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Appetitive and Consummatory Sexual Behaviors in the Male Rat Following Castration and Testosterone Replacement: The Role of the Ventral Tegmental Area

Soraya Centeno

A Thesis

In

The Department

of

Psychology

Presented in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy at
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ABSTRACT

Appetitive and Consummatory Sexual Behaviors in the Male Rat Following Castration and Testosterone Replacement: The Role of the Ventral Tegmental Area

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This thesis investigated the role of gonadal steriods in the expression of appetitive and consummatory aspects of sexual behavior in the male rat, with particular attention to the involvement of the ventral tegmental area (VTA). Initial experiments established the time course of the loss and reinstatement of appetitive and consummatory behaviors following castration and subsequent testosterone (T) treatment, and further the excitatory role for sexual experience in "buffering" males against the inhibitory effects of castration. Additional experiments were conducted to examine the activation of different brain regions, including the VTA, following chemosensory, somatosensory, and hormonal stimulation in castrated, T-treated males, using Fos immunocytochemistry. Neural activation of the VTA was observed following following multiple ejaculations, and subsequent studies revealed populations of dopamine and GABA neurons that had been activated differentially by copulatory stimulation. Lesions of the VTA disrupted both appetitive and consummatory sexual behaviors, but animals with more sexual experience prior to the lesions were less affected. Finally, intracranial implants of crystalline estradiol (E) into the VTA facilitated mounting in a majority of castrated males. T produced a smaller effect, whereas progesterone or cholesterol had no effect. These data indicate that the VTA plays a role in the hormonal stimulation of copulatory behavior. The effectiveness of E was particularly surprising, given the lack of neuronal aromatase and classical estrogen receptor in this region. However, the presence of glial aromatase

and estrogen receptor β in this region could account for these effects, and suggest a novel mechanism by which dopamine and GABA neurons in this region might be regulated.

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DEDICATION

I would like to dedicate this thesis to my beloved father Pedro Antonio, whose support, understanding, and sacrifices during the last twelve years have been necessary for the completion of this work. I would also like to mention my sister, Angela and my brother Toni for believing in me and understanding my absence from home during some of our critical adulthood years.

I also dedicate this work to the memory of my beloved mother Maria.

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GENERAL INTRODUCTION

Castration inhibits the ability of males of a variety of species to respond to cues from sexually receptive females and to initiate copulatory behavior (Cooper & Aronson, 1974; Davidson, 1966; 1980; Hart, 1968; 1974; Meisel & Sachs, 1994; Phoenix, Slob, & Goy, 1973; Sachs & Barfield, 1976). This deterioration can be observed as a progressive decline in the proportion of males that copulate, and also as a decrease in the number of penile intromissions or ejaculations that males achieve during each copulatory test. However, the rate of decline of copulatory behavior following castration is not constant and can be delayed in male rats by increasing the value of the sexual incentive. This can be achieved, for example, by allowing males to copulate with females that pace the copulatory contact rather than with females that can not pace (Madlafousek, Hlinak, & Beran, 1976).

A principal focus of this thesis was an examination of male rat copulation following castration and testosterone (T) treatment as a function of the amount of sexual experience males received prior to and after castration. Administration of T, either by systemic injection or subdermal implants of silastic capsules, reinstates copulation in castrated males (Davidson, 1966; Malmnas, 1973; Smith, Damassa & Davidson, 1977). The restoration of copulatory behavior in the male rat follows a relatively typical pattern in which the last behaviors to disappear, e.g., mounting, are the first to re-appear, followed by the re-appearance of intromissions and then ejaculations (Baum & Vreeburg, 1973; Davidson, 1966; Larsson, Sodersten, & Beyer, 1973; Whalen & Luttge, 1971). The restoration of consummatory behaviors in long-term castrated males, depends on the

dose of androgen given to the animal, the route of administration (e.g., systemic versus central injections), and the schedule of behavioral testing (Beach & Holtz-Tucker, 1949; Christensen & Clemens, 1974; Smith, Damassa, & Davidson, 1977). Following castration and subsequent daily systemic injections of T, castrated male rats given weekly tests of sexual behavior are able to copulate to ejaculation after 4-5 weeks. Doses of T ranging from 25-500μg are all capable of reinstating copulatory behaviors lost after castration, with 25-50μg being the threshold maintenance dose necessary to keep copulatory behaviors at preoperative levels nine weeks following castration (Beach & Holtz-Tucker, 1949). In contrast, castrated male rats are able to ejaculate as early as two weeks after a single injection of 10 mg of T (Nucci & Beach, 1970).

Early studies described an increase in attempted mounts while complete mounts declined after castration (Beach & Holtz-Tucker, 1949). Stone (1939) reported however that the incidence of anogenital investigation of receptive females by castrated males remained stable as copulatory behaviors declined. Other appetitive sexual behaviors such as conditioned level-changing or bar-pressing for a second-order sexual incentive, or precopulatory behaviors such as pursuit of females are also decreased by castration, but to a smaller extent (Everitt & Stacey, 1987; Mendelson & Pfaus, 1989; Stone, 1939). The behavioral changes observed in male copulation following castration and subsequent T treatment may be dependent upon the action of androgens and/or estrogens, given that T is converted to estrogen (E) by aromatization process and into dihydrotestosterone (DHT) by 5-alpha reductase. The following section reviews the literature on the effects of T metabolites on male copulation.

Effects of T Metabolites on the Expression of Male Copulatory Behavior

Sodersten (1973) examined the effects of E on the reinstatement of sexual behaviors in prepubertal and sexually experienced castrated male rats. Following daily subcutaneous injections of 50 μg of E for a period of 26 days castrated males in both conditions showed a return of mounts and intromissions. Some ejaculations were observed in the sexually experienced animals, but no ejaculations were observed in the prepubertally castrated males as a result of this treatment. The absence of the ejaculatory pattern in the prepubertal group was correlated with a reduction in growth of the testes and sexual accessory glands (Taylor, Weiss, & Komitowski, 1983). A higher dose of E (100 μg) increased the proportion of sexually castrated experienced male rats that copulated to ejaculation, but failed to reinstate ejaculations in the prepubertal castrated rats. Compared to castrated males treated with T, sexually experienced castrated E-treated males tended to have longer intromission and ejaculation latencies, and a greater number of mounts and intromissions prior to ejaculation (Sodersten, 1973). In another study, Pfaff (1970) demonstrated the return of mounts and intromissions, but not ejaculations, in sexually inexperienced castrated males after daily injections of only 10 μg of E for a period of 30 days. These data suggest that low doses of E may reinstate mounts and intromissions in castrated male rats, regardless of their sexual experience, but the return of the complete pattern of sexual behaviour, including ejaculations, requires higher doses of E and/or a different hormone.

