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THE ROLE OF ESTROGEN IN THE RETENTION OF COPULATORY BEHAVIOR AFTER CASTRATION IN B6D2F1 MALE HOUSE MOUSE (MUS MUSCULUS)

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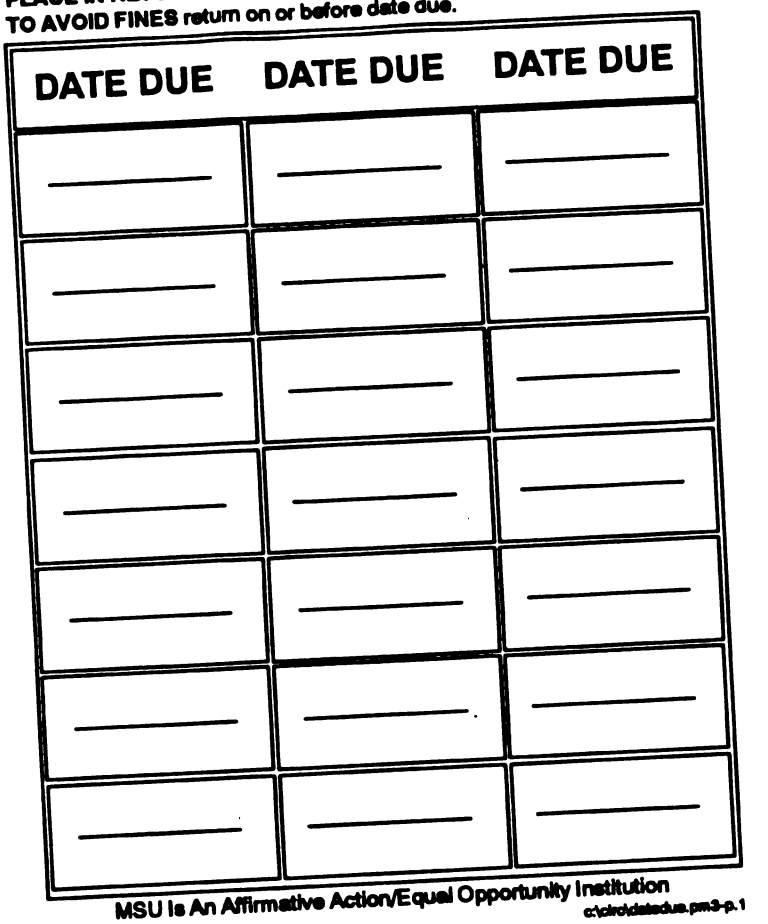

THE ROLE OF ESTROGEN IN THE RETENTION OF COPULATORY BEHAVIOR AFTER CASTRATION IN B6D2F1 MALE HOUSE MOUSE (MUS MUSCULUS)

By

Kevin Sinchak

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ABSTRACT

THE ROLE OF ESTROGEN IN THE RETENTION OF COPULATORY BEHAVIOR AFTER CASTRATION IN B6D2F1 MALE HOUSE MOUSE (MUS MUSCULUS)

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In the B6D2F1 hybrid strain of house mouse, (Mus musculus), most males continue to achieve an ejaculatory reflex for six to twelve months after castration (continuers), while others stop copulating within a few weeks after castration (noncontinuers)(Clemens et al., 1988; McGill & Manning, 1976). The present study investigated: 1) if continued copulation after castration in B6D2F1 males is dependent on steroid hormone stimulation; 2) if continuer and noncontinuer males differ in steroid hormone physiology.

Serum levels of testosterone (T), and estradiol (E2) were measured in intact and castrated continuer and noncontinuer males. Castration reduced, but did not eliminate T from the circulation of B6D2F1 males. In contrast, castration did not affect serum E2 levels. However, continuer and noncontinuer males did not differ in serum T or E2 levels. The adrenal gland is not the source of these nongonadal androgens and estrogens, since removal of the adrenals had no effect on serum T and E2 levels in castrated males. The importance of nongonadal estrogens in the maintenance of copulation in continuer males was determined by blocking the aromatization of androgens to estrogens with an aromatase inhibitor, 1,4,6-androstatriene-2-17-dione (ATD) in continuer males. Inhibition of estrogen synthesis by ATD reduced the percentage of continuer males that achieved ejaculation and intromission, but had no effect on percentage of males that mounted. Continuer and noncontinuer males appear to differ in their responsiveness to estrogens, since they do not differ in circulating levels E2 and

continued copulation is dependent on synthesis of nongonadal estrogens.

Since copulation in continuers appears to be estrogen dependent, and continuers and noncontinuer appear to differ in responsiveness to estrogens, aromatase activity (AA), and estrogen receptor (ER) levels were measured in the preoptic area (POA), hypothalamus (HYP) and amygdala (AM) to determine if continuer and noncontinuer males differ in estrogen physiology. In general continuers and noncontinuers did not differ in AA or ER levels. Castration reduced but did not eliminate AA in POA, HYP and AM. Castration did not affect nuclear ER levels in the POA and HYP but reduced nuclear ER levels in the AM. The data support the hypothesis that sexual behavior of castrated B6D2F2 male mice continues to be influenced by nongonadal E2. Although continuer and noncontinuer males appear to differ in responsiveness to estrogens, they could not distinguished by serum T, or E2 levels, or AA or ER levels in the POA, HYP and AM.

To

Mom and Dad

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LIST OF ABBREVIATIONS

AA = aromatase activity

ADX = adrenalectomy

AE = androstenedione

AH = anterior hypothalamus

AM = amygdala

ANOVA = analysis of variance

AOB = accessory olfactory bulb

ATD = 1,4,6-androstatriene-2-17-dione

BC = bulbocavernosus

cmAM = corticomedial amygdala

cont = continuer

DHT = dihydrotestosterone

E2 = 17ß-estradiol

ER = estrogen receptor

ERc = cytosolic estrogen receptor

ERn = nuclear estrogen receptor

ERt = total estrogen receptor

HYP = hypothalamus

int = intact

ir = immunoreactivity

LOT = lateral olfactory tract

mAM = medial amygdala

MOB = main olfactory bulb

mPON = medial preoptic

MVPC = medioventral pars compacta nucleus

N-K = Newman-Keuls' multiple range test

nonc = noncontinuer

POA = preoptic area

RIA = radioimmunoassay

SDN = sexually dimorphic nucleus

SNB = spinal nucleus of the bulbocavernosus

S-R = steroid hormone-receptor complex

T = testosterone

TP = testosterone propionate

VNO = vomeronasal organ

INTRODUCTION

Expression of copulatory behavior in males of most mammalian species is dependent upon the presence of gonadal hormones (reviews: Meisel & Sachs, 1994; Sachs & Meisel, 1988). However, the effect of castration on copulatory behavior differs between species as well as between individuals within a species or strain. For example, some males continue to achieve an ejaculatory reflex for up to a year in dogs, cats, goats, rats, rhesus monkeys and the B6D2F1 hybrid strain of house mouse (Beach, 1970; Clemens, Wee, Weaver, Roy, Goldman, & Rakerd, 1988; Hart, 1968; Hart, 1975; McGill & Manning, 1976; Phoenix, Slob, & Goy, 1973; Rosenblatt & Aronson, 1958; Stone, 1927). In contrast, other castrated individuals within these same species stop copulating within days or weeks after castration. Why some males continue to copulate after castration and other do not is not understood. In the case of the B6D2F1 house mouse (Mus musculus), most males continue to achieve an ejaculatory reflex for six to twelve months after castration, while others stop ejaculating within a few weeks after castration (Clemens et al., 1988; McGill & Manning, 1976). In the present study, genetically homogenous hybrid B6D2F1 male mice that continue to copulate after castration and those that do not were studied to determine if they differ in their steroid hormone physiology. Therefore, a review of the effects of steroid hormones on copulatory behavior as well as the effects of steroids on steroid hormone sensitive neural circuits that regulate copulatory behavior in male mammals will be provided as a background for this set

of experiments.

Effects of Orchidectomy and Steroid Hormones on Sexual Behaviors

Effects of Castration on Copulation

Removal of the testes eliminates the major source of the steroid hormone testosterone (T). Castration generally reduces or abolishes the expression of male sexual behavior. The loss of copulatory behaviors after castration follows a typical pattern, in which ejaculatory behavior is first to cease, followed by intromission, then mount behavior, and finally precopulatory behaviors such as ultrasound production (Clemens & Pomerantz, 1981; Dizinno & Whitney, 1977; Nunez, Nyby, & Whitney, 1978; Sachs & Barfield, 1976). However, the length of time that copulatory behavior persists after castration as well as the levels of copulatory behavior expressed after castration vary among species as well as among individuals of a given species or specific strain. For example, in the C57Bl/6J strain of house mouse, nearly all males stopped exhibiting an ejaculatory reflex 7 weeks after castration, while up to 80% of the castrated males in the B6D2F1 hybrid strain of house mouse (derived from the cross of a DBA/2J male and a C57Bl/6J female) continued to exhibit an ejaculatory reflex for several months after castration and some continued for over a year after castration (Clemens et al., 1988; McGill, 1965; McGill & Haynes, 1973; McGill & Manning, 1976; McGill & Tucker, 1964). DBA/2J males, the paternal strain of B6D2F1 hybrids, exhibited a behavioral response to castration that is between C57Bl/6J and B6D2F1 males. DBA/2J males showed a rapid decline in ejaculatory behavior seven weeks after castration, like C57

males, however, a few individuals continued to achieve an ejaculatory reflex very sporadically up to 25 weeks after castration (Clemens et al., 1988).

In these strains of house mouse, the retention of copulatory behavior is under genetic influence. The persistance of copulation after castration in B6D2F1 males is due to heterozygosity between loci (genes), and not due to heterozygosity of alleles (genes)(hybrid vigor). A hybrid of two inbred strains has a new combination of paired alleles. Some of these alleles remain homozygous, while many are now heterozygous. This new combination of alleles ultimately affects phenotypes that are the product of multiple gene expression. It has been demonstrated that the exact recombination the autosomal alleles is not sufficient to produce persistance of copulation after castration. Males resulting from the reciprocal cross of the B6D2F1 parental strains, D2B6F1, exhibit a loss of copulatory behavior after castration that resembled the parental strains (McGill & Manning, 1976). These data indicate that genetic information on the sex chromosomes interacts with the autosomes to maintain copulatory behavior after castration. The importance of the interaction of the autosomal loci was demonstrated by the production of recombinant inbred strains of B6D2F1 house mice, BXD strains (Coquelin, 1991). After systematically inbreeding B6D2F1 males over twenty generations, several new inbred strains of BXD strains were produced, each with a new combination of fixed homozygous alleles at all loci. When tested for retention of copulatory behavior after castration, males from two of the six recombinant BXD strains continued to copulate 5 months after castration (Coquelin, 1991). Thus, the interaction of autosomal and sex loci determine the maintenance of copulatory behavior after castration in the house mouse.

Variability in the retention of the ejaculatory reflex is seen in a number of other species

as well. For example, in dogs (Canis familiaris), some males stop copulating within one to two months after castration, while other males continue to intromit and achieve copulatory lock which is associated with ejaculation five years after castration (Beach, 1970; Hart, 1968). This kind of variability has also been observed in goats, rams, and rhesus monkeys (Clegg, Beamer, & Bermant, 1969; Hart, 1975; Michael & Wilson, 1974; Phoenix et al., 1973).

Castration and the Effects of Androgen and Estrogen Replacement on Male Copulatory Behavior

Testosterone

In mammals, copulatory behavior may be maintained or restored after castration by treating males with exogenous testosterone (T): deer mice, (Clemens & Pomerantz, 1981; Clemens & Pomerantz, 1982), house mouse (Dizinno & Whitney, 1977; Larsson, 1979; Nunez et al., 1978; Wee, Weaver, & Clemens, 1988), rabbits (Macmillan, Desjardins, Kirton, & Hafs, 1969), rats (Beach & Holz-Tucker, 1949; Bloch & Davidson, 1968; Whalen & Luttge, 1971), guinea pigs (Grunt & Young, 1952), gerbils (Yahr, Newman, & Stephens, 1979), hamsters (Whalen & DeBold, 1971), dogs (Beach, 1970), sheep (Clegg et al., 1969), and rhesus monkeys (Michael & Wilson, 1974; Phoenix et al., 1973). Administration of smaller doses of testosterone propionate (TP) is required right after castration to maintain sexual behavior at pre-castration levels compared to the higher doses of TP required to restore sexual behavior if lost after castration (Davidson, 1972; Yahr et al., 1979).

Individual differences in sexual capacity and responsiveness to T have been demonstrated. Within guinea pigs and rats, individual intact males exhibit varying levels (high vs low) of sexual behavior (e.g. number of ejaculations per test, latencies or duration of

particular behavior etc...)(Grunt & Young, 1952; Larsson, 1966). After castration, high activity males required fewer T treatments to restore ejaculation than the low activity group (Larsson, 1966). Further, these differences in intact levels of behavior were not due to differences in circulating levels of T. Low and high sexually active males given an equivalent dose of TP after castration exhibited the their respective intact levels of sexual activity after castration (Grunt & Young, 1952; Larsson, 1966; Whalen, Beach, & Kuehn, 1961). Additionally, superphysiological doses of TP given to castrated males did not increase their levels of sexual behavior beyond intact levels (Larsson, 1966; Riss & Young, 1954). Therefore, these data indicate that intact levels of copulatory behavior are dependent on the capacity of the individual to exhibit sexual behavior, not on the absolute concentration of T. However, these individuals demonstrate differences in the threshold levels of hormone stimulation required to initiation copulatory behavior (responsiveness) to the activation effects of hormones.

Metabolism of Testosterone

Although T restores and maintains copulatory behavior in males, T may not be the hormone that facilitates behavior. T may be converted into more metabolically active androgens or estrogens. The metabolism of T to 17ß-estradiol (E2) or dihydrotestosterone (DHT) is regulated by enzymes which are members of the large superfamily of P450 cytochromes of which hundreds of isoforms are known to occur naturally (Juchau, 1990). The P450 isoforms that are responsible for the aromatization of C19 steroids to estrogens and α- and ß-reduction of androgens belong to family XI isoforms. Aromatase is the P450 enzyme that is responsible for converting T and adrostenedione (AE) to E2 and estrone

respectively. Aromatase, converts the Δ-4, 3 ketone-A ring of aromatizable androgens to an aromatic benzene ring of estrogens. The reductase enzymes are responsible for the conversion of T and AE to the α- or ß-reduced androgens, DHT and androstanedione. These reduced androgens are formed when T or AE are reduced at the carbon-5 position. The reduction of T and AE is a nonreversible reaction, and once reduced, these androgens cannot be aromatized or converted to estrogens.

Effects of Estrogen and Reduced Androgen on Copulatory Behavior in Castrated Males

Responsiveness to metabolites of T varies from species to species as well as among strains. For example, E2 treatment restores or maintains copulatory behavior in castrated males of the following strains of house mice (*Mus musculus)*: CD-1 mice, Swiss-Webster, B6D2F1, but does not restore ejaculatory behavior in deer mice (Peromyscus maniculatus bairdi) (Clemens & Pomerantz, 1982; Dalterio, Bartke, & Butler, 1979; Edwards & Burge, 1971; Wallis & Luttge, 1975; Wee et al., 1988). E2 also maintains or restores copulatory behavior in castrated male rats, guinea pigs, and cats (Antliff & Young, 1956; Davidson, 1969; Feder, Naftolin, & Ryan, 1974; Green, Clement, & deGroot, 1957; Sodersten, 1973).

DHT is able to restore or maintain copulatory behavior after castration in B6D2F1, and Swiss-Webster strains of house mouse, and the deer mouse (Clemens & Pomerantz, 1981; Clemens & Pomerantz, 1982; Luttge & Hall, 1973; Sinchak & Clemens, 1990; Wallis & Luttge, 1975). However, DHT treatment is unable to maintain or restore copulatory behavior in the CD-1 strain of house mouse, as well as rats, hamsters, gerbils, pigs or sheep (Baum, Kingsbury, & Erskine, 1987; Baum & Starr, 1980; Christensen, Coniglio, Paup, & Clemens, 1973; Feder, 1971; Levis & Ford, 1989; Luttge & Whalen, 1970, Luttge, 1973 #45;

McDonald, Beyer, Newton, Brien, Baker, Tan, Sampson, Kitching, Greenhill, & Pritchard, 1970; Parrott, 1986; Sodersten, Eneroth, & Hansson, 1988; Whalen & Luttge, 1971; Yahr & Stephens, 1987).

Although treatment of castrate males with either androgens or estrogens alone may maintain or restore behavior, it is likely that both estrogenic and androgenic stimulation facilitate copulatory behavior in mice as well as other species. For example, E2 and DHT act in a synergistic manner to activate intact levels of copulatory behavior in castrated males of the CD-1 strain of house mouse (Wallis & Luttge, 1975). Further, in castrated deer mice that respond to DHT alone, blocking the conversion of T to E2 or DHT reduced the effectiveness of exogenous T to activate copulatory behavior (Clemens & Pomerantz, 1981; Clemens & Pomerantz, 1982). The synergism of androgens and estrogens in activating copulatory behavior has also been demonstrated in the rats and hamsters (Baum & Vreeburg, 1973; DeBold & Clemens, 1978; Larsson, Sodersten, & Beyer, 1973b). Although E2 treatment restores copulatory behavior in castrated male rats, if castrated males are adrenalectomized, the same dose of E2 is unable to restore copulatory behavior (Gorzalka, Rezek, & Whalen, 1975). However, if these same male are then treated with TP, ejaculatory behavior is restored which suggests that adrenal androgens may facilitate copulation (Gorzalka et al., 1975). Further evidence of synergism was demonstrated in castrated male rats given subthreshold doses of E2 that did not facilitate copulation. When DHT was implanted discretely in the lateral septum or medial amygdala, these E2 treated males started copulating (Baum, Tobet, Starr, & Bradshaw, 1982).

Aromatization of Androgens to Estrogen and Behavior

The "aromatization hypothesis" for activation of copulatory behavior was formulated from a combination of studies that demonstrated that 1) aromatizable androgens were more effective than nonaromatizable androgens in activating copulatory behavior in castrated males of several species (Beyer, Larsson, Perez-Palacios, & Morali, 1973; Beyer & Rivaud, 1973; Luttge & Hall, 1973; McDonald et al., 1970; Whalen & Luttge, 1971), and 2) E2 either restores copulatory behavior or facilitates the effects of DHT or other nonaromatizable androgens to activate copulatory behavior in a number of species (see above). The basic premise was the aromatization of androgens (T) to estrogens (E2) is required for the activation of copulatory behavior. This aromatization hypothesis was supported by numerous studies in several species that showed the ability of T to restore copulatory behavior in castrates could be inhibited by either blocking its aromatization to E2 with systemic aromatase inhibitors or by blocking estrogenic stimulation with an estrogen receptor antagonist (Alexandre & Balthazart, 1986; Beyer, Morali, Naftolin, Larsson, & Perez-Palacios, 1976; Carroll, Weaver, & Baum, 1988; Christensen & Clemens, 1975; Floody & Petropoulos, 1987; Luttge, 1975; Morali, Larsson, & Beyer, 1977).

In summary, in most male mammals, castration causes a reduction in sexual activity by a reduction in gonadal steroid hormones (mainly T). Sexual behaviors may be restored in these males by replacing gonadal hormones. Although T is the most effective in restoring copulatory behavior to castrate males, its metabolites E2, and DHT appear to be the metabolites that promote copulation. Alone, E2 restores copulatory behavior in castrate males of numerous species, however, concurrent administration of DHT and E2 works better to restore copulatory behavior to intact levels. The fact that copulation in some males is

dependent on the presence of gonadal hormones while it is not in others suggests two possible reasons for the different effects of castration on copulatory behavior among individuals. One possibility is that continued copulation in castrated males is independent of steroid hormone facilitation. The other possibility is that castrated males that copulate are more responsive to circulating steroid hormones that are present after castration.

Effects of castration on steroid hormone levels

Castration reduces but does not eliminate T from systemic circulation. Although this general profile occurs in all species studied to date, relative levels of nongonadal steroids vary among species. For example, T concentrations in castrates vary from 0.45% (rats) to nearly 12.3% (hamster) of intact levels (Table 1). In the B6D2F1 male mice, castration reduced T levels to approximately 10% of intact levels (Clemens et al., 1988).

In contrast, for some mammalian species, E2 levels are not affected by castration while in others they are slightly reduced. For example, E2 concentrations after castration vary from 26.2% (horse) to 156.1% (ferret) of intact levels (Table 2). This differential effect of castration on T and E2 levels has been observed in rats, rhesus monkeys, and ferrets indicating that serum E2 is maintained by an unknown nongonadal source (Carroll et al., 1988; Roselli & Resko, 1984; West, Roselli, Resko, Greene, & Brenner, 1988).

