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INTERACTIONS OF HORMONES, AGING AND SEXUAL EXPERIENCE ON MASCULINE SEXUAL BEHAVIOR AND HORMONE RECEPTOR EXPRESSION IN THE HYPOTHALAMUS

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INTERACTIONS OF HORMONES, AGING AND SEXUAL EXPERIENCE ON MASCULINE SEXUAL BEHAVIOR AND HORMONE RECEPTOR EXPRESSION IN THE HYPOTHALAMUS

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DEDICATION

I dedicate this dissertation to my parents, 吴敖根 and 吴家箴 (Jiangzhen and Aogen Wu), for their love, their serving as strong role models for my life, and their emotional support during my graduate studies in the U.S. I am extremely grateful to my husband, 刘存 (Cun Liu), for his encouragement and support of my work over the past six years. Finally, I express my gratitude to Joanne Click, my "American mom," for her friendship and support.

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INTERACTIONS OF HORMONES, AGING AND SEXUAL EXPERIENCE ON MASCULINE SEXUAL BEHAVIOR AND HORMONE RECEPTOR EXPRESSION IN THE HYPOTHALAMUS

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Age-related declines of androgens and libido in males have been observed for decades. This dissertation sought to elucidate the mechanisms by which hormones may act differentially upon their receptors in the hypothalamus of aging compared to young males. I also examined how sexual experience modulates the ability of hormones to facilitate sexual behavior with aging. Experiment one measured androgen receptors (AR) and estrogen receptor α (ERα) cells in male rats at young, middle-aged and old age. I found that AR cell numbers in hypothalamic regions studied underwent significant age-related increases. Numbers of heavily ERα labeled cells, but not total ERα cells, increased with age. This study demonstrates that the aging brain has the

capacity to synthesize hormone receptors which is increased possibly due to decreased testosterone concentrations. Experiment two examined the effect of sexual experience on serum hormones and cells of AR and ERα in hypothalamic regions in young and middle-aged males. The results showed that AR cell numbers increased with aging but did not change with experience. No age- or experience-related alteration in ERα expression occurred. However, serum testosterone increased and estradiol decreased with age. Experience increased total and free testosterone. Interactions of age and experience on total testosterone, estradiol, and luteinizing hormone were found. These results show long-lasting effects of sexual experience on hormones, but not on their receptors in the hypothalamus. Experiment three investigated effects of exogenous testosterone on sexual behavior in young and middle-aged males. The results showed a decline in sexual behavior parameters with age. After castration with testosterone treatment, there were few differences in sexual behavior measures between young and middle-aged males. AR cell numbers were higher and ERα cell numbers lower in testosterone compared to vehicle-treated males of both ages, and few effects of age occurred. These findings indicate that testosterone and aging interact in a complex manner to control numbers of cells expressing hormone receptors in the brain and on the subsequent control of sexual behavior. This insight provides a better understanding of the relationship between molecular changes in the brain and behavior, and suggests new therapeutic targets to human testosterone treatment.

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CHAPTER 1. INTRODUCTION

Life expectancy has increased dramatically since the turn of the 20th century due to improved standards of hygiene and to the development of advanced theories and techniques of modern medicine. Currently, about one-fifth of the world's population confronts the major topic of aging. As a result of growing emphasis on aging populations, improvements in quality of life are now becoming as important as attempts to increase life expectancy.

Visible signs of aging that are oftentimes observable in middle-aged adults include abdominal obesity, decreasing energy, mood changes, muscle wasting, thin skin and skin wrinkles, poor sleep, cognitive changes, etc. For males, one of the signs that may affect quality of life the most is lessened sexual desire or performance. A series of studies have focused on this problem.

MALE SEXUAL BEHAVIOR

Male sexual behavior has been thought to contain two components: 1) Sexual desire: it drives the male to search and get near to a receptive female; 2) Consummation: it refers to direct body contact including mounting, intromissions and ejaculations (Beach, 1956, Roselli et al., 2003). Later, mounting behavior has been recategorized as a measure of motivation, as opposed to

consummation. Dr. Clark (1984) has redefined components of male sexual behavior to include motivation (approaching and mounting), erectile, and ejaculatory responses (Clark et al., 1984). In this dissertation, I categorize masculine sexual behavior into two components: motivation (mounting) and copulation (intromission and ejaculation).

TESTOSTERONE AND MALE SEXUAL BEHAVIOR

Studies of hormones in male sexual behavior have led to the recognition that androgens play a critical role in the regulation of these processes. Numerous studies have specifically elucidated testosterone's primary role in regulating masculine motivation and copulatory behavior in young human and experimental animals. In humans, a lowering of testosterone has been observed to lead to a consistent and significant reduction in the level of sexual motivation within 3 to 4 weeks. Moreover, with testosterone treatment, normal sexual interest is restored [for review see (Bancroft, 2005)]. Because it is difficult to measure sexual activity in humans, research has been undertaken to measure the relationship between parameters of sexual behavior and testosterone in rats. In young rats, with the reduction of testosterone, copulatory behavior disappeared completely within several days to a couple of weeks (Davidson, 1966, Chambers et al., 1991) and, after testosterone exposure, was restored within 7 to 10 days (McGinnis et al., 1989, Chambers et al., 1991). However, in an earlier study, it was shown that for

intact male rats who could complete copulatory behavior, there was no correlation between the behavior measures and testosterone levels and that behavior parameters did not change significantly with different serum testosterone within the physiological range achieved by implantation with different sizes of testosterone capsules in castrated rats (Damassa et al., 1977). This indicates the existence of a threshold of sexual behavior in response to testosterone. Once beyond that threshhold, extra testosterone does not improve sexual performance very much. In the same report in young rats (Damassa et al., 1977), less than one-third the normal levels of testosterone was needed for castrated male rats to maintain normal sexuality. The beyond-threshold “extra” testosterone may be required for other testosterone targets since different organs expressing androgen receptor have varying threshold values of plasma testosterone to exhibit response (Fielder et al., 1989).

Metabolism of Testosterone

There are two major pathways for testosterone metabolism in the brain: one is the aromatization to estradiol by P450 aromatase and the other is the 5-alpha-reduction to DHT (Dihydrotestosterone). As a result, testosterone can activate estrogen receptors by estradiol and activate the androgen receptor directly or indirectly by DHT which is more potent for binding the androgen receptors.

Estradiol

The hypothesis that testosterone exerts its effects upon masculine sexual behavior via conversion to estradiol has been supported by numerous studies that found that an aromatase inhibitor or antiestrogen can block testosterone induction of copulation (Luttge, 1975, Morali et al., 1977, Zumpe et al., 1993, Vagell and McGinnis, 1997, Clancy et al., 2000, Rosario et al., 2004, Taziaux et al., 2007); high dose of estradiol capsule implantation can restore partner preference for a receptive over a non-receptive female (Merkx, 1984, Bakker et al., 1993) and that estradiol can restore copulatory behavior in castrated experienced male rats, albeit at supraphysiological levels (Davidson, 1969, Sodersten, 1973).

Dihydrotestosterone (DHT)

The second metabolite of testosterone, DHT, has high affinity for androgen receptors and appears to mediate many of the effects of testosterone on many, although not all, aspects of masculine sexual behavior. Studies have found that while DHT fails to initiate partner preference to females compared to rats with combined DHT and estradiol treatment in experienced male rats (Bakker et al., 1993) and sexual behavior in castrated inexperienced male rats (McDonald et al., 1970), DHT does restore masculine copulatory behavior in experienced male rats (Whalen and Luttge, 1971, Butera and Czaja, 1989), adult male hamsters (Romeo et al., 2001), guinea pigs (Alsum and Goy, 1974),

monkeys (Phoenix, 1974, Michael et al., 1987) and whiptail lizards (Wade et al., 1993). In these reports, very high does of DHT were required and were found to be less effective than testosterone in supporting copulatory behavior, thus suggesting some complementary role of DHT together with estradiol. However, DHT is considered to be the active androgen, functioning primarily in the peripheral nervous system, which is responsible for preventing erectile failure seen in castrated rats (Lugg et al., 1995) although its function in the central nervous system has been reported (Baum et al., 1982).

Estradiol plus DHT

Although both estradiol and DHT have limited ability to fully restore masculine sexual behavior, when a combination of estradiol and DHT were administrated, they could easily restore masculine sexual behavior to the level comparable to testosterone treatment (Baum and Vreeburg, 1973, Larsson et al., 1973, Feder et al., 1974, Beyer et al., 1976, Davis and Barfield, 1979b, Baum et al., 1982).

A widely accepted hypothesis is that estrogen stimulates the neurons in the central nervous system for male sexual behavior, while androgen primarily acts in the peripheral nervous system to stimulate the penis development that is necessary for vaginal penetration (Larsson et al., 1973, Lieberburg and McEwen, 1975). Since local aromatase activity is regulated by an androgen receptor-dependent mechanism (Roselli and Resko, 1984), DHT facilitates estradiol

production in the central nervous system. Blockade of AR in the brain leads to a decrease of local estradiol synthesis and, as a result, sexual behavior is affected. However, there are several points that challenge this hypothesis:

1) Estradiol injection or capsule implantation is able to fully restore sexual behavior only at a very high level (beyond physiological level) in castrated sexually experienced rats (Davidson, 1969, Sodersten, 1973). Within physiological range, estradiol could not or only weakly stimulate mounting, motivation part of masculine sexual behavior, in castrated animals [rats: (Pfaff, 1970, Larsson et al., 1976, Hawkins et al., 1988, Vagell and McGinnis, 1997); guinea pigs: (Alsum and Goy, 1974)].

2) One argument about why estradiol-treated castrated adult male rats do not ejaculate is that the lack of penile development may lead to the lack of ejaculatory behavior, and estradiol cannot stimulate erection in castrated male rats (Hart, 1973). In fact, when the ejaculatory behavior was induced by a high dose of estradiol in castrated male rats, the penile size or morphology was not stimulated significantly (Paup et al., 1975) and DHT could maintain the number of penile papillae and weight of the penile but failed to induce ejaculation (Hart, 1973). Also, intracranial DHT implantation and systemic given estradiol can stimulate sexual behavior without stimulating penile spine growth (Baum et al., 1982). Thus, it is clear that masculine behavior is dissociated with peripheral morphology.

3) The level of mounting achieved in males with testosterone treatment was significantly greater than that observed with estradiol treatment (Roselli and Chambers, 1999), suggesting that the androgenic component of testosterone is involved.

4) For copulatory behavior, motivation is a necessary, but not sufficient, condition. Blocking local synthesis of estradiol leads to blocking of the entire copulatory behavior. That may also be the reason why it is hard to measure the effect of nonaromatized androgen directly on copulation.

5) DHT, a nonaromatized androgen, treatment can stimulate copulatory behavior in experienced male rats (Whalen and Luttge, 1971, Butera and Czaja, 1989).

6) At the physiological level, estradiol and DHT cannot fully restore masculine sexual behavior compared to testosterone treatment in castrated male rats (McGinnis and Dreifuss, 1989).

These observations, taken together, suggest that androgens, especially testosterone that is not aromatized, also contribute to sexual motivation and copulatory behavior.

Hormone Levels in the Brain

In 1959, Phoenix and colleagues found that sexual behavior is regulated by testosterone in guinea pigs (Phoenix et al., 1959), which indicated that the brain is a target of sex steroid hormones. Since then, numerous studies were

done to investigate hormones' effects on both brain structure and function, such as brain morphology [e. g. (Raisman and Field, 1973)], brain nuclear volume [e. g. (Gorski et al., 1978)] and sexual behavior as mentioned previously.

However, the actual levels of hormones in the brain were not measured until 1990's. Before that time, limitations in technology prevented the quantification of brain hormone levels directly. Therefore, research was limited to in vitro studies to investigate brain testosterone and estradiol levels. [e.g. (Dessi-Fulgheri et al., 1983)].

At present, methods measuring testosterone and estradiol levels in the brain include radioimmunoassay (Alomary et al., 2001, Amateau et al., 2004) and chromatography (Vallee et al., 2000, Higashi et al., 2006). Hormone levels in the brain are expressed as hormone level per wet tissue weight. Brain testosterone levels in aging men without neuropathology were reported as is 1.0 ng/g (Rosario et al., 2004). In rodents, testosterone levels in the whole brain were reported as 30-35 pmol/g in young male mice (Pinna et al., 2005) and 0.4-0.5 ng/g in young male Sprague-Dawley rats (Liu et al., 2003). Estradiol levels were shown to be approximately 0.1 ng/g for aging men without neuropathology (Rosario et al., 2004). To my knowledge, no data have been reported for brain estradiol levels in male rodents.

Only two studies measured brain and serum testosterone levels side by side using the liquid chromatography–mass spectrometry (LC–MS) and negative chemical ionization gas chromatography/mass spectrometry (Vallee et al., 2000,

Higashi et al., 2006). In the paper of Higashi et al., serum testosterone concentration was ~3 ng/ml and the whole brain testosterone concentration was ~1 ng/g (Higashi et al., 2006). It appears that serum testosterone is approximately three times higher than the whole brain testosterone. Therefore brain testosterone levels are quite high relative to serum.

NEUROTRANSMITTERS REGULATING SEXUAL BEHAVIOR

Preoptic Area

Several studies have substantiated that an area of the brain that plays a fundamental role in the control of male copulatory behavior, penile erection and ejaculation is the preoptic area (POA, sometimes inclusive of the anterior hypothalamus, and referred to as the preoptic anterior hypothalamic area, POAH). Lesions of the preoptic area have been found to block sexual behavior completely in male rats (Christensen et al., 1977) while intracerebral implants of either testosterone or estradiol into the POA of castrated male rats can stimulate masculine sexual behavior (Christensen and Clemens, 1974, Huddleston et al., 2007). Additionally, very high aromatase activity, which is regulated by testosterone, has been found in this area (Roselli and Resko, 1984). When neuron activity was recorded, the firing rate of the neurons in this area increased significantly starting with the introduction of a female through a copulatory series until ejaculation, and decreased during the postejaculatory interval (Shimura et

al., 1994). This latter result suggests a role of the POA in motivational and consummational aspects of masculine sexual behavior. As a result, a lot of research on sexual behavior focuses on neurotransmitters in the POA.

Neurotransmitters

A number of neurotransmitters are known to influence the testosterone activation of masculine behavior pathway including norepinephrine, glutamate, dopamine, serotonin, GABA and nitric oxide. These neurotransmitters are all released into the POA, and their receptors are localized in this region. Administration of agonists or antagonists of these transmitter receptors affects male sexual behavior regulated by testosterone. Although it is beyond the scope of this dissertation provide a comprehensive review of all neurotransmitters and their effects on male sexual behavior, I provide a brief overview of a few representative neurotransmitters (see Table 1.1 to Table 1.5). This section is included to highlight the point that masculine sexual behavior involves a complex circuit of steroid-sensitive neurons within and projecting to the POA, that communicate by several central neurotransmitters.

Table 1.1. Norepinephrine and Masculine Sexual Behavior

Norepinephrine facilitates sexual behavior.

Facilitating masculine sexual behavior	POA contains high quantities of norepinephrine ((Versteeg et al., 1976, Mas et al., 1987), which is partly released from locus coeruleus to medial preoptic area (Anselmo-Franci et al., 1997). Injection of norepinephrine into the medial preoptic area can facilitate both motivation and the copulatory part of sexual behavior (Mallick et al., 1996).
Effects of agonists and antagonists	Prazosin, the adrenergic receptors alpha 1 adrenergic receptor antagonist, increased ejaculation latency. In contrast, yohimbine, by blocking to presynaptic alpha 2 adrenergic receptor to increase norepinephrine, decreased ejaculation latency and post-ejaculatory interval (Clark et al., 1985b).
Modulation by testosterone	The yohimbine-facilitated sexual behavior is testosterone-independent (Clark et al., 1985c). While testosterone treatment in castrated hamsters produced less norepinephrine in medial preoptic area (Tomaselli et al., 2001), in the whole preoptic area in male rats, norepinephrine levels were not altered by castration or testosterone (Gabriel et al., 1988)

Table 1.2. Glutamate and Masculine Sexual Behavior

Glutamate facilitates masculine sexual behavior.

Facilitating masculine sexual behavior	Copulation is accompanied by increased extracellular glutamate in medial preoptic area with a peak observed at the time of ejaculation ((Dominguez et al., 2006b).
Effects of agonists and antagonists	Injection of glutamate uptake inhibitors into this area decreased ejaculation latency and postejaculatory interval. The glutamate receptor subtype NMDA receptor appears to play a key role of mediating effects of glutamate in the POA. Administration of an NMDA receptor antagonist in male rats prior to sexual experience reduced or eliminated facilitation of sexual performance induced by previous experience with female rats (Fleming and Kucera, 1991, Powell et al., 2003).
Modulation by testosterone	Glutamate-responsive neurons are also testosterone-sensitive (Yang et al., 2002), but in male rat hypothalamus, NMDA receptor binding did not change after castration or testosterone treatment (Brann et al., 1993). This suggests that a mechanism other than receptor binding is altered by exposure to testosterone. Non-NMDA glutamate receptors may also play a role, as subunits of AMPA Glu1 and GluR 2/3 were significantly higher in medial preoptic area in castrated rats treated with estradiol or testosterone and in the intact than in the castrated males (Diano et al., 1997).

Table 1.3. Dopamine and Masculine Sexual Behavior

Dopamine facilitates masculine sexual behavior.

Facilitating masculine sexual behavior	Dopamine facilitates male sexual behavior and its levels are high in preoptic area (Mas et al., 1987). Sexual motivation and copulation in male rats are accompanied by increased extracellular dopamine in medial preoptic area (Mas et al., 1987, Mas et al., 1995).
Effects of agonists and antagonists	Rats taking dopamine precursor L-dopa and dopamine receptor agonist apomorphine have lower intromission latency and ejaculate sooner and with fewer intromissions (Paglietti et al., 1978). Administration of dopamine receptor agonist apomorphine directly in medial preoptic area restored mounting behavior in long-term castrated males (Scaletta and Hull, 1990).
Modulation by testosterone	With respect to steroids, both sexual behavior and dopamine release can be abolished by castration (Hull et al., 1995). It was reported that estradiol treatment helped maintain a high dopamine level in medial preoptic area in castrated male rats that could be related to intromission, while DHT could induce an elevation of dopamine after exposure to receptive female (Putnam et al., 2003).

Table 1.4. Serotonin and Masculine Sexual Behavior

Serotonin inhibits masculine sexual behavior.

Facilitating masculine sexual behavior	Serotonin inhibits masculine sexual behavior probably via action of binding 5-HT1B receptors (Fernandez-Guasti et al., 1992). Serotonin-stained fibers were observed surrounding medial preoptic nuclei at a low to medium density while the lateral cell-sparse part of medial preoptic nuclei was filled with a dense plexus of serotonin fibers (Simerly et al., 1984). Serotonin and serotonin metabolite 5-hydroxyindoleacetic acid were released at ejaculation in the preoptic area (Mas et al., 1987, Mas et al., 1995, Lorrain et al., 1997) and their levels decreased until before the next ejaculation (Lorrain et al., 1997).
Effects of agonists and antagonists	Conflicting results were reported about serotonin's function in medial preoptic area. Injection of Cyp, serotonin receptor antagonist, in medial preoptic area shortened ejaculation latency (Verma et al., 1989) whereas another lab observed no effect on sexual behavior by elevating serotonin in medial preoptic area (Lorrain et al., 1997).
Modulation by testosterone	Testosterone increases 5-HT1A receptors (Mendelson and McEwen, 1990) but not 5-HT2A receptors (Sumner and Fink, 1998) binding in medial preoptic nucleus in castrated male rats. Serotonin metabolism was not affected by castration or testosterone treatment (Gabriel et al., 1988).

Table 1.5. GABA, Nitric Oxide and Masculine Sexual Behavior

GABA inhibits masculine sexual behavior.

Facilitating masculine sexual behavior	Injection of the GABA antagonist bicuculline into the medial preoptic area reduced ejaculation latency and post-ejaculatory interval whereas GABA agonist muscimol depressed masculine sexual behavior in male rats (Fernandez-Guasti et al., 1986b).
Modulation by testosterone	This effect of bicuculline in the medial POA can synergize with sub-threshold dosages of testosterone to facilitate masculine sexual behavior (Fernandez-Guasti et al., 1986a). $GABA_A$ alpha2 and alpha5 subunit mRNA expression in the medial preoptic nucleus in male mice tended to increase 3 weeks after chronic anabolic androgenic steroid treatment and tended to decrease at the 6^{th} week (no significant difference) (Penatti et al., 2005). Castrated male rats showed lower GABA release in the medial preoptic area than intact and castrated rats with testosterone treatment (Tin Tin Win et al., 2002). The rate of GABA turnover decreased 6 hours after castration in medial preoptic area in male rats (Yoo et al., 2000).

Nitric oxide facilitates masculine sexual behavior

Effects of agonists and antagonists	Administration of neuronal nitric oxide synthase (nNOS) inhibitor in the preoptic area reduces mount rates (Sato et al., 1998) and also reduces the ejaculation percentage (Benelli et al., 1995, Lagoda et al., 2004) in male rats. Treatment of rats with L-arginine, a nitric oxide precursor, has been observed to facilitate mount and ejaculation sexual behavior in male rats (Benelli et al., 1995, Sato et al., 1998).
Modulation by testosterone	It was reported that nNOS expression was increased by testosterone and depleted by castration in preoptic area (Putnam et al., 2003, Sanderson et al., 2008).

Summary

Neurotransmitters' actions are interactive and complex but each neurotransmitter can affect masculine sexual performance alone. The regulation of neurotransmitters on sexual behavior is affected by testosterone or its metabolites estradiol and DHT.

HORMONE RECEPTORS AND SEXUAL BEHAVIOR

Androgen receptors and estrogen receptors may mediate the actions of testosterone or estradiol by functioning as ligand-activated nuclear transcription factors and thus alter the expression of specific sets of hormone-responsive genes.

Androgen Receptors

Testosterone can affect neurons by binding to various receptors: (1) Nuclear receptors for transcriptional regulation and (2) Membrane-bound receptors to influence second messengers system. Although it is often difficult to distinguish between nuclear versus membrane actions, it is clear that one or both types of ARs are important for sexual behavior. Androgens may act in a nongenomic manner through more than one mechanism, such as MAP kinase, cAMP and PKA, or a G protein-coupled receptor that has not been unambiguously identified (Heinlein and Chang, 2002). Studies of this latter

androgen action are limited to human prostate cancer, and to my knowledge, no data on its regulation of sexual behavior have been reported. So references to “androgen receptors” in this dissertation refer specifically to nuclear androgen receptors.

The androgen receptor is closely related to masculine sexual behavior because:

1) Androgen receptor is necessary for the restoration of masculine sexual behavior. Vagell et al. reported that inhibition of ARs with hydroxyflutamide blocked the restoration of male copulatory behavior and partner preference by testosterone (Vagell and McGinnis, 1998).

2) Androgen receptor is involved in the interaction between testosterone and neurotransmitters. In medial preoptic area, 51% and 45.2% AR colocalized with subunits of AMPA GluR2/3 and GluR1 respectively (Diano et al., 1997). Although Hull’s lab failed to find colocalization of tyrosine hydroxylase and AR neurons in several dopaminergic cell groups in the preoptic area and hypothalamus (Sato and Hull, 1999, Hull et al., 2004), they found that colocalization of AR and neuronal nitric oxide synthase is 52.5% in medial preoptic area and 60.3% in anteroventral periventricular nucleus (AVPV) (Sato et al., 2005).

3) Sexual activity status appears to be related to the androgen receptor density in reproductive brain areas. Castration depletes male sexual behavior and androgen receptor expression in hypothalamus while testosterone treatment

can restore sexual behavior and androgen receptors (McGinnis et al., 1989, Krey and McGinnis, 1990). Sexually active male rats have significantly higher AR density than sexually exhausted male rats who did not want to mate although the serum testosterone levels were similar in both groups (Fernandez-Guasti et al., 2003). Further studies in that lab have shown that a reduction in sexual behavior after sexual satiety was accompanied by an AR density decrease only in the medial preoptic area-medial part of the brain (Phillips-Farfan and Fernandez-Guasti, 2008). Therefore, AR density in reproductive brain areas is considered to play a key role in the increase of testosterone on behavioral performance.

4) Androgen receptor mediates local estradiol production. Local aromatase activity in POA is regulated by an androgen receptor-dependent mechanism (Roselli and Resko, 1984), blockade of AR leads to decrease of local estradiol synthesis and further decrease effects of estradiol on sexual behavior.

Estrogen Receptors

At present, two kinds of estrogen receptor, estrogen receptor α (ERα) and estrogen receptor β (ERβ), have been cloned and sequenced in mammals. In the brain, these receptors are widely distributed but they are regionally specific and mostly do not overlap. ERα is expressed dominantly in the hypothalamus and preoptic area while ERβ is expressed abundantly in the cerebral cortex, hippocampus and medial preoptic area (Shughrue et al., 1997) Although research found that ERβ plays a critical role in sexual behaviors (Temple et al.,

2003), masculine sexual behavior may be regulated mainly by ERα indirectly by neurotransmitters such as dopamine, glutamate and nitric oxide released from interneurons (Hull et al., 2004, Dominguez et al., 2006b). A study done using knockout mice found that ERα is primarily involved in masculinization while ERβ has a major role in defeminization of sexual behaviors (Kudwa et al., 2006). Male ERβKO mice are fertile and are able to exhibit normal sexual behavior (Krege et al., 1998, Ogawa et al., 1999) whereas in ERαKO mice sexual behavior is disrupted (Ogawa et al., 1997, Wersinger et al., 1997). Because this dissertation focuses on age and behavioral changes in ERα, the following discussion will be specifically about this molecule.

ERα also interacts with the other neurotransmitters relating to sexual behavior. In medial preoptic area of both male and female rats, 48.5% and 45.2% ERα colocalized with subunits of AMPA GluR2/3 and GluR1, respectively (Diano et al., 1997). Number of tyrosine hydroxylase immunoreactive neurons in the AVPV is higher in wild-type than in ERαKO male mice (Simerly et al., 1997). Hull's lab found that in preoptic area and hypothalamus, tyrosine hydroxylase and ERα did not colocalize in the neurons in several dopaminergic cell groups (Sato and Hull, 1999, Hull et al., 2004), but they observed that colocalization of ERα and neuronal nitric oxide synthase is 52.5% in medial preoptic area and 77.0% in anteroventral periventricular nucleus in male rats (AVPV) (Sato et al., 2005).

Interaction of AR and ERα

Estrogen and androgen affect each other's function through actions on each other's receptors. Estrogen increases the number of AR and the duration of ligand occupancy (Roselli and Fasasi, 1992, Handa et al., 1996). Increase of AR by estradiol is also reported in other tissues such as brain cortex (Kumar and Thakur, 2004), immature rat uterus (Weihua et al., 2002) and rat prostate (Nakhla et al., 1997, Richter et al., 2007). Testosterone decreased ERα in preoptic area, ventromedial and arcuate nuclei of hypothalamus in neonatal male rats [protein, (Kuhnemann et al., 1995)], in cerebral cortex in both young and old male mice [transcriptional level, (Thakur and Sharma, 2007)], in VMH of female whiptail lizards [mRNA, (Crews et al., 2004)], in rat prostate gland [mRNA, (Asano et al., 2003)], and in primate mammary epithelial cell [mRNA, (Zhou et al., 2000)].

Both AR and ERα are densely localized in the preoptic area. In medial preoptic area of male rats, 81% of ERα neurons also express AR while 31% of AR neurons are also ERα positive (Greco et al., 1998). The overlapping distribution of AR and ERα may mediate synergistic actions of estrogen and androgen on the neuroendocrine system at the transcriptional level (Jaussi et al., 1992). Their actions may also be antagonistic since the estradiol/ERα complex can inhibit transcriptional activity of AR (Kumar et al., 1994).

It is interesting that the 200-250g, male castrated Long-Evans rat with one 5-mm 0.5% estradiol plus two 10-mm DHT capsules could produce comparable

level of AR and ERα to those in rats with two 10-mm testosterone capsules. But the former could not perform masculine sexual behavior and the latter mated very well (McGinnis and Dreifuss, 1989). This indicates the critical role of testosterone/AR complex in stimulating sexual behavior.

AGING

Sexual Behavior and Testosterone with Aging

It is clear that there is a decline in sexual interest, arousability and activity in aging men (Schiavi et al., 1990) and other male mammals (Bronson and Desjardins, 1977, Chambers and Phoenix, 1983, Smith et al., 1992). At the same time, reports on testosterone in male mammals are relatively consistent, showing age-related declines in humans (Harman, 2001), rhesus monkeys (Downs and Urbanski, 2006), mice (Bronson and Desjardins, 1977) and rats [Sprague-Dawley (Downs and Urbanski, 2006); Wistar (Taylor et al., 1996, Bernardi et al., 1998); Brown Norway (Chen et al., 1994, Gruenewald et al., 2000), Fischer 344 (Chambers et al., 1991, Luine et al., 2007)].

Exogenous Testosterone and Aging Males

Various observations on masculine sexual behavior and androgen in young male rats have led to the hypothesis that androgen deficiency may lead to

a decline in sexual desire and activity in aging males. However, additional studies have demonstrated that when testosterone levels in old male rats were brought back to the level of that in young rats, the decline of sexual arousal (Gray et al., 1981) and copulatory activity (Hsu et al., 1986, Chambers et al., 1991) could not be restored. Together, these studies show that the results of testosterone deficiency and replacement in young adult males cannot lead to the absolute prediction of the consequences of deficiency and replacement in older males. There are many possibilities involved in the failure of restoring sexual behavior in aging males, such as: (1) Erosion of negative feedback of the hypothalamic-pituitary-gonadal system which causes a decrease in the release of the luteinizing hormone and further leads to the decline of testosterone production [Human: (Mitchell et al., 1995), Rat: (Haji et al., 1981, Chambers et al., 1991, Bonavera et al., 1997)]; (2) Increase of sex hormone binding globulin, making less testosterone bio-available [Human: (Field et al., 1994, Yasui et al., 2007)]; (3) Increase of aromatase activity with aging leading to more testosterone being converted into estradiol [Humans: (Pirke and Doerr, 1975), Rats: (Lupo-di Prisco and Dessi-Fulgheri, 1980)].

Of particular relevance to this dissertation is to address the possibility that the aging individual's neural sensitivity to exogenous androgen may change in old males. Two of the possibilities are:

(1) The metabolism of androgen changes with aging;

(2) Androgen receptor expression (its number and/or density) in the brain regions is influenced by aging.

(1) The metabolism of testosterone changes with aging

It is important to consider local synthesized estradiol and DHT in preoptic area (POA) of the brain, where much of the regulation of reproduction and sexual behavior is controlled.