DHT is another major T metabolite, which results from the conversion of T via 5-alpha reduction (Martini, 1982). Failure to reinstate ejaculation with E treatment among

prepubertal castrated males has been attributed to the undervelopment of the penile spines in the male rat's penis (Wilson & Gloyna, 1970). It was suggested that the rapid decrease in circulating androgens following castration might interfere with the development of the penile spines. Wilson and Glyona (1970) demonstrated the effects of DHT on the growth of accessory sex organs and suggested that DHT might be a potent stimulator involved in the full development of the penis. This finding has been supported by subsequent studies (Johnston & Davidson, 1972; Larsson, Sodersten, & Beyer, 1973; Sodersten, 1973). In addition, even though daily administration of 125 μg DHT for a period of 3 weeks has consistently failed to restore intromissions and ejaculations in castrated male rats (Baum, 1979; McDonald, Beyer, Newton, et al., 1970; McGinnis & Dreifuss, 1989), a higher dose of DHT (1 mg/day) for a period of 26 days has been reported to restore copulatory behaviors, including ejaculations, in 30% of castrated male rats (Paup et al., 1975). On the other hand, the copulatory behavior observed in castrated males rats treated with both DHT (1mg) and E (0.05-50μg) for 26 days was not significantly different from the behaviors displayed by intact untreated males or castrated T (1mg/day) treated males (Baum & Vreeburg, 1973; Larsson, Sodersten, & Beyer, 1973). This suggests that a combined action of androgen peripherally and estrogen centrally is critical for the normal expression of male copulatory behavior in the rat.

Brain Regions Associated with Male Copulation

The neural circuitry mediating the interactions between chemosensory inputs and hormonal signals have been extensively investigated by centrally administering steroids to particular brain areas. The medial preoptic area (mPOA), the bed nucleus of the stria terminalis (BNST), and the medial amygdala (MEA) are all involved in the processing of

chemosensory signals. These structures contain a high number of steriod receptors (e.g. androgen receptors (AR), estrogen receptors (ER), and progesterone receptors (PR) (Coolen, Peters, & Veening, 1996; Sar & Stumpf, 1975; Simmerly, Chang, Muramatsu, & Swanson, 1990). T implants in the mPOA facilitate the expression of mounts, intromissions, and ejaculations in castrated male rats (Christensen & Clemens, 1974; Davidson, 1966; Lisk, 1967; Smith, Damassa & Davidson, 1977; Wood & Newman, 1995). However, this behavioral facilitation does not tend to be as consistent (i.e., behavior is not always observed across test sessions) and/or vigorous (i.e., fewer mounts, intromissions, or ejaculations per test session) as the sexual behaviors observed in intact male rats. Implants of estradiol (E) in the mPOA proved to be more effective than T in facilitating mounting behavior (Christensen & Clemens, 1975), while DHT implants in the mPOA had no effects in rats.

The process of aromatization by the brain in which T gets converted into E has been suggested as a necessary step for the occurrence of male rat sexual behaviour (Luttge, 1975; Luttge, Hall, Wallis, & Campbell, 1975). This hypothesis has been supported by studies investigating the behavioral effects of aromatizable versus non-aromatizable androgens (Larsson, 1979; McGinnis & Dreifuss, 1989; Sodersten, 1973), and of aromatase inhibitors and antiestrogens (Beyer, Morali, Naftolin, Larsson, & Perez-Palacios, 1976; Christensen & Clemens, 1975; Luttge, 1975). Studies investigating the effects of aromatizable androgen (T) versus non-aromatizable androgens (DHT) in castrated male rats have shown that T completely reinstates mounts, intromissions and ejaculations in castrated male rats (McGinnis & Dreifuss, 1989; Sodersten, 1973), but that DHT reinstates only mounting behavior (Larsson, 1978; McGinnis & Dreifuss,

1989). Studies investigating the effects of intracranially applied aromatase inhibitors such as 1,4,6-androstatriene-3, 17-dione (ATD) on sexual behaviour have consistently reported a decrease in the percentage of intact males displaying mounts, intromissions and ejaculations (Balthazart & Surlemont, 1990; Balthazart, Foidart, & Hendrick, 1990; Brand, Krooner, Mos, & Slob, 1991; Christensen & Clemens, 1975; Floody, & Petropoulos, 1987; Kaplan & McGinnis, 1989; Sodersten, Eneroth, Hansson, et al., 1988; Watson, & Adkins-Regan, 1989).

Peripheral administration of antiestrogens has been also used to demonstrate the necessity of E for the expression of male copulatory behaviors (Beyer, Morali, Naftolin, Larson, & Perez-Palacios, 1976; Luttge, 1975; Vagell & McGinnis, 1997). Antiestrogens block estrogen receptors, inhibiting estrogen receptor (ER) binding. Luttge (1975) studied the effects of Nitronifene citrate (CI-628), an antiestrogen, on T-treated castrated male rats, and reported a significant reduction in the proportion of males displaying intromissions and ejaculations compared to castrated T-treated males. However, blocking ERs by systemic administration of the antiestrogen RU 58668 inhibited the restoration of 50-kHz ultrasonic vocalizations and scent marking, but did not affect copulatory behaviors (i.e., intromissions and ejaculations) or partner preference in T treated castrated male rats, even though the level of brain nuclear ER occupation was significantly reduced to the level found in castrated males (Vagell & McGinnis, 1997). These findings indicate that the activation of nuclear ERs is necessary for the restoration of some, but not all copulatory behaviors. Those other behaviors might be androgen receptor-dependent.

The Importance of Sexual Experience

As noted earlier, male copulation is modulated not only by hormones but also by

the amount of sexual experience with receptive females (Agmo, 1976; Dahlof & Larsson, 1978; de Jonge, Burger, van Haaren, Overdijk, & van de Poll, 1987; Drori & Folman, 1964; Gray et al, 1976; Herz, Folman, & Drori, 1969; Lisk & Heimann, 1980; Manning & Thompson, 1976; Thor & Flannelly, 1977). Compared to sexually inexperienced males, sexually experienced males have larger testes (Drori & Folman, 1964), heavier penises (Herz, Folman, & Drori, 1969), lighter body weights (Siegel, Nunez, & Wade, 1981) and increased secretions from accessory glands (Drori & Folman, 1964). Sexual experience has been shown to block the disruptive effects of anosmia (Thorn & Flannelly, 1977), castration (Lisk & Heiman, 1980), penile deafferentation (Lodder, 1975), age (Gray, Smith, Dorsa, & Davidson, 1981), and novelty stress (Pfaus & Wilkins, 1995) on copulatory behavior in male rats. Intact, sexually experienced males also prefer the odors of receptive females over those of non-receptive females, whereas sexually naive or castrated males do not display a significant preference (Carr, Loeb, & Dissinger, 1965; Carr, Loeb, & Wyllie, 1966).

The amount of sexual experience and pacing behavior receptive females show during copulation can buffer the effects of castration in sexually experienced male rats. For example, Madlafousek, Hlinak and Beran (1976) demonstrated that intense female precopulatory behavior (e.g., hopping behavior) could delay the deterioration of copulatory behavior in castrated male rats. Following castration, sexually experienced males tested with females that displayed lordosis and a full complement of proceptive behaviors that included pacing, ear-wiggling, hopping, and darting, maintained their copulatory behaviors for 8 weeks, whereas experienced castrated males, tested with females that displayed only lordosis, lost all copulatory behaviors by the third week after

castration.