However, levels of nongonadal E2 or T do not correlate with the maintenance of copulatory behavior after castration among species. For example, castrated hamsters have the highest relative level of T compared to intact males, and castrated rats maintain E2 equivalent to intacts, yet both species stop copulating shortly after castration, while males of other species with less relative T and/or E2 levels continue to copulate.

Nonetheless, the fact that steroid hormones are available after castration suggests that it is possible that continued copulation after castration is dependent on these nongonadal steroids. Although T levels are reduced, it is still available to provide androgenic stimulation, as well as being a substrate for aromatization to E2. Furthermore, the fact that endogenous E2 levels are less affected by castration (Table 2) than T (Table 1), support the idea that species whose copulatory behavior is dependent upon estrogenic stimulation would be less likely to show an effect of castration on copulation than those species that require androgens.

Sources of Nongonadal Steroids

Nongonadal steroids may originate from several sources. One possible source is the adrenal gland. However, since copulation often continues after castration and adrenalectomy in a number of species (Cooper & Aronson, 1958; Schwartz & Beach, 1954; Thompson, McGill, McIntosh, & Manning, 1976), other sources may also exist if this behavior is hormone dependent.

Another potential source of nongonadal androgens and estrogens is the brain. A considerable amount of steroid metabolism takes place in the brain. Local tissue metabolism (reviewed later) as well as the possibility of de novo synthesis of steroid from cholesterol are potential mechanisms by which the brain could synthesize metabolically active steroids that affect behavior. Steroids produced by de novo synthesis or by in situ metabolism of circulating steroid are referred to as neurosteroids (Baulieu, 1981). Three of the four enzymes needed for the metabolism of cholesterol to estrogen have been demonstrated in the adult rat brain (Corpechot, Robel, Axelson, Sjovall, & Baulieu, 1981; Le Goascogne, Robel, Gouezou, Sananes, Baulieu, & Waterman, 1987; Robel & Baulieu, 1995). While the fourth,

Table 1

Mean (+/- s.e.m.) circulating levels of testosterone (T) (ng/ml) in intact and castrated males of several mammalian species, and the levels circulating T in the castrate as a percent of intact T levels.

Species	Intact	Castrate	% of Intact
B6D2F1 Mouse	1.63 (0.20)[2]	0.18 (0.9)	11.0
Rat	3.80 (1.10)[7]	0.070 (0.030)	1.8
	2.67 (0.19)[5]	0.012 (.0018)	0.45
	3.80 (0.86)[8]	0.030 (0.020)	0.79
Ferret	7.01 (2.67)[1]	0.030 (0.010)	0.43
	2.07 (1.58)[6]	0.042 (0.023)	2.03
Rhesus Monkey	5.00 (0.80)[4]	0.30 (0.10)	6.0
	17.00 (1.50)[4]	0.10 (0.10)	0.58
	4.32 (1.20)[10]	0.31 (0.04)	7.18
Horse	2.10 (0.10)[3]	0.20 (0.03)	9.5
Hamster[LD]	4.30 (0.90)[9]	0.53 (0.20)	12.3

Note. [LD]Hamsters housed in long day light cycle (14 hour light/10 hour dark).
[1]Carroll, et al., 1988; [2]Clemens, et al., 1988; [3]Ganjam, et al., 1975; [4]Goodman, Hotchkiss, Karsch & Knobil, 1974; [5]Handa, Reid & Resko, 1986; [6]Kastener & Apfelbach, 1987; [7]Roselli, et al., 1984; [8]Roselli, et al., 1993; [9]Sisk & Turek, 1983; [10]West, et al., 1988.

Table 2

Mean (+/- s.e.m.) circulating levels of estradiol (E2) (pg/ml) in intact and castrated males of several mammalian species, and the levels circulating E2 in the castrate as a percent of intact E2 levels.

Species	Intact	Castrate	% of Intact
Rat	36.7 (2.3)[7]	39.0 (2.5)	106.1
	3.9 (1.4)[8]	2.6 (1.1)	66.7
Ferret	8.2 (2.6)[1]	12.1 (7.2)	156.1
Rhesus Monkey	19.0 (6.0)[10]	14.0 (4.0)	73.7
Horse	43.9 (2.3)[3]	11.5 (2.0)	26.2

Note. [1]Carroll, et al., 1988; [3]Ganjam & Kenney, 1975; [7]Roselli, et al., 1984; [8]Roselli, et al., 1993; [10]West, et al., 1988.

17α–hydroxylase ($P450_{17\alpha}$) with 17,20-desmolase activity, which converts pregnenolone to dehydroepiandrosterone (DHEA) has not been demonstrated in mammalian brain, there is circumstantial evidence for its presence: DHEA levels maintain a circadian rhythm and are higher in the brain of castrated, adrenalectomized rats than in the plasma, suggesting DHEA may be synthesized in the brain (Corpechot, et al., 1981; Robel, et al., 1987).

Because steroid hormones interact on so many levels within the body to organize and regulate important metabolic and behavioral functions, it is not surprising that steroid synthesis takes place at multiple sites. While gonadal synthesis of steroid hormones and copulatory behavior are associated in mammals, it is possible that multiple sources of steroid hormone may influence copulatory behavior.

Neural Circuits Important for Male Copulatory Behavior: Their Metabolism of, and Responsiveness to Steroid Hormones

In the central nervous system (CNS) several neural pathways are involved in the regulation of reproductive behavior. The best understood mechanism by which steroid hormones exert their influence on copulatory behavior is through regulating genomic activity of these neurons that contain steroid receptors in these pathways. As previously mentioned, the brain is able to synthesize and metabolize steroid hormones. Much of this activity is located in brain regions within the neural circuits that regulate copulation. The following section will focus on and review the steroid sensitive neural pathways of the limbic system that are involved in the regulation of male copulatory behavior, and how steroid hormones regulate copulatory behavior and steroid physiology (receptor mechanisms and metabolism of steroids) within the CNS. Since the studies performed in the dissertation investigate the

role of estrogen in maintenance of copulatory behavior in male B6D2F1 males after castration, emphasis will be place on the role of estrogen.

Both external cues (e.g. female odor, or behavioral cues) as well as internal cues (e.g. hormonal or dietary status) that modulate the sexual motivation appear to converge in the medial preoptic area (mPOA). In turn, the mPOA modulates three types of behavioral responses: 1) Appetitive or sexual motivation, 2) Somatomotor regulating copulation, and 3) genital reflexes. In general, each of these behavioral responses is regulated by a "series" of brain regions that comprise a neural circuit that receives, integrates, and relays information that eventually results in behavioral output. The activity of nearly every brain region involved in these circuits is either directly or indirectly influenced by steroid hormones. The following section will first focus on the behavioral neural circuits in the limbic system that regulate male sexual behavior, and then review the steroid hormone physiology of brain regions that comprise these circuits.

Chemosensory Pathways

Olfactory Bulbs and Copulatory Behavior

In rodents, he olfactory bulbs are the most rostral portions of the brain. There are two anatomically distinct regions to the olfactory bulb: the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB). Each region receives and integrates different types of olfactory information. The MOB receives airborne chemical information that stimulate olfactory neurons located in the nasal mucosa of the nasal cavity. The AOB receives and integrates chemical stimuli that are taken into the oral cavity and reach the vomeronasal organ (VNO) through the nasopalatine ducts in the roof of the mouth. During precopulatory ano-

genital investigation, the male picks up chemical cues from the female that stimulate both olfactory systems. These chemical cues reveal the reproductive status of the female and affect the sexual arousal state and motivation of the male. Sensations from olfactory and vomeronasal organ (VNO) receptors send chemosensory information to the MOB and AOB respectively via axons that project through the cribiform plate of the ethmoid bone and into the olfactory bulbs. These olfactory and VNO projections synapse with dendrites of mitral and tufted cells in the glomerular layer of the MOB and AOB respectively (Barber & Raisman, 1974). There is evidence that some integrative function occurs in the olfactory bulbs, since chemosensory information is supplemented by trigeminal afferent information and possibly by projections of the nervus terminalis (Silver, 1987; Wirsig & Leonard, 1986).

Bilateral removal of olfactory bulbs abolished expression of male copulatory behavior in mice and hamsters (Devor, 1973; Doty, Carter, & Clemens, 1971; Edwards & Burge, 1973; Lisk, Zeiss, & Ciaccio, 1972; Murphy, 1980; Murphy & Schneider, 1970; Rowe & Edwards, 1972; Winans & Powers, 1974). However, vomeronasal information from AOB appears to be more important for the expression of copulatory behavior than olfactory stimuli of the MOB. For example, peripherally induced anosmia that eliminates olfactory stimuli to the MOB, but not to AOB, does not affect expression of male copulatory behavior in Swiss-Webster mice (Edwards & Burge, 1973; Rowe & Edwards, 1972). Whereas, removal of the VNO causes a decrease in the percent of males that achieve intromission and ejaculate (Clancy, Coquelin, Macrides, Gorski, & Noble, 1984). Eliminating chemosensory input to the MOB in the hamster by irrigating the nasal cavity with zinc sulfate, reduced copulatory behavior in some studies but not in others (Devor, 1973; Lisk et al., 1972; O'Connell & Meredith, 1984; Powers, Fields, & Winans, 1979; Powers & Winans, 1975; Winans &

Powers, 1977). Likewise, removal of chemosensory input to the AOB by cutting the vomeronasal nerve or removing the VNO affects copulation in less than 50% of the animals (Meredith, 1986; O'Connell & Meredith, 1984; Powers et al., 1979; Powers & winans, 1975; Winans & Powers, 1977). In the male rat, olfactory bulbectomy mainly produces a reduction in the percent of males that achieve ejaculation (Larsson, 1969; Larsson, 1975; Lumia, Zebrowski, & McGinnis, 1987; Meisel, Lumia, & Sachs, 1982; Meisel, Lumia, & Sachs, 1986). Evidence in rats suggests that these olfactory cues are important in identifying the female's reproductive state and stimulating sexual arousal of the male. For example, male rats that continue to copulate after olfactory bulbectomy, no longer exhibit a preference of an estrous female over a nonestrus female. Moreover, these males take longer to achieve intromission (intromission latency (IL)), which is suggestive that sexual arousal may be reduced (Edwards, Griffis, & Tardival, 1990).

Projections of the Main and Accessory Olfactory Bulbs

In the male hamster, MOB and AOB efferents project to corticomedial nuclei of the amygdaloid complex via separate fiber bundles within the lateral olfactory tract (LOT) (Scalia & Winans, 1975). Efferent fibers of the MOB carry olfactory information to the "olfactory amygdala" and the AOB efferents carry VNO information to the medial amygdaloid group or "vomeronasal amygdala" (Scalia & Winans, 1975). In turn, each of these nuclei, projects to the bed nucleus of the stria terminalis (BNST) and to the medial preoptic area/anterior hypothalamus (POA-AH) via the stria terminalis (Winans, Lehman, & Powers, 1982).

Amygdala (AM)

The anatomy, projections, and behavioral properties of amygdala have not been worked out in the mouse. Therefore, most of the data presented will be from other closely related rodents, the rat and hamster, which demonstrate the roles of different regions of the amygdala in chemosensory regulation of motivation (arousal) and facilitation of copulatory behavior.

The amygdala can be divided into five major anatomical nuclei: cortical amygdala (olfactory), medial amygdala, basomedial amygdala, lateral amygdala and a central amygdala (De Olmos, Alheid, & Beltramino, 1985). Two regions of the amygdala have been investigated as to their roles in expression of male copulatory behavior: basolateral amygdaloid nuclei (basomedial and lateral nuclei) and corticomedial amygdaloid nuclei (cortical and medial amygdala nuclei).

The basolateral amygdala appears to be part of a neural circuit that is involved in regulation of chemoinvestigative behavior which appears to affect sexual motivation but not the ability to copulate. For example, male rats and hamsters with lesions of the basolateral amygdala copulate if presented with a female (Harris & Sachs, 1975; Kevetter & Winans, 1981; Lehman, Winans, & Powers, 1980). However, male rats that have been trained to associate the secondary stimulus of bar pressing to gain access to a receptive female, will not bar press to gain access to an estrous female if a lesion has been placed in the basolateral amygdala (Everitt, Cador, & Robbins, 1987). Thus, sexual motivation that is stimulated by primary cues (female odor, vocalizations, or behavior) is not disrupted by these lesion, but association of secondary stimuli for facilitating sexual motivation is impaired (Everitt et al., 1987). It appears that the amygdala plays a role in regulating the motivational states of a

number of other behaviors including aggressive, fear and ingestive behaviors (reviewed Everitt, 1989; Everitt et al., 1987).

In contrast, lesions of the corticomedial amygdala cause varying amounts of deficits in expression of copulatory behavior or motor output, and sexual motivation or arousal in rats and hamsters. For example, in hamsters, lesions in the rostral region of the corticomedial amygdala eliminate both chemoinvestigatory and copulatory behavior, whereas lesions in the caudal corticomedial amygdala produce variable and more short-lived effects on expression of copulatory behavior (Lehman & Winans, 1982; Lehman et al., 1980). Caudal lesions increased mount latency and ejaculatory latency shortly after surgery, however, these latencies returned to control levels three weeks after surgery. In rats, corticomedial amygdala lesions increased the ejaculatory latency and decreased the number of ejaculations achieved by rats (Giotonio, Lund, & Gerall, 1970). While another study suggested that the effects of these lesions were actually more subtle in that copulatory deficits only arose in male rats if the stimulus female was only primed with estrogen versus a female primed with estrogen and progesterone (Perkins, Perkins, & Hitt, 1973).

Projections of the Amygdala

In the hamster, the caudal corticomedial amygdala, which receives information from the AOB, sends projections via the stria terminalis to the medial preoptic area of the hypothalamus (mPOA) (Kevetter & Winans, 1981). The rostral corticomedial amygdala, which receives afferents from the MOB, projects efferents via the ventral fiber pathway (VP) to the caudal medial bed nucleus of the stria terminalis (BNST) which then sends efferents to the mPOA (Lehman, Powers, & Winans, 1983).

Stria Terminalis and Bed Nucleus of the Stria Terminalis (BNST)

Lesions in either the BNST or stria terminalis in the male rat produce increased ejaculatory latencies as seen with corticomedial amygdala (cmAM) lesions, as well as increased number of intromissions to ejaculation, and interintromission intervals (Emery & Sachs, 1976; Giotonio et al., 1970; Paxinos, 1976; Valcourt & Sachs, 1979).

In the hamster, the caudal portion of the cmAM projects directly to the medial preoptic area/anterior hypothalamus (mPOA-AH) via the stria terminalis (Kevetter & Winans, 1981). Cutting the stria terminalis caused temporary deficits as seen with caudal cmAM lesions (Lehman et al., 1983). In contrast, the rostral portion of the cmAM projects via the VP to the caudal medial BNST, which in turn projects to the mPOA-AH (Lehman et al., 1983). Cutting the ventral fiber pathway caused severe deficits in copulatory behavior similar to rostral cmAM lesions, while cutting both stria terminalis and ventral fiber pathway eliminated copulatory behavior in hamsters (Lehman et al., 1983). Further, these pathways have been shown to be selectively activated by sexually relevant olfactory stimuli. For example, expression of the immediate-early gene c-fos (which increases in some cells when stimulated) was increased in the POA and mAM when male rats were exposed to stimuli associated with reproduction (Baum & Everitt, 1992). By unilaterally cutting pathways of olfactory and vomeronasal information received by mAM, induction c-fos by sexually relevant information in the ipsilateral POA was attenuated (Baum & Everitt, 1992; Krettek & Price, 1978).

Thus, it appears that the BNST and stria terminalis do very little processing of sexual relevant olfactory information. These regions appear to act mainly as a relays of information, since lesions and knife cuts in these regions produce basically the same effects that lesions of

their respective nuclei in the amygdala produce in the rat and hamster (Emery & Sachs, 1976; Lehman et al., 1983; Lehman & Winans, 1983; Paxinos, 1976; Valcourt & Sachs, 1979).

Medial Preoptic Area and Hypothalamus

Lesions of the mPOA-AH cause major deficits in male copulatory behavior in all mammalian species studied thus far which includes mice (Meisel & Sachs, 1994). The roles of the mPOA are: 1) integrate afferent information; 2) affect the transition from precopulatory behavior (*e.g.* sexual arousal behaviors, female investigation, ultrasound production) to initiation of copulatory behavior (mount, intromission, and ejaculatory behaviors); 3) modulate arousal state of the male 4) regulate penile reflexes

The mPOA receives information indirectly from almost all sensory systems of the animal via limbic and brainstem sites: 1) olfactory input via the medial amygdala and bed nucleus of the stria terminalis, 2) auditory, visual and somatosensory input from the neocortex via the ventral subiculum and lateral septum, 3) visceral and genital input from the central tegmental field, nucleus of the solitary tract, and the A1 noradrenergic region of the brain stem (Simerly & Swanson, 1986). The mPOA also has reciprocal connections with all these regions, and therefore may modulate sexually relevant information coming from these regions.

A major role of the mPOA is to initiate copulatory behavior to sexually relevant stimuli. For example, in the Swiss-Webster strain of house mouse, lesions of the mPOA eliminated intromissions, and ejaculations in all males tested, and mounting in all but one male was eliminated (Bean, Nunez, & Conner, 1980). In contrast, arousal and motivation was far less affected. For example, the proportion of males displaying ano-genital investigation was not affected by mPOA lesions, but the latency to start ano-genital investigation was increased

(Bean et al., 1980). Furthermore, ultrasound production by the male to a stimulus female or bedding soiled by female urine was not disrupted by mPOA lesions. These same effects on sexual behaviors were seen in B6D2F1 male mice by blocking protein synthesis in the POA with cyclohexamine (Quadagno, Albelda, McGill, & Kaplan, 1976). Males that received implants of cycloheximide in the POA still investigated the genital region of the female, but significantly fewer of these males mounted, gained intromission and ejaculated.

In other species, more discrete lesions reveal that POA-AH may regulate several measures of copulatory behavior. In the rat, dorsal parastriatal lesions of the POA decrease the percent of male that ejaculated (Arendash & Gorski, 1983). Further, dorsal and ventral lesions each eliminated ejaculation, while ventral lesions disrupted initiation of copulation (Kondo, Shinods, Yamanouchi, & Arai, 1990). In some species, small lesions in the POA eliminate copulatory behavior. For example, in the hamster, lesions of the region of the POA that receives caudal medial amygdala efferents eliminate copulation (Powers, Newman, & Bergondy, 1987). Further, in the gerbil small lesions that eliminate the sexually dimorphic nucleus (SDN) (a nucleus of cells that is larger and more darkly staining in males than it is in females) also eliminates copulatory behavior (Commins & Yahr, 1985b). In contrast, lesions of the SDN in the rat do not affect expression of copulatory behavior unless the rat was inexperienced (de Jonge, Louwerse, Ooms, Evers, Endert, & van de Poll, 1989).

Evidence that the POA plays a role in initiating and directing sexual behavior to female comes from studies with rats and monkeys where POA lesions eliminated copulation. Males with these POA lesions still engage in precopulatory behaviors (e.g. investigate, pursue and climb over females) and sometimes mount females (Hansen & Drake af Hagelsrum, 1984; Heimer & Larsson, 1966/67; Meisel, 1983). Further, both male rats and rhesus monkeys with

POA lesions continue to bar press to gain access to an estrous female, but exhibit very little female directed sexual contact. Further, rhesus monkeys with POA lesions continue to masturbate which means sexual motivation, motor output and reward pathways are still functional, but no longer directed towards the female. Thus, the mPOA appears to regulate initiation of female directed copulatory behavior.

There is also evidence to suggest the mPOA is part of neural circuits that regulate penile reflexes and seminal emission, and that dopaminergic innervation of the mPOA regulates these responses (Bazzett, Eaton, Thompson, Markowski, Lumley, & Hull, 1991; Hull, 1995; Hull, Eaton, Markowski, Moses, Lumley, & Joucks, 1992). Projections from the mPOA appear to facilitate penile reflexes, since stimulation of the POA increased penile reflexes (Hughes, Everitt, Lightman, & Todd, 1987). Furthermore, lesions in regions that mPOA project to also influence copulatory behavior and penile reflexes (paraventricular nucleus of the hypothalamus (PVN), median raphe, and nucleus paragigantocellularis) (Chiba & Murata, 1985; Marson & McKenna, 1990; Marson, Platt, & McKenna, 1993; Monaghan, Arjomand, & Breedlove, 1993; Monaghan & Breedlove, 1991; Yells, Hendricks, & Prendergast, 1992).

The projections of the mPOA have not been delineated in the mouse, therefore, most of the information presented will be from the rat. The mPOA projects to numerous regions, and appears to be able to modulate or regulate motivational, arousal, consummatory behaviors and penile reflexes.

Most regions that project to mPOA receive reciprocal projections from the mPOA. Therefore, olfactory information important for motivation and arousal coming from these rostral brain regions may be modulated by these reciprocal efferents of the mPOA.