Estradiol: In preoptic area (POA) aromatase activity decreases with aging [rat: (Chambers et al., 1991); Japanese quail: (Dellovade et al., 1995)]. In fact, there is a significant positive correlation between circulating testosterone levels and preoptic-hypothalamic aromatase activity [young male hamster: (Petterborg et al., 1991); young and old male rat: (Roselli et al., 1986)], such that with aging, it would be predicted that with declining serum testosterone, there would be a decrease in aromatase activity in POA. However, it was shown in aged male rats that exogenous testosterone could increase brain aromatase activity acutely to the level of young males (Roselli et al., 1986, Chambers et al., 1991). In other words, testosterone treatment of aging males can restore aromatase activity and bring hypothalamic estradiol to the level of young males.

DHT: Notably, the rate of conversion of testosterone to dihydrotestosterone (DHT) is the same in hypothalamus of both young and old rats (Piva et al., 1993). Activity of the enzyme, 5-alpha reductase, does not significantly change with aging in Japanese quail (Balthazart and Schumacher,

1984) and rats (Gao et al., 2002) and is not affected by testosterone treatment or castration in rats (Melcangi et al., 1987, Gao et al., 2002). The conclusion that DHT does not change significantly with aging is in agreement with these observations (Pirke and Doerr, 1975, Simpkins et al., 1981, Field et al., 1994, Labrie et al., 1997).

(2) Hormone receptors expression changes with aging

Few studies have reported how hormone receptors change with aging. Haji reported that in hypothalamus, both AR and ERα receptor decrease with aging (Haji et al., 1981) and Greenstein observed the lower affinity of DHT binding reaction in aging male rat hypothalamus (Greenstein, 1979). Because of the limit of technology, these two studies measured the unbounded hormone receptor in cytosol after castration. It is reported that AR mRNA (rat) and AR synthesis (mice) expressed in the hypothalamus decreased in the old group compared to the young (Kerr et al., 1995, Thakur et al., 2000). For ERα, its expression in the medial preoptic area does not change with aging (Madeira et al., 2000). Age-related alteration in both AR and ERα expression in preoptic area has not been reported. As a result, investigation of both AR and ERα expression in preoptic areas at different ages needs to done.

SEXUAL EXPERIENCE

One important factor that also needs to be considered during behavioral experimentation is an animal's sexual experience. Research has shown that after sexual experience males are more efficient in both motivational and copulatory behaviors. For example, experience with females made mice significantly prefer the odor of female urine odor rather than that of water (Ogawa et al., 1996). Rats with experience demonstrated a greater preference for receptive females than for males (Matuszczyk and Larsson, 1994). While rats were getting more sexual experience, they showed significantly increasing pre-contact vocalizations (Bialy et al., 2000). Compared with naïve rats, sexually experienced rats could achieve an ejaculation with fewer mounts and intromissions (Larsson, 1959) and they had lower mount latency, intromission latency and ejaculation latency (Dewsbury, 1969, Dahlof and Larsson, 1978, Frankel, 1981, Pfaus and Wilkins, 1995, Bialy et al., 2000) and shorter post-ejaculatory intervals (Larsson, 1959).

Experience also alters regulation of masculine behavior by sex hormones. Experienced animals keep all or part of masculine behavior even after castration, including sexual motivation in rats (Fielder et al., 1989, Witt et al., 1995) and copulatory behavior in cats (Rosenblatt and Aronson, 1958) and hamsters (Lisk and Heimann, 1980). DHT treatment can stimulate copulatory behavior in experienced but not naïve male rats (McDonald et al., 1970, Whalen and Luttge, 1971, Beyer et al., 1976, Butera and Czaja, 1989).

Experience-facilitated sexual performance may be due to neuronal plasticity in the area regulating masculine behavior. Behavioral experience can cause elevated metabolic capacity in the limbic system (Sakata et al., 2002) and plasticity of excitatory synapses in medial preoptic area in vitro (Malinina et al., 2006). Ejaculation has also been found to generate more neural activation in medial preoptic area in experienced males as compared to naïve males (Lumley and Hull, 1999). Furthermore, experienced rats, as compared to non-experienced males, showed greater sexual performance after brain impairment caused by lesions of the medial preoptic area (de Jonge et al., 1989, Liu et al., 1997), lesions of the medial amydala (de Jonge et al., 1992, Kondo, 1992) as well as in lesions of the bed nucleus of stria terminalis (Claro et al., 1995). These latter regions are interconnected to POA and are part of the masculine sexual behavior circuitry. Since sexual experience changes the sensitivity of sex hormones, it may also affect hormone receptor expression but no investigation has been reported on this relationship.

TWO SUB-AREAS OF INTEREST

We have already discussed the critical role of preoptic area on masculine reproductive behavior. Importantly, the preoptic area is a heterogeneous structure which contains several sub-areas such as medial preoptic nucleus (MPN), the median preoptic nucleus, lateral preoptic nucleus, periventricular

preoptic nucleus, and anteroventral periventricular nucleus (AVPV). Two of these sub-areas, AVPV and MPN, were the focus of this dissertation.

AVPV

The AVPV is located bilaterally around the third ventricle in anterior and ventral parts of POA, as its name (anteroventral periventricular nucleus) implies. This area is closely related to reproductive function:

1) The AVPV is sexually dimorphic, a process that is first observed shortly after birth in rats, and remaining dimorphic through adulthood (Davis et al., 1996). The AVPV is nearly twice as large and has higher cell density in female rats than in males (Bleier et al., 1982). However, males have denser projections from the bed nuclei of the stria terminalis (BST) (which carries olfactory information to hypothalamus) to AVPV since postnatal day 10 while females have none (Hutton et al., 1998). Sexually dimorphic volume of AVPV can be reversed by castration of neonatal male rats whereas ovariectomy of females had no effect (Davis et al., 1996).

2) The AVPV is abundant in many of the neurotransmitters that are involved in regulation of masculine sexual behavior and their expressions are also sexually dimorphic.

Glutamate: Female rats express NMDA receptor 1 and AMPA receptor subtypes abundantly in AVPV (Gu et al., 1999).

Dopamine: male rats had tyrosine hydroxylase (the rate-limiting enzyme for dopamine synthesis) -stained cells and fibers in AVPV although it was less than in females (Simerly et al., 1985).

Serotonin: Moderate and high densities of 5-HT labeled fibers were observed surrounding AVPV although few of them were found in AVPV in both male and female rats (Simerly et al., 1985).

Nitric oxide: At birth, the AVPV in males contains neuronal nitric oxide synthase that is three times higher than in female rats (Edelmann et al., 2007).

These items of evidence showed that AVPV is an intersection under the control of a variety of neurotransmitters.

3). The AVPV is crucial for the regulation of hypothalamic-pituitary-gonadal (HPG) axis.

a. It regulates the GnRH/LH surge in female rats that occurs just prior to ovulation. Small electrolytic lesions in this area led to inhibition of ovulation and persistent vaginal estrus in female rats, although AVPV does not contain GnRH neurons (Wiegand and Terasawa, 1982). Anterograde tracing experiments demonstrated that the AVPV sent projections directly to GnRH neuron area (Gu and Simerly, 1997).

b. In males, kisspeptins, a regulator of HPG function (Irwig et al., 2004), are expressed in AVPV in mice [Kiss1 mRNA, (Gottsch et al., 2004); protein, (Clarkson and Herbison, 2006)]. Kiss1 mRNA expression is increased by

testosterone treatment and abolished by castration exclusively in AVPV (Smith et al., 2005).

4) Although little is known about the AVPV function in males, it has among the highest density of nuclear hormone receptors (Simerly et al., 1990, Simerly, 1998, Wu et al., 2009).

Overall, the AVPV is an important component of neural circuitry that is involved in the mechanism by which steroid hormones regulate reproductive function.

MPN

The MPN is a central integrative site for the regulation of masculine reproductive functions. There are several lines of evidence to prove its key role.

1) The MPN, especially the central part of MPN (MPNc), is also sexually dimorphic. But as opposed to the AVPV, this part of the preoptic area is larger in males than in female rats (Simerly et al., 1984, Simerly and Swanson, 1988). Sexual dimorphic volume of MPNc [also identified by Gorski as sexually dimorphic nucleus of the preoptic area (SDN-POA) (Jacobson and Gorski, 1981, Handa et al., 1985)] is testosterone-dependent prenatally and postnatally (Jacobson and Gorski, 1981, Ito et al., 1986). This is thought to be the result of brain masculinization by sex hormone, which is sex-different during gestation and early life (Dohler and Wuttke, 1975, Ward and Weisz, 1980, Weisz and Ward, 1980).

2) The projections from the MPN to other parts, such as the BST, are also sexually dimorphic, being stronger in males than in females (Simerly and Swanson, 1988, Hutton et al., 1998).

3) The MPN receives indirect input from widely distributed areas including those receiving sensory cues (Simerly and Swanson, 1986) and sends massive projections to structures such as AVPV, ventromedial nuclei, arcuate and other areas regulating reproduction function including the patterning of copulation (Davidson, 1966, Simerly and Swanson, 1986).

4) The MPN is abundant in almost every neurotransmitter that is involved in regulation of masculine sexual behavior and application of their agonists and antagonist in MPN could directly affect sexual behaviors (see earlier sections).

5) The MPN is a site strongly related to the regulation of masculine sexual behavior. While lesion-induced impairment of the MPN led to decreased sexual performance in rats (Arendash and Gorski, 1983), electrical stimulation of MPN made rats achieve ejaculation with lower latency and fewer intromissions (Malsbury, 1971, Rodriguez-Manzo et al., 2000).

QUANTIFICATION OF HORMONE RECEPTORS

Research on hormone receptors in the non-human primate and rodent brain has been underway for more than 30 years. At the same time, there have been major changes in experimental technologies. Autoradiography,

hybridization histochemistry, ribonuclease protection assay, western blot and hormone receptor knock-out mice have been used to measure hormone receptor levels. The current dissertation utilized immunohistochemistry of AR and ER immunoreactive cell numbers, along with quantification by stereologic counting. The stereology techniques allow us to examine or reexamine more accurately effects of aging, hormone treatment, and experience on nuclear hormone receptor numbers at the level of the protein. This is important because:

1) The subregion of POA can be identified by Nissl counterstaining, providing a more accurate outline which is different in individuals. A 3-D analysis evaluates the volume of regions of interest in an unbiased manner.

2) The techniques measures protein as opposed to mRNA. Since the protein is the functional unit, this provides a different type of information about numbers of cells that have translated the molecule.

3) The distribution of protein can be observed within the neuron. The number and density of neurons expressing the protein of interest can be measured, which provides more meaningful endpoints than the total protein expressed in one area.

4) Analysis is time-independent, brain sections can be reanalyzed and results can be compared.

5) Protein can be examined according to different categorization, such as lightly or heavily immunorlabeled.

HYPOTHESIS OF THIS STUDY

I hypothesize that sex hormone receptor expression in POA will differ in aged and young males, and that this underlies behavioral and physiological differences with aging.

Therefore, testosterone treatment in castrated males of different ages is predicted to have differential effects on levels of hormone receptors in POA, and that this will be manifested in age-related differences in the maintenance and/or restoration of sexual behavior. Based on differences in effects of sexual experience on hormones, brain and behavior, I also predict that sexual experience will partially maintain expression of nuclear hormone receptors in the POA of aging rats.

Specific Aims of This Study Are as Follows:

1. To examine whether sex steroid hormone receptor expression changes with aging in the specific preoptic areas that regulates male reproductive functions and sexual behaviors. For this purpose, I measured androgen receptor and estrogen receptor expression in preoptic areas as well as serum hormone levels in intact Sprague-Dawley rats in young, middle-aged and old groups.

2. To establish whether sexual experience is involved in hormone receptor expression in preoptic areas in both young and aging males. I investigated androgen receptor and estrogen receptor expression in preoptic areas as well as serum hormone levels in intact naïve Sprague-Dawley rats or rats with sexual experience in young and middle-aged groups.

3. To discover whether testosterone treatment at castration can improve sexual performance and alter steroid hormone receptors in POA of aging males. To this end, young and aged males were castrated and given testosterone at the time of castration and effects on sexual performance and androgen and estrogen receptors were measured.

CHAPTER 2. AGE-RELATED CHANGES IN HYPOTHALAMIC ANDROGEN RECEPTOR AND ESTROGEN RECEPTOR α IN MALE RATS

ABSTRACT

The control of reproductive function involves actions of sex steroids upon their nuclear receptors in the hypothalamus and preoptic area (POA). Whether hypothalamic hormone receptors change their expression in aging male mammals has not been extensively pursued, although such changes may underlie functional losses in reproductive physiology occurring with aging. We performed a stereological analysis of immunoreactive androgen receptor (AR) and estrogen receptor alpha (ERα) cells in two POA nuclei of male Sprague-Dawley rats [anteroventral periventricular nucleus (AVPV) and medial preoptic nucleus (MPN)], at young (3 mo), middle-aged (12 mo) and old (20 mo) ages. Serum testosterone and estradiol levels were assayed. Testosterone concentrations decreased significantly and progressively with aging. Estradiol concentrations were significantly higher in middle-aged than either young or old rats. Stereologic analyses of the POA demonstrated that AR-immunoreactive cell numbers and density in the AVPV and MPN were significantly higher in old compared with young or middle-aged rats. No change in the total number or

density of ERα immunoreactive cells was detected with age, although when cells were subdivided by intensity of immunolabeling, the most heavily-labeled ERα cells increased in number and density with aging in the AVPV. There are several interpretations to our finding of substantially increased AR cell numbers during aging, including a potential compensatory increase of the AR under diminished testosterone concentrations. These results provide further information about how the neural targets of steroid hormones change with advancing age.

INTRODUCTION

In mammals, the process of aging results in an increased probability of infertility or reproductive dysfunction (Wise et al., 2002, Mobbs, 2004). However, the mechanisms underlying these processes are not well understood. Nevertheless, it is clear that the three levels of the hypothalamic-pituitary-gonadal (HPG) axis exhibit age-related changes. From the hypothalamic perspective, reproductive systems comprise the neural circuits regulating gonadotropin-releasing hormone (GnRH) neurons, together with the central nervous pathways that control reproductive physiology. Any of these neural systems may undergo age-associated alterations, changes to which may underlie some of the reproductive losses during aging.

Critical to reproductive function are actions of sex steroid hormones upon their receptors in the nervous system. Several groups have assayed

concentrations of gonadal steroid concentrations during aging, although results differ depending upon species and even strain. Reports on testosterone in male mammals are relatively consistent, showing age-related declines in humans (Harman et al., 2001), rhesus monkeys (Downs and Urbanski, 2006) and rats [Sprague-Dawley (Roselli et al., 1986); Wistar (Taylor et al., 1996, Bernardi et al., 1998); Brown Norway (Chen et al., 1994, Gruenewald et al., 2000), Fischer 344 (Chambers et al., 1991, Luine et al., 2007)]. Results for estradiol and aging in males are conflicting. In Fischer 344 rats (Fujita et al., 1990, Luine et al., 2007) and Wistar rats (Herath et al., 2001) serum estradiol concentrations increase with aging, while for Sprague-Dawley rats (Goya et al., 1990) and Brown Norway rats (Gruenewald et al., 2000) serum estradiol concentrations do not change with aging. In primates, a significant age-related increase of plasma estradiol was reported in normal adult men (Drafta et al., 1982, Vermeulen et al., 2002) but not in rhesus macaques (Chambers et al., 1982, Chambers and Phoenix, 1992). Thus, further information on whether and how serum estradiol levels change with aging is needed. Notably, in adult male rats (Sprague-Dawley) circulating estradiol concentrations are relatively high, being comparable to those in diestrous females (see data below, and (Smith et al., 1975, Oliveira et al., 2004), consistent with important and physiologically-relevant roles of estrogens on reproductive functions (Larsson et al., 1973, Vagell and McGinnis, 1997).

Understanding the expression of steroid hormone receptors in the brain is crucial for elucidating the causes and consequences of reproductive aging. In

young adults, brain nuclei that are abundant in nuclear steroid hormone receptors including, but not limited to, androgen receptor [AR; (Simerly et al., 1990) and the estrogen receptors (ER), ERα and ERβ (Shughrue et al., 1997, Mitra et al., 2003)] mediate effects of testosterone and estradiol, respectively, and play key roles in the control of reproductive physiology and behavior. Observations that sociosexual behaviors decline in male rats with aging (Gray, 1978, Chambers et al., 1991, Smith et al., 1992, Taylor et al., 1996), and that these cannot be rescued by replacing steroid hormones to young levels (Hsu et al., 1986, Taylor et al., 1996), are consistent with both steroid-dependent and -independent neurobiological changes including potential alterations in numbers or responsiveness of steroid hormone receptors in the brain. However, with the exception of pathological disorders, the aging brain does not appear to undergo a wholesale loss of neurons (Finch, 2003). Rather, there appear to be changes to or losses in specific cell phenotypes that may underlie functional losses. This point can be applied to those neurons expressing nuclear steroid hormone receptors, although to date, there are few published reports on how AR and ER expression change with aging in male hypothalamus. Thus, studies on changes in hormones and neural receptors in aging mammals will contribute to better understanding these biological processes.

In the current study, we used immunohistochemistry and stereology to quantify the number of cells expressing AR and ERα in the hypothalamus of aging male rats. Two preoptic brain regions were chosen because of their robust

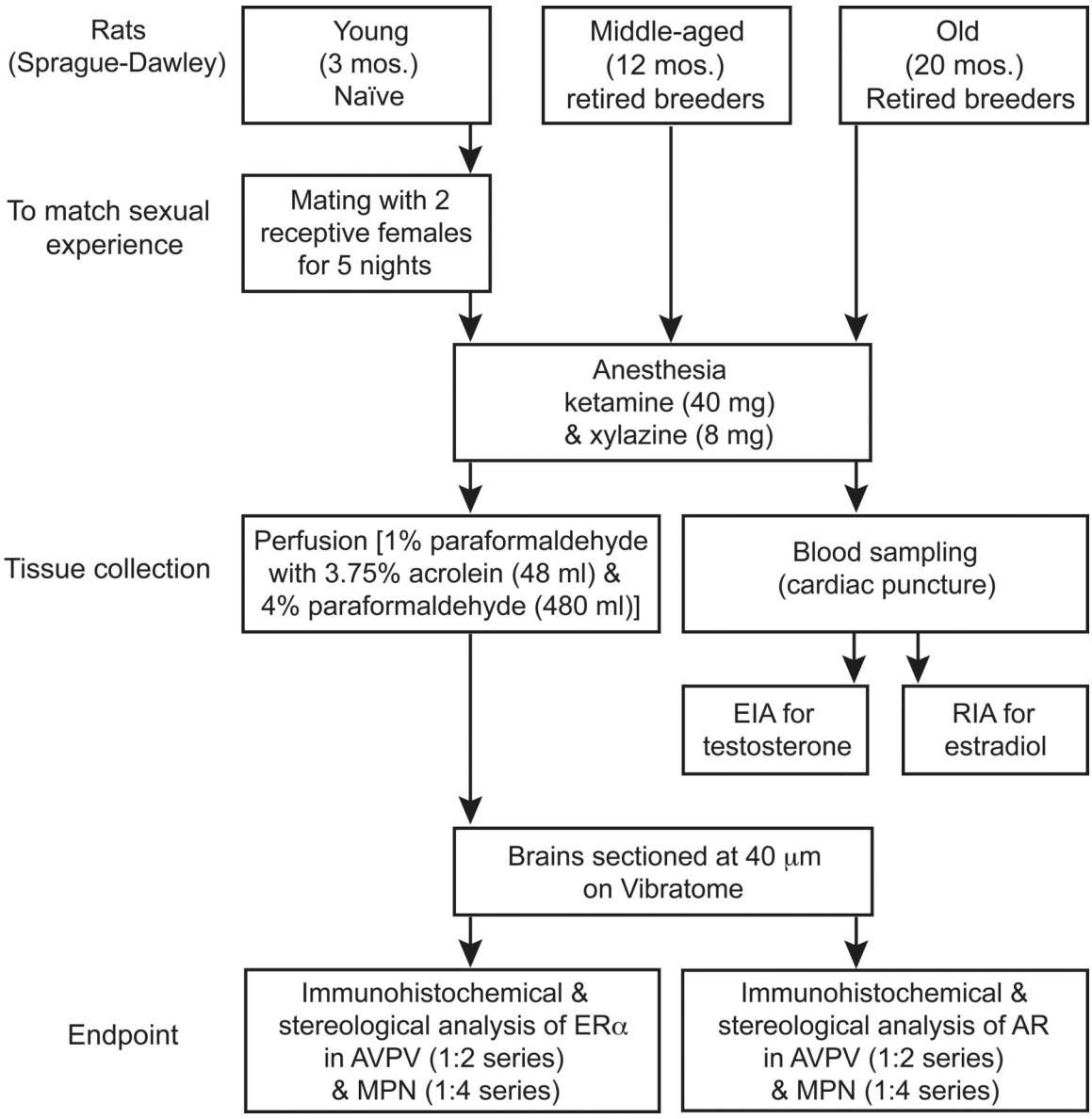
expression of both ERα and AR: two, the anteroventral periventricular nucleus (AVPV) and medial preoptic nucleus (MPN), play functional roles in reproductive physiology and/or behavior (Wiegand and Terasawa, 1982, Simerly and Swanson, 1988).

METHODS

Animals

Male Sprague-Dawley rats were purchased at 3 months (young, N=7), 12 months (middle-aged, N=8) and 20 months (old, N=8), from the rat colony at the Animal Resource Center, University of Texas at Austin (UT). This rat colony originally derived from a Harlan Sprague-Dawley colony and is regularly introduced with new Harlan Sprague-Dawley rats. The middle-aged and old rats were retired breeders. Although exact sexual experience was not known, our animal colony allows a male to mate with two females periodically over the course of three to four months. It is not possible to match this exact life history of sexual experience in young rats, but for the young group, we placed each male rat in a cage with two receptive female rats (rotated among males) at least five nights in a row. Mating was confirmed by checking for sperm in the females' vaginas. Rats were housed in an AAALAC-approved facility (two per cage, cage dimensions 47 X 20 X 25 cm) with Rat Sterilizable Diet (Harlan Teklad LM -485 7012, Madison, WI) and water available *ad libitum*. The light cycle was 12 h light,

Figure 2.1. Experiment One Flowchart



12 h dark cycle (lights on 2300h), and temperature was 21 ± 1ºC. All animal procedures performed herein were approved by the UT-Austin IACUC (Protocol number: 05031102) and studies were performed following the *Guide for the Care and Use of Experimental Laboratory Animals*.

Perfusion

Animals were deeply anesthetized with ketamine (100 mg/ml) and xylazine (20 mg/ml) (0.4 ml + 0.4 ml, respectively). Rats were perfused (48 ml/min) sequentially with 0.9% saline (24 ml), 0.9% saline with 10% heparin (24 ml), and 1% paraformaldehyde with 3.75% acrolein (48 ml), followed by 4% paraformaldehyde (480 ml)(Chakraborty et al., 2003a). All fixatives were dissolved in PBSA (phosphate-buffered saline A: 0.08 M, PO_4: 0.12 M, pH=7.3). The brains were removed from the skull and post-fixed for 3 hours in 4% paraformaldehyde and then transferred into PBSA with 0.05% sodium azide for storage at 4°C. Tissue sections (40 µm-thick) were cut on a vibrating microtome (Leica VT 1000S, Leica Microsystems, Nussloch, Germany) and stored in PBSA with sodium azide at 4°C.

Immunohistochemistry

For tissue processing and analyses, tissues were henceforward recoded so that the experimenter was blind to age group. Sections at the level of the POA

were rinsed in TBST (Trizma-buffered saline/Triton X, Trizma base: 0.1 M, NaCl: 0.15 M, Triton X-100: 0.1%, pH = 7.3) at room temperature on a shaker. Although there were too many sections to process in a single run, animals from each age group were equally represented in every run. Sodium borohydride [1% in PBSB (phosphate-buffered saline B: saline: 0.16 M, PO_4: 0.01M, pH=7.3)] was used to clear the acrolein for 20 minutes. Sections were washed until no bubbles were observed. Then, the sections were treated to eliminate any endogenous peroxide activity (3:1 methanol:3% H_2O_2, 20 minutes at room temperature). For AR immunohistochemistry, sections were then washed, and incubated in the AR antibody PG21-36 (1:2000; generously provided by Dr. Gail S. Prins, University of Illinois-Chicaco). This antibody is a rabbit polyclonal raised against amino acids 1-21 of the rat AR and has been extensively characterized by Prins *et al*. (Prins et al., 1991) and Zhou *et al.* (Zhou et al., 1994). These laboratories have demonstrated that preabsorption of this antibody with the antigen resulted in an abolition of immunoreactivity, and western blots showed strong immunoreactivity at the expected molecular weight of 110 kDa. Furthermore, application of the antibody to AR-negative tissue (rat spleen) resulted in no detectable immunoreactivity (Prins et al., 1991). In one of those published studies, castration resulted in a loss of nuclear ARir, a finding that could be attributable either to an increase/maintenance of ARir by peripheral testosterone that is lost after castration, or to the possibility that the antibody only binds to liganded AR

receptor (Zhou et al., 1994). However, in the current studies, all males were gonadally intact and had detectable concentrations of serum testosterone.

In our study, the primary antibody incubation was performed in 10% normal goat serum (NGS) and 0.1% Triton-X overnight at 4°C on a shaker. The sections were then washed and incubated in biotinylated anti-rabbit immunoglobulin G (IgG, 1:600, Vector Laboratories, Burlingame, CA) for 2 hours followed by rinsing in TBST. After rinsing, sections were incubated in ABC (Vector Laboratories) for 1 hour. They were rinsed in buffer and developed using 3,3-diaminobenzidine (DAB) as a chromogen. Sections were rinsed, dried at room temperature and then Nissl-stained (Toluidine blue)(Chakraborty et al., 2003b, Salama et al., 2003), and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA). Controls were also run with the primary antibody omitted, and no specific binding was observed.

The procedure of ERα immunohistochemistry was identical to that described above for AR immunohistochemistry with the following exceptions. The assay buffer was PBSB. The ERα antibody was the rabbit polyclonal anti-estrogen receptor alpha antibody (1:20,000, C1355, Upstate Biotechnology, Waltham, MA) that was produced against the last 15 amino acids of ERα. This region has no homology to the corresponding region of ERβ, so detection of immunoreactivity should reflect the presence of ERα receptor only. In addition, Friend *et al.* (Friend et al., 1997) showed using immunoblots that the antibody recognizes the recombinant ERα of the appropriate molecular weight, and

recognizes both liganded and unliganded ERα (Friend et al., 1997). Specific binding was eliminated in that study by preabsorption with the antigen. Previously published work from our own laboratory, using ovariectomized female rats, showed the ability of the antibody to detect nuclear ERα both in estradiol- and vehicle-replaced animals, again suggesting that the antibody can bind to liganded and unliganded ERα (Chakraborty et al., 2003a, Chakraborty et al., 2003c). In the present study, when either primary antibody, AR or ERα, was omitted as a negative control, no immunoreactivity was detected.

Stereological Analysis

A stereological analysis was performed according to methods described in detail previously (Chakraborty et al., 2003a, Chakraborty et al., 2005). For the AVPV, every other section was used to provide a 1:2 series for analysis of each nuclear receptor. For the MPN, every fourth section was used to provide a 1:4 series of each receptor. A wet-mount of fresh tissue showed that average tissue thickness was 40.9 μm. For each rat, 6-7 sections containing AVPV, and 8-9 sections containing MPN were chosen. The sections were carefully matched for rostral-caudal landmarks among all the animals, and the AVPV and MPN were identified in Nissl-stained sections by comparing anatomical landmarks to an atlas of the rat brain (Swanson, 1998). Quantitative analyses were done using a computer-assisted morphometry system that consisted of a MAC 5000 Manual joystick control (Ludl Electronic Products Ltd. Hawthorne, NY), MicroFire S99808

video camera (Optronics, Goleta, CA), Dell computer (Dimension 4550 series, Austin, TX), and Cintiq 15X screen (Wacom, Vancouver, WA) and Stereo Investigator® software. Using the Stereo Investigator® software (MicroBrightField, Williston, VT), closed contours were drawn to surround the region of interest (AVPV and MPN) at low magnification (4X) using the laboratory Olympus BX-61 microscope. Each section mount thickness was measured within the contour. A buffer zone at the top and bottom of the tissue, where the slice thickness is most likely to have been affected by tissue processing and is not representative of the tissue specimen from the volume, was set at 3 μm for all experimental stereology. For each rat, the volume of the regions of interest in each section was extrapolated based on the contours and tissue thickness (Volume = regional area x thickness). The Stereo Investigator software randomly placed 135 μm × 80 μm grids ("disector frames") within each contour. Within these disector frames, the DAB-stained ERα or AR labeled nuclei were counted within a 40 μm × 40 μm counting frame ("optical disectors"). The counting criteria were: 1) only nuclei that came into focus as we focused down from the top of the counting frame were counted; 2) only nuclei falling entirely within the counting frame or touching/crossing the top or right lines of the counting frame were counted. Nuclei touching/crossing the bottom or left lines were excluded. These counting rules ensured that the same cells would not be counted twice. In addition, because the diameter of nuclei is approximately 10 μm, tissue thickness is 40 μm, and either a 1:2 series (or AVPV) or 1:4 series (MPN) was used, nuclei

could not be double-counted. Based on these parameters, the number and density (# immunoreactive cells/volume of each nucleus) of AR-immunoreactive (ARir) nuclei or ERαimmunoreactive (ERαir) nuclei falling within the regions was quantified. The coefficients of error and variation of the estimates were calculated as per Schmitz and Hof (Schmitz and Hof, 2000) and shown in Tables 2.1 and 2.2. CE's were low and never exceeded 0.09 (see Tables 2.1 and 2.2). Photomicrographs were taken to produce the figures, and images were subjected to only minor adjustments using Adobe Photoshop 7.0 (Adobe, San Jose, CA), with any adjustments applied equally to tissues from rats of different ages to avoid any bias, and so as not to alter the appearance from the original tissues.

During the course of stereological analyses we noted that ARir and ERαir cell nuclei fell into two qualitatively distinct classes that we have defined as "heavily labeled" and "lightly labeled" (Figure 2.2) similar to a recent report (Phillips-Farfan et al., 2007). Criteria for "heavily labeled" cells included a markedly more intense nuclear coloration (black) that was largely evenly distributed through the nucleus (nuclei indicated by thick arrows in Figure 2.2). Criteria for "lightly labeled" cells were a notably less intense coloration (gray) often characterized by a spotty appearance (nuclei indicated by thin arrows in Figure 2.2). We used an internal scale of cell density from 1-6, with 1=most lightly immunolabeled and 6=most heavily immunolabeled that we used in characterizing the cells during analysis. Cells that were in the range 1-3 were considered lightly-labeled and those 4-6 were heavily-labeled. There was virtually

Table 2.1. Optical Fractionator Analysis of AR Immunoreactive Cell Number and Volume

AVPV

	Young	CE	Middle-aged	CE	Old	CE
Heavily Labeled ARir Cell Number	329 ± 74	0.07	758 ± 350	0.07	2680 ± 556	0.09
Lightly Labeled ARir Cell Number	1249 ± 126	0.08	2098 ± 581	0.07	3830 ± 601	0.07
Total ARir Cell Number	1578 ± 149	0.08	2856 ± 881	0.09	6510 ± 1147	0.07
Volume (μm^3) x 10^7	6.95 ± 0.91	0.05	6.80 ± 0.64	0.07	7.02 ± 0.73	0.07

MPN

	Young	CE	Middle-aged	CE	Old	CE
Heavily Labeled ARir Cell Number	7098 ± 1001	0.04	12732 ± 2378	0.03	34446 ± 3871	0.03
Lightly Labeled ARir Cell Number	19648 ± 1163	0.03	22299 ± 2581	0.03	32770 ± 693	0.04
Total ARir Cell Number	26747 ± 1333	0.03	35031 ± 4118	0.03	67216 ± 3992	0.07
Volume (μm^3) x 10^8	5.99 ± 0.18	0.03	6.26 ± 0.17	0.03	6.18 ± 0.11	0.03

Data shown are mean ± SEM. Abbreviations: ARir: androgen receptor immunoreactivę;AVPV: anteroventral periventricular nucleus; MPN: medial preoptic nucleus; CE: Coefficient of error.

