# The Role of Androgens in Cognitive Performance in Adulthood and Aging

A Dissertation

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2009

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CERTIFICATE OF APPROVAL

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Acknowledgements:

I would like to thank many people for their help with the work contained in this dissertation. First, I must thank Jacob Raber Ph.D. for his mentorship, undying support, and friendship during my entire graduate school experience. I learned more about the scientific method through working on (so many!) projects with Dr. Raber than I could ever have expected. Dr. Raber has an incredibly open mind and helped me to always see the benefits that my experimental results might have for knowledge. Many thanks to my dissertation advisory committee for their advice on my proposed studies and the results: Jeri Janowsky Ph.D., Steven Kohama Ph.D., Charles Meshul Ph.D., Jacob Raber Ph.D., and Andrey Ryabinin Ph.D. I must also mention specific Behavioral Neuroscience faculty past and present who have had a significant impact upon my mind and knowledge during my training, and who have supported my grant writing and manuscript preparations: Jeri Janowsky Ph.D., Kim Neve Ph.D, John Crabbe Ph.D., Robert Stackman Ph.D., Chris Cunningham Ph.D., and Matt Lattal Ph.D. I must specially thank Steven Kohama Ph.D. for his help with my first manuscript as a graduate student. I would also like to thank the members of the Raber Lab past and present for their help with experiments, and for their willingness to criticize my experimental results and scientific interpretations of data. Specifically I must thank Angela Rizk, Timothy Pfankuch, and Peter Van Meer for their help with experiments, and Jessica Siegel, Laura Villasana, and Brian Piper for their input. A fond thanks to CHAR / B.O.F.S. without whom I would have become a complete science nerd with no prospects of a balanced life (Kimberlee Stafford, my BFF and sometimes attorney, who

taught me my first molecular biology lessons, Steve Tate who has been my unconditional friend for the last 13 years, and Steven Wetzel who is a great friend and an artistic inspiration). In addition, I must give my special appreciation to JAS for her love, support, and companionship. Last, but certainly not least, I want to thank my parents Maureen Benice and Ted Benice Jr. for all of their love, support, mentorship, and attitude-adjustments over the last 36 years. Without their consistent encouragement I would never have entered graduate school much less finished this document.

Figure 3 is adapted from an image in the public domain:
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These experiments were supported by the following grants:
NIH R01-AG20904, EMF-AG-NS-0201, T32-NS007466-05, 5T32AG023477-02, RR-00163, NASA NNJ05HE63G, Alzheimer's Association IIRG-05-14021

Chapter 1: General introduction

**Androgens and their mechanisms of action:**

Androgens are a class of cholesterol-derived hormones, or 'steroid hormones', that are produced from cholesterol by a series of P450 enzyme catalyzed reactions (Figure 1). Androgens are so-called for their ability to interact with the nuclear androgen receptor (AR), a cytosolic ligand activated transcription factor that influences gene expression in many tissues including the brain (Lu et al., 1998). The main androgens, according to this definition, are testosterone (T), and dihydrotestosterone (DHT). Although both T and DHT bind the AR, DHT does so with approximately five-times greater affinity (Grino et al., 1990). AR is widely expressed in the brain and has high expression levels in brain areas that contribute to sexual behavior (Lu et al., 1998). However, AR is also expressed in brain areas that are important for learning and memory such as the amygdala, cortex, and hippocampus (Simerly et al., 1990). Although a complete list of genes that are androgen-responsive under baseline conditions in the brain is not currently available, in ischemic brain 421 genes are up-regulated and 239 genes are down-regulated in DHT replaced adult male rats compared to their castrated counterparts (Cheng et al., 2007). Interestingly, the expression of neurotransmitter receptors and other cell signaling molecules that might be of direct importance to cognition are among these. Specifically, expression of brain-derived neurotrophic factor (BDNF) mRNA, a strong modulator of synaptic strength and plasticity, was increased 5-fold by DHT in this circumstance (Cheng et al., 2007).

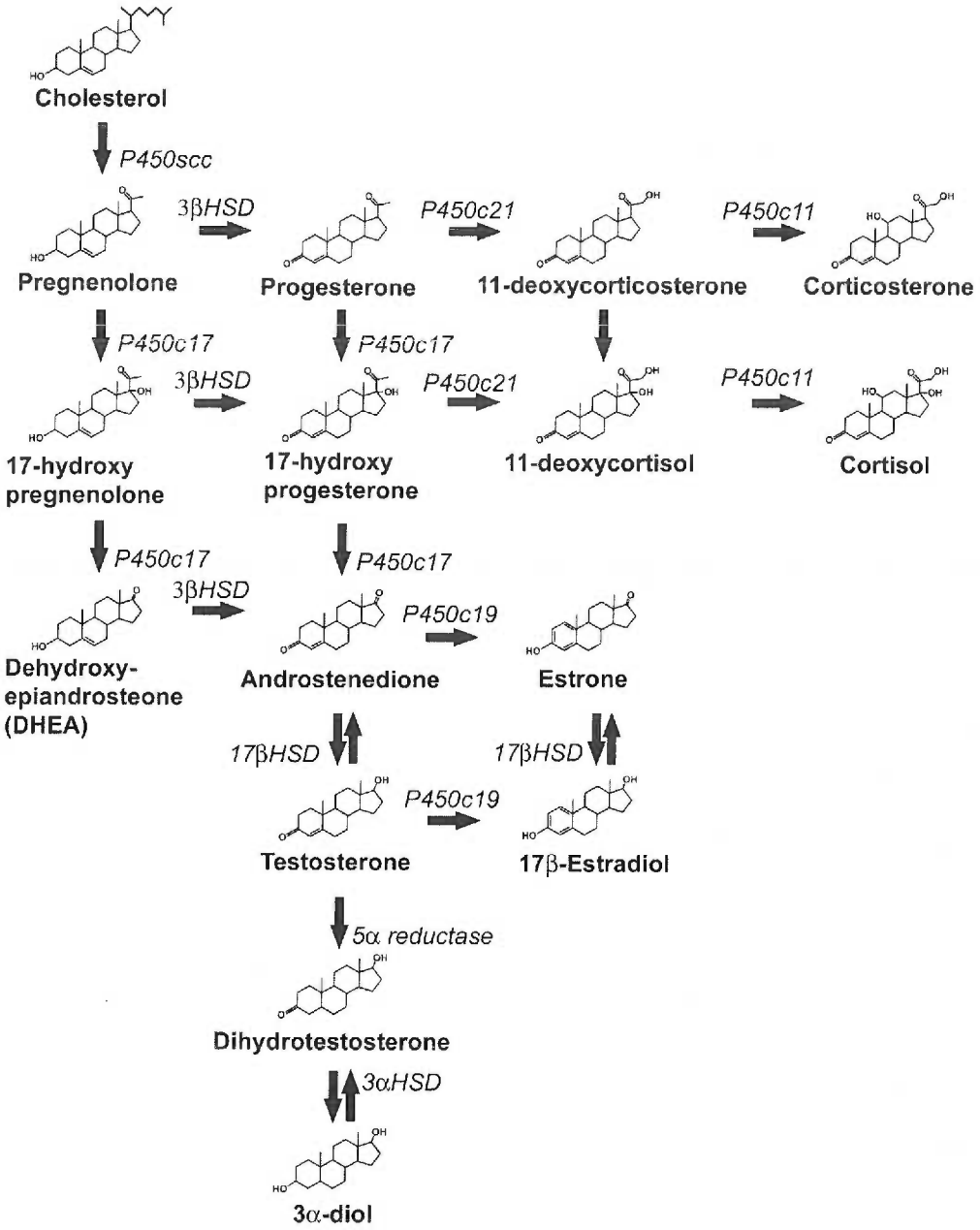


*Figure 1.* Major steroid hormone synthesis pathways, showing chemical structures and synthetic enzymes responsible for each metabolic step. P450scc = Cytochrome P450 cholesterol side-chain cleavage; P450c17 = Cytochrome P450 17α-hydroxylase, c17,20 lyase; P450c21 = 21-hydroxylase; 3βHSD = 3β hydroxyl-steroid dehydrogenase; 17βHSD = 17β hydroxysteroid dehydrogenase; 5α reductase = 5α hydroxysteroid reductase; P450c11 = 11β hydroxylase.

In addition to the classical nuclear receptor pathway, androstenedione and its androgenic metabolites can also affect brain function by other means (Foradori et al., 2008). Ligand-bound ARs can act as protein scaffolds in the cytosol to affect signaling pathways including the mitogen-activated protein kinase (MAPK) pathway and others (Heinlein and Chang, 2002). It has been suggested that other forms of androgen receptors exist beside the classical nuclear receptor, including a cell membrane-bound receptor (Michels and Hoppe, 2008). Finally, the metabolites of the endogenous classical AR agonist DHT can directly affect gamma-amino butyric acid (GABA)-mediated neurotransmission since DHT can be converted to 5α-androstan-3α,17β-diol (3α-diol) which potently and allosterically increases GABA-stimulated chloride influx through type-A GABA receptors (Mellon and Griffin, 2002). Also, DHT can be converted to 5α-androstane-3β,17β-diol (3β-diol), which can activate the estrogen receptor β to affect a different but possibly overlapping set of genes (Handa et al., 2008). *In this dissertation, levels of androgens are manipulated by castration and replacement with exogenous hormones. Since circulating androgen levels were affected globally by these manipulations, any or all of the above androgen actions could play a role in the reported behavioral effects.*

**Anatomical sites and physiological regulation of androgen production:**

T is the most abundant androgen in mammals, with well described metabolic pathways (Payne and Hales, 2004), and the main points will be summarized here. T is produced primarily in the Leydig cells of the testes in males, but in females its production is shared by the ovaries and the adrenal gland. Despite the ability of both

sexes to synthesized T, the level of T circulating in the blood is ~10-times higher in males compared to females. Part of this difference may be due to the lower rate of conversion of androstendione to T and the greater conversion of T to 17β-estradiol in females. The Leydig cells of testes express all of the requisite enzymes to produce T *de novo*, as do the ovaries and adrenal gland. Similarly, the testis of males and adrenal gland of females express the 5-alpha reductase, 3-alpha hydroxysteroid dehydrogenase (3α-HSD), and 3-beta hydroxysteroid dehydrogenase (3β-HSD) enzymes required to metabolize T into DHT, and DHT then into 3α-diol, and 3β-diol respectively. Importantly, T can also be converted to 17β-estradiol by the CYP19 (aromatase) enzyme which is expressed in many tissues including gonads, liver, skin, and brain (Roselli, 2007).

In adult male rodents and humans, T production is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Figure 2), which produces a pulsatile pattern of circulating T secretion. HPG axis control of T pulsatility begins with the activation of neurons that are widely distributed from the basal forebrain to the medial-basal hypothalamus that release gonadotropin-releasing hormone (GnRH) into the median eminence of the pituitary (Gill et al., 2008). GnRH then activates the release of luutenizing hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotropic cells of the anterior pituitary into the general circulation (Robaire B et al., 1995). In the testes, LH acts on Leydig cells via the LH receptor to increase cyclic AMP (cAMP) and subsequently the activation of protein kinase A (PKA). PKA phosphorylates key steroidogenic proteins, leading to the increase in T production (Stocco et al., 2005). However, other extra-testicular molecules, such as epidermal growth factor, can also initiate or enhance T synthesis in Leydig cells (Stocco et al., 2005). T can then circulate

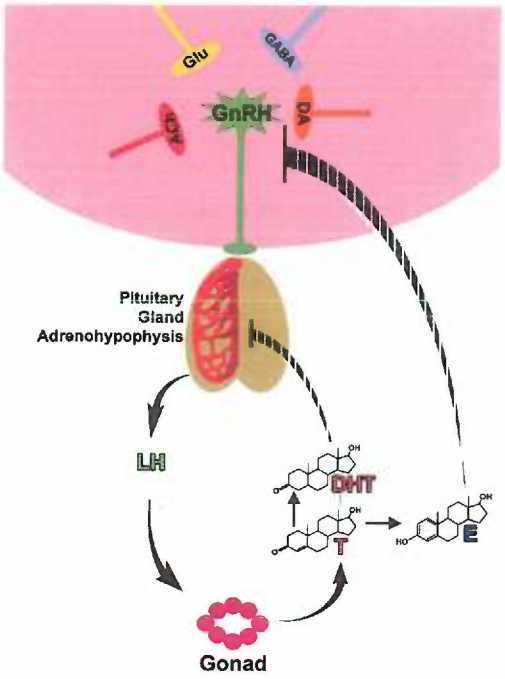


*Figure 2.* Diagram of the hypothalamic-pituitary-gonadal (HPG) axis that is responsible for regulation of androgen synthesis in the gonads (lydig cells in males, follicle cells in females). Glu = Glutamate; GABA = Gamma-aminobutyric-acid; DA = Dopamine; Ach = Acetylcholine; GnRH = Gonadotropic releasing hormone; LH = Leutinizing hormone; T = T; DHT = DHT; E = 17β-Estradiol; Black arrows indicate stimulation, broken arrows indicate inhibition.

back to the pituitary to produce negative feed- back upon the pituitary gonadotropes and reduce LH release (Robaire et al., 1995). Also, if T is converted to 17β-estradiol, either centrally or peripherally, it can then also alter HPG axis by regulating GnRH expression (Wintermantel et al., 2006) and neuron firing patterns (Nunemaker et al., 2002). In addition, T production is modulated by circadian mechanisms (Blank and Desjardins, 1986) and follows a significant diurnal rhythm in both rodents (Prendergast et al., 2009) and humans (Brambilla et al., 2009). However, in humans the peak of T production is in the morning and is in the evening in rodents (Brambilla et al., 2009). Moreover, the role of circadian mechanisms in T production is greater in so-called 'photoperiodic' animals such as Siberian hamsters and voles compared to 'non-photoperiodic' animals such as rats and mice, though manipulations such as olfactory bulbectomy can induce hormonal photosensitivity in rats (Nelson et al., 1982b, Yellon and Tran, 2002). The SCN

expresses AR and the periodicity of cell activity in the SCN is altered by castration and restored by replacement with either T or DHT (Karatsoreos et al., 2007). Also, SCN mediated circadian behaviors such as initiation of the active cycle in rodents is altered by androgen manipulations (Karatsoreos et al., 2007).

In addition to the main steroidogenic organs (gonads, liver, adrenals, skin), most steroid hormones can also be synthesized *de novo* in the brain from cholesterol (Mellon and Griffin, 2002, Payne and Hales, 2004). The notable exception is the corticosteroid pathway where the enzyme CYP21, which converts progesterone to the precursor of corticosterone and also converts 17α-hydroxyprogesterone to the precursor of cortisol, is absent from the brain (Payne and Hales, 2004). In addition, the enzymes 5α-reductase and 3α-HSD are also present in the brain and together can produce 3α-diol from T (Celotti et al., 1997, Kohchi et al., 1998). The steroids synthesized in the brain *de novo* or from steroid precursors are called 'neurosteroids' and are mostly produced secreted by glial cells, although neurons are also capable of synthesizing them (Tsutsui et al., 2000). The most studied neurosteroids are pregnenolone, DHEA, allopregnanolone, and 3α-diol which have been implicated in controlling behaviors such as aggression (Robel et al., 1995) and cognitive performance (Flood et al., 1988, Reddy et al., 1998, Frye and Sturgis, 1995, Darnaudery et al., 2002, Frye et al., 2004). Importantly, neurosteroids can be produced in the absence of peripheral steroid sources, thus castration likely does not remove all androgens from the brain (Robel et al., 1995). Also, androgen precursors such as DHEA are produced in the adrenal glands and can be carried by the blood circulation into the brain to be converted into androgens.

**Androgenic mechanisms in behavioral development:**

T production during gestation is considered the central event that leads to a fetus' anatomical differentiation into the male phenotype, in a process termed 'masculinization', since genetically female fetuses can also be masculinized by androgen manipulation during certain developmental periods (Morris et al., 2004). In addition to the T-dependent masculinization process, early T exposure also blocks aspects of the female differentiation pathway in a process termed 'defemininization' (Schwarz and McCarthy, 2008). In contrast, female differentiation is thought to be the default developmental pathway (Schwarz and McCarthy, 2008), which is supported by experiments blocking hormonal signaling in developing male rats and mice, and by human genetic disorders such as androgen insensitivity. These situations produce a female phenotype despite the presence of the XY male genotype (Zuloaga et al., 2008). Masculinization involves gross anatomical differentiation, including the production of male genitalia, but it also produces lasting differentiation in the brain, setting the stage for male-typical behavior in adulthood (Schwarz and McCarthy, 2008, Zuloaga et al., 2008).

Although cognition is the focus of this dissertation, the role of androgens in the development of sexual behavior must be introduced since sexual behavior is the most well studied and understood aspect of sex-hormone-dependent brain 'organization'. Masculinization and defeminization of sexual behavior has been studied in several species including rodents and primates, and the biochemical pathways responsible for these two processes are relatively well understood and highly species dependent. In rodents, male-typical sexual behaviors include mounting, intromission, and ejaculation,

which are stimulated by elevated T levels and normally occur only in adults (Schwarz and McCarthy, 2008). Female-typical sexual behaviors, including lordosis, are stimulated by increased 17β-estradiol levels and normally occur only in adult females (Schwarz and McCarthy, 2008). The promotion of the development of the former (masculinization) and suppression of the latter (defeminization) behavior in rodents is dependent upon the developmental activity of the enzyme CYP17 (aromatase), which converts T to 17β-estradiol in the brain during development (Schwarz and McCarthy, 2008). The fact that 17β-estradiol does not masculinize the adult brain, only the developing brain, is because the hormone only acts during a 'sensitive period' in development when neurons are actively dividing and undergoing an apoptotic 'pruning' process. However, androgenic mechanisms also play a role since male mice and rats that lack the androgen receptor show less masculinized behaviors (Zuloaga et al., 2008). In non-human primates however, aromatization of T is not necessary for masculinization and defeminization of the brain (Wallen, 2005). Prenatal administration of DHT to developing monkeys at least partially masculinizes the sexual behavior of females (Wallen, 2005).

Although less studied compared to sexual behaviors, there is also evidence that cognition is influenced by androgens during development. Although the processes of masculinization and defeminization are not clearly demonstrated for cognitive behaviors, adult sex-differences in cognitive behaviors have been documented. In humans, the most well-known finding is that males outperform females on a mental rotation task that requires spatial abilities (Puts et al., 2008). In addition, elevated androgen levels during development, caused by congenital adrenal hyperplasia, tends

to 'masculinize' spatial rotation performance (Puts et al., 2008). A more recent report has suggested that adult women outperform men in an object location memory task (Levy et al., 2005), and verbal memory performance (de Frias et al., 2006).

In animals, male rats may have an advantage over females in both spatial reference memory in the Morris water maze task (Veng et al., 2003) and trial-dependent spatial memory in the radial arm maze (Roof, 1993), but sex differences in these types of tasks have not always been observed, perhaps due to differences in the age of the subjects or the testing procedures (Bucci et al., 1995, Healy et al., 1999, Roof and Stein, 1999). The same controversial male advantage in spatial memory task performance has been reported to exist in mice (Isgor and Sengelaub, 1998, Gresack and Frick, 2003), but not in all studies (Benice et al., 2006), and may be dependent on the way the task is administered (Lamberty and Gower, 1988, Berger-Sweeney et al., 1995). One study has suggested a male advantage in object recognition memory (Frick and Gresack, 2003), but this result was not replicated by one of our own studies (Benice et al., 2006). Sex differences in young adults are not well documented for passive avoidance memory tasks, but we found that old female mice perform worse than age-matched males (Benice et al., 2006).

Developmental androgen production may play a significant role in patterning cognitive sex differences in rodents. For example, administration of T to developing female rats can improve spatial reference memory performance in adulthood (Isgor and Sengelaub, 2003), whereas neonatal castration impairs spatial reference memory performance in males (Isgor and Sengelaub, 2003). Although these effects might rely mostly on aromatization of T to 17β-estradiol, AR signaling does play a role since

dominant negative mutations of the AR in rats (Zuloaga et al., 2008) and mice (Rizk et al., 2005) results in disrupted spatial memory performance. Similar studies to elucidate the developmental role of androgens in novel object recognition or passive avoidance memory performance have yet to be performed. *Finally, It is possible that androgen exposure during early development might affect cognitive performance in old age. In addition, having higher androgen levels in old age might be protective against cognitive decline. One aim of this dissertation is to explore how sexual differentiation affects the rate of decline in cognitive performance with aging.*

**Role of androgens in adult cognitive behavior:**

In addition to influencing the brain in early development, androgens also play a role in adult cognition. Although the literature as a whole supports such a role in humans, the effects of androgens on cognition are complex and thus there are significant disagreements between studies. The reasons for these disparate results seem to stem from methodological differences in controlling systematic variables such as sex, age, hormone status, species, the cognitive tasks used and the cognitive domains that the tasks likely assess. In humans, suppressing T production in men is reported to disrupt performance on a verbal memory task in healthy young men (Cherrier et al., 2002). However, in older men the evidence is less clear. For example, a cross-sectional study reported lower verbal memory scores in men with prostate cancer undergoing androgen-deprivation treatment compared to older men without prostate cancer (Bussiere et al., 2005), but a longitudinal study including only prostate cancer patients failed to find significant changes (Salminen et al., 2003). In addition, in the latter

study, androgen-deprivation improved performance on some cognitive tasks measuring object recognition and semantic memory (Salminen et al., 2003). Similarly, measures of spatial performance have been shown to improve with T supplementation in healthy older men, (Cherrier et al., 2001), but not in healthy young men (Cherrier et al., 2002).

For women, the role of androgens in cognition is even more controversial. A recent study suggests that increased T can be detrimental to performance on some memory tasks, but again, the results are confounded by the comparison of healthy women to women of a patient population (Schattmann and Sherwin, 2007). Studies of T supplementation in post-menopausal women have largely failed to show therapeutic effects on cognitive performance (Hogervorst et al., 2005, Wierman et al., 2006). Nevertheless, past studies have shown some improvements in cognitive performance (Sherwin, 1988) as well as improvements in sexual function and mood (Sands and Studd, 1995) in post-menopausal women given androgen supplements. However, the effects of T on cognition in women and men is complicated by the ready aromatization of T to 17β-estradiol. Also, it is possible that androgens are metabolized differently in men and women, and that androgenic mechanisms might operate differently in male and female brains resulting in sexually differential cognitive responses to androgen treatments. To date, no studies have directly assessed the potential of androgens, apart from aromatization, as a target for cognitive enhancement in women. Few studies have measured cognitive effects of androgen manipulations in young women.

