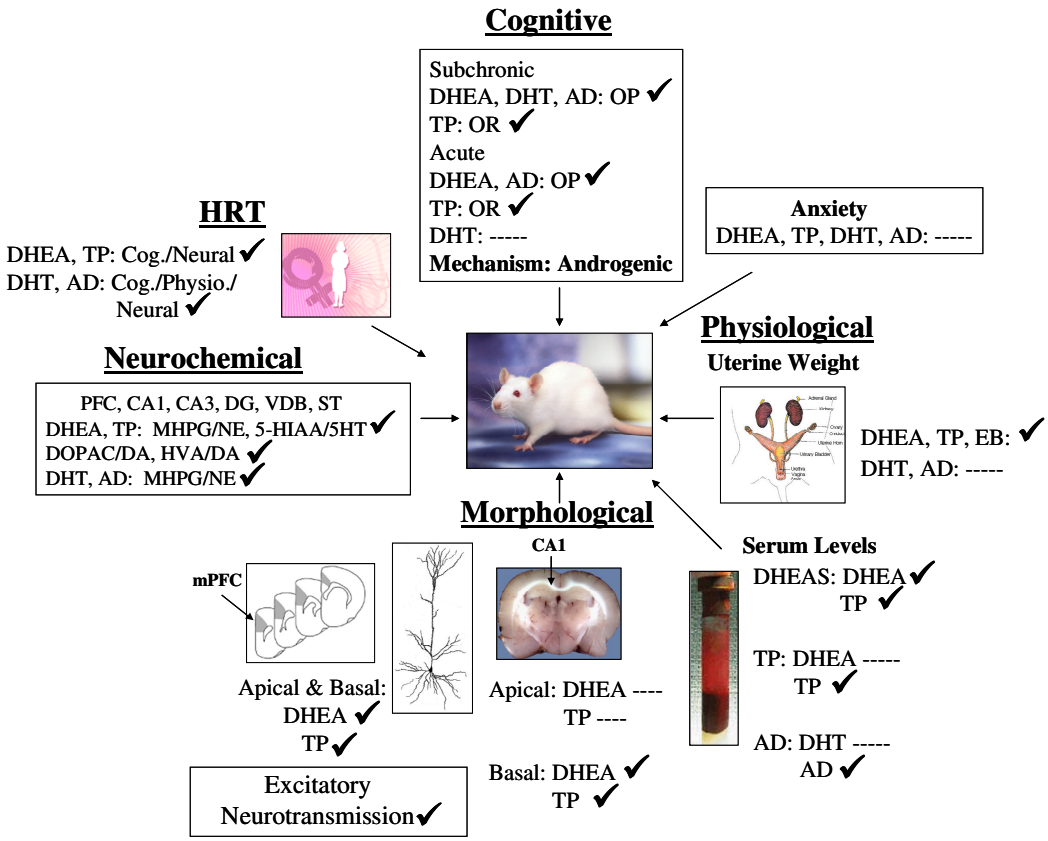



**Figure 36. Summary and model of androgen replacement therapy.**
Cognitive, physiological, morphological, and neurochemical effects of androgens from the current work is summarized. Androgen replacement therapy based on these findings is proposed. (cog.: cognitive; physio.: physiological; HRT: hormone replacement therapy; ✓: increased or enhanced; -----: no difference).

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