Conditioned level changes represent an appetitive aspect of male copulation, indicating males' level of arousal during copulation with receptive females. The conditioning to the bilevel chambers is acquired by their being associated with the repeated exposure to receptive females, occuring 5 min after the introduction of the male into the chamber. In male rats, it has been shown that the development of conditioned level changing is dependent on the presence of estrous odors within the chamber (van Furth & van Ree, 1996), and in Long-Evans rats this behavior is additionally dependent on the conditional presentation of receptive females (Mendelson & Pfaus, 1989). In addition, the rates of appetitive level changing displayed during a 5-min period prior to the introduction of a receptive female rat into a bilevel chamber decreases very slowly over a three-month period following castration, suggesting that this specific appetitive component might be less dependent on androgen actions, and can be buffered by the males' prior experience with receptive females (Mendelson & Pfaus, 1989).

The amount of sexual desire, arousal, and performance reported by surgically castrated human males or hypogonadal males varies from a total loss to no apparent change in behavior, depending on the patient's age and prior sexual experience at the time of the surgery (Davidson, Kwan, & Greenleaf, 1982). The motivation required by human males to act on sexually relevant stimuli seems to be influenced by androgen levels, as well as by social factors. It has been reported that 31% of sexually experienced, surgically castrated human males retained the ability to engage in intercourse ten years following castration (Heim, 1979; 1981). It has also been suggested that these castrated sexually active human males could be engaging in sexual activity for reasons other than

sexual desire or pleasure (e.g., to satisfy their partners or self-image).

The interaction between sexual experience and androgenergic activity in the maintenance of appetitive and consummatory aspects of male sexual behavior has not been investigated systematically. The preceding review would appear to suggest that such an interaction could be critical in helping to resolve questions that arise from the literature about hypogonadal human males, particularly with regard to the ability of some men to maintain sexual desire, arousal, and copulation, relative to other men who lose these abilities. In summary, the importance of sexual experience on male copulation has been associated with the amount of sexual stimulation obtained through genital and/or somatosensory stimuli and with the activation of particular brain areas associated with each type of stimulus.

Sensory Stimulation and Male Copulatory Behavior

The sensory stimulation obtained during male rat copulation comes primarily from chemosensory and somatosensory cues. Chemosensory cues are received during anogenital investigation and licking of the female and auto-grooming following intromission. Somatosensory cues are received via the penis and the pelvic muscles each time males mount, intromit, and ejaculate with receptive females. Brain areas involved in the processing of these chemosensory and somatosensory stimuli include the limbic system and hypothalamus.

The role of olfactory and pheromonal communication in the facilitation of male rat copulation has been demonstrated previously (Brown & McDonald, 1985; Carr, Loeb, & Wilie; 1966; Doty, 1976; Krames & Mastromatteo, 1973; Pfaff & Pfaffmann, 1969a; 1969b; Vanderbergh, 1994). Odors emanating from sexually receptive female rats serve

as an attractant for males, depending upon the males' prior sexual experience (Carr, Loeb, & Wylie, 1966; Pfaff & Pfaffmann, 1969a; 1969b). A typical preference paradigm presents animals with two specific odors and compares the amount of time spent investigating each odor (Doty, 1975). This paradigm is principally concerned with the preferences of different groups of subjects (e.g. intact versus castrated males) for competing odors (e.g., from receptive versus non-receptive females). Findings from these studies show that intact, sexually experienced males prefer the odor of receptive females to that of non-receptive females, whereas naive, gonadally intact males or castrated males show no preference. This indicates that the behavioral preference of male rats for female estrous odors is facilitated by prior sexual experience (Carr, Loeb, & Dissinger, 1965; Landauer, Wiese, & Carr, 1977; Pfaff & Pfaffmann, 1969b). Indeed, mesolimbic dopamine release in the nucleus accumbens of males sensitizes with repeated exposure to estrous odors (Mitchell & Gratton, 1991). Furthermore, sexually experienced, intact males display a significantly higher preference for estrous and ovariectomized females' odors over those of castrated males, or sexually naïve intact males (Brown, 1977).

The role of olfactory stimuli and the excitatory influence of previous sexual experience on male rat copulation has been examined by surgically removing the olfactory bulb or infusing zinc sulfate ($ZnSO_4$) into the nasal cavity of male rats to produce anosmia. Evidence from recent studies shows the excitatory role of volatile estrous odors in triggering sexual arousal (i.e. an increase in the mean number of non-contact erections (NCE)) (Sachs, 1997; Sachs, Akasofu, Citron, Daniels, & Natoli, 1994). In the presence of an inaccessible receptive female, visual and auditory stimuli are not sufficient to evoke NCE (Sachs, 1997). Furthermore, deafferentation of the olfactory bulb

of the male rat and hamster decreases sexual performance in copulatory tests with females, and reduces the frequency of NCEs, suggesting the existence of a mechanism that might process the volatile chemosensory cues from receptive females into sexual copulatory behaviors (Edwards & Davis, 1997; Larsson, 1979; Winans & Powers, 1977).

The facilitatory role of olfactory stimuli present during copulation on the expression of male rat sexual behavior has also been demonstrated by Van Furth and Van Ree (1996), who showed that male Wistar rats exposed to bilevel chambers with either receptive females, non-receptive females, or no females, displayed increased anticipatory level-changing. The development of anticipatory level-changing by male rats was disrupted following $ZnSO_4$ treatment, suggesting that the olfactory stimuli from animals copulating previously in the bilevel chambers are critical for the development of anticipatory level changes (Van Furth & Van Ree, 1996). Sexual experience also attenuates the debilitating effects of anosmia induced by $ZnSO_4$ infusions on male sexual performance. In contrast, the frequency of mounts, intromissions, and ejaculations among naive anosmic male rats decreases following $ZnSO_4$ treatment (Thor & Flannelly, 1977). For example, the ejaculation frequency observed among intact control males and $ZnSO_4$-treated anosmic males has been found to be the same when all males were sexually experienced (Cain & Paxinos, 1974).

The vomeronasal system (VNS) is involved in the regulation of neuroendocrine functions and reproductive behaviors elicited by pheromones by processing chemosensory signals. One of the target areas innervated by the VNS is the mPOA, which receives putative chemosensory-related inputs from the MEA and BNST. Lesions of the mPOA eliminate mounts, intromissions, and ejaculations (Arendash & Gorski,

1983; Bazzett, Lumley, Bitran, et al., 1992) and also disrupt certain appetitive behaviors (e.g., maze running). However, lesions of the mPOA do not affect the proportion of males showing anogenital investigation and pursuit (Edwards & Einhorn, 1986; Hansen, Kohler, Goldstein, & Steinbusch, 1982), or attraction to female odors (Powers, Newman, & Bergondy, 1987). This suggests that the mPOA is involved in the facilitation of copulatory behaviors and, perhaps to a lesser extent, the expression of certain appetitive behaviors (especially those not dependent on olfactory stimulation). However, electrophysiological studies have shown that the mPOA is activated by females' odors. Pfaff and colleagues found an increase in the activation of neurons within the mPOA of male rats following exposure to female rat urine odors (Pfaff & Gregory, 1969; Pfaff & Pfaffman, 1969). Additional appetitive behaviors such as instrumental responses and partner preference are altered by manipulations of the mPOA. Infusions of β-endorphins into the medial preoptic area-anterior hypothalamic area (mPOA/AHA) abolish males' preference for an estrous over an anestrous female. In addition, infusions of α-melanocyte stimulating hormone (MSH) into the mPOA/AHA increase the mean number of instrumental responses for an estrous female presented under a second-order schedule of reinforcement (Hughes, Everitt, & Herbert, 1990).