In rodents, the majority of evidence suggests that androgens modulate performance on so called 'spatial working memory' or 'trial-dependent spatial memory' tasks in which animals must use new spatial information that is learned in each trial

(Spritzer et al., 2007). In contrast, there is little evidence supporting any effect of androgen manipulations on performance of spatial 'reference memory' tasks in which the same information is repeatedly learned (Spritzer et al., 2007). Castration disrupts acquisition of T-maze delayed alternation performance in rats, in which the animals must remember which location in the maze they entered on the preceding trial and choose the other arm to receive a reward (Kritzer et al., 2001). Castration also causes rats to commit more errors in a T-maze delayed matching to place task, which also involves remembering the location chosen on the preceding trial, but animals instead choose to enter the same location during the current trial, with increasing delays between the first and second trials (Gibbs, 2005). These impairments are reversed by replacement with T, but not by giving 17β-estradiol, suggesting that the effects are androgenic and not estrogenic (Kritzer et al., 2001, Gibbs, 2005). Castration in rats also increases working-memory errors in the radial arm maze (Spritzer et al., 2007). The radial arm maze task requires animals to remember which maze locations have already been visited in the current trial in order to avoid the error of re-entering those locations. Similarly, castration disrupts delay-dependent spatial memory in a Morris water maze delayed matching to place (DMTP) task in rats, and these impairments are reversed by replacement with T (Sandstrom et al., 2006). In this task, animals must remember a spatial location within a pool of opaque water and then return to that location after a delay. However, it is unclear whether this result is mediated by aromatization to 17β-estradiol. Although the effects of androgens on spatial cognitive performance in male rodents is fairly consistent, androgens can also modulate other behaviors such as measures of anxiety and avoidance learning in rats (Frye and Lacey, 2001) and little is

known about how androgens might affect non-spatial abilities in mice. *One major aim of this dissertation is to elucidate the effects of androgen manipulations on both spatial and non-spatial cognitive performance in male mice. To do this, cognitive performance in castrated mice is assessed on a variety of behavioral tasks including open field and elevated zero maze to assess measures of anxiety, trial-dependent spatial memory, spatial reference memory, novel object recognition, and passive avoidance memory. It is hypothesized that removal of androgens worsens cognitive performance specifically in a trial-dependent spatial memory task. Then, castrated mice are given androgen replacement with DHT and are tested in the cognitive task most sensitive to castration. This experiment provides evidence that androgens, apart from aromatization, support cognitive performance.*

**Potential mechanisms for androgen effects on cognition:**

There are several mechanisms whereby androgens could modulate cognitive performance in adult animals. Androgens play a significant role in the developmental patterning and maintenance of morphological sex differences in brain regions important for cognition, such as the hippocampus and amygdala (Isgor and Sengelaub, 2003, Cooke, 2006). Also, changes in androgen levels have been linked to neurochemical alterations in aspects of several brain neurotransmitter systems such as glutamate, GABA, dopamine, and acetylcholine in adult animals (Pouliot et al., 1996, Nakamura et al., 2002, Bianchi et al., 2004, Foradori et al., 2007, Kritzer et al., 2007). Finally, androgens have been recently implicated in the survival of newly generated granule-cell neurons in the adult hippocampus, a brain area highly implicated in learning and

memory (Spritzer and Galea, 2007). This latter possibility is the focus of this dissertation.

Adult neurogenesis in the brain provides a potential mechanism whereby androgens could modulate cognitive task performance. Until recently, it was assumed that adult brains do not have the ability to produce new neurons. However, it was discovered in the last century that neurons are produced throughout life in the brains of many species, including rodents and humans, in specific brain regions (Altman and Das, 1965, Eriksson et al., 1998, Ming and Song, 2005). To date, adult neurogenesis in the brain has been definitively demonstrated in the sub-ventricular zone of the lateral ventricles (SVZ) and the sub-granular zone (SGZ) of the granule-cell layer in the dentate gyrus of the hippocampus (Ming and Song, 2005). The new cells produced in the SVZ migrate along the rostral migratory stream to locate in the olfactory bulb, where they differentiate into interneurons. The adult-born neurons originating in the SGZ of the dentate gyrus may participate in cognitive performance, and this dissertation focuses on adult neurogenesis in the hippocampus. Since the hippocampus plays an important role in cognitive function, the production of new neurons in this region in adults suggests that they participate in normative cognitive performance. Although the precise way in which adult hippocampal neurogenesis (AHN) participates in learning and memory is not yet clear, there is evidence linking AHN with performance on cognitive tasks in rodents including spatial memory tasks (Raber et al., 2004b, Villasana et al., 2006). In addition, several theories have arisen that model the potential role for adult hippocampal neurogenesis (AHN) in cognitive processes (discussed below). Importantly, recent studies demonstrate that changes in circulating androgen levels (Spritzer and Galea,

2007), or neurosteroids (Mayo et al., 2001), in rats can alter the survival rate of new hippocampal neurons . However, no evidence exists to link this observation with cognitive performance. *This dissertation investigates androgen effects on adult neurogenesis in the SGZ of male mice, and the potential role that AHN plays androgenic cognitive effects. To do this, the numbers of immature neurons and proliferating neural progenitor cells are quantified in castrated mice that have been previously tested in a cognitive task that is sensitive to castration. If AHN plays a role in the cognitive effects of castration and androgen replacement, then the number of immature neurons and proliferating progenitor cells should likewise be affected by castration. In addition, if AHN mediates androgenic cognitive effects, then inhibition of AHN should likewise affect cognitive performance. To test this, mice received either cranial irradiation or sham-irradiation to inhibit AHN, and they were then tested in a cognitive task that is sensitive to castration and androgen replacement.*

**Anatomy of the dentate gyrus and its importance in spatial memory:**

The dentate gyrus is an integral part of the hippocampal formation (for review see Amaral et al., 2007). The dentate gyrus has three-layer structure: the molecular layer which consists solely of the apical dendrites of the granule cell neurons, the granule cell layer (GCL) which consists of granule cell neurons and inhibitory basket neurons, and the polymorph layer which includes many cell types, mostly inhibitory interneurons, and the axons of the granule cells (Figure 3). The GCL is the seat of AHN and contains ~1 million mature granule cell neurons in rats and mice, which is many

times the number of neurons in layer II of the entorhinal cortex, the main source of efferent excitatory input to the dentate gyrus.

The granule cells, the projection neurons of the dentate gyrus, send unmyelinated axons into the polymorph layer of the dentate gyrus and to the CA3 pyramidal layer of the hippocampus. These so-called 'mossy fibers' consist of an axonal process branching into a large number of collaterals that terminate in the polymorph layer. The terminals of granule cell axons end in large excitatory synapses, with glutamate as the primary neurotransmitter, that innervate the cell bodies and proximal dendrites of the CA3 pyramidal neurons as well as the dendrites of interneurons in the

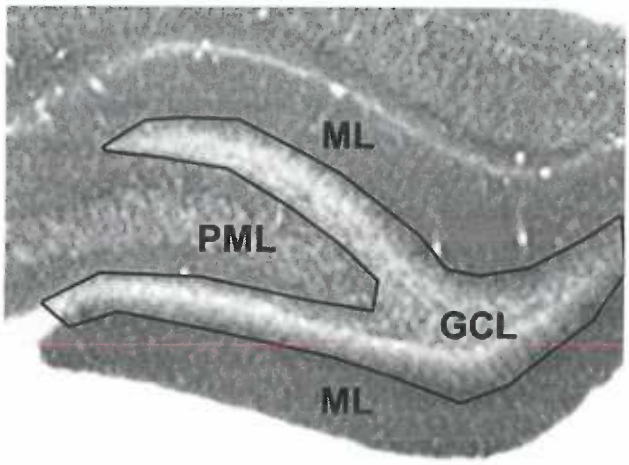


*Figure 3:* Diagram of the anatomical divisions within the dentate gyrus, GCL=granule cell layer, ML= molecular layer, PML=polymorph layer

polymorph layer. The mossy fiber synapses are thought to have a very strong excitatory influence on CA3 pyramidal cells, and one mossy fiber axon can apply excitatory input to CA3 neurons and inhibitory interneurons. However, it is unlikely that two CA3 pyramids will receive input from the same group of granule cell neurons (Kresnor, 2007). The dendrites of granule cells extend in a highly arborized fashion into the molecular layer where they form asymmetric synapses on dendritic spines with

glutamatergic 'perforant path' efferents originating in layer II of the entorhinal cortex and acetylcholinergic efferents from the medial septum, as well as symmetrical inhibitory synapses with inhibitory inputs from GCL basket cells and interneurons in the polymorph layer.

The dentate gyrus can be considered the main input 'gateway' for sensory input entering the hippocampus, and is crucial for spatial memory (for review see Kresnor, 2007). Lesions of the dentate gyrus cause severe deficits in performance in the radial arm maze, DMTP, t-maze alternation, and contextual fear conditioning, with performance resembling complete hippocampal lesions. This is interesting since these are cognitive tasks in which performance is disrupted by castration (see above). *Thus, in this dissertation, it is hypothesized that castration-induced changes in the production of new neurons in the dentate gyrus via AHN may underlie cognitive androgen effects*

**Developmental physiology of adult hippocampal neurogenesis:**

AHN occurs throughout life, however the rate of neuron production decreases dramatically with aging (Kempermann et al., 2002) possibly contributing to age-related cognitive decline. The process of generating new neurons is composed of four stages: proliferation, differentiation, migration, and maturation (Hagg, 2005, Ming and Song, 2005, Suter and Krause, 2008). During proliferation, neuronal stem-cells located in the sub-granular zone (SGZ) between the GCL and the hilus of the dentate gyrus, undergo mitosis. The newly generated daughter cells can then differentiate into 'transit-amplifying cells', or neural progenitors, which are more committed to a neuronal lineage. Neural progenitors can either continue to divide and generate daughter cells or

differentiate further into either immature neurons or glia. Newly formed immature neurons, if they survive, can then migrate up into the GCL from the SGZ. In parallel with this migration process, the immature neurons undergo maturation processes including axon extension and synaptic formations that lead to fully functional connections to the CA3 terminal field.

Each stage of neurogenesis can be affected by factors intrinsic to the brain such as hormones and neurochemicals, and by extrinsic factors such as behavioral experience, stress, exercise and learning. For example, the source of immature granule-cell neurons that are generated in the dentate gyrus by AHN is a pool of pluri-potent stem cells that are partially committed to the neuronal lineage. These stem cells are glial-fibrillary-acidic-protein (GFAP) and nestin-expressing radial glial cells, which differentiate into either a neuronal or glial phenotype. A sub-population of neural stem cells actively proliferate to generate other pluri-potent stem cells. Various endogenous growth factors have been shown to support stem-cell proliferation, including fibroblast growth factor (FGF2) and transforming growth factor-a (TGF-$\alpha$), among others (Hagg, 2005). Also, a confluence of neurotransmitter signaling could account for the localization of stem cell proliferation to the SGZ (Hagg, 2005).

Some stem cells differentiate further into the so-called 'transit-amplifying cells' or 'neural precursor cells', which form the pool of precursors that generate immature-neurons. The neural precursors can continue to proliferate and generate additional neural precursors. As with stem-cells, proliferation of neural precursors is modulated by the availability of growth factors and neurotransmitters (Hagg, 2005, Ming and Song, 2005). In addition, 17$\beta$-estradiol has been shown to transiently increase proliferation

(Ormerod et al., 2003). Some life experiences and activities can also influence proliferation. Physical exercise, environmental enrichment, and learning in some cognitive tasks increase proliferation (Ming and Song, 2005). In contrast, stressful experiences leading to increased glucocorticoid secretion decrease proliferation (Ming and Song, 2005). However, and DHT may not influence neural precursor proliferation (Spritzer and Galea, 2007).

In the SGZ, a majority of neural precursors differentiate into neurons instead of glia. Much work has focused on elucidating the factors modulating neuronal differentiation in embryonic stem-cells (ES cells), as this information is crucial to the development of neuronal-replacement technologies (Suter and Krause, 2008). For ES cells in culture, the important factors seem to parallel those that are important during embryonic development such as bone morphogenic protein inhibition and Notch signaling (Suter and Krause, 2008). In addition, 17β-estradiol increases neuronal determination over glia in rat embryonic stem cell culture, but not in cultures derived from adult animals (Brannvall et al., 2002). Compared to other phases of AHN, little is known about how androgens affect differentiation.

Following determination, new neurons migrate into the GCL of the DG and adopt a mature phenotype complete with appropriate synaptic connections to efferent and afferent targets. During the first month following determination, immature neurons express DCX allowing for their easy immunohistochemical identification. However, less than 50% of new neurons normally survive the first month after neuronal determination (Lehmann et al., 2005). Survival is enhanced following exposure to novel or enriched environments, learning during the performance of some cognitive tasks and with

neurotrophin availability (Ming and Song, 2005). Increased exposure to glucocorticoids, which occurs in stressful situations, decreases the rate of survival of new neurons. A recent hypothesis suggests that, like mature neurons, new neurons require active efferent inputs to survive (Lehmann et al., 2005). In support of this, factors such as exercise and spatial learning which enhance new neuron survival also activate the hippocampus (Lehmann et al., 2005). In addition, T and DHT in male rats have recently been shown to increase survival without affecting proliferation of neural progenitors (Spritzer and Galea, 2007). *This dissertation aims to build on these findings by counting immature neurons and proliferating progenitor cells in the dentate gyrus of adult male castrated or sham-operated intact mice. In addition, the effects of castration on AHN are linked to cognitive performance by measuring these markers of AHN in mice that have been tested in a castration-sensitive cognitive task. The hypothesis is that castration reduces the number of immature neurons in the GCL without affecting the number of proliferating progenitors. In addition, it is expected that inhibition of AHN by cranial irradiation would disrupt cognitive performance in the same cognitive task.*

There are two overarching methods used to measure the effects of hormones or other manipulations on AHN, each having certain strengths and weaknesses (for review, see Ming and Song, 2005). One method involves treating animals with labeled nucleotides, which takes advantage of the fact that neural precursor proliferation requires DNA synthesis, thereby 'marking' cells that are produced at the time of treatment. For example, Altman et al used [$^3$H]thymidine which was injected into rats at various ages and visualized in the GCL by autoradiography. More modern methods use brominated or iodinated nucleotides, such as bromodeoxyuridine (BrdU) to mark

dividing cells, then image the generated cells with immunohistochemistry. Using this method, one can measure the rate of precursor proliferation by killing the treated animals 24-48 hours following treatment and counting the number of marked cells, and one can measure cell survival by killing the animals and counting marked cells at some distal time-point (often ~30 days post-injection). The main advantage of this technique is that one knows the exact date when the marked cells were generated and can therefore accurately follow them to see the effects of factors introduced during different stages of the neurogenesis processes such as proliferation and maturation. However, in the context of behavioral experiments, this can also be a disadvantage since one must know the appropriate moment to inject the animals in order to visualize the cells involved in influencing behavior. Also, the injection procedure itself, which may be stressful or novel, may modify neurogenesis and behavioral outcomes. Another disadvantage of this technique is the difficulty with knowing the lineage of the marked cells, since only nuclei are labeled. This necessitates the parallel use of the second main technique for measuring neurogenesis, neuron- and glia-specific markers to determine the phenotype of counted cells. This second technique uses immunohistochemistry or genetic techniques to label endogenous factors that are specific markers for cells in various stages of neurogenesis. In the present studies, we used immunohistochemistry to label cells expressing doublecortin, a specific marker for immature neurons, and Ki-67-specific marker for actively dividing cells. The main advantages of this technique for the present studies are that the animals do not need to be injected, and we can measure the cumulative effect of hormonal manipulations and behavioral experience on the number of proliferating progenitors and immature neurons

without worrying about which cells should be labeled. The disadvantages are that we cannot know the 'birth-date' of the observed cells and it is difficult to know what stage of neurogenesis is modified by the manipulations. Nonetheless, this endogenous marker technique is preferable because of the above-listed limitations of nucleotide techniques in the context of behavioral experiments.

**The role of adult hippocampal neurogenesis in cognition:**

Although the precise role of AHN in performance on cognitive tasks is not well understood, many studies have linked AHN to cognition. Inhibiting AHN by focal brain irradiation can disrupt performance on cognitive tasks that are sensitive to hippocampal lesions (so called 'hippocampus-dependent tasks), such as spatial memory tasks (Raber et al., 2004b, Villasana et al., 2006, Acevedo et al., 2008b). Genetic manipulations that decrease AHN also disrupt spatial memory in adult mice (Zhang et al., 2008). Similarly, inhibiting AHN by administration of anti-mitotic drugs impairs the ability of rats to acquire trace eyeblink conditioning, a hippocampus-dependent form of learning, but not delay eyeblink conditioning, which is not hippocampus-dependent (Shors et al., 2001). However, other studies have failed to show a decrement in spatial memory performance and other tasks with decreased AHN (Shors et al., 2001, Wojtowicz et al., 2008), or have suggested that decreased AHN can actually improve performance (Saxe et al., 2007). One study suggested that increased AHN in mice is not needed for the enhancing effects of environmental enrichment on spatial memory (Meshi et al., 2006). Inhibition of AHN by irradiation improved performance on a spatial

'working memory' radial arm maze task while having no effect on performance in a spatial 'reference memory' task (Saxe et al., 2007).

Interestingly, cognitive performance on these tasks that are sensitive to changes in neurogenesis are likewise affected by castration in male rats (Gibbs, 2005, Spritzer et al., 2007), indicating that AHN might mediate the cognitive effects of androgen manipulations. The role of AHN in spatial memory might also be time-dependent. One study gave evidence that the process of spatial memory formation relies on a complex pattern of both apoptosis of new neurons early in learning, and survival of new neurons later in the learning process (Dupret et al., 2007).

Regardless of the complicated empirical evidence, some hypotheses have been put forward to explain how AHN might influence cognition. These hypotheses, drawn mostly from computational models, focus on how the formation of immature neurons in the GCL might change the network properties of the hippocampus. In general they support a beneficial effect of AHN on memory, but diverge on what feature of AHN is important in changing hippocampal function. One hypothesis suggests that AHN results in a net addition of new neurons which can increase the memory capacity of the hippocampus and prevent interference between old and new memories (Wiskott et al., 2006). Another focuses on replacement of old neurons with new ones, which might allow replacement of old memories by new ones or prevent confusion between memories that are highly similar (Becker, 2005). Some evidence suggests that immature neurons may be more excitable than mature neurons since they have a high resting potential, and acquire long-term potentiation more easily (Aimone et al., 2006). However, agreement on the latter hypothesis is not universal (Karten et al., 2006).

Nonetheless, a recent hypothesis suggests that immature neurons might allow the dentate gyrus to encode time into new memories by exhibiting overlapping activation during two or more temporally proximal events. Thus, linkage of the encoding of these events occurs in the dentate gyrus, at least while they retain an enhanced excitability (Aimone et al., 2006). However, a downside of this mechanism is that large numbers of immature neurons might lead to too much overlap between new memories, thereby disrupting cognitive performance. Although there is scant evident to empirically support these hypotheses, they will hopefully shape future experiments that will shed light on how AHN affects cognition.

**Changes in androgen production with aging:**

With aging comes a significant alteration in the levels of circulating androgens in both males and females (Brann and Mahesh, 2005), and some evidence links this alteration with age-related cognitive changes (Janowsky, 2006, Driscoll and Resnick, 2007). However, the role of declining androgens levels in age-related cognitive decline remains unclear (Fonda et al., 2005). In addition to reduced total androgen levels, there is an age-related increase in production of the sex-steroid binding globulin, which results in a net decrease in bio-available 'free' androgens age in older men (Harman et al., 2001), and post menopausal women (Laughlin et al., 2000). In males, the circulating T levels decrease steadily over the last half of life, at an average rate of -1.6% per year, which can lead to a state of partial hypogonadism (Sampson et al., 2007). On average however, the overall reduction in T levels is modest. In contrast, females experience a dramatic reduction in circulating 17β-estradiol levels with menopause, starting in middle

age (Brann and Mahesh, 2005). However, T levels decrease most substantially between 20 and 40 years of age, with a shallower age-related decline thereafter (Wierman et al., 2006). The majority of evidence does not support a menopause-related change in androgen levels, however there is a significant decline following hysterectomy (Wierman et al., 2006). This is important since controlled studies of estrogen replacement in aging women have failed to demonstrate cognitive improvement with therapy (Wisniewski et al., 2002). Thus, it is possible that the age-related loss of androgens, not estrogens, plays a significant role in cognitive aging in females. However, it is important to note that there are significant species-differences in age-related reproductive aging. Whereas female humans experience a sudden loss of sex-hormone secretion with menopause, female rodents initially experience a prolonged increase in gonadal hormone secretion and then later a prolonged reduction, ending with much reduced but still existing levels (constant estrous), or consistently high levels (constant diestrous) (Hung et al., 2003). Despite these differences, there is a prolonged withdrawal of androgens in aging rodents that models age-related androgen loss in humans.

The traditionally held mechanisms underlying reproductive aging are currently being challenged in this field and additional mechanisms are coming to light. It is becoming clear that the mechanisms regulating age-related reductions in sex-hormone levels are complicated and likely involve functional degradation at all levels of the HPG axis. Although the mechanisms probably differ somewhat between males and females, they are similar in important ways and these common mechanisms are introduced here. There is evidence for an age-related degradation of GnRH neuronal function that may

be due to aging of the GnRH neurons themselves or changes in their afferent excitatory and inhibitory drive (Brann and Mahesh, 2005). This degradation leads to reduced frequency and amplitude of GnRH stimulation of the pituitary. In parallel, sex-steroid production in the gonads is reduced by loss of secretory capacity and numbers of Leydig cells (males) and follicle cells (females). In addition, the disinhibition of pituitary FSH and LH release, due to age-related decreases in androgens, is less than expected in aging males assuming normal pituitary function. Thus, there may also be pituitary dysfunction with age (Brann and Mahesh, 2005). To date, there has been limited, if any, reports of age-related reductions in the expression of steroidogenic enzymes. In fact, there is a reported increase in aromatase expression in skin and adipose tissue in aging females that contributes to age-related risks for development of breast and ovarian cancers (Cleland et al., 1985). However, since sex-steroids, including androgens, can be synthesized *de novo* in brain tissue, it is important to consider the potential for an age-related decline or compensatory increase in steroidogenic enzyme expression in the brain. *The first aim of this dissertation is to measure sex differences in the rate of cognitive decline in aging in mice. The hypothesis is that male mice would be relatively protected against cognitive decline with age since they have higher levels of circulating androgens. Similarly, it was expected that supplementing aged female mice with T or DHT would enhance cognitive performance. This result would suggest that androgenic mechanisms in the brain might provide a therapeutic target to combat age-related cognitive decline in women.*

**Summary and Dissertation Goals:**

Androgens are ubiquitous steroid hormones that can affect brain function via a variety of mechanisms. Changes in the levels of circulating androgens are associated with alterations in cognitive performance in male and female animals, both during development and in adulthood. One potential mechanism for beneficial effects of androgens on cognitive performance is androgenic support of the survival of immature neurons and neural progenitors in the dentate gyrus of the hippocampus. In addition, if androgens are beneficial to cognition, then they may present a therapeutic target to combat cognitive aging. This dissertation seeks to explore these possibilities in a series of studies, asking the following overarching questions:

1) Is there a sex-difference in age-related cognitive decline in C57BL/6J mice that might be consistent with a protective role of circulating androgens? I hypothesize that, since female mice experience a more severe age-related decline in cognitive performance on a range of cognitive tasks, perhaps because they have lower levels of circulating androgens.

2) Are androgens important for the maintenance of cognition in male mice? To the extent that androgenic pathways are necessary for the support of normal performance on cognitive tasks with age, removing androgens in a healthy adult mouse should cause impairment. I hypothesize that castration of adult male mice causes a decrement in performance on cognitive tasks sensitive to age in female mice, and a deficit in trial-dependent spatial memory as has been observed in rats.

4) Do androgens play a role in supporting AHN and can this potential link explain androgenic cognitive effects? AHN provides a potential mechanism whereby androgens

support cognition. I hypothesize that a) androgens are required for the maintenance of normal levels of AHN in the GCL of the hippocampus, b) the measures of proliferation and neurogenesis would correlate with cognitive task performance, and c) inhibiting AHN by cranial irradiation would impair performance on cognitive tasks that are sensitive to castration.

5) Can androgen supplementation with T or the non-aromatizable DHT improve cognitive performance in old female mice? I expect that supplementation with DHT or T, can improve spatial reference memory and passive avoidance performance, supplying evidence that androgenic pathways serve to maintain cognition during aging, and that they might supply a therapeutic target for age-related cognitive decline.

Chapter 2: Sex-differences in age-related cognitive decline in C57BL/6J mice

*Adapted from:*

**Benice T.**, Rizk A., Kohama S., Pfankuch T., and Raber J. Sex-differences in age-related cognitive decline associated with increased microtubule-associated protein 2 and synaptophysin. *Neuroscience* (2006), 137(2): *413-423.*

**Introduction:**

Understanding the neurobiological effects of aging is becoming increasingly critical since the average age of the human population is increasing. Age-related cognitive decline (ACD) is a particularly insidious problem that can drastically affect quality of life independent of overt physiological disease (Evans et al., 1989). ACD is associated with declines in spatial and non-spatial learning and memory (Small et al., 1995), and with marked impairments in executive functions such as working-memory and attention (Tisserand and Jolles, 2003). Studies of brain morphology and function in ACD have revealed declines in regional brain volume and cortical activation in the pre-frontal cortex and medial-temporal lobe, including the hippocampus (O'Brien et al., 1997, Resnick et al., 2003).