A main projection from the mPOA appears to regulate the initiation of copulatory motor patterns as discussed above. This efferent projection of the mPOA is to the midbrain region via the medial forebrain bundle (MFB) (Swanson, 1976). This efferent pathway appears to project laterally from the mPOA to join the MFB and then project caudally to the midbrain, since only knife cuts lateral to the mPOA and lesions of the MFB caudal to the mPOA disrupt copulatory behavior (Caggiula, Antelman, & Zigmond, 1974; Hendricks & Scheetz, 1973; Scouten, Burrell, Palmer, & Cegavske, 1980; Szechtman, Caggiula, & Wulkan, 1978).

Another projection of the mPOA regulates penile reflexes and possibly seminal emission. This mPOA efferent projects to the PVN. In turn, the PVN projects to a group of motoneurons in the lumbosacral region of the spinal cord, the spinal nucleus of the bulbocavernosus (SNB) (Wagner & Clemens, 1991). This circuit from the mPOA to PVN to SNB appears to regulate penile reflexes (Argiolas, Melis, & Gessa, 1987; Bitran, Hull, Holmes, & Lookingland, 1988; Bjorklund, Lindvall, & Novin, 1975; Hull, Bitran, Pehek, Warner, Band, & Holmes, 1986).

Regions of the Brain that Concentrate Steroid Hormones and the Effects of Intracerebral Implants of Steroid Hormones

Lesions studies in conjunction with tract tracing studies have aided in elucidating distinct neural circuits that regulate copulatory behavior. However, these studies do not reveal which hormones are acting at which particular sites to facilitate copulatory behavior.

Brain regions that concentrate androgens

In the rat, neurons which concentrate T or DHT were determined by autoradiography (Sar & Stumpf, 1975). Both T and DHT produced similar patterns of accumulation. Androgens were accumulated in most of the brain regions known to be involved in copulatory behavior. In the telencephalon, T is accumulated in the olfactory bulbs, amygdala, with heaviest accumulation in the medial amygdala. In the diencephalon, heavy accumulations of androgens were seen in the mPOA, BNST, PVN, and lateral POA, and lighter accumulation in the anterior hypothalamus. In the mouse, although the olfactory bulbs were not were not sampled, the pattern of androgen accumulating cells in the telencephalon and diencephalon was similar to the rat (Luttge, 1975; Sheridan, 1978; Sheridan, Howard, & Gandelman, 1982). Additionally, there are numerous other regions that are not associated with copulatory behavior that also concentrate androgens in the telencephalon and diencephalon in both the mice and rats.

In the rat, androgen concentrating cells were also found throughout the midbrain and brainstem of the rat. These neurons were located in the central gray nucleus, tegmental nuclei, magnocellular reticular nuclei, and pontine nuclei (Sar & Stumpf, 1975; Sar & Stumpf, 1977). In addition, motor nuclei of the spinal cord (SNB and DLN) that are important for copulation concentrate androgens (Breedlove & Arnold, 1983).

Brain Regions that Concentrate Estrogens

In mice and rats, estrogen concentrating cells are located from the most rostral regions of the telencephalon to the brainstem (Sheridan, 1978; Stumpf & Sar, 1974/1975; Stumpf, Sar, & Keefer, 1974/1975). The regions that concentrate the most estrogen are also areas

that are important for regulating copulatory behavior. For example, the olfactory bulb, medial and cortical AM, BNST, lateral septum, mPOA, periventricular POA, anterior hypothalamus, diagonal band of Broca, VMH, dorsal raphe, and dorsomedial nucleus gigantocellularis contain cells that concentrate estrogen.

Effects of T microimplants

Microimplants of crystalline T in the mPOA (which likely stimulate BNST also) are the most effective stimulating copulatory behavior in rats, hamsters, and ferrets (Davidson, 1966; Tang & Sisk, 1991; Wood & Newman, 1993b). Wood and Newman (1993) were able to pinpoint with greater accuracy to regions of the brain that were being stimulated by their T microimplants by observing androgen receptor (AR) immunoreactivity (ir) in the area around the implants. AR-ir in castrate males is very weak compared to intact males, however, where the T had diffused from the implant and activated cells, AR-ir was as robust as intact males. By this method, they were able to determine that implants of T in the AM that were most effective in facilitating copulatory behavior were those that increased immunoreactivity in the dorsal medial nucleus of AM near the optic tract. It should be noted that sexual behavior may be reinstated by the microimplants, however, it is not restored to the level of the intact, in part due to the lack of peripheral steroid hormone stimulation (Wood & Newman, 1993b).

Although T implants elucidate the regions of the brain that are targets for the behavioral effects of steroids, they do not reveal whether androgenic or estrogenic stimulation is responsible for the behavioral effects. Implant studies using DHT and E2 demonstrate that the responsiveness to androgenic and estrogenic facilitation of copulatory behavior is site

specific. E2, but not DHT implants are effective in facilitating copulatory behavior in the mPOA or rats, and hamsters (Baum et al., 1982; Christensen & Clemens, 1974; Christensen & Clemens, 1975; Lisk & Greenwald, 1983). In contrast, DHT appears to be effective at stimulating copulatory behavior when implanted into the AM and lateral septum (Baum et al., 1982). However, E2 also facilitates copulation when implanted in the AM (Rasia-Filho, Peres, Cubilla-Gutierres, & Lucion, 1991).

Neural aromatase activity and copulatory behavior

Since T or E2 implants in the POA facilitate copulatory in male rats, and the ability of the anterior hypothalamic region of the brain to aromatize androgens to estrogens was established, it was suggested that the local aromatization of the T to E2 was responsible for the activation of copulatory behavior (Naftolin, Ryan, & Petro, 1972).

Christensen and Clemens (1975) demonstrated that local aromatization of T to E2 in the POA was necessary to activate mounting behavior in castrated male rats. Either T or E2 implanted directly into the POA-AH restores mount behavior in male rats that have stopped copulating. However, if 1,4,6-androstatriene-2-17-dione (ATD) (an aromatase inhibitor) is implanted 20 minutes prior to implantation of T, the facilitatory effects of T are blocked, however, ATD does not block the facilitatory effects E2 implants. Thus, it appears that estrogenic stimulation derived from the local aromatization of T to E2 within the POA-AH is necessary for expression of copulatory behavior.

Neural aromatase activity and the effects of castration

The ability to aromatize androgens to estrogens in discrete brain regions suggests that

estrogen concentrations within these regions may be independent of circulating levels of estrogens. Therefore, these local estrogen concentrations would be dependent on the amount of available substrate (aromatizable androgens) and/or the activity or quantity of the enzymes involved in the reaction.

In mammals, localization of brain regions that aromatized androgens to estrogens has been determined by dissecting discrete brain regions and measuring the rate of conversion of AE to estrone by radioassay. Aromatase activity was measured in regions of the brain that are associated with copulation, however, AA varies among these brain regions in the hypothalamus (HYP) and limbic systems (Roselli, Horton, & Resko, 1985; Selmonoff, Brodkin, Weiner, & Liiteri, 1977). Within the limbic system, the BNST had the greatest levels of AA followed the mAM and cAM. Neither the lateral septum nor the medial septum had measurable levels of AA. In the HYP, periventricular preoptic nucleus and POA had the greatest levels of AA. Intermediate levels of AA were measured in the anterior hypothalamus, periventricular anterior hypothalamus, VMH, and SCN. Low levels of AA were measured in lateral hypothalamus, dorsomedial nucleus and arcuate-median eminence region.

Other methods have been employed to localize aromatase and its activity. The use of antibodies to localize aromatase by immunohistochemistry has worked well in confirming localization AA in nonmammalian species, however, in mammals,
it has not been effective (Balthazart, Foidart, Surlemont, & Harada, 1991; Balthazart, Foidart, Surlemont, Vockel, & Harada, 1990; Sanghera, Simpson, McPhaul, Kozlowski, Conley, & Lephart, 1991; Shinoda, Kideo, Hisao, Soawa, & Shiotani, 1989). For example, aromatase immunoreactivity is absent or extremely sparse in the POA and BNST of mice and rats where AA has been demonstrated by other methods (Roselli, Ellinwood, & Resko, 1984; Roselli et

al., 1985; Roselli & Resko, 1984; Schleicher, Stumpf, Drews, & Sar, 1986a; Schleicher, Stumpf, Morin, & Drews, 1986b; Sheridan, 1978).

There are two distinct categories of AA. One type is regulated by gonadal hormones, while the other is not influenced by gonadal hormones (Callard, Mak, & Solomon, 1986; Reddy, Naftolin, & Ryan, 1973; Roselli et al., 1984; Roselli et al., 1985; Roselli, Horton, & Resko, 1987a; Roselli & Resko, 1984; Roselli & Resko, 1986; Roselli & Resko, 1989; Roselli, Salisbury, & Resko, 1987b). For example, in the rabbit, castration increased AA in block dissections of the HYP and amygdala and anterior hippocampus (Reddy et al., 1973). However, in the male rat, when AA was measured by taking more discrete dissections of the brain, levels of AA are reduced in the BNST, mAM, periventricular preoptic nucleus, medial preoptic nucleus, VMH, anterior hypothalamus and suprachiasmatic nucleus following castration (Roselli et al., 1985). In contrast, AA was not influenced by castration or T replacement in the cortical amygdala, periventricular nucleus of the anterior hypothalamus, arcuate nucleus/median eminence region, lateral preoptic nucleus, and dorsal medial nucleus. Thus, potential for aromatization of androgens to estrogen in specific brain nuclei exists after castration.

There is also evidence that regulation and distribution of neural AA in the adult ferret is determined by organizational effects of steroid hormones on the fetal brain in utero (Krohmer & Baum, 1989). Organizational effects of steroids are also responsible for influencing levels of sexual and aggressive behavior in adult mice which are activated by estrogenic stimulation (vom Saal, Grant, McMullen, & Laves, 1983). Therefore, since castration does not eliminate aromatizable androgens, differences among individuals that continue to copulate after castration and those that do not may be related to levels of AA

activity in brain regions important for copulation.

Estrogen Receptors

One way in which steroid hormones alter cellular activity (and behavior) is via activation of receptors classified as ligand activated transcription factors that regulate genomic activity (Evans, 1988). For example, E2 binds to an estrogen receptor (ER) forming a steroid-receptor complex (S-R). Two S-R's dimerize and then attach to specific gene regulatory binding sites on the DNA within the nucleus where they alter the gene's activity. This in turn, leads to a change in protein synthesis and presumably behavior. Since estradiol restores and maintains sexual behavior in castrated males of a number of species and the effects of can be blocked in castrated male by ER antagonists, activation of ER's most likely play a role in male copulatory behavior (Callard et al., 1986; Carroll et al., 1988; Christensen & Clemens, 1974; Christensen & Clemens, 1975; Gorzalka et al., 1975; Wee et al., 1988).

Location of estrogen receptor in copulatory behavior neural circuits

ER's have been identified throughout the neural circuits that regulate copulatory behavior of males in numerous mammals (Commins & Yahr, 1985a; Fox, Ross, Handa, & Jacobson, 1991; Koch & Ehret, 1989; Lieberburg, MacLusky, & McEwen, 1980; Nordeen & Yahr, 1983; Shughrue, Bushnell, & Dorsa, 1992; Vito, DeBold, & Fox, 1983; Whalen & Olsen, 1978: Wood, 1992; Wood & Newman, 1993b). The location of ER's has been determined by biochemical assays (receptor binding) and histochemical techniques (uptake of radiolabeled ligand (reviewed earlier); immunocytochemistry and In situ hybridization for mRNA).

By immunocytochemistry ER-ir cells have been visualized in the mPOA, ventral region of the lateral septum, medial division of the BNST, lateral region of VMH, arcuate nucleus, and ventral premammillary nucleus. In the amygdala ER-ir was seen in the anterior cAM, mAM, central AM, basal AM magnocellular division (Fox et al., 1991; Koch & Ehret, 1989; Wood, Brabec, Swann, & Newman, 1992; Wood & Newman, 1993b).

Measuring ER

ER levels within a region may be quantified by two basic methods, each with it own set of limitations. ER levels may be determined within a region by an estrogen receptor exchange assay (MacLusky, Roy, Shanabrough, & Eisenfeld, 1986; Roy & McEwen, 1977). This assay measures the total amount of ER, however, since the tissue is homogenized, precision of cell location is lost.

ER levels are measured in two populations: nuclear ER (ERn) and cytosolic ER (ERc). These populations of ER's are the product of differential centrifugation. ERn are located in the nuclear pellet of the centrifugation and are presumed to be ER's that are dimerized S-R and bound to DNA. Since these are thought to be bound to the DNA, ERn levels may indicate the amount of estrogenic stimulation the target tissue is receiving. In contrast, ERc's represent the population of receptors that is detected in the cytosolic fraction of the centrifugation. These receptors may be bound to estrogen, but are not bound to the DNA and are not regulating gene expression at that time (Gorski, Welshons, Sakai, Hansen, Walent, Kasis, Shull, Stack, & Campen, 1986). Immunocytochemical evidence suggests that there is a significant proportion of ER's in the cytoplasm, and therefore, the ERc fraction may not be as artifactual as once thought (Wood & Newman, 1993b). Total ER (ERt) of a region

may be calculated by summing the ERn and ERc, if both populations of ER are expressed as a ratio of DNA in the pellet. Using this method, the dynamics of ER regulation and activity of a region can be determined. However, determining in which cells ER levels are measured in is not possible.

In contrast, in situ hybridization, a method which visualizes the levels of ER mRNA per cell, allows for the possibility of determining in which cells the transcription of ER message is being altered and under what conditions. On the other hand, determining the levels of ER mRNA does not indicate the amount of ER that has been transcribed or the functional state of the ER population.

Like in situ hybridization, immunocytochemistry visualizes the location of ER-ir cells. Additionally, this method may be used in conjunction with other immuno labeling to visualize other substances (AR, neuropeptides, c-fos etc...) that are colocalized in the cell or with tract tracing methods to determine afferents and efferents of the cells (Wood et al., 1992; Wood & Newman, 1993a). Furthermore, the localization of the ER within the cell can be visualized as well, although it is difficult to determine the activity of ER and the ratios of bound and unbound receptor.

Effects of castration and hormonal regulation of ER

In male rats, gonadal hormones cause a down regulation of ER. Castration does not affect the number of cells that express ER mRNA in the periventricular POA, MPN or BNST. In fact there is a trend towards increased number of cells that express ER mRNA following castration (Lisciotto & Morrell, 1993; Morrell, Wagner, Malik, & Lisciotto, 1995). Further, the relative amount of mRNA per cell increased in castrated males (Lisciotto & Morrell,

1993; Simerly & Young, 1991). When castrated males were treated with T, ER mRNA levels were restored to the level of intact males. Although in situ hybridization demonstrates that ER mRNA is increased by castration, it tells nothing about the actual levels or state of the ER. ER assays demonstrate that castration causes a reduction in ERn in POA, HYP (HYP) and AM, but an increase in ERc (Roselli, Thornton, & Chambers, 1993). These data suggest ERt may increase, but less estrogenic stimulation is produced due to the reduction in ERn. This repartitioning of ER is supported by immunocytochemistry. In intact males, ER-ir is practically entirely confined to the nucleus, however in castrate males, ER-ir is found in both cytoplasmic and nuclear regions of the cell (Wood & Newman, 1995; Wood & Newman, 1993b).

Summary and Purpose

In the genetically homogeneous B6D2F1 hybrid strain of house mouse, some males continue to copulate after castration while others do not. Although it appears that continued copulation after castration in B6D2F1 males is independent of gonadal hormones, there is evidence that suggests that steroid hormones, in particular estrogens, may be necessary for the continued copulation after castration. First, estrogenic stimulation appears important since E2 restores copulatory behavior to B6D2F1 males that have stopped copulating. Second, castration does not eliminate T from the circulation of B6D2F1 males, and in other species E2 levels are not affected by castration. Third, since B6D2F1 males are genetically homogenous, these differences in behavioral responsiveness to E2 are likely due to differences in steroid physiology of steroid-regulated behavioral neural circuits in the adult. These physiological differences among individuals, could be brought by pre- or neonatal steroid

hormones which organize neuronal function and morphology of steroid responsive regions of the brain. Both distribution and levels of ER and aromatase have been shown to been under the influence of the pre- or neonatal organizational effects of steroid hormones. Therefore, levels of aromatase and/or ER may differ in regions of the brain that regulate copulatory behavior among B6D2F1 males that differ in expression of copulatory behavior after castration. The following experiments investigate the role of estrogen related physiology in the maintenance of copulatory behavior after castration. The results indicate that retention of behavior after castration is dependent on aromatization of nongonadal androgens to estrogens, and that the source of these nongonadal hormones is not the adrenal gland.

EXPERIMENT 1: LEVELS OF SERUM STEROID HORMONES IN INTACT AND CASTRATED CONTINUER AND NONCONTINUER MALES

In castrated B6D2F1 continuer and noncontinuer males, serum T levels do not differ (Clemens et al., 1988). Although castration reduced T to less than 10% of intact levels, it was not eliminated entirely. In rats and other species, castration has little or no affect on E2 levels (Table 2). Therefore, it is possible that serum steroids of nongonadal origin maintain copulatory behavior after castration in B6D2F1 males. Since T is the precursor molecule for the synthesis of both DHT and E2, and administration of DHT or E2 to noncontinuers restores copulatory behavior (Sinchak & Clemens, 1990; Wee et al., 1988), the possibility exists that circulating levels of DHT or E2 may account for behavior differences between continuer and noncontinuer males. Thus, continuers and noncontinuers may differ in the conversion of T to DHT and/or E2 which would result in a difference in the circulating levels of one or both of the neurally active metabolites of T.

A difference in circulating levels of DHT or E2 between continuer and noncontinuer males would affect the levels of steroid hormone stimulation that would facilitate copulation. Differences in DHT levels could have effects by two avenues. First, differences in DHT could directly affect the amount of androgenic stimulation received that facilitates copulatory behavior. Second, since DHT regulates aromatase activity in some regions of the central nervous system, differences in DHT may result in changes in the rate at which estrogen is synthesized (Roselli et al., 1985; Roselli & Resko, 1984). Furthermore, E2 levels may differ

between males that continue to copulate and those that do not in the absence of differences in DHT levels.

The purpose of this study was to determine if nongonadal T, DHT, and/or E2 are present after castration to stimulate copulatory behavior, and if so do levels of these hormones differ between continuers and noncontinuer B6D2F1 males.

METHODS

Subjects:

Sixty-day old male B6D2F1 hybrid house mice (Mus musculus) (the cross of a C57BL/6J female and a DBA/2J male), were purchased from Jackson Laboratory, Bar Harbor, ME. All males were individually housed in 7 x 11 x 4h (inches) plexiglas cages while in the colony and maintained on a 14:10 light-dark cycle with lights out at 11:30AM. Food and water were available ad libitum except during behavioral testing.

Sexual Behavior Tests

Behavioral testing was performed during the dark phase of the light cycle under red illumination between 12:30-5:30PM. Behavioral scoring and testing procedures were as described previously (Clemens, et al., 1988) except that the experimental males were tested in their home cages (Wee & Clemens, 1989). The home cage of the male was placed in the testing room 30 minutes prior to the start of testing. The cage top was removed and replaced with an inverted cage top to prevent animals from escaping. Testing started by placing a hormone-primed stimulus female into the male's cage. If the male did not achieve intromission or display intromissive-like behavior within 10 minutes after introduction of the

female, the first female was replaced by another stimulus female. Again if the male failed to intromit within 10 minutes, the female was replaced by a third and final stimulus female. If intromission was not achieved within 30 minutes, the male's test was terminated.

If intromission did occur, the test continued until 1) the male ejaculated, 2) 40 minutes elapsed between succesive intromissions, or 3) 4 hr passed from the time of first intromission, at which time the test was stopped even if the male was still sexually active. The stimulus female was replaced with another stimulus female, if she became unreceptive anytime during the course of testing. The pine chip bedding (Alpha Chips) in the home cage was changed after the completion of each behavioral test so that the male had two-week old bedding at the time of behavioral testing. However, during extended periods without behavioral testing, the bedding was changed on a weekly basis. The data were recorded with a TRS-80 Model 4P computer using software described by Rakerd et al., (1985).

Adult intact C57BL/6J female mice were used as stimulus females. They were group housed in the same colony room as the experimental males. Sexual receptivity was induced in the females by sequential subcutaneous injections of 17ß-estradiol benzoate (60μg) 48 and 24 hours before testing and progesterone (0.6mg) 4 hours before testing. All hormones were delivered in 0.03ml sesame oil.

Six biweekly tests for copulatory behavior with a sexually receptive female were given to the males prior to castration or sham surgery. Surgeries were performed under methoxyflurane anesthesia (Metofane; Pittman-Moore, Inc.) one week after the sixth behavioral test. Since continuous testing is not necessary for the maintenance of copulatory behavior in castrated B6D2F1 males (Wee & Clemens, 1989), behavior testing resumed twelve weeks after surgery and continued on a biweekly schedule until 38 weeks after

castration to assure that maintenance of copulatory behavior in castrated males was robust. Prior to sacrifice, males were grouped according to their surgical treatment and post-castration behavior exhibited in the last six behavioral tests after surgery. The criteria used for determining behavioral groups were:

Intacts - Intact males that received sham castration surgery and exhibited copulatory behavior in all of the last six behavior tests (int).