Electrolytic lesions of the BNST significantly reduce male hamsters' chemoinvestigatory behavior, even though the majority of males continue to copulate normally (Powers, Newman, & Bergondy, 1987). These findings suggest a direct role for this area in the expression of certain appetitive behaviors. In contrast, lesions of the corticomedial amygdala, but not the basolateral amygdala, have been shown to decrease the number of ejaculations achieved by male rats (Harris & Sachs, 1975), increase the

ejaculation latencies, and reduce the male's chemosensory investigation of the female (Lehman & Winans, 1982; McGregor & Hebert, 1992). Previous studies investigating the excitatory role of olfactory stimulation in male Syrian hamsters have suggested that the chemosensory input that the mPOA receives from the VNS, and from the interconnections between the MEA and BNST, is necessary for the complete expression of male copulatory behavior (Coolen & Wood, 1999; Wood & Newman, 1995). Lesions of the BNST resulted in males displaying more intromissions preceding ejaculation, fewer ejaculations, longer intervals between intromissions, and longer postejaculatory refractory periods than intact males (Claro, Segovia, Guillamon, & Del Abril, 1995; Valcourt & Sachs, 1979). Bilateral lesioning of the medial nucleus of the amygdala eliminates copulation in the male hamster, and decreases grooming and anogenital investigation of the female (Lehman, Winans, & Powers, 1980). Finally, lesions of the VNS in male rats increase the number of intromissions prior to ejaculation, increase the ejaculation latency, and decrease the proportion of males that ejaculate (Saito & Moltz, 1986). Overall, these findings demonstrate the different role of specific brain areas during male sexual behavior, and the importance of their interconnection to achieve typical levels of male copulation.

Immunocytochemical visualization of Fos, the protein product of the immediate early gene c-fos, has been used widely in rats, hamsters, and gerbils as a marker for the activation of neurons in response to a variety of sexual stimuli, including olfactory sensory stimuli (Coolen, Peters, & Veening, 1997; Heeb & Yahr, 1996; Hoffman, Smith, & Fitzsimmons, 1992; Morgan, & Curran, 1995; Oboh, Paredes, & Baum, 1994). Precopulatory behavior such as investigation of the receptive female's genital area results

in an increase in Fos-like immunoreactivity (Fos-IR) in the posteromedial subdivision of the bed nucleus of the stria terminalis (BNSTpm), and the posterodorsal subdivision of the (MEApd), and subparafascicular nucleus (SPF) of male rats (Coolen, Peters & Veening, 1997). In addition, intromissions and ejaculations, but not mounts, have been shown to induce Fos expression within particular brain areas such as the mPOA, PdPN, and MEApd (Baum & Everitt, 1992; Coolen, Peters, & Veening, 1997; Heeb & Yahr, 1996; Roberston, Pfaus, Atkinson et al., 1991). In summary, these findings suggest a differential neural activation following appetitive behaviors and following mounts (i.e., BNSTpm and MEApd) versus intromissions and ejaculations (i.e., mPOA, PdPN, and MEApd). An additional brain area associated with the motivational aspects of male behavior has been the VTA. None of the studies discussed reported neural activation within the VTA following either appetitive and/or consummatory sexual behaviors. The following section reviews the literature extant on this brain area and the ways in which it pertains to motivated behaviour in general and male copulatory behavior in particular.

The role of the VTA in Goal-Directed Behavioral Responses

The limbic system refers to the amygdala, hippocampus, and other forebrain structures, and their connections to the hypothalamus and to the midbrain, in particular to the ventral tegmental area (VTA), via the medial forebrain bundle (MFB) (Mogenson, Jones, & Yim, 1980). Many studies have demonstrated the involvement of limbic forebrain structures and the hypothalamus in goal-directed behavioral responses (Mogenson, 1977; Mogenson & Calaresu, 1975; Livingston & Hornykiewicz, 1978; Stevenson, 1969; Yim & Mogenson, 1980). In cats, attack responses are elicited by electrical stimulation of the amygdala and lateral hypothalamus (Flynn, 1967; Hess,

1957) and the VTA (Bandler, Chi, & Flynn, 1972). Lesioning of the VTA disrupts attack responses induced by electrical stimulation of the amygdala and lateral hypothalamus (Proshansky, Blander, & Flynn, 1974), and electrical lesioning of the lateral hypothalamus attenuates attack responses and results in the degeneration of fibers originating in the VTA (Chi & Flynn, 1971). Similar results have been observed for ingestive behaviors in rats. Lesioning of the MFB-LH, a region which elicits drinking and feeding with electrical stimulation, also results in the degeneration of fibers originating in the VTA (Huang & Mogenson, 1972). In order to understand the role of the VTA in goal-directed behavioral responses, the involvement of the dopaminergic (DAergic), GABAergic and opioid systems within the VTA and their forebrain targets is reviewed in the following sections. In addition, the current literature on the effects of hormone implants in the VTA on male copulation is reviewed within this section.

VTA and DA

The VTA is the source of mesocorticolimbic dopaminergic (A10) neurons projecting to the NAcc, the basolateral and central subnuclei of the amygdala, lateral septum, olfactory tubercle, and medial prefrontal cortex (Lindvall & Bjorklund, 1978; Moore and Bloom, 1978; Thierry, Blanc, Sobel, Stinus, & Glowinski, 1973; Ungerstedt 1971). Injections of dopamine (DA) into the NAcc initiate locomotor responses in rats tested in open fields (Costall & Naylor, 1976; Jones, Wu, & Mogenson, 1978). Injections into the NAcc of apomorphine, a non-selective DA receptor agonist, or amphetamine, which causes DA release and blockade of DA reuptake, also increase locomotor responses (Costall & Naylor, 1976; Kelly, Seviour, & Iversen, 1975). Furthermore, the hyperactivity observed with high doses of amphetamine is abolished when the DAergic

projections to the NAcc from the VTA are destroyed by injections of 6-hydroxydopamine (Kelly, Seviour, & Iversen, 1975). Findings from these studies implicate the mesolimbic DA projections from the VTA to the NAcc in locomotor responses. In addition to locomotor responses, the DA projections from the VTA are associated with the incentive properties of reward-related stimuli (Berridge & Robinson, 1998). Although, DA increases significantly in the NAcc and VTA in response to eating and drinking (Yoshida, Yokoo, Mizoguchi et al., 1992), within the NAcc the DA increase depended on the type of food ingested, arguing against an exclusive role for motor or reward processes in determining DA activity (Blackburn, Phillips, Jakubovic, & Fibiger, 1986).