There is evidence for sex-differences in cognitive aging. Women experience a faster rate of decline with age in visual-spatial skills than men (Small et al., 1995) and are also more sensitive to the deleterious effects of apolipoprotein E4 (apoE4, a risk factor for ACD) on cognition (Mortensen and Høgh, 2001). Finally, women are 3-4 times more likely to develop Alzheimer's disease than men (Gao et al., 1998). These

observations, combined with the fact that women possess a longer average lifespan, underscore the importance of understanding the role of sex-differences in ACD.

Rodent models have been used successfully to study the behavioral and neurophysiological changes associated with cognitive aging, but the majority of studies have focused on males. For example, aged male rats have altered hippocampal function (Wilson et al., 2003), and they show marked impairments in spatial place learning as measured in the Morris water maze (Zyzak et al., 1995). In addition to hippocampal dysfunction, aged male rats and mice are impaired in tests that measure frontal cortex mediated cognitive functions (Barense et al., 2002, Magnusson et al., 2003). Regardless of the focus on males in the cognitive aging literature, some studies have repeatedly shown age-related declines in learning and memory in female rats (Markowska, 1999, Bimonte et al., 2003, Foster et al., 2003) and mice (Frick et al., 2000, Grootendorst et al., 2004). Likewise, in transgenic models of Alzheimer's disease, females show amyloid plaque deposition at a younger age compared to males (Lee et al., 2002b), as well as an earlier onset of cognitive impairment compared to males in some paradigms (King et al., 1999). In addition, a few studies have compared the effects of aging on cognition between male and female rats (Markowska, 1999), and have suggested that females decline in cognitive function sooner than males. However, performing these studies in mice is important since mice are most often used in genetic models. One study in mice reported sex-differences in age-related impairments on one measure of Morris water maze performance (Frick et al., 2000). However, as males tend to perform better than females on tests of spatial performance in general (Berger-Sweeney et al., 1995), it is important to also test for potential sex-differences in

cognitive decline using assays that do not involve spatial navigation. We have developed a battery of tests that are sensitive to sex-differences in cognition in mice, and used it here to investigate sex-differences in ACD in mice. We hypothesized that female mice would show a higher rate of ACD compared to male mice as measured by novel location and novel object recognition memory, spatial reference memory, and passive avoidance memory.

**Experimental Procedures:**

Mice:

Twenty young (10 male/10 female, 3-4 months old), twenty-four middle-aged (12 male/12 female, 10-12 months old), and twenty-four old (12 male/12 female, 18-20 months old) C57BL/6J mice (Jackson Laboratories, ME) were kept on 12:12 hr light-dark schedule (lights on at 6 am) with lab chow (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO) and water given *ad libitum*. The mice were purchased at 2 months of age and were raised in our colony with minimal handling. Animals were experimentally naive prior to testing and were housed singly beginning 24 hours prior to the first behavioral test. All procedures conformed to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Oregon Health and Science University.

Determination of estrous cyclicity:

Since estrous cyclicity can influence learning and memory (Warren and Juraska, 1997), vaginal lavage was collected from middle-aged and old mice, and was analyzed as described (Nelson et al., 1982a) with minor modifications in order to assess the cycling status of the aging mice. Vaginal smears were collected every day for 7 days (old mice) or 17 days (middle-aged mice) following the completion of behavioral testing. Smears were put immediately onto slides (Superfrost/Plus, Fisher Scientific, Pittsburgh, PA) and were allowed to dry. They were then stained (Hema 3 system, Fisher

Scientific, Pittsburgh, PA) and cell types were analyzed to determine the phase of cyclicity.

Behavioral testing:

The sequence of behavioral testing was: open field activity (to measure exploratory activity and anxiety-like behavior), elevated zero maze (to measure anxiety-like behavior), location and object novelty recognition (to measure object recognition memory), Morris water maze (to measure spatial learning and memory), and passive avoidance (to measure hippocampus and amygdala-dependent learning and memory).

*Open field activity:*

Open field activity was assessed individually for 10 minutes in a brightly lit enclosure (16 inch x 16 inch square) equipped with a 16 x 16 array of infrared photocells for measuring horizontal movements and quantified automatically by computer (Hamilton-Kinder, Poway, CA). The following parameters were analyzed as outcome measures: Distance moved and percent time spent in the center of the enclosure. Percent time spent in the center of the enclosure was used as a measure of anxiety-like behavior (Clement et al., 2002). After each assessment of open field activity, the equipment was cleaned with 5% acetic acid to remove residual odors.

*Elevated zero maze:*

In addition to the open field, anxiety-like behavior was also assessed in the elevated zero maze (Clement et al., 2002). The elevated zero maze (Hamilton-Kinder,

Poway, CA) consisted of a circular arena (53.34 cm) with two enclosed areas and two open areas of equal size. Mice were placed in the closed part of the maze and allowed free access to all four areas for 10 minutes. A video tracking system (Noldus, Leesburg VA) was used to analyze the mouse movements, and the percentage time spent in the open areas and the number of entries into the open areas was analyzed as outcome measures.

*Novel object and novel location recognition (NO/NL):*

The novel location recognition test assesses the ability of mice to recognize a novel spatial arrangement of familiar objects and is sensitive to hippocampal damage (Save et al., 1992). The novel object recognition test assesses the ability to recognize a novel object in the environment and is unaffected by hippocampal lesions (Ennaceur and Aggleton, 1997). For three consecutive days, the mice were individually habituated to a 16 X 16 inch square-shaped open field with clear plexiglass walls for 5 min (Hamilton-Kinder, Poway, CA). On the fourth day, the mice were first trained in three consecutive 10-min familiarization trials, and then were tested in two consecutive 10-min trials, each with a 5 minute inter-trial interval. For the familiarization trials, three plastic toy objects were placed in the open field (one in each of three corners), and an individual animal was allowed to explore for 10 minutes. All familiar objects were exchanged with replicas in subsequent trials. After three familiarization trials, the mouse was tested in a location novelty recognition test in which one of the familiar objects was moved to a novel location in the arena. The same object was moved to the same new location for every mouse tested. The mouse was then tested in an object novelty

recognition test in which a novel object replaced one of the familiar objects. All objects and the arena were thoroughly cleaned with 5% acetic acid between trials to remove odors.

The time spent exploring each object during the familiarization training and the testing trials was recorded by a trained observer. 'Exploration' was defined as approaching the object nose-first within 2-4cm. The time spent exploring each object, as a percentage of the total exploration time, was calculated for each trial. The difference between the percent time spent exploring the object in the novel location (trial 4) and the percent time spent exploring the same object in its original location on the previous trial (trial 3) was calculated to measure location novelty recognition. The percent time spent exploring the novel object during trial 5 was calculated to measure object novelty recognition.

*Spatial reference memory (Morris water maze):*

*Training:* A circular pool (diameter 140 cm) was filled with opaque water (24°C) and mice were trained to swim to a submerged platform in order to escape from the water. First they were trained with the platform clearly marked by a beacon on the visible platform component (non-spatial training, days 1 and 2), and then they were trained with the beacon removed in the hidden platform component (spatial training, days 3-5) during which the mice had to navigate by using the available spatial cues in the room (posters on the walls and assorted apparatus on benches). There were two daily sessions 3.5 hr apart, each consisting of three 60 second trials (with 10-15 min inter-trail intervals). Mice that failed to find the platform within 60 seconds were led to

the platform by the experimenter where they were allowed to stay for 3 seconds. During the visible platform training, the platform was moved to a different quadrant of the pool for each session. For the hidden platform training, mice were assigned to four groups using a randomized block design (quadrant was the blocking factor) in order to avoid any quadrant bias, and the platform location was kept constant for each group. The hidden platform location was the same as the location in the first visible platform session. Mice were placed into the water facing the wall at the side of the pool in 9 different locations around the pool circumference, and the starting location was changed for each trial. Recordings of the swimming patterns of the mice were sampled six times/second with a Noldus Instrument Ethovision video tracking system (Noldus Information Technology, Sterling, VA). To measure performance in both the visible and hidden trials, the escape latency, cumulative distance to the target (distance to the platform location summed over all samples), and average swim speed were analyzed as outcome measures for each session.

*Probe Testing:* The probe trials were designed to examine the extent of spatial discrimination learning (spatial bias) at three different time points during hidden platform training. To do this, the platform was removed from the pool approximately 1 hour after the last hidden platform training trial of each day of hidden training (total of 3 probe trials), and the time that mice spent swimming in the target quadrant (where the platform was located during hidden platform training), and in the three non-target quadrants (right quadrant, left quadrant, opposite quadrant), as well as the cumulative distance to the area where the platform had been located (cumulative distance to target) was measured over a 60 second trial. Differences in preferential searching were indicated by

differences in cumulative distance to target (swimming closer to where the target had been located during training).

*Passive avoidance learning and memory:*

Passive avoidance learning was measured with a step-through box consisting of a brightly lit compartment and an identical dark compartment connected with a sliding door (Hamilton-Kinder, Poway, CA). Mice were placed individually in the bright compartment and, after a habituation period of 5 seconds, the door was opened into the darkened compartment. Mice, being averse to bright light, were naturally inclined to enter the darkened compartment. When they did so, the door quickly shut and a slight foot-shock was delivered (0.3 mA for 3 seconds). Each mouse was trained with repeated trials (2-5 minute inter-trial interval) until it met a learning criterion of three consecutive 120-second trials without entering the darkened compartment, or up to ten trials, whichever came first. Twenty-four hours later, each mouse was once again placed into the bright compartment, and the latency to re-enter the dark compartment was recorded up to 300 seconds. The number of trials to criterion was used to measure acquisition, and time before entering the dark chamber 24 hours after training was used to measure retention of the avoidance behavior.

Statistical Analysis:

*General*: All data were tested for normality and homogeneity of variance prior to deciding whether parametric or non-parametric tests would be used. To measure the effects of age and sex on exploratory activity, anxiety-like behavior, location and object

novelty recognition, water maze probe trial performance, and passive avoidance acquisition, two-way (3X2) ANOVA was used with age and sex as between-subjects factors. To compare water maze performance curves, three way ANOVAs were used with age and sex as between-subjects factors and session# as a within-subjects factor. Visible and hidden performance curves were compared separately. Since passive avoidance test-day performance is not normally distributed, a Kruskal-Wallis non-parametric test compared overall between the groups and Mann-Whitney U-tests were used for post-hoc tests where appropriate. To compare MAP2 and synaptophysin immunoreactivity, three-way ANOVAs were used with age and sex as between-subjects factors and brain-area as a within-subjects factor. Tukey's HSD post-hoc comparisons were used where appropriate to analyze results from significant ANOVA tests. All tests were conducted using SPSS (SPSS Inc, Chicago, IL) and were considered significant with $p < 0.05$.

*Factor Analysis*: Principle components factor analysis was performed to determine the relationships between performances on the various tasks on the level of individual mice, and was undertaken for two reasons: 1) to determine whether control measures for exploratory or anxiety-like behavior contribute significantly to performance on learning and memory tasks, and 2) to approximate to what extent the learning and memory tasks measure the same underlying abilities in the mice. Measures entered into the model were: percentage time in the center of the open-field, % time in the open arms of the elevated zero maze, % time spent exploring the novel object, % time spent exploring the novel location, latency to enter the dark compartment on passive avoidance test

day, cumulative distance to the target and swim velocity during both the visible and hidden water maze training sessions (average across sessions), and cumulative distance to the target during water maze probe trials (averaged across the three probe trials). Principle components analysis was performed using SPSS, and factors with eigenvalue > 1.0 were considered significant. The verimax rotated matrix was used to interpret the factor loadings.

**Results:**

Reproductive Status:

Middle-aged and old female mice were classified into cycling or acyclic groups based on whether at least one cycle was observed from vaginal smears collected immediately following the testing period. The twelve middle-aged mice as a group were transitioning to acyclicity, as seven were cycling, one had an elongated cycle, and 2 were acyclic during the 17-day test period. The status of 2 mice could not be determined due to poor cytology. In contrast to middle-aged mice, all old female mice were found to be acyclic.

Exploratory activity and anxiety-like behavior:

Middle-aged mice were more active in the open field compared to either young or old mice (ANOVA effect of age, $F(2,62)=13.7$, $p<0.01$, Tukey $p<0.05$ Table 1). Young mice spent more time in the center of the open field compared to old mice (ANOVA effect of age, $F(2,62)=4.75$, $p<0.05$, Tukey $p<0.05$). Also, females spent more time in the center of the open field compared to males (ANOVA effect of sex, $F(1,62)=4.23$, $p<0.05$). In the zero maze, young mice spent more time in the open arms than middle-aged mice (ANOVA effect of age, $F(2,62)=8.12$, $p<0.01$, Tukey $p<0.05$). This was not due to differences in locomotor activity on the zero maze since there was no age or sex difference in distance moved (ANOVA $p>0.05$).

Table 1. Comparison of exploratory activity, anxiety-like behavior, and sensorimotor function between female and male young, middle-aged, and old mice

| | Young (3-4 months) | | Middle Age (10.5 - 12 months) | | Old (18 - 20 months) | |
|---|---|---|---|---|---|---|
| | Male (n=10) | Female (n=10) | Male (n=12) | Female (n=12) | Male (n = 12) | Female (n=12) |
| Open-field Distance Moved (m) † | 5.5 ± 0.23 | 6.0 ± 0.32 | 7.8 ± 0.40 | 9.7 ± 0.67 | 3.7 ± 0.44 | 3.5 ± 0.58 |
| Open-field %time in center (% of total time) ‡,† | 14 ± 0.94 | 14 ± 1.4 | 9.3 ± 3.3 | 13 ± 4.5 | 6.2 ± 1.6 | 11 ± 2.7 |
| Zero-maze entries into open areas (# of entries) | 12 ± 2.1 | 10 ± 3.0 | 12 ± 1.6 | 17 ± 3.1 | 9.2 ± 1.5 | 10 ± 1.3 |
| Zero-maze %time in open arms (% of total time) † | 20 ± 3.2 | 26 ± 4.6 | 9.1 ± 1.2 | 9.7 ± 1.2 | 21 ± 4.9 | 12 ± 2.2 |

All values are mean ± SEM.

† - Main effect of age, $p < 0.05$

‡ - Main effect of sex, $p < 0.05$

Age impairs location novelty recognition :

Old mice had reduced location novelty recognition compared to middle aged or young mice (ANOVA effect of age, $F(2,62)=7.21$, $p<0.01$, Tukey $p < 0.05$), but there was no effect of sex (Figure 4A). Young, middle-aged, and old mice showed equal recognition of the novel object, with no age or sex difference (Figure 4B).

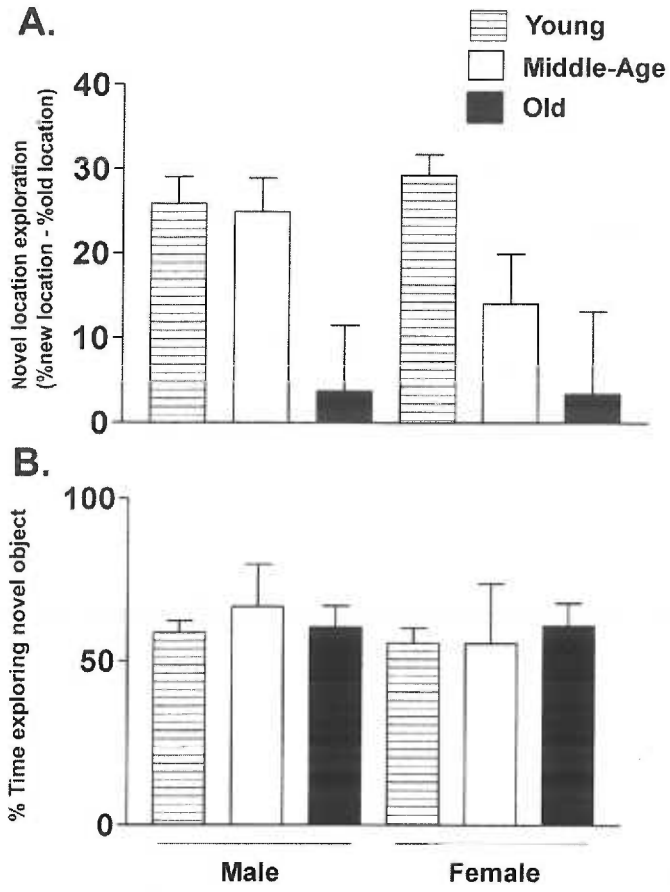


*Figure 4.* Old mice are impaired in location (A), but not object (B), novelty recognition. (young mice n =10 each sex, middle-age and old n=12 each sex). Error bars are SEM.

Female mice have greater age-related spatial cognitive impairments than male mice: Since swim velocity can influence cumulative distance to the target during training sessions, swim velocity was used as a covariate in all statistical analyses. For the visible training (Figure 5A/5B), mice showed significant performance improvement from session-to-session ($F(3, 186)=107.4$, $p<0.001$). However, across sessions old mice swam further from the platform than either middle-age or young mice (ANOVA effect of age, $F(2, 62)=10.5$, $p<0.01$, Tukey $p<0.05$). Also, females swam slightly but significantly further from the platform across sessions compared to males (ANOVA effect of sex, $F(1, 62)=4.80$, $p=0.04$).

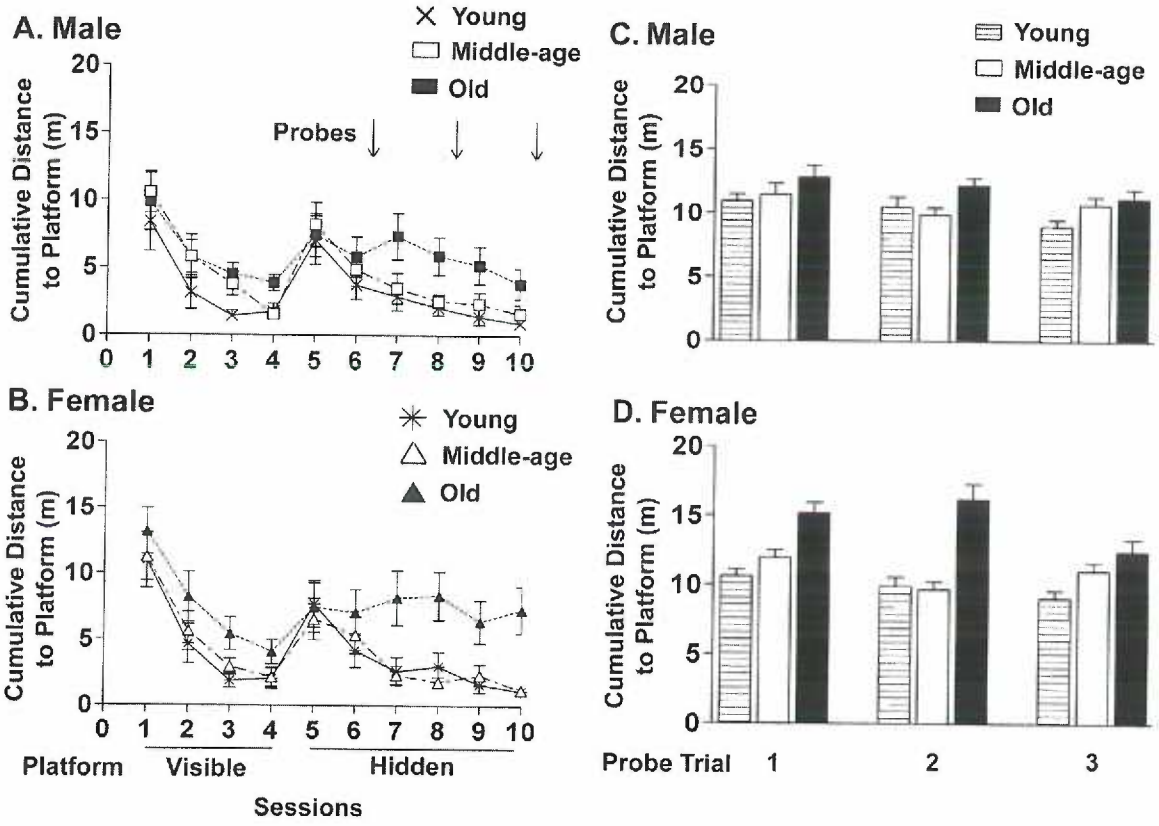


*Figure 5.* Aged female mice show a worse spatial cognitive decline compared to male mice. Young (n = 10 each sex), middle-aged (n = 12 each sex) and old (n = 12 each sex) male and female mice were trained in the visible platform (non-spatial) and hidden platform (spatial) components, and spatial bias was tested using a probe trial after each day of hidden platform training. Shown are the cumulative distance to the target platform during visible and hidden training sessions for young, middle-aged, and old males (A), and females (B), as well as cumulative distance to the target during probe trials for males (C), and females (D). Error bars are SEM.

For hidden training (Figure 5A/5B), old mice did not show a significant performance improvement from session to session, whereas both young and middle aged mice did improve (ANOVA session X age interaction, $F(10, 305)=3.27$, $p<0.01$). Although a sex X age interaction failed to reach significance ($p=0.14$), a sex-difference in the hidden training performance of the old mice did appear when the data was

analyzed separately by sex (two-way ANOVAs with session as within-subjects factor and age as between-subjects factor). For males there was no age-difference in the slope of the hidden performance curves indicating that the old males learned at a similar rate compared to middle-aged and young males (ANOVA session X age interaction n.s., $p>0.2$). In contrast, old females showed a decreased rate of performance improvement compared to middle-aged and young females (ANOVA session X age interaction, $F(10, 150)=2.72$, $p<0.05$).

This sex-difference in age-related performance deficits in the spatial component of the water maze was also reflected in the probe trial performance (Figure 5C/5D). Overall, mice improved in probe trial performance between days indicating that they progressively learned to use a spatial strategy (ANOVA effect of day, $F(2,122)=8.82$, $p<0.01$, Tukey $p<0.05$). Averaged across the three probe trials, old mice swam further from the platform than middle-aged and young mice, and middle-aged mice swam further from the platform than young mice (ANOVA effect of age, $F(2,61)=35.8$, $p<0.01$, Tukey $p<0.05$). There was no overall sex difference in probe trail performance.

Importantly, the effect of age on probe-trial performance was different between the sexes (ANOVA age X sex interaction $F(2,61)=4.15$, $p<0.05$). Whereas for males, the age-difference was small and was only significant between young and old mice (Tukey $p<0.05$ Figure 5C), for females the age-difference was large and was significant between all three age-groups (Tukey $p<0.05$ Figure 5D). In addition, whereas males and females performed similarly in young and middle-age, old females swam significantly further from the platform than old males during the probe trials (Tukey $p<0.05$).

Old female mice are impaired in passive avoidance retention:

For this task, there was no significant age or sex-difference in the number of trials to criterion, (acquisition). In contrast, old female mice showed marked impairment in retention of the avoidance behavior 24 hours after training compared to any other group (Kruskal-Wallis test, $\chi^2$=25.1, p<0.01, Mann-Whitney U test p<0.01 all tests Figure 6): Eleven of the twelve old female mice entered the dark chamber during testing, whereas only 3/12 old males, 2/12 middle-aged males, 4/12 middle-aged females, 1/12 young males, and 2/12 young females entered the dark chamber.