Table 2.2. Optical Fractionator Analysis of ERα Immunoreactive Cell Number and Volume

AVPV

	Young	CE	Middle-aged	CE	Old	CE
Heavily Labeled ERαir Cell Number	1871 ± 327	0.09	4199 ± 526	0.08	4641 ± 1015	0.06
Lightly Labeled ERαir Cell Number	3715 ± 683	0.03	3717 ± 295	0.06	3952 ± 681	0.07
Total ERαir Cell Number	5586 ± 913	0.07	7916 ± 654	0.06	8593 ± 1380	0.06
Volume (μm^3) x 10^7	8.05 ± 0.97	0.05	8.24 ± 0.43	0.05	7.94 ± 0.39	0.05

MPN

	Young	CE	Middle-aged	CE	Old	CE
Heavily Labeled ERαir Cell Number	4850 ± 1555	0.07	12488 ± 1795	0.05	13947 ± 3000	0.04
Lightly Labeled ERαir Cell Number	27471 ± 4300	0.04	35082 ± 4340	0.03	25715 ± 3907	0.03
Total ERαir Cell Number	32839 ± 5405	0.04	47570 ± 5545	0.04	39663 ± 6419	0.03
Volume (μm^3) x 10^8	6.66 ± 0.41	0.03	6.91 ± 0.29	0.04	6.95 ± 0.51	0.03

Data shown are mean ± SEM. Abbreviations: ERαir: estrogen receptor α immunoreactive; AVPV: anteroventral periventricular nucleus; MPN: medial preoptic nucleus; CE: Coefficient of error.

Figure 2.2. Criteria for Assignment of Cells as Heavily and Lightly Immuno-labeled

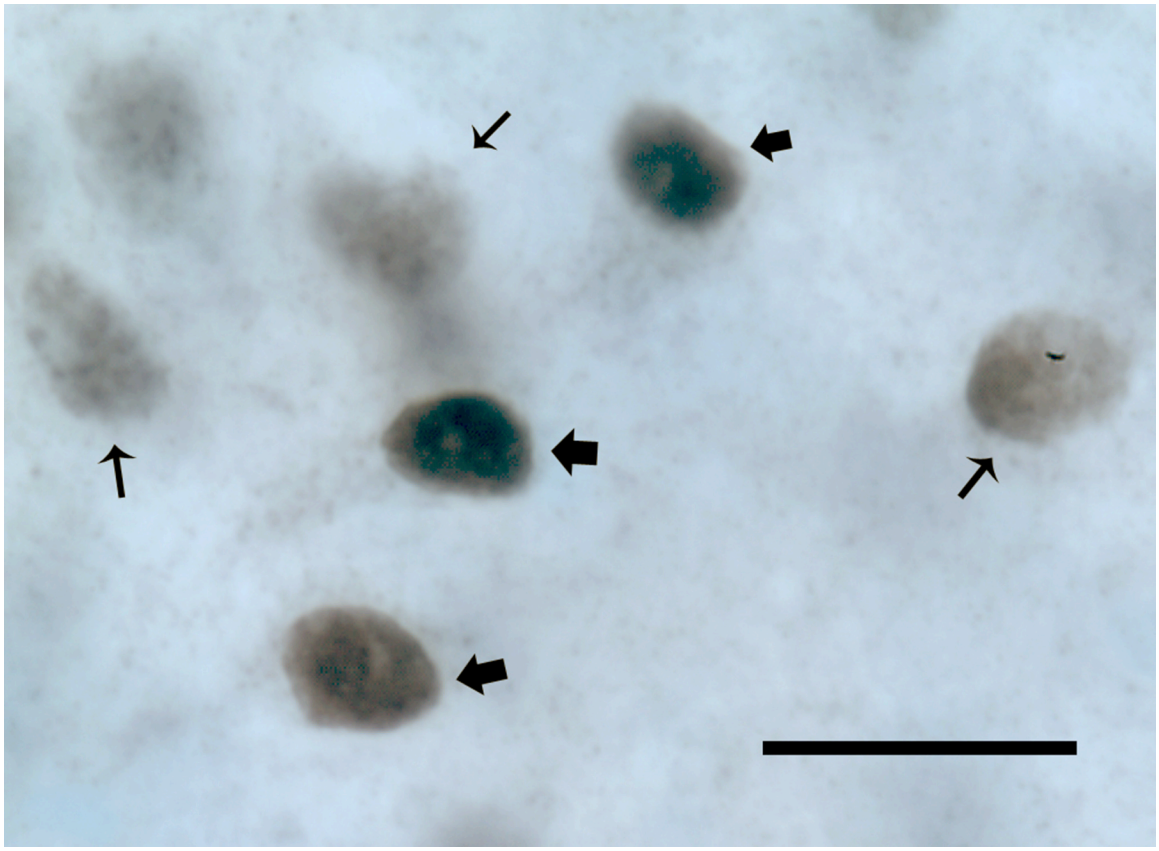


Figure 2.2. Data shown are for ERα, and similar criteria were applied to the AR analysis. The nuclei indicated by large arrows are identified as heavily labeled based on black coloration and relatively homogeneous distribution of label. Nuclei numbers indicated by small arrows are identified as lightly labeled, as they are gray in color and have a spotty, uneven appearance. Cells were easily categorized into one of the two classes using an internal scale of 1 to 3 = lightly labeled, 4 to 6 = heavily labeled. Scale bar = 50 µm.

no ambiguity in the assignment of cells to one of the other of these two classes, as most cells fell into one of the two extremes. Therefore, in stereological analyses we subdivided immunoreactive cells in these two classes separately, and also combined data for analysis and presentation.

Serum Hormone Concentrations

A terminal blood sample was collected from the anesthetized rat by cardiac puncture just prior to euthanasia by perfusion. The serum was separated by centrifugation (8000 rpm, 5 min) and stored at –80 °C for hormone assays.

Enzyme immunoassay (EIA) of serum testosterone

Testosterone concentrations were measured by a single testosterone enzyme immunoassay using the EIA kit DSL-10-4000 according to the method described by Diagnostic Systems Laboratories, Inc. (Webster, TX). Duplicate samples were run at a volume of 50 µl each. The minimum detectable level of testosterone was 0.04 ng/ml per tube. Intra-assay variability was 2.00%.

Radioimmunoassay (RIA) of serum estradiol

Estradiol concentrations were measured in a single ultra-sensitive estradiol RIA using the DSL-4800 RIA kit according to the method described by

Diagnostic Systems Laboratories, Inc. (Webster, TX). Duplicate samples were run at a volume of 200 µl each. The minimum detectable level of estradiol was 2.2 pg/ml per tube. Intra-assay variability was 1.41%.

Statistical Analysis

Statistical analysis was done with each rat as the unit of analysis. Using SPSS statistical software (13.0) (SPSS Inc., Chicago, Illinois), effects of age were evaluated on the following endpoints: number of ARir or ERαir cell numbers (subdivided into heavily-labeled, lightly-labeled, and total, as described above), regional volume of each brain nucleus, ARir or ERαir density (calculated as cell numbers/volume), and serum hormone levels. First, datasets were tested for homogeneity of variance and normality. For datasets that met these criteria, comparisons were made by one-way ANOVA followed by Tukey post hoc analysis when indicated by a significant main effect. Otherwise the nonparametric Kruskal-Wallis H test was applied and followed by Mann-Whitney U test in the event of significant main effects. Correlations between serum testosterone with total AR- and ERα-immunoreactive cell numbers or density were evaluated by regression analysis using SPSS. In all cases, the criterion for statistical significance was $p < 0.05$.

RESULTS

Stereology of AR in AVPV and MPN of Aging Male Rats

Representative photomicrographs of the expression and distribution of AR are shown in Figures 2.3 and 2.4, and demonstrate the robust levels of AR in the nucleus of cells in the AVPV and MPN. Stereologic analysis of ARir cell numbers was performed in the two regions, taking into consideration whether nuclei were lightly or heavily labeled. As shown in Figure 2.5, numbers of ARir cells increased significantly with age from young to middle-aged to old for heavily-labeled, lightly-labeled and total numbers of ARir cells. In all cases, numbers and densities of ARir cells were significantly higher in old than young male rats, and in most cases (with the sole exception of lightly labeled AR cell numbers in AVPV) old rats had higher AR cell numbers and densities than middle-aged rats. Middle-aged rats also had higher AR numbers and densities than young rats for lightly labeled AR cell density in MPN (Figure 2.5).

Stereology of ERα in AVPV and MPN of Aging Male Rats

Representative photomicrographs of the AVPV and MPN are shown in Figures 2.3 and 2.6, showing the regions of analysis and the expression and distribution of ERα. The data demonstrate that ERαir is abundant in the selected

Figure 2.3. Photomicrographs of Representative ERα and AR Immuno-labeled Sections.

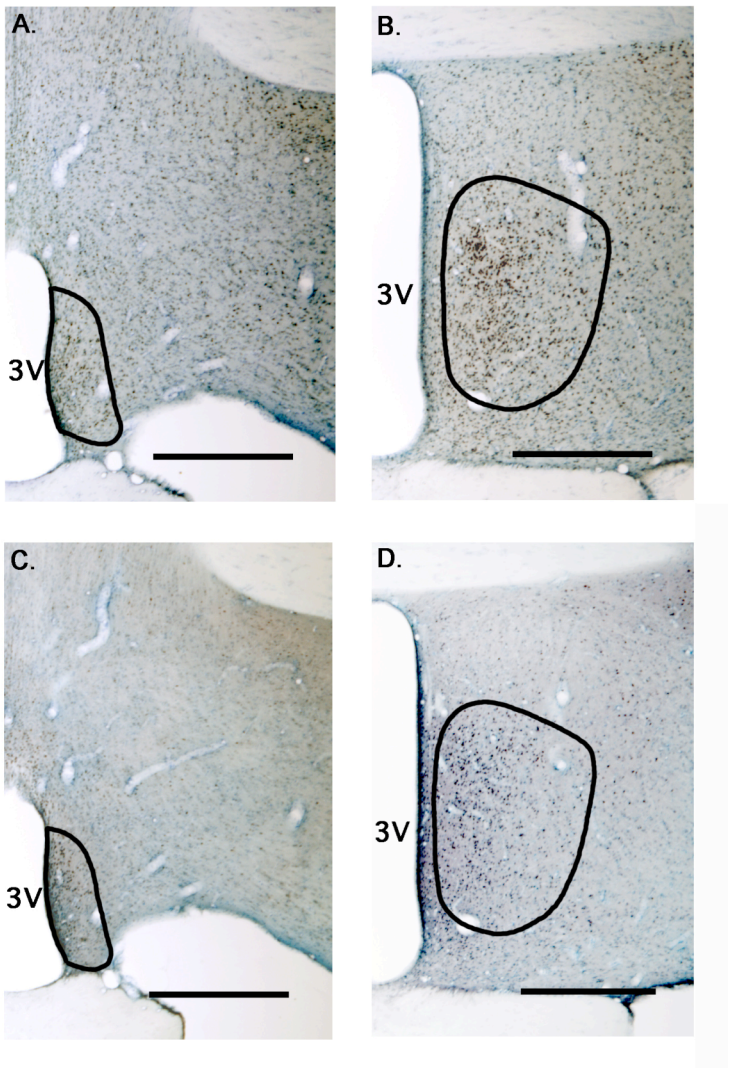


Figure 2.3. The POA is shown at 4x magnification at the level of the AVPV (panels A, C) and the MPN (panels B, D) of a representative old male rat. Sections shown in A and C were adjacent, as were B and D. Sections were labeled for AR immunoreactivity (top row) or ERα immunoreactivity (bottom row), and counterstained by Nissl labeling. The contours of the AVPV and MPN are drawn based on Nissl staining and in comparison to Swanson's rat brain atlas (1998). (Note that during the actual stereological analysis, contours were drawn according to the Nissl staining which is out of focus in the figure.) Scale bar = 500 μm.

Figure 2.4. Photomicrographs of AR Immunoreactivity in Young, Middle-aged and Old Rats

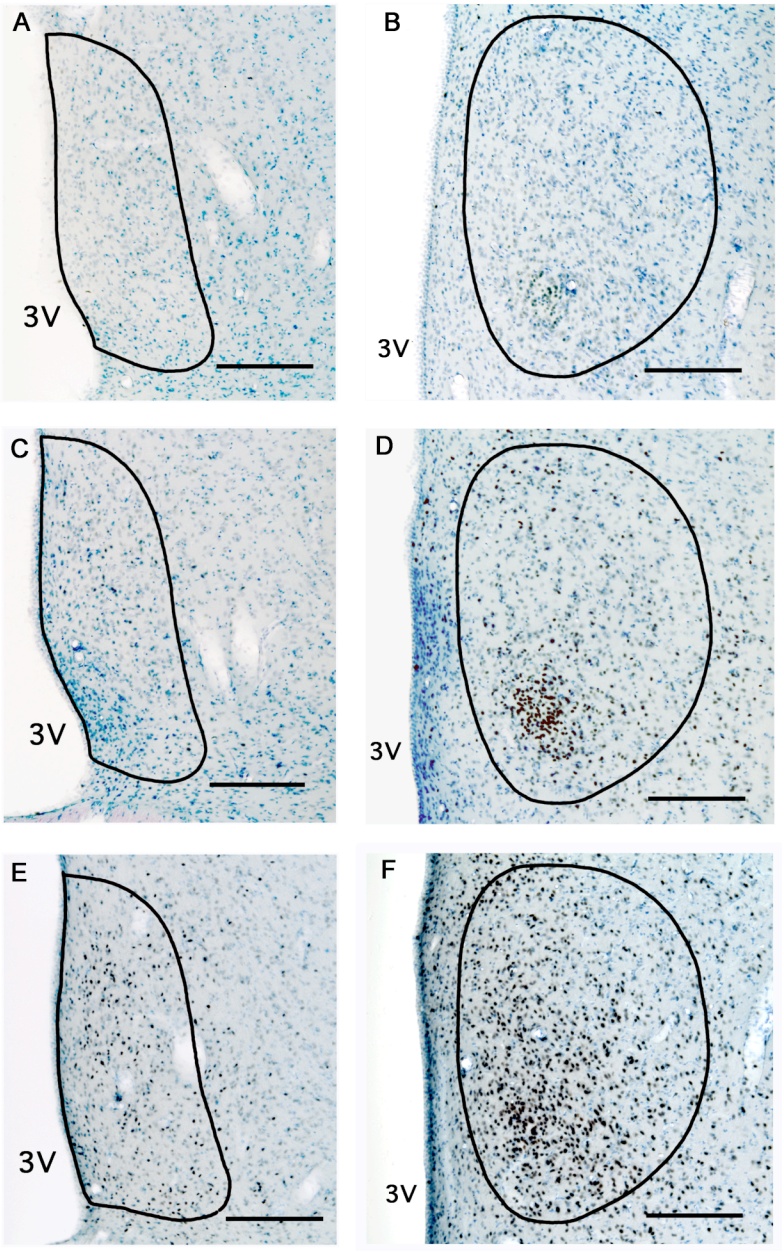


Figure 2.4. Representative sections from a young (panels A, B) middle-aged (panels C, D) and old (panels E, F) male rat of the two brain regions [AVPV (left column) and MPN (right column)] are shown. The contours of the AVPV and MPN are drawn based on Nissl staining and according to Swanson's rat brain atlas (1998). Scale bar = 200 μm.

Figure 2.5. Stereologic Analysis of AR Immunoreactive Cell Numbers and Density

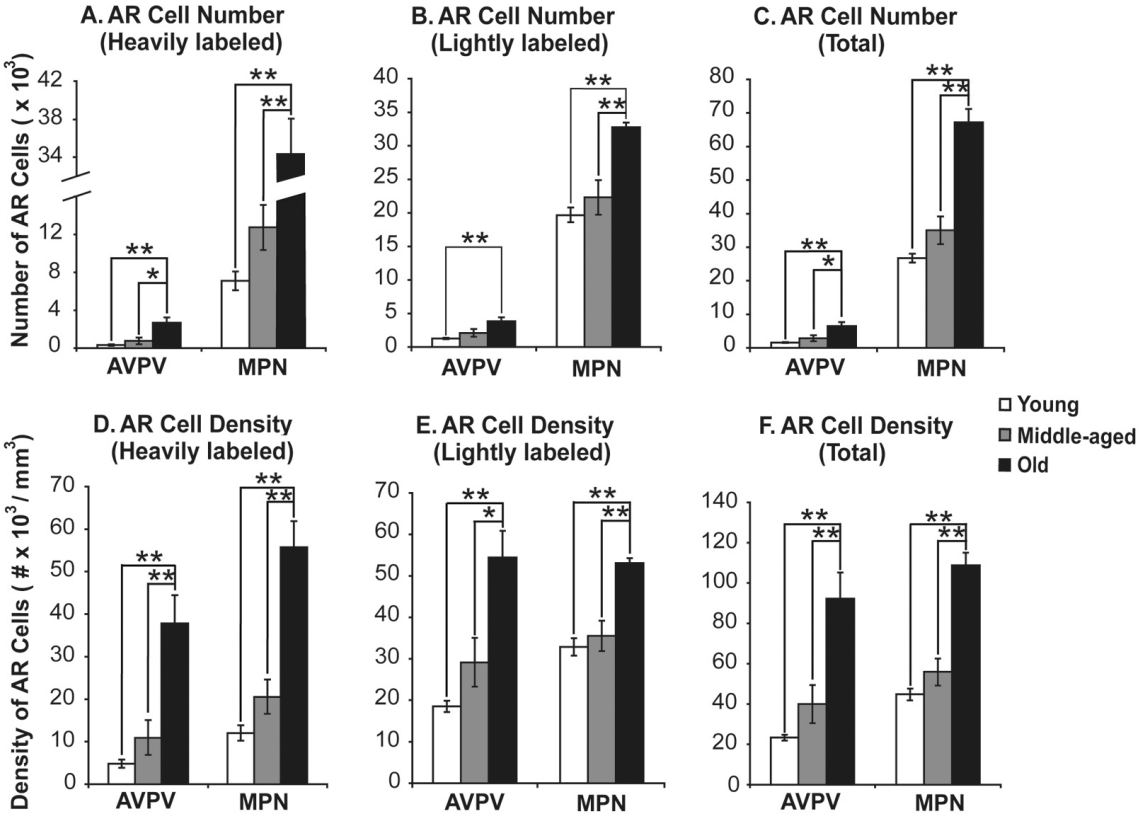


Figure 2.5. AR immunoreactive cell numbers were counted in young, middle-aged and old rats in AVPV and MPN. There were significant age-related increases detected for heavily labeled (A), lightly labeled (B) and total numbers (C) of AR cells. Similar observations were made for AR density in the two regions (panels D, E and F for heavily, lightly and total AR cell densities, respectively). *, $p < 0.05$; **, $p < 0.01$.

hypothalamic areas. Stereologic analyses of ERαir cell numbers were performed, taking into consideration whether cell nuclei were lightly or heavily labeled (see Figure 2.2). No age-related change in the total number of ERαir cell numbers and densities were detected (Figure 2.7). When cells were subdivided into the lightly- or heavily-immunolabeled subgroups, there were no significant age differences on lightly-labeled cells (Figure 2.7). However, a significant effect of age on the heavily-labeled ERα cell numbers was detected in AVPV ($p < 0.05$), with a trend in the MPN ($p = 0.065$). Post-hoc analyses of significant main effects showed that in the AVPV (but not the MPN) numbers of heavily-labeled ERαir cells were significantly higher in middle-aged than in young rats and higher in old than young rats. An effect of age on the density of heavily labeled ERα immunoreactive cells was found only in AVPV ($p < 0.05$), with a trend in MPN ($p = 0.065$). Post-hoc analysis of the AVPV showed this effect was attributable to a difference between the young and middle-aged groups.

Serum Testosterone and Estradiol Concentrations

Serum testosterone levels declined significantly with age ($p < 0.001$). Testosterone concentrations were significantly lower in the old group than in the young or middle-aged rats ($p < 0.001$ for both; Figure 2.8A). There was also a significant difference between middle-aged and young rats ($p < 0.05$).

Serum estradiol levels varied significantly with age ($p < 0.001$) and were highest in middle-aged rats and lowest in the old rats (Figure 2.8B). A significant

Figure 2.6. Photomicrographs of ERα Immunoreactivity in Young, Middle-aged and Old Rats.

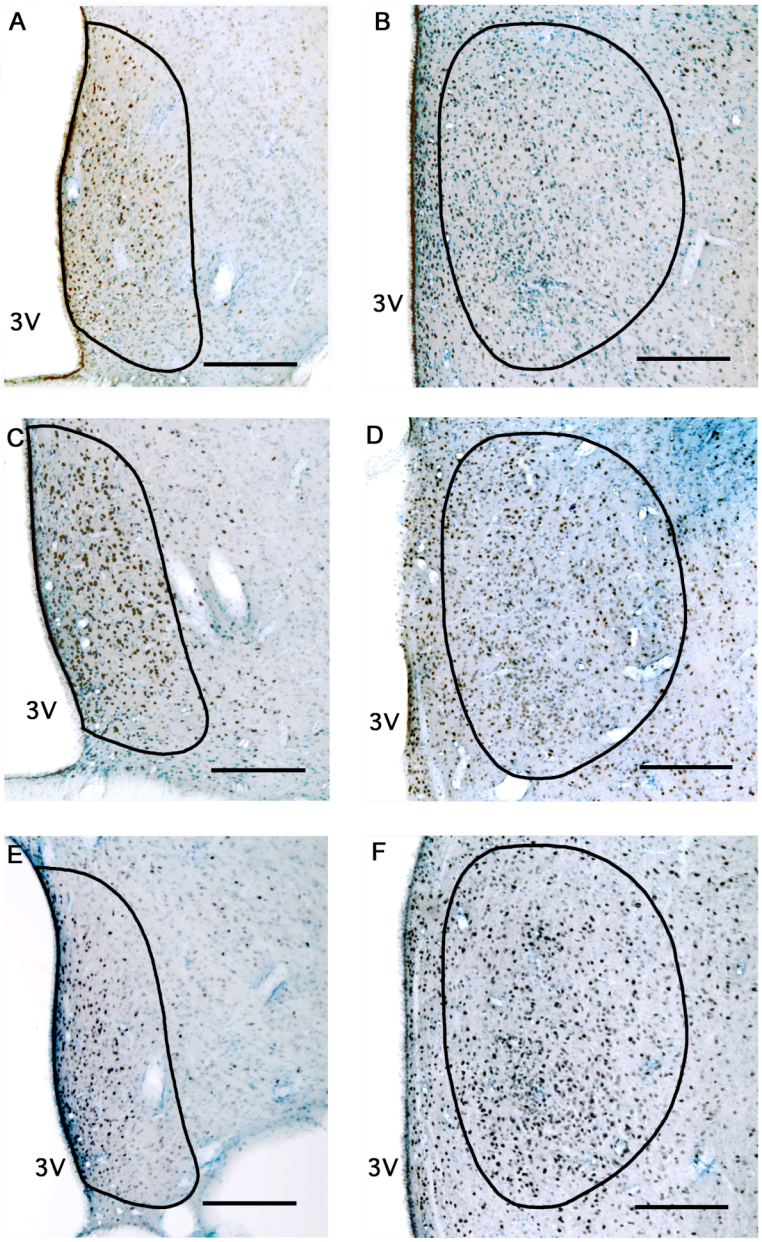


Figure 2.6. Representative sections from a young (panels A, B) middle-aged (panels C, D) and old (panels E, F) rat of the three brain regions [AVPV (left column) and MPN (right column)] are shown. The contours of the AVPV and MPN are drawn based on Nissl labeling and in comparison to Swanson's rat brain atlas (1998). Scale bar = 200 μm.

Figure 2.7. Stereologic Analysis of ERα Immunoreactive Cell Numbers and Density.

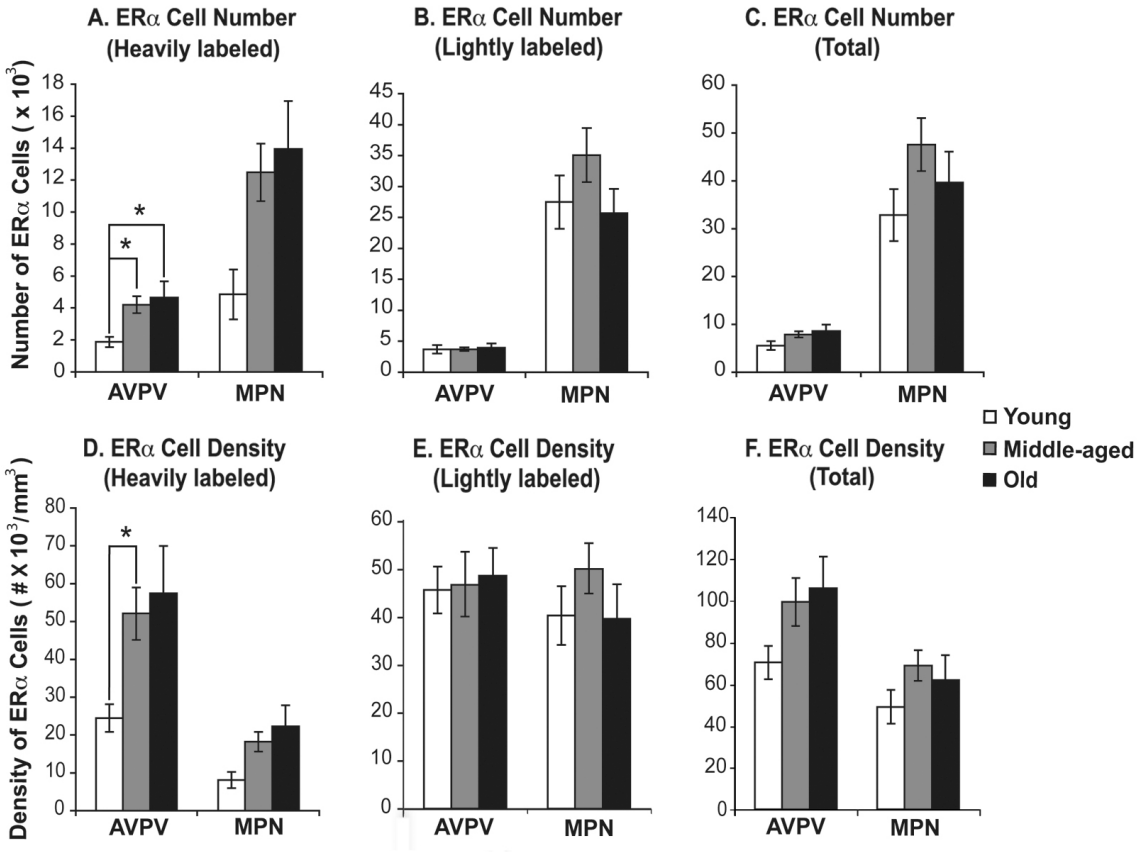


Figure 2.7. ERα immunoreactive neuron numbers were quantified in young, middle-aged and old rats in the AVPV and MPN. Data are shown for heavily labeled (A), lightly labeled (B) and total (C) ERαir cell numbers. Although there were no differences with age in total or lightly labeled ERαir cells, there was a significant increase in heavily labeled cells with age from young to middle-aged in the AVPV. Panels D, E and F present similar data for ERα cell densities in aging rats. The only significant difference in density was found for heavily labeled ERαir cells, with an increase from young to middle-aged rats in the AVPV. *, $p < 0.05$; **, $p < 0.01$.

difference between middle-aged and old rats was detected ($p < 0.001$), as was a significant difference between old and young rats ($p < 0.01$).

Correlations between Androgen Receptor and Serum Testosterone Levels

Based on observations for overall age-related increases in ARir cells, and decreases in serum testosterone concentrations, regression analyses were performed between ARir cells (total number and density) and serum testosterone levels (Figure 2.9) irrespective of age. Both ARir cell number and density in AVPV and MPN were negatively correlated with serum testosterone levels ($p < 0.05$ for all).

DISCUSSION

In this study, we demonstrated a 3 to 4-fold age-related increase in AR immunoreactive cells in two preoptic brain areas, the AVPV and MPN, in male Sprague Dawley rats. During this same period, total serum testosterone levels decreased profoundly. Thus, there is a significant inverse relationship between ARir cells and circulating testosterone. Although ERαir cells increased modestly in these same two regions with age, effects were only significant for the most heavily-labeled cells. Based on the result that serum estradiol levels peaked in middle age, I speculate that the regulation of expression of ERα by estradiol in male rats of increasing age may be controlled by differing mechanisms, and at

Figure 2.8. Serum Testosterone and Estradiol Concentrations in Aging Male Sprague-Dawley Rats.

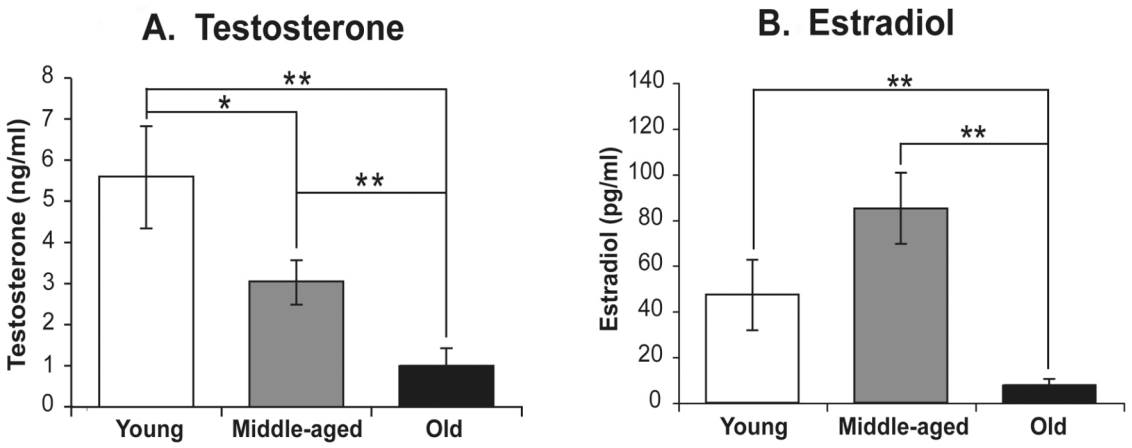


Figure 2.8. Serum testosterone (A) and estradiol (B) concentrations are shown. Both hormones were affected significantly by age. *, $p < 0.05$; **, $p < 0.01$.