Continuers - Castrated males that exhibited the ejaculatory reflex on the last behavior test and on 4 of 5 preceding tests (cont).

Noncontinuers - Castrated males that did not exhibit copulatory behavior in any of the last 6 behavior tests (mount, intromissive and ejaculatory) (nonc) (Figure 1).

Those males that did not meet the criteria for the above experimental groups were eliminated from the study. All assays had eight animals per experimental group and were performed without knowledge of experimental group and were decoded after statistical analyses were performed.

Steroid Hormone Radioimmunoassay (RIA)

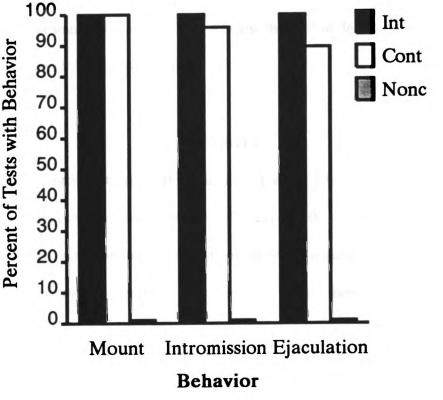
Within one week after their last behavioral test, the mice were sacrificed by decapitation. Trunk blood was collected from each animal individually and allowed to coagulate in an ice bath. The blood was centrifuged and serum was collected and quickly frozen. The coded serum samples were shipped on dry ice to the Hormone Assay Core of the Population Research Center at University of California, Los Angeles to determine the concentrations of T, DHT and E2 by RIA. The brains of these males were excised and placed on aluminum foil on dry ice to quickly freeze the brains and stored at -80°C for ER and AA

assays (Exp 3).

To monitor recovery, tracer amounts of [^{3}H]T/E2, DHT/estrone or androstenedione were added to alternate serum samples and then the serum was extracted with diethyl ether (10:1 v/v). The organic phase was separated from the aqueous phase and dried under a stream of dry, filtered air. The dried extract was then solubilized in 0.5ml of nanograde isooctane. Samples were then applied to celite chromatography columns for fractionation (Abraham, 1977).

T and E2 were analyzed in an [^{125}I]-RIA with reagents obtained from ICN Biomedicals, Inc. (Costa Mesa, CA) and counted in a micromedic 4/600 gamma counter with automatic data reduction software (RIA AID; Robert Maciel and Associates, Inc., Arlington, MA 02174). Standard curves were calculated using the four parameter logistic option. DHT was analyzed in a [^{3}H]-RIA utilizing charcoal separation methods with reagents from ICN Biomedicals, Inc, and counted in a LS355 liquid scintillation counter. Data were calculated using the software described above.

Figure 1. The percent of the last six copulatory behavior tests with behavior (mount, intromission, and ejaculation) for intact (Int) and castrated B6D2F1 continuers (Cont). Each bar represents the total number of tests with behavior for all males within a behavioral group divided by the total number of tests for all males. The noncontinuer group is not represented since sexual behavior was not exhibited by these males (refer to text for definitions of behavioral groups).


100
90
80
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Percent of Tests with Behavior
Int
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Intromission
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Figure 1

The within assay coefficient of variation at the ED90, ED50, and ED20 were respectively for T (10.8, 16.4, and 16.8%), for DHT (7.4, 16.1, and 11.5%), and for E2 (12.6, 12.2, and 5.5). Between assay error was not applicable, since all hormones were run in one assay. The limits of detection for the RIA were as follows: T, 0.07ng/ml; DHT, 0.05 ng/ml; E2, 19.20 pg/ml.

RIA data were analyzed by parametric 1 way analysis of variance (ANOVA) followed by post hoc Newman-Keuls' multiple range test (N-K) for ANOVA's with F values associated with p's $\leq .05$. For statistical purposes, values below the limit of detection of the assay were assigned the value of the limit of detection.

RESULTS

Castration significantly reduced but did not eliminate T in the serum of continuer and noncontinuer males compared to intact males ($F(2,21) = 9.88$, $p < .05$; N-K $p < .01$) (Table 3). T levels did not differ between castrated continuer and noncontinuer behavioral groups (Table 3). T levels were below the limits of detection in only three of the castrate males (2 noncontinuers and 1 continuer).

Castration did not affect the level of serum E2 in either continuer or noncontinuer males compared to intact males, nor were there differences between the continuer and noncontinuer behavioral groups, ($F(2,21) = 0.108$) (TABLE 3). E2 levels were below the limits of detection in only 1 noncontinuer male.

Castration did not affect the level of serum DHT in either continuer or noncontinuer males compared to intact males, nor were there differences between the continuer and noncontinuer behavioral groups, ($F(2,21) = 1.95$) (TABLE 3). DHT levels were below the

limits of detection in 4 intact, 4 noncontinuer, and 6 continuer males. Therefore the results of this assay may not reflect actual physiological values.

SUMMARY

Results of this experiment indicate that nongonadal T and E2 hormones are present after castration in B6D2F1 males. Serum T levels were decreased by castration but were not different between castrated continuer and noncontinuer males as seen previously (Clemens, et al., 1988). Serum E2 and DHT levels were not affected by castration in either continuer or noncontinuer B6D2F1 males.

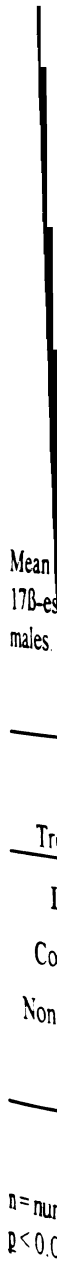

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Table 3

Mean (+/- s.e.m.) concentration of testosterone (T), dihydrotestosterone (DHT), and 17ß-estradiol (E2) in serum of intact and castrated continuer and noncontinuer B6D2F1 males.

		Steroid		
Treatment	n	T (ng/ml)	DHT (ng/ml)	E2 (pg/ml)
Intact	8	3.426 (1.045)	0.095 (0.018)	83.8 (12.6)
Continuer	8	0.140 (0.022)**	0.070 (0.014)	90.0 (16.7)
Noncontinuer	8	0.137 (0.023)**	0.058 (0.006)	96.4 (25.8)

n = number of subjects per group. ** = significantly less than intact serum steroid level (N-K $p < 0.01$).

EXPERIMENT 2: DETERMINATION IF MAINTENANCE OF COPULATORY BEHAVIOR AFTER CASTRATION IN B6D2F1 MALES IS DEPENDENT ON THE AROMATIZATION OF NONGONADAL ANDROGENS TO ESTROGENS

The fact that castration does not affect serum E2 level or eliminate T from the circulation of B6D2F1 males may indicate that maintenance of copulatory behavior after castration is facilitated by the continued presence of these hormones. E2 stimulation has been shown to be critical for the expression of copulatory behavior. For example, in noncontinuer B6D2F1 males, E2 treatment restores copulatory behavior (Wee et al., 1988). Further, in castrated male rats, the aromatization of androgens to estrogens in the POA-AH is necessary for the facilitation of mounting behavior (Christensen & Clemens, 1975). Therefore, it is possible that endogenous nongonadal estradiol may facilitate copulation in castrated continuer B6D2F1 male mice.

To test whether non-gonadal estrogens play a role in the retention of the ejaculatory reflex after castration in the male B6D2F1 house mouse, the aromatase inhibitor, ATD, that blocks the enzymatic aromatization of testosterone to 17ß-estradiol, was administered to continuer males.

METHODS

Male mice (Mus musculus) of the B6D2F1 hybrid strain purchased from Jackson Laboratories (Bar Harbor, ME) were housed individually and maintained on a 14L:10D light-dark cycle, with lights on at 2130 hours. Commercial chow (Breeder Blox) and water were

available ad libitum except during testing. The males were allowed to adjust to the colony room two weeks prior to the beginning of the experiment and were approximately 45 days of age at the start of behavioral testing. A total of 77 males completed the 47-week experiment.

Adult Swiss Webster females were used as stimulus females for behavioral testing and were made sexually receptive by sequential, subcutaneous injections of estradiol benzoate (60 μg, 48 hours prior to testing) and progesterone (0.6mg, 4 hours prior to testing). Both hormones were administered in .03cc of sesame oil vehicle. The females were grouped house in the same colony room as the experimental males.

Surgical Procedures

The males were anesthetized using methoxyflurane (Metofane; Pittman-Moore, Inc.) for both castration and silastic implant surgeries. Castration was performed via a single midline scrotal incision. Silastic capsules were implanted subdermally between the shoulder blades through a small incision made in the nape of the neck.

Silastic Capsules

Silastic capsules (1.47 i.d. x 1.96 o.d. mm) 17mm in length were filled with ATD (Steraloids, Wilton, NH) or no steroid (Blank). The ends of the capsules were plugged with Dow silastic silicone cement. Capsules were dried overnight in a 37° C oven and weighed prior to implantation. The capsules were not incubated before implanting.

Behavioral Testing

Behavioral testing was conducted between 1200 and 1700 hours under red light illumination. Behavioral scoring and testing procedures were similar to Clemens et al. (1988), a brief summary is presented here. Testing of the experimental males took place in their home cage rather than an arena (Wee & Clemens, 1989). The pine chip bedding (Alpha Chips) in the home cage was changed after the completion of each behavioral test so that the male had two week old bedding at the time of behavioral testing. However, during extended periods without behavioral testing, the bedding was changed on a weekly basis. The data were recorded on a TRS-80 Model 4P computer (Rakerd, Brigham, & Clemens, 1985).

Males were given 6 biweekly tests for copulatory behavior, and castrated one week after the sixth behavioral test. Four biweekly tests commenced one week after surgery, then the males were not tested for 5 weeks. Behavioral testing was resumed and a final series of eleven biweekly tests were given. Within 24hrs after the eighth test after surgery, the castrated males were divided into three behavioral groups based on their performance in the last four copulatory behavior tests. Assignment to these three groups was based upon three criteria:

1) Continuers displayed ejaculatory reflex in at least 3 of the 4 tests;

2) Intermediates displayed ejaculatory reflex in less than 3 of the 4 tests;

3) Noncontinuers displayed no ejaculatory reflex in the last 4 tests.

Males were randomly assigned to either the ATD or blank implant treatment group and subsequently implanted subcutaneously with a 17mm blank or ATD filled silastic capsule. Implant treatment continued through the last 3 biweekly behavioral tests. At the time of the implant, all males were treated for mites with 0.04% rotenone solution (Canex, Pittman-

Moore, Inc.) in mineral oil vehicle. A stripe of the solution was painted on the back of each male with a cotton swab applicator.

Statistical Analysis

Percentage data for the last six behavioral tests were analyzed by nonparametric statistics (Siegel, 1956). The effects, within treatment group over time, of ATD or sham treatment on the percent of males that mounted, intromitted or ejaculated for the three weeks prior to and after treatment were analyzed by Cochran's Q test. For Cochran's Q tests with $p \leq 0.5$, within group comparisons of percent of males that exhibited behavior were made between the last test prior to silastic capsule implantation (-24hr test) and each of the three tests after implantation by post hoc Fisher exact probability test. Between treatment group comparisons of blank and ATD silastic implanted within the continuer, intermediate, and noncontinuer behavioral groups were analyzed by Chi^2 analysis for independent samples between the -24hr test and the three post-implant behavioral tests. Since, the number of males that continued to show intromissive and ejaculatory behaviors after ATD treatment were so few, only the mount latency data of the ATD and sham treated continuer groups were analyzed by parametric two-way ANOVA.

RESULTS

There was a main effect of time from implantation on expression of the ejaculatory reflex within each treatment (Cochran Q ATD - $p < 0.001$ 1t; 26.67, df = 5; Shams - $p < 0.05$ 1t; 9.8889, df = 5). ATD significantly reduced the percent of continuer males that achieved an ejaculatory reflex 4 and 6 weeks after initiation of ATD treatment when compared to their

-24hr pre-ATD implant test performance (Fisher exact test: $p = 0.041$; $p = 0.0045$ 1t; respectively). The percent of sham continuer males achieving an ejaculatory reflex on the test conducted 6 weeks after implant surgery was significantly reduced compared to their -24 hour preimplant test (Fisher exact test: $p < 0.049$, 1t). However, the percent of ATD treated continuer males that achieved an ejaculatory reflex was significantly less than the blank implant continuer group 4 and 6 weeks after implantation (Fisher exact test: $p = 0.042$ and $p = 0.011$ respectively) (Figure 2).

The percent of continuers that achieved intromission within each treatment was affected over time after implantation (Cochran Q: ATD - $p < 0.005$ 1t; 19.69, df = 5; Shams - $p < 0.025$ 1t; 12.5, DF = 5). ATD significantly reduced the percent of continuer males that achieved intromission 4 and 6 weeks after ATD treatment when compared to their -24hr pre-ATD implant test performance (Fisher exact test: $p = 0.037$; $p = 0.012$ 1t; respectively). The percent of sham continuer males that achieved intromission on the test conducted 6 weeks after implant surgery, was significantly reduced compared to their -24 hour preimplant test (Fisher exact test: $p = 0.049$, 1t). However, the percent of ATD treated continuer males that achieved an ejaculatory reflex was significantly less than the blank implant continuer group 4 weeks after implantation (Fisher exact test: $p = 0.043$) (Figure 2).

The percent of continuers that mounted after implantation was not affected over time (Cochran Q: ATD - $p < 0.30$ 1t; $Q = 5.0$, df = 5; Shams - $p < 0.30$ 1t; $Q = 5.0$, df = 5). Further, ATD and blank continuer implant groups did not differ in the percent of tests with a mount ($X^2 = 9.859$ df = 11) or the latency to mount ($F = 0.073$; df = 1,88) over all six tests prior to and after implantation. However, there was a significant effect of the repeated testing on the latency to mount for both ATD and blank continuer implant groups post implantation

($F = 4.041$; $df = 3,88$; $p < 0.025$) (Figures 2 & 3).

There was a main effect of repeated testing over the last six tests (three prior to implantation and three post implantation) on the percent of intermediate males that achieved an ejaculatory reflex, achieved intromission or mounted (Cochran Q: ejaculatory reflex: $p < 0.001$ 1t; $Q = 21.67$, df. = 5; intromission: $p < 0.001$ 1t; $Q = 21.67$, $df = 5$; mount: $p < 0.02$ 1t; $Q = 14.32$, $df = 5$) (data not presented). However, compared to last test prior to implantation (-24hr test), ATD had no effect on the percent of intermediates that achieved an ejaculatory reflex or mounted 2, 4, and 6 weeks after implantation (Fisher exact test: ejaculatory reflex: $p = 0.297$, $p = 0.11$, and $p = 0.11$; mount: $p = 0.50$, $p = 0.12$, and $p = 0.12$ respectively). Compared to -24hr test, ATD reduced the percent of intermediates that achieved intromission 4 and 6 weeks after implantation (Fisher exact test: 2 weeks $p = 0.322$, $p < 0.05$, and $p < 0.05$ respectively) (data not presented).

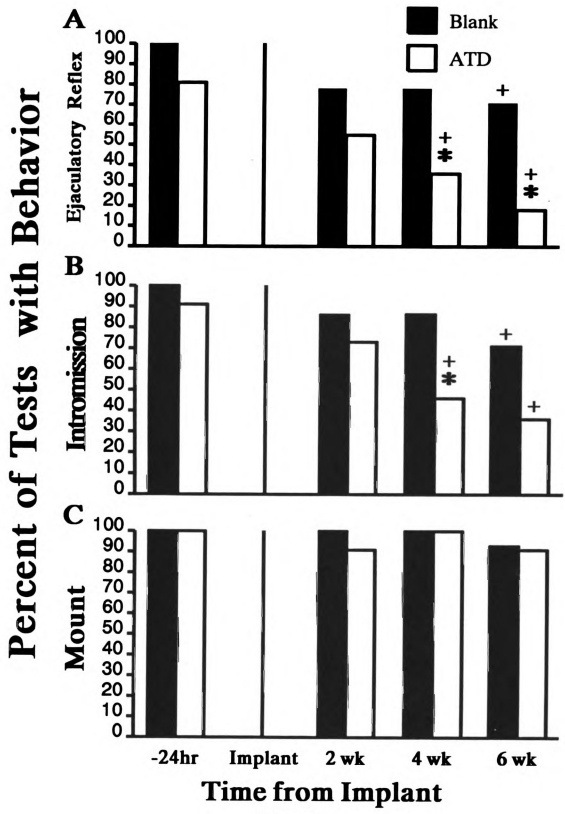
Repeated testing over the last six tests did not affect the percent of ATD noncontinuer males that achieved an ejaculatory reflex, achieved intromission or mounted (Cochran Q: ejaculatory reflex, $p > 0.30$ 1t; $Q = 5.0$, $df = 5$; intromission: $p > 0.10$ 1t; $Q = 8.07$, $df = 5$; mount: $p > 0.20$ 1t; $Q = 7.27$, $df = 5$) (data not presented).

SUMMARY

The results of the present study are consistent with the idea that nongonadal estrogens contribute to the maintenance of copulatory behavior after castration in male B6D2F1 hybrid house mice. When synthesis of E2 was inhibited with ATD, fewer continuer B6D2F1 males achieved ejaculations and intromissions when compared to their pretreatment test as well as to continuers that received blank silastic capsules. In contrast, inhibition of estrogen synthesis

had no effect on the percent of continuer males that mounted nor on their latency to mount.

Figure 2. Percent of continuer B6D2F1 males that expressed behavior A) ejaculatory reflex, B) Intromission, C) Mount per test 24 hours (hr) prior to and 2, 4, and 6 weeks (wk) after ATD or blank silastic capsule implant. * = significantly less than blank silastic implant group on that test ($p < 0.05$). + = significantly less than preimplant (-24 hour) test within treatment group ($p < 0.05$).


A
Blank
ATD
Ejaculatory Reflex
100
90
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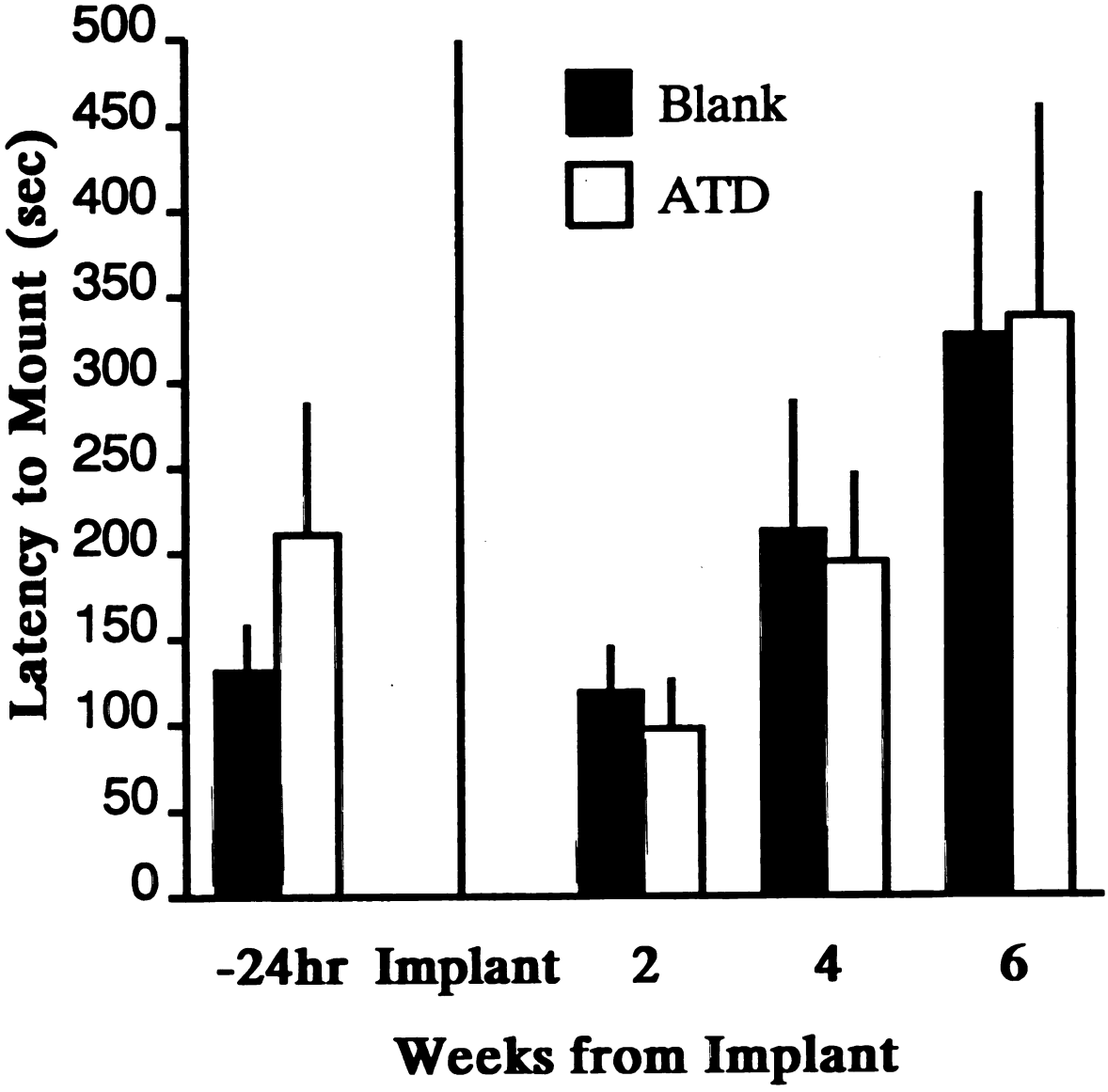
+
*
B
Intromission
C
Mount
-24hr
Implant
2 wk
4 wk
6 wk
Time from Implant
Percent of Tests with Behavior

Figure 2

Figure 3. Mount latency (sec) +/- (s.e.m.) for blank silastic capsule and ATD treated continuer B6D2F1 males 24 hours (hr) prior to and 2, 4, and 6 weeks (wk) after ATD or blank silastic capsule implant.