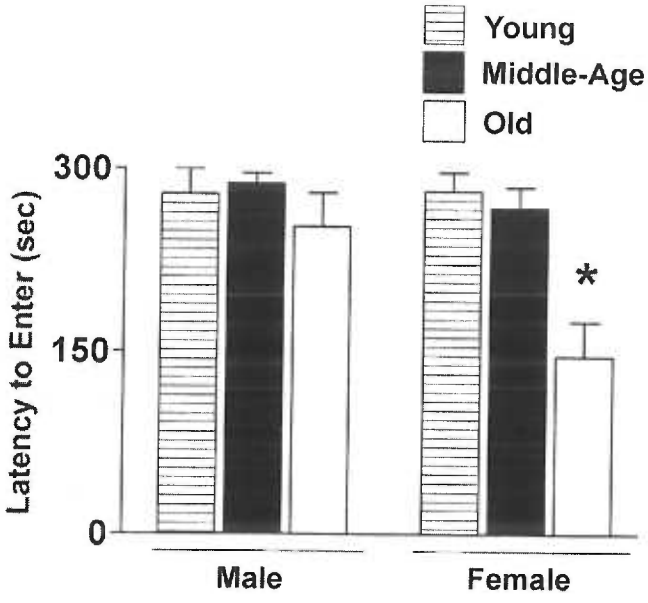


*Figure 6.* Old female mice show impaired passive avoidance behavior 24 hours after training. Shown is the latency to enter the dark chamber on test day, 24 hours after training for male and female young (n=10 each sex), middle-aged (n = 12), and old mice (n=12).. There was no age or sex-differences in the number of trials to criterion during training (acquisition).

*- p < 0.05 by Mann-Whitney U-test

Error bars are SEM.

Principle Component Analysis:

The principle components factor analysis yielded 3 factors with eigenvalue > 1.0, which explained a total of 61.7% of the variance among the measures entered into the model (Table 2). Passive avoidance performance, location novelty performance, water

Table 2. Principle component analysis- component loadings for behavioral measures[1]

| Behavioral Measure | Factor 1 | Factor 2 | Factor 3 |
|---|---|---|---|
| Open Field (% time in center) | -0.236 | -0.187 | -0.749 |
| Zero Maze (% time in open arms) | -0.167 | **0.530** | -0.031 |
| Novel Object Recognition | -0.059 | -0.220 | **0.881** |
| Novel Location Recognition | **-0.617** | 0.115 | 0.306 |
| Visible Water Maze Performance | **0.797** | 0.098 | 0.025 |
| Hidden Water Maze Performance | **0.833** | 0.051 | 0.340 |
| Visible Water Maze Swim Velocity | 0.133 | **0.765** | 0.047 |
| Hidden Water Maze Swim Velocity | 0.116 | **0.886** | -0.032 |
| Mean Water Maze Probe Performance | **0.808** | -0.018 | 0.302 |
| Passive Avoidance Test Performance | **-0.664** | -0.027 | 0.015 |
| Eigenvalues | 3.107 | 1.704 | 1.359 |
| % of Model Variance Explained | 31.07 | 17.04 | 13.59 |

[1]Loadings higher than 0.500 are in bold.

maze probe-trial performance, and averages for both visible and hidden training session performance all loaded onto Factor 1, indicating a common underlying ability being measured by all of these tests. The directions of the component loadings in Factor 1 were such that increasing values of the factor indicate better cognitive performance (increasing latency to enter the dark chamber for passive avoidance, increasing % time exploring the novel location, and decreasing cumulative distance to target for water maze measures). Swim velocity in both visible and hidden sessions loaded on Factor 2,

indicating that swim velocities in the water maze measured an attribute in these mice independent from other water maze measures.

Open field percentage time in center and elevated zero maze percentage time in the open arms loaded separately onto factors 2 and 3 respectively indicating that these two anxiety tests measured different underlying attributes in the mice. Interestingly, Factors 3 also received loading from novel object performance, indicating that there may be an anxiety-related component to performance in the object recognition task.

**Discussion:**

This study demonstrates that the performance of female C57BL/6J mice declines more severely with age in spatial learning and memory and passive avoidance memory tasks compared to age-matched C57BL/6J males. Furthermore, this age-related decline occurs principally between 10-12 months (middle-age) and 18-20 months of age (old). In contrast, female and male mice declined similarly with age in location novelty recognition. Object novelty recognition, as performed in this study, did not decline with age in either sex.

We observed that female mice showed greater age-related decline in spatial water maze performance compared with males, which is consistent with prior reports in rats (Markowska, 1999) and mice (Frick et al., 2000). Frick et al. (2000) reported age-related declines in spatial water maze performance in both male and female mice and observed greater declines in females at 17 months of age in the path-length measure. However, in this study there were no sex differences in escape latency or probe-trial performance for 17 or 25 month-old mice, whereas clear differences were found in our study. There are various possibilities for explaining the disparate results. Frick et al. (2000) tested C57BL/6NIA mice, which may have genetic differences from the C57BL/6J mice that were tested in our study. The discrepancy could also be due to differences in task administration. Whereas Frick et al. (2000) trained mice in the hidden component before the visible component, we did the opposite. In addition to spatial strategies, mice must learn cortically-mediated non-spatial "strategies" in order to be successful in finding the hidden platform (Hoh et al., 2003). Such strategies probably involve learning that the platform exists, and that swimming away from the wall

increases the likelihood of finding the platform (Hoh et al., 2003). Although non-spatial strategies may be learned equally well during either visible or hidden training, it is possible that prior training in the visible component allows the mice to learn non-spatial strategies before subsequently having to learn the spatial strategies. We would speculate that Frick et al. (2000) masked sex-differences in spatial learning by requiring the mice to simultaneously learn spatial *and* non-spatial strategies.

Similar to water maze performance, female mice had a greater age-related decline in passive avoidance retention compared to male mice. To our knowledge, this is the first study to compare age-related passive avoidance performance deficits in male and female mice. It is well known that passive avoidance retention is disrupted by lesions to the amygdala in rodents (Swartzwelder, 1981). However, there is also evidence that it is sensitive to hippocampal lesions, since Ambrogi et al. (1997) found that unilateral reversible tetrodotoxin lesions of the ventral hippocampus, given prior to training, were sufficient to disrupt passive avoidance retention. Likewise, passive avoidance performance loaded on the same factor as water maze hidden training session performance and probe trial performance in the principle components analysis. This suggests that a similar, perhaps hippocampal mediated, ability was gauged by passive avoidance and the hidden water maze. Therefore, our passive avoidance results are consistent with greater age-related hippocampal deficit in female mice.

We found that aging in C57BL/6J mice is associated with impairments in location novelty recognition but not object novelty recognition. This pattern of deficits is consistent with hippocampal dysfunction. Save et al. (1992) demonstrated that hippocampal lesions impair location novelty recognition in a paradigm wherein rats

recognized a change in configuration of five objects in an open field. In addition, object recognition in a two-object paradigm was unaffected by fornix lesions (Ennaceur et al., 1997). Although, to the best of our knowledge, similar studies have not been conducted in mice, it is reasonable to expect that location novelty recognition is similarly hippocampus dependent in mice. Interestingly, in our study both old male and female mice were impaired in location novelty recognition, whereas only old female mice were impaired in water maze and passive avoidance learning, both of which are also sensitive to hippocampal lesions (Morris et al., 1982, Stubley-Weatherly et al., 1996). One explanation for these results could be that old mice have a decreased drive to explore novelty. However, this is unlikely since there was no significant effect of age or sex on total exploration time during the familiarization trials (data not shown), and all groups showed preference for the novel object. Although location novelty recognition is hippocampus-dependent, it is possible that other brain circuitry, involved in this task, is equally susceptible to age-related degradation in males and females. Further studies are required to determine if this is the case.

Principle components analysis revealed that measures of water maze, passive avoidance, and location novelty recognition performance gauge a common underlying ability in the mice studied, and that this underlying ability is unrelated to differences in anxiety-like behavior. However, this common underlying ability remains elusive. One interpretation could be that it reflects "hippocampus-dependent learning and memory" since water maze, passive avoidance, and location novelty recognition are all hippocampus-dependent tasks. However, we must note that both visible and hidden water maze performance were correlated. This may be due to the fact that, although

they did learn, old mice performed slightly worse than young or middle-aged mice in the visible water maze component. This observation probably points to a disruption of non-spatial task learning in the old mice that may have contributed to worse hidden water maze performance. Since the water maze, passive avoidance, and location novelty recognition performance were all degraded by age, another possibility is that the factor analysis simply highlighted some of the underlying cognitive abilities in mice that are susceptible to age-dependent degradation. Another interesting finding is that measures of anxiety-like behavior in the open field and the elevated zero maze seemed to measure different aspects of anxiety, confirming earlier reports of a dissociation in measures of anxiety in these two tests in genetic mutant mice (Rizk et al., 2004).

In conclusion, old, reproductively senescent, female C57BL6/J mice show greater age-related decline in spatial performance and passive avoidance retention compared to aged-matched males, unrelated to anxiety-like behavior. It is tempting to speculate that exposure to higher levels of androgenic hormones T and DHT throughout life in male mice might contribute to resilience of males against ACD. Future studies should elucidate this possibility.

Chapter 3: DHT modulates trial-dependent spatial memory in adult male mice

*Adapted from:*

**Benice T.**, Raber J. Dihydrotestosterone modulates spatial working memory performance in male mice. *Journal of Neurochemistry, In Press.*

**Introduction:**

Androgens are steroid hormones produced mostly in the testes of male mammals and in the adrenal glands of females. Androgens are ligands for the androgen receptor (AR), a cytosolic ligand-activated transcription factor that can influence developmental traits (Hebbard et al., 2003) and also modify physiology and behavior in adulthood (Frye and Seliga, 2001). The highly abundant androgen testosterone (T) circulates in the blood and can be metabolized into dihydrotestosterone (DHT), both of which have the ability to change gene transcription via the AR in various tissues, including the brain. T can also modulate brain function via aromatization to 17β-estradiol, thereby influencing estrogen receptor-mediated gene transcription. Since T readily crosses the blood brain barrier, the potential for androgens to affect cognition has generated much interest in recent years. This is especially true in light of evidence suggesting that reduced androgenic actions in the brain might contribute to cognitive deficits in circumstances such as prostate cancer treatment (Cherrier et al., 2003), Alzheimer's disease (Raber, 2004), and healthy aging (Janowsky, 2006).

There is clear evidence for androgen effects on human cognition in adulthood, and these effects may be domain specific. Suppressing T production in men impairs

verbal memory in some (Cherrier et al., 2002), but not all studies (Salminen et al., 2003). Spatial cognition is likewise affected by manipulations of T levels (Cherrier et al., 2001), but not always seeming to depend on the age of the study population (Cherrier et al., 2002). In some cases, the effect of T supplementation on verbal memory appears to be due to aromatization to 17β-estradiol, whereas the effect on spatial memory is independent of aromatization (Cherrier et al., 2005). Androgens may also improve other cognitive domains such as working memory (Janowsky et al., 2000). Conflicting findings between studies may be due to the choice of cognitive tasks, differences in hormone manipulations, or differences in the populations studied.

In rodents, androgen manipulations modulate trial-dependent spatial memory, in which animals must use new spatial information that is learned in each trial, but not spatial reference memory where the same information is used in each trial to solve the task (Spritzer et al., 2007). Androgens can also modulate other behaviors such as measures of anxiety and avoidance learning in rats (Frye and Lacey, 2001), however, relatively little is known about how androgens might affect non-spatial abilities in mice.

In this study, we asked: 1) whether castration disrupts trial-dependent spatial memory in mice, 2) if castration affects cognitive domains other than trial-dependent spatial memory, and 3) if castration and androgen replacement specifically affect trial-dependent spatial memory performance following various delays between learning and retrieval.

**Experimental Procedures:**

Mice:

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4-6 weeks of age. All mice were housed in groups of 5 with water and food (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO) provided *ad libitum*, and with a constant 12h on/12h off light cycle (on 6:00 am, off 6:00 pm). All procedures conformed to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Oregon Health and Science University.

Surgeries, hormone treatments, and measurement of hormone levels:

All mice underwent surgery at 8-weeks of age and were behaviorally tested at 5-months of age. For the experiment involving DHT replacement, capsules were present from the time of surgery until death, which occurred shortly after the completion of training. All mice were experimentally naive prior to testing and were housed singly beginning 24 hours prior to the first behavioral test to minimize the potential effects of social influences on behavioral performance. Mice were randomly assigned to receive 1) sham surgery (intact), 2) Castration, 3) Castration with DHT replacement. Sham and castration groups were implanted with empty SILASTIC capsules (inner diameter = 1.57mm, outer diameter = 3.18mm, length 2.5 cm). The DHT group received capsules containing DHT (Sigma, St. Louis, MO). DHT was used as it cannot be aromatized to 17β-estradiol, thus ruling out aromatization in any effects of replacement. The method of DHT treatment was based upon previous studies (Raber et al., 2002).

Mice were deeply anesthetized using isofluorane, and a 0.5 cm – 1.0 cm incision was made in the scrotum. Animals receiving sham castration had their testes exposed without removal whereas the castration and castration+ DHT groups had their testes removed by epididymis transection. The wound was then closed with surgical sutures. Implants were embedded immediately following castration or sham surgery. For this, a 0.5 cm opening was made in the skin between the shoulder-blades of each mouse and the capsule was placed subcutaneously. The wound was then sutured closed. To measure the effectiveness of hormone treatment, plasma samples were taken following behavioral testing and were subjected to ELISA analysis for concentrations of T and DHT using commercially available kits (Alpha Diagnostic International, San Antonio, TX) according to the supplier's instructions. For plasma samples, mice were decapitated and trunk blood was taken into Eppendorf tubes containing ~10μL of 5% EDTA solution. The blood was centrifuged for 10 minutes at 16,000 x g. The plasma was then separated and stored at -20°C until use.

Behavioral Experimental Design:

In the first experiment, we investigated whether castration could affect trial-dependent spatial memory performance with a 1-hour retention interval. To do this, castrated ($n$ = 10) and sham-operated intact ($n$ = 10) mice were tested in the DMTP procedure with a 1-minute retention interval and a 1-hour retention interval.

In the second experiment, we studied whether cognitive domains other than trial-dependent spatial memory could be affected by castration. To do this, castrated ($n$ = 30) and sham-operated ($n$ = 30) mice were given placebo capsules and were tested in a

behavioral battery used in our laboratory to measure cognition in mice. This battery of tests is able to measure different cognitive domains, and has been described in detail (Benice et al., 2006). In addition, we measured anxiety-like behavior and motor coordination since these can also indirectly affect performance on cognitive tasks (Benice et al., 2006). Anxiety-like behavior was assessed using the open field and elevated zero maze tests. Object recognition memory, spatial reference memory, and passive avoidance memory were assessed following an early (5-minute), intermediate (1-hour) or long (24-hour) retention interval, with equal numbers of castrated and sham-operated mice being tested at each time point in a between-subjects design. The behavioral tests were performed in the order indicated in Table 3. Because of health problems, 4 mice did not receive all of the tests in the battery. The total number of mice in each group is shown in Table 3.

In the third experiment, we investigated whether castration-induced changes in trial-dependent spatial memory in the delayed matching to place (DMTP) procedure (defined below) could be reversed by hormonal replacement with DHT. Here, castrated ($n$ = 9), sham-operated ($n$ = 9), and castrated with DHT replacement ($n$ = 12) mice were tested in the DMTP procedure. The DMTP procedure was chosen based on the results of the first two experiments. For this, mice were tested following various retention intervals (1 minute, 15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, and 24 hours), counter-balanced to assess the effects of androgen manipulations on trial-dependent spatial memory performance following a variety of increasing retention intervals.

In each DMTP experiment, an additional group of mice received a 'handling only' treatment in which the mice were picked up and replaced to the home cage a number of

times equivalent to the mice that were tested in the DMTP. No data was collected here for these mice; they were included as part of further studies described in Chapter 4.

Delayed Matching to Place Test (DMTP):

This test was used to assess trial-dependent spatial memory after increasing delays between learning and retrieval. There were two separate DMTP experiments that involved separate groups of mice. In both experiments mice learned to locate a submerged platform in a circular pool of opaque water (144 cm diameter). The platform was either unmarked to promote the use of a spatial search strategy, (Hidden Training) or was marked with a visual cue attached to the platform to promote the use of a non-spatial strategy (Visible Training). In both DMTP experiments, separate groups of mice underwent hidden and visible training. During each trial, mice were placed into the water at one of 5 locations against the wall of the pool, in a pseudo-random order. They swam for 120 seconds or until they found the platform. If they did not find the platform within 120 seconds, they were led to it by the experimenter. They were allowed to remain on the platform for 10 seconds.

*DMTP Experiment 1:*

There were 2 sessions per day, each with a different platform location determined in pseudorandom order out of 12 possible platform locations. Each session consisted of a learning trial and a retrieval trial separated by a retention interval. There was a 7-day training phase during which the retention interval was 1 minute. Following

the training phase, there was a 2-day test phase in which the retention interval was increased to 1 hour.

*DMTP Experiment 2:*

This experiment tested trial-dependent spatial memory with various retention intervals between 1-minute and 24 hours. The procedure was the same as in experiment 1 (above) except there was a 22-day test phase in which the retention interval was increased to either 15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, or 24 hours. For practical reasons, the 3 hour, 6 hour, and 24 hour retention interval sessions occurred within 1 session per day. The order of the retention intervals was pseudo-randomized. The inter-session interval was 2 hours during the days with 2 sessions and 16-22 hours during the days with 1 session.

*Data analysis:*

The swim pattern of the mice during each trial was recorded by a video tracking system (Ethovision v2.3, Noldus Information Technology, Wageningen Netherlands), and the latency to find the platform location, in seconds, was calculated since there was no difference in swim speed between the groups. Spatial memory was assessed by the retention score, which was calculated as the difference between the swim time or path-length in the learning trial and the swim time or path-length in the retrieval trial, divided by the total swim time for the session. In other words, the retention score reflected the improvement in swim time between the learning and retrieval trials as a percentage of the total swim time during the session. Thus, better spatial memory corresponded to

higher retention scores. During the training phase, efficacy of training was assessed by examining the Retention Score averaged across the last four training sessions (Sandstrom et al., 2006), and this was used as the baseline DMTP performance. During the testing phase, the retention scores were averaged across all sessions for a given retention interval for each mouse. In the second DMTP experiment, we also analyzed percentage time spent swimming in the periphery of the water maze 10-12 cm in from the outer edge of the pool (thigmotaxis), to account for potential effects of anxiety on DMTP performance (Mendez et al., 2008).

Open Field Test:

The open field task was performed as described in **chapter 2** (pg. 38).

Elevated Zero Maze:

The elevated zero maze task was performed as in **chapter 2** (pg. 38-39).

Novel object recognition memory:

The novel object recognition memory test was performed as described in **Chapter 2**, with some modification. For three consecutive days, the mice were individually habituated to a 16 X 16 inch square-shaped open field with clear plexiglass walls for 5 min (Kinder Scientific). On the fourth day, the mice were first trained in three consecutive 10-min familiarization trials, each with a 5-minute inter-trial interval. There was then a 10-min test trial which occurred after a 5-minute, 1 -hour, or 24-hour retention interval. For the familiarization trials, three plastic toy objects were placed in

the open field (one in each of three corners), and an individual animal was allowed to explore for 10 minutes. All familiar objects were exchanged with replicas in subsequent trials. For the test trial a novel object replaced one of the familiar objects. The same object was always replaced for all mice. All objects and the arena were thoroughly cleaned with 5% acetic acid between trials to remove odors.

The time spent exploring each object during the familiarization training and the testing trials was recorded using a video tracking software that can track the nose point of the mice (Ethovision XT, Noldus, Leesburg VA). 'Exploration' was counted when the nose was in an 'object zone' 3-cm surrounding an object, but the center point of the animal was outside this object zone. This technique has been validated in our lab and allows for reliable scoring of exploration times in the novel object recognition test (Benice and Raber, 2008). The time spent exploring each object, as a percentage of the total exploration time, was calculated for each trial. The percentage time spent exploring the replaced object, averaged over the three training trials, was defined as the baseline preference to account for any potential location bias toward or against the location of the novel object. The percent time spent exploring the novel object during the test trial was calculated to measure novel object recognition memory.

Reference Memory Water Maze:

This test was similar to the delayed matching to place test, except that the location of the hidden platform remained constant from session to session (Benice et al., 2006). Mice received 5 days of training with 2 sessions per day, one in the morning and one in the afternoon with a 2-3 hour inter session interval. There were three 60-

second training trials per session with a 10-15 minute inter-trial interval. Mice that failed to find the platform within 60 seconds were led to the platform by the experimenter where they were allowed to stay for 10 seconds. The water maze was divided into 4 imaginary quadrants for the purpose of testing. The mice were assigned to 4 groups, each of which was trained to find the hidden platform in one of the 4 quadrants according to a randomized block design to avoid any potential quadrant bias. Following the 5 days of training, spatial reference memory was measured using a probe trial, which occurred 5 minutes, 1 hour, or 24 hours following the last training trial. For the probe trial, the platform was removed from the pool and mice were allowed to swim for 60 seconds, starting directly across from the target location.

Video tracking (Ethovision 2.3, Noldus) was used to calculate the time spent swimming before finding the platform (Escape Latency) for each mouse in each trial. To measure learning, the average Escape Latency was calculated within each session for each mouse. Escape latency was used as there were no potentially confounding differences in swim velocity between groups. Spatial reference memory was assessed for each mouse during the probe trial by calculating the distance from the mouse to the target location summed across all samples taken during the trial (Cumulative Distance to the Target). Mice with better spatial reference memory swim closer to the platform location during the probe trial, and thus lower Cumulative Distance indicates better memory. Cumulative Distance to the Target is a sensitive measure of spatial reference memory (Benice et al., 2006). To represent baseline performance in the spatial reference memory analysis, the average cumulative distance to the target during the first hidden session was used.

Roto-Rod:

The Roto-Rod was used to test potentially confounding differences in a variety of proprioceptive, vestibular, and fine-tuned motor abilities (Benice et al., 2006). In this task, mice balanced on a 7 cm diameter rotating rod (Kinder Scientific). After a 1 minute adaptation period without rotation, the rod was accelerated by 5 rpm every 15 seconds and the latency to fall from the rod was recorded. Each mouse received 3 days of roto-rod testing with 3 trials per day and a ~20 min inter-trial interval. The average fall latency for all trials was used as the measure of motor coordination.

Passive Avoidance:

Passive avoidance was performed as described in **Chapter 2**, with some modification. Learning was measured with a step-through box consisting of a brightly lit compartment and an identical dark compartment connected with a sliding door (Kinder Scientific). Mice were given one training trial in which they were placed individually in the bright compartment and, after a habituation period of 5 seconds, the door was opened into the darkened compartment. Mice, being averse to bright light, are naturally inclined to enter the darkened compartment. When they did so, the door quickly shut and a slight foot-shock was delivered (0.3 mA for 3 seconds). After a retention interval of 5 minutes, 1 hour, or 24 hours, each mouse received a test trial. During the test trial each mouse was once again placed into the bright compartment, and the latency to re-enter the dark compartment was recorded up to 300 seconds. The latency to enter the dark compartment during the training trial was used as a baseline measure, and time

before entering the dark chamber during the test trial was used to measure retention of passive avoidance behavior.

Statistical Analysis:

Independent-samples Student's t-tests were used to analyze open field, elevated zero maze, and roto-rod data. Three-way mixed ANOVA was used to measure the effects of training (baseline vs. test performance as a within subjects factor), androgen manipulation (between-subjects factor), and retention interval (between-subjects factor) on object recognition memory, spatial reference memory, and passive avoidance memory. Two-way mixed ANOVA was used to analyze the effects of training session (within-subjects factor) and castration (between-subjects factor) on escape latencies during training sessions in the reference memory water maze test. For the DMTP procedure, two-way mixed ANOVA was used to analyze the effects of androgen manipulation (between-subjects factor) and retention interval (within-subjects factor) on measures of spatial memory and thigmotaxis. One-way ANOVA was used to analyze hormone assay results. Duncan's post-hoc tests, which apply corrections for multiple comparisons, were used to assess differences between individual groups to examine significant effects or interactions where appropriate. All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL), and $p < 0.05$ was considered significant for all tests.