Figure 2.9. Correlations between Total Androgen Receptor Cell (Number and Density) and Serum Testosterone Concentrations

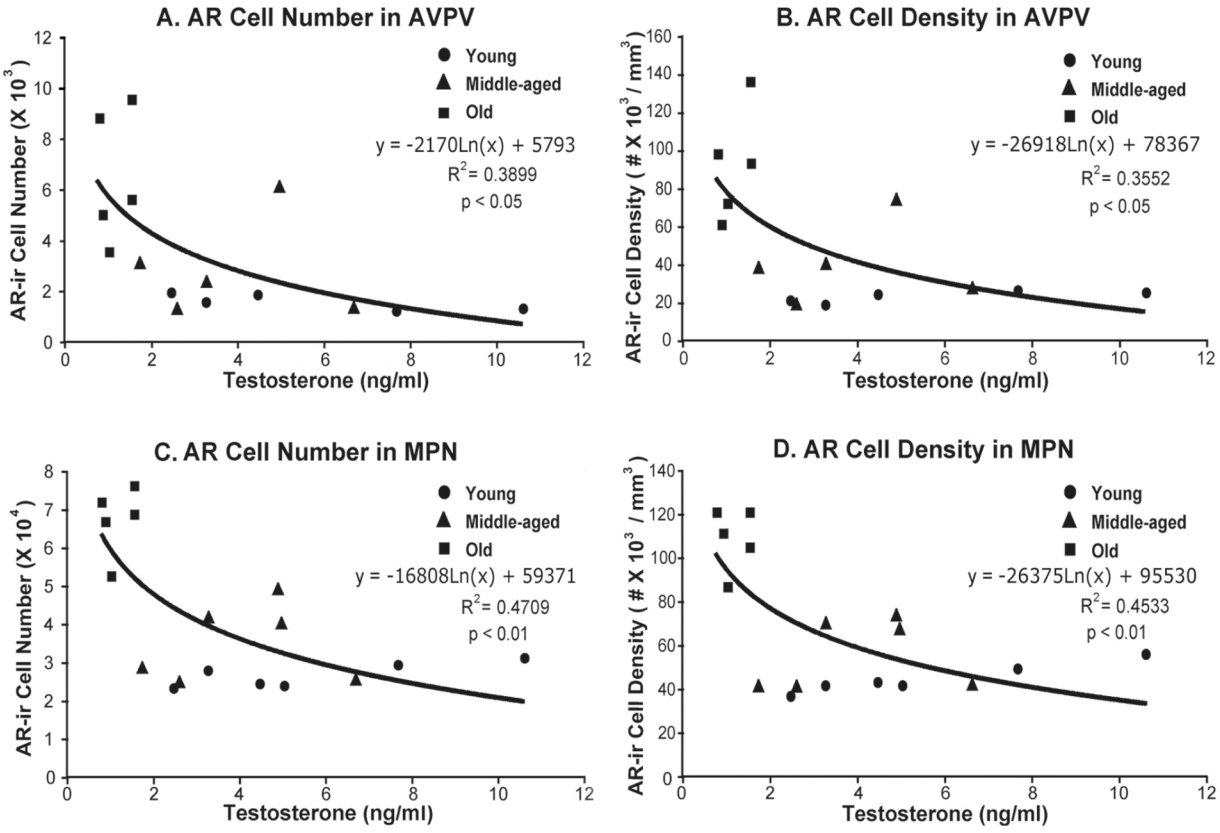


Figure 2.9. Regression analyses were performed for ARir numbers and density in AVPV (panels A, B), MePO (panels C, D) and MPN (panels E, F). There were significant negative correlations between serum testosterone concentrations and ARir cells in AVPV ($p < 0.05$), MePO ($p < 0.05$) and MPN ($p < 0.01$).

the very least, that there is a non-linear relationship between the serum hormone and its nuclear receptor. These results demonstrate the capacity of the aging male brain to continue to express nuclear hormone receptors, and even its ability to increase the numbers of cells that synthesize these proteins.

Roles of Two Preoptic Regions in Male Reproduction

The regions of interest in the current study were chosen based on their reported roles in reproductive physiology and behavior, evidence for sexual dimorphisms, along with our own pilot analyses showing their abundant amounts of both AR and ERα in aging male rats. The AVPV is a sexually dimorphic nucleus (Simerly, 1998) the function of which in male rats is not well-understood, although it appears to be necessary for female ovulatory function (Wiegand and Terasawa, 1982). The AVPV is smaller in male than female rats, and this effect is organized perinatally by sex differences in steroid hormones (Polston et al., 2004). Finally, the AVPV has abundant expression of both AR and ERα (Simerly et al., 1990, Chakraborty et al., 2003a, Chakraborty et al., 2003c). The MPN is a central integrative site for the regulation of male sexual behavior. It receives inputs from sensory systems (Simerly and Swanson, 1986) and projects to structures that are involved in copulatory behavior (Simerly and Swanson, 1988). Like the AVPV, neonatal steroid hormones organize the size of the MPN, which is larger in males than females (Bloch and Gorski, 1988, Portillo et al., 2006), and both AR and ERα are expressed in this region (Chakraborty et al., 2003a,

Chakraborty et al., 2003c, Portillo et al., 2006). Thus, these two regions were chosen from the many hypothalamic-preoptic regions that express AR and ERα as a first step in understanding how these receptors change during male aging.

Androgen Receptor Cell Numbers Increase Robustly with Aging in POA, while Serum Testosterone Concentrations Decrease

Quantitative stereological analyses of numbers and densities of cells expressing AR immunoreactivity in AVPV and MPN demonstrated abundant labeling and similar densities in all two regions. Although there were some small differences between the regions, in general, AR cell numbers and density were highest in old rats, with this age group having roughly 3-4 times higher total AR cells compared to the young group, which had the lowest AR expression. During this same period, total serum testosterone decreased from the young to the old group, with young rats having approximately four times higher testosterone levels than old rats. Indeed, our regression analysis showed a significant inverse relationship between serum testosterone and expression of total ARir cell numbers and density in the AVPV and MPN. This result is surprising, as other studies evaluating the relationship between androgen receptor and circulating testosterone have reported the opposite, namely, that the presence or administration of androgen maintains or increases the nuclear AR (Coolen and Wood, 1999), and castration decreases AR mRNA (Handa et al., 1996), or changes its localization from nuclear to cytoplasmic (Krey and McGinnis, 1990,

Wood and Newman, 1993). These relationships do not appear to be the case in aging male rats.

There are several interpretations of our data that are not mutually exclusive. First, our testosterone assay measured total testosterone (both bound and free), leaving open the possibility that there may be differential age-related changes in free, as opposed to total testosterone. To our knowledge this issue has not been addressed in aging male rats, although human studies demonstrate that both total and free testosterone decline with age (Feldman et al., 2002, Kang et al., 2003). Ongoing studies are investigating this question in a new cohort of animals. A second and related possibility is that age-related differences in sex hormone binding globulin, which increases with age (Feldman et al., 2002, Kang et al., 2003), decreases the amount of bioavailable testosterone, and as a consequence, there is a compensatory increase of the AR. A third possibility is related to the sexual experience of the rats. Fernandez-Guasti reported that sexual activity in male Wistar rats reduced hypothalamic AR immunoreactivity in MPN compared to naive rats, but had no effect on total androgens (Fernandez-Guasti et al., 2003). In the current study, although males were sexually experienced they were used at time points post-experience that differed considerably. Our young rats were perfused 1-2 weeks after they gained sexual experience while middle-aged and old rats were retired breeders that were used 4- or 12-months, respectively, after retirement. Fourth, although evidence in young male rodents discussed above indicates a positive correlation between

serum androgen and AR expression in hypothalamus-POA, it is possible that this relationship becomes disconnected or dysregulated when rats reach middle age. In fact, our results are suggestive of a compensatory increase of the AR in response to diminished testosterone concentrations. Fifth, testosterone may be synthesized within the brain [rat: (Hojo et al., 2004); human: (Stoffel-Wagner et al., 1999)] and this may change with aging, or compensate for the age-related loss in peripheral steroids. Finally, the aging brain may increase its synthesis of the AR independent of influences by gonadal steroids. We are currently performing experiments on male rats that are castrated and given testosterone (or vehicle) replacement to test what role, if any, testosterone plays in the regulation of its receptor with aging, or if the age-related increase in AR is a function of aging that is independent of peripheral gonadal hormones.

Estrogen Receptor α Immunoreactive Cells Undergo Limited Changes with Age, whereas Serum Estradiol Peaks at Middle Age

The results of our stereological analyses showed small and primarily non-significant age-related increases in total ERα in the POA of male rats. This finding is consistent with the report of Madeira et al. (Madeira et al., 2000), who showed no difference in ERα cell numbers in the aging male MPN. In addition, Jarry's laboratory reported that ERα mRNA did not change significantly in the POA of aging Wistar male rats (Bottner et al., 2007), a result consistent with our relative lack of change of the ERα protein. We also quantified ERαir cells in the

AVPV, in which we found significant increases in heavily-labeled ERα cells, but not lightly labeled or total numbers of cells, similar to the MPN. As discussed above for the AR, we do not know whether there is any functional difference between heavily- and lightly-labeled ERαir cells, although this observation is not unique to our laboratory, as the classification of ERα cells into lightly- and heavily-immunolabeled was published by another group (Phillips-Farfan et al., 2007). Heavily-immunolabeled cells may express more estrogen receptors, may have a different post-translational status (e.g., phosphorylation states), and/or may be differentially sensitive to estradiol. The main interpretation of our results, however, is that total ERα immunoreactive cell numbers do not change significantly in the POA of aging male rats.

Despite our finding of little change in total ERα cell numbers, serum estradiol levels were about two-fold and eight-fold higher in middle-aged than young or old males rats, respectively, indicating that there is no obvious relationship between serum estradiol level and ERαir cell numbers. Thus, if ERαir cell numbers are negatively regulated in male rats, as they are in females (Lauber et al., 1990), there is a disconnection in this regulation with aging. Notably, our current results on estradiol are not consistent with previous reports showing either no change or an overall increase in estradiol with aging (Chambers et al., 1982, Drafta et al., 1982, Fujita et al., 1990, Goya et al., 1990, Chambers and Phoenix, 1992, Gruenewald et al., 2000, Herath et al., 2001, Vermeulen et al., 2002, Luine et al., 2007). We do not know the reason for this

difference but it may be attributable to species/strain, and/or sexual experience. Finally, in considering the regulation of nuclear receptors in the POA, it is necessary to understand the role of local aromatization of testosterone to estradiol. Roselli *et al.* (Roselli et al., 1986) reported that hypothalamic aromatase activity was unchanged in aging male rats, and there may be sufficient estrogen in the POA of aged males, even with their lower testosterone levels, to maintain ERα protein expression at a relatively similar level.

It should be noted that there is a second nuclear receptor for estrogens, the estrogen receptor beta (ERβ), which is expressed in the MPN and AVPV of male and female rats (Zhang et al., 2002, Chakraborty et al., 2003b, Bu and Lephart, 2007). In female rats, aging is associated with a decrease in ERβ immunoreactive cell numbers in the AVPV (Chakraborty et al., 2003b). ERβ mRNA in another hypothalamic region of female rats, the suprachiasmatic nucleus, declined with age, although it did not change in periventricular preoptic nucleus (roughly equivalent to AVPV), MPN or paraventricular nucleus (Wilson et al., 2002). To our knowledge, age-related changes in ERβ in male brains have not been reported. We were unable to quantify ERβ in the current study due to failure of available antibodies to work in our tissues, but this is an interesting future area of research, particularly considering the changes of serum estradiol in aging mammals discussed above.

SUMMARY AND CONCLUSIONS

Data from the present study add to previous observations showing an abundant number of cells expressing AR and ERα in AVPV and MPN, and extend these findings to the aging male brain. ERα immunoreactivity increased modestly with age, an effect largely limited to the most heavily immunoreactive cells, as total ERαir cell numbers and density did not change with aging. Numbers and density of ARir cells in these two preoptic regions increased robustly with aging, a period during which circulating testosterone declines precipitously. Indeed, a significant negative correlation between serum testosterone concentrations and AR cell numbers was detected in the AVPV and MPN, regardless of chronological age. These findings provide quantitative evidence for differential expression of nuclear hormone receptors in the aging compared to the young adult male POA.

CHAPTER 3. SEXUAL EXPERIENCE CHANGES SEX HORMONES BUT NOT HYPOTHALAMIC STEROID HORMONE RECEPTOR EXPRESSION IN YOUNG AND MIDDLE-AGED MALE RATS

ABSTRACT

Testosterone is well known to regulate sexual behavior in males, but this is dependent upon prior sexual experience. Aging is associated with decreased libido and changes in testosterone, but the role of experience in these age-related processes has not been systematically studied. We examined effects of age and experience on serum hormones (total testosterone, free testosterone, estradiol, LH) and on numbers of androgen receptor (AR) and estrogen receptor α (ERα) immunoreactive cells in the hypothalamus. Extensive sexual experience was given to male rats at 4 months of age, which were euthanized at either 4 months (young) or 12 months (middle-aged (MA)). Comparable sexually naïve male rats were handled and placed into the testing arena but did not receive any sexual experience. Thus, we had four groups: young-naïve, young-experienced, MA-naïve and MA-experienced. Serum hormone levels were assayed, and numbers of AR and ERα cells were quantified stereologically in the medial preoptic nucleus (MPN) and the anteroventral periventricular nucleus (AVPV).

Sexually experienced males had significantly elevated serum testosterone and free testosterone in both age groups. Both total and free testosterone were higher, and estradiol lower, in middle-aged than young rats. Experience did not alter either AR or ERα expression in the preoptic brain regions studied. Aging was associated with increased expression of AR, but no change in ERα. These results show that sexual experience can induce short-term and long-term alterations in serum hormones but these effects are not manifested upon their receptors in the hypothalamus.

INTRODUCTION

Both the appetitive (motivational) and consummatory (copulatory) aspects of male sexual behavior are related to the actions of steroid hormones upon their receptors located in a network of interconnected limbic brain regions, principally the preoptic area (Davidson, 1966, Dominguez and Hull, 2005). Most work in this area has focused on testosterone's actions on the androgen receptor (AR), because castration is associated with the reduction and eventual disappearance of appetitive and consummatory sexual behaviors, which can be restored by testosterone treatment (Davidson, 1966, McGinnis et al., 1989, Chambers et al., 1991). Moreover, castration decreases AR expression in the preoptic area, and testosterone treatment restores it (Krey and McGinnis, 1990, Handa et al., 1996). Estrogen receptors (ER) also play a role in this process, as exogenous estradiol

given to castrated rats can restore sexual behavior, although supraphysiological levels are necessary to provide a complete restoration (Davidson, 1969, Sodersten, 1973). Aromatase inhibitors block testosterone induction of copulation, again highlighting the role of estradiol in this process (Morali et al., 1977, Zumpe et al., 1993, Vagell and McGinnis, 1997, Clancy et al., 2000).

Less attention has focused upon the role of sexual experience in copulatory behavior. This is important, behavioral experience can change the brain, causing elevated metabolic capacity in the limbic system (Sakata et al., 2002) and plasticity of excitatory synapses in medial preoptic area (Malinina et al., 2006) that may modulate the ability of hormones to act upon preoptic brain regions to control behavior. A role for previous sexual experience has been shown for both motivational (Matuszczyk and Larsson, 1994, Kelliher and Baum, 2002) and consummatory components of behavior [cats (Rosenblatt and Aronson, 1958) and hamsters (Lisk and Heimann, 1980)]. Although conflicting results were found for the effect of experience in the timing of the loss of mating behaviors in rats (Rabedeau and Whalen, 1959, Bloch and Davidson, 1968, Retana-Marquez and Velazquez-Moctezuma, 1997), it was shown that male rat copulatory behavior is more efficient after sexual experience. Compared to naïve, sexually experienced rats could achieve an ejaculation with fewer mounts and intromissions; those males with prior sexual experience had lower mount, intromission and ejaculation latencies (Dewsbury, 1969, Dahlof and Larsson, 1978, Frankel, 1981, Pfaus and Wilkins, 1995, Bialy et al., 2000) and lower post-

ejaculatory interval (Larsson, 1959). These effects are mediated by preoptic brain regions and their inputs as supported by evidence that experienced rats as compared to non-experienced males, showed greater sexual performance after brain impairment caused by lesions of the medial preoptic area (de Jonge et al., 1989), medial amydala (de Jonge et al., 1992, Kondo, 1992) and the bed nucleus of stria terminalis (Claro et al., 1995).

During aging, there is a decline in male sexual performance (Chambers and Phoenix, 1983, Smith et al., 1992, Nicolosi et al., 2004), but the role of sexual experience in this process has not been systematically studied. This is important because experience may interact with aging to affect reproductive behavior and function, and cause structural and neurochemical changes in the nervous system. For example, neuropeptide Y levels, a neurotransmitter involved in sexual behavior (Clark et al., 1985a) and gonadotropin-releasing hormone secretion (Kalra and Crowley, 1992), were significantly higher in middle-aged sexually experienced male rats than naïve middle-aged animals, an effect that was not found in young males (Clark, 1994). Middle-aged naive but not experienced male rats responded to a receptive female with significantly rising testosterone during mating while there was no difference between young naïve and young experienced male (Frankel, 1981). Further, compared to sexually naive males, experienced male rats have greater copulation-induced Fos-Like immunoreactivity in the medial preoptic nucleus (Lumley and Hull, 1999). Beyond

these and a few additional studies, little is known about how hormones, brain and sexual behavior are affected by sexual experience during aging.

Previously, we showed an age-related increase in AR, but little change in ERα, in two preoptic regions: the medial preoptic nucleus (MPN) and the anteroventral periventricular nucleus (AVPV; (Wu et al., 2009). We hypothesized that sexual experience may increase sensitivity to steroid hormones by affecting their secretion or changing their actions upon their nuclear hormone receptors in hypothalamus. Using a male rat model, we compared effects of identical sexual experience in young and middle-aged animals on hormones and expression of AR and ERα in the preoptic area.

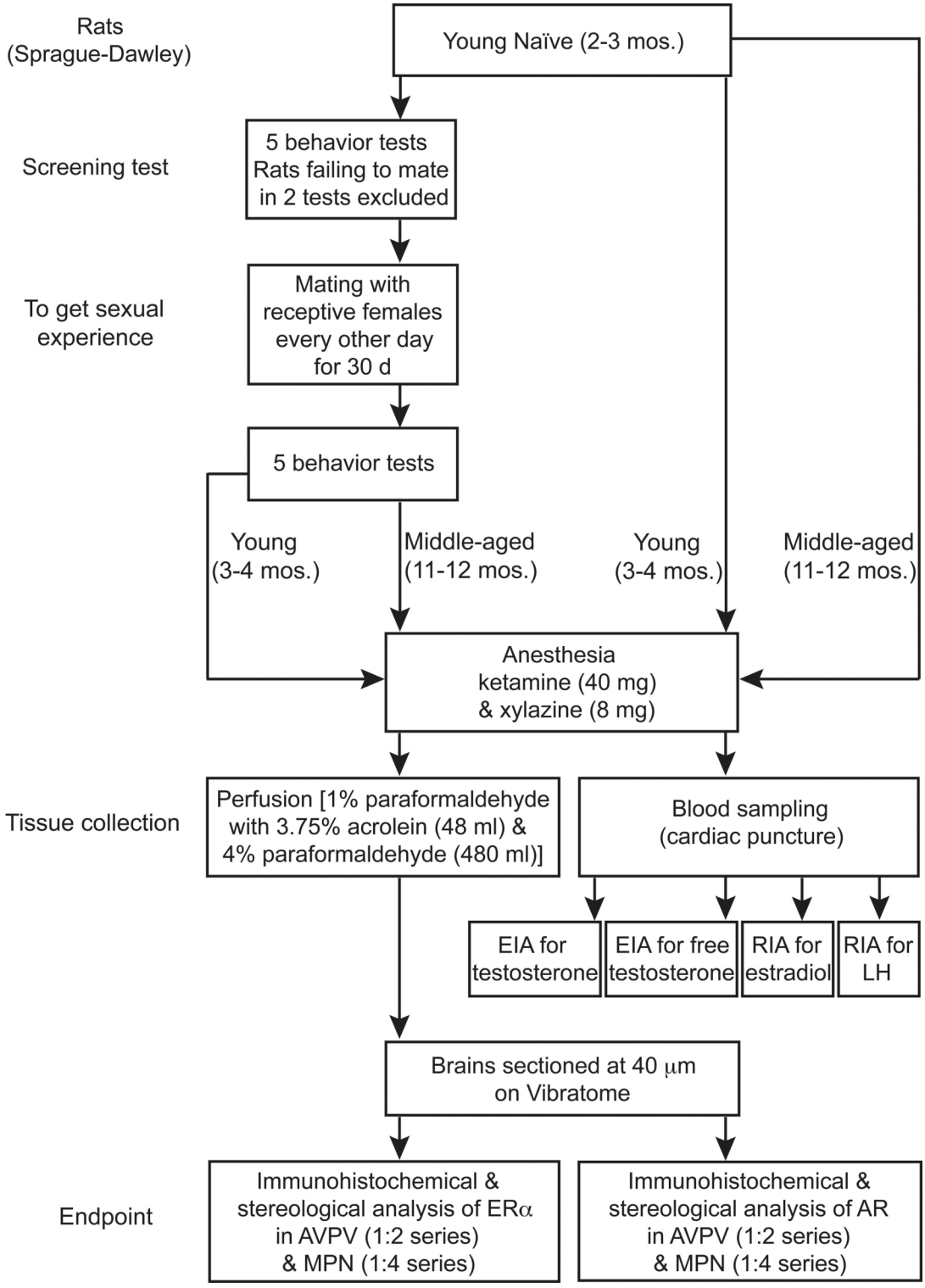
METHODS

Animals

At start of the experiments, 54 male Sprague-Dawley rats without sexual experience were purchased at 2-3 months from Harlan Sprague-Dawley Inc (Indianapolis, Indiana; Stock/Strain: Hsd:Sprague Dawley®™ SD®™).

24 young female Sprague-Dawley rats (2-3 months) were purchased from Harlan Sprague-Dawley Inc. After ovariectomy, they were implanted with estradiol [one silastic capsule (1.98-mm I.D. × 3.18-mm O.D. × 5-mm length; Dow Corning Corporation, Midland, MI) packed with 5% crystalline 17b-estradiol

Figure 3.1. Experiment Two Flowchart



(Sigma-Aldrich, St. Louis, MO)]. These female rats were given 500 μg progesterone (Sigma-Aldrich, St. Louis, MO) in sesame oil (0.1 ml) injection three hours before the mating tests. Only females who showed lordosis after being mounted by male rats were used in mating trials.

Rats lived in an AAALAC-approved facility (two same-sex same-experience animals per cage, cage dimensions 47 × 20 × 25 cm) with Rat Sterilizable Diet (Harlan Teklad LM -485 7012, Madison, WI) and water available ad libitum. The light cycle was 12 h light, 12 h dark cycle (lights on 2300h), and temperature was kept at 21 ± 1ºC. All animal procedures were approved by the UT-Austin Institutional Animal Care and Use Committee (Protocol number: 08030101) and studies were performed following *the Guide for the Care and Use of Experimental Laboratory Animals*.

Sexual Experience

Mating tests were performed under red dim lights, beginning 3 hours after lights out (1400h-1700h). Every female habituated to the chamber for 10 minutes first. Every male habituated the chamber for 20 minutes before the female was introduced. 20-minute mating tests took place in a transparent Plexiglas chamber [38 cm (L) × 32 cm (W) × 46 cm (H)]. The test was terminated at 20 minutes. Mating tests were recorded using a Sony Handycam Hi-Fi Stereo Video 8 XR camcorder (Sony Corporation of America, New York, NY). As control, naïve group were put in clean test chambers without a female for 20 minutes.

At ~3 months of age, male rats were randomly assigned to one of two experimental groups: Naïve (n = 24) or Sexual experience (n = 30). For the experience group, male rats were initially given five mating tests every other day with different receptive females (Figure 3.2). Those males who mated in only one of the five tests or did not mate were excluded. The rest of the males in this group (n = 22) continued to receive sexual experience by staying with a receptive female overnight 15 times (every other day for 30 days) with females rotating among males. Ejaculations were confirmed by checking the females' vaginas the next morning for the presence of sperm. All the males ejaculated for each of the 15 nights. Another 5 mating tests were performed for experienced rats to confirm mating ability (Figure 3.2). All the rats mated in 3 to 5 of the 5 mating tests.

Both naïve and experienced groups were then further randomly subdivided into two groups (Figure 3.2): those to be perfused at either young or middle-aged list stages. This resulted in four experimental groups: young-naïve group (n = 12), young-experienced (n = 11), middle-aged-naïve (n = 12), middle-aged-experienced (n = 11).

Perfusion

Young-experienced rats were perfused the day after the last mating test. Young-naïve group were perfused at the same time relative to placement in the mating chamber without a female. Middle-aged rats (naïve or experienced) rats were perfused 8 months after testing or handling. For perfusions, rats were

Figure 3.2 The General Experimental Design

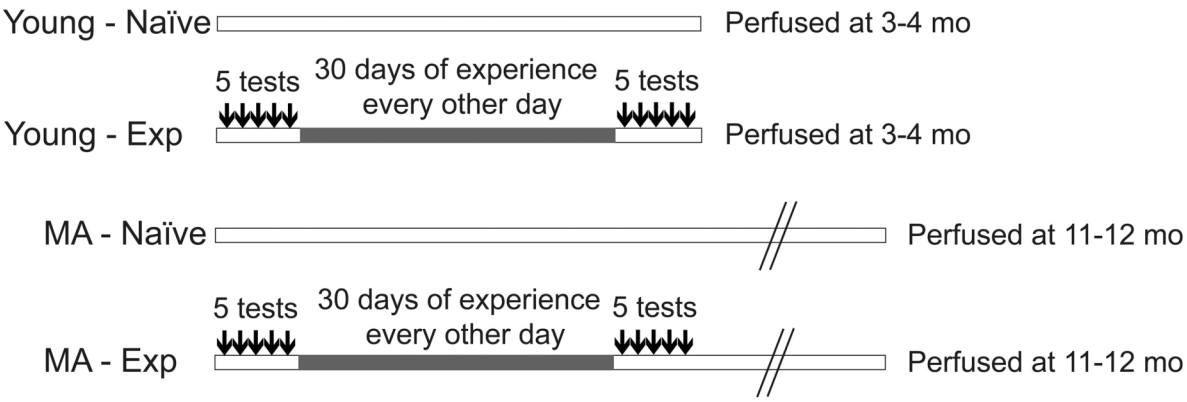


Figure 3.2. The general experimental design is shown. Arrows indicate days of five consecutive mating tests in the experience groups given in two sessions, interspersed by a thirty day period in which males were placed with a receptive female every other day. Naïve rats were placed into the testing chambers for equivalent periods of time but did not have access to a female. Young rats were perfused one day after the last testing or handling period. Middle-aged rats were perfused 8 months after testing. MA: middle-aged; Exp: experienced; mo: months of age.

anesthetized with ketamine (40 mg) and xylazine (8 mg) (i.p. injection). Then, the thoracic cavity was opened, and a blood sample (6 ml) was drawn from the ventricle for subsequent hormone assays. Animals were subsequently perfused (48 ml/min) sequentially with 0.9% saline (24 ml), 0.9% saline with 10% heparin (24 ml), and 1% paraformaldehyde with 3.75% acrolein (48 ml), followed by 4% paraformaldehyde (480 ml) (Wu et al., 2009). PBSA (phosphate-buffered saline A: 0.08 M, PO4: 0.12 M, pH=7.3) was used to dissolve all fixatives. After perfusion, the brains were carefully removed and post-fixed for 3 hours in 4% paraformaldehyde and then transferred into PBSA with 0.05% sodium azide for storage at 4°C. A vibrating microtome (Leica VT 1000S, Leica Microsystems, Nussloch, Germany) was used to cut brain into sections (40 μm-thick) that later were stored in PBSA at 4°C until use.

Immunohistochemistry

For AR immunohistochemistry, sections were incubated in the rabbit polyclonal AR antibody N-20 (1:1500; Santa Cruz Biotechnology, Inc.) developed in 1996 against a peptide mapping at the N-terminus (amino acids 299–315) of AR of human origin and which has been validated extensively by preabsorption of the antibody with the antigen, western blotting, and other controls (Chlenski et al., 2001, McKinnell et al., 2001).

The ERα antibody was a purified rabbit polyclonal antibody generated against the final 14 C-terminal amino acids (TYYIPPEAEGFPNTI) of the rat ER

that has also been very well validated for use in neural, reproductive and other tissues (Friend et al., 1997, Moffatt et al., 1998, Chakraborty et al., 2003a, Wu et al., 2009).

For immunohistochemistry, all the sections were processed in two runs. For each run, animals from each age and experience group were equally represented. In order to make the experimenter blind to age and experience group, sections IDs were then recoded. Sections containing the preoptic area were selected in a 1:2 series (AVPV region) or a 1:4 series (MPN region; see below for details) and rinsed in PBSB (phosphate-buffered saline B: saline: 0.16 M, PO4: 0.01M, pH=7.3) All steps, unless noted, occurred at room temperature (22°C) on a platform. Procedures of immunohistochemistry, tissue handling and Nissl staining were done identically to our previously published work (Wu et al., 2009).

Stereological Analysis

A stereological analysis was performed according to methods described in detail previously (Chakraborty et al., 2005, Wu et al., 2009). For the AVPV, a 1:2 series (5-6 sections) and for MPN a 1:4 series (8-9 sections) was used. A wet-mount of fresh tissue showed that average tissue thickness was 40.9 μm. The sections were carefully matched for rostral-caudal landmarks among all the animals, and the regions were identified in Nissl-stained sections by comparing anatomical landmarks to an atlas of the rat brain (Swanson, 1998). Quantitative

analyses were done as previously described (Wu et al., 2009). For stereology, closed contours were drawn to surround the region of interest (AVPV and MPN) at low magnification (10X and 4X, respectively) using the laboratory Olympus BX-61 microscope. A buffer zone at the top and bottom of sections was set at 3 μm for all experimental stereology. For each rat, the regional volume of the AVPV and MPN was extrapolated based on the contours and tissue thickness (Volume = regional area × thickness). The Stereo Investigator software randomly placed 135 μm × 80 μm grids ("disector frames") within the AVPV contour and 120 μm × 180 μm grids within the MPN contour. Within these disector frames, the DAB-stained AR- or ERα- labeled nuclei were counted within a 50 μm × 50 μm counting frame ("optical disectors"). Based on these parameters, the number and density (# immunoreactive cells/volume of each nucleus) of AR-immunoreactive (AR-ir) or ERα-immunoreactive (ERα-ir) nuclei falling within the regions was quantified. The coefficients of error and variation of the estimates were calculated as per Schmitz and Hof (Schmitz and Hof, 2000) and shown in Tables 3.1. CE's were low and never exceeded 0.09 (see Tables 3.1). Photomicrographs were taken to produce the figures, and images were subjected to only minor adjustments of contrast using Adobe Photoshop 7.0 (Adobe, San Jose, CA). In order to avoid any bias, any adjustments were applied equally to tissues from rats of different ages.