The inhibitory effects of drug withdrawal on DA neurons' activity within the VTA have been previously examined (Rasmussen & Czachura, 1995; Shen & Chiodo, 1993). Acute withdrawal after repeated ethanol treatment reduces the number of spontaneously active dopaminergic (DAergic) neurons in the VTA (Shen & Chiodo, 1993). Furthermore, nicotine withdrawal leads to increased firing rates of midbrain DA neurons, which return to normal levels following exposure to nicotine (Rasmussen & Czachura, 1995). Findings from these studies demonstrate the involvement of the VTA and its DA projections to the NAcc in the expression of ingestive behaviors and drug addiction.

Numerous studies have examined DA activity in the NAcc during male copulation (Blackburn, Pfaus, & Phillips, 1992; Damsma, Pfaus, Wenkstern, Phillips, & Fibiger, 1992; Fiorino, Coury, & Phillips, 1997; Fiorino & Phillips, 1999; Hull, Bitran, Pehek et al., 1989; Mas, Fumero, Fernandez-Vera, & Gonzalez-Mora, 1995; Pfaus, Damsma, Nomikos et al., 1990; Pfaus & Phillips, 1991; Pleim, Matochik, Barfield, &

Auerbach, 1990; Wenkstern, Pfaus, & Fibiger, 1993). DA levels in the NAcc increase during the anticipatory phase of male sexual behavior (e.g., when a male is placed into the mating chamber and exposed to olfactory cues remaining in the chamber after previous copulation tests (Pfaus, Damsma, Nomikos et al., 1990). Furthermore, DA levels in the NAcc increase during the first ejaculatory series and then decrease following each ejaculation, although not returning to basal levels during the PEI (Blackburn, Pfaus, & Phillips, 1992). Progressively smaller increases in DA within the NAcc are observed during subsequent ejaculatory series leading to sexual exhaustion (Fiorino, Coury, & Phillips, 1997; Blackburn, Pfaus, & Phillips, 1992). In addition, blocking DA receptors by bilateral infusions of haloperidol, a DA antagonist, in the NAcc decreases conditioned level changing (Pfaus & Phillips, 1991). Bilateral infusion of d-amphetamine into the NAcc attenuates the decrease in instrumental behavior (i.e. lever-pressing for a neutral light which had gained reinforcing properties through its prior association with the presentation of a receptive female) observed following lesions of the basolateral amygdala (Everitt, Cador, & Robbins, 1989). Finally, NMDA lesions of the NAcc have been recently shown to decrease the proportion of males displaying non-contact erections, intromissions, and ejaculations (Kippin, Sotiropoulos, Badih, & Pfaus, 2000). Findings from these studies suggest that DAergic projections from the VTA to the NAcc might be involved in the control of both appetitive and consummatory sexual behaviors in the male rat.

Electrophysiological studies have shown that the firing rate of DA neurons in the VTA is enhanced by male copulation (Hull, Bitran, Pehek et al., 1989). Microinjection into the VTA of apomorphine, a DA agonist, results in an increase in the latency for

males to initiate copulation, and a decrease in the number of mounts, intromissions, and ejaculations (Hull, Bitran, Pehek et al., 1989). In contrast, microinjection of *cis*-Flupenthixol, a DA antagonist, shortens the latency for males to begin copulating (Hull, Bitran, Pehek et al., 1989). These findings suggest the involvement of impulse-regulating autoreceptors on cell bodies of the A10 mesocorticolimbic dopamine neurons in determining the effects of DA agonists or antagonists in the VTA.

VTA and GABA

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the adult mammalian central nervous system (Bormann, 1991; Matsumoto, 1989; Mott & Lewis, 1994). Evidence from anatomical studies has shown that GABA is contained in a subpopulation of neurons in the mesolimbic pathway from the VTA to the NAcc (Kalivas, 1993; Kalivas, Churchill, & Klitenik, 1993; Van Bockstaele & Pickel, 1995). VTA neurons also receive inhibitory GABA inputs from the NAcc (Wolf, Olpe, Avirith, & Hass, 1978; Yim & Mogenson, 1980). Several studies have shown that administration of GABA into the VTA inhibits the discharge rate of DAergic neurons (Yim & Mogenson, 1980; Suaud-Chagny, Chergui, Chouvet, & Gonon, 1992). Electrophysiological studies have shown that $GABA_B$ receptors located on VTA DAergic neurons are hyperpolarized by the $GABA_B$ receptor agonist, baclofen (Mueller & Brodie, 1989). Intracellular recordings demonstrate that $GABA_A$ agonists also hyperpolarize DAergic neurons in the VTA (Johnson & North, 1992; Olpe, koella, Wolf, & Hass, 1977; Seabrook, Howson, & Lacey, 1990). In addition, application of the $GABA_A$ antagonists, picrotoxin or bicuculline, increases the discharge rate of DA neurons (Johnson & North, 1992; Yim & Mogenson, 1980). Finally, injection of picrotoxin to the VTA increases

locomotor activity (Wolf, olpe, Avrith, & Hass, 1978; Yim & Mogenson, 1980). However, pre-treating the NAcc with spiroperidol, a DA receptor antagonist, attenuates the increase in locomotor activity. These findings suggest that mesolimbic DAergic neurons are disinhibited by picrotoxin, increasing the release of DA from the axon terminals and thereby increasing locomotor responses. Accordingly, GABA inputs on DA neurons must therefore be inhibitory.

Although previous studies have demonstrated the presence of GABA interneurons within the VTA and their facilitative role in locomotor responses, the role of GABA within the VTA in the expression of male sexual behaviors remains unclear at present. However, many studies have investigated the role of the GABAergic system in other brain sites associated with male copulation such as the mPOA. Microinjection of bicuculline methiodide into the mPOA of intact males decreases the PEI and ejaculation latency, but has no effect on the number of mounts, intromissions, or ejaculations (Fernandez-Guasti, Larsson, & Beyer, 1986). An increase in the number of mounts and intromissions was observed among castrated male rats when bicuculline was administered in combination with subthreshold doses of TP (150μg/kg/day) (Fernandez-Guasti, Larsson, & Beyer, 1986). Injection of muscimol or ethanolamine-O-sulphate, both GABA agonists, abolished all consummatory behaviors (Fernandez-Guasti, Larsson, & Beyer, 1986). In addition, intraperitoneal injection of the $GABA_A$ agonist, 3-amino-1-propanesulfonic acid, or the $GABA_B$ agonist baclofen, decreased the number of mounts, intromissions, and ejaculations in intact males (Ågmo & Paredes, 1985). Findings from these studies demonstrated that GABA neurotransmission within the mPOA inhibits male copulation.