**Results:**

Hormone Levels:

Castration and DHT replacement altered both circulating T and DHT levels compared to sham-operated intact mice (Figure 7). There was an effect of treatment on both T ($F(2,67) = 9.76$, $p < 0.001$) and DHT ($F(2,72) = 10.18$, $p < 0.001$) levels. Post-hoc analysis revealed that castration significantly reduced levels of both T ($p < 0.05$) and DHT ($p < 0.05$) compared to sham-operated intact mice. DHT treatment resulted in levels of DHT that were no different from sham-operated intact levels, and were significantly higher than castrated levels ($p < 0.05$). DHT treatment resulted in T levels that were significantly lower than sham-operated intact mice ($p < 0.05$). T levels were not different between DHT treated mice and castrated mice.

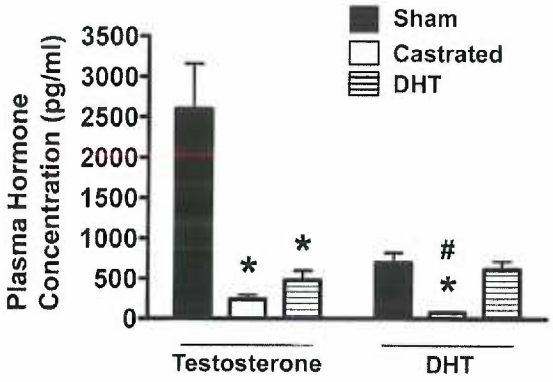


*Figure 7.* Plasma hormone concentrations of sham-operated (Sham), castrated, and DHT treated mice.

* - $p < 0.05$ by Duncan's post hoc test compared to sham-operated control mice.

# - $p < 0.05$ by Duncan's post-hoc test compared to DHT-treated mice.

Castration disrupts spatial memory following a 1-hour retention interval:

The average swim speeds for mice that received hidden training in the DMTP, averaged across all trials, were 18.1 ± 0.83 cm/sec for sham operated intact, 18.8 ± 1.24 cm/sec for castrated, and 20.1 ± 1.24 cm/sec for DHT-treated mice. There were no significant differences in swim speed.

When the platform location was made visible with a beacon, there was no effect of castration on performance (Figure 8A), indicating no difference in task learning between intact and castrated animals. All mice quickly learned to navigate towards the beacon. The average latencies to escape the pool were near asymptote after the first session of training (Figure 8B).

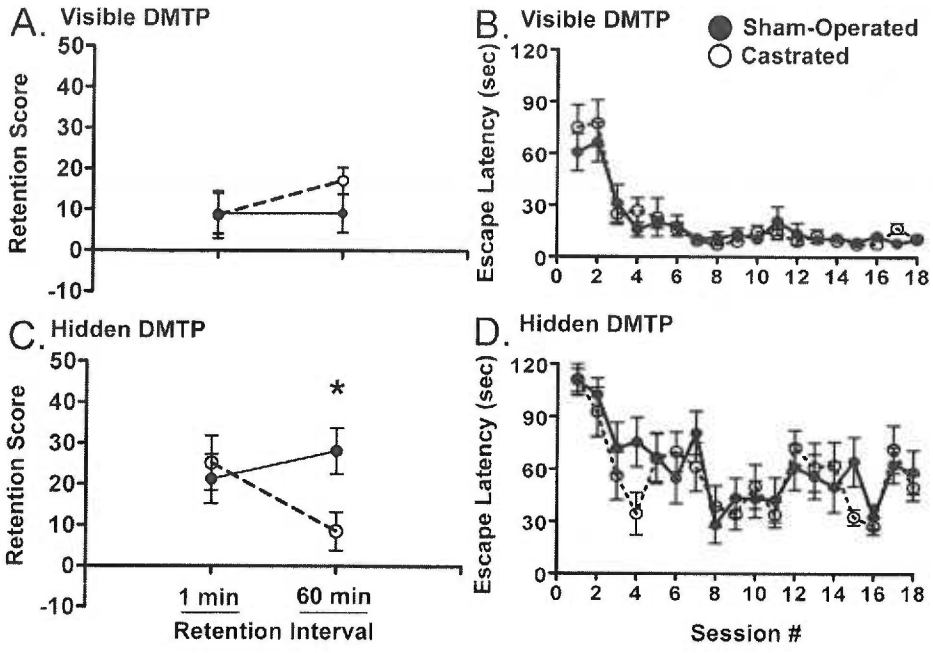


Figure 8. Effects of castration on DMTP performance. Castration did not affect retention scores in visible DMTP (A) or latencies to find the platform during visible learning trials (B). Compared to sham-operated intact mice, castrated mice showed disrupted hidden DMTP retention scores following a 60 minute retention interval, but not a 1 minute retention interval (C). Ability to find the hidden platform during hidden DMTP training trials was not changed by castration (D).

* - $p < 0.05$, castration vs. sham-operated intact mice at the 60 min retention interval by Duncan's Post-Hoc test following ANOVA.

For the group undergoing spatial DMTP, there was no difference in latency to find the hidden platform on the first trial of each session when the platform location was unknown (Figure 8D), indicating that non-spatial strategies were unaffected by castration. ANOVA analysis of Retention Scores showed no main effect of castration, and no main effect of retention interval. However, there was an interaction between castration and retention interval ($F(1,18) = 4.82$, $p < 0.05$). Post-hoc analysis indicated that castration had no effect on DMTP performance during the training phase following a 1 minute retention interval, but castrated mice showed significantly lower Retention Scores following a 1-hour retention interval ($p < 0.05$) (Figure 8C). Similar results were found when retention scores were calculated using path-length instead of escape latency. In this case there was also a significant castration X retention interval interaction ($F(1,18) = 4.45$, $p < 0.05$) with castrated mice having lower retention scores compared to sham-operated mice ($p < 0.05$).

Effects of castration on anxiety-like behavior and motor coordination:

There was a small but significant effect of castration on time spent exploring the center of the open field ($t = 3.22$, $p < 0.05$), and activity in the open field ($t = 3.12$, $p < 0.05$) with castrated mice being less active and spending less time in the center of the arena compared to sham-operated intact mice. However, there was no significant treatment effect on the elevated zero maze. Castration also did not affect motor coordination in the roto-rod test (Table 3).

**Table 3.** Behavioral performance of sham-operated intact and castrated mice in a behavioral test battery assessing measures of activity, spatial memory, non-spatial memory, and motor coordination.

| Behavioral Task | Outcome Measure | Behavioral Dimension Tested | Retention Interval | Sham Mean ± SEM | Castrate Mean ± SEM |
|---|---|---|---|---|---|
| (1) Open Field | Distance Moved (cm) | Locomotor Activity | NA | 1920 ± 72.2 (n = 10) | 1550 ± 90.2 [1] (n = 10) |
| | % Time in the Center | Anxiety-Like Behavior | NA | 15.2 ± 1.07 (n = 10) | 10.6 ± 0.95 [1] (n = 10) |
| (2) Elevated Zero Maze | Distance Moved (cm) | Locomotor Activity | NA | 918 ± 36.9 (n = 10) | 945.2 ± 30.2 (n = 10) |
| | % Time in the Open Areas | Anxiety-Like Behavior | NA | 15.3 ± 1.03 (n = 10) | 12.6 ± 1.32 (n = 10) |
| (3) Novel Object Recognition | % Time Spent Exploring the Novel Object | Object Recognition Memory | Baseline (Old Object) | 38.6 ± 3.16 (n = 30) | 41.0 ± 3.53 (n = 30) |
| | | | 5 min | 61.2 ± 3.22 [2] (n = 10) | 55.7 ± 6.24 [2] (n = 10) |
| | | | 60 min | 46.8 ± 5.61 [2] (n = 10) | 61.4 ± 6.26 [2] (n = 10) |
| | | | 24 hours | 37.7 ± 3.93 (n = 10) | 41.6 ± 6.35 (n = 10) |
| (4) "Reference Memory" Morris Water Maze | Probe Trial Cumulative Distance to the Target (m) | Spatial Reference Memory | Baseline (Session) | 19.7 ± 1.16 (n = 29) | 19.7 ± 1.25 (n = 30) |
| | | | 5 min | 13.5 ± 0.744 [2] (n = 10) | 14.1 ± 0.641 [2] (n = 10) |
| | | | 60 min | 11.9 ± 1.17 [2] (n = 9) | 13.8 ± 1.18 [2] (n = 10) |
| | | | 24 hours | 13.5 ± 0.870 [2] (n = 10) | 10.9 ± 1.19 [2] (n = 10) |
| (5) Roto Rod | Latency to Fall | Motor Coordination | NA | 45.3 ± 1.37 (n = 29) | 49.0 ± 1.40 (n = 30) |
| (6) Passive Avoidance | Latency to Enter the Dark Compartment (sec) | Emotional Memory | Baseline (Training) | 10.1 ± 3.19 (n = 26) | 6.38 ± 2.64 (n = 30) |
| | | | 5 min | 36.0 ± 6.65 [2] (n = 9) | 38.9 ± 7.75 [2] (n = 10) |

| | | | |
|---|---|---|---|
| | 60 min | 53.2 ± 9.74 [2] (n = 9) | 46.9 ± 12.2 [2] (n = 10) |
| | 24 hours | 32.7 ± 13.3 [2] (n = 8) | 57.9 ± 16.3 [2] (n = 10) |

**1**- $p < 0.05$ by Duncan's post-hoc test, compared to sham-operated intact

**2**- $p < 0.05$ by Duncan's post-hoc test, compared to corresponding baseline (training)

The order of behavioral testing is indicated numerically in column 1.

Novel object recognition memory performance unaffected by castration:

For novel object recognition memory, there was a significant effect of training ($F(1, 54) = 38.9$, $p < 0.001$) with mice showing significant preference for the novel object. There was no effect of retention interval. However, there was a significant training X retention interval interaction ($F(2, 54) = 9.87$, $p < 0.001$). Post hoc tests indicated that there was a greater training effect following a 5-minute retention interval compared to a 24-hour retention interval ($p < 0.05$). One sample t-tests revealed that preference for the novel object was significantly greater than baseline preference following a 5-minute ($t(19) = 5.21$, $p < 0.001$) and 60-minute ($t(19) = 3.76$, $p < 0.001$) retention interval, but not following a 24-hour retention interval. However, there was no effect of castration on novel object recognition memory (Table 3).

Spatial reference memory water maze performance unaffected by castration:

For spatial reference memory, there was a significant effect of session during training ($F(9, 513) = 46.4$, $p < 0.001$). Escape latencies decreased across the 10 training sessions, indicating that mice learned to locate the hidden platform over the course of training (Figure 9). In addition, there was no effect of castration on acquisition

of the task during the training sessions. For the probe trials, there was a significant effect of training ($F(1, 54) = 188$, $p < 0.001$) indicating that mice showed significant improvement in cumulative distance to the platform between the first hidden session and the probe trial. However, there was no effect of castration and no effect of retention interval on probe trial performance (Table 3).

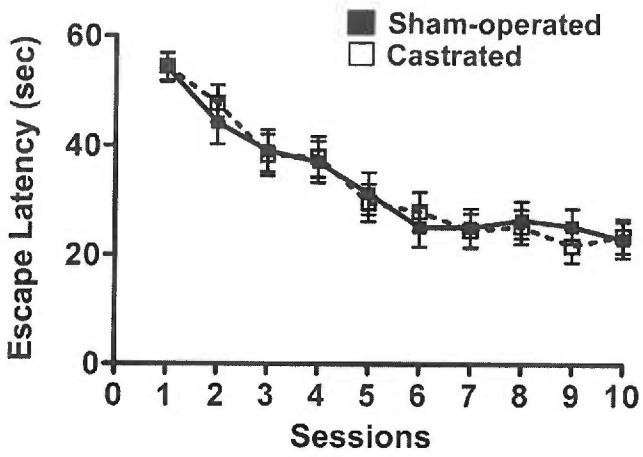


*Figure 9.* Spatial reference memory training performance of sham-operated intact and castrated mice. Both sham-operated intact and castrated mice improved performance over the 10 sessions of training, but there was no effect of castration.

Passive avoidance memory performance unaffected by castration:

For the passive avoidance memory task, there was a significant effect of training ($F(1, 54) = 44.6$, $p < 0.001$). Mice also remained significantly longer in the lit chamber during the test trial compared to the training trials. However, there was no effect of castration or retention interval on passive avoidance memory (Table 3).

Castration impairs, and DHT recovers, spatial memory in the DMTP task following a 24 hour retention interval:

There was a main effect of retention interval ($F(6, 162) = 4.82$, $p < 0.001$), but no main effect of androgen manipulation on performance in the DMPT task (Figure 10).

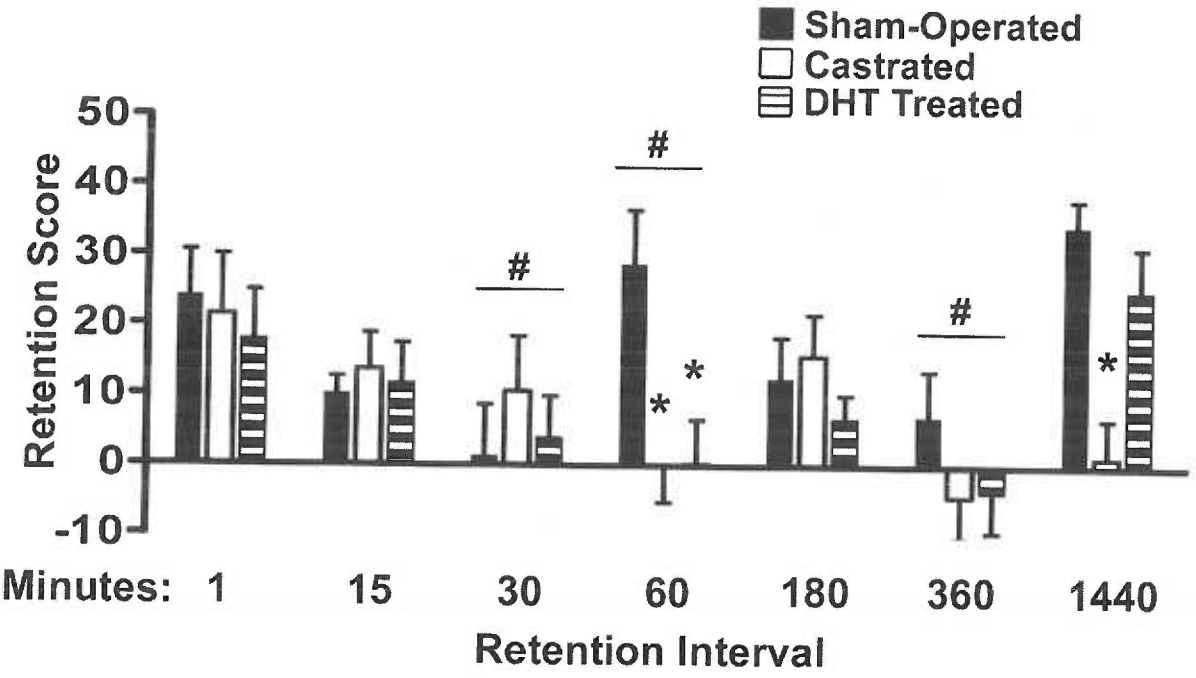


*Figure 10.* Effects of castration and DHT replacement on DMTP performance at various retention intervals. Compared to the sham-operated intact group, both castrated mice and DHT-treated mice had significantly reduced retention scores at the 60-minute retention interval. In contrast, at the 24 hour retention interval, castrated mice had a lower retention score than sham-operated intact mice, but this impairment was recovered in the DHT-treated mice.

# - $p < 0.05$ collapsed across treatment groups compared to the 1 minute retention interval, Duncan's post-hoc test following ANOVA.

* - $p < 0.05$ within a retention interval compared to sham-operated intact mice, Duncan's post-hoc test following ANOVA.

Post-hoc analysis revealed that retention scores were significantly reduced compared to the 1-minute baseline retention interval at the 30-minute, 1-hour, and 6-hour retention intervals ($p < 0.05$, Duncan's post-hoc test). In contrast, retention scores were equally high compared to the 1-minute retention interval at the 15-minute, 3-hour, and 24-hour

retention intervals. There was also an androgen manipulation X retention interval interaction ($F(12, 162) = 2.17$, $p = 0.015$). Post-hoc analysis of treatment-induced differences at each retention interval revealed that compared to the sham-operated intact mice, both the castrated and DHT-treated mice had significantly reduced retention scores following a 1-hour retention interval ($p < 0.05$, Duncan's post-hoc test). However, following a 24-hour retention interval, the castrated mice had significantly lower retention scores compared to the sham-operated intact mice ($p < 0.05$, Duncan's post-hoc test), whereas the DHT treated mice performed as well as the sham-operated intact mice. Also, retention scores were significantly higher in the DHT treated mice compared to castrated mice following the 24-hour retention interval. Retention scores at all other retention intervals, including the 1-minute baseline retention interval, were not significantly different between the androgen treatment groups. There were no significant effects of either castration or retention interval on either retention scores or learning trial escape latencies for the visible platform condition.

There was no effect of androgen treatment ($p = 0.19$) or retention interval ($p = 0.20$), and no interaction ($p = 0.32$), on thigmotaxis.

**Discussion:**

There were three main findings in this study: 1) trial-dependent spatial memory in the DMTP task was sensitive to changes in DHT levels in adult male mice, but this effect was dependent upon the retention interval elapsing between learning trials and retrieval trials 2) behavioral tasks assessing cognitive domains other than spatial memory were unaffected by androgen manipulation, and 3) anxiety-like behavior was affected by androgen manipulation in the open-field test, but not the elevated zero-maze test. Overall, these results are consistent with rat studies of androgen effects on cognition. However, this study adds to the available literature by indicating that the effects of androgens on trial-dependent spatial memory are complex and vary depending upon the time interval elapsed between learning and retrieval.

Castrated mice performed as well as sham-operated intact mice on the DMTP task when the retention interval between learning and retrieval was 1 minute. In contrast, castrated mice had deficits when the retention interval was increased to 1 hour. This was observed during both DMTP experiments. These data are consistent with recent data showing that castration impairs DMTP performance following 1 hour retention interval in rats (Sandstrom et al., 2006). This likely reflects an effect of castration on spatial memory since there was no castration-induced difference in non-spatial strategies or performance in the visible version of the DMTP task. In addition, the effect of androgen deprivation on spatial memory in male rodents is not limited to the DMTP task. Castration causes performance deficits in the t-maze DMTP task (Gibbs, 2005), the t-maze alternation task (Kritzer et al., 2001), and the radial arm maze (Spritzer et al., 2007), all of which measure trial-dependent spatial memory. Combined,

these results lend strong support to a role for androgens in trial-dependent spatial memory performance.

Similar to other studies (Sandstrom et al., 2006, Spritzer et al., 2007) we found that castration did not affect spatial memory performance in the reference memory water maze task, in which animals repeatedly relearn a given location. Thus, our data are consistent with the idea that androgens mainly affect trial-dependent spatial memory where new information is learned in each trial, as opposed to the ability to use already learned spatial information, at least under the conditions of our study. In contrast, castration did not affect performance in the passive avoidance where animals were also only exposed to learned information only once. This could be because passive avoidance may assess a different cognitive domain from spatial memory (Benice et al., 2006), and may be dependent on a different brain circuitry (Swartzwelder, 1981). Thus, our data suggests that androgens may affect new memories in a subset of cognitive domains, as opposed to having general cognitive effects. However, we cannot rule out the possibility that the order of testing in experiment 2 may have modulated the sensitivity of some tasks to castration. For example, castration-related changes in hippocampal synaptic density which might otherwise result in disruption of PA performance (Leranth et al., 2003), might be equalized by previous reference memory water maze testing (Frick et al., 2004). In addition, task difficulty or other procedural differences between cognitive tests may have influenced the sensitivity of performance to castration.

Although we did not observe castration-induced cognitive performance disruption in any test besides DMTP, we cannot rule out potential androgen effects on other

cognitive domains under other testing conditions. For instance, mice lacking apolipoprotein E, an important lipid-transport molecule in the brain, do show spatial reference memory deficits with castration compared to intact counterparts (Pfankuch et al., 2005). In addition, others have found reduced passive avoidance memory in castrated rats (Frye and Seliga, 2001). Since the period of androgen withdrawal was much shorter in this prior study, compared to the 12 weeks in our study, perhaps there are differential effects of castration on passive avoidance following acute versus long-term androgen withdrawal. There could also be a species difference in effects of castration on passive avoidance memory. Rats were used in the previous study (Frye and Seliga, 2001) whereas mice were used in this study. Consistent with this notion, no effects of castration on passive avoidance memory was found in mice lacking apolipoprotein E or expressing apoE3 or apoE4 (Pfankuch et al., 2005). We also found no effect of androgen withdrawal on novel-object recognition memory. Again, novel object recognition memory may rely upon a different brain circuitry compared to spatial memory tasks (Duva et al., 1997). It is interesting to note that retention interval modified performance in the novel object recognition memory task, but not the spatial reference memory task or the passive avoidance memory task. This indicates that the longest retention interval used in this study (24 hours) may not be long enough to observe significant reduction in memory in some tasks. Thus, we cannot rule out a potential effect of castration on spatial reference memory or passive avoidance memory following retention intervals exceeding 24 hours.

Compared to sham-operated intact controls, castration resulted in increased anxiety-like behavior in the open field test as observed in a previous study (Frye and

Seliga, 2001). However, we did not observe increased anxiety-like behavior in the elevated zero-maze test. We have previously reported that these two tests may assess different aspects of anxiety in mice, perhaps because of the drastically different enclosures used and the ability of mice to hide more effectively in the closed areas of the zero maze than in the periphery of the open field (Benice et al., 2006). Thus androgen manipulation may only be effective in modifying some aspects of anxiety, but not others. Nonetheless, androgens can affect the stress response via attenuation of corticotrophin-releasing factor (CRF) in the hypothalamus (Gomez et al., 2004) and by modulating arginine vasopressin (AVP) production in stress-sensitive brain areas such as the amygdala and bed nucleus of the stria terminalis (Vaiu et al., 2001). Since CRF (Muller et al., 2003) and AVP signaling in the brain are central to expression of anxiety-like behavior in mice, it is likely that the loss of androgen suppression underlies increased anxiety-like behavior in castrated mice. Although we cannot rule out an influence of anxiety on the spatial memory impairments observed in castrated mice, it is unlikely. We observed no significant difference in thigmotaxic swimming, a measure of anxiety (Mendez et al., 2008), during training or testing in the DMTP test. Also, the observed increase in anxiety-like behavior in the open field test was a small effect and the effect in the zero maze was not significant. Nonetheless, CRF signaling has been implicated in memory processes (Contarino et al., 1999), as has AVP (van Wimersma Greidanus et al., 1986), thus castration-induced changes in stress-related peptides might contribute to changes in spatial memory.

Since spatial memory performance in the DMTP test was robustly affected by castration, we further investigated the role of androgens in this process by comparing

the performance of castrated mice to that of sham-operated intact mice and castrated mice given androgen replacement with the potent non-aromatizable androgen DHT. Also, this was the first study to measure DMTP performance following a variety of retention intervals ranging from 1-minute to 24-hours. As seen in the first experiment, castration caused a deficit in spatial memory retention following a 1-hour retention interval. In addition, castration caused a deficit in spatial memory following a 24-hour retention interval, but not following shorter retention intervals of 1-minute, 15-minutes, or 30-minutes. In the case of the intermediate retention intervals, it appears that the lack of castration effect may be due to a floor-effect, since sham-operated controls also had low retention scores. DHT replacement did not reverse the castration-effect following a 1-hour retention interval. One possible interpretation of these data is that castration disrupted the formation of long-term spatial memories, and that DHT replacement restored this formation. In this case, perhaps the DHT replaced mice were still unable to adequately retrieve the memories 1-hour after the learning trial. This contrasts with a recent report showing that T replacement reversed the DMTP performance deficit following a 1-hour retention interval in castrated male rats (Sandstrom et al., 2006). Our result using non-aromatizable DHT suggests that this previously reported effect of T may have been be due to aromatization of T to 17β-estradiol. Another possibility is that, since DHT binds to the androgen receptor with greater affinity compared to T, differences in androgenic efficacy between DHT and T may cause differential effects on trial-dependent spatial memory. Perhaps a different dose of DHT or a different time-course of treatment might restore DMTP performance following a 1-hour retention interval. Importantly, DHT did normalize DMTP performance following the 24-hour

retention interval. This indicates that spatial memory is sensitive to androgen replacement following long delays between learning and retrieval. Since we performed the hormone manipulations prior to training in the DMTP task, we cannot rule out the possibility that castration and DHT replacement may have subtly affected learning during the training phase, leading to the observed differences in performance during the testing phase. Also, although the DHT levels of the replaced mice measured following behavioral testing were no different from intact levels, we cannot rule out that the DHT levels may have changed over time. This may have affected the behavioral results.