Table 3.1 Optical Fractionator Analysis of AR and ERα Immunoreactive Cell Number and Volume

AVPV

	ARir Cell Number	CE	Volume (μm^3) x 10^7	CE	ERαir Cell Number	CE	Volume (μm^3) x 10^7	CE
Young-Naïve	1492 ± 407	0.09	6.37 ± 1.03	0.08	7795 ± 1172	0.08	7.40 ± 0.78	0.06
Young-EXP	1329 ± 212	0.09	6.51 ± 1.18	0.08	7489 ± 1184	0.08	8.21 ± 1.36	0.05
MA-Naïve	2569 ± 488	0.09	6.81 ± 1.48	0.08	7154 ± 1229	0.08	7.82 ± 1.46	0.06
MA-EXP	3394 ± 546	0.07	6.87 ± 0.86	0.06	7955 ± 1401	0.06	7.81 ± 1.03	0.05

MPN

	ARir Cell Number	CE	Volume (μm^3) x 10^8	CE	ERαir Cell Number	CE	Volume (μm^3) x 10^8	CE
Young-Naïve	24966 ± 5396	0.04	7.14 ± 0.57	0.02	21586 ± 3339	0.04	6.67 ± 0.25	0.03
Young-EXP	25493 ± 4319	0.02	6.99 ± 0.28	0.03	18417 ± 3562	0.06	6.67 ± 0.17	0.03
MA-Naïve	42054 ± 3920	0.02	7.54 ± 0.31	0.02	29268 ± 5973	0.04	7.25 ± 0.19	0.03
MA-EXP	39752 ± 3191	0.02	7.47 ± 0.34	0.02	34492 ± 6143	0.05	7.32 ± 0.54	0.03

Data shown are mean ± SEM. Abbreviations: ARir:androgen receptor immunoreactive; ERαir : estrogen receptor α immunoreactive; EXP: experienced; MA, middle-aged; AVPV: anteroventral periventricular nucleus; MPN: medial preoptic nucleus; CE: Coefficient of error.

Serum Hormone Concentrations

Blood samples collected at the time of perfusion were centrifuged (8000 rpm, 5 min) and the serum collected and stored at –80 °C for hormone assays.

Enzyme immunoassay (EIA) of serum total testosterone

Total testosterone concentrations were measured by a single enzyme immunoassay in two plates using the EIA kit DSL-10-4000 according to the method described by Diagnostic Systems Laboratories, Inc. (Webster, TX). Duplicate samples were run at a volume of 50 µl each. The minimum detectable level of testosterone was 0.04 ng/ml per tube. Intra-assay variabilities of two plates were 7.61% and 7.48% respectively. Inter-assay variability was 0.79%.

Enzyme immunoassay (EIA) of serum free testosterone

Free testosterone concentrations were measured by two testosterone enzyme immunoassays using the EIA kit DSL-10-49000 according to the method described by Diagnostic Systems Laboratories, Inc. Duplicate samples were run at a volume of 50 µl each. The minimum detectable level of testosterone was 0.19 pg/ml per tube. Intra-assay variabilities were 2.38% and 4.59% respectively. Inter-assay variability was 9.98%.

Radioimmunoassay (RIA) of serum estradiol

Estradiol concentrations were measured by a single ultra-sensitive estradiol RIA using the DSL-4800 RIA kit according to the method described by Diagnostic Systems Laboratories, Inc. Duplicate samples were run at a volume of 200 µl each. The minimum detectable level of estradiol was 2.2 pg/ml per tube. Intra-assay variability was 1.86%.

Radioimmunoassay (RIA) of serum luteinizing hormone (LH)

LH in serum samples was determined by a single double-antibody RIA. This RIA was performed using the rat LH RP-3 standard, iodinate and antibody from the National Hormone and Pituitary Program of the NIDDK (kindly provided by Dr. A.F. Parlow). Single samples were run. The assay sensitivity was 0.03 ng/tube at 85% binding. Intra-assay variability was 5.76%.

Statistical Analysis

Statistical analysis was done with each rat as the unit of analysis. Using JMP statistical software (7.0) (SAS institute, Cary, NC), effects of age and experience were evaluated on the following endpoints each for the AVPV and MPN: AR-ir and ERα-ir cell numbers, regional volume, and immunoreactive cell density (calculated as cell numbers/volume). Effects of age and experience on serum hormone levels were similarly analyzed. First, datasets were tested for

homogeneity of variance and normality. Then comparisons were made by two-way ANOVA followed by post hoc analysis when indicated by a significant interaction. Otherwise data were square root transformed to normalize variation across a broad range of values and then analyzed by two-way ANOVA. The criterion for statistical significance was $p < 0.05$.

RESULTS

AR Immunoreactivity in AVPV and MPN

Representative photomicrographs of AR immunoreactivity in the AVPV and MPN (Figure 3.3) are shown in young and middle-aged male rats with or without sexual experience, and demonstrate robust expression of AR-ir in these regions. Stereologic analyses of AR-ir cell numbers and densities in AVPV and MPN were performed (Figure 3.4). A significant main effect of age was detected on AR-ir cell numbers and densities in both of these preoptic regions (AVPV: AR-ir cell numbers $P < 0.01$, AR-ir cell densities $P < 0.05$; MPN: AR-ir cell numbers $P < 0.01$, AR-ir cell densities $P < 0.001$). Specifically, middle-aged rats had higher numbers and densities of AR-ir cells in AVPV and MPN compared to young rats. There was no significant effect of experience on AR cell number and density in AVPV and MPN (Figure 3.4).

Figure 3.3. Photomicrographs of AR Immunoreactivity in the AVPV and MPN

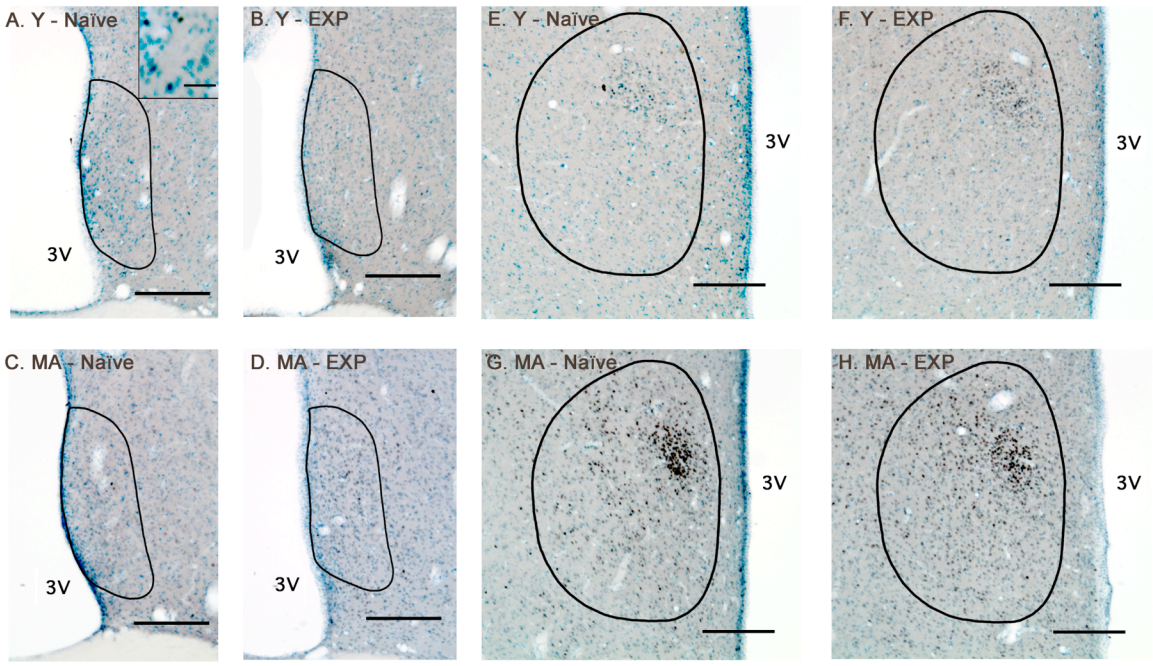


Figure 3.3. Photomicrographs of AR immunoreactivity in the AVPV (A – D) and MPN (E – H) are shown for representative young-experienced (A, E), young-naïve (B, F), middle-aged-experienced (C, G) and middle-aged-naïve (D, H) male rats. The contours of the AVPV or MPN are drawn based on Nissl staining and according to Swanson's rat brain atlas (1998). Inset shows AR immunoreactivity in the AVPV under higher magnification. Scale bar in each panel = 200 µm. Scale bar in the inset = 30 µm. Abbreviations: MA, middle-aged; EXP, experienced; 3V, third ventricle.

Figure 3.4. Stereologic Analysis Results of AR Immunoreactive Cell Number, Regional Volume and Cell Density

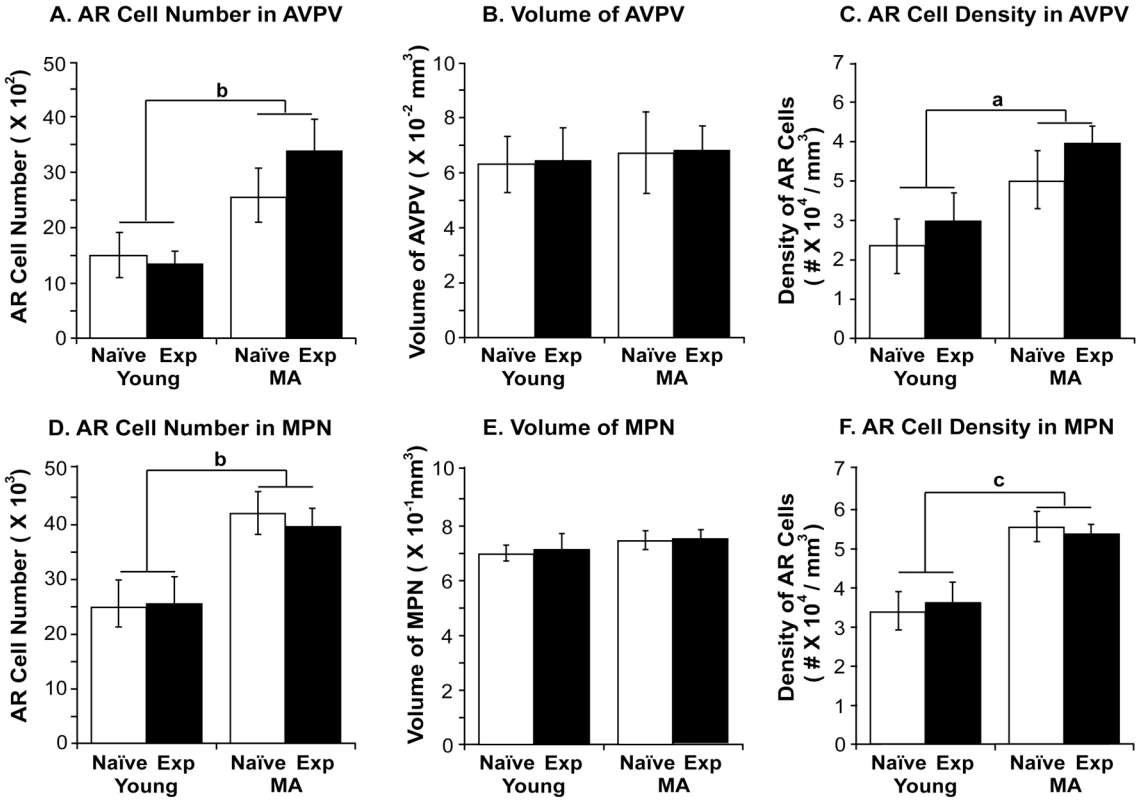


Figure 3.4. Stereologic analysis results of AR-immunoreactive cell number, regional volume and cell density (number of AR-ir cells/regional volume) are shown for young-experienced, young-naïve, middle-aged-experienced and middle-aged-naïve rats in the AVPV (A, B, C) and MPN (D, E, F). Although there was no effect of experience, aging was associated with significantly higher numbers and density of AR cells in both AVPV and MPN. The bars represent the mean ± SEM. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$. N=6 rats per group.

ERα Immunoreactivity in AVPV and MPN

Representative photomicrographs of ERα immunoreactivity in the AVPV and MPN are shown in Figure 3.5 for young and middle-aged naïve and experienced rats. Although expression of ERα cells was abundant, stereological analyses of ERα-ir cell numbers and densities showed no significant effect of either age or sexual experience (Figure 3.6).

Serum Hormone Concentrations

Serum testosterone, free testosterone, estradiol and luteinizing hormone (LH) in were assayed in young and middle-aged male rats (Figure 3.7). A significant main effect of age on serum testosterone was detected ($P < 0.001$) with the middle-aged group having higher levels of testosterone than young rats. Experience also had a significant effect on serum testosterone ($P < 0.0001$) with experienced groups having higher serum testosterone. In addition, there was a significant interaction of age and experience ($P < 0.05$). Specifically, the young naïve group had lower testosterone than the other three groups ($P < 0.01$, $P < 0.0001$, $P < 0.05$ vs. young-experienced, MA-experienced, and MA-naïve, respectively). The MA-naïve rats also had lower testosterone than MA-experienced rats ($P < 0.05$).

For serum free testosterone, a significant main effect of age was detected ($P < 0.0001$) with middle-aged rats having higher levels of free testosterone.

Figure 3.5. Photomicrographs of ERα immunoreactivity in the AVPV and MPN

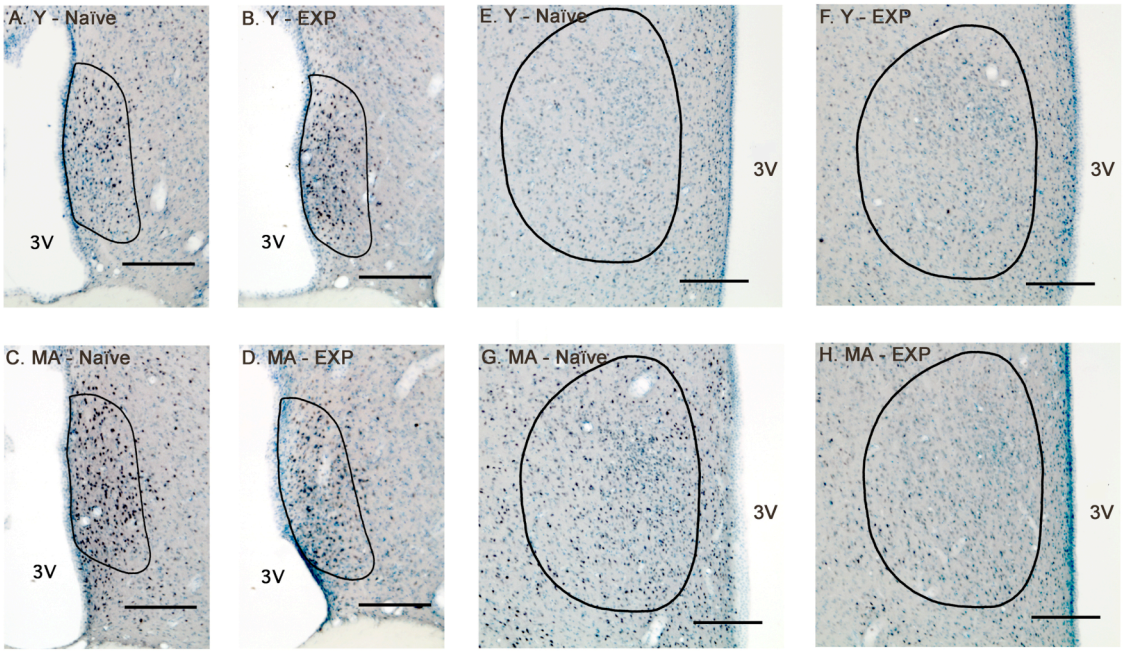


Figure 3.5. Photomicrographs of ERα immunoreactivity in the AVPV (A – D) and MPN (E – H) are shown for representative young-experienced (A, E), young-naïve (B, F), middle-aged-experienced (C, G) and middle-aged-naïve (D, H) male rats. The contours of the AVPV or MPN are drawn based on Nissl staining and according to Swanson's rat brain atlas (1998). Scale bar = 200 μm. Abbreviations: MA, middle-aged; EXP, experienced; 3V, third ventricle.

Figure 3.6. Stereologic Analysis Results of ERα Immunoreactive Cell, Regional Volume and Cell Density

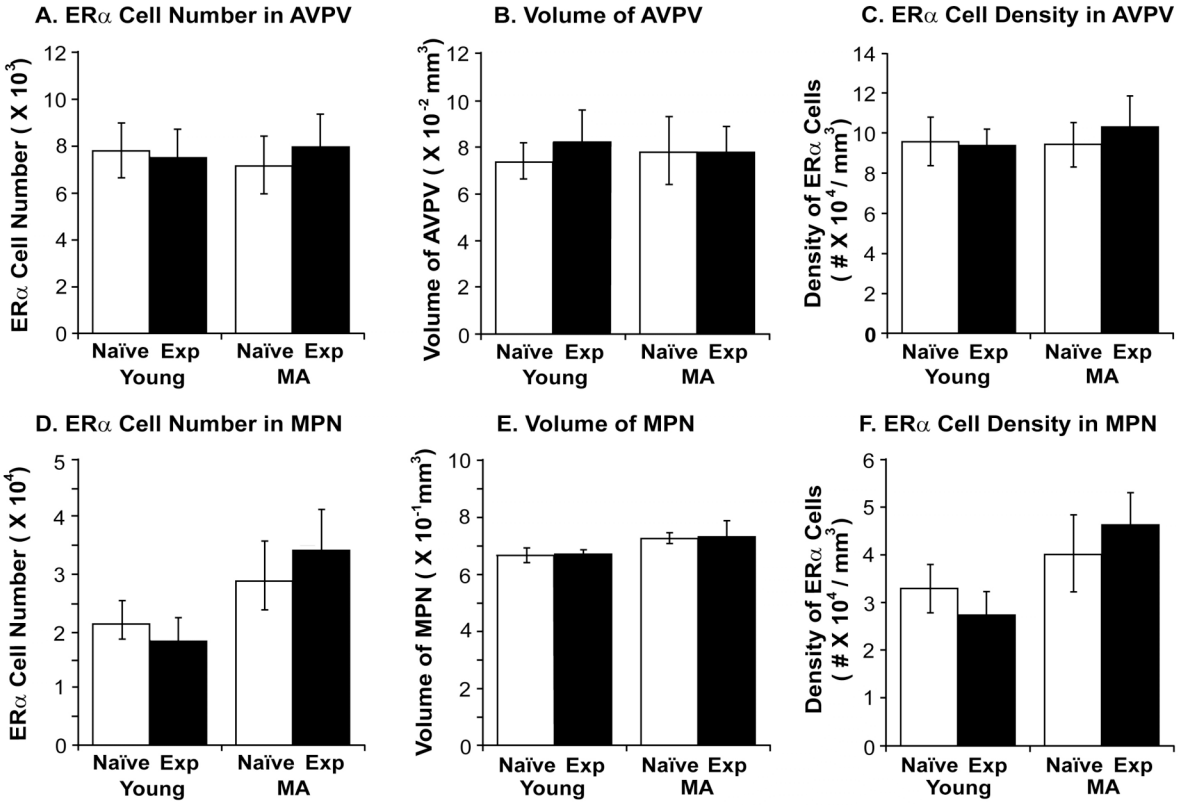


Figure 3.6. Stereologic analysis results of ERα immunoreactive cell, regional volume and cell density (#ERαir cells/regional volume) are shown for young-experienced (n=7), young-naïve (n=6), middle-aged-experienced (n=6) and middle-aged-naïve (n=6) rats in the AVPV (A, B, C) and MPN (D, E, F). There was no significant effect of age or experience on these endpoints.

Figure 3.7. Serum Total Testosterone, Free Testosterone, Estradiol and LH

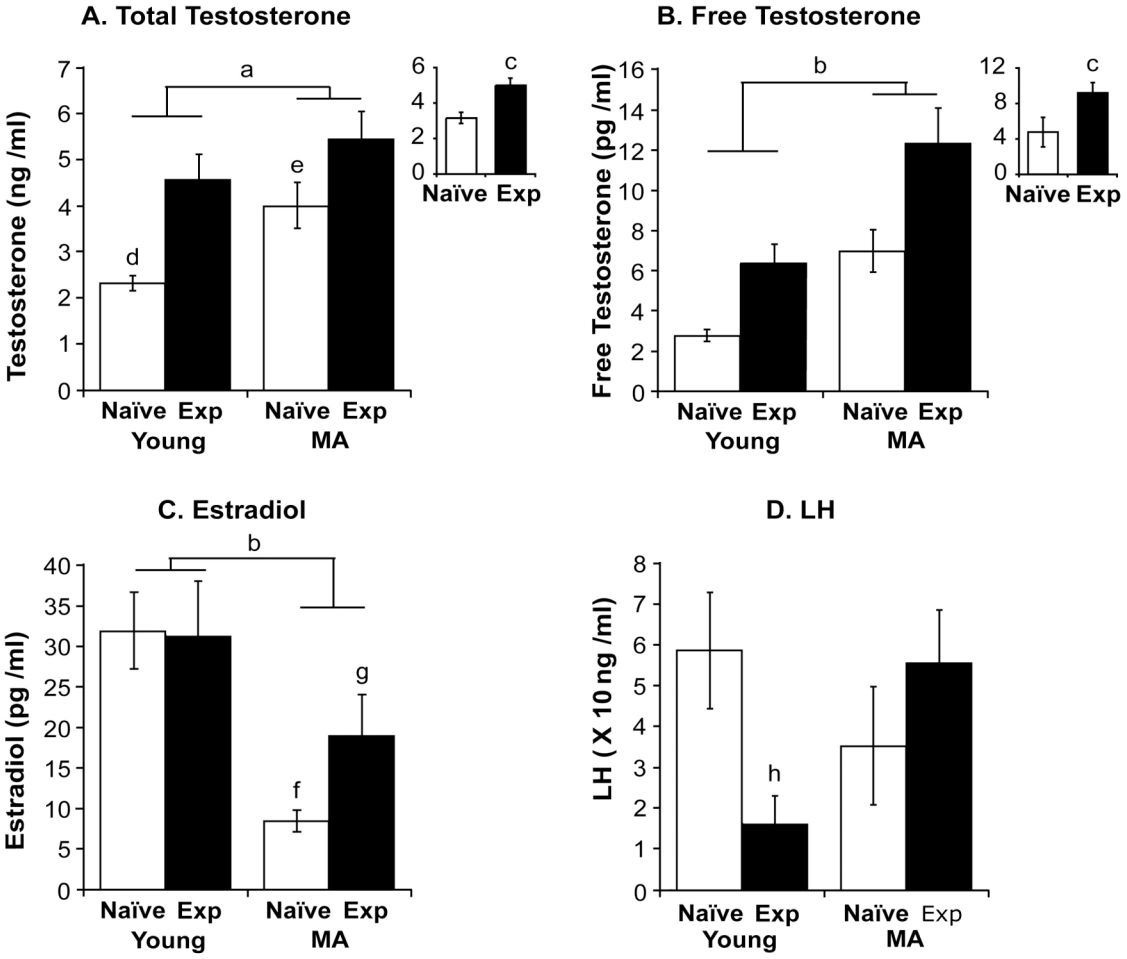


Figure 3.7. Serum total testosterone (A), free testosterone (B), estradiol (C) and LH (D) were assayed in young and middle-aged (MA) male rats with or without experience. For both total and free testosterone, significant main effects of age and experience were detected, with levels higher in middle-aged than young rats, and in experienced (EXP) than naïve groups. Estradiol levels were significantly lower in middle-aged than young rats. The interaction of age and experience was also significant. For LH, a significant interaction of age and experience was found. The bars represent the mean ± SEM. a, $P < 0.001$ young vs. MA; b, $P < 0.0001$ young vs. MA; c, $P < 0.0001$ vs. naive group; d, $P < 0.01$, $P < 0.05$, $P < 0.0001$, vs. young-EXP, MA-naive and MA-EXP respectively; e, $P < 0.05$ vs. MA-EXP; f, $P < 0.05$, $P < 0.01$ vs. young-EXP, MA-EXP respectively; g, $P < 0.05$ vs. young-naïve; h, $P < 0.05$, $P < 0.05$ vs. young-naïve, MA-EXP respectively. $P < 0.01$). The middle-aged-experienced group also had significantly lower estradiol than the young-naïve group ($P < 0.05$).

Experience also had a significant effect, with the experienced groups having significant higher free testosterone than naïve groups ($P < 0.0001$). The interaction of age and experience was not significant.

The main effect of age on serum estradiol was significant ($P < 0.001$) with middle-aged rats having lower levels than young rats. Although there was not a significant effect of experience, the interaction of age and experience was significant ($P < 0.05$). Specifically, the MA-naïve group had significantly lower estradiol concentrations than young-experienced and young-naïve rats ($P < 0.05$).

For serum LH, only the interaction of age and experience was found to be significant ($P < 0.05$). The young-experienced group had significantly lower LH concentrations than the young-naïve and middle-aged experienced groups ($P < 0.05$ for both comparisons).

DISCUSSION

The current study compared the effects of age and experience on the expression of steroid hormone receptors, AR and ERα, in preoptic areas. We observed an increase in expression of AR with aging, but no change in ERα, in agreement with a previous study (Wu et al., 2009). However, experience did not alter either AR or ERα expression in the preoptic brain regions studied. Serum hormones, by contrast, were affected by both age and sexual experience. With

respect to age, we found that both total and free testosterone were higher, and estradiol lower, in middle-aged than young rats. This finding contrasts with the literature (Roselli et al., 1986, Goya et al., 1990, Gruenewald et al., 2000, Luine et al., 2007, Wu et al., 2009), with differences potentially due to the nature and timing of sexual experience of the animals. In addition, experience significantly elevated serum testosterone and free testosterone in both young and middle-aged groups, a finding that is consistent with previous reports [rat: (Edinger and Frye, 2007); lizard: (Crews et al., 1997)]. An interaction of age and experience was observed for testosterone, estradiol and LH. Together, these results suggest that the extensive sexual experience given in the current study has long-lasting effects on serum hormones but not on their nuclear receptors in the preoptic area.

In this study, young and middle-aged rats had same extensive experience at the age of 3-4 months. These animals differed in the lag between experience and euthanasia, as well as their chronological age. When we originally designed the experiment, we also considered keeping the level of experience constant, but delaying the behavioral tests until middle-aged rats were 12 months of age. However, we could not pursue this route because rats that are not given any sexual experience until that age are very inconsistent in their mating behavior, often not mating at all [unpublished observations; (Frankel, 1981)]. Therefore, present observations must be interpreted in light of the matched experienced, but differences in age and time after experience.

Hormones, Aging and Experience

An unexpected finding was that there was an age-related increase of total and free testosterone in the rats. This is contrary to our previous report (Wu et al., 2009) and others' (Roselli et al., 1986, Taylor et al., 1996, Bernardi et al., 1998, Luine et al., 2007) that serum testosterone decreases with aging. Although the significant age-related increase in testosterone may be partially attributable to differences in circadian patterns of circulating testosterone in middle-aged rats (Kalra and Kalra, 1977, Bremner et al., 1983, Clark, 1994, Fentie et al., 2004) which are blunted compared to young rats (Simpkins et al., 1981, Fentie et al., 2004), we collected the blood samples at a consistent time, at the end of the lights-on phase of our light cycle, when the peak of testosterone is anticipated to occur (Fentie et al., 2004). Therefore, the age-related increase of testosterone is more likely to be related to the timing of sexual experience relative to when animals were killed. Although sexual experiences were matched, young rats were euthanized one day after the last mating (or control) trial, whereas middle-aged rats were euthanized eight months later.

We also observed an age-related decline in estradiol concentrations. Again, this result is different from our previous work (Wu et al., 2009) and that of other labs (Fujita et al. 1990, Luine et al., 2007, Herath et al., 2001). It is also interesting to note an experience-related difference of estradiol in the middle-aged but not the young group, with levels of estradiol being significantly lower in naïve than experienced rats at this age. To our knowledge, this has not been

reported previously. Again, we believe that our results on estradiol differ from earlier studies due to the extensive and carefully matched sexual experience in our animals.

For LH, there were no overall effects of age or experience, but an interaction was found. Young-experienced rats had lower LH levels than young-naïve or middle-aged experienced rats. It is possible that young rats are more sensitive to negative feedback effects of testosterone than middle-aged rats, and that this is further altered by experience. Previously, middle-aged rats have been reported to have diminished LH regulation by testosterone [Human: (Mitchell et al., 1995); Rat: (Haji et al., 1981, Chambers et al., 1991, Bonavera et al., 1997)]. Additional differences in LH may also be attributable to the pulsatile nature of this hormone's release. There can be considerable individual variability in LH levels depending upon when during a pulse the sample is collected. This may also explain the relatively high variability in LH levels compared to variability in steroid hormones.

AR and ERα Expression: Effects of Age and Experience

The increase in expression of AR with aging we observed is in agreement with our previous study (Wu et al., 2009). However, our finding of a lack of effect of experience on AR-ir cell numbers differs from previous reports showing that mating was associated with decreased AR density in a time-dependent manner (Fernandez-Guasti et al., 2003, Romano-Torres et al., 2007). However, in those

reports, only young sexually active males were used. Therefore, some differences in results may be attributable to the extent of experience between the studies.

We also did not observe any age- and experience-related difference in the expression of ERα. This lack of change with aging is consistent with our previous observation in experienced rats (Wu et al., 2009). With respect to experience, Phillips-Farfán et al. (Phillips-Farfan et al., 2007) showed that mating resulted in an increase of ERα, a finding that differs from our current result. However, the nature of experience was different between the two studies. Evidence that ERα expression may not be strongly related to masculine sexual behavior is confirmed by the demonstration that blockade of ERα with RU 58668, an antiestrogen, did not inhibit the restoration of copulatory behavior or partner preference in testosterone-treated gonadectomized male rats (Vagell and McGinnis, 1998).

Neural Mechanisms of Experience-facilitated Behavior

Sex steroid hormones can affect sexual behaviors in males. At the same time, each sexual experience can alter hormones level by rapidly increasing testosterone level [rats: (Frankel, 1981); rhesus monkeys: (Herndon et al., 1981)] although whether acute experience-induced increases in testosterone can be accumulated is unknown. Therefore, the interactions between experience and effects of hormones on the brain provide a “chicken and egg” dilemma in

understanding how these factors modulate one another, and whether one (or the other) provides the causal influence.