500
450
400
350
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250
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Latency to Mount (sec)
Blank
ATD
-24hr
Implant
2
4
6
Weeks from Implant

Figure 3

EXPERIMENT 3: DETERMINATION OF AROMATASE ACTIVITY IN POA, HYP AND AM OF INTACT AND CASTRATED CONTINUER AND NONCONTINUER B6D2F1 MALES

Since inhibition of aromatase activity reduced copulatory behavior in continuer males, and continuers and noncontinuers do not differ in serum steroid levels, it is possible that continuers and noncontinuers differ in the levels of neural aromatase in regions of the brain that are important for copulatory behavior which could result in difference in E2 concentration within those regions among continuers and noncontinuers. Since aromatization of T to E2 implanted directly into the POA-anterior hypothalamus (AH) is all that is necessary to restore mounting to castrated male rats that have stopped copulating, it is possible that noncontinuers lack the, or have a reduced ability, to convert aromatizable androgens to estrogens in the POA or other regions of the brain important for copulation (Christensen & Clemens, 1974; Christensen & Clemens, 1975).

In rats, levels of aromatase activity (AA) within the POA-AH are dependent on gonadal androgens (Roselli et al., 1984; Roselli et al., 1985; Roselli & Resko, 1984). Castration reduces AA in the POA of the rat and AA is restored with androgen treatment. Therefore, even though serum E2 concentrations are maintained after castration, it is possible that estrogenic stimulation is reduced in POA-AH because castration lowers local AA as well as the availability of aromatizable androgens (T). Thus, it is possible that behavioral differences between continuer and noncontinuer B6D2F1 male house mice produced by castration may be due to differences in the ability to aromatize androgens to estrogens within

the POA.

The medial amygdala (mAM) has also been shown to concentrate estrogens, contain ER, aromatize androgens, express aromatase immunoreactivity, and project efferents to the POA that are part of a neural regulatory pathway of male copulatory behavior (Akesson, Simerly, & Micevych, 1988; Callard et al., 1986; Koch & Ehret, 1989; Roselli et al., 1985; Sanghera et al., 1991; Sheridan, 1978; Simerly, Chang, Muramatsu, & Swanson, 1990; Stumpf & Sar, 1974/1975; Winans et al., 1982). In rodents, the amygdala (AM) receives olfactory and vomeronasal information from the main and accessory olfactory bulbs respectively (Scalia & Winans, 1975). Reproductively relevant olfactory bulb sensory information increases expression of the immediate-early gene c-fos in mAM in male rats (Baum & Everitt, 1992). Further, this olfactory and vomeronasal information received by mAM is in part responsible for mating induced increases in c-fos levels of the POA and for the regulation of male copulatory behavior (Baum & Everitt, 1992; Krettek & Price, 1978). Thus, it is likely that estrogen acting in the mAM facilitates male copulatory behavior, and that in B6D2F1 males castration may induce differences in estrogen synthesis in the AM between continuers and noncontinuers.

The following experiment investigates the possibility that continuers and noncontinuers differ in the ability to aromatize available androgens to estrogen in regions of the brain that are important for copulatory behavior (POA, hypothalamus (HYP) and AM) by measuring aromatase activity (AA) in these brain regions.

METHODS

Subjects

The males for this study were the same males from experiment one.

Brain Tissue Preparation

The brain was quickly excised from the cranium, frozen on dry ice, and shipped overnight to Dr. Roselli in Oregon to measure AA, cytosolic ER (ERc), nuclear ER (ERn) and total ER (ERt). On the day of the assays (2 days after sacrifice) the brains were placed ventral side up on a cold platform and three sequential coronal cuts were made: 1) approximately 1mm anterior of the optic chiasm, 2) immediately posterior to the optic chiasm and 3) immediately posterior to the mammillary bodies. The POA dissection extended from the medial septum to the caudal border of the optic chiasm, bilaterally to the lateral border of the supraoptic nucleus and dorsally to the superior border of the third ventricle. The HYP dissection extended from the optic chiasm to the mammillary bodies, bilaterally to the optic tract and dorsally to the superior border of the third ventricle. The AM dissection consisted of the temporal cortex lying immediately adjacent to the HYP and having the same anterior to posterior boundaries.

Aromatase Activity Assay

AA was quantified with a radiometric assay that measures the stereospecific loss of tritium from the C-1ß position of [^{3}H-1ß]androstenedione and its incorporation into ^3H_2O which is produced in proportion to the amount of estrogen formed (Roselli et al., 1984). This assay has been previously validated for mouse brain (Wozniak, Hutchinson, & Hutchison,

1992).

Briefly, brains tissues were homogenized in 250 µl ice-cold TEGD buffer (10mM Tris, 1.5mM EDTA, 10% glycerol, 1 mM dithiothreitol, pH 7.4). The homogenates were centrifuged at 1,000x g for 10 min. The pellets from this first spin (1,000x g pellets) were reserved for the ERn assay. The low-speed supernatants were harvested and centrifuged for 10 min. at 106,000x g to generate cytosols (106,000x g supernatants) and mixed mitochondrial-microsomal pellets (106,000x g pellets). These pellets were suspended in 30 volumes of phosphate buffer (10 mM KPO_4, 100 mM KCl, 1 mM EDTA; pH 7.4) and sonicated. Aliquots were then incubated for 1 h at 37°C with 0.3 µM [^{3}H-ß1]androstenedione. The reactions were stopped with 10% trichloroacetic acid containing 20 mg/ml charcoal, and the ^3H_2O generated was purified on small Dowex columns. The reaction exhibited Michaelis-Menton kinetics (Vmax = 89.8 fmol/h·mg protein; Km = 9.0 nM).

Aromatase activity data were analyzed by parametric 3x3 way ANOVA (brain region by behavioral group) followed by post hoc one way ANOVA and N-K for ANOVA's with F values associated with p's ≤ .05. Behavioral group codes were not revealed until statistical analyses were complete.

RESULTS

There was a main effect of behavioral group on AA levels ($F(2,63) = 22.39$, $p < 0.0001$). Further, there was a main effect of brain region on AA ($F(2,63) = 122.08$, $p < 0.0001$). However, there was no significant interaction of between behavioral group and brain region ($F(4,63) = 2.31$, $p = 0.068$). Between brain regions within each behavioral

group, AA levels in the AM were greater than both POA and HYP, and POA AA levels were greater than HYP (intact, $F(2,63) = 56.35$, $p < 0.001$; continuer, $F(2,63) = 38.59$, $p < 0.001$; noncontinuer, $F(2,63) = 31.74$, $p < 0.001$; all N-K, $p < 0.05$).

Within brain region, AA was significantly decreased in the POA, HYP, and AM of castrated continuer and noncontinuer B6D2F1 males compared to intact males, (POA, $F(2,63) = 17.59$, $p < 0.001$; HYP, $F(2,63) = 17.71$ $p < 0.001$; AM, $F(2,63) = 7.98$, $p < 0.001$; all N-K $p < 0.05$) (Figure 4). Within each brain region AA was not different between castrated continuer and noncontinuer males.

SUMMARY

The results of this experiment support the idea that aromatizable androgens of nongonadal origin may be converted to estrogens in regions of the brain important for copulation. Following castration, AA in POA, HYP, and AM was reduced but not eliminated. In none of the three brain regions was the level of AA correlated with the maintenance of copulatory behavior after castration in continuer and noncontinuer B6D2F1 males.

Figure 4. Mean (+/- s.e.m.) aromatase activity levels (fmol/h/mg protein) in the POA, HYP, and AM for intact (Int), and castrated B6D2F1 continuers (Cont) and noncontinuers (Nonc). * = significantly less than intact treatment group (N-K $p < 0.05$). + = significantly greater than other brain regions within behavioral group (N-K $p < 0.05$).

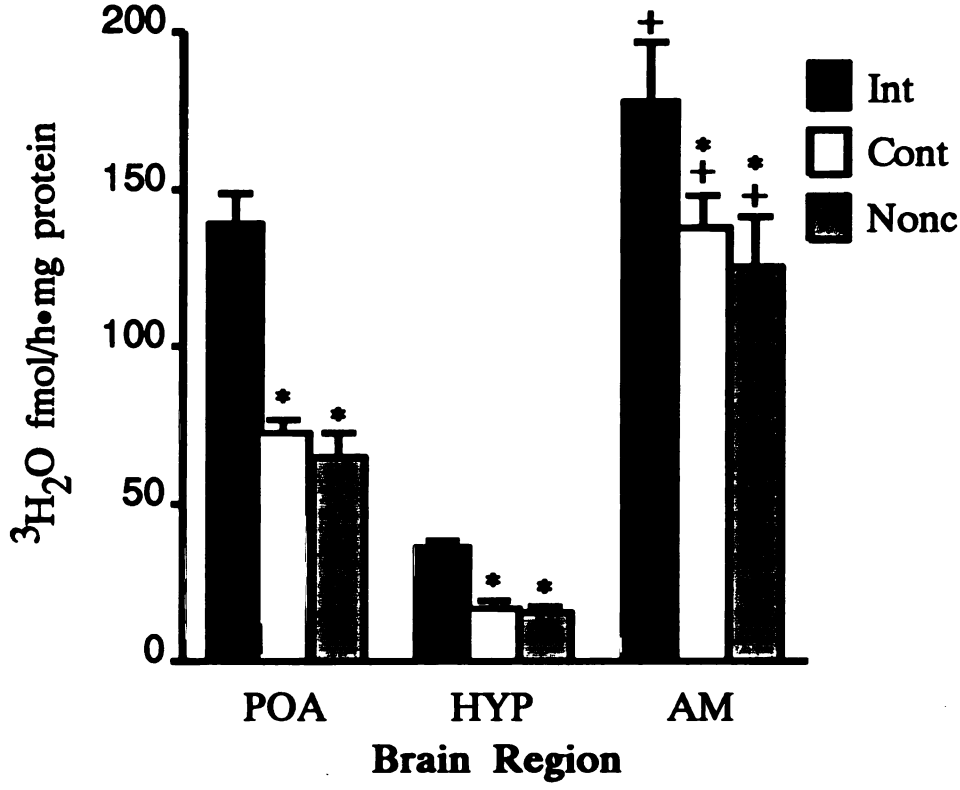
200
150
100
50
0
^3H_2O fmol/h•mg protein
Int
Cont
Nonc
POA
HYP
AM
Brain Region

Figure 4

EXPERIMENT 4: ESTROGEN RECEPTOR LEVELS IN THE POA, HYP AND AM OF INTACT AND CASTRATED CONTINUER AND NONCONTINUER B6D2F1 MALES

EXPERIMENT 4A: THE EFFECTS OF CASTRATION AND BEHAVIORAL STATUS ON ESTROGEN RECEPTOR LEVELS IN THE POA, HYP AND AM OF INTACT AND CASTRATED CONTINUER AND NONCONTINUER B6D2F1 MALES

Since continuers and noncontinuers do not differ in serum T, E2, or DHT levels, or in neural aromatase activity, it seems likely that they may differ in their responsiveness to these hormones. One measure of responsiveness to E2 that may differ between continuers and noncontinuers is the levels of estrogen receptors (ER) in regions of the brain important for copulatory behavior.

One way in which steroid hormones alter cellular activity (and behavior) is via activation of receptors that regulate genomic activity. For example, E2 binds to an estrogen receptor (ER) forming a steroid-receptor complex (S-R). Two S-R's dimerize and then attach to specific gene regulatory binding sites on the DNA within the nucleus where they alter the gene's activity. This in turn, leads to a change in protein synthesis and presumably behavior.

In earlier rat studies, nuclear estrogen receptor (ERn) levels in POA were reduced by castration and were associated with circulating androgen levels (Krey, Kemel, & McEwen, 1980). This reduction in ERn in the mPOA was also seen in continuer and noncontinuer B6D2F1 males, however, ERn levels did not differ between these behavioral groups (Clemens et al., 1988). Unfortunately, ERc levels were not measured in these B6D2F1 males, which

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may have revealed possible differences in the dynamics of ER regulation in the POA between continuer and noncontinuer males (Clemens et al., 1988). For example, testicular hormones appear to down regulate ER. In rats, castration increased ER mRNA levels, which were returned to intact levels in some brain regions by T and/or E2 treatment (Lauber, Mobbs, Muramatsu, & Pfaff, 1991; Lisciotto & Morrell, 1993; Simerly & Young, 1991). Alternatively, castration may elicit a slower turnover or degradation rate of ER protein. The actual amount of ER synthesized, used and degraded per unit of time cannot be determined by our method of analysis. However, if there were difference in ER turnover between continuers and noncontinuers it would be revealed in the ERn to ERc ratios.

In addition, only ERn in the mPOA was measured by Clemens et al., (1988). It is known that the AM of a number of species contains ER, synthesizes estrogen from circulating androgens and is responsive to estrogen (Lieberburg & McEwen, 1977; Roselli & Resko, 1984; Sanghera et al., 1991; Schleicher et al., 1986b; Sheridan, 1978; Simerly et al., 1990; Stumpf & Sar, 1974/1975; Winans et al., 1982).

The purpose of this experiment was to extend the findings of Clemens, et al., (1988) to determine if there are differences in ER levels and dynamics in POA, HYP and AM between castrated continuer, noncontinuer, and intact B6D2F1 male house mice by measuring ERn, ERc, and ERt levels.

METHODS

Estrogen Receptor Assay

ERc and ERn measurements were made using modifications of previously described methods (MacLusky et al., 1986; Roy & McEwen, 1977). Cytosols (106,000x g pellets)

were adjusted to 225µl total volume. To measure ERc, 100µl aliquots were incubated for 2h at 0-4C with 2 nM[2,4,6,7 ^{3}H]-E2 with or without a 200-fold excess of radioinert E2.

To measure ERn, the crude nuclear pellets were mixed with Cellex (5mg/25 µl TEGD buffer) and washed twice with 200µl TEGD buffer followed each time by centrifugation at 15,600 x g for 5 min in an IEC Centra-M microcentrifuge (International Equipment Co., Needham Heights, MA). ERn were salt extracted by suspending the washed pellets in 115 µl TEGDB buffer (TEGD buffer + 0.5 mM bacitracin) and an equal amount of TEGDB buffer containing 0.8 M KCl was added to give a final salt concentration of 0.4 M. After a 30 min extraction period, the tubes were centrifuged at 15,600 x g for 5 min. Aliquots (100 µl) of the supernatant (nuclear extract) were incubated for 5h at 25C with 2nM [^{3}H]E_2 with or without a 200-fold excess of radioinert E2. The DNA contents of the washed nuclear pellets were estimated by the diphenylamine method (Giles & Myers, 1965).

Bound [^{3}H]E2 was separated from free steroid on Sephadex LH-20 columns. Specific binding was calculated by subtracting nonspecific binding (measured in the presence of excess radioinert E2) from total binding (measured in the absence of excess radioinert E2). Results for both ERc and ERn are expressed as femtomoles (fmol) of [^{3}H]E_2 bound per mg DNA. Total binding (ERt) was calculated as the sum of ERc + ERn.

Estrogen receptor assay data were analyzed by parametric 3x3 way ANOVA (brain region by behavioral group) followed by post hoc one way ANOVA and N-K for ANOVA's with F values associated with p's $\leq$ .05. Behavioral group codes were not revealed until statistical analyses were complete.

RESULTS

Nuclear Estrogen Receptors

There was a main effect of brain region on ERn levels, ($F(2,59) = 3.361$, $p < 0.05$) with a significant interaction between region and behavioral group ($F(4,59) = 2.462$, $p = 0.05$).

Between brain regions within intact males, ERn levels in the AM were greater than POA and HYP ($F(2,59) = 7.286$, $p < 0.01$; N-K $p < 0.01$). However, there were no differences in ERn levels between brain regions of the continuer ($F(2,59) = 0.312$) and noncontinuer ($F(2,59) = 0.767$) males.

ERn levels in the AM were significantly decreased in the castrated continuer and noncontinuer behavioral groups compared to intact males ($F(2,59) = 7.19$, $p < 0.01$; all N-K $p < 0.05$) however, ERn levels were not significantly different between intacts and castrated continuers and noncontinuers in the POA and HYP (POA, $F(2,59) = 0.310$; HYP, $F(2,59) = 0.264$) (Figure 7). Within each brain region ERn levels were not significantly different between castrated continuer and noncontinuer males.

Cytosolic Estrogen Receptors

There was a main effect of behavior group and brain region on ERc (behavior, $F(2,59) = 23.074$, $p < 0.001$; region, $F(2,59) = 14.221$, $p < 0.001$). However, there was not an interaction between behavioral group and brain region ($F(4,59) = 1.100$).

Between brain regions, ERc levels were significantly greater in the AM compared to the POA and HYP of continuer and noncontinuer males (continuer, $F(2,59) = 13.91$; $p < 0.005$; noncontinuer, $F(2,59) = 6.201$; $p < 0.005$; all N-K $p < 0.05$). In intacts, ERc levels

in the AM were greater than the POA (intacts, $F(2,59) = 5.524$; $p < 0.05$; N-K $p < 0.05$) (Figure 6).

Within brain region, ERc levels of castrated continuer and noncontinuer males were significantly greater in POA and AM compared to intact males (POA, $F(2,59) = 4.56$, $p < 0.05$; AM, $F(2,59) = 8.00$, $p < 0.005$, all N-K $p < 0.05$). In HYP there was a main effect of behavioral group on mean ERc levels ($F(2,59) = 4.06$; $p < 0.05$). ERc were significantly increased in HYP of noncontinuers compared to intacts (N-K $p < .05$), while ERc levels in the continuers were not significantly different from either noncontinuers or intacts (Figure 6).

Total Estrogen Receptors

There were main effects of behavioral group on ERt ($F(2,59) = 10.46$, $p < 0.001$) and brain region ($F(2,59) = 23.45$, $p < 0.001$), but no significant interaction between behavioral group and brain region ($F(4,59) = 0.570$).

Between brain regions, AM ERt levels within each behavioral group were significantly greater than in both POA and HYP (N-K $p < 0.05$), however there were no differences in ERt levels between POA and HYP.

Within brain region, both continuer and noncontinuer males had greater ERt levels in the POA, HYP, and AM than intact males (POA, $F(2,59) = 3.568$, $p < 0.05$, HYP, $F(2,59) = 3.934$, $p < 0.05$; AM, $F(2,59) = 4.514$, $p < 0.05$; all N-K $p < .05$) (Figure 7).

SUMMARY

Castration did not affect the level of ERn in POA and HYP, while in the AM ERn were significantly reduced after castration. Overall, castration increased ERt in POA, HYP,

and AM. Although castration altered steroid levels, AA, and ER dynamics, none of these changes were associated with maintenance of copulatory behavior after castration in continuer males compared to noncontinuer males.

Figure 5. Mean (+/- s.e.m.) nuclear estrogen receptor (ERn) levels (fmol/mg DNA) in the POA, HYP, and AM for intact (Int) and castrated B6D2F1 continuers (Cont) and noncontinuers (Nonc). * = significantly less than intact treatment group, (N-K $p < 0.05$). + = significantly greater than other brain regions within behavioral group (N-K $p < 0.05$).