VTA and Gonadal Hormones

Early autoradiographic studies revealed the absence of intracellular estrogen (ER) and certain androgen receptors (AR) within the substantia nigra (SN), VTA, and retrorubral fields (RRF) and their major striatal, cortical and limbic targets (ER: Pfaff & Keiner, 1973; AR: Heritage, Stumpf, Sar, & Grant, 1981). These findings suggested that the facilitative effects observed on male copulation following T or E manipulations within those areas were due to genomic actions exerted on midbrain DA neurons (Mitchell & Stewart, 1989). However, subsequent studies using immunocytochemical and in situ hybridization techniques have identified subpopulations of intracellular ARs in the VTA, suggesting that specific subsets of midbrain neurons might be direct targets of gonadal hormones (Don Carlos, Monroy, & Morrell, 1991; Simmerly, Chang, Muramatsu, & Swanson, 1990). Furthermore, a recent study using double label immunocytochemistry has mapped intracellular colocalized ER or AR and TH, a selective marker for DA containing neurons, in the VTA of intact, adult male rats (Kritzer, 1997). ERs were present within subpopulations of cells in the ventrolateral paranigral VTA (VTApn), and ARs were numerous in the VTApn and parabrachial VTA (VTApb). Furthermore, nearly every androgen receptor-bearing cell in the VTA was TH immunopositive (Kritzer, 1997). These findings suggest that DA systems in the VTA of male rats could be regulated by androgens or by aromatized E.

Rationale for the Current Research

The sexual behavior of male rats follows a stereotyped pattern wherein a series of mounts and intromissions leads to ejaculation. Following such a series, there is a predictable refractory period before resumption of sexual activity. Although it has been

known that castration leads to deterioration or disappearance of copulatory behavior in rats and in other species (Cooper & Aronson, 1974; Davidson, 1966; 1980; Hart, 1968; Miesel & Sachs, 1994; Phoenix, Slob, & Goy, 1973; Sachs & Barfield, 1976), little attention has been paid to the time course of this deterioration, and to the experential factors that might augment or inhibit those effects. Furthermore, although the androgen-dependent nature of male sexual behavior was one of the first endocrine phenomena to be discovered (Berthold, 1849), many aspects of this interaction have still not been elaborated. This thesis examined the time course of androgen reinstatement of appetitive and consummatory sexual behaviors in long-term castrated male rats that had different amounts of sexual experience prior to, or following, castration.

In Chapter 1, the progressive decline of appetitive and precopulatory sexual behaviors (i.e., level changes, pursuits, anogenital investigations, and attempted mounts) and consummatory sexual behaviors (i.e., mounts, intromissions, and ejaculations) were examined in male rats following castration. The subsequent reinstatement of these behaviors was examined following daily treatment with testosterone (T). The effect of sexual experience in the decline and reinstatement of sexual behaviors following castration and T replacement was also examined in male rats that had 1 versus 10 prior sexual experiences to ejaculation. Findings from these experiments established the necessary behavioral, hormonal, and experiential parameters for subsequent studies.

Brain regions activated by copulatory stimulation in gonadally intact male rats have received extensive study using Fos immunocytochemistry. Several forebrain structures that are activated are also important in the hormonal stimulation of sexual behavior in the rat, most notably the mPOA, BNSTpm, and the MEApd (Baum & Everitt,

1992; Coolen, Peters, & Veening, 1997). Experiments in Chapter 2 examined Fos activation within these and other regions following different genitosensory (i.e., mounts, intromissions, and ejaculations) and olfactory stimuli. In addition to Fos activation observed in areas previously associated with male copulation, findings from this study revealed Fos activation in the VTA of male rats following multiple ejaculations. Subsequently, a series of experiments was designed to further investigate the role of the VTA in male copulation.

The VTA, and in particular its DA projections to the NAcc, is associated with goal-directed behaviors (Mogenson & Calaresu, 1975; Mogenson, 1977; Yim & Mogenson, 1980; Shen & Chiodo, 1993; Ramussen & Czuchura, 1995). Building on the data of Chapter 2, Experiment 1 of Chapter 3 examined the neurochemical identity of cells activated following specific copulatory activity, using double immunocytochemistry to colocalize Fos and either tyroxine hydroxylase (TH), or Fos and γ–Aminobutyric acid (GABA) within the VTA of intact male rats after sexual testing with receptive females. To further understand the overall role of the VTA in male copulation, the effects of NMDA lesions on male sexual behaviors were investigated in Experiment 2 of Chapter 3. Lesions of the mPOA are known to disrupt consummatory behaviors without disrupting the expression of anogenital investigation or pursuits (Edwards & Einhorn, 1986; Hansen, Kohler, Goldstein, Steinbusch, 1982). Lesions of the VTA were expected to disrupt the appetitive aspects leading to the receptive female, given the previous association of this brain region with goal-directed behavioral responses. The effects of the VTA lesions were also examined in sexually experienced and relatively inexperienced males (with either 1 or 10 prior tests to ejaculation with receptive females prior to the lesions). The final question addressed in Chapter 3 concerned the existence of a

mechanism of action within the VTA regulated by gonadal steriods. Previous evidence has shown the localization of androgen receptor (AR), progesterone receptor (PR), and estrogen receptor-beta (ER-β) within the VTA (Kritzer, 1997; Pfaff & Keiner, 1973; Simmerly, Chang, Muramatsu, & Swanson, 1990). Accordingly, Experiment 3 of Chapter 3 investigated the effects of intracranial implants of crystalline steroid hormones in the VTA on the copulatory activity of castrated male rats. Cannulae containing T, estradiol (E), progesterone (P), or cholesterol (CH) were implanted bilaterally into the VTA of long-term castrated males that received extensive sexual experience prior to castration, and the reinstatement of sexual behavior was examined at 4-day intervals following in the implant.

The results of these studies were discussed in terms of the ability of steroid hormones to induce sexual arousability by actions in several brain regions, including the VTA, and the role of sexual experience in the sensitization of mechanisms associated with competent sexual activity. Mechanisms by which steroid hormones may act in the VTA are also proposed.

CHAPTER I

APPETITIVE AND CONSUMMATORY SEXUAL BEHAVIORS FOLLOWING CASTRATION AND TESTOSTERONE REPLACEMENT: THE ROLE OF EXPERIENCE

Experiment 1. Effects of Castration on Appetitive and Consummatory Behaviors.

The first step toward investigating the role of castration and T replacement in the expression of appetitive and consummatory male sexual behaviors is to determine systematically the length of time taken by male rats to cease these behaviors following castration, in the absence of any hormonal treatment. A considerable amount of work has been done in the hormonal regulation of copulatory behavior and there is substantial information regarding the effects of castration and T replacement. The restoration of copulation in long-term castrated males depends on the dose of androgen given to the animal, the route of administration (i.e., systemic versus central injections), and the schedule of behavioral testing (Beach & Holtz-Tucker, 1949; Christensen & Clemens, 1974; Smith, Damassa, & Davidson, 1977).

Interestingly, although previous studies have examined the role of testosterone in the restoration or maintenance of copulatory behavior in male rats, few have described the gradual decline in sexual behavior following castration and the gradual recovery after daily T administration in a systematic fashion (e.g., as in Beach & Holz-Tucker, 1948; Stone, 1939). Furthermore, most of those studies have examined the decline of copulatory behavior and not of the appetitive aspects of male sexual behavior. The study of the appetitive aspects of sexual behavior in the male rat has provided information about how levels of sexual excitement and arousal vary as a function of T, and in a

manner that is not necessarily confounded with the level of sexual receptivity of the female partner (Beach & Holz-Tucker; Stone, 1939; Everitt & Stacey, 1987). The purpose of the initial study of Chapter 1, therefore, was to examine both the appetitive and consummatory aspects of male rat sexual behavior following castration and its reinstatement following daily administration of TP. This study examined the progressive decline and gradual return of appetitive (level changes), precopulatory (pursuit behavior, anogenital investigation and attempted mounts) and consummatory (mounts, intromissions and ejaculations) sexual behaviors in male rats following castration and T replacement respectively. Appetitive, precopulatory and consummatory behaviors were examined in sexually experienced castrated males that were tested with sexually receptive females at four-day intervals, beginning one week after castration.