Most studies on sexual experience have compared naïve and experienced groups. However, exactly how much experience an animal has gained makes a difference. For example, rats showed significantly increasing pre-contact vocalizations while they were getting more sexual experience (Bialy et al., 2000). In addition, rats mated 16 times had significant higher metabolic capacity in sexual behavior related brain areas than naïve males or those who only mated 3 times (Sakata et al., 2002). These data indicate that experience changes brain activity. In fact, repetitive electrical stimulation can evoke short-term plastic changes in MPN neurons in vitro (Malinina et al., 2006), and more neurons are activated in the MPN after ejaculation in males with 7 sexual experiences than those one with only one experience (Lumley and Hull, 1999). We hypothesize that as experience is acquired, communication of neurons and the synthesis of key proteins in the hypothalamus is altered. That is, experience may increase neural plasticity, a hypothesis that can explain why in term of masculine sexual behavior experienced rats had smaller effects of brain impairment caused by lesions of the medial preoptic area (de Jonge et al., 1989, Liu et al., 1997), medial amydala (de Jonge et al., 1992, Kondo, 1992) and the bed nucleus of stria terminalis (Claro et al., 1995). However, our current results suggest that this plasticity does not appear to involve numbers of AR- or ERα-immunoreactive cells in the preoptic area, as these did not change with experience.

Hull, Dominguez and colleagues (Dominguez et al., 2006a) have proposed that testosterone stimulates masculine sexual behavior through a complex network of neurons in the preoptic area, including neuronal nitric oxide, dopamine, and glutamate. One study showed that previous but not acute sexual experience increased the number of nNOS-ir cells and nNOS protein concentrations in the MPN, although testosterone level was not measured in that study (Dominguez et al., 2006a). Based on the fact that the presence of a receptive female significantly enhances testosterone levels in experienced but not naïve males (Bonilla-Jaime et al., 2006) and 50-60 % nNOS colocalized with AR and 53-77% with ERα in AVPV and MPN (Sato et al., 2005), the upstream mechanism may be related to increased secretion of testosterone and/or increased sensitivity to testosterone. Although we observed an effect of experience on increased serum testosterone in both young and middle-aged male rats, an earlier study (Damassa et al., 1977) showed that for intact male rats who could complete copulatory behavior, there was no correlation between the behavior measures and testosterone levels. A second possibility is that experience modulates sensitivity to testosterone by increasing hormone receptor expression. Although, we did not observe any experience-induced changes in either AR or ERα cell numbers in AVPV or MPN, this does not exclude the possibility that there are post-translational properties of these receptors (e.g., phosphorylation) that may alter their function, something that could not be distinguished by the current immunohistochemical approach. In addition, or

alternatively, it is possible that other regions of the brain than the AVPV or MPN may change their expression of these molecules, but this was beyond the scope of the current study. It will also be interesting to look at neurotransmitters and their receptors involved in masculine sexual behavior (e.g., nitric oxide, dopamine, glutamate systems) to ascertain effects of experience and aging on these steroid-sensitive circuits.

SUMMARY AND CONCLUSIONS

We report that sexual experience increases testosterone and free testosterone in both young and middle-aged males. Sexual experience also interacts with aging to alter testosterone, estradiol and LH. However, we did not find experience-related difference in expression of AR or ERα immunoreactive cell numbers in two preoptic regions, the AVPV or MPN. This investigation is important because sexual experience can induce short-term and long-term alterations in hormones. It also suggests that males with matched sexual experience in young adulthood may differ in the time from last experience and in chronological age, but have a similar phenotype of expression of steroid hormone receptors in some preoptic brain regions.

CHAPTER 4. CHANGES IN ANDROGEN RECEPTOR, ESTROGEN RECEPTOR α, AND SEXUAL BEHAVIOR WITH AGING AND TESTOSTERONE IN MALE RATS

ABSTRACT

Reproductive aging in male rats is characterized by changes in circulating steroid hormones, including testosterone and estradiol, together with a diminution in sexual behavior. A link among these processes is the androgen and estrogen receptors in the hypothalamus, which also undergo age and hormone regulation. We hypothesized that the behavioral decline with age may be due to changes in sex steroid hormone receptors in the brain, with differential age responsiveness to testosterone. To test our hypothesis, young (3 mo) and middle-aged (12 mo) adult male Sprague-Dawley rats were given two mating trials (pre-tests), then castrated and immediately implanted with Silastic capsules containing either cholesterol vehicle (V) or testosterone (T). Two weeks later, two additional mating trials were given (post-tests). Then, rats were perfused, stereological counting of androgen receptor (AR) and estrogen receptor α (ERα) immunoreactive cells was performed in two hypothalamic regions, the anteroventral periventricular nucleus (AVPV) and the medial preoptic nucleus

(MPN). Results of behavioral pre-tests in the intact males showed significant age-related diminutions in all sexual behaviors measures in the middle-aged compared to young males. In the post-tests, sexual behaviors were for the most part similar between the ages, with similar effects of testosterone treatment, although mount frequency and intromission frequency continued to be lower in middle-aged males. AR-immunoreactive cell density did not differ by age in both the AVPV and MPN, but was significantly higher in the T-treated males. ERα cell density did not vary by age in AVPV and MPN, but was significantly lower in the T-males. Final, measures of total and free testosterone showed mostly comparable levels in the two age groups. These studies suggest that hormones and aging interact in a complex manner to control numbers of cell expressing steroid hormone receptors in the brain and on the subsequent control of sexual behavior.

INTRODUCTION

In numerous species including rats, monkeys and humans, there is an age-related decline in sexual performance that may be attributable to physiological or psychological changes (Chambers and Phoenix, 1983, Smith et al., 1992, Nicolosi et al., 2004). Much of the loss of male sexual function with aging is attributed to the well-documented decline in serum testosterone that is conserved across species [humans (Harman et al., 2001); rhesus monkeys

(Downs and Urbanski, 2006); rats: Sprague-Dawley (Roselli et al., 1986, Wu et al., 2009), Wistar (Taylor et al., 1996, Bernardi et al., 1998), Brown Norway (Chen et al., 1994, Gruenewald et al., 2000), Fischer 344 (Chambers et al., 1991, Luine et al., 2007). Additional evidence for the androgen-dependence of masculine sexual behavior is provided by studies showing that after castration, male rats gradually lose their copulatory ability (Smith et al., 1992).

However, not all of the loss of sexual function with aging is due to testosterone, based on evidence that testosterone replacement in middle-aged or old castrated rats failed to reinstate sexual performance (Chambers and Phoenix, 1984, Chambers et al., 1991, Sato et al., 1998). A plausible hypothesis is that the ability of the organism to respond to testosterone may decline with age, due to changes in actions of testosterone on its androgen receptor (AR) in brain regions involved in the control of masculine sexual behaviors and the hypothalamic-pituitary-testicular axis (Vagell and McGinnis, 1998). Circulating testosterone levels are thought to regulate expression of the AR, as castration can eliminate AR immunostaining and administration of testosterone can restore AR expression in the brain of a variety of species (Wood and Newman, 1993, Iqbal et al., 1995, Lu et al., 1998, Lu et al., 1999), often rapidly (within hours) in a dose-dependent manner (Lu et al., 1998, Lu et al., 1999). However, these latter studies all used young animals as models. Whether exogenous testosterone can affect AR expression in the same manner in aging males is unknown; if testosterone is not as efficacious as in young males, this may explain why

previous studies reported a lack of restoration of sexual behavior by testosterone in aging males.

Previously, we showed that while middle-aged males had lower testosterone levels than young males, they had higher numbers of AR immunoreactive cells within hypothalamic regions involved in reproduction, including the anteroventral periventricular nucleus (AVPV) and the medial preoptic nucleus (MPN) (Wu et al., 2009). Therefore, we hypothesized that testosterone replacement immediately after castration in middle-aged rats would lower expression of AR similar to those detected in young rats. Further, we predicted that this treatment would maintain or even improve sexual performance in aging males.

Some effects of testosterone on masculine behaviors and physiology are due to its aromatization to estradiol (Morali et al., 1977, Zumpe et al., 1993, Vagell and McGinnis, 1997, Clancy et al., 2000), but little is known about how this changes with age. Thus, we investigated the effects of aging, testosterone treatment, and their interactions on sexual behavior and expression of AR and ERα in the hypothalamus. Together, these studies are intended to provide novel information about the potential roles of these hormone receptors, and their regulation by testosterone, during reproductive aging.

METHODS

Animals

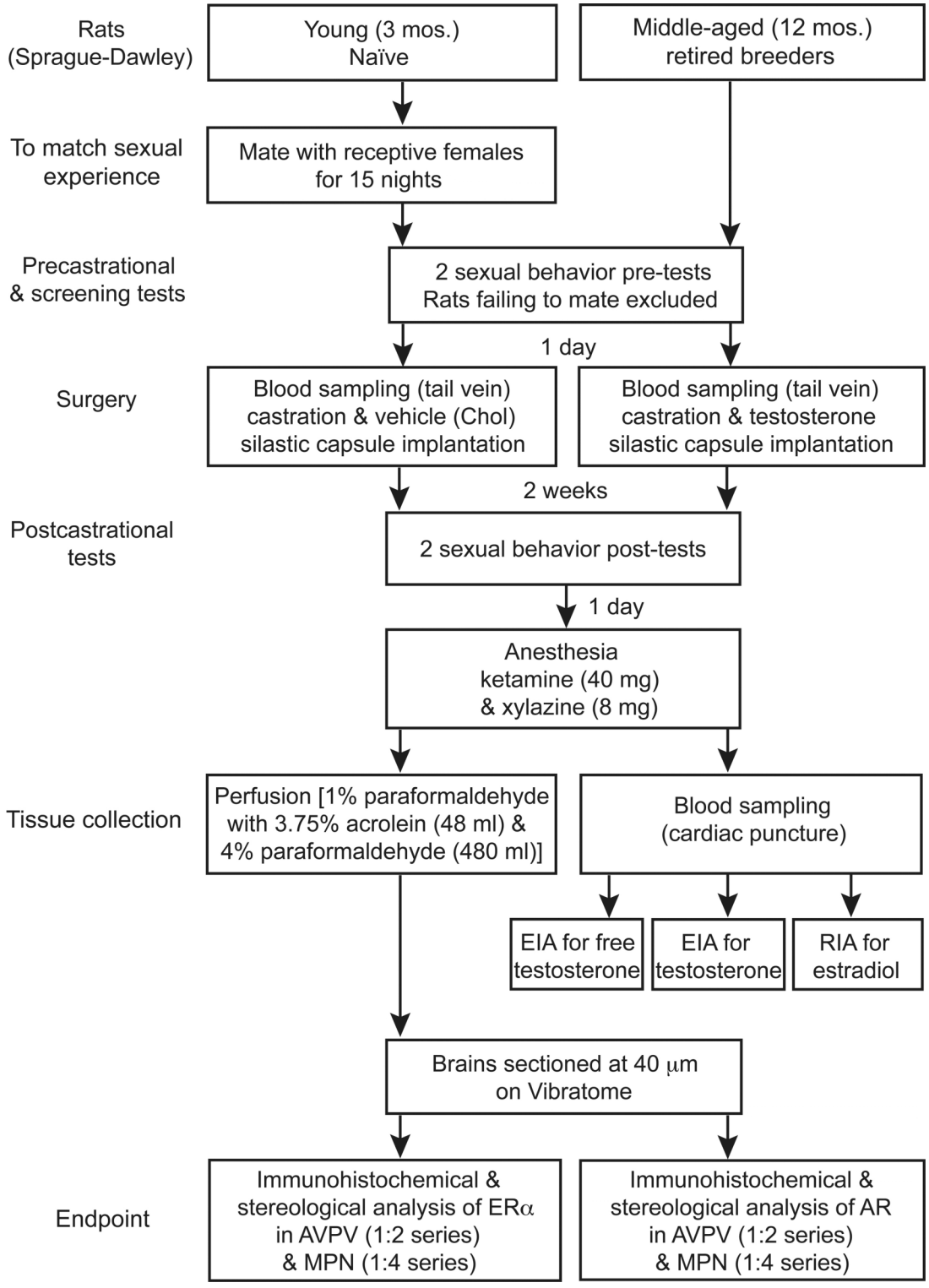
Males

At start of the experiments, male Sprague-Dawley rats were purchased at 2-3 months (young, N=18) and 10-11 months (middle-aged, N=38) from Harlan Sprague-Dawley Inc (Indianapolis, Indiana; Stock/Strain: Hsd:Sprague Dawley®™ SD®™). The middle-aged rats were retired breeders. The exact sexual experience of the middle-aged rats was not known, but Harlan allows a male to mate with females periodically over the course of three to four months. It is impossible to match perfectly this exact life history of sexual experience in young rats, but in order to give them experience, we placed each male rat in a cage with a receptive female rat (rotated among males) twenty nights in a row. Mating was confirmed by checking for sperm in the females' vaginas. Records showed that all young male rats mated for an average of 15 nights and consecutively for the last 8 nights.

Females

Female Sprague-Dawley rats were purchased at 2-3 months (N=37) Harlan Sprague-Dawley Inc (Indianapolis, Indiana; Stock/Strain: Hsd:Sprague Dawley®™ SD®™). They were ovariectomized and estradiol-implanted [one

Figure 4.1. Experiment Three Flowchart



silastic capsule (1.98-mm I.D. × 3.18-mm O.D. × 5-mm length; Dow Corning Corporation, Midland, MI) packed with 5% crystalline 17b-estradiol (Sigma-Aldrich, St. Louis, MO)]. These female rats received 500 µg progesterone (Sigma-Aldrich, St. Louis, MO) in sesame oil (0.1 ml) injection three hours before the mating trials. Only females who showed lordosis with male rats were used in mating trials.

Rats were housed in an AAALAC-approved facility (two same-sex animals per cage, cage dimensions 47 × 20 × 25 cm) with Rat Sterilizable Diet (Harlan Teklad LM-485 7012, Madison, WI) and water available ad libitum. The light cycle was 12 h light, 12 h dark cycle (lights on 2300h), and temperature was 21 ± 1ºC. All animal procedures were approved by the UT-Austin Institutional Animal Care and Use Committee (Protocol number: 08030101) and studies were performed following the Guide for the Care and Use of Experimental Laboratory Animals.

Pre-tests for Mating Behaviors

All the male rats were given two pre-tests (one day off between tests) to measure the baselines for each parameter of the mating behaviors, with different receptive females rotated among males.

Mating tests were performed under dim red light, beginning 3 hours after lights out (1400h-1700h). Every female was habituated to the chamber for 10 minutes. Every male was habituated the chamber for 20 minutes before the

female was introduced. 20-minute mating trials took place in a transparent Plexiglas cage [76 cm (L) × 32 cm (W) × 46 cm (H)]. The following parameters of male sex behavior were recorded in each test following the descriptions of Ågmo et al. (Agmo, 1997): mount latency, time from introduction of the female to the first mount; intromission latency, time from introduction of the female to the first intromission; ejaculation latency, time from the first intromission to ejaculation; postejaculatory interval (PEI), time from ejaculation to the first intromission of the next series; mount frequency, number of mounts before the first ejaculation; intromission frequency, number of mounts with penile insertion before the first ejaculation; ejaculation frequency, number of ejaculations before the end of test; inter-intromission interval, the first ejaculation latency divided by the number of intromissions before the first ejaculation, intromission ratio, dividing the number of intromissions by the number of mounts plus number of intromissions. The test was terminated at 20 minutes or after a second ejaculation, whichever came first. Mating trials were recorded using a Sony Handycam Hi-Fi Stereo Video 8 XR camcorder (Sony Corporation of America, New York, NY). The videotaped trials were further analyzed by using JWatcher v1.0 (www.jwatcher.ucla.edu, Dan Blumstein's Lab, University of California Los Angeles & The Animal Behaviour Lab, Macquarie University, Sydney) computer software to quantify the behavior of each experimental animal (Crews et al., 2007).

All the young males finished ejaculation in both of two pre-tests (n = 18). For middle-aged male rats, only those who ejaculated successfully in at least one

of the two pre-tests were chosen to participate in further experiments (n = 27). Only these latter rats are described in the remaining sections.

Castration and Hormone Implantation

Young and screened middle-aged males were randomly assigned to one of two experimental groups: gonadectomized with testosterone implants and gonadectomized with vehicle implants. Testosterone implants were made by filling 1.5 cm-long silastic capsules (1.98-mm I.D. × 3.18-mm O.D. × 5-mm length; Dow Corning Corporation, Midland, MI) with crystalline testosterone [4-Androsten-17b-ol-3-one (Sigma-Aldrich, St. Louis, MO)]. Vehicle implants were made by filling 1.5 cm-long silastic capsules with cholesterol (Sigma-Aldrich, St. Louis, MO). The ends of implants were sealed with 100% silicone Household Adhesive (DAP Inc. Baltimore, MD). For the castration and implantation surgery, animals were anesthetized with Isoflurane (Baxter Healthcare Corporation, Beerfield, IL) via a gas anesthesia machine. Bilateral orchidectomy was performed, after which a blood sample (~ 0.5 ml) was collected from the rat's tail lateral vein to get a pre-castration hormone level. Then each rat was implanted subcutaneously either with two T capsules or two V capsules. Rats were treated post-operatively with 5 mg/kg Rimadyl. They were allowed to recover for two weeks.

Post-tests for Mating Behaviors

Two weeks after implantation, rats were given two mating tests (post-tests) to measure each parameter of the mating behaviors after treatment, each time with different receptive females. Behaviors were scored as described for the pre-tests. There was one day off between the two tests.

Perfusion

On the day after post-tests, rats were deeply anesthetized with ketamine (40 mg) and xylazine (8 mg). The thoracic cavity was opened and a blood sample (8-9 ml) was drawn from the ventricle for subsequent hormone assays. Animals were perfused (48 ml/min) sequentially with 0.9% saline (24 ml), 0.9% saline with 10% heparin (24 ml), and 1% paraformaldehyde with 3.75% acrolein (48 ml), followed by 4% paraformaldehyde (480 ml) (Wu et al., 2009). PBSA (phosphate-buffered saline A: 0.08 M, PO4: 0.12 M, pH=7.3) was used to dissolve all fixatives. We removed the brains from the skull and post-fixed them for 3 hours in 4% paraformaldehyde and then transferred the brains into PBSA with 0.05% sodium azide for storage at 4°C. Then brains were cut on a vibrating microtome (Leica VT 1000S, Leica Microsystems, Nussloch, Germany) to get tissue sections (40 μm-thick) for further experiments. Sections were stored in PBSA at 4°C until use.

Immunohistochemistry

For AR immunohistochemistry, sections were incubated in the rabbit polyclonal AR antibody N-20 (1:1500; Santa Cruz Biotechnology, Inc.) raised against a peptide mapping at the N-terminus (amino acids 299–315) of AR of human origin [Genetic locus: AR (human) mapping to Xq11.2-q12; AR (mouse) mapping to 9 A5.3]. Previous work has shown high specificity of this antibody for the AR based on preabsorption of this antibody with the antigen, western blots, and other controls (Chlenski et al., 2001, McKinnell et al., 2001).

The ERα antibody was purified rabbit polyclonal antibody generated against the final 14 C-terminal amino acids (TYYIPPEAEGFPNTI) of the rat ERα, validation of which has been described in detail previously (Friend et al., 1997, Moffatt et al., 1998, Wu et al., 2009).

All the sections were processed in two IHC runs per antibody. For each run, animals from each age and treatment group were equally represented. In order to make the experimenter blind to age and treatment group, sections IDs were then recoded. Sections containing the preoptic area were selected in a 1:2 series (AVPV region) or a 1:4 series (MPN region; see below for details) and rinsed in PBSB (phosphate-buffered saline B: saline: 0.16 M, PO4: 0.01M, pH=7.3). DAB immunohistochemistry was performed as described previously in detail using either the AR or ERα antibody (Wu et al., 2009). Sections were mounted on slides, dehydrated and coverslipped as published (Wu et al., 2009).

Stereological Analysis

A stereological analysis was performed according to methods described in detail previously (Chakraborty et al., 2005, Wu et al., 2009). For the AVPV, a 1:2 series (5-6 sections) and for MPN a 1:4 series (8-9 sections) was used. A wet-mount of fresh tissue showed that average tissue thickness was 40.89 μm. The sections were carefully matched for rostral-caudal landmarks among all the animals, and the regions were identified in Nissl-stained sections by comparing anatomical landmarks to an atlas of the rat brain (Swanson, 1998). Quantitative analyses were done as previously described (Wu et al., 2009). The number and density (# immunoreactive cells/volume of each nucleus) of AR-immunoreactive (AR-ir) or ERα-immunoreactive (ERα-ir) nuclei falling within the regions was quantified. The coefficients of error (Cruz-Orive/Geiser) and variation of the estimates were calculated as described by Schmitz and Hof (Schmitz and Hof, 2000) and were between 0.02 and 0.1 per group (see Table 4.1). Photomicrographs were taken to produce the figures, and images were subjected to only minor adjustments of contrast using Adobe Photoshop 7.0 (Adobe, San Jose, CA). In order to avoid any bias, any adjustments were applied equally to tissues from rats of different ages.

Serum Hormone Concentrations

Blood samples collected at the time of castration surgery or perfusion were centrifuged (8000 rpm, 5 min) and the serum collected and stored at –80 °C for hormone assays.

Table 4.1. Optical Fractionator Analysis of AR and ERα Immunoreactive Cell Number and Volume

AVPV

	ARir Cell Number	CE	Volume (μm^3) x 10^7	CE	ERαir Cell Number	CE	Volume (μm^3) x 10^7	CE
Young- T	3682 ± 792	0.07	6.69 ± 1.01	0.07	6898 ± 396	0.06	8.54 ± 0.97	0.06
Young- V	779 ± 223	0.10	5.58 ± 0.90	0.07	5683 ± 1463	0.08	4.36 ± 0.85	0.07
MA-T	3503 ± 492	0.08	8.14 ± 0.89	0.06	5380 ± 1592	0.07	6.09 ± 1.25	0.07
MA-V	463 ± 144	0.10	5.88 ± 0.59	0.07	9253 ± 1396	0.06	8.34 ± 0.86	0.05

MPN

	ARir Cell Number	CE	Volume (μm^3) x 10^8	CE	ERαir Cell Number	CE	Volume (μm^3) x 10^8	CE
Young- T	40663 ± 4698	0.03	6.78 ± 0.16	0.03	9910 ± 2080	0.10	6.80 ± 0.07	0.03
Young- V	5074 ± 1525	0.09	6.12 ± 0.29	0.02	42339 ± 4001	0.04	6.03 ± 0.05	0.03
MA-T	30418 ± 4497	0.04	7.37 ± 0.22	0.03	7040 ± 434	0.07	7.10 ± 0.22	0.03
MA-V	4734± 1681	0.10	6.78 ± 0.18	0.03	38133 ± 4906	0.02	7.02 ± 0.18	0.03

Data shown are mean ± SEM. Abbreviations: ARir:androgen receptor immunoreactive; ERαir : estrogen receptor α immunoreactive; AVPV: anteroventral periventricular nucleus; MPN: medial preoptic nucleus; MA, middle-aged; T, testosterone; V, vehicle; CE: Coefficient of error.

Enzyme immunoassay (EIA) of serum testosterone

Testosterone concentrations were measured by a single testosterone enzyme immunoassay using the EIA kit DSL-10-4000 according to the method described by Diagnostic Systems Laboratories, Inc. (Webster, TX). Duplicate samples were run at a volume of 50 µl each. The minimum detectable level of testosterone was 0.04 ng/ml per tube. Intra-assay variability was 2.60%.

Enzyme immunoassay (EIA) of serum free testosterone

Free testosterone concentrations were measured by two testosterone enzyme immunoassays using the EIA kit DSL-10-49000 according to the method described by Diagnostic Systems Laboratories, Inc. Duplicate samples were run at a volume of 50 µl each. The minimum detectable level of testosterone was 0.19 pg/ml per tube. Intra-assay variability was 1.95% and 1.65%. Inter-assay variability was 8.82%.

Radioimmunoassay (RIA) of serum estradiol

Estradiol concentrations were measured by a single ultra-sensitive estradiol RIA using the DSL-4800 RIA kit according to the method described by Diagnostic Systems Laboratories, Inc. Duplicate samples were run at a volume of 200 µl each. The minimum detectable level of estradiol was 2.2 pg/ml per tube. Intra-assay variability was 1.86%.

Statistical Analysis

Statistical analysis was done with each rat as the unit of analysis. Using JMP statistical software (7.0) (SAS institute, Cary, NC), effects of age and treatments were evaluated on the following endpoints: regional AR-ir and ERα-ir density (calculated as cell numbers/volume), serum hormone levels, and behavioral parameters. First, datasets were tested for homogeneity of variance and normality. Then comparisons were made by two-way ANOVA followed by post hoc analysis when indicated by a significant interaction. Otherwise data were square root transformed to normalize variation across a broad range of values and then analyzed by two-way ANOVA. For calculation of percentage of ejaculations, Fisher's Exact Test was used (McGinnis et al., 1996). The criterion for statistical significance was $p < 0.05$.

RESULTS

Sexual Behavior of Male Rats

Representative examples of the numbers and timing of male copulatory behaviors (mount, intromission, ejaculation) are shown for one representative male per group [young-T, young-V, middle-aged (MA)-T, and MA-V] in Figure 4.2.

All of the young males (n = 18) mated to ejaculation in the two pre-tests. Twenty-seven of the original 38 MA males mated to ejaculation in at least one

pre-test, and subsequent data shown are from those 27 males. The percentage of ejaculations was calculated as the percentage of males ejaculating in each trial (Figure 4.3). The young group was significantly different from the MA group in both pre-test 1 [Fisher's Exact test (1, N = 45) P = 0.0316] and in pre-test 2 [Fisher's Exact test (1, N = 45) P = 0.0085]. Following castration and hormone treatment, the percentage of ejaculations was significantly lower in the young-V and MA-V rats in both post-tests compared to the testosterone groups (Figure 4.3).

The effects of castration, testosterone treatment and aging on male sexual behaviors are shown in Figure 4.4. For the pre-tests in intact rats, the middle-aged group had a significantly longer mount latency, intromission latency, ejaculation latency, postejaculatory interval, mount frequency and intromission frequency ($P < 0.05$, $P < 0.001$, $P < 0.001$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$ respectively; Figure 4.4). For the post-tests, a significant main effect of age was found only for mount frequency and intromission frequency ($P < 0.01$, $P < 0.01$), with the young group having higher frequencies than the MA group. Intromission latency, ejaculation latency, postejaculatory interval, mount frequency and intromission frequency were all significantly affected in the post-tests by hormone treatment ($P < 0.001$, $P < 0.0001$, $P < 0.0001$, $P < 0.001$, $P < 0.0001$;), with shorter latencies and higher frequencies for the testosterone than the vehicle groups (Figure 4.4 insets). For young-T and MA-T rats, sexual behaviors were compared between the pre-test and post-test was also compared using t-test.

Figure 4.2. Copulatory Behaviors of Representative Rats from Each Group

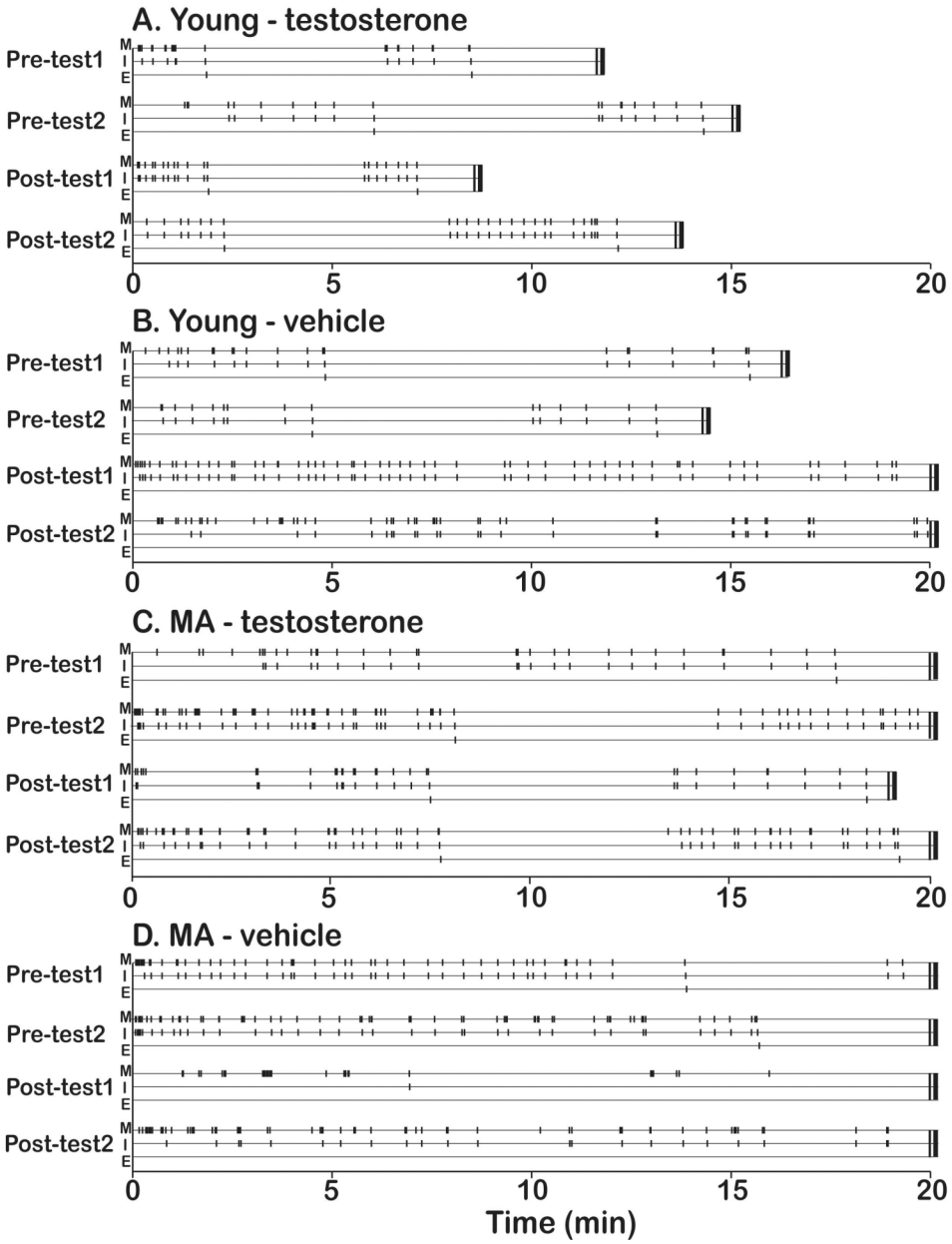


Figure 4.2. Copulatory behaviors of representative rats from each group are shown. Pre-test 1 and Pre-test 2 took place 3 days and 1 day before castration and implantation. Post-test 1 and Post-test 2 were 14 and 16 days after castration and implantation. In each test, three lines show the timing of mounts (M), intromissions (I) and ejaculations (E). Events are indicated by tick marks according to the time of each event in the 20-minute test. Tests were terminated after two ejaculations or 20 minutes.