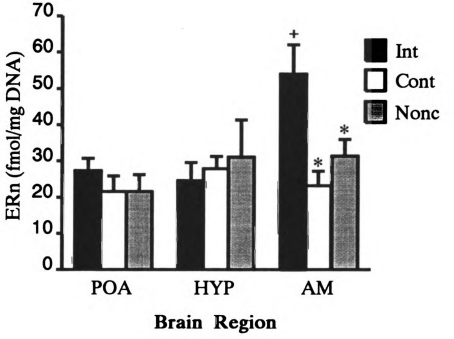

70
60
50
40
30
20
10
0
ERn (fmol/mg DNA)
+
*
*
Int
Cont
Nonc
POA
HYP
AM
Brain Region

Figure 5

Figure 6. Mean (+/- s.e.m.) cytosolic estrogen receptor (ERc) levels (fmol/mg DNA) in the POA, HYP, and AM for intact (Int) and castrated B6D2F1 continuers (Cont) and noncontinuers (Nonc). * = significantly greater than intact treatment group (N-K $p < 0.05$). + = significantly greater than other brain regions within behavioral group (N-K $p < 0.05$). ▲ = significantly greater than HYP within behavioral group (N-K $p < 0.05$).

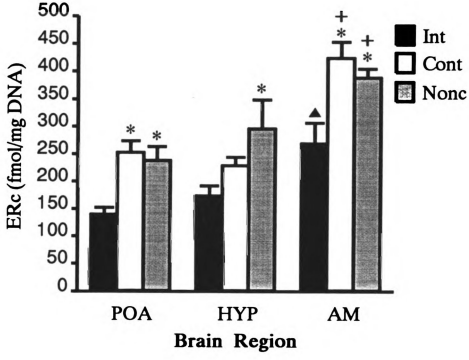

500
450
400
350
300
250
200
150
100
50
0
ERc (fmol/mg DNA)
Int
Cont
Nonc
POA
HYP
AM
Brain Region

Figure 6

Figure 7. Mean (+/- s.e.m.) total estrogen receptor (ERt) levels (ERn + ERc) (fmol/mg DNA) in the POA, HYP, and AM for intact (Int) and castrated B6D2F1 continuers (Cont) and noncontinuers (Nonc). * = significantly greater than intact treatment group (N-K $p < 0.05$). + = significantly greater than other brain regions within behavioral group (N-K $p < 0.05$).

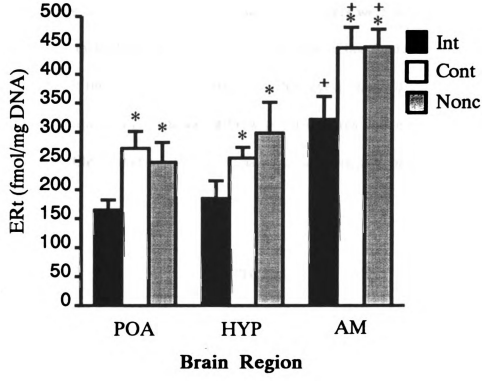

500
450
400
350
300
250
200
150
100
50
0
ERt (fmol/mg DNA)
Int
Cont
Nonc
POA
HYP
AM
Brain Region

Figure 7

EXPERIMENT 4B: COMPARISON OF ER MEASURED IN THE POA, HYP, AND AM BETWEEN NUCLEAR PELLETS PURIFIED WITH SUCROSE AND NUCLEAR PELLETS NOT PURIFIED IN INTACT AND CASTRATED B6D2F1 MALES

In EXP 4A castration did not affect the level of ERn in the region of the POA of continuer or noncontinuer B6D2F1 males. These data are in contrast to earlier work, where mPOA ERn levels of castrated continuers and noncontinuers were significantly decreased compared to intact males (Clemens et al., 1988). Because of this discrepancy, EXP 4B was designed to verify the effects of castration on ERn levels obtained in EXP 4A, and to determine if ER measures are equivalent between procedures where the nuclear pellet is sucrose purified (Clemens et al., 1988) compared to not purifying the nuclear pellet (EXP 4A).

METHODS

Male B6D2F1 hybrid house mice, 60 days old, were castrated or sham castrated and housed individually. Five weeks after surgery the male mice were sacrificed by decapitation. Their brains were excised from the cranium, frozen on dry ice and shipped to Dr. Roselli. Each experimental group consisted of six animals.

In order to compare Clemens et al., (1988) preparation of the nuclear pellet by sucrose purification (sucrose purified) to our washed pellet procedure in EXP 4A (non-sucrose purified), each brain tissue sample was homogenized in 250 µl ice-cold TEGD buffer (10mM Tris, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, pH 7.4) and centrifuged at 1,000

x g for 10 min. The 1,000 x g pellet (crude nuclear pellet) was either washed twice with TEGD buffer as in EXP 4A (non-sucrose purified) or purified through sucrose before extraction as in Clemens et al., (1988). For sucrose purification, the pellets were first resuspended in 25 µl of low sucrose buffer (1mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, 1mM DTT, 10% glycerol, pH 6.8) that contained 5mg of Cellex 410 (BioRad Laboratories, Richmond, CA.). An additional 200 µl high sucrose buffer (1mM KH_2PO_4, 2.1 M sucrose, 3 mM $MgCl_2$, 1mM DTT, 10% glycerol, pH 6.8) was added to make a 2 M sucrose solution. This suspension was centrifuged at 60,000 x g for 10 min. to obtain a purified nuclear pellet. Both the washed and purified nuclear pellets were extracted with 0.4 M KCl and aliquots (100 µl) used to measure ERn. Receptor measurements were performed using 2 nM [2,4,6,7-^{3}H]-E2 in the absence (total binding) or presence (nonspecific binding) of a 200-fold excess cold E2. Bound [^{3}H]-E2 was separated from free steroid on Sephadex LH-20 minicolumns. The DNA contents of the washed nuclear pellets were estimated by the diphenylamine method (Giles & Myers, 1965).

Statistical Analysis

The DNA content of the nuclear pellet was analyzed with a 3x2x2 way ANOVA to make comparisons between brain region, purification procedure, and surgical treatment and analyzed by post hoc N-K for ANOVA's with $p \leq .05$. The ERn levels of the nuclear pellet were analyzed with a 3x2x2 way ANOVA to make comparisons between brain region, purification procedure, and surgical treatment and analyzed by post hoc N-K for ANOVA's with $p \leq .05$. Significant main effects of purification were analyzed post hoc by 1 way ANOVA within brain regions and N-K's for ANOVA's with $p < 0.05$. Individual

measurements that did not produce positive values were omitted from the statistical analyses.

RESULTS

DNA Recovery

There was a main effect of sucrose purification of the nuclear pellet with no interaction with surgical treatment, brain region or surgical treatment and brain region (ANOVA purification - $F(1,60) = 59.7$, $p < .0001$; $p = 0.78$; purification-surgical treatment - $F(1,60) = 1.49$, $p = .2382$; purification-brain region - $F(2,60) = 1.798$, $p = .1745$; purification-surgical treatment-brain region - $F(2,60) = 1.072$, $p = .3489$)(Figure 8). Sucrose purification significantly reduced the mean amount of DNA (μg) recovered from the nuclear pellet within each brain region (N-K $p < 0.05$). The mean (+/- s.e.m.) amount of DNA (μg) recovered from the purified nuclear pellet was 63% (+/- 4.3) of the total amount extracted from the nonpurified nuclear pellet (Figure 8).

There was a main effect of brain region on DNA recovery levels (ANOVA $F(1,60) = 3.253$, $p = 0.0456$) with no interaction with surgical treatment (ANOVA $F(2,60) = 1.933$, $p = 1.537$) (Figure 8). There was no main effect of surgical treatment on the recovery of DNA from the nuclear pellet (ANOVA surgical treatment - $F(1,60) = 0.077$) (Figure 8).

ERn

There was a main effect of purification of the nuclear pellet on the mean concentration of ERn (fmol/mg DNA) (ANOVA $F(1,42) = 5.907$, $p = 0.0194$). Purification had no effect on ERn levels in POA and HYP, (ANOVA POA, $F(1,42) = 1.099$; HYP, $F(1,42) = 0.737$). However, in the AM there was a significant effect of purification (ANOVA AM, $F(1,42) =$

5.351; $p < 0.05$) (Figure 9).

There was a main effect of brain region on the levels of ERn (ANOVA $F(2,42) = 9.318$, $p = 0.0004$). The AM had greater levels of ERn compared to POA and HYP (N-K, $p < 0.05$).

There was no main effect of castration (5 weeks) on the mean level of ERn (fmol/mg DNA) (ANOVA $F(1,42) = 0.085$).

Summary EXP 4B

Sucrose purification reduced the amount of DNA recovered from the nuclear pellet, and reduced ERn levels in the AM but not in the POA and HYP compared to nonpurified nuclear pellets. In spite of these reductions in DNA recovery and AM ERn levels by sucrose purification, sucrose purified and nonpurified nuclear pellets produced equivalent results in ERn levels with respect to surgical treatment and brain region. These findings are consistent with our results from EXP 4A.

SUMMARY

The results of EXP 4 support the notion that brain regions important for copulatory behavior in castrated B6D2F1 males continue to receive estrogenic stimulation that may facilitate the maintenance of copulatory behavior. However, changes in ER levels after castration were not associated with the maintenance of copulatory behavior in castrated continuer B6D2F1 males. Differences in the effects of castration on ERn levels in POA of B6D2F1 continuer males between Clemens et al. (1988) and EXP 4A were not due to differences in preparation of the nuclear fraction prior to measurement of ERn.

Figure 8. Mean (+/- s.e.m.) levels of DNA (μg) in the nonpurified (N) and sucrose purified (S-P) nuclear pellet of brain tissue from the POA, HYP, and AM of intact (Int) and castrated (Cast) B6D2F1 males.

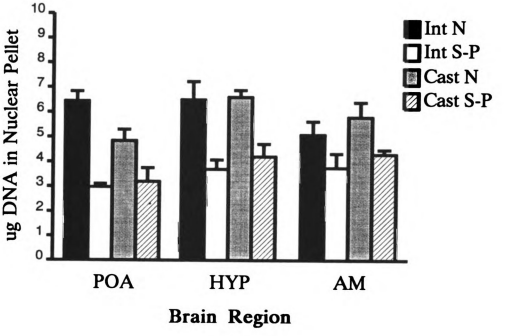
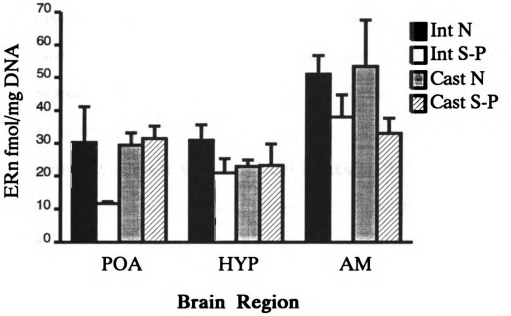

ug DNA in Nuclear Pellet
10
9
8
7
6
5
4
3
2
1
0
Int N
Int S-P
Cast N
Cast S-P
POA
HYP
AM
Brain Region

Figure 8

Figure 9. Mean (+/- s.e.m.) nuclear estrogen receptors (ERn) levels (fmol/mg DNA) of nonpurified (N) and sucrose purified (S-P) nuclear pellet of brain tissue from the POA, HYP, and AM of intact (Int) and castrated (Cast) B6D2F1 males.


70
60
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AM
Brain Region

Figure 9

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EXPERIMENT 5: DETERMINATION OF AROMATASE ACTIVITY LEVELS IN POA, HYP AND AM OF INTACT AND CASTRATED C57BL/6J, DBA/2J AND B6D2F1 MALES

Continuer and noncontinuers cannot be distinguished by levels of serum androgens or 17ß-estradiol nor by levels of AA or ER levels in the POA, HYP, and AM (EXP 1-4). However, in mice, reproductive behavior, physiology, neuronal anatomy and the effects of castration on these phenotypes are known to be influenced by genotype. For example, castration abolishes copulatory behavior in both C57Bl/6J and DBA/2J strains of mice. However, compared to DBA's, C57 males stop achieving an ejaculatory reflex sooner after castration. In contrast, most B6D2F1 males continue to copulate for six months to over a year after castration (Clemens et al., 1988; McGill & Manning, 1976). Other related behaviors also differ between these strains. DBA, C57 and B6D2F1 males differ in expression and retention of aggressive behavior after castration (Sinchak & Clemens, 1988; Wee, Weaver, Sinchak, & Clemens, 1985). Additionally, DBA and C57 males differed in levels of infanticide (Svare, Kingsley, Mann, & Broida, 1984).

DBA, C57 and B6D2F1 strains also differ from one another physiologically and morphologically in a number of reproductively related measures, as well as their responses to castration. For example, testicular weight, spermatogenesis, cholesterol concentration, testosterone levels and glycolipid make-up differ between DBA/2J males and C57Bl/10J strains (Bartke, 1974; Bartke & Shire, 1972; McCluer, Deutsch, & Gross, 1983; Shire & Bartke, 1972). The weight of the bulbocavernosus (BC) muscle does not differ among intact

C57Bl/6J, DBA/2J and B6D2F1 males, however, castration reduces the mass of the BC in C57 and B6D2F1 males compared to DBA males (Wagner, Popper, Ulibarri, Clemens, & Micevych, 1994). Furthermore, DBA males have fewer motoneurons in the spinal nucleus of the bulbocavernosus (SNB). Levels of CGRP in cells of the SNB also differ among these strains after castration (Wagner et al., 1994; Wee & Clemens, 1987).

The POA-anterior hypothalamus is important for regulation of reproductive behavior and physiology which includes copulatory behavior, scent marking, and secretion of gonadal hormones (Bean et al., 1980; Quadagno et al., 1976; Yahr, Commins, Jackson, & Newman, 1982). A major role of the mPOA is to facilitate copulatory behavior in response to sexually relevant stimuli. For example, in the Swiss-Webster strain of house mouse, lesions in the mPOA eliminated intromissions, and ejaculations (Bean et al., 1980). These same effects on copulatory behavior occur in B6D2F1 male mice by blocking protein synthesis in the POA with cyclohexamine (Quadagno et al., 1976).

Interestingly, regional POA morphology differs between C57 and DBA males. In the medial preoptic nucleus (mPON), DBA/2J male mice have a set of densely packed, darkly staining cells (medioventral pars compacta (MVPC)) that are not seen in C57BL/6J males (Robinson, Fox, & Sidman, 1985). The function of this set of neurons in not known. However, these gross morphological differences among strains that differ in expression of reproductive behavior suggest that other differences in cell physiology may exist between these strains that regulate behavior. Since retention of copulatory behavior after castration in B6D2F1 males appears estrogen dependent, and C57BL/6J and DBA/2J males stop copulating shortly after castration, the following experiment investigates if there are differences in estrogen synthesis (AA) in the POA, HYP, and AM of intact and castrated

C57BL/6J, DBA/2J, and B6D2F1 mice.

METHODS

Subjects

Adult male inbred C57BL/6J and DBA/2J, and hybrid house mice (Mus musculus), 60 days old, were purchased from Jackson Laboratory, Bar Harbor, ME. All males were individually housed while in the colony and maintained on a 14:10 light-dark cycle with lights out at 11:30AM. Food and water were available ad libitum. One week after arrival, each male was either castrated or sham castrated 18 weeks prior to removal and microdissection of the brain for AA assay (6 per treatment group).

Procedures for Aromatase Activity Assay

Procedures of tissue preparation and aromatase assay are detailed in EXP 3.

Brain Tissue Preparation

Briefly, the mice were sacrificed by decapitation, and each brain was quickly excised from the cranium and blocked by removal of the olfactory bulbs and cerebellum with a razor blade. The blocked brain was positioned dorsal side down on aluminum foil and placed on dry ice to rapidly freeze the brain. The brains were stored overnight on dry ice. Trunk blood was also taken for each animal upon decapitation. On the following day, (3 days after sacrifice), the brains were placed dorsal side down on ice and the preoptic area (POA), hypothalamus (HYP) and amygdala (AM) dissected.

Aromatase Activity Assay

AA was quantified with a radiometric assay that measures the stereospecific loss of tritium from the C-1ß position of [^{3}H-1ß]androstenedione and its incorporation into ^3H_2O which is produced in proportion to the amount of estrogen formed (Roselli et al., 1984).

Briefly, brains tissues were homogenized in 250 µl ice-cold TEGD buffer (10mM Tris, 1.5mM EDTA, 10% glycerol, 1 mM dithiothreitol, pH 7.4). The homogenates were centrifuged at 1,000x g for 10 min. The low-speed supernatants were harvested and centrifuged for 10 min. at 106,000 x g to generate cytosols (106,000 x g supernatants) and mixed mitochondrial-microsomal pellets (106,000 x g pellets). These pellets were suspended in 30 volumes of phosphate buffer (10 mM KPO_4, 100 mM KCl, 1 mM EDTA; pH 7.4) and sonicated. Aliquots were then incubated for 1 h at 37°C with 0.3 µM [^{3}H-ß1]androstenedione. The reactions were stopped with 10% trichloroacetic acid containing 20 mg/ml charcoal, and the ^3H_2O generated was purified on small Dowex columns. The reaction exhibited Michaelis-Menton kinetics in all three strains of mice (C57Bl/6J: intact, Vmax = 78.34 fmol/h·mg protein, Km = 10.10 nM; castrate, Vmax = 31.2 fmol/h·mg protein; Km = 5.92 nM; DBA/2J: intact, Vmax = 103.1 fmol/h·mg protein, Km = 4.37 nM; castrate, Vmax = 52.76 fmol/h·mg protein; Km = 24.3 nM; B6D2F1: intact, Vmax = 88.31 fmol/h·mg protein, Km = 7.47 nM; castrate, Vmax = 42.42 fmol/h·mg protein; Km = 4.97 nM).

Statistical Analysis

Aromatase activity data within brain region were analyzed by parametric 2 way ANOVA (genetic strain by gonadal status) followed by post hoc Student-Newman-Keuls' multiple range test (SNK) for ANOVA's with F values associated with p's $\leq$ .05.

RESULTS

In the POA, compared to intact DBA males, AA levels were lower in intact C57 and B6D2F1 males (ANOVA $F(2,30) = 7.84$, $p < 0.05$; SNK, $p < 0.05$)(Figure 10). Castration reduced AA in POA all three strains ($F(1,30) = 249.90$, $p < 0.05$; SNK, $p < 0.05$), and eliminated the differences in AA among the strains ($F(2,30) = 3.78$, $p < 0.05$; SNK, $p > 0.05$) (Figure 10).

Similarly, in the HYP, compared to intact DBA males, AA levels were lower in intact C57 and B6D2F1 males (ANOVA $F(2,30) = 4.61$, $p < 0.05$; SNK, $p < 0.05$)(Figure 10). Castration reduced AA in POA all three strains ($F(1,30) = 119.90$, $p < 0.05$; SNK, $p < 0.05$), and eliminated the differences in AA among the strains ($F(2,30) = 1.21$, $p = 0.312$) (Figure 10).

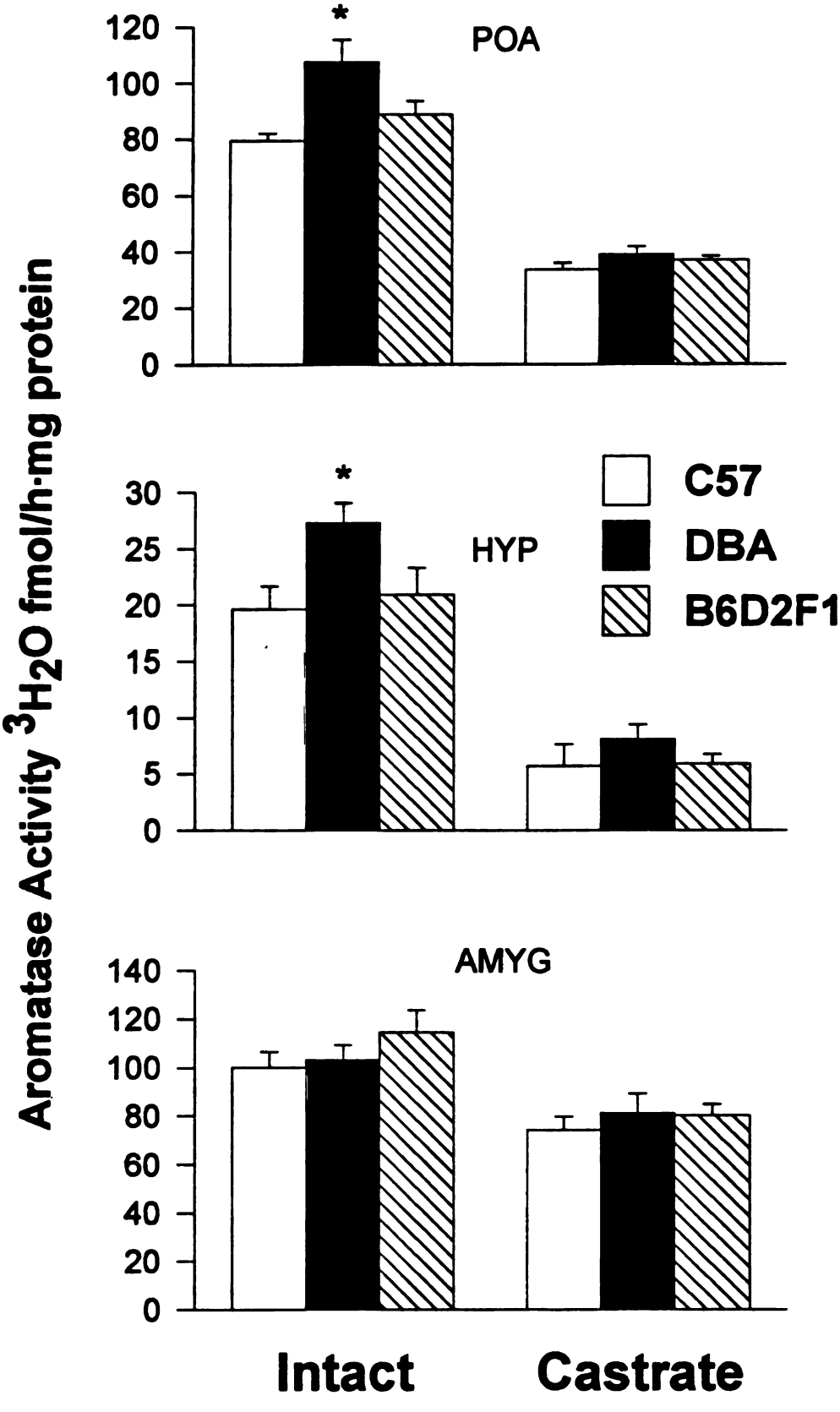
In the AM, AA levels did not differ among the three strains (ANOVA $F(2,30) = 1.104$, $p = 0.3446$). Castration significantly reduced AA in all three strains ($F(1,30) = 24.003$, $p < 0.05$), and as in the other brain regions, there was no difference in AA among the three strains of mice ($F(2,30) = 0.412$, $p = 0.6662$)(Figure 10).