In conclusion, this study supports a role for androgens in trial-dependent spatial memory performance in male mice. Similar to previous studies, we found no effect of castration on performance in a spatial 'reference memory' task in which the same spatial information is repeatedly learned. In addition, other tasks that assessed cognitive domains such as object recognition memory and emotional memory were unaffected by castration in the conditions of our study. Androgen replacement with the potent AR agonist DHT resulted in reversal of spatial memory impairments following a long retention interval but not following a relatively short retention interval. Taken together, these results suggest that the effects of androgens on cognition may differentially affect spatial memory performance after long delays between learning and retrieval.

Chapter 4: Effects of castration and spatial learning on measures of neurogenesis in adult male mice

*Adapted from:*

**Benice T.**, Raber J. Effects of castration and spatial learning on measures of neurogenesis in adult male mice. *Neuroscience* - Submitted

**Introduction:**

New neurons are produced throughout life in the hippocampal granule cell layer (GCL), a brain region important for spatial learning and memory (Kresnor, 2007). This process of AHN proceeds through stages that include proliferation of progenitor cells, differentiation into neurons, migration of new neurons into the GCL, and maturation of connectivity with afferent and efferent targets (Ming and Song, 2005). Many factors have been identified that increase AHN during these stages either singly or in combination such as environmental enrichment (Kempermann et al., 2002) and hippocampus-dependent learning (Shors, 2004). In contrast, stress (Mirescu and Gould, 2006) decreases the production of new neurons in the GCL. Neurochemical factors such as growth factors (Rai et al., 2007, Zhao et al., 2007), neurotrophins (Lee et al., 2002a), and neurotransmitters (Cameron et al., 1998), can likewise affect the rates of proliferation or survival of new neurons. In addition, steroid hormones such as corticosterone (Mayer et al., 2006, Mirescu et al., 2006), estrogens (Ormerod et al., 2003), and androgens have recently been suggested to affect AHN (Spritzer and Galea, 2007).

Androgens also alter hippocampus-dependent memory formation (Raber et al., 2002, Bimonte-Nelson et al., 2003, Driscoll et al., 2005). In rodents, the hippocampus is required for performance on spatial memory tasks (Morris et al., 1982, Duva et al., 1997, Xavier et al., 1999). Although castration does not affect spatial 'reference' memory performance where the same information is learned repeatedly, castration disrupts newly formed spatial memories in a delay-dependent manner (Sandstrom et al., 2006, Spritzer et al., 2007). Compared to intact rats, castrated rats show more working memory errors in the radial arm maze task (Spritzer et al., 2007), and they exhibit lower retention scores with increasing retention intervals in the DMTP task (Sandstrom et al., 1998). The mechanism for castration induced spatial memory deficits unknown, but some have suggested a role for changes in neurotransmitter levels (Nakamura et al., 2002, Romeo et al., 2005, Leonard et al., 2007), or structural changes such as modulation of spine synapse density in the hippocampus (Leranth et al., 2004a, Leranth et al., 2004b). It is also possible that AHN mediates androgen effects on spatial memory. Adult hippocampal neurogenesis has been implicated in spatial memory performance in mice (Kempermann et al., 2002, Bizon et al., 2004, Raber et al., 2004b). However, the link between androgen-induced changes in neurogenesis and spatial memory has not been investigated.

In this study, we asked whether: 1) castration modulates markers of hippocampal neurogenesis in male mice 2) if androgens are required to produce effects of DMTP training on markers of neurogenesis and 3) if changes in markers of neurogenesis might account for the effects of castration on spatial memory in the DMTP. To do this we used unbiased stereology to measure the number of doublecortin-expressing immature

neurons (DCX+) and Ki-67-expressing proliferating progenitor cells (Ki-67+) in the dentate gyrus of castrated and intact male mice that were previously tested in either a spatial version of the DMTP, a non-spatial version of the DMTP, or that received equivalent amounts of handling only. Similar to rat studies, we previously found that castration disrupted spatial memory following a 1-hour retention interval, but had no effect following a 1-minute retention interval and had no effect on visible DMTP performance (see DMTP Experiment 1 in **Chapter 3**). Here, we hypothesized that castration would reduce the number of immature neurons and proliferating progenitor cells in the GCL, and that the number of immature neurons and proliferating progenitor cells would correlate with spatial DMTP performance. We also hypothesized that hidden DMTP training would increase the number of immature neurons in the GCL compared to either visible DMTP training or handling only, and that castration would inhibit these effects. To test these hypotheses, the number of immature neurons and proliferating progenitor cells were counted in the brains of mice previously tested in the first DMTP experiment in **Chapter 3**. Finally, if neurogenesis mediates castration-induced disruption of spatial DMTP performance, we hypothesized that inhibition of neurogenesis by cranial irradiation with $^{137}Cs$ would have a similar effect.

**Experimental Procedures:**

Mice:

The mice used for the neurogenesis studies were the same mice that were behaviorally tested in the first DMTP experiment of **Chapter 3**. In addition, there was a separate group of mice that received an equivalent amount of handling without being tested in the DMTP test. For the irradiation studies, additional male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4-6 weeks of age. All mice were housed in groups of 5 mice with water and food (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO) provided *ad libitum*, and with a 12h light:12h dark cycle (on 6:00 am, off 6:00 pm). All procedures conformed to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Oregon Health and Science University.

Surgeries, hormone treatments, and measurement of hormone levels:

See **Chapter 3** for methods.

Immunohistochemistry:

Within 24 hours of the last DMTP trial (see **Chapter 3** for the DMTP methods), mice were deeply anesthetized with a cocktail of 100 mg/kg ketamine, 10 mg/kg xylazine, and 2 mg/kg acepromazine and were then perfused transcardially with PBS followed by 4% paraformaldahyde pH 7.4. Brains were removed and post-fixed overnight in 4% paraformaldahyde at 4°C, transferred to 30% sucrose solution for

cryoprotection and stored at -80°C until sectioning. Serial, coronal sections (50 μm) were collected through the entire dentate gyrus for each brain and ten to twelve sections per mouse, with a 200 μm inter-section distance comprising the entire dentate gyrus, were collected onto Superfrost microscope slides (Fisher Scientific). Sections were then stained for fluorescent visualization of either DCX, a marker of immature neurons, or Ki-67, a marker of dividing cells (Ming and Song, 2005). Following an antigen retrieval step according to manufacturer's instructions (H-3000, Vector Laboratories), sections were incubated for 2 hours at room-temperature in a blocking solution (5% normal donkey serum in PBS containing 0.05% Triton X-100 and 0.2% bovine serum albumen (PBT)), followed by incubation overnight at 4°C in either goat-anti-doublecortin (1:200, Santa Cruz Biotechnology) or mouse-anti-Ki67 (1:400, BD Pharmingen) primary antibodies. Following 4 washes with PBT followed by 1 wash with PBS (10 min each), sections were incubated for 2 hours at room temperature in either donkey-anti-goat-IgG or donkey-anti-mouse-IgG antibodies (both diluted at 1:50) conjugated to Texas-red for fluorescent visualization (Jackson Immunoresearch). Following 4 washes with PBT and 1 wash with PBS (10 min each), the sections were covered with anti-fade solution containing a DAPI counter-stain (Vectashield, Vector Laboratories), and were cover-slipped (Fisher Scientific). Slides were stored in the dark at 4°C until they were imaged.

Confocal Microscopy and Unbiased Stereology Analysis:

The total number of DCX+ and Ki-67+ cells in the bilateral granule cell layer (GCL) of the dentate gyrus was estimated, using the optical fractionator technique

(West et al., 1991). Brains from a subgroup of behaviorally tested mice in the first DMTP experiment of **Chapter 3** that received hidden DMTP training (sham-operated $n$ = 4, castrated $n$ = 3), visible DMTP training (sham-operated $n$ = 4, castrated $n$ = 4), or handling only (sham-operated n = 4, castrated $n$ = 4) were selected at random for analysis. Due to higher inter-subject variability in the number of Ki-67+ cells, an additional 2 subjects were counted in each DMTP training group. For DCX, eight to ten stacks of 5 images each (dissectors), with a 2 μm inter-image distance, were taken bilaterally within the GCL of each brain section. Images were collected using the 40x objective lens of an Olympus spinning disc confocal microscope (IX81, Olympus Imaging Corp.), equipped with Slidebook software (Intelligent Imaging Solutions). A square counting frame (75 μm x 75 μm) was laid over the computer screen with the GCL visible within, and cells were counted when they appeared within the frame in one image of the 5-image stack but not in the preceding image. DCX+ cells were counted only when the staining appeared surrounding a distinctly labeled DAPI counter-stained nucleus. This way, a total of 80-120 individual dissectors were counted within each brain to obtain an unbiased and accurate average density of DCX+ cells/$mm^3$ within the GCL of each mouse. Due to the very low density of Ki-67+ cells observed in each section, the total number of cells were counted bilaterally. Ki-67 cells were counted when the nuclear Ki-67 stain overlapped a DAPI counter-stained nucleus. Thus, the entire GCL for each section constituted the dissectors for approximating the density of Ki-67+ cells.

The Cavalieri principle was used to estimate the total volume of the GCL, by outlining the DAPI counter-stained GCL in the Slidebook software to obtain the GCL

area in each section bilaterally. The sum of these areas was multiplied by the inter-section distance (200 μm) and the total number of cells was then obtained by multiplying the average density measurement by the total volume estimate.

Cranial irradiation:

Mice received ionizing radiation from a $^{137}Cs$ source as described, with some modifications (Villasana et al., 2006). Following an i.p. injection of anesthesia (80 mg/kg ketamine (Sigma) and 20 mg/kg xylazine (Sigma), mice were placed in adjustable Plexiglas cylinder tubes (Fisher Scientific) containing multiple holes for breathing. The tubes were vertically placed inside 5 cm thick lead shielding with the back of the mouse head facing the source of the irradiator through a 2 cm diameter hole bored through the lead brick. Dosimetry was performed by putting dosimetry chips ($n$ = 10 in total) in the Plexiglass cylinder tubes near the location of the heads of the mice. Dosimeters were shipped offsite for assessment of the received radiation dose. The obtained data was then used to calculate the required irradiation time. The mice were sham-irradiated or irradiated at 10 Gy in a Mark 1 Cesium Irradiator (Shepherd and Associates, San Fernando, CA) at a dose rate of 236.8 Rad/min. The cerebellum, eyes, and body were shielded with lead and the experimental procedure was identical between the sham-irradiated and irradiated animals. The mice were behaviorally tested starting 3 months following irradiation.

Delayed Matching to Place Test (DMTP) for Irradiated Mice:

This test was used to assess the status of memory for a spatial location after increasing delays between learning and retrieval. All mice were experimentally naive prior to testing and were singly housed beginning 24 hours prior to the first behavioral test, to minimize the potential effects of social influences on behavioral performance. Mice learned to locate a submerged platform in a circular pool of opaque water (144 cm diameter), either by navigating using extra-maze cues located in the room (Hidden Training) or by swimming to a cue attached to the platform (Visible Training). Separate groups of mice underwent hidden training or visible training.

Mice were trained in the DMTP procedure for 2 sessions per day (2-3 hour inter-session interval), each with a different platform location determined in pseudorandom order out of 12 possible platform locations. Each session consisted of a learning and a retrieval trial separated by a retention interval. There was a 7-day training phase during which the retention interval was 1 minute. Following the training phase, there was a test phase in which the retention interval was increased to either 1-hour or 24 hours. There were four sessions per retention interval. During each trial, mice were placed into the water at one of 5 locations, against the wall of the pool, in a pseudo-random order. They swam for 120 seconds or until they found the platform. If they didn't find the platform within 120 seconds, they were led to it by the experimenter. They were allowed to remain on the platform for 10 seconds.

The swim pattern of the mice during each trial was recorded by a video tracking system (Ethovision v2.3, Noldus Information Technology, Wageningen Netherlands), and the latency to find the platform location, in seconds, was calculated since there was no difference in swim speed between the groups. Spatial memory was assessed by the

retention score, which was calculated as the difference between the swim time in the learning trial and the swim time in the retrieval trial, divided by the total swim time for the session. In other words, the retention score reflected the improvement in swim time between the learning and retrieval trials as a percentage of the total swim time during the session. Thus, better spatial memory corresponded to higher retention scores. During the training phase, efficacy of training was assessed by examining the retention score averaged across the last four training sessions (Sandstrom et al., 2006). Retention score during the first training session was used as the baseline performance to assess the effect of learning. During the testing phase, the retention scores of each mouse were averaged across all sessions for a given retention interval.

Statistical Analysis:

The total number of DCX+ cells, total number of Ki-67+ cells, and total volume estimate were analyzed using two-way ANOVA to measure the effects of castration and DMTP training type. An additional analysis was performed to determine if there was a significantly variable distribution of DCX+ or Ki-67+ cells through the rostral-caudal extent of the GCL for either castrated or sham-operated mice. For this, each section for each subject was coded into bins based on it's location relative to the most rostral point of the GCL (0-250 μm, 250-500 μm, 500-750 μm, etc. to 2750-3000μm), and the effect of bin and castration on density of DCX+ and Ki-67+ cells was measured with a linear mixed effects analysis. For the DMTP procedure, two-way mixed ANOVA was used to analyze the effects of irradiation (between-subjects factor) and retention interval (within-subjects factor) on retention scores. Duncan's post-hoc tests, which apply corrections

for multiple comparisons, were used to assess differences between individual groups where appropriate. Pearson's correlation analysis was used to examine the relationships between DMTP performance measures and neurogenesis markers. Independent-samples Student's t-tests measured the effect of castration on plasma levels of T and DHT. All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL), and $p < 0.05$ was considered significant for all tests.

**Results:**

Hormone Levels:

ELISAs were used to verify that castration and hormone replacement treatments were effective in modulating circulating hormone levels (Figure 11). Sham-operated intact mice had significantly higher levels of both T ($p < 0.001$) and DHT ($p < 0.001$) compared to castrated mice.

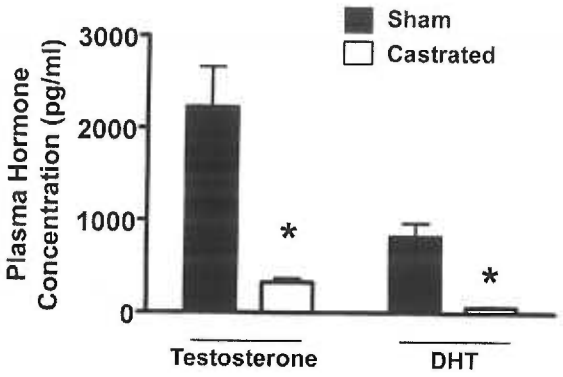


*Figure 11.* Plasma hormone concentrations of sham-operated (Sham) and castrated mice. Castration greatly reduced, but did not completely remove, circulating plasma levels of both T and DHT (DHT). * - $p < 0.001$ by unpaired Student's t-test compared to sham-operated control.

Castration and visible DMTP training reduced the number of DCX+, but not Ki-67+ cells, in the GCL:

DCX+ immature neurons (Figure 11C) and Ki-67+ proliferating progenitor cells (Figure 12E) were clearly visualized in the GCL of the dentate gyrus (Figure 12A). For DCX+ cells, there was an effect of castration ($F$ (1, 17) = 5.47, $p$ = 0.03) with castrated mice having fewer DCX+ cells than sham-operated mice. There was also an effect of DMTP training type ($F$ (2, 17) = 11.6, $p$ = 0.001) with mice receiving visible DMTP training having fewer DCX+ cells compared to mice that received either hidden DMTP training or handling only (Figure 12D). Castration did not modulate the effect of visible

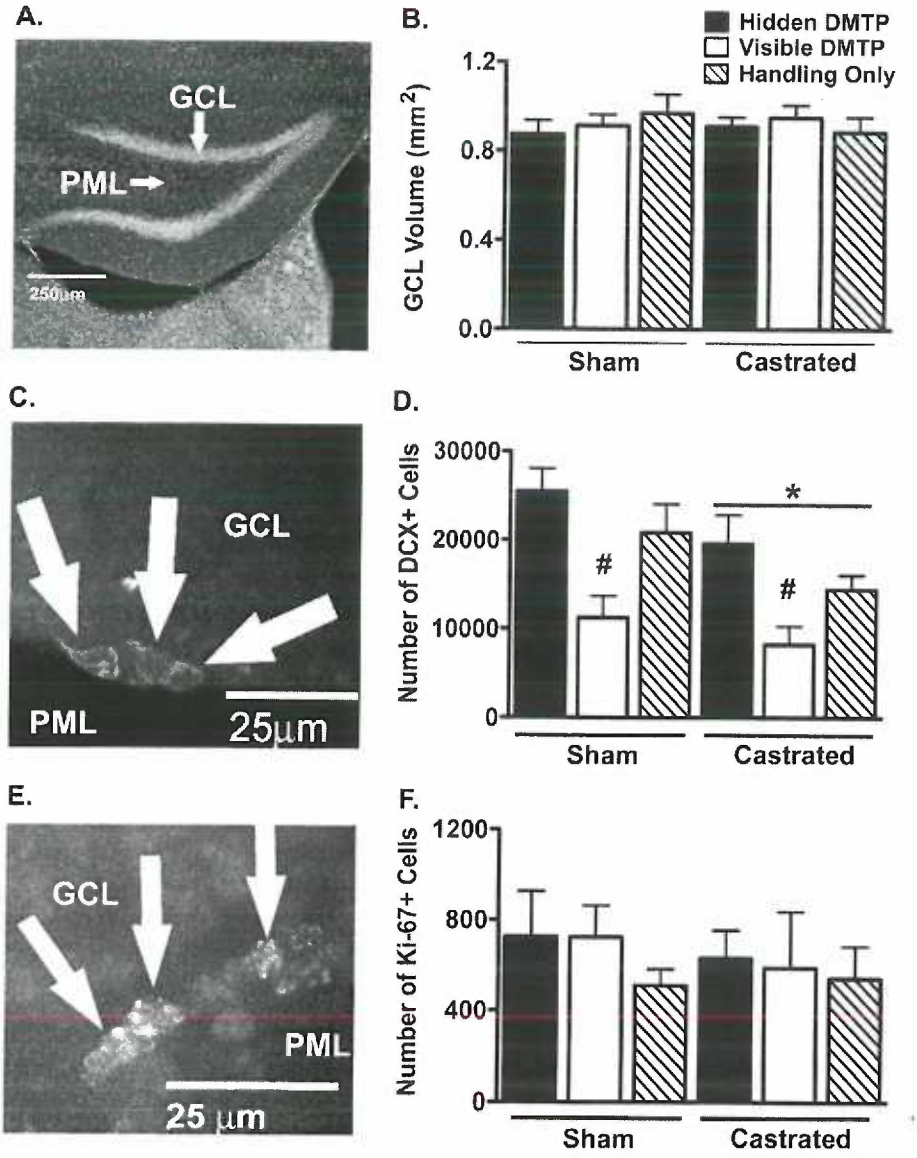


*Figure 12.* Effects of castration and DMTP training experience on the volume estimates (A, B) in the granule cell layer (GCL) of the dentate gyrus, number of DCX+ immature neurons (C,D), and number of Ki67+ proliferating progenitors (E,F). Arrows point to profiles of DCX+ immature neurons (C), Ki-67+ proliferating progenitors (E) in images taken in the region 250-500μm from the most rostral end of the GCL. DAPI counter-staining was used to visualize the granule cells in the dentate gyrus and measure the volume of the GCL (A). GCL = granule cell layer, PML = polymorph layer.

* significant effect of castration ($p < 0.05$) by two-way ANOVA

# $p < 0.05$ by Duncan's post-hoc test compared to either hidden DMTP or handling only.

DMTP training on the number of DCX+ cells. For Ki-67+ cells, there were no effect of either castration or DMTP training type (Figure 12F). Neither castration nor DMTP training type affected the GCL volumes (Figure 12B). Linear mixed effects analysis showed no effect of bin (location rostral-caudal within the GCL) on either the density of DCX+ or Ki-67+ cells, and no castration X bin interaction.

No relationship between neurogenesis markers and hidden DMTP performance:

Pearson's correlations revealed no significant relationship between the numbers of DCX+ immature neurons and hidden DMTP performance at either the 1-minute ($r$ = 0.205, $p$ = 0.66) or 1-hour ($r$ = 0.31, $p$ = 0.50) retention interval. Likewise, there were no significant relationships between the numbers of Ki-67+ proliferating progenitors and hidden DMTP performance at either the 1-minute ($r$ = 0.122, $p$ = 0.80) or 1-hour ($r$ = 0.47, $p$ = 0.29) retention interval. In addition, a Student's t-test showed that the hidden DMTP performance of the castrated mice used here was significantly worse than the sham-operated control mice with a 1-hour retention interval between learning and retrieval ($t(5) = 2.96$, $p < 0.05$) (see DMTP experiment 1 of **Chapter 3** for details of the behavioral experiment).

$^{137}$Ce irradiation did not disrupt spatial memory in the DMTP:

If neurogenesis mediated the effects of castration on hidden DMTP performance, we hypothesized that inhibiting neurogenesis by irradiation would mimic the effects of castration (Figure 13). The irradiation treatment had a clear effect in reducing the number of DCX+ neurons in the GCL 3-months later (Figure 13A). There was an effect

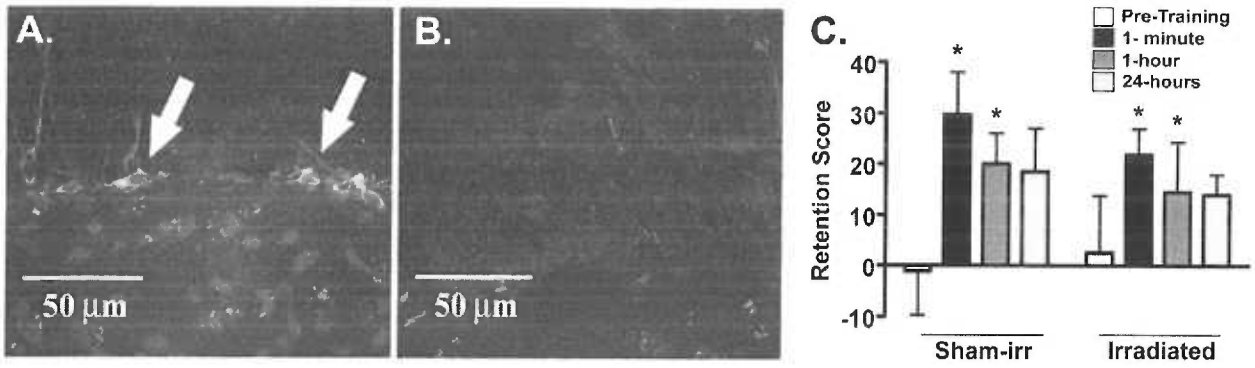


*Figure 13.* Effects of neurogenesis inhibition by $^{137}Cs$ irradiation on hidden DMTP performance. Irradiation drastically reduced the number of DCX+ immature neurons in the GCL (A) compared to sham irradiated controls (B). Compared to the first session of DMTP training (Pre-Training), both sham-irradiated controls (Sham-irr, $n$ = 10) and irradiated mice ($n$ = 10) showed significant improvement in retention scores following a 1-minute and 1-hour, but not 24-hour, retention interval (C). However, irradiation did not affect retention scores following any retention interval.