Materials and Methods

Animals

Forty adult male Long-Evans hooded rats from Charles River Canada (St. Constant, Québec, Canada) weighing 250-280g upon arrival were used as subjects. Twenty adult female Long-Evans hooded rats from the same supplier weighing 240-250g, were used as stimulus females. All animals were housed two per cage in plastic shoebox cages and on a reversed 12-hour light-dark cycle with lights off at 08h00. Food (Purina Rat Chow) and water were available ad libidum. The animal colony was maintained at a constant temperature of 21°C.

Females were anesthetized with a mixture of ketamine hydrochloride (50 mg/ml) and xylazine hydrocholride (4 mg/ml), mixed at a ratio of 4:3 ml, respectively, injected intraperitoneally (i.p.) in a volume of 1 ml/kg of body weight, and ovariectomized

bilaterally via lumbar incisions. Once ovaries were removed and the fallopian tubes ligated, the incisions in the peritoneal walls and the skin were sewn shut with surgical silk. The animals recovered under a heat lamp overnight, then were returned to their home cages. All animals were given a week to recuperate after surgery. Subsequently, artificial estrus was induced by subcutaneous injections of 10 μg of estradiol benzoate (dissolved in 0.1ml reagent-grade sesame oil) 48 hours prior to each copulatory test, and 500 μg of progesterone (dissolved in 0.1ml reagent grade sesame oil) 4 hours before each test. To assure a high level of sexual experience, these females were given 10 tests of sexual behavior with sexually experienced males prior to behavioral testing as stimulus females.

Sexual Behaviors

Mammalian sexual behavior is composed of appetitive, precopulatory, and consummatory phases (Pfaus, 1996; 1999). The following paragraph describes the appetitive and precopulatory behaviors examined in this thesis:

Appetitive Behaviors:

a) *Conditioned level changes.* The number of times that the male changes levels in the bilevel chamber during the 5 minute habituation period without the female.

Precopulatory:

b) *Anogenital investigations.* The number of times the male investigates the female's anogenital region.

c) *Pursuit of the female.* The male follows the receptive female around the bilevel testing chamber after the female is introduced into the chamber, counted as number of level changes per mount.

d) *Attempted mounts.* The male approaches the female from the back, presses against her, lifts up one of its front legs and place it momentarily on her back, but does not mount fully or display pelvic thrusting.

The consummatory aspects include all copulatory responses (i.e., mounts, intromissions, and ejaculations; Davidson, Kwan & Greenleaf, 1982). Between mounts and intromissions, the male typically displays anogenital investigation of the female and pursuit. Following an intromission the male usually grooms his genitals, although genital grooming might also occur following mounts without intromission. After a series of mounts and intromissions, ejaculation follows. At this point the male will first groom his genitals and then begin a period of inactivity referred to as the post-ejaculatory interval (PEI), which may last for several minutes. Under laboratory conditions, male rats can have 2-4 ejaculatory series in a 30-min test session (Meisel & Sachs, 1994; Pfaus, Mendelson, & Phillips, 1990). The following paragraphs describes each of the consummatory behaviors examined in this thesis:

a) *Mounts.* The male, while clasping the female in the lateral-lumbar region, holds to the female's hindquarters with its forepaws and slowly moves its hindquarters in piston-like fashion (Stone, 1939; Beach, 1942; 1956; Beach & Holtz-Tucker, 1949; Smith, Damassa, & Davidson, 1977; Davidson, 1980; Larsson, 1979).

b) *Intromissions.* The male mounts the female, gains vaginal penetration and then dismounts abruptly without an obvious backward thrust.

c) *Ejaculations.* This pattern of behavior is characterized by an intromission of long duration along with simultaneous abdominal and pelvic tightening, after which the male grooms his penis and becomes behaviorally quiescent.

Procedure

Intact males and ovariectomized, hormone-primed females were pre-exposed to bilevel chambers for five consecutive days (20 min/day). This habituation procedure has been shown to increase the proportion of males that become vigorous copulators (Pfaus & Wilkins, 1995). Following this pre-exposure period, intact males were given sexual experience with sexually-experienced receptive females. During this training period, males were placed in the bilevel chamber for 5 minutes, after which the female was introduced for a 30-min copulatory test. Appetitive measures (e.g., frequency and latency of level changes), precopulatory measures (e.g., the frequency and latency of anogenital investigations, pursuit behaviours and attempted mounts), and consummatory measures (e.g., frequency and latency of mounts, intromissions, and ejaculations) were recorded for each male during each session. Males were given 10 training sessions at 4-day intervals prior to castration. Only animals that ejaculated at least twice during the last 5 training sessions were included in the study. Measures of sexual behavior during the last training session were used as baselines.

Following the 10 testing trials all male rats were castrated under methoxyflurane anaesthesia within a one-week period. One midline incision was made to the scrotum, the

testes were ligated and removed, and the scrotum was sewn shut with surgical silk. All animals were given a week to recuperate prior to testing. The procedure used during the testing phase was identical to that used previously during the training period. Copulatory tests were performed in the bilevel chambers at 4-day intervals for a 16-week period. The last session prior to treatment with T was used as a post-castration baseline measure. The castrated males were then given daily subcutaneous injections (30 μg) of testosterone proprionate (TP; Steraloids) and exposed to a receptive female every four days for an additional 52 days. TP injections were always administered between 08:00 and 11:00h, and copulatory tests were conducted between 12:00 and 16:00h.

Behavioral and Statistical Analysis

Tests were videotaped and subsequently scored using a computerized behavioral scoring program ("Male Reproductive Behaviour, Data Acquisition/Analysis Program", by S. Ogawa, revised by Steve Cabilio). The mean number of appetitive level changes, precopulatory behaviors (anogenital investigation, pursuit of the female, and attempted mounts), and consummatory behaviors (mounts, intromissions and ejaculations) observed following castration and subsequent hormone treatment were analyzed using within group analyses of variance (ANOVAs). Significant main effects and interactions were followed by post hoc analyses of individual means using the Tukey method. Chi-square analysis was used for proportions of rats showing appetitive and consummatory behaviors before and after hormone treatment (i.e., pre-castration baseline versus test session 24; and post-castration baseline versus day 24 of TP treatment). The level of significance used for all comparisons was $p < 0.05$.

Results

Sexual Behaviors Following Castration

Appetitive and Precopulatory Behaviors. Figure 1 shows the percentage of animals displaying appetitive and precopulatory behaviors following castration. The proportion of males that attempted to mount decreased significantly over the 24 test sessions [χ^2(df=1) = 58.56, p<.0001] (i.e., T0 versus T24). However, the proportion of males showing anogenital investigations, pursuit of the female, and level changes, did not decline significantly after castration.