SUMMARY

These data demonstrate that AA levels in some brain regions of intact male mice are influenced by genotype, while in another brain region AA did not vary among genotypes analyzed here. DBA/2J males had higher levels of AA in the POA and HYP compared to C57BL/6J and B6D2F1 males, however, in the AM, AA did not differ among the strains. Castration reduced AA levels in all three regions of the brain in all three strains and eliminated

differences among the strains in AA levels in the POA and HYP. Although the behavioral and physiological significance of different AA levels among strains of intact male mice is unclear, these differences in AA do suggest that there are physiological differences among these strains that may account for differences in behavioral and physiological responses among strains of mice.

Figure 10. Mean (+/- s.e.m.) aromatase activity levels (fmol/h/mg protein) in the preoptic area (POA), hypothalamus (HYP), and amygdala (AM) for intact (Int), and castrated C57BL/6J, DBA/2J and B6D2F1 male house mice. * = significantly greater than other strains within surgical treatment group (SNK, $p < 0.05$).


Aromatase Activity ^3H_2O fmol/h·mg protein
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DBA
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Figure 10

EXPERIMENT 6: SERUM CONCENTRATIONS OF TESTOSTERONE, ESTRADIOL AND DIHYDROTESTOSTERONE IN B6D2F1 INTACT, CASTRATE, AND CASTRATE-ADRENALECTOMIZED MALES

INTRODUCTION

A possible source of gonadal-like steroids in castrated B6D2F1 males is the adrenal gland. However, some castrated B6D2F1 males, as well as cats and dogs, continue to copulate after adrenalectomy (Beach, 1970; Cooper & Aronson, 1958; Schwartz & Beach, 1954; Thompson et al., 1976). Thus, other sources of steroid hormones may exist, since maintenance of copulatory behavior in B6D2F1 males appears estrogen dependent.

As reviewed earlier, the brain is a potential source of steroid hormone synthesis and E2 production (review, (Corpechot et al., 1981; Le Goascogne et al., 1987; Robel, Bourreau, Corpechot, Dang, Halberg, Clarke, Haug, Schlegel, Synguelakis, Vourch, & Baulieu, 1987; Robel, Synguelakis, Halberg, & Baulieu, 1986). The ability of the brain to produce measurable circulating levels of E2 is not unprecedented, since the brain of the zebra finch has been shown to be the birds major source of estrogen synthesis (Schlinger & Arnold, 1991; Schlinger & Arnold, 1992).

To determine if there are tissues other than the adrenal gland that synthesize T, DHT or E2, serum concentrations of T, DHT and E2 were measured by RIA in intact, castrate, and castrate-adrenalectomized B6D2F1 males.

METHODS

Subjects

Sixty-day old male B6D2F1 hybrid house mice (Mus musculus) were purchased from Jackson Laboratory, Bar Harbor, ME. All males were individually housed while in the colony and maintained on a 14:10 light-dark cycle with lights out at 11:30AM. Food and water were available ad libitum except during behavioral testing.

Surgical procedures

At approximately 150 days of age, all males were either castrated or sham castrated. Castration surgeries were performed under methoxyflurane anesthesia (Metofane; Pittman-Moore, Inc.). Adrenalectomies were performed six months after castration or sham castration surgery. Castrated and sham castrated males were either bilaterally adrenalectomized or sham adrenalectomized to produce the following groups:

1) sham castrated/sham adrenalectomized

2) sham castrated/adrenalectomized

3) castrated/sham adrenalectomized

4) castrated/adrenalectomized.

Males in the castrated/adrenalectomized group were divided into two recovery groups. Blood samples were taken from one set of males 3 days after adrenalectomy, and from the second set 14 days after adrenalectomy.

Each male was given a 0.05cc IP injection of 30mg/ml of pentobarbital. If this dose did not produce an adequate anesthetic state, methoxyflurane (Metophane; Pittman-Moore) inhalant was administered. Adrenalectomies were performed under a dissection microscope

at 10X. An incision was made on the lateral dorsal side of the abdomen just caudal to the ribs. The tissue ventral to the adrenal gland was clamped with toothed forceps and ligated ventral to the forceps with 0-4 silk. The musculature of the abdomen was sutured with catgut, and the skin was closed with wound clips. In a rare case where bleeding occurred that could be controlled, a small piece of Gel Foam was inserted in the abdominal cavity rostral to the kidney and the abdominal cavity was sutured closed.

Steroid Hormone Radioimmunoassay (RIA)

Males were anesthetized and approximately 1ml of blood was collected from the right ventricle of each animal individually and allowed to coagulate in an ice bath. The blood was centrifuged and serum was collected and quickly frozen. The coded serum samples were shipped on dry ice to the Hormone Assay Core of the Population Research Center at University of California, Los Angeles to determine the concentrations of T, DHT and E2 by RIA.

To monitor recovery, tracer amounts of [^{3}H]T/E2, DHT/estrone or androstenedione were added to alternate serum samples and then the serum was extracted with diethyl ether (10:1 v/v). The organic phase was separated from the aqueous phase and dried under a stream of dry, filtered air. The dried extract was then solubilized in 0.5ml of nanograde isooctane. Samples were then applied to celite chromatography columns for fractionation (Abraham, 1977).

T and E2 were analyzed in an [^{125}I]-RIA with reagents obtained from ICN Biomedicals, Inc. (Costa Mesa, CA) and counted in a micromedic 4/600 gamma counter with automatic data reduction software (RIA AID; Robert Maciel and Associates, Inc., Arlington,

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MA 02174). Standard curves were calculated using the four parameter logistic option. DHT was analyzed in a [^{3}H]-RIA utilizing charcoal separation methods with reagents from ICN Biomedicals, Inc, and counted in a LS355 liquid scintillation counter. Data were calculated using the software described above.

The within assay coefficient of variation were less than 6% for each assay. Between assay error was not applicable, since all hormones were run in one assay. The limits of detection for the RIA were as follows: T, 0.07ng/ml; DHT, 0.05 ng/ml; E2, 19.20 pg/ml.

RIA data were analyzed by parametric 1 way ANOVA followed by post hoc Newman-Keuls' multiple range test (N-K) for ANOVA's with F values associated with p's $\leq$ .05. For statistical purposes, values below the limit of detection of the assay were assigned the value of the limit of detection.

RESULTS

Serum T levels were reduced by adrenalectomy alone, castration alone and the combination of castration and adrenalectomy ($F(4,35) = 19.3$, $p < .0001$; N-K $p < .05$) (Figure 11). It appeared that castration alone reduced T levels further than adrenalectomy (N-K $p < 0.05$), however, T levels were not significantly different between adrenalectomized and the castrated/adrenalectomized groups. T levels were below the limits of detection in four of the castrated/sham males, one castrated/adrenalectomized 3 day males and four castrated/adrenalectomized 14 day males.

In contrast, castration, adrenalectomy, and the combination of castration and adrenalectomy had no effect on serum E2 levels compared to intact males ($F(4,36) = 0.463$, $p = 0.7627$) (Figure 12). E2 levels were below the limits of detection in only 1 intact male.

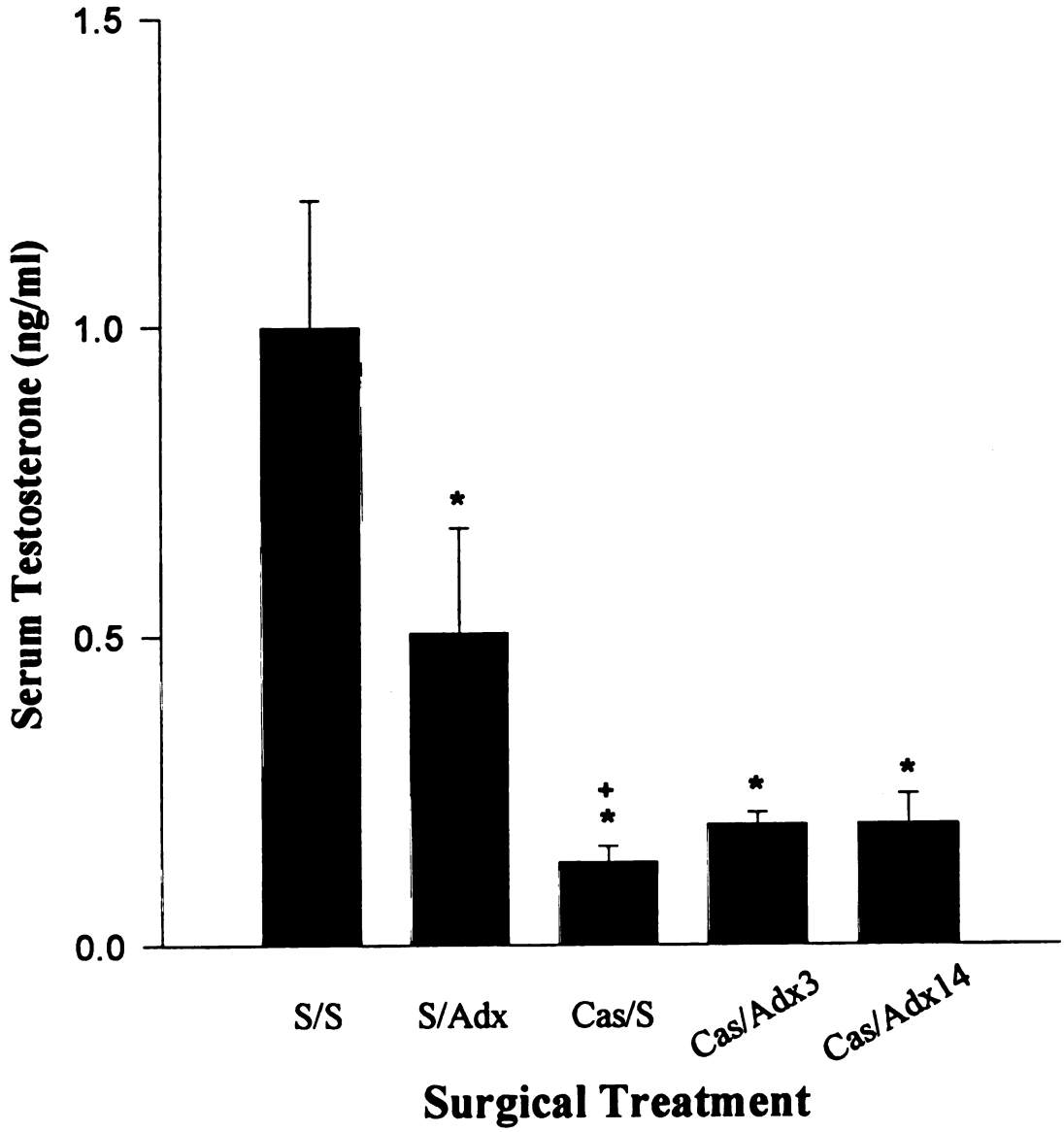
Castration, adrenalectomy and the combination of castration and adrenalectomy did

not affect the level of serum DHT compared to intact males ($F(4,38) = 0.883$, $p = 0.4833$) (Figure 13). DHT levels were below the limits of detection in 2 intact males, one castrated/sham male, six castrated/adrenalectomized 3 day males and seven castrated/adrenalectomized 14 day males. Therefore the results of this assay may not reflect actual physiological values.

SUMMARY

Results of this experiment support the idea that steroid hormones (T, E2, and DHT) are present after removal of the testes and adrenal glands. Serum T levels were decreased by adrenalectomy alone and even further by castration, however, the combination of castration and adrenalectomy was not additive in their effects on reduction of serum T levels. In contrast, the serum concentrations of E2 and DHT, the metabolites of T, were not affected by adrenalectomy, castration, or the combination of castration and adrenalectomy.

Figure 11. Mean (+/- s.e.m.) concentration of testosterone (T) in serum of sham castrate/sham adrenalectomized (S/S), sham castrate/ sham adrenalectomized (Cas/S), castrate/3 day adrenalectomized (Cas/Adx3), castrate/14 day adrenalectomized (Cas/Adx14) B6D2F1 males. * = significantly less than S/S group (N-K $p < 0.05$). + = significantly less that S/Cas group (N-K $p < 0.05$).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1.0
0.5
0.0
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+
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*
*
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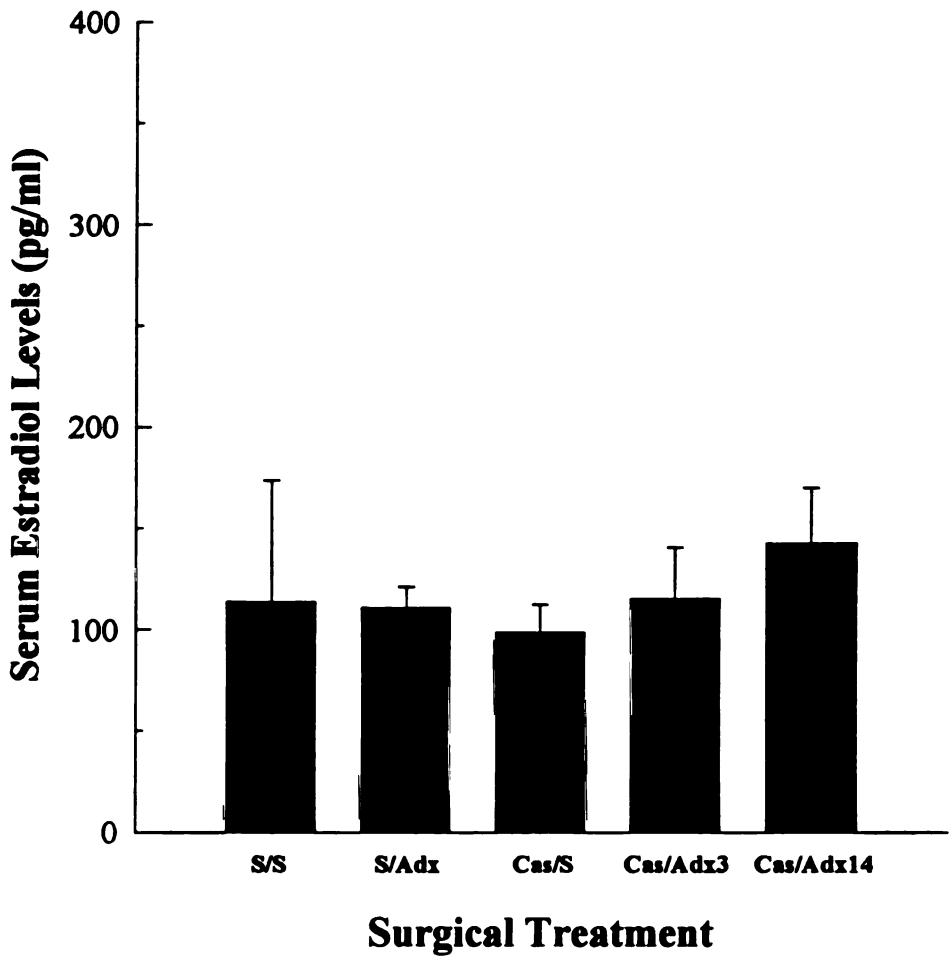
S/Adx
Cas/S
Cas/Adx3
Cas/Adx14
Surgical Treatment

Figure 11

Figure 12. Mean (+/- s.e.m.) concentration of 17ß-estradiol (E2) in serum of sham castrate/sham adrenalectomized (S/S), sham castrate/ sham adrenalectomized (Cas/S), castrate/3 day adrenalectomized (Cas/Adx3), castrate/14 day adrenalectomized (Cas/Adx14) B6D2F1 males.


400
300
200
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Serum Estradiol Levels (pg/ml)
S/S
S/Adx
Cas/S
Cas/Adx3
Cas/Adx14
Surgical Treatment

Figure 12

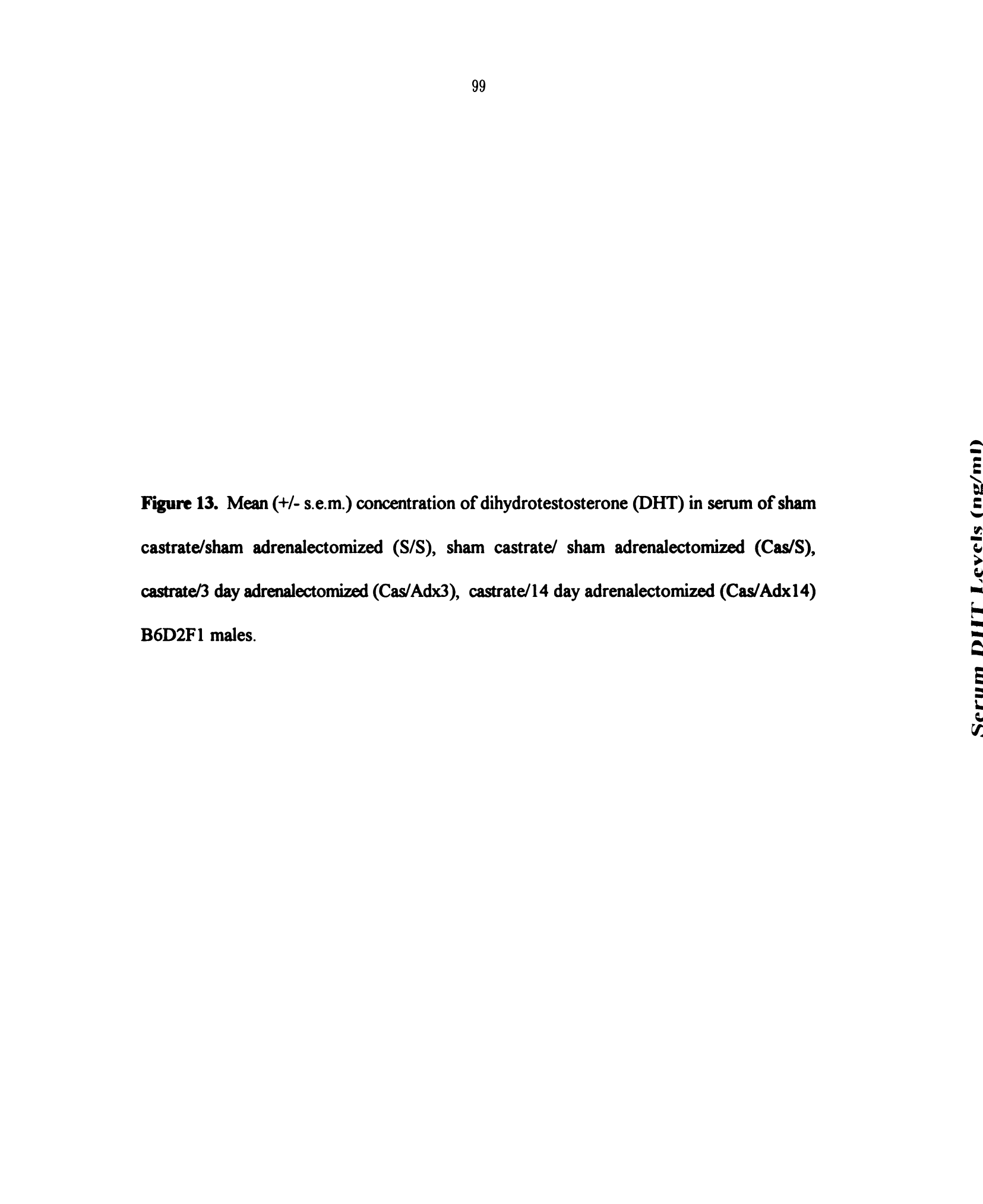

Figure 13. Mean (+/- s.e.m.) concentration of dihydrotestosterone (DHT) in serum of sham castrate/sham adrenalectomized (S/S), sham castrate/ sham adrenalectomized (Cas/S), castrate/3 day adrenalectomized (Cas/Adx3), castrate/14 day adrenalectomized (Cas/Adx14) B6D2F1 males.