*$p < 0.05$ by Duncan's post-hoc test following ANOVA, compared to Pre-Training.

of retention interval ($F(3, 60) = 3.18$, $p = 0.03$), where the retention scores following a 1-minute and a 1-hour retention interval were significantly higher than the baseline performance (Duncan post-hoc test $p < 0.05$). However, contrary to our hypothesis, irradiation had no effect on hidden DMTP performance following any of the retention intervals ($F(1, 20) = 0.501$, $p = 0.49$).

**Discussion:**

There were three major findings in this study: 1) castration resulted in a significant reduction in the number of immature neurons in the GCL, 2) castration did not modulate the effects of DMTP training experience on the number of immature neurons or proliferating progenitors, 3) inhibition of neurogenesis by $^{137}Cs$ irradiation had no effect on spatial memory in the DMTP procedure. These results suggest that castration reduces neurogenesis in the GCL in addition to disrupting spatial memory in the DMTP test, but the change in neurogenesis may not play a major role in the cognitive impairment. It also indicates that androgens do not play a major role in the effects of hippocampus-dependent learning on neurogenesis. Although the literature has mixed results regarding the role of neurogenesis in spatial memory, our data supports that the role is small, at least for the DMTP water maze procedure.

Compared to sham-operated mice, castrated mice had fewer DCX+ immature neurons in the GCL of the dentate gyrus. In contrast, castration had no effect on the number of Ki-67+ proliferating progenitor cells. This result is consistent with the idea that androgens provide some survival support to developing dentate granule cells, influence neuronal differentiation, change neuronal maturation rate, or a combination, without influencing precursor proliferation (Galea et al., 2006). Similar to this, castration in rats reduced the number of bromodeoxyuridine (BrdU) labeled cells in the GCL 30-days, but not 24-hours following labeling, which was recovered by androgen replacement (Spritzer and Galea, 2007). However, a limitation of this study was that the neuronal lineage of the surviving BrdU-labeled cells could not be directly verified. Our study verifies that androgens affect the generation of new neurons, since DCX is

expressed only in immature neurons. However, since we did not measure the number of new glia produced during the experiment we cannot determine whether the effect was on survival or differentiation. In addition, since DCX is expressed during a limited period in neuronal development (Ming and Song, 2005), it is possible that castration increased the maturation rate of the immature neurons thereby reducing the number expressing DCX. The current data cannot conclude which of these alternatives is the case. Nonetheless, our data adds to accumulating evidence that the effects of androgens on new-born neurons are probably species independent. This effect has been observed in songbirds (Absil et al., 2003), meadow voles (Ormerod and Galea, 2003), rats (Spritzer and Galea, 2007), and now mice. This is important as it suggests that the promotion of neurogenesis by androgens may also occur in humans.

The number of immature neurons found in the hippocampus was strongly influenced by the type of DMTP. Surprisingly, compared to handling only, visible DMTP training decreased and hidden DMTP failed to increase the number of immature neurons. Since DMTP training experience did not affect the proliferating progenitor population, the differences in immature neuron numbers are likely due to changes in neuronal differentiation, survival, or maturation rate, not proliferation rate. From this we might conclude that handling only and hidden DMTP training affected these processes equally, while visible DMTP training reduced either neuronal differentiation or survival. However, it is also possible that visible training increased maturation rate leading to reduced numbers of new neurons expressing DCX. This contrasts with previous studies in which, compared to naïve controls, hippocampus-dependent learning in a reference memory water maze increased the survival of cells that were labeled with BrdU one

week prior to training (Gould et al., 1999, Prickaerts et al., 2004). However, it is not clear that the control rats in these studies experienced equivalent handling, thus handling by itself could increase survival of new neurons. Alternatively, prolonged DMTP training in the water maze might reduce neurogenesis by increasing corticosterone production (Mohapel et al., 2006). Compared to handling only, stress may have reduced DCX+ neuron number in the visible DMTP training group, and also inhibited the expected increase in DCX+ neuron number in the hidden DMTP group. Furthermore, we used DCX to label new neurons whereas others have used BrdU, which allows for birth-dating of newly generated cells, but does not include cells that were generated prior to, or after, BrdU injection and involves the potential stress of the injection, which by itself might affect neurogenesis. In contrast, our study measured the total number of immature neurons existing after training regardless of birth-date, thus our results probably include a larger population of cells than those measured in the BrdU studies. This could be important since the spatial-learning induced cell survival enhancement is dependent upon the age of the immature neurons (Epp et al., 2007), and initial stages of learning can promote cell death (Dupret et al., 2007).

While the mechanism underlying the effects of behavioral experience on adult hippocampal neurogenesis are not completely clear, factors such stress (Mayer et al., 2006, Wong and Herbert, 2006), hippocampal activation (Smith et al., 2005, Toda et al., 2008), and neurotrophins certainly play a role (Lee et al., 2002a, Scharfman et al., 2005). Androgens are known to modulate the hypothalamic-pituitary-adrenal axis response to stressful stimuli (McCormick et al., 2002). The neurosteroid metabolite of DHT, 3$\alpha$-diol, is a potent positive modulator of GABA neurotransmission (Frye et al.,

2001) and may thereby affect afferent activation of hippocampal circuits. Also, castration can reduce the expression of some neurotrophic peptides in the brain (Katoh-Semba et al., 1994, Bimonte-Nelson et al., 2003). In these ways, androgens have potential to modulate the effects of learning on neurogenesis. However, we found that castration did not change the effect of DMTP testing on immature neuron number. This indicates that, although DMTP experience strongly modulated neurogenesis, androgens were not required for these effects. However, it is possible that androgens might play a role in the neurogenesis-modulating effects of other experiences such as environmental enrichment or exercise.

The removal of androgens by castration disrupted spatial memory in the DMTP task, and also reduced the number of immature neurons in the GCL of the same mice. If the reduction in neurogenesis mediated the disruption of spatial memory, we hypothesized to find that a) the number of immature neurons in the GCL would correlate with hidden DMTP performance, and b) reduction of the number of immature neurons by irradiation would likewise disrupt spatial memory in the DMTP. Contrary to our hypotheses, there was no significant relationship between DCX+ neuron number and retention scores following either a 1-minute or a 1-hour retention interval in the hidden DMTP. Similarly, cranial irradiation, which potently reduces the number of DCX+ immature neurons in the GCL (Raber et al., 2004a), had no significant effect on spatial memory. From this we conclude that, at least for the water maze DMTP task used in this study, androgens likely affect spatial memory by other mechanisms apart from reducing neurogenesis. The evidence implicating neurogenesis in performance on spatial memory tasks is complex. Many studies (Raber et al., 2004b, Rola et al., 2004,

Snyder et al., 2005, Shi et al., 2006, Villasana et al., 2006), but not all (Wojtowicz et al., 2008), using irradiation to suppress neurogenesis have reported disruption in spatial learning and memory in the reference memory version of the Morris water maze. In the same vein, manipulations such as environmental enrichment or voluntary exercise, which enhance adult hippocampal neurogenesis, also improve spatial memory performance (Alaei et al., 2007, Luo et al., 2007, Mello et al., 2008). However, inhibition of neurogenesis prior to such manipulations suggest that neurogenesis may not mediate the associated spatial memory improvements (Meshi et al., 2006). To complicate matters further, a recent study reported improved spatial 'working memory' in the radial arm maze following inhibition of neurogenesis (Saxe et al., 2007). To our knowledge, this study is the first to report the effects of irradiation on spatial water maze DMTP performance. In addition to not supporting a role for neurogenesis in castration-induced spatial memory deficits, our data is also consistent with the idea that neurogenesis does not modulate spatial memory in the DMTP. However, we cannot rule out the possibility that cranial irradiation might affect brain function in other ways to compensate for changes in neurogenesis.

Androgens alter brain morphology in ways that might account for the cognitive effects of androgens. Although not true for the hippocampus as a whole, the volume of the granule cell layer of the dentate gyrus (GCL) and the size of the pyramidal neurons in the CA1 and CA3 regions are larger in males than females, and administration of T to neonatal females has been shown to masculinize these traits (Isgor and Sengelaub, 1998). Similarly, neonatal castration of male rats reduces the GCL volume to a smaller female-typical size (Isgor and Sengelaub, 1998). The same relationship appears to hold

in the amygdala, with male rats having larger cell size and regional volumes than females, and neonatal T administration eliminating the sex-difference (Cooke, 2006). In adult rats, changes in androgen levels appear to have little effect on GCL volume in the hippocampus (Isgor and Sengelaub, 1998), but can eliminate sex differences in amygdalar volume (Morris et al., 2008). In contrast to regional volumes, the density of CA1 apical dendritic spines in the stratum radiatum in the hippocampus in adult male rats is reduced by castration and normalized by replacement with either T or DHT (Leranth et al., 2003). The same is true in adult female ovariectomized rats (Leranth et al., 2004a). Although clearly androgen sensitive, it is not known at present how changes in these hippocampal and amygdalar morphological traits contribute to cognitive performance.

Androgen manipulations might affect performance on some cognitive tasks by altering neurotransmitter systems. For example, administration of the neuro-active steroid 3$\alpha$-diol recovers a castration-induced passive avoidance performance deficit in adult male rats (Frye and Seliga, 2001). Also, androgens might affect cognition by altering some of the electrophysiological characteristics of hippocampal N-methyl-D-aspartate type glutamate receptors (NMDAR), which has been shown in hippocampal slice preparations from adult male rats (Pouliot et al., 1996). Androgens might also interact with acetylcholinergic pathways since castration reduces brain levels of choline-acetyltransferase, the enzyme synthesizing acetylcholine (Nakamura et al., 2002), and worsens the negative effects of scopolamine treatment on errors in the radial arm maze (Daniel et al., 2003). In addition, increased dopaminergic signaling is implicated in the disruptive effect of castration on T-maze alternation performance (Kritzer et al., 2001).

Finally, T, via aromatization to 17β-estradiol, maintains normal mRNA expression of the serotonin type 2A receptor in the cortex of male rats (Sumner and Fink, 1998). However, a direct link between this receptor and cognitive task performance has yet to be made.

In conclusion, this study adds to a growing literature suggesting that changes in androgen status can affect AHN in the mammalian hippocampus, mainly by modulating the survival of immature neurons. Although castration in mice can also disrupt spatial memory in the water maze DMTP task, the current data does not support a straight-forward link between spatial memory and changes in adult hippocampal neurogenesis.

Chapter 5: Testosterone and dihydrotestosterone differentially improve cognition in aged female mice

*Adapted from:*

**Benice T.**, Raber J. Testosterone and dihydrotestosterone differentially improve cognition in aged female mice. *Learning & Memory*- In Press.

**Introduction:**

Age-related cognitive decline (ACD) refers to a generalized disruption of cognitive function that occurs during the aging process in the absence of overt neurodegenerative disease (Small et al., 1995, Tisserand and Jolles, 2003). ACD is associated with declines in multiple cognitive domains including spatial cognition (Driscoll et al., 2005), verbal memory (Small et al., 1995), attention (Tisserand and Jolles, 2003), and executive function (Hanninen et al., 1997). Along with functional cognitive decline, there are associated age-related disruptions in cortical and hippocampal volumes (Driscoll et al., 2003), volumes of cortical gray-matter and cerebrospinal fluid, (Smith et al., 2007) and white matter integrity (Silbert et al., 2008). As the overall population of the aged is rapidly increasing, it becomes important to find ways to prevent and treat ACD.

Hormone replacement therapy with estrogens has received much attention in recent years as a possible treatment to slow ACD in aging women. Despite clear beneficial effects of HRT in preclinical and epidemiological research (Eberling et al., 2002, Wolf and Kirschbaum, 2002, Tinkler and Voytko, 2005), controlled clinical trials have suggested that the potential benefits in aging women are complicated by many

factors (Sherwin and Henry, 2008). In contrast, the cognitive effects of supplementation with androgens such as T or DHT in aged female animals have been little studied.

In rodents, there is a clear sex-difference in the rate of ACD (Markowska, 1999, Frick et al., 2000, Benice et al., 2006), as assessed by tasks that measure hippocampus and amygdala-dependent learning and memory tasks. Hippocampal function can be measured using spatial memory tasks such as the Morris water maze (Morris et al., 1982). Female rats experience faster declines in hippocampus-dependent spatial memory performance compared to males (Markowska, 1999). In mice, females show greater age-related impairments in the spatial 'reference' memory water maze task compared to males (Frick et al., 2000, Benice et al., 2006). In mice that express human apolipoprotein E4, a major risk factor for sporadic Alzheimer's disease, aged females show greater impairments than aged males in spatial reference memory performance (Raber et al., 2000). Amygdala function in rodents can be measured using the passive avoidance task (PA) (Swartzwelder, 1981). Compared to males, female mice also show greater age-related declines in PA performance (Benice et al., 2006). Since males have higher levels of circulating androgens compared to females at all ages, it is possible that androgens may confer some protection against ACD. Thus, androgenic mechanisms may provide a therapeutic avenue to treat ACD in aged females. In support of this, brief supplementation with T or DHT or Selective Androgen Receptor Modulators (SARMs) antagonized the spatial reference memory impairments associated with neuronal apolipoprotein E4 expression in 6-month old female mice (Raber et al., 2002, Acevedo et al., 2008c).

To characterize the effects of androgen supplementation on cognitive performance in aged female mice, we treated 22-24 month old female mice with T, non-aromatizable DHT, or placebo, and tested behavior in a battery of tests designed to assay hippocampus-dependent, hippocampus-independent, and amygdala-dependent learning and memory. We also measured anxiety-like behavior and motor coordination as these behaviors can potentially indirectly contribute to measures of differences in learning and memory (Benice et al., 2006). We hypothesized that supplementation with either T or DHT, compared to placebo treatment could enhance cognitive performance on a range of cognitive tasks in aged female mice. Further, we expected that those tasks in which the performance of aged female mice declines faster than males (Spatial reference memory and passive avoidance memory) would be especially benefitted by T or DHT supplementation. Since DHT cannot be aromatized to 17β-estradiol, beneficial effects of DHT would be interpreted as involving an androgenic mechanism apart from aromatization.

**Experimental Procedures:**

Mice:

Female C57BL/6J mice were bred in our colony and housed 3-5 per cage until they were 20-22 months of age at which point surgery took place (see procedure below). Food (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO) and water were provided *ad libitum*, and there was a constant 12h on/12h off light cycle (on 6:00 am, off 6:00 pm). Mice were singly housed starting 2 days before the first behavioral test to eliminate potential social effects (including hierarchy in the home cage and stress of removing a mouse from the cage on remaining mice in the cage) on behavioral performance. Behavioral testing commenced 6-weeks following surgery and lasted 4 weeks. Within 48 hours of the end of the last behavioral test, blood samples were taken to measure plasma hormone levels (see procedure below).

Hormone supplementation and measurement of hormone levels:

The mice were 20-22 months of age at the time of surgery. Each mouse was deeply anesthetized using isofluorane in O2, and a 0.5cm incision was made in the skin between the shoulder blades. A SILASTIC capsule (inner diameter = 1.57mm, outer diameter = 3.18mm, length 2.5 cm) containing either T, DHT (Sigma, St. Louis, MO), or remaining empty (placebo) was implanted subcutaneously and the wound sutured. To measure circulating hormone levels following implantation, plasma samples were taken following behavioral testing and were subjected to ELISA analysis for concentrations of T and DHT using commercially available kits (Alpha Diagnostic International, San Antonio, TX) according to the supplier's instructions. Blood taken from heart puncture

was placed into Eppendorf tubes containing ~10μL of a 5% EDTA solution. The blood was centrifuged for 10 minutes at 16,000 x g and the plasma was then separated and stored at -20°C until assay.

Behavioral Testing:

*Open field test:*

The open field task was performed as described in **chapter 2** (pg. 38).

*Novel Object and Novel Location recognition (NO/NL):*

The NO/NL tasks were performed as described in **chapter 2** (pg. 40).

*Spatial reference-memory (Morris water maze):*

The Morris water maze task was performed as described in **chapter 2** (pg. 40-41).

*Roto-Rod:*

Roto-Rod was used to test potential effects of hormone supplementation on a variety of proprioceptive, vestibular, and fine-tuned motor abilities. In this task, mice balanced on a 7 cm diameter rotating rod (Kinder Scientific). After a 1 minute adaptation period without rotation, the rod was accelerated by 5 rpm every 15 seconds and the latency to fall from the rod was recorded. Each mouse received 3 days of roto-rod testing with 3 trials per day and a ~20 min inter-trial interval. The average fall latency for all trials was used as the measure of motor coordination.

*Passive avoidance learning and memory (PA):*

The PA task was performed as described in **chapter 2** (pg. 42).

Statistical Analysis:

For plasma hormone levels, open field test, and the location and object novelty recognition tasks, the effect of hormone treatment as a between-subjects factor was measured by one-way fixed ANOVA. Reference-memory water maze training performance was analyzed using two-way mixed ANOVA with hormone treatment as the between-subjects factor and training session as the within-subjects factor. For the water-maze probe trials, we expected a-priori to see higher preference for the target quadrant over the other four quadrants. Thus, target quadrant preference in the water maze probe trials was measured by comparing percentage time spent swimming in the target quadrant vs. all other quadrants using an a-priori planned contrast f-test. Roto-rod performance was analyzed in the same fashion as water maze training performance, except trial number was the within-subjects factor. The effects of hormone treatment on passive avoidance acquisition and memory performance were analyzed using the Kruskal-Wallis non-parametric test. For the ANOVA analyses, Duncan's post- hoc test was used to measure differences between individual groups where appropriate. Kruskal-Wallis tests were followed by Mann-Whitney U tests to compare individual groups.

**Results:**

Hormone Levels:

Both T ($F(2, 16) = 572$, $p < 0.001$) and DHT ($F(2, 16) = 77.1$, $p < 0.001$) supplementation successfully elevated hormone levels over placebo (Figure 14). T levels were significantly higher in the mice supplemented with T compared to either placebo or DHT-treated mice ($p < 0.05$ by Duncan's post-hoc test). Similarly, DHT levels were highest in the mice supplemented with T compared to either DHT or placebo-treated mice ($p < 0.05$ by Duncan's post-hoc test). Finally, DHT levels were also higher in the DHT-treated mice compared to placebo-treated mice ($p < 0.05$ by Duncan's post-hoc test).

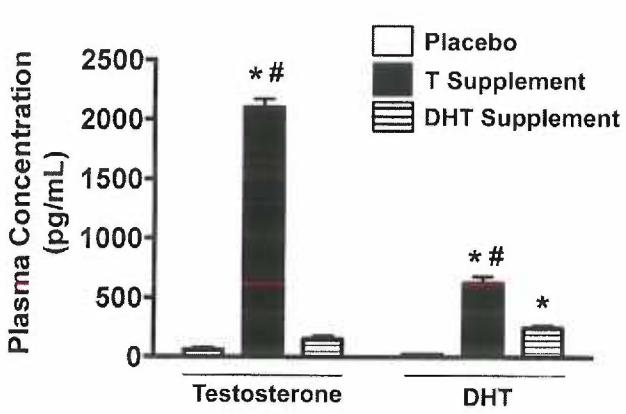


*Figure 14.* Plasma T and DHT levels for mice supplemented with either T (n = 6), DHT (n = 6), or given placebo (n = 5) (empty capsules).

* - $p < 0.05$ by Duncan's post-hoc test vs. placebo

#- $p < 0.05$ by Duncan's post-hoc test vs. DHT supplement.

Open Field:

Neither T nor DHT affected measures of anxiety or activity in the open field (Figure 15A). There was no effect of hormone treatment on percentage time spent in the center of the open field ($F(2, 28) = 1.18$, $p = 0.32$). There was also no effect of hormone treatment on total distance moved in the open field ($F(2, 28) = 1.81$, $p = 0.18$).

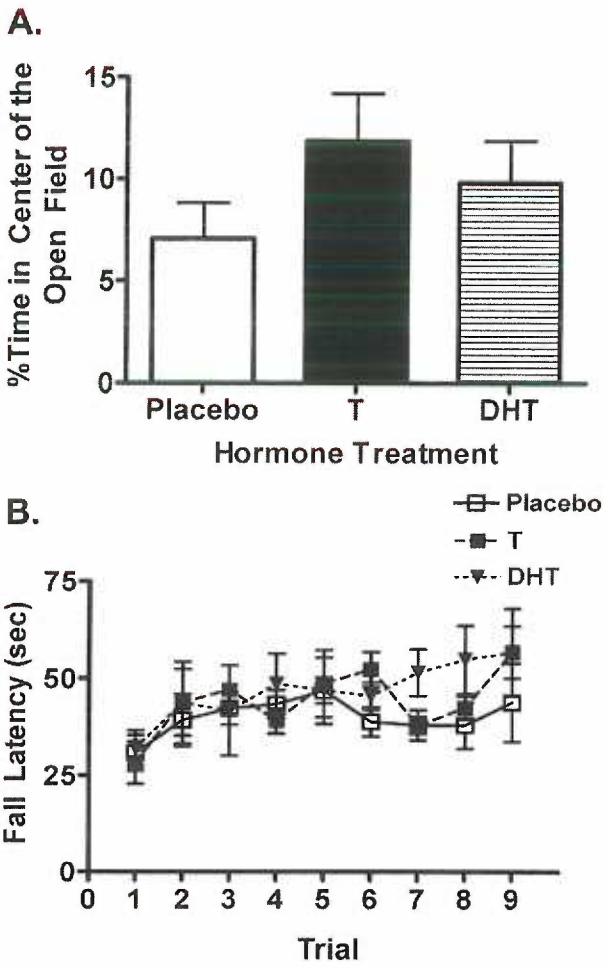


*Figure 15.* Measures of anxiety in the open field (a), and motor coordination in the roto-rod (b) in aged female mice treated with either T, DHT, or placebo. Neither T nor DHT supplementation significantly changed anxiety. Although all groups showed significant improvement in fall latency, there was no effect of either T or DHT supplementation.

Novel object and novel location memory (NO/NL):

Treatment with T or DHT did not improve object recognition (Figure 16). There was no effect of hormone treatment on either the novel object recognition score ($F(2, 28) = 0.515$, $p = 0.60$) or the novel object recognition score ($F(2, 28) = 0.68$, $p = 0.51$).

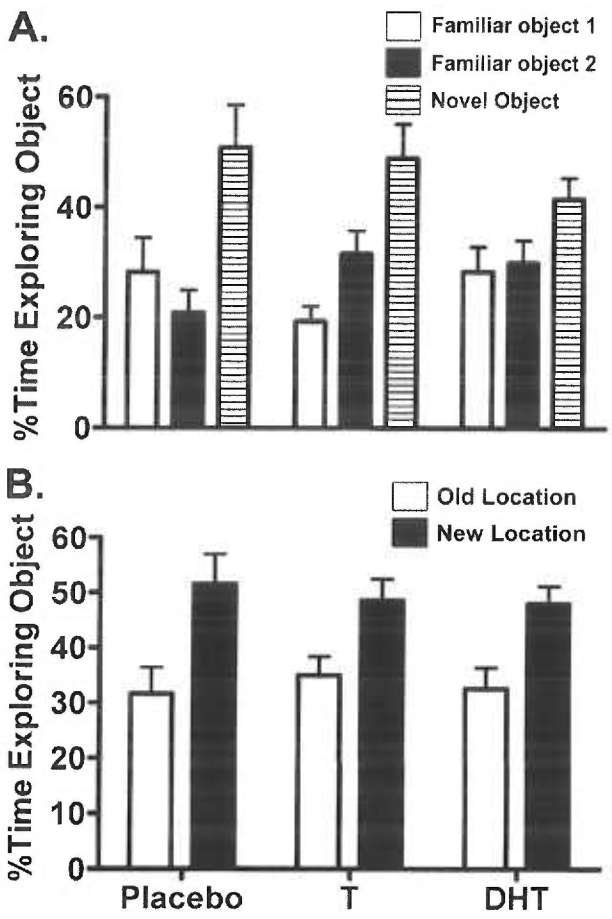


*Figure 16.* Novel object (A) and novel location (B) recognition performance in aged female mice treated with either T, DHT, or placebo. Preference for the novel object during trial 5 (novel object test), and for a familiar object in a novel location vs. the old location, were unaffected by T or DHT supplementation.