Figure 4.3. The Percentage of Ejaculations

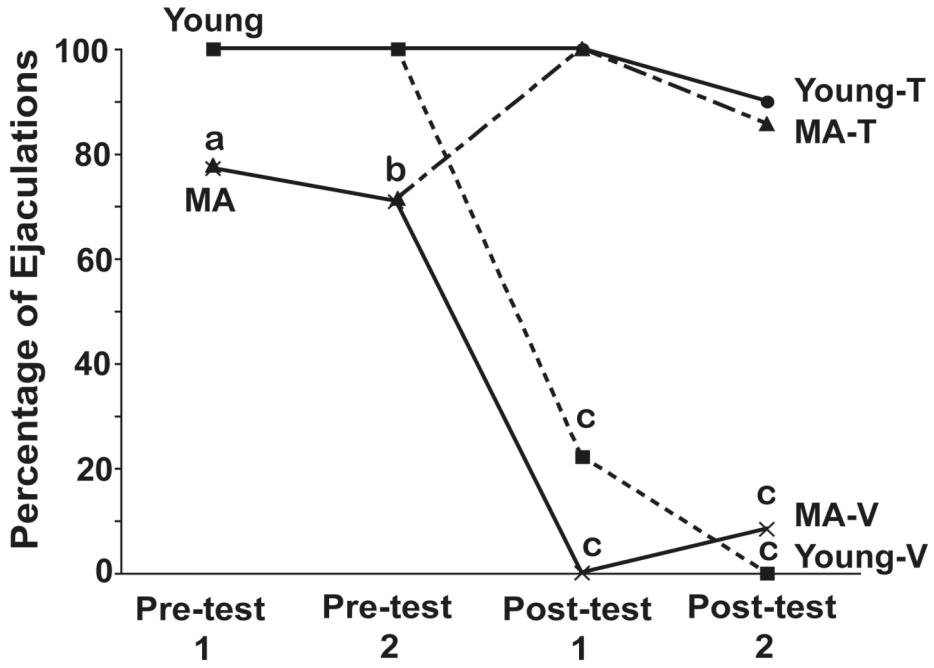


Figure 4.3. The percentage of ejaculations, calculated as measured by the percentage of males ejaculating, is shown. Data for the pre-tests are shown combined for each age. For the post-test, data for the four groups are shown separately. In Pre-test 1 and Pre-test 2, the percentage of ejaculations in young males was significantly higher than in middle-aged males (a: $P < 0.05$; b: $P < 0.01$). In Post-test 1 and Post-test 2, "c" indicates $P < 0.001$ compared to the corresponding young-T group. Sample sizes were: Young-T (n=9), Young-V (n=9), MA-T (n=14), and MA-V (n=13). Abbreviations: MA, middle-aged; T, testosterone.

Figure 4.4. Male Copulatory Behaviors

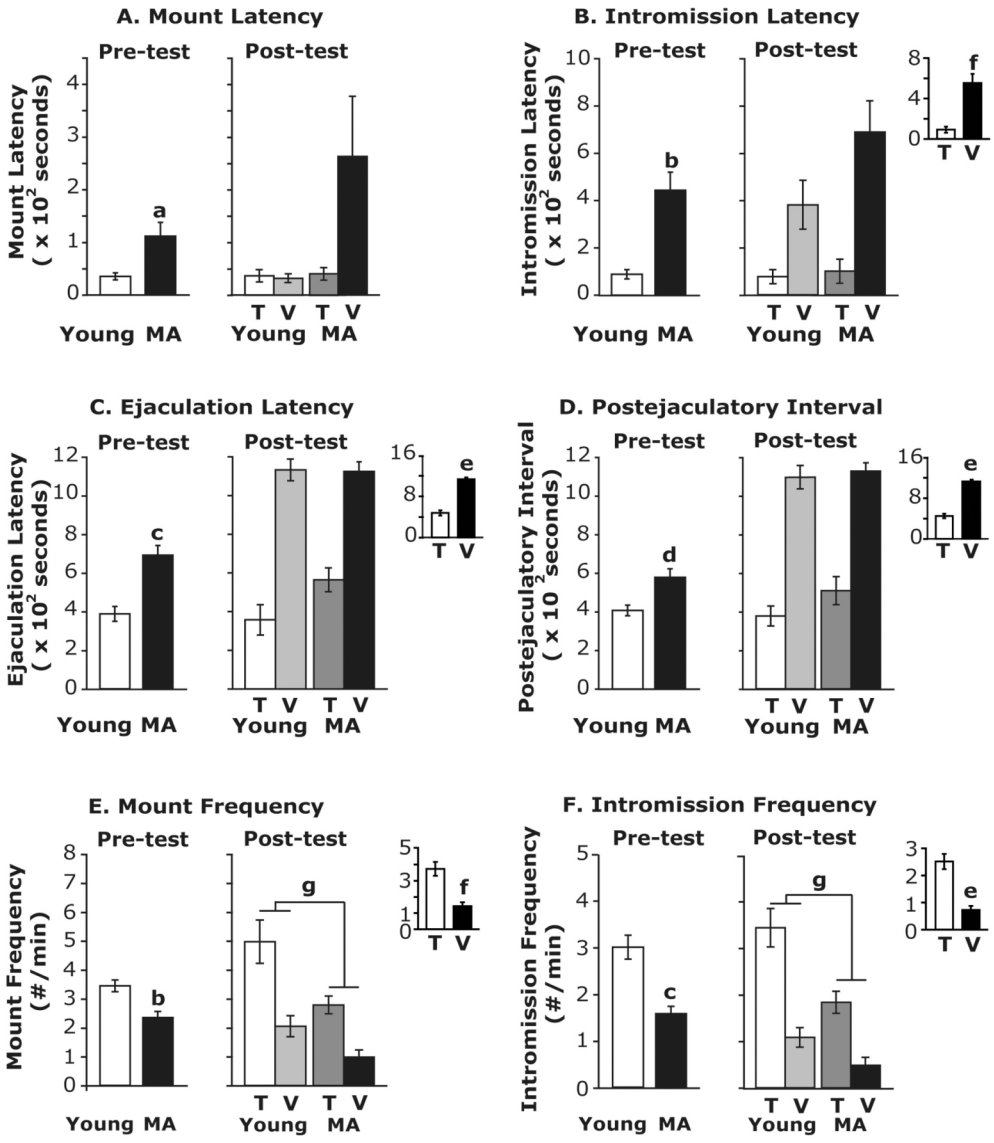


Figure 4.4. Male copulatory behaviors are shown for mount latency (A), intromission latency (B), ejaculation latency (C), post ejaculatory interval (D), mount frequency (E) and intromission frequency (F). Data are shown combined for the intact rats at the two ages in the two pre-tests. Data for the combined post-tests are shown for each age in castrated (T or V) rats. The bars represent the mean ± SEM. a, $P < 0.05$ vs. young males in the pre-test; b, $P < 0.01$ vs. young males in the pre-test; c, $P < 0.001$ compared young males in the pre-test; d, $P < 0.0001$ vs. testosterone group; e, $P < 0.01$; f, $P < 0.001$. Abbreviations: MA, middle-aged; T, testosterone; V, vehicle.

None of the sexual behavior measures differed before and after testosterone treatment in the young male group. Testosterone treatment significantly decreased intromission latency in MA males relative to levels in the pre-test ($P < 0.05$).

Other parameters of male sexual behavior (ejaculation frequency, inter-intromission interval and intromission ratio) were measured. In pre-tests, ejaculation frequencies and intromission ratio were significantly higher in young group than the MA ($P < 0.01$, $P < 0.001$ respectively). Inter-intromission interval was significantly shorter in the young group than the MA ($P < 0.01$). After castration, ejaculation frequencies, inter-intromission interval and intromission ratio were highly affected by treatment ($P < 0.0001$, $P < 0.05$, $P < 0.001$) but not age with higher ejaculation frequency, intromission ratio and lower inter-intromission interval in the testosterone than the vehicle groups (data not shown).

AR Immunoreactivity in AVPV and MPN

Representative photomicrographs of AR-immunoreactivity in the AVPV and MPN are provided in Figure 4.5. Quantitative stereological analyses of AR-ir cell densities were performed, and results shown in Figure 4.6. There was no effect of age on AR cell density. A significant main effect of treatment in both AVPV and MPN was found ($P < 0.0001$, $P < 0.0001$), with significantly greater density of AR cells in the testosterone than the vehicle treated groups of both ages (Figure 4.6, insets).

ERα Immunoreactivity in AVPV and MPN

Representative photomicrographs of ERα-immunoreactivity in the AVPV and MPN are shown in Figure 4.7. Stereologic analyses of ERα -ir cell densities were performed (Figure 4.6). There was a significant main effect of treatment on ERα cell density in AVPV ($P < 0.01$) and MPN ($P < 0.0001$), with ERα cell density being significantly lower in the testosterone than the vehicle groups of both ages. There was no effect of age on ERα cell density.

Serum Hormone Concentrations

In intact rats (Figure 4.8A), serum total testosterone levels tended to be higher in the middle-aged than the young group but this was not significant. Seventeen days after castration and hormone (T or V) treatment, there was no effect of age but a significant effect of treatment ($P < 0.001$). Vehicle animals had significantly lower testosterone levels than the testosterone group. In addition, the testosterone levels in the T-replaced group were in a similar range to pre-castration levels (c.f. Figures 4.8A and 4.8B).

Free testosterone concentrations in intact rats did not differ between the middle-aged and the young group (Figure 4.8C). Following castration and hormone (T or V) treatment, there were significant main effects of both age and treatment (Figure 4.8D, $P < 0.05$, $P < 0.01$). For age, serum free testosterone was significantly higher in the young than the middle-aged groups. For treatment,

Figure 4.5. Photomicrographs of AR Immunoreactivity in the AVPV and MPN

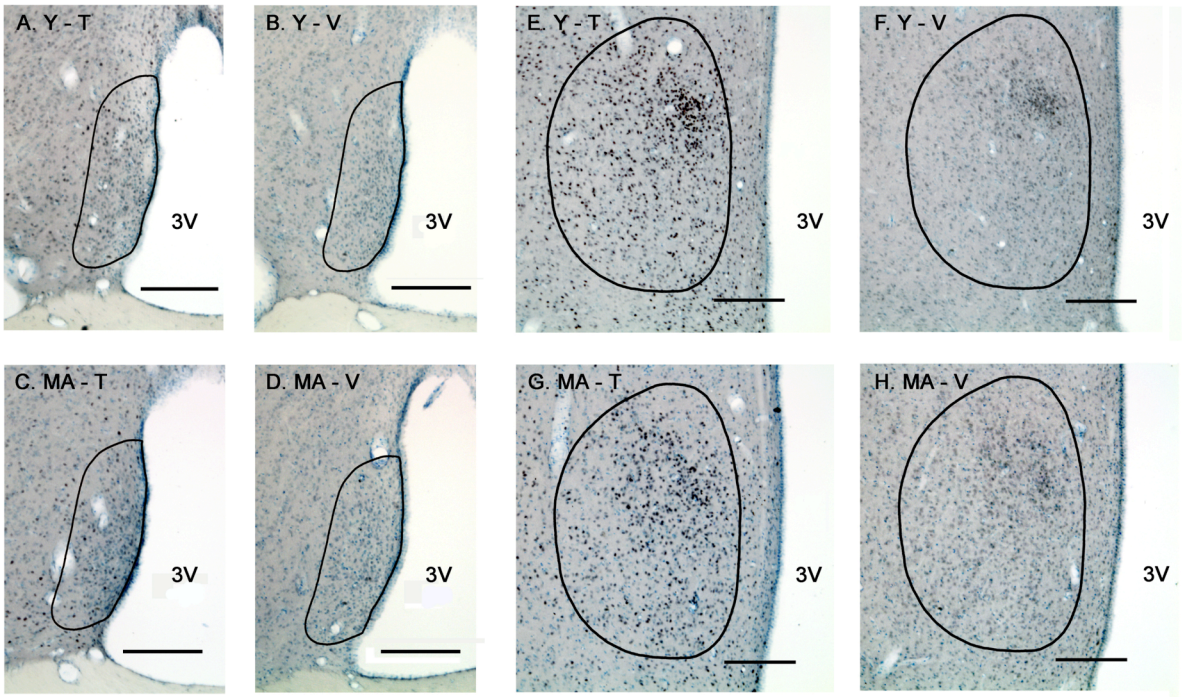


Figure 4.5. Photomicrographs of AR immunoreactivity in the AVPV (left) and MPN (right) of young and middle-aged rats are shown. Rats were perfused at the completion of the two post-tests, 17 days after castration and hormone (testosterone or vehicle) treatment. Representative sections are shown for the AVPV and MPN, respectively, of young-T (A, E), young-V (B, F), middle-aged-T (C, G) and middle-aged-V (D, H) male rats. The contours of the AVPV or MPN are drawn based on Nissl staining and according to Swanson's rat brain atlas (1998). Scale bar = 200 μm. Abbreviations: MA, middle-aged; T, testosterone; V, vehicle; 3V, third ventricle.

Figure 4.6. Stereologic Analysis Results of AR or ERα immunoreactive Cell Density

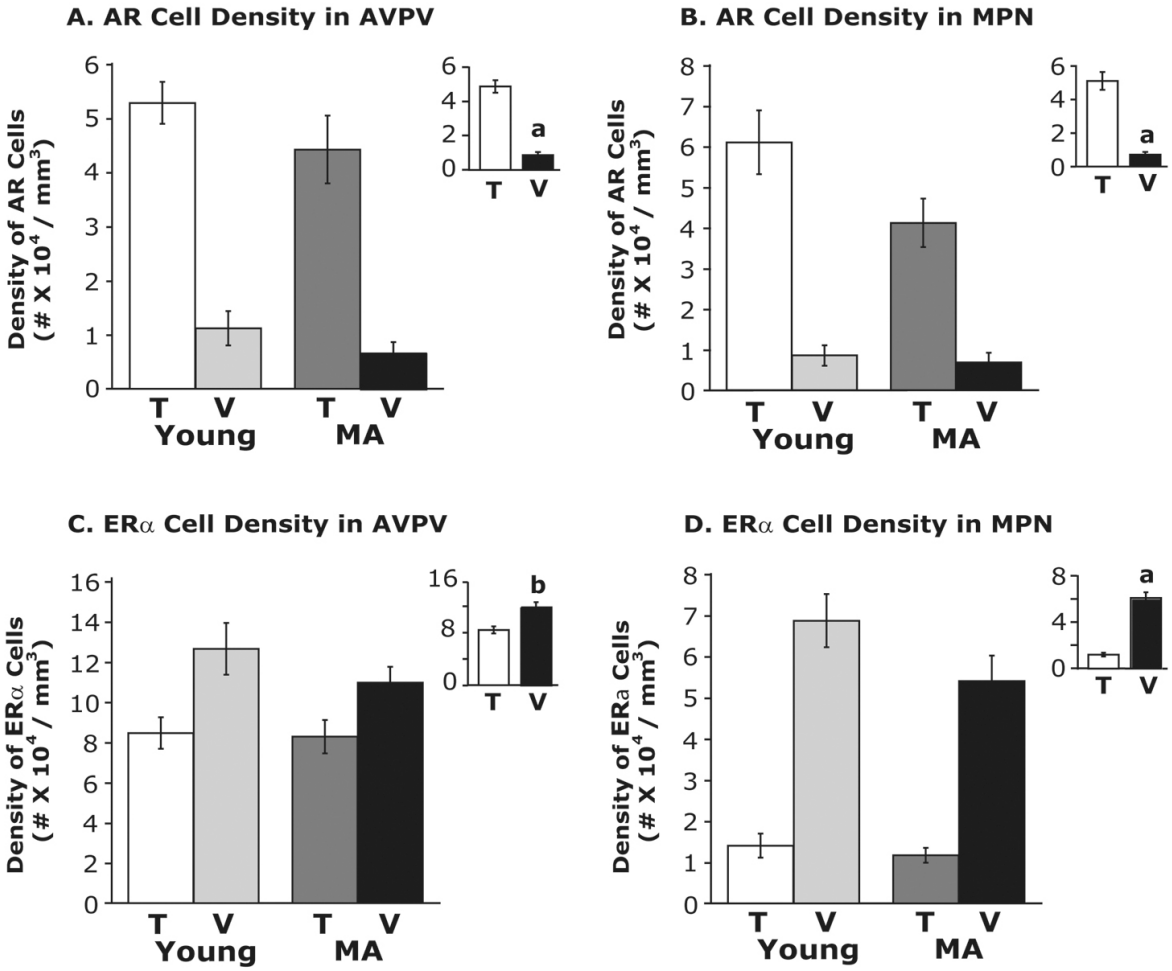


Figure 4.6. Stereologic analysis results of AR or ERα immunoreactive cell density (number of immunoreactive cells/regional volume) are shown for AR-ir cell density in the AVPV (panel A, n=8 rats per group); ARir cell density in the MPN (panel B, n=8 rats per group); ERαir cell density in the AVPV (panel C, n=6 rats per group); ERαir cell density in the MPN (panel D, n=6 rats per group). Insets show the significant main effect of testosterone. The bars represent the mean ± SEM. Significant effects of testosterone are shown in the insets (a, $P < 0.0001$; b, $P < 0.01$ for the vehicle compared to the testosterone group). Abbreviations: AVPV, anteroventral periventricular nucleus; MPN, medial preoptic nucleus; T, testosterone; V, vehicle; MA, middle-aged.

Figure 4.7. Photomicrographs of ERα Immunoreactivity in the AVPV and MPN

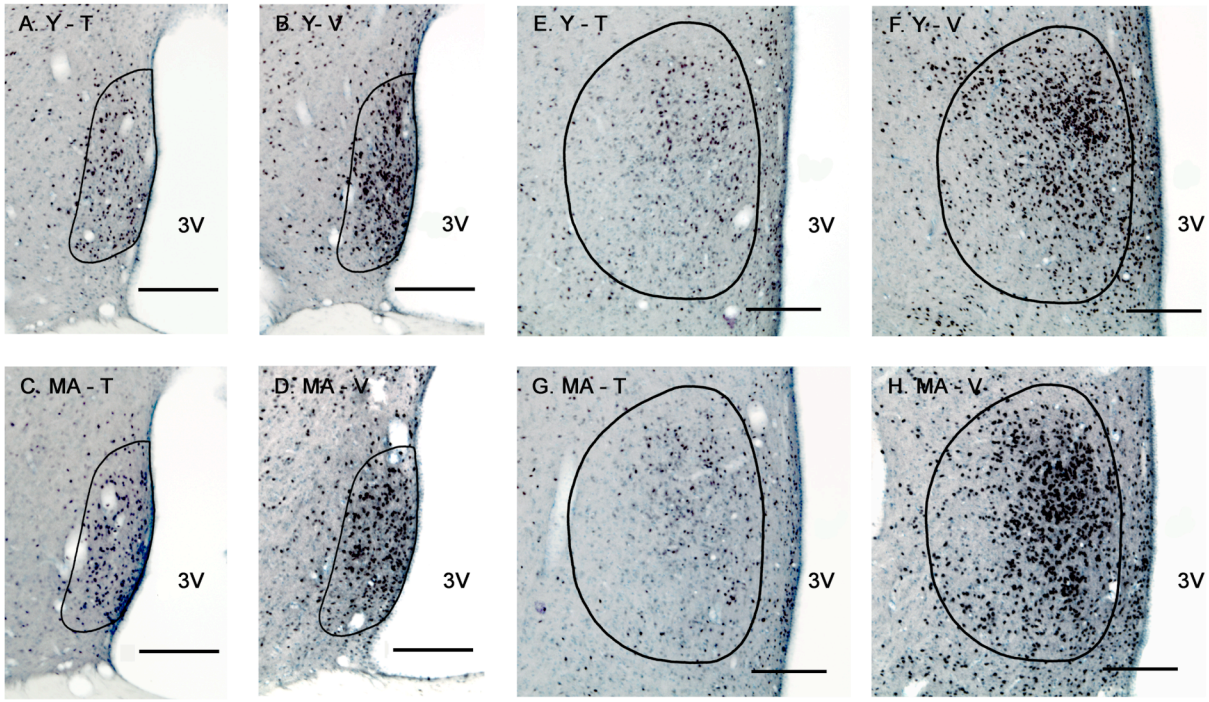


Figure 4.7. Photomicrographs of ERα immunoreactivity in the AVPV (left) and MPN (right) of the same young and middle-aged rats described in Figure 4.5 are shown. Representative sections from the AVPV or MPN, respectively, of young-T (A, E), young-V (B, F), middle-aged-T (C, G) and middle-aged-V (D, H) male rats are presented. The contours of the AVPV or MPN are drawn based on Nissl staining and according to Swanson's rat brain atlas (1998). Scale bar = 200 μm. Abbreviations: MA, middle-aged; T, testosterone; V, vehicle; 3V, third ventricle.

Figure 4.8. Serum Total and Free Testosterone were Assayed in Young and Middle-aged Male Rats before and after Castration and Hormone Treatment

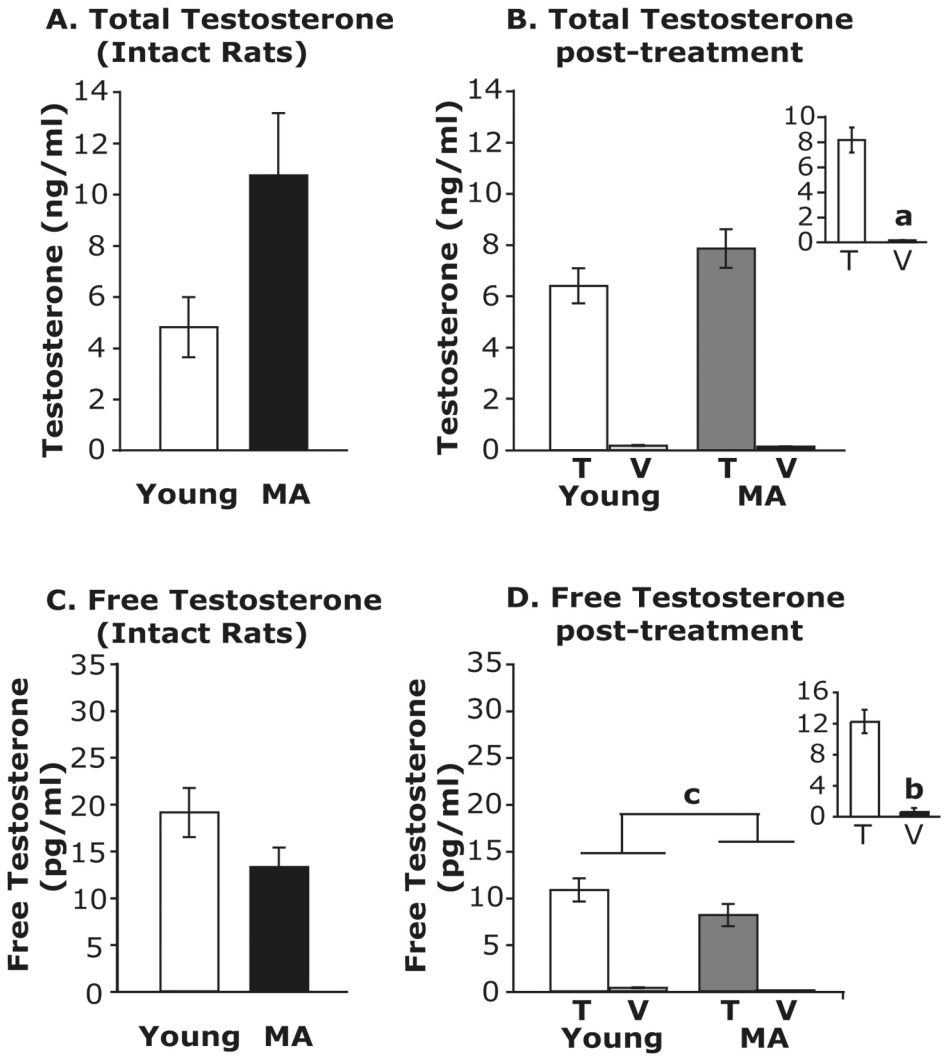


Figure 4.8. Serum total and free testosterone were assayed in young and middle-aged male rats before and after castration and hormone treatment. Hormones from intact young and middle-aged rats are shown in panels A and C. Hormones in the four groups 17 days after castration and treatment are shown in panels B and D. Insets in panel B and D show the significant main effect of testosterone treatment (a, $P < 0.001$; b, $P < 0.01$). In panel D, a significant main effect of age on serum free testosterone is shown (c, $P < 0.05$). The bars represent the mean ± SEM. Abbreviations: T, testosterone; V, vehicle; MA, middle-aged.

the free testosterone levels were higher in the testosterone than the vehicle groups.

Serum estradiol levels were measured in intact rats, with levels similar between the young and middle-aged groups (Young: 8.96 ± 1.62; MA: 6.77 ± 1.33). Due to a technical error, serum estradiol levels could not be measured reliably in the post-castration groups.

DISCUSSION

In these studies, we examined the effects of testosterone on masculine sexual behavior and on androgen receptor and estrogen receptor α cells in the preoptic area of the brain in young and middle-aged male rats. Aging was associated with a significant decline in sexual behavior in intact males, in agreement with reports from previous studies (Chambers and Phoenix, 1983, Smith et al., 1992). Testosterone replacement to rats at the time of castration, with testing 2 weeks later, resulted in the exhibition of most masculine sexual behaviors at a similar level to those in the young pre-test (intact) rats. Animals receiving the vehicle at the time of castration had a loss of these behaviors, with the exception of mount latency in the young group. There were also some age differences in the post-castration behavioral tests, with mount and intromission latency both lower in the middle-aged than the young group. Finally, AR- and ERα-immunoreactive cell densities were quantified in the hypothalamus of rats

after testing and treatments. Gonadectomy of adult male rats caused a decrease in AR immunoreactive density and an increase in ERα immunoreactive density in both the MPN and AVPV that was similar in the two age groups.

Masculine Sexual Behavior, Hormones, and Aging

In the current study, we observed an age-associated decline in sexual behavior, but little difference in testosterone concentrations. Although it is well-accepted that testosterone is important for masculine sexual behavior, the exact relationship between testosterone concentrations and sexual behavior is still controversial, and our results and others' suggest a dissociation among at least some of these processes. It was reported in young rats by Damassa et al. (Damassa et al., 1977) that less than one-third the normal levels of testosterone needed to be administered to castrated male rats to maintain normal sexuality. Smith et al. (Smith et al., 1992) found that intact male rats that could complete copulatory behavior showed no correlation between the behavioral measures and testosterone levels. Further, the behavioral parameters did not vary significantly with different serum testosterone concentrations within the physiological range achieved by different testosterone implantation in castrated rats.

The ability of testosterone to regulate sexual behavior differs further with aging. Although declines in plasma testosterone concentrations have typically been associated with a loss of sexual performance in aging males [human:

(Harman et al., 2001); rats: (Roselli et al., 1986, Gruenewald et al., 2000)], it was reported that when testosterone levels in old male rats were brought back to levels similar to those in young rats, the decline of sexual arousal (Gray et al., 1981) and copulatory activity (Hsu et al., 1986, Chambers et al., 1991) could not be restored or maintained. Indeed, in the current study, the middle-aged rats had similar pre-castration levels of total and free testosterone as the young rats, yet the middle-aged groups had significant declines in some sexual behavior measures. Together, these findings indicate that low levels of serum testosterone in aging males are not exclusively responsible for the diminution of sexual behaviors.

The one behavior that was not regulated in a predictable way by testosterone was mount latency. Castrated young male rats had similar mount latencies whether replaced with vehicle or testosterone. Middle-aged rats had longer mount latencies in the pre-test, and after castration, this behavior was facilitated by testosterone but not vehicle. These results suggest that mount latency, a behavior that has previously been shown to be modulated by testosterone (James and Nyby, 2002), is testosterone-independent only at the young age, and indicates a potential age-related change in the neural circuitry underlying this motivational behavior. We hypothesize that the prior sexual experience of the rats may influence the maintenance of male sexual responsiveness: although both young and middle-aged males were experienced, the timing of our experiment relative to the last experience was shorter in the

young than the middle-aged group. This hypothesis can be supported by the fact that naïve castrated rats did not show any mounting and intromission behavior in another study (Witt et al., 1995). A role for sexual experience was previously reported in young rats: for experienced animals, those males that were sexually active had significantly higher AR density than sexually exhausted male rats (Fernandez-Guasti et al., 2003). This suggests that sexual status also plays a role in AR expression in the hypothalamus.

Steroid Hormones in Intact Aging Male Rats

It is interesting to note that in the present study testosterone and free testosterone concentrations prior to testing did not differ significantly between the young and middle-aged intact males. We also found no difference in serum estradiol concentrations in these animals. The results are somewhat different from previous publications, including our own, showing an age-related decline in serum testosterone, and an increase in serum estradiol, from young to middle-aged rats (Roselli et al., 1986, Chambers et al., 1991, Chen et al., 1994, Taylor et al., 1996, Bernardi et al., 1998, Gruenewald et al., 2000, Luine et al., 2007, Wu et al., 2009). This discrepancy may be due to the behavioral status and/or sexual experience of the experimental animals. Although all of our rats were experienced, middle-aged rats were retired breeders, whereas young rats were given extensive experience and then used in our tests shortly thereafter. Furthermore, in the current study sexually inactive subjects were excluded if they

did not mate in at least one of the two pre-tests. In aging male rats, animals that have mated have been observed to have higher testosterone levels than non-mated counterparts (Frankel, 1984). This suggests the potential role of sexual experience in the control of sex steroid hormone levels.

Steroid Hormones and Behavior in Castrated Male Rats

In the present experiments, castration and testosterone implantation resulted in concentrations of total and free testosterone that were similar to intact levels. Nevertheless, exogenous testosterone maintained or even facilitated aspects of sexual behavior. This may be interpreted to mean that exogenous testosterone may be more behaviorally effective than the same mean level of endogenous testosterone in intact males, especially in shortening mount and intromission latencies. This phenomenon was first observed and discussed by Damassa et al. (Damassa et al., 1976) who demonstrated that a given circulating testosterone level that resulted from implanted capsules could be about twice as effective on accessory sex glands and plasma LH as the same mean level originating from the testes. There are three interpretations for these differences between serum endogenous and exogenous testosterone: 1) circulating testosterone concentrations may not be completely representative of the testosterone concentration in regions of the brain, including preoptic area, that control sexual behavior; 2) Circulating serum testosterone in intact males includes peaks (Bartke et al., 1973) and troughs whereas testosterone capsules

probably provide more continuous release. The consequences of pulsatile vs. continuous testosterone may include a difference in neural actions of testosterone; 3) Our rats were given testosterone implantation at the time of castration, thereby maintaining hormone levels. Other studies have evaluated effects of testosterone treatment following a longer post-castration delay. Differences in these experimental paradigms may differentially increase or decrease neural steroid hormone receptors, thereby producing behavioral differences. This hypothesis is consistent with the observation that the anti-androgen, Sch 16423, was able to block the restoration of masculine sexual behavior but had only a comparatively weak effect on the maintenance of copulatory behavior (McGinnis and Mirth, 1986). Moreover, considerably higher amounts of testosterone are required for restoration of sexual behavior in castrates, as opposed to its maintenance when treatment is initiated immediately (Damassa et al., 1977).