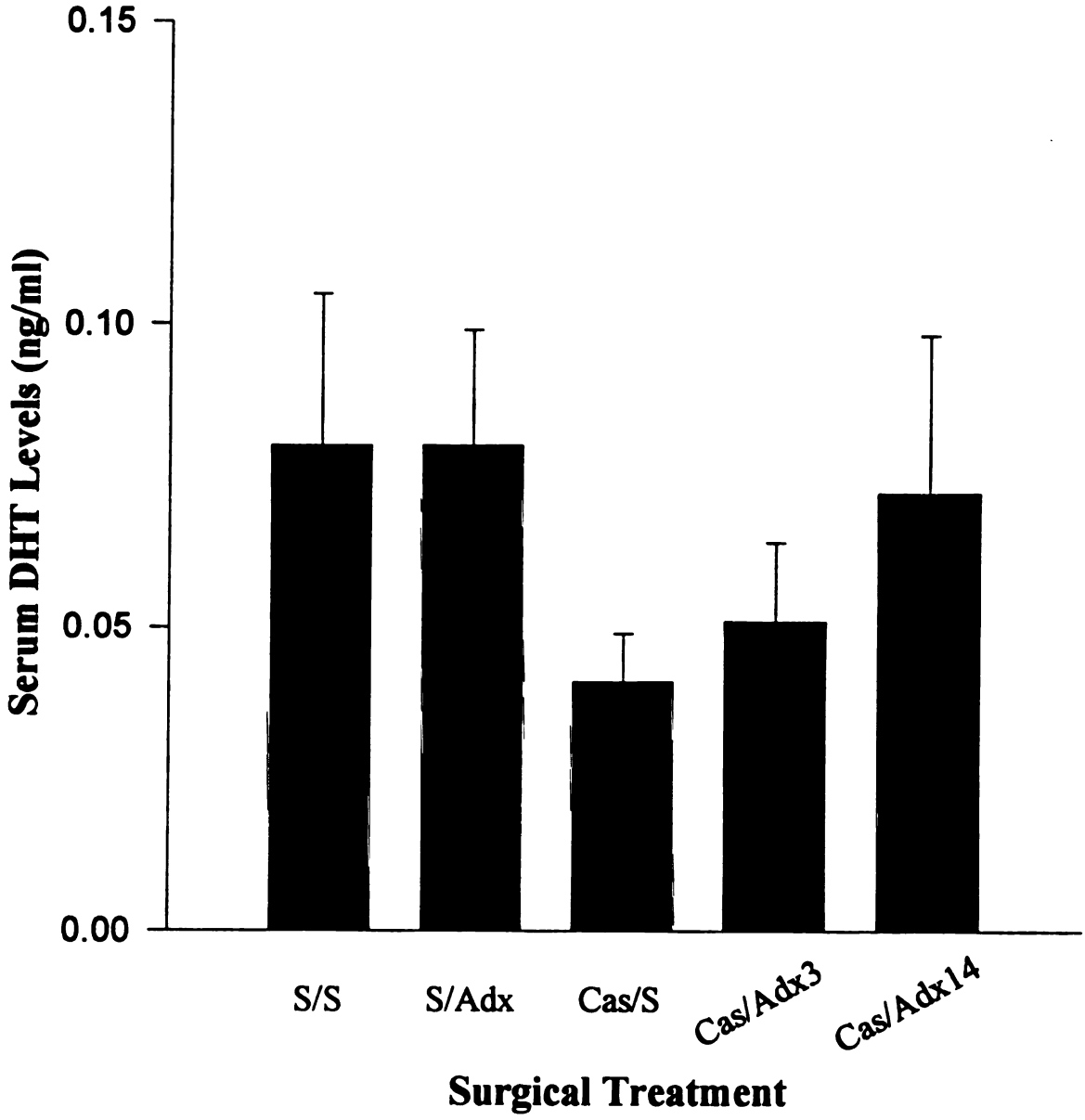
0.15
0.10
0.05
0.00
Serum DHT Levels (ng/ml)
S/S
S/Adx
Cas/S
Cas/Adx3
Cas/Adx14
Surgical Treatment

Figure 13

GENERAL DISCUSSION

The results of the present studies are consistent with the idea that nongonadal steroids, in particular estrogens, contribute to the maintenance of copulatory behavior after castration in male B6D2F1 hybrid house mice.

The ability to achieve an ejaculatory reflex and intromission after castration in B6D2F1 male house mice is dependent on the aromatization of nongonadal androgens to estrogens that are present after castration. Although circulating T levels are reduced by castration, or castration and adrenalectomy, E2 levels remain unaffected. The ability to convert androgens to estrogens in regions of the brain important for copulatory behavior (POA, HYP, and AM) was reduced but not eliminated after castration. Likely, it is in these regions where the aromatase inhibitor ATD is having its behavioral effects. Furthermore, estrogenic stimulation appears to be maintained in POA and HYP after castration, and is present in the AM as well, but at reduced levels. However, continuer and noncontinuer males did not differ in serum T, E2 and DHT levels, AA or ER levels in the POA, HYP and AM.

Effects of Castration on Steroid Hormone Levels in Continuer and Noncontinuer Males

Serum T levels were decreased by castration but were not different between castrated continuer and noncontinuer males as seen previously (Clemens, et al., 1988). However, serum E2 levels were not affected by castration in either continuer or noncontinuer B6D2F1

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males. This differential effect of castration on T and E2 levels has been observed in rats, rhesus monkeys, and ferrets indicating that serum E2 is maintained by an unknown nongonadal source (Carroll, Weaver & Baum, 1988; Roselli & Resko, 1984; West, Roselli, Resko, Greene & Brenner, 1988). Since T is not eliminated from the circulation after castration, it may facilitate behavior via androgenic stimulation and act as a substrate to be aromatized to E2. More interestingly though, castration does not affect E2 levels which supports the notion that copulation after castration may be maintained by estrogenic stimulation. Although retention of copulatory behavior after castration was not associated with higher serum levels of T, E2 or DHT in continuers, it is possible that continuer B6D2F1 males are more responsive to hormone stimulation than noncontinuers. This notion is also consistent with findings on other individual differences, e.g. males with "high sex drives" respond differently to the same level of hormones as males with "low sex drive" (Grunt & Young, 1952; Larsson, 1966; Whalen et al., 1961).

Effects of Adrenalectomy on Testosterone and Estradiol Levels

Adrenalectomy reduces serum T levels, but not to the same extent that castration does. However, adrenalectomy and castration were not additive: removal of both adrenals and testes did not decrease T levels any further than castration alone. Therefore, although the adrenals appear to contribute to circulating levels of T, their effect on circulating levels of T may be via indirect regulation of steroidogenesis in the testes.

As seen with the testes, the adrenal glands do not appear to regulate circulating levels of E2. Removal of both adrenals and testes also did not affect E2 levels. Thus, based on these data the adrenal gland does not contribute significantly to circulating E2 levels either by

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providing aromatizable androgens or as a site for aromatization of androgens to estrogens. This lack of adrenal contribution to E2 levels has been seen in the zebra finch where AA was not detectable in the adrenals (Schlinger & Arnold, 1991; Schlinger & Arnold, 1992). However, substrates that can be aromatized to estrogens are synthesized in the zebra finch adrenal which may be aromatized elsewhere (the brain) to contribute to circulating E2 levels (Schlinger & Arnold, 1991).

That circulating levels of T and E2 remain after adrenalectomy and castration in B6D2F1 males demonstrates that continued copulation after removal of both adrenals and testes as seen in B6D2F1 mice, cats and dogs may still be under the influence of steroid hormones and independent of adrenal hormones (Beach, 1970; Cooper & Aronson, 1958; Schwartz & Beach, 1954; Thompson et al., 1976). However, in the rat, adrenals appear to secrete androgens necessary for the ability of E2 to activate copulatory behavior in castrated males (Gorzalka et al., 1975).

As reviewed earlier, a potential source of nongonadal-nonadrenal androgens and estrogens is the brain. Local metabolism of circulating steroid hormones in neural tissue has been well established. However, the de novo synthesis of neurosteroids from cholesterol within neural tissue is a potential mechanism by which the brain could be influenced by metabolically active steroids that affect behavior (Baulieu, 1981; Corpechot et al., 1981; Le Goascogne et al., 1987; Robel & Baulieu, 1995).

The Effects of Inhibiting Aromatase on Copulatory Behavior

When castrated males were treated with ATD, fewer continuer B6D2F1 males achieved ejaculations and intromissions compared to their pretreatment test as well as

continuers that received blank silastic capsules. The results are consistent with the idea that nongonadal estrogens contribute to the maintenance of copulatory behavior after castration in male B6D2F1 hybrid house mice.

The inhibitory effects of ATD on copulation in continuer B6D2F1 males probably resulted from the ability of ATD to block neural aromatization of androgens to estrogens (Brodie, Marsh, Wu, & Brodie, 1979; Lieberburg, Wallach, & McEwen, 1977). The importance of local neural aromatization of T to E2 for the activation of copulatory behavior has been demonstrated in castrated rats. ATD implanted directly in the POA inhibits the ability of T implants in the POA to restore mounting behavior (Christensen & Clemens, 1975). Castrated B6D2F1 males retain aromatase activity in POA as well as the amygdala and hypothalamus where non-gonadal T may be converted to estrogen (EXP 3). Thus, it is likely that ATD treatment inhibited aromatase activity in these brain regions of continuer B6D2F1 males which reduced the amount of estrogen available to bind with estrogen receptors that are maintained in the POA, HYP and AM of castrated B6D2F1 males even after castration (EXP 5). However, since ATD was administered systemically, both peripheral and CNS aromatization of androgens to estrogens were inhibited. Thus, estrogens that normally originate from the periphery that may have acted in the CNS to facilitate copulatory behavior would be reduced as well. Therefore, the possibility that estrogen originating in the periphery may be in part responsible for maintenance of copulation after castration in continuer B6D2F1 male mice cannot be excluded.

In contrast, ATD did not affect the percent of continuer males that mounted, nor their latency to mount which is in contrast to the rat (Christensen & Clemens, 1975). While ATD did not block mounting, it is still possible that E2 plays some role in this behavior, since it is

unlikely that ATD blocked all estrogen production. Alternatively, mount behavior may be maintained in ATD treated continuer males via androgenic stimulation, since androgens are not completely eliminated by castration (EXP's 1 and 6). Furthermore, since ATD is a steroid it is possible that ATD may have some action of its own (Christensen & Clemens, 1975; Kaplan & McGinnis, 1989; Landau, 1980). This is unlikely, however, since ATD treatment did not facilitate mounting in noncontinuer B6D2F1 males.

Effects of Castration on Aromatase Activity Levels in Continuer and Noncontinuer Males

Following castration, AA in POA, HYP, and AM was reduced but not eliminated. In none of these three brain regions was the level of AA associated with the maintenance of copulatory behavior after castration in continuer and noncontinuer B6D2F1 males. Although reductions in neural AA and aromatizable androgens (T) indicate that local neural concentrations of estrogens may be reduced after castration, nonetheless, these results in conjunction with ATD inhibiting copulation support the notion that maintenance of copulatory behavior after castration may be due to estrogenic stimulation derived from the aromatization of nongonadal androgens in regions of the brain important for copulatory behavior.

As seen in other species, AA in B6D2F1 males appears to be regulated by gonadal hormones (Connolly, Roselli, & Resko, 1990; Roselli et al., 1984; Roselli et al., 1985; Roselli et al., 1987a; Roselli & Resko, 1984; Roselli & Resko, 1986; Roselli & Resko, 1989; Roselli et al., 1987b; Weaver & Baum, 1991). The decline in AA levels in the POA, HYP, and AM following castration may indicate that AA in B6D2F1 males is regulated by gonadal

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androgens as seen in other species (Roselli et al., 1984; Roselli & Resko, 1984; Roselli & Resko, 1986; Roselli & Resko, 1989). In other species, AA in parts of the AM is independent of T levels, while in other regions of the AM they are not (Roselli et al., 1984). Because the AM dissection in the present study contained a mixture of these regions, it cannot be determined if regulation of AA in regions of the AM of B6D2F1 males is independent of gonadal hormones.

Effects of Castration on Estrogen Receptor Levels in Continuer and Noncontinuer Males

Nuclear Estrogen Receptors (ERn)

Castration did not affect ERn levels in the POA and HYP, but did significantly reduce ERn levels in the AM. It should be noted, that ERn levels (fmol/mg DNA) were greater in the AM than in either the POA or HYP of intact males, and that after castration, ERn levels were equivalent in all three brain regions. Although present after castration, ERn levels in the POA, HYP, and AM were not associated with expression of copulatory behavior in continuers and noncontinuers, but suggest that estrogenic stimulation is still present (Gorski et al., 1986).

Maintenance of ERn levels after castration in the POA of B6D2F1 males mice is in contrast with those in rats. In the rat, ERn levels in POA were reduced by castration and associated with circulating androgen levels (Krey et al., 1980). However, it is unclear in castrated B6D2F1 mice, whether ERn in POA and HYP are affected by circulating nongonadal estrogens. In the AM, ERn levels may be associated with T concentrations in the B6D2F1 male since both were reduced after castration, as reported in rats (Krey et al., 1980;

Roselli et al., 1993).

The present observation that POA ERn levels are unchanged after castration in B6D2F1 males contrasts with our previous report that ERn in POA declined in castrated B6D2F1 males (Clemens et al., 1988). Differences in preparation of the nuclear fraction prior to measurement of ERn do not account for differences between the two studies. Purification and nonpurification of the nuclear pellets prior to KCL extraction produce similar results in measuring ERn levels between brain regions and with respect to the effects of castration. However, sucrose purification reduced the total amount of DNA extracted from the nuclear pellet by an average of 37 percent compared to the nonpurified nuclear pellet. Further, sucrose purification may affect ERn levels in that the concentrations of ERn (fmol/mg DNA) were reduced in the AM compared to nonpurified samples; however, sucrose purification did not affect ERn levels in the POA or HYP (EXP 4B). Sucrose purification may also artificially increase ERc levels (expressed as fmol/mg DNA) since the ratio of ERc to mg DNA would be greater due to decreased DNA recovery.

It is unlikely that differences in ERn between the two studies were due to the use of frozen versus fresh tissue, since fresh tissue appears to yield greater ERn levels than frozen tissue (MacLusky, et al., 1986), and ERn levels in the POA of intact males were comparable between the studies (between 20-30 fmol/mg DNA). Nonetheless, the differences that occurred between the two studies may have been due to differences in the dissections, heterogeneity of the tissue, and/or the length of time the animals were castrated.

In contrast to EXP 4A, although there appeared to be a slight reduction, castration did not significantly reduce ERn levels in the AM in EXP 4B. It is possible the effects of castration on ERn levels in the AM are time dependent since the assays were run 5 (EXP 4B)

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and 38 (EXP 4A) weeks after castration. Alternatively, the effects of castration may have been obscured by a lower number of animals assayed, and/or tissue heterogeneity.

Cytosolic and Total Estrogen Receptors (ERc & ERt)

Our results suggest that ERt levels were down-regulated by testicular hormones. Castration increased ERt levels in the POA, HYP, and AM. These increased ERt levels mainly reflect changes in ERc levels, since ERc levels are 3 to 7 times greater than ERn levels (EXP 4A). ERc levels were increased in the HYP of noncontinuers compared to intacts, while ERc levels in the HYP of continuers were not significantly different from either intact or noncontinuer males. Down regulation of ERt in B6D2F1 males by gonadal hormones may parallel regulation of ER mRNA levels seen in the male rat where castration increased ER mRNA in numerous nuclei (Lisciotto & Morrell, 1993; Morrell et al., 1995). In some brain regions it appears that both androgens and estrogens may play a role in regulation of ER mRNA levels, while in other brain regions gonadal hormones do not affect ER mRNA (Lauber et al., 1991; Simerly & Young, 1991). An alternative explanation for increased ERt is that castration elicits a slower turnover or degradation rate of ER protein. However, the actual amount of ER synthesized, used and degraded per unit of time cannot be determined by our method of analysis. Thus, gonadal hormones appear to regulate ER levels and dynamics, but the mechanism of action has yet to be determined.

Effects of Genotype on Aromatase Activity in C57BL/6J, DBA/2J and B6D2F1 Male Mice

The data demonstrate that AA levels in some brain regions of intact male mice are

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influenced by genotype, while in others it is not. In the POA and HYP, DBA/2J males have greater levels of AA than C57Bl/2J and B6D2F1 males. However, levels of AA in the AM do not differ among the three strains of mice. Although the behavioral and physiological significance of different AA levels among strains of intact male mice is unclear, these differences in AA do suggest that there are physiological differences among these strains that may account for differences in behavioral and physiological responses among strains of mice. In contrast, AA levels in all three strains were reduced by castration and differences among the strains in AA levels were eliminated. Therefore, strains that continue to copulate after castration and those that do not could not be distinguish by their levels of AA in POA, HYP and AM.

Effects of Castration on Aromatase Activity Levels in C57Bl/6J, DBA/2J and B6D2F1 Males

Following castration, AA in POA, HYP, and AM was reduced in males of all three strains. Furthermore, castration eliminated differences in AA levels that were seen between the three strains of intact males. Thus, we could not distinguish between these three strains of males based on AA levels in regions important for copulatory behavior (POA, HYP, and AM) after castration.

The decline in AA levels in the POA, HYP, and AM following castration may indicate that AA in C57Bl/6J, DBA/2J and B6D2F1 males is regulated by gonadal androgens as seen in other species (Connolly et al., 1990; Roselli et al., 1984; Roselli & Resko, 1984; Roselli & Resko, 1986; Roselli & Resko, 1989; Roselli et al., 1987b; Weaver & Baum, 1991). However, in rats, AA in parts of the AM is independent of T levels, while other regions of the

AM are not (Roselli et al., 1984). Because our AM dissection contained a mixture of these regions, we cannot determine if regulation of AA in regions of the AM of male mice in these three strains is independent of gonadal hormones.

That the levels of AA were not different between the strains after castration may reflect the limited ability of our assay to discriminate differences at the cellular level. On the other hand, the failure to see differences in AA levels between strains that continue copulate after castration and those that do not suggests that other estrogenic processes that may facilitate reproductive behavior may differ between the strains. These processes would not be revealed by our analysis.

Differences Between Continuer and Noncontinuer B6D2F1 Males

Since circulating levels of T, DHT and E2 do not differ between continuers and noncontinuers, continuer B6D2F1 males appear to be more responsiveness to steroids than noncontinuer males. This difference in responsiveness cannot be accounted for at the level of the steroid receptor since aromatase activity and estrogen receptor levels in the POA, amygdala and hypothalamus do not differ between continuers and noncontinuers (EXP 4). Alternatively, continuers and noncontinuers may differ in their responsiveness to androgenic stimulation, since androgens appear important for copulation. For example, DHT restores copulatory behavior to noncontinuer B6D2F1 males (Sinchak & Clemens, 1990). Furthermore, estradiol and DHT given simultaneously restore copulatory behavior in the CD-1 strain of house mouse as well as rats better than either hormone individually (Baum, Sodersten, & Vreeburg, 1974; Baum & Vreeburg, 1973; Larsson, Sodersten, & Beyer, 1973a; Larsson et al., 1973b; Wallis & Luttge, 1975).

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Summary and Conclusions

The present studies demonstrate that maintenance of copulatory behavior after castration in B6D2F1 male house mice is dependent on the aromatization of nongonadal androgens to estrogens that are present after castration. Although a reduction in circulating T levels occurs after castration, or castration and adrenalectomy, E2 levels are not affected. The ability to convert androgens to estrogens is reduced but not eliminated after castration in regions of the brain important for copulatory behavior (POA, HYP, and AM). Likely, it is in these regions where the aromatase inhibitor ATD is having its behavioral effects. Furthermore, estrogenic stimulation appears to be maintained after castration in POA and HYP, and present in the AM, but at reduced levels as reflected by ERn levels.

It is not surprising that numerous sites within the body synthesize steroid hormones, since steroids interact at numerous levels to organize and regulate vital metabolic and behavioral functions. Therefore, although gonadal steroid hormone synthesis and copulatory behavior are associated in the B6D2F1 house mouse, our data support the idea that non-gonadally produced steroid hormones facilitate copulatory behavior in the castrate B6D2F1 male house mouse.

Although continuers and noncontinuers could not be distinguished by serum T, E2 or DHT levels, AA or ER levels in the POA, HYP and AM, the ability to study a genetically homogenous strain that behaves differently after castration is an important model to the determination of the role of steroid hormones and their mechanisms that regulate behavior.

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