Reference Memory Morris Water Maze:

Performance during the training phase of the water maze task was unaffected by hormone treatments (Figure 17A). Swim speed during the visible training sessions did not differ between the treatment groups ($F(2, 28) = 2.42$, $p = 0.11$). However, there was a trend toward a hormone treatment effect on swim speed during the hidden training sessions ($F(2, 28) = 2.69$, $p = 0.09$). There was an effect of session on escape latency in both the visible ($F(3, 75) = 3.95$, $p = 0.01$) and hidden sessions ($F(5, 130) = 7.87$, $p < 0.001$), indicating that the mice improved performance during training. The same effect of session was also found for the total distance moved in both the visible (F (3,

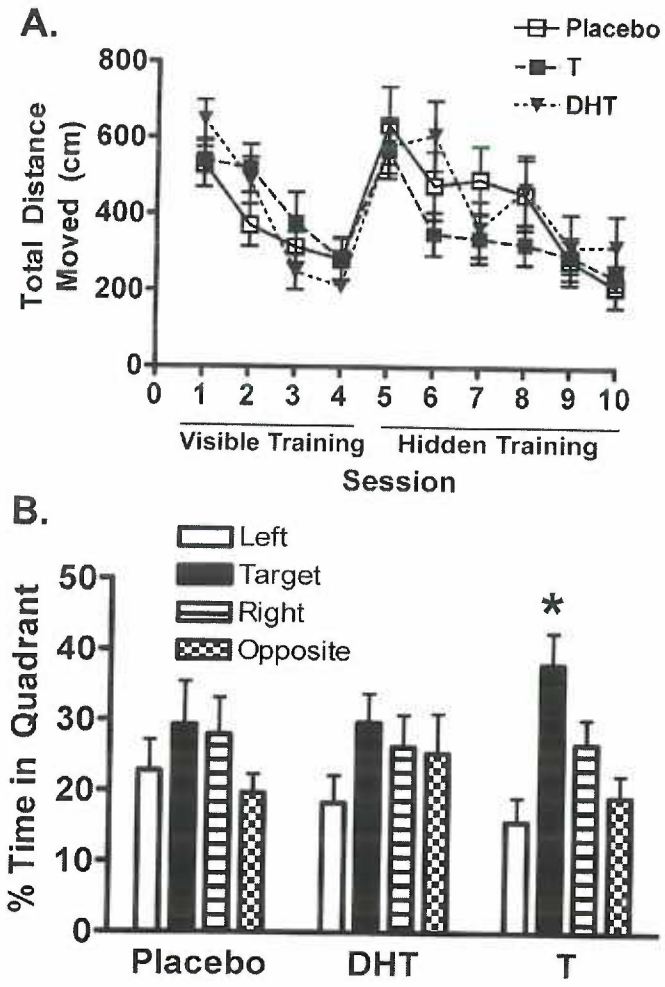


*Figure 17.* Learning during the visible and hidden training trials (A) and memory retention during the third and final probe trial (B) of the reference memory Morris water maze in aged female mice treated with either T, DHT, or placebo. Mice in all groups showed significant performance improvement over the training sessions during both visible and hidden training. However, performance was unaffected by either T or DHT supplementation. In contrast, mice that received T supplementation achieved significant spatial bias whereas placebo and DHT treated mice did not. No significant preference for the target quadrant of the maze was observed during either the first or second probe trials for any group. * - $p < 0.05$, a-priori planned contrast.

75) = 8.42, $p < 0.01$) and hidden ($F(5, 130) = 13.1$, $p < 0.001$) sessions. However, there was no effect of hormone treatment on escape latency (or total distance moved) in either visible ($F(2, 26) = 0.75$, $p = 0.48$) or hidden ($F(2, 26) = 1.23$, $p = 0.31$), and there were no significant interactions between session and hormone treatment. In contrast to the training performance, T-treated mice showed improved performance during the probe trial memory test after the last day of training (Figure 17B). The T-treated females show spatial bias for the target quadrant indicating spatial learning of the target location after day 3 of hidden training ($p < 0.05$ by a-priori planned contrast), but not after day 1

or day 2. In contrast, neither placebo- or DHT-treated females ever showed spatial bias for the target quadrant.

Roto-Rod:

There was significant an effect of training trial on roto-rod performance ($F(8, 200) = 3.87$, $p < 0.001$) indicating improvement in performance over the 9 training trials (Figure 15B). However, there was no effect of hormone treatment ($F(2, 25) = 1.71$, $p = 0.30$), and no interaction between hormone treatment and training trial.

Passive Avoidance (PA):

DHT treatment, but not T treatment, significantly improved passive avoidance memory performance (Figure 18). There was no hormone treatment effect on test acquisition ($\chi^2 = 2.07$, $p = 0.35$). However, there was an effect of hormone treatment on passive avoidance memory retention 24 hours after training ($\chi^2 = 6.87$, $p = 0.03$). Compared to placebo, DHT significantly increased latency to enter the dark compartment during the memory retention test trial ($Z = 2.54$, $p = 0.02$), but T did not ($Z = 1.67$, $p = 0.12$). However, there was no significant difference in passive avoidance memory performance between T and DHT treated females ($Z = 0.93$, $p = 0.48$). For the placebo-treated mice, 6 out of 8 mice entered the dark compartment during the test trial. In contrast, only 1 out of 10 DHT-treated mice entered the dark compartment. The performance of T-treated mice was in between placebo- and DHT-treated mice with 4 out of 10 mice entering the dark compartment. Similar to latency to enter, chi-squared tests revealed that a smaller proportion of DHT-treated mice entered the dark

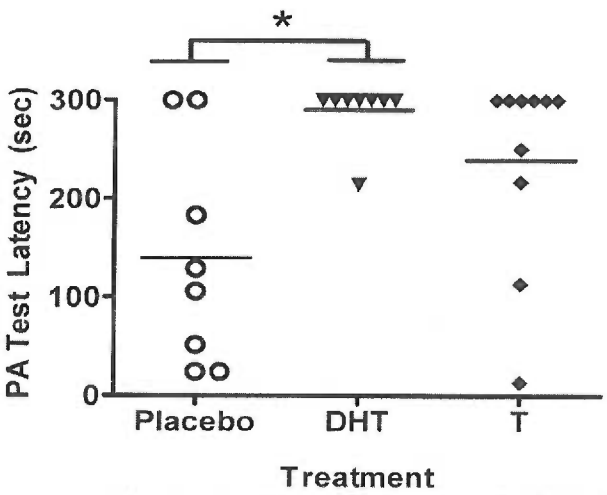


*Figure 38.* Passive avoidance (PA) memory performance 24 hours following training. Compared to either T or placebo treated mice, mice that received DHT supplementation showed significantly elevated latencies to enter the dark chamber (PA test latency) 24 hours after training, indicating improved passive avoidance memory. In contrast, performance was not improved in T treated mice compared to placebo. Note- Each data point in the figure represents each subject.

* - $p < 0.05$ by Mann-Whitney U-test

compartment compared to placebo ($\chi^2 = 7.9$, $p < 0.05$), but there were no significant differences between either T and placebo or between T and DHT.

**Discussion:**

There were three major findings from this study in 22-24 month-old female mice: 1) compared to placebo-treatment, supplementation with T, but not DHT, improved spatial reference memory performance in the Morris water maze , 2) compared to placebo treatment, supplementation with DHT improved passive avoidance memory performance whereas T only marginally improved performance, and 3) hormone supplementation had no effect on either location or object novelty recognition memory performance, open field measures of anxiety, or roto-rod performance. These data are the first to explore the cognitive-enhancing effects of androgen hormone supplementation in aged female rodents. The data support the hypothesis that androgen hormone supplementation is beneficial for cognitive performance in aged female mice. Also, androgenic mechanisms may present different targets for treatment of separate aspects of age-related cognitive decline.

The aged female mice in this study had significant impairments in spatial reference memory, as measured by the Morris water maze task. Although they showed improvement in total distance moved during the training phase, they failed to show any target quadrant preference during any probe trial indicating an impairment in spatial memory. This is in agreement with previous findings using the same strain of female mice aged 18-20 months-old (Benice et al., 2006). Treatment with T, but not DHT or placebo, improved target quadrant bias on the third-and-final probe trial indicating a significant improvement in spatial memory. In addition, since there was no effect of either T or DHT on the roto-rod task, it is unlikely that performance differences in the water maze are due to motor effects of androgens. Similar results have been recently

reported in aged male rats using a 'working-memory' radial-arm water maze task (Bimonte-Nelson et al., 2003). In addition to reducing the number of errors in a trial, T, but not DHT, increased message levels of brain-derived neurotrophic factor in the hippocampus (Bimonte-Nelson et al., 2003). This was interpreted to mean that conversion of T to 17β-estradiol was the likely pathway for T's effects on brain and behavior. This idea is supported by a large literature indicating that 17β-estradiol can improve spatial memory task performance (Galea et al., 2008). Estrogenic mechanisms could also be involved in the pathway for T's effects on spatial reference memory in the current study. However, T can simultaneously be metabolized into DHT in addition to 17β-estradiol, so cognition may be enhanced by a combination of estrogenic and androgenic signaling.

In contrast to spatial reference memory performance, supplementation with DHT, but not T, greatly improved passive avoidance memory performance. However, T supplementation did improve performance slightly compared to placebo. The present result are in agreement with previous data suggesting that DHT can, under some circumstances, improve passive avoidance memory in rats (Frye and Lacey, 2001, Frye et al., 2004). Also, in adult male rats, DHT treatment reverses castration-induced impairment in passive avoidance memory (Frye and Seliga, 2001). However, ours is the first study to show that DHT can improve cognitive performance in aged female mice. Other studies using an inhibitor of DHT metabolism, suggest that the beneficial effects of DHT in male rats may be due to the actions of its neuro-active metabolite 3α-diol (Frye et al., 2004). 3α-diol is an allosteric positive modulator of GABA induced chloride ion flux through GABA type A (GABA-A) receptors and this mechanism has been

proposed to explain its effect on passive avoidance memory in male rats. The beneficial effect of 3α-diol is also attributable, at least in part, to post-training memory consolidation effects. Conversion to 3α-diol may also mediate the beneficial effects of DHT on passive avoidance memory in aged female mice. However, we cannot rule out potential mediation by direct genomic or non-genomic actions of the androgen receptor. For example, androgen receptor activation has been linked to dendritic spine-synapse density (Leranth et al., 2004a), adult hippocampal neurogenesis (Spritzer and Galea, 2007), and central neurotransmitter systems including glutamate (Pouliot et al., 1996, Foradori et al., 2007), dopamine (Kritzer et al., 2007), acetylcholine (Nakamura et al., 2002), and corticotrophin-releasing factor (Gomez et al., 2004).

Neither T nor DHT supplementation affected performance on measures of novel object/novel location recognition memory. Within the limits of our study, these results indicate that behavioral performance on this task is androgen-insensitive in aged female mice. Whereas age-related disruptions in water maze reference memory and passive avoidance memory performance are greater in female mice compared to male mice, age-related decline in novel object or novel location recognition is unaffected by sex (Benice et al., 2006). Although not directly related to androgen levels, these results support the idea that age-related decline in novel object or novel location recognition may be unaffected by reduced androgen levels. Also, NL recognition declines with age in mice but NO recognition does not (Benice et al., 2006). Thus, an androgen-induced performance enhancement in NO performance would not necessarily be expected. In addition, in adult male testicular feminization mutation mice, which have impaired androgen receptor signaling, both NO and NL recognition is intact (Rizk et al., 2005).

However, adult female ovariectomized rats show improvements in NO performance when given T or DHT immediately following training, indicating that androgen supplementation can improve NO performance in some circumstances (Frye and Lacey, 2001). However, the NO task in that study is different from the one used in the current study in that it was likely hippocampus-dependent (Hammond et al., 2004).

Androgen supplementation did not affect anxiety in the open field test. Androgens are linked to stress responsiveness and anxiety-like behavior through its actions on corticotrophin-releasing hormone and arginine-vasopressin neurons in the hypothalamus and amygdala (McCormick et al., 2002, Gomez et al., 2004). In general, androgens are considered anxiolytic, due in part to the effects of the 3$\alpha$-reduced neurosteroid 3$\alpha$-diol on GABA-A receptors (Frye and Seliga, 2001, Patchev et al., 2004). However, our data suggests that the anxiolytic effects of androgens, which have been shown exclusively in adult animals, may not be potent in aged animals. Furthermore, there is little data concerning androgen-induced anxiolysis in female rodents. Our data suggests that sex-differences should be examined closely in future studies of mood elevation using androgen-based therapies.

In conclusion, in aged female mice, hormone supplementation with T improved spatial reference memory and DHT improved passive avoidance memory. Neither treatment affected anxiety in the open field. These data support that androgen-based therapies might benefit cognition in elderly female populations. As specific androgen-related mechanisms differentially benefit aspects of age-related cognitive decline, future studies are warranted to determine the molecular mechanisms underlying these differential therapeutic effects.

## Chapter 6: General Discussion

There are three main findings in this dissertation that will be highlighted here as they are of general interest. First, although changing androgen levels do not always result in cognitive performance changes in mice, when the changes do occur they support a constructive role for androgens in cognitive performance. This suggests that androgens typically act as a 'good guy' in the milieu of neurochemicals that modulate cognitive performance in mice. However, the effects of androgen manipulations in these studies were not uniform across all cognitive tasks used, or between age and sex. Thus, androgens have complex actions on cognitive behaviors in mice, as well as in humans as was outlined in **Chapter 1**. Second, performance in some, but not all, cognitive tasks is improved in 2 year old female C57BL/6J mice by supplementation with the potent non-aromatizable androgen DHT. This finding suggests that androgenic pathways might provide therapeutic value against age-related cognitive decline in women. However, the effects of T and DHT supplementation were not uniform in my hands and the complexities of androgen supplementation must therefore be taken into account. In a similar vein, not all androgenic effects are likely to be positive in women. Finally, although androgen levels appear supportive of neurogenesis in the dentate gyrus, and despite the good evidence in support of a link between cognition and AHN, the direct modulators of androgenic cognitive effects probably lie elsewhere. I suggest that future basic studies should strive to elucidate the effects of androgens on the physiology, chemistry, and activation patterns in the brain during behavioral performance. In addition, these studies might be most interesting in the context of challenges to the

brain such as stroke, head trauma, irradiation, exposure to nerve agents, neurodegenerative disease, and aging. Finally, the complex temporal effects in DMTP performance that resulted from androgen manipulations might provide a nice way to validate the mediators of androgenic effects on cognitive performance.

In these studies, androgen levels were manipulated in several different ways: they were manipulated pseudo-experimentally by comparing males and females at different ages where females have lower androgen levels than males, androgen levels were increased exogenously in aged females by 6-weeks of either T or DHT supplementation, and adult males were castrated, with some being given DHT androgen replacement. In each case, lower total circulating androgen levels were associated with worse cognitive performance on at least one measure compared to animals with higher levels. Thus, the findings of this dissertation support the idea that androgens are constructive for cognitive performance in both male and female mice, at least under the circumstances of these studies. However, not all cognitive tasks were affected equally by the androgen manipulations. Male and female mice showed equivalent performance at all ages in the novel location and novel object recognition task, and this task was also unaltered by castration in adult male mice. Spatial reference memory performance in the water maze was disrupted by age in both male and female mice; the disruption was moderately greater in the female mice. In contrast, there was no effect of castration on performance in adult male mice. Similarly, although supplementation with T was effective in improving performance on this task in the old female mice, the improvement was modest and DHT had no effect. Androgens seemed to support passive avoidance memory performance since old female mice were

impaired compared to old males, but castration had no effect on this task in adult males. Thus, it seems that even in a single inbred strain of mice factors such as subject sex, subject age, and the types of tasks measured are important in determining the effects of androgens on cognitive performance. Thus it is perhaps important to look to the anatomical and physiological consequences of androgen manipulations to find common denominators to link these complex findings together. In addition, it is possible that castration affected some other pathway only indirectly linked to androgen removal or that was altered as a consequence of androgen removal. Two possibilities are LH and FSH, which increase in circulation due to removal of the androgenic negative feedback on their release from the pituitary (Robaire B et al., 1995). This may be important as increased LH levels have been associated with disruption of spatial cognition in mice (Casadesus et al., 2006, Casadesus et al., 2007).

When old female mice were treated for six-weeks with T or DHT, cognitive performance improved, though not on the same tasks. Again, increased levels of circulating androgens were associated with improvements, not declines, in cognitive performance. However, it is important to recognize that performance in these old mice was likely at a floor-level with little room to decline further. Also, T treatment resulted in very high circulating DHT levels, yet the cognitive effects were not the same as with DHT treatment alone. This points again to the complex nature of hormonal effects on behavior and the need to better understand the underlying neurophysiological effects of androgens. Nonetheless, this finding supports the idea that androgen pathways such as androgen nuclear receptor activation and GABA type-A receptor neuromodulation might provide a therapeutic target to combat age related cognitive decline. Androgens have

been prescribed clinically to treat age-related disorders such as declining mood and libido in women, with some success (Hogervorst et al., 2005, Wierman et al., 2006). Also, a few studies have reported on the cognitive effects of androgen supplementation with mixed results (Wierman et al., 2006). Unfortunately, the findings in these studies have been confounded by the choice of either pre- or post-menopausal subjects often using estrogenic hormone-replacement therapy (Wierman et al., 2006). Thus, it is difficult to know if a lack of cognitive benefit is because of ineffectiveness of androgens per se, or because of a negative synergism with estrogens. The results from my studies suggest that the latter is at least possible since the striking passive avoidance memory performance improvement was seen with DHT treatment only, not with T treatment in which there is the opportunity for the production of estrogens via T aromatization. To my knowledge, no studies have investigated the potential benefit of DHT or other strictly androgenic hormone therapy on cognition in elderly women.

In addition to the equivocal androgen therapy effects in women, there has been concern over the safety of such therapies. Androgen treatment might cause unwanted side-effects such as hair-growth, acne, virulization of the larynx and other sensitive tissues. Also of concern is the potential for estrogen, derived from the androgen supplement, to exacerbate breast or endometrial cancer risk in elderly women. However, the U.S. Endocrine Society best practices bulletin downplays these concerns (Wierman et al., 2006, Braunstein, 2007). At least with supplementation of T in the normal physiological or slightly superphysiological range for young women, there is little evidence for these side effects above control levels (Braunstein, 2007). In addition, T supplementation has been often used to combat cancer, and has been shown in animal

models and clinical research to reduce cancer risk (Braunstein, 2007). Thus, these potential problems should not stand in the way of controlled trials using strictly androgenic agents to combat age-related cognitive decline. In addition, new drugs have been developed that can increase androgen receptor activity only in brain tissue. These selective androgen receptor modulators (SARMS) allosterically modulate the actions of tissue-specific coactivators and/or cosupressors at the androgen receptor (Omwancha and Brown, 2006). Such agents selective for bone have been effective in treating muscle and bone atrophy in rat models without effects on prostate (Sun et al., 2006, Miner et al., 2007), however clinical trials have not been done. In addition, brain-specific SARMS can improve cognitive performance in female mice expressing apolipoprotein E4 (Acevedo et al., 2008a), similar to treatment with DHT (Raber et al., 2002). Thus, these agents might be an excellent treatment for age-related cognitive decline as they can avoid the potential peripheral side effects of androgen hormone treatment.

One possible mechanism by which androgens could help maintain cognitive performance in adulthood and into old age is by protecting the survival of adult-born immature neurons in the GCL of the dentate gyrus. My studies found that removing androgens by castration slightly but significantly reduced the number of doublecortin-expressing immature neurons in the GCL. This is consistent with the evidence to date that androgens are a survival factor for adult-born immature neurons in the hippocampus of male rats (Spritzer and Galea, 2007). It was expected that if the immature neurons in the GCL were mediating the effects of castration on spatial trial-dependent memory performance in the water maze, then removal of these neurons by cranial irradiation should have had a similar effect. In fact, this was not found. Thus, it is

unlikely that androgens support cognition solely by enhancing the survival of immature neurons. Thus, it can be concluded from my studies that androgens can have a significant effect on cognition and they can have a significant effect on AHN, but there is little evidence for a direct link between the two.

During my training, I have discovered a personal craving to see my research move out of pure research and into applied science. I feel that my hard work would be more personally rewarding if I could make tangible impacts on the human condition through medical research. Relative to the potential for androgens as a drug target for ACD, I would like to pursue studies that can be used as preclinical evidence toward future clinical trials. Thus, I would suggest using the robust deficit in PA performance observed in female mice, and the strong DHT replacement effects, to begin the working toward the development of novel drugs to target ACD. The first aim of this research would be to elucidate what brain areas are most important in generating the age- and sex-dependent deficits in PA performance in mice, and what mechanisms allow DHT to provide benefits in these brain areas. For the second aim, I think that all translational research efforts should definitely benefit from the lessons of the past, including the failures of recent (and expensive) clinical trials in stroke medicine (Davis, 2006). Therefore, it would be important from a preclinical perspective to determine the cross-species applicability of the mouse research by performing similar experiments in non-human primates and humans, and to choose appropriate outcome measures in those studies to reflect species-differences in age-related cognitive problems. Finally, the last aim would be to develop novel drugs to specifically modulate androgen receptors in the therapeutically important brain areas, while having no effect on brain areas that might

produce unwanted side-effects. Beside the problem of side effects, I believe this latter point is important for applied androgen research because even early-stage clinical trials require more funding than grants can afford, and novel drug development provides the intellectual property that private investors find attractive. Speaking to the practicality of these aims, I have some knowledge and expertise that might allow me to pursue the first with some collaboration within my field, but I have little experience with the issues involved in the second and third aims. Clearly, I would need to interest experts in these fields and seek strong collaborations with them to perform those studies. To do that I would likely need to be armed with more than just the studies reported in this dissertation and I would like to give some idea of how I would plan to accomplish that.

For the first aim, I would hypothesize that androgens, acting directly on androgen receptors in the hippocampus and amygdala, cause increased synaptogenesis and enhanced LTP in those brain regions that directly modulate behavioral performance in PA. For the first experiment, I would reproduce the DHT effect in aged female mice using a brain-selective androgen receptor modulator (SARM), since this acts directly on androgen receptors in the brain and has better clinical applicability compared DHT treatment. Moreover, I would try to avoid treatments that invade the blood-brain-barrier, such as i.c.v or site-directed injections, since these have little direct clinical application. For the second experiment, I would be interested in establishing what brain areas are activated by learning and retrieval in the PA task in male and female mice of various ages, and with androgen treatment, by studying the pattern of immediate-early-gene (IEG) expression in the brain. Since the dorsal hippocampus and the amygdala are involved in PA performance (Swartzwelder, 1981, Ambrogi Lorenzini et al., 1997), I

would hypothesize that the hippocampus and amygdala would show higher-than-background numbers of neurons expressing these markers after training and after the test trials, and that these numbers would decrease with age and correlate with performance outcomes. Moreover, I would expect that castrating aged males would reduce the number of IEG-expressing neurons and disrupt PA performance, whilst giving SARM treatment would reverse the effect. In addition, I would expect that giving SARM supplements to aged females would increase IEG-expressing neuron number and improve PA performance. With the important brain areas in hand, I would use electron-microscopy with a unbiased stereological technique to quantify the number of symmetrical and asymmetrical synapses in those regions as a function of sex, age, and androgen status. I would then want to know whether changes in synapse number correspond a measurable electrophysiological effect in the tissue. LTP has been observed in the hippocampus (Staubli and Lynch, 1987) and amygdala (Shaban et al., 2006) so those would be obvious targets to study. Finally, if enhanced LTP and neuronal activation due to increased synapse number are responsible for the benefits of SARM treatment on PA performance, I would expect that treatments which block LTP and neuronal activation would likewise block the beneficial effects of SARM treatment.

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