AR Immunoreactivity in Aging Males

The expression of AR mRNA and protein in the hypothalamus varies with age and hormone status (Wood and Newman, 1993, Iqbal et al., 1995, Lu et al., 1998, Lu et al., 1999, Walker et al., 2009, Wu et al., 2009). Castration diminishes AR expression while testosterone treatment can restore their expression (Krey and McGinnis, 1990, Wood and Newman, 1993). We previously reported that AR immunoreactive cell density increased with age in the preoptic area of intact male

rats (Wu et al., 2009), whereas serum testosterone levels decreased during this period. That study suggested an inverse relationship between age and AR immunoreactivity. The current experiment differed from the previous in several ways. First, we did not find any differences in total testosterone between the young and middle-aged rats, either while intact or after castration plus hormone treatment. Second, the animals from the two studies differed in sexual experience, and in addition, they were pre-screened for sexual activity only in the present study. Therefore, our current result showing no significant age difference in AR immunoreactive cell density in the preoptic area of male rats is consistent with the relative lack of a behavioral difference in these animals in the post-tests.

In the present study, we implanted testosterone capsules at the castration. The importance of the timing of hormone treatment has already been discussed above for effects on behavior, but timing may also have consequences on expression of ARs. Whether testosterone treatment at the time of castration can maintain AR expression to the level before castration in MA is still unknown since immunohistochemistry in the current study was performed only on castrated animals. Comparisons with our earlier work suggests that levels of AR in the AVPV and MPN are in a similar range to those in young and middle-aged intact rats (Wu et al., 2009) but a side-by-side comparison of intact and castrated rats is merited in the context of aging.

ERα Immunoreactivity in Aging Males

Some effects of testosterone on sexual behavior may be mediated through its aromatization to estradiol and subsequent actions on estrogen receptors. Further, estradiol facilitates masculine sexual behavior (Baum and Vreeburg, 1973, Larsson et al., 1976). There is some evidence that hormones and aging affect expression of the ERα in male rats. A castration-induced increase of ERα mRNA has been reported in the MPN (Handa et al., 1996). Clancy et al. (Clancy et al., 2000) reported that counts of ER-immunoreactive cells in the medial preoptic area were significantly lower in testosterone-compared to control-treated male rat, and this was region specific, as a difference was not detected in the bed nucleus of the stria terminalis or the lateral septum. Roselli reported that in the whole preoptic area, both young and old rats expressed a significantly lower level of ERα (Roselli and Resko, 1993) after castration. However, the current study suggests no difference with age, but a robust effect of testosterone treatment, on ERα cell density. In the MPN and AVPV, ERα cell density was substantially higher in the vehicle compared to the testosterone treated rats of both ages. Although we were unable to reliably measure estradiol levels in our animals at the end of the study, we speculate that testosterone regulates ERα levels either indirectly through AR-expressing cells, or directly through its aromatization to estradiol. It has been reported that some cells may co-express AR and ERα (Greco et al., 1998) so these actions are not necessarily mutually exclusive.

SUMMARY AND CONCLUSIONS

Data from the present study add to previous observations showing that in castrated adult male rats, compared to the vehicle, testosterone maintains masculine sexual behavior; it increases AR expression in the AVPV and MPN; and it decreases ERα immunoreactivity in the MPN and the AVPV. Although effects of aging were small, several significant differences were observed, particularly on mount frequency, intromission frequency; and on free testosterone. These studies suggest that hormones and aging interact in a complex manner to control expression of molecules in the brain and on the subsequent control of sexual behavior.

CHAPTER 5. GENERAL DISCUSSION

This research has focused on understanding the role of hormone receptors, AR and ERα, their regulation by aging and sexual experience, and the effects of testosterone on their expression in male rats.

In the first experiment, I performed a stereological analysis of AR and ERα in two preoptic areas (AVPV and MPN), in young, middle-aged, and old male rats. Data from my first experiment, which support previous observations showing that the AR and ERα are expressed abundantly in AVPV and MPN, extend those findings to the aging male brain. ERα immunoreactivity increased modestly with aging, an effect limited largely to the most heavily immunoreactive cells while total ERα immunoreactive cell numbers and density did not change with aging. Numbers and density of AR immunoreactive cells in the two preoptic regions increased robustly with aging, a period during which circulating testosterone declines precipitously. Indeed, a significant negative correlation between serum testosterone concentrations and AR cell numbers was detected in the AVPV and MPN, regardless of chronological age. As a whole, these results were unexpected and did not support my hypothesis that age-related decline in testosterone is associated with the decrease of AR expression in the POA of males. The data suggest that the regulation of AR by testosterone may be different in aged than young males.

In the second experiment, I compared the effects of age and experience on the expression of steroid hormone receptors, AR and ERα, in the AVPV and MPN of young and middle-aged male rats. In this experiment, the sexual experience was carefully matched and controlled, with animals being either completely naïve or given extensive sexual experience at 3-4 months of age. I discovered that sexual experience increases testosterone and free testosterone in both young and middle-aged males. Sexual experience also interacts with aging to alter testosterone, estradiol and LH. However, I did not find an experience-related difference in expression of hormone receptors. This investigation is important because it shows that sexual experience can induce short-term and long-term alterations in hormones. It also suggests that males with matched sexual experience in young adulthood may differ from the time of the last sexual experience but have a similar phenotype of expression of steroid hormone receptors in some preoptic brain regions.

In the third experiment, I examined the effects of testosterone on masculine sexual behavior and on AR and ERα expression in the preoptic area (AVPV and MPN) of the brain in young and middle-aged male rats. Data showed that in castrated adult male rats with testosterone treatment, there was no significant age-related difference in masculine sexual behavior measures between young and middle-aged males except for mount frequency and intromission frequency. Testosterone treatment increased AR immunoreactive cells in the AVPV and MPN; and it decreased ERα immunoreactivity in the MPN

and only slightly in the AVPV. Although effects of aging were small, several significant differences were observed including free testosterone and ERα immunoreactive cell density in the MPN. These studies suggest that hormones and aging interact in a complex manner to control numbers of cells expressing key molecules in the brain and on the subsequent control of sexual behavior.

SYNERGISTIC EFFECT OF SEX HORMONES ON MASCULINE SEXUAL BEHAVIOR

The synergistic effect of testosterone and estradiol and the relative importance of androgen and estrogen receptors in activation of the motivational and performance aspects of copulatory behavior are well known. However, the underlying mechanism remains unclear.

Physiological estradiol may induce only part of masculine sexual behavior, such as mounting (Pfaff, 1970, Larsson et al., 1976), which is considered to be a measure of sexual motivation as opposed to consummation. Furthermore, in male rats, estrogen appears to facilitate activity in neural tissues controlling not only mounting but also lordosis (Baum and Vreeburg, 1973, Larsson et al., 1976), which is a feminine sexual behavior. In both male rats and females, the ventromedial hypothalamic nucleus region (VMH) is the primary site for the hormonal activation of lordosis behavior. Estradiol benzoate implants located in the region of the VMH were significantly more effective in stimulating lordosis

responses in males than in rats with implants in the preoptic area, or those receiving blank implants (Davis and Barfield, 1979a). After receiving estradiol, NADPH-d activity, which shows the nitric oxide synthase activity (Hope et al., 1991), is higher in the VMH of estradiol-treated male rats than in the control animal (Okamura et al., 1994). Although the VMH has high androgen receptor and estrogen receptor expression (Yokosuka et al., 1997, Holmes et al., 2008), its role in regulating masculine sexual behavior has largely been overlooked. McGinnis and her colleagues found that implants of hydroxyflutamide, the androgen receptor blocker, into the VMH prevented the restoration of male sexual behavior by testosterone. This inhibition was even more effective than comparable implants of hydroxyflutamide in the MPN (McGinnis et al., 1996). An additional study done by the same lab showed that animals receiving hydroxyflutamide in the VMN had reduced preference for receptive females whereas hydroxyflutamide in MPN had no effect on the restoration of partner preference (Harding and McGinnis, 2004). However, in mice, testosterone intracranial implant at VMH failed to stimulate any sexual behavior (vocalization, mounting) whereas stimulatory effects of testosterone implants in the MPN were observed (Nyby et al., 1992). This suggests that the regulation of testosterone through AR in VMH is necessary, but not sufficient, to induce masculine sexual behavior.

Androgens can have paradoxical effects on different areas in the brain. For example, testosterone has different regional regulation of binding of oxytocin,

whose secretion is stimulated by sexual activity in both males and females [for review see (Neumann, 2008)]. In mice, castration could approximately double the binding of the oxytocin receptor in the VMH in castrated males as compared to either intact or the testosterone-treated castrates, whereas in the lateral septum, binding to oxytocin receptors was higher with testosterone treatment (Insel et al., 1993). A second example is that testosterone has different regional effects on expression of kiss1 mRNA which encodes kisspeptins to stimulate GnRH release from hypothalamus [for review see (Dungan et al., 2006)]. In male mice, kiss1 mRNA expression was reduced by castration and then increased by testosterone treatment in AVPV whereas in arcuate this expression increased with castration then decreased with exogenous testosterone (Smith et al., 2005).

Combined with the fact that non-aromatized androgen can stimulate neuron activity in POA to induce masculine sexual behavior (Butera and Czaja, 1989) and non-aromatized androgen inhibits lordosis (Baum and Vreeburg, 1973), my assumption is that non-aromatized androgen may exert the inhibitory effect in VMH on estrogen-activated lordosis behavior of males. In other words, non-aromatized androgen may inhibit lordosis in VMH but stimulate mounting in POA.

My hypothesis about the synergistic effect of testosterone and estradiol on masculine sexual behavior is as follows: in males, estrogen exerts its effect on POA and VMH to induce BOTH masculine and feminine behaviors associated with sexual motivation (mounting and lordosis, respectively). Non-aromatized

androgen works on VMH and POA with differential effects: it activates neurons in POA to induce masculine motivation and then copulatory behavior, but inhibits neuron activity in VMH, thus inhibiting feminine sexual behavior.

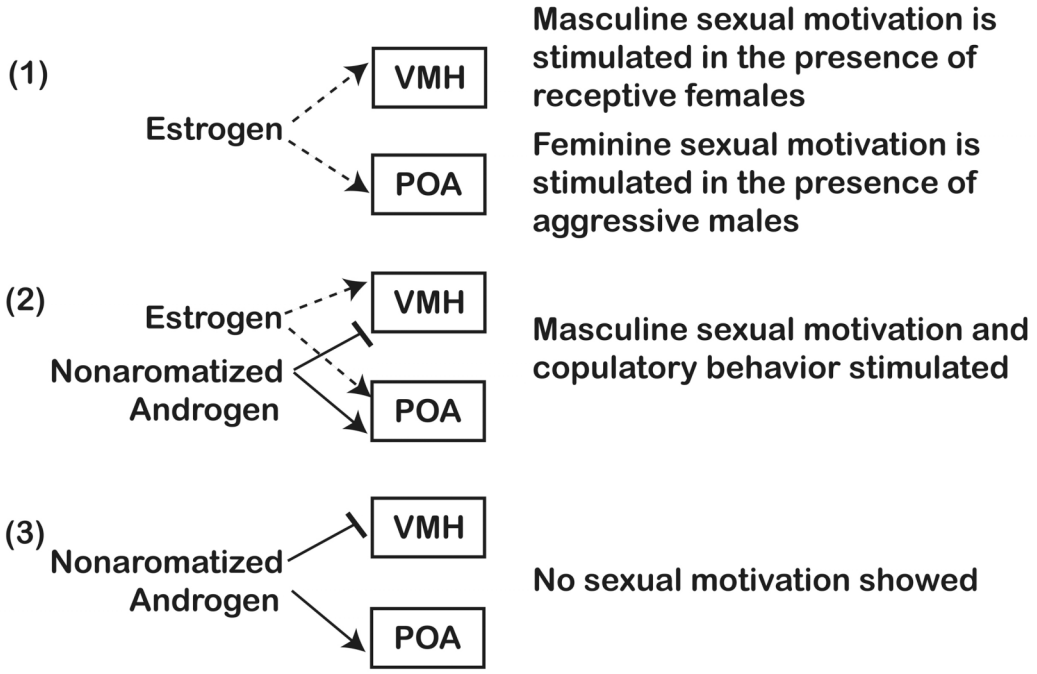
With this hypothesis, I predict that (Figure 5.1):

(1) Estradiol-treated castrated males may show male or female sexual motivation that is dependent on his partner. In the presence of a receptive female, approaching and mounting will be induced in estradiol-treated castrated males whereas lordosis can be stimulated in the presence of an aggressive male. Thus a physiological level of estrogen treatment should not improve a castrated male rat's partner preference, which has already been reported (Vagell and McGinnis, 1998);

(2) For non-aromatized androgen and estradiol treatment, non-aromatized androgen strengthens masculine motivation and further stimulates copulatory behavior by inhibiting female behavior in the VMH. In the POA, its action in males is only on masculine behavior;

(3) For those males that are treated only with non-aromatized androgen, the motivational part of sexual behavior cannot be induced so that no sexual performance is observed.

Figure 5.1. Synergistic and Antagonistic Effect of Sex Hormones on Sexual Behavior



INTERACTIONS OF ANDROGEN AND AGING ON ARS, AND CONSEQUENCES ON SEXUAL BEHAVIOR

Testosterone appears to regulate masculine sexual behavior by acting through intracellular nuclear receptors and causing the induction of protein synthesis. Although I discussed earlier that sexual motivation might be stimulated by estradiol, local aromatase activity is regulated by an androgen receptor-dependent mechanism (Roselli and Resko, 1984). Thus, AR is related also to sexual motivation. Many studies suggest the key role of androgen receptors in regulating masculine sexual behavior:

(1) Intracranial implantation of hydroxyflutamide, the AR blocker, at MPN or VMH can significantly prevent the restoration of masculine sexual behavior induced by testosterone treatment (McGinnis et al., 1996).

(2) A study showed that sexual behavior maintenance and restoration all depended on the post-transcriptional function of androgen receptors because the application of anisomycin, a protein synthesis inhibitor, in MPN could block expression of nearly all androgen-dependent behaviors except partner preference and vocalization (McGinnis and Kahn, 1997).

(3) In young castrated rats, copulatory behavior was restored within 7 to 10 days after testosterone exposure [Fischer 344 rat, (Chambers et al., 1991); Long-Evans Rat, (McGinnis et al., 1989)]. This time course is consistent with the time needed to produce new protein.

(4) AR density is significantly reduced in the MPN medial and central parts 48 hours after sexual satiety, coinciding with a drastic inhibition in ejaculation (Romano-Torres et al., 2007).

The results of the first and second experiments of my dissertation showed that aging animals synthesized more AR in AVPV and MPN than the young group and that there is a negative correlation between serum testosterone and AR densities in AVPV and MPN. Based on these results, I predicted that in middle-aged rats exogenous testosterone treatment at the time of castration would lower expression of AR similar to that observed in young rats. However, by comparing AR density in intact rats (first and second experiments) and in testosterone-treated rats (third experiment), I found that castration resulted in very low and comparable levels of AR in young and middle-aged rats, and that exogenous testosterone, in fact, increased expression of AR similarly in the two age groups. Therefore, acute testosterone treatment may only increase AR expression and the underlying mechanism may be different with the one involving in increasing AR density with gradually decreased testosterone due to aging.

However, it has been shown that acute testosterone treatment in old male rats cannot restore sexual arousal (Gray et al., 1981) and copulatory activity (Hsu et al., 1986, Chambers et al., 1991). This may be due to the inability of testosterone to restore androgen receptors in the preoptic area and hypothalamus although this assumption has not been investigated. Since

different androgen target organs have varying threshold values of plasma testosterone to enable them to exhibit a response (Fielder et al., 1989), there may be a threshold for restoring androgen receptors that differs with aging. The other possibility is that the abolishment of androgen receptors after castration in middle-aged males may make it difficult for them to respond to testosterone, thus inhibiting the increase of AR.

Before starting the third experiment, I hypothesized that testosterone treatment at the time of castration in middle-aged rats with higher AR immunoreactive cell number would bring sexual performance in middle-aged males up to a level similar to that of the young group with testosterone treatment. In this way, I maintained testosterone levels at the time of castration. During the pre-castration tests of intact rats, the middle-aged group had a significantly longer latency for mount, intromission, ejaculation, a longer postejaculatory interval, as well as lower mount and intromission frequencies. During the post-castration tests, a significant main effect of age was not found except for mount frequency and intromission frequency with the young group having higher frequencies than the middle-aged group. The middle-aged males also had significantly lower free testosterone than the young group although no age-related difference of testosterone level was detected. These results supported my hypothesis and, in addition, indicated the importance of the interaction between androgen and aging in regulating androgen receptors and masculine sexual behavior.

ESTROGEN RECEPTOR AND SEXUAL MOTIVATION

By comparing ERα density in intact rats (first and second experiment) and testosterone-treated castrated rats (third experiment), I found that exogenous testosterone regulated ERα expression in an area-specific manner. In AVPV, testosterone maintained ERα expression in the young group while castration tended to increase ERα density. In MPN, testosterone treatment decreased ERα density in both young and middle-aged groups and castration tended to increase ERα only in the young group. The latter observation is consistent with the report that castration increased ERα mRNA in MPN in young naïve male rats (Handa et al., 1996).

However, I did not find any significant age- or experience-related differences in expression of ERα in the two preoptic areas even though aging and experience have been observed to be related to an alteration of sexual behavior (see Introduction). It appears that ERα density in preoptic area is not closely related to sexual performance. In support of this, blockade of ERα with RU58668, an antiestrogen, did not inhibit the restoration of copulatory behavior or partner preference in testosterone-treated castrated male rats (Vagell and McGinnis, 1998). Moreover, experienced ERα knockout mice preferred female urine at the same level as wild-type males (Ogawa et al., 1996). ERα knockout

mice showed disrupted, but not eradicated, male sexual behavior (Eddy et al., 1996, Ogawa et al., 1997, Wersinger et al., 1997).

In male rats, Cross and Roselli demonstrated that a single injection of estradiol can increase chemoinvestigation (genital sniffs) and mounting frequency and reduce the mount latency within 15-35 minutes of treatment in sexually experienced castrated rats (Cross and Roselli, 1999). This rapid time course of estradiol's behavioral effects is not consistent with the need for new protein synthesis, which requires hours to days. These studies indicate that estradiol may stimulate masculine sexual motivation through a nongenomic pathway by binding to membrane estrogen receptors that may be associated with estradiol-induced activation of the MAPK cascade (Nabekura et al., 1986, Lagrange et al., 1997, Toran-Allerand et al., 2002) or with GPR30, a G-protein coupled estrogen receptor [for review see (Prossnitz et al., 2008)]. Rapid effects of estradiol include changes in the ionic conductance of the postsynaptic membrane, such as potassium conductance, and this effect is maintained even after elimination of synaptic input and inhibition of protein synthesis (Nabekura et al., 1986). Studies showing that infusion of a potassium channel inhibitor, tolbutamide, in POA of castrated male rats could decrease mount latency, but not other behavior measures, to the level of the intact males (Chamberlin et al., 2006, McDevitt et al., 2008) confirmed the role of estrogen membrane receptors in regulation of male sexual motivation.

SEXUAL EXPERIENCE AND MOTIVATION

It is noteworthy that in the third experiment, young castrated male rats maintained their mounting behavior. This is consistent with observations by other investigators that mounting is not affected by either surgical castration or medical castration (by applying GnRH analogs) in young experienced male rats (Fielder et al., 1989). However, naïve castrated rats did not show any mounting and intromission behavior (Witt et al., 1995) and for rats with only two sexual pre-tests, only a few mounts were observed (Baum and Vreeburg, 1973). These results indicate that how much experience an animal has gained makes a difference. This is consistent with the report that rats showed significantly increasing pre-contact vocalizations while they were gaining more sexual experience (Bialy et al., 2000). Also, DHT treatment can stimulate copulatory behavior in experienced but not naïve male rats (McDonald et al., 1970, Whalen and Luttge, 1971, Beyer et al., 1976, Butera and Czaja, 1989). However, the underlying mechanism explaining the experience-facilitated sexual behavior is still unknown. Here, I propose a hypothesis:

Experienced rats can synthesize estradiol locally in the brain without testosterone.

I discussed before that estradiol can stimulate sexual motivation. Traditional thought about estradiol synthesis is that in males it is produced by aromatization from testosterone primarily in the testes and other tissues such as in adipocytes, brain, adrenal cortex and arterial wall. In male rats, aromatase

activity was decreased by 60% in POA with castration (Roselli and Resko, 1984). Since both testosterone and aromatase activity decrease after castration, what keeps the sexual motivation in young experienced castrated male rats is unknown. However, in recent years, estrogen has also been found to be synthesized in hippocampal neurons (Prange-Kiel et al., 2003, Kretz et al., 2004). It was reported that in male rats, the hippocampal synthesized estradiol is converted from pregnenolone, instead of testosterone, by cytochromes P45017alpha and P450 aromatase. The concentration of estradiol synthesized in the hippocampus can be about six times higher than the serum estradiol basal level (Hojo et al., 2004). In that report, the fact that NMDA treatment increased the synthesis of estradiol from hippocampus slices indicated that local synthesis of estradiol is mediated by the excitatory neurotransmitter glutamate. At the same time, it has been shown that stimulation of NMDA receptors (which are abundant in hippocampus) in each sexual experience is important to enhance sexual performance immediately as well as in the future. NMDA antagonist MK-801 and CGP40116, administered before each sexual experience, was found to inhibit experience facilitation to the sexual performance (Fleming and Kucera, 1991, Bialy et al., 2000, Powell et al., 2003).

In this hypothesis:

Sexual experience → NMDA receptor stimulation → estradiol synthesis in hippocampus → sexual motivation

However, in aged rats, NMDA receptors decreased in nearly all areas of the brain, especially in the cerebral cortex and hippocampus (Tamaru et al., 1991). A lot of NMDA mediated response decreases with aging in male rats beginning in middle age, such as norepinephrine release from hippocampus (Gonzales et al., 1991). Thus, it is no surprise that an experience-induced decrease of mount latency was not observed in middle-aged experienced males in my experiment.

There are other hypotheses that can explain experience-facilitated sexual motivation. For example, experienced castrated male rats may maintain motivation through a pathway involving cytochrome oxidase in MPN. Crew's lab found heterosexual housing experience elevated cytochrome oxidase in the preoptic area and led to retention of mounting after castration in whiptail lizards (Sakata et al., 2002). Cytochrome oxidase accounts for most oxygen consumption and causes ATP production [Review (Wong-Riley, 1989)]. This further leads to the closing of the ATP-sensitive K^+ channel and then induces mounting behavior. Two studies showed that direct infusion of tolbutamide, an ATP-sensitive K^+ channel inhibitor, into the POA significantly reduced mount latency of castrated male rats (Chamberlin et al., 2006, McDevitt et al., 2008).

TWO KINDS OF REGULATION OF ANDROGEN RECEPTORS BY TESTOSTERONE

In the third experiment, castration led to a decrease of AR density in the two preoptic areas while castration plus testosterone treatment maintained expression of AR. This is consistent with what was reported previously (Krey and McGinnis, 1990). However, in the first experiment of this study, I observed significantly higher AR density in MPN and AVPV in 20-month old male rats and a significant negative correlation between AR expression and serum testosterone level. Along with the obvious difference between the studies that rats were intact (experiment one) or castrated and given either testosterone or vehicle (experiment three) I have tried to interpret the differential results in two ways:

1. Compensation Effects

Unlike menopause in females, there is a gradual decline of testosterone in males that takes months in rats. The process involves enough time for the system to produce more specific protein to compensate for the loss. However, castration causes a sharp drop of testosterone in days. There may be two different responses to those two types of testosterone depletion.

2. Biphasic Pattern of AR Regulation

Handa and his colleagues examined AR mRNA levels after short-term and long-term castration. Their results showed that in male rat there was a significant increase of AR mRNA four days after castration but a significant decrease two months after castration (Handa et al., 1996). This indicated a biphasic and time-dependent pattern of autologous regulation of AR mRNA in the preoptic area.

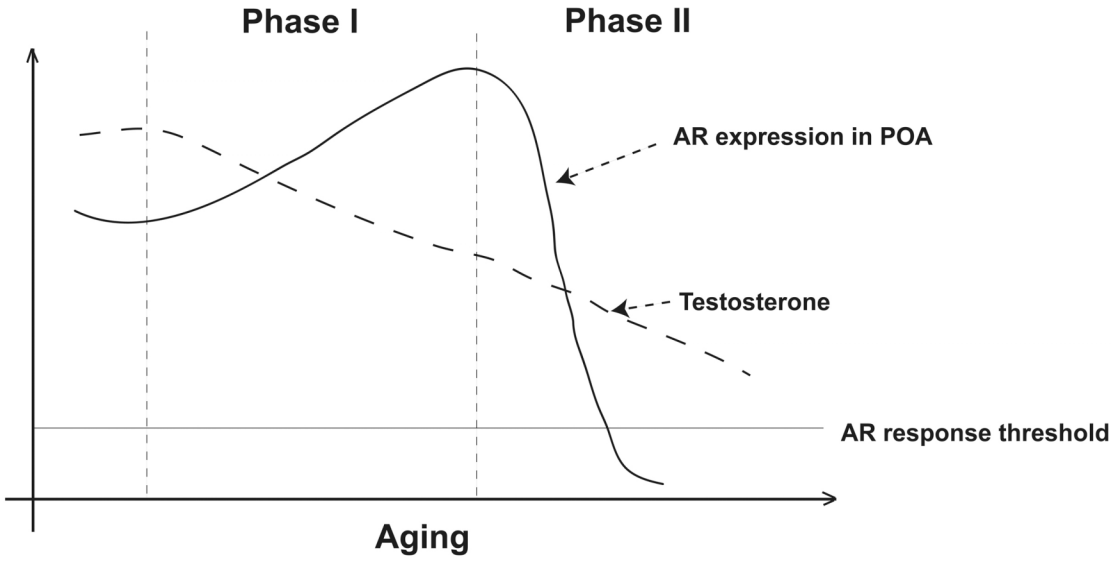
I hypothesize that AR expression alteration with aging also follows a biphasic and time-dependent pattern.

So there are two aging phases (see Figure 5.2):

In Phase I, serum testosterone level starts to decline in middle age and, in compensation, more ARs are synthesized in response to the diminishing testosterone levels. Indeed, in experiment one, testosterone and AR expression were negatively correlated. During this aging process, decreased masculine behavior is due to decreased testosterone, as masculine behavioral performance can be brought back to the level of the young group by testosterone treatment.

In Phase II, testosterone continues to decline and less testosterone is available to maintain the AR level through transcriptional initiation. AR density starts to decrease. Decreased masculine behavior is due to a decrease in testosterone and a decrease in AR. The effect of exogenous testosterone on masculine behavior performance is attenuated. When AR expression decreases to below the AR response threshold, testosterone treatment cannot restore the AR and, as a result, cannot rescue masculine behavior.

Figure 5.2. Biphasic Pattern of AR Regulation with Aging



In my study, I observed only an increase of AR density with aging and a negative correlation between AR and testosterone, which may be due to early phase aging. In the old group, rats were only 20 months old, not old enough, and what I observed may have been Phase I of aging when AR expression increases.

CASTRATION, SEXUAL SATIETY AND AGING

My results showed that castration leads to a decline of male copulatory behavior as well as a long-term decrease of AR and increase of ERα in MPN. It is very interesting that Femandez-Guasti's lab reported that sexual satiety also caused a decrease of AR and an increase of ERα in MPN (Fernandez-Guasti et al., 2003, Phillips-Farfan et al., 2007).

Sexual satiety is a phenomenon by which, after repeated ejaculations, an inhibition of male sexual behavior takes place (Beach and Jordan, 1956). Twenty-four hours after sexual satiety, about two-thirds of the males showed a complete inhibition of sexual behavior while one-third exhibited a single ejaculation with prolonged latencies and did not resume mating afterwards (Rodriguez-Manzo et al., 2000, Fernandez-Guasti and Rodriguez-Manzo, 2003). Male rats require 7 to 15 days of sexual inactivity to recover their full mating capacity (Beach and Jordan, 1956, Romano-Torres et al., 2007). However, during the sexual satiety process and recovery time, serum testosterone did not change significantly (Fernandez-Guasti et al., 2003, Romano-Torres et al., 2007).

Therefore, it is assumed that the alteration of sexual behavior depends on changes of expression of hormone receptors.

According to a previous report, AR decreased in the MPN in males that were sacrificed after one ejaculation and further decreased in MPN in males that were sacrificed after reaching sexual satiety (mating with female 5-8 times for 4 hours) (Fernandez-Guasti and Rodriguez-Manzo, 2003). This suggests a cumulative decrease in AR expression with ejaculation. However, ERα expression in MPN did not change with one ejaculation. It increased significantly when animals reached sexual satiety and were not able to perform very well no matter how many ejaculations they needed to obtain sexual satiety (Phillips-Farfan et al., 2007). Thus, it appears that the increase of ERα expression in MPN is an "on/off" phenomenon that reflects a male's sexual capacity. Whether ERα expression can cause the inhibition of sexual behavior needs more investigation.

The results shown in these sexual satiety studies are similar to my observation after castration. Therefore, we can hypothesize that the abolishment of testosterone as well as repeated ejaculations may cause an alteration of AR and ERα expression in MPN that, in turn, may induce further changes in sexual performance.

Age-related sexual decline may also be due to alteration of AR and ERα expression although I observed an increase of AR density in POA with aging. However, if the biphasic pattern of AR expression with aging is true, we can expect to observe a decrease of AR expression with aging. Although this study

did not observe an increase of ERα expression in MPN with aging, I did find that heavily stained ERα density increased with aging, which can be considered a forerunner of increased ERα expression. It is also possible that the heavily stained ERα cells represent those with a greater number of ERα molecules per nucleus, or a differential post-translational state such as receptor phosphorylation, which affects activity of the ERα (Bunone et al., 1996). This is a subject for future investigation.

CONCLUSION

Taken together, these three experiments tell a story about how aging and sexual experience regulate sex hormone and hormone receptor expression and, ultimately, contribute to the control of sexual behavior. Specifically, androgen receptors, interacting with testosterone, play a key role in stimulating masculine behavior. Changes in number of cells expressing hormone receptors, caused by other factors such aging and sexual experience, lead directly to changes in sexual behavior.

This insight offers us a better understanding of the relationship that goes on between molecular changes in the brain and behavior. This information encourages us to give additional attention to human testosterone treatment and suggests new therapeutic targets. The long-term goal is to enhance the quality of life for men who will encounter this problem as they age.

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