Figure 2 shows the mean number of appetitive and precopulatory behaviors displayed by males following castration. The mean number of anogenital investigations [F (24, 936) = 4.482, p<.001], and pursuits of the female [F (24, 936) = 8.404, p<.001] were found to be significantly different across the 24 test sessions following castration. (Figure 2). Post-hoc comparisons of the individual means revealed a significant difference in the mean number of anogenital investigations between baseline and test sessions 3-6, 8, and 19. In addition, post-hocs showed a significant decrease in the mean number of pursuits between baseline and test sessions 7-24. The mean number of attempted mounts and level changes remained unchanged over the 24 test sessions.

Consummatory behaviors. The percentage of males displaying consummatory behaviors is shown in Figure 3. The proportion of males showing mounts, intromissions, and ejaculations significantly decreased from the pre-castration baseline measure to the post-castration final copulatory session as follows: mounts [χ^2(df = 1) = 12.93, p<.001]; intromissions [χ^2 (df = 1) = 14.89, p<.001]; and ejaculations [χ^2(df = 1) = 15.71, p<.001]. The animals continued to engage in mounting behavior for two or three more sessions after

they stopped intromitting and ejaculating (Figure 3). A decrease in the mean number of mounts, intromissions and ejaculations was also observed over the 96 days of post-castration testing (Figure 4). A within-group ANOVA detected an overall significant difference for all three consummatory behaviors measured across the 24 test sessions following castration compared to pre-castration levels: for mounts [$F(24, 936)=10.69$, $p<.001$]; for intromissions [$F(24, 936)=10.22$, $p< .001$]; and for ejaculations [$F(24, 936) = 42.81$, $p< .001$]. Post hoc analysis revealed a significant difference between baseline and test sessions 3-24 for mounts and intromissions, and test sessions 6-7 and 9-24 for ejaculations. These findings showed that the effects of castration on male copulation can be observed starting 12 days following castration.

Sexual Behaviors Following Hormone Treatment

Appetitive and Precopulatory Behaviors. Figure 5 shows the proportion of animals that engaged in appetitive and precopulatory behaviors following TP (30 μg) replacement. The mean number of these behaviors following TP treatment is shown in Figure 6. The percentage of males showing pursuit of the female was significantly higher on days 16 and 40, compared to post-castration baseline measure on day 0 [for day 16: $\chi^2(df = 1) = 52.97$, $p<.001$; for day 40: $\chi^2(df = 1) = 59.85$, $p<.001$]. In addition, the proportion of animals showing attempted mounts was significantly higher on days 12-20 and days 36-40 following TP treatment, but then gradually decreased to post-castration baseline levels by day 52. The proportion of males displaying appetitive level changes and anogenital investigations did not change across the 52 days of TP treatment.

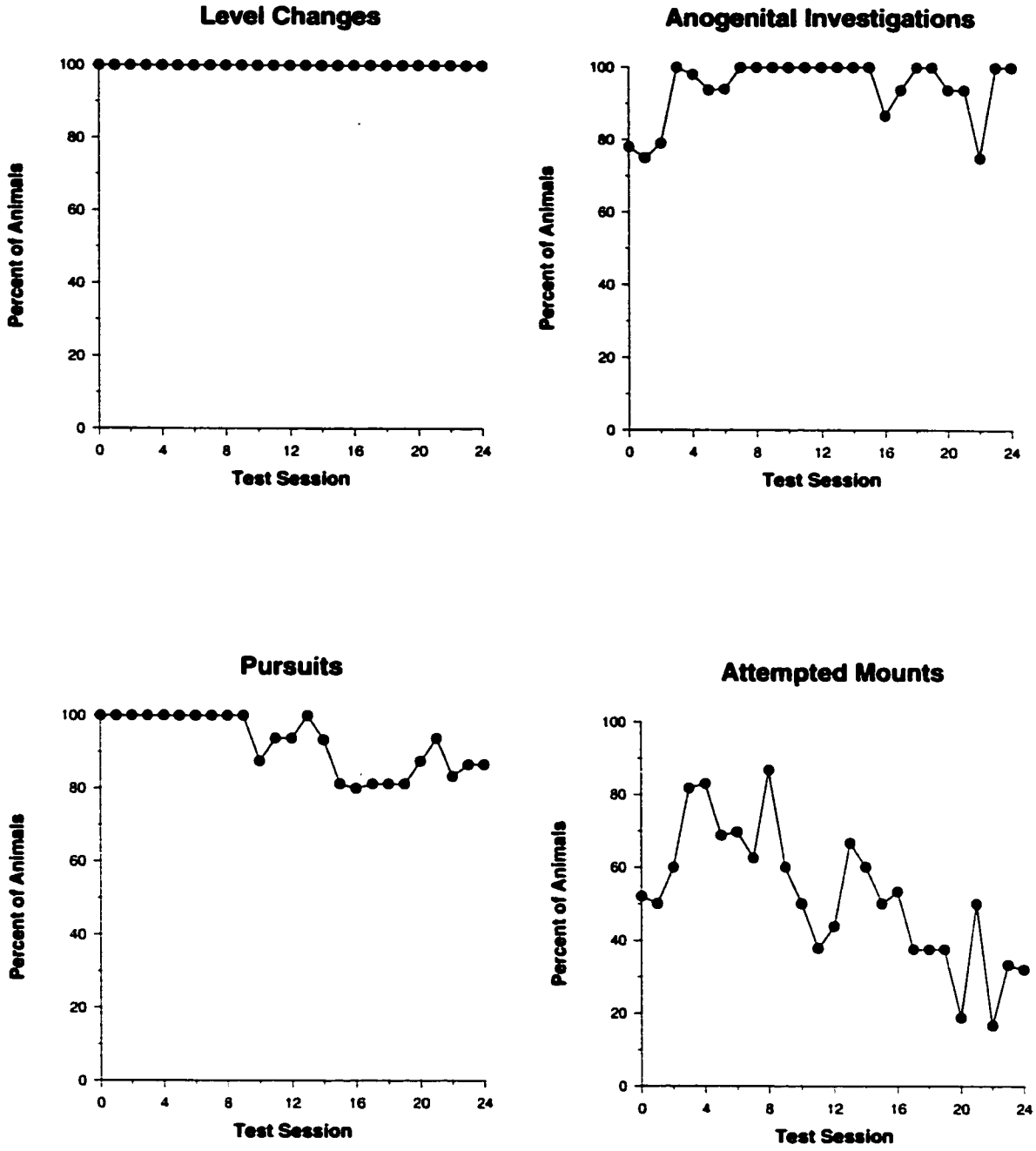


Figure 1. Percentage of sexually experienced males displaying appetitive conditioned level changing, and precopulatory behaviors (anogenital investigations, pursuits, and attempted mounts) following castration. Males were tested with receptive females at 4-day intervals for a period of 96 days (i.e., 24 test sessions). (Day 0: Baseline measure prior to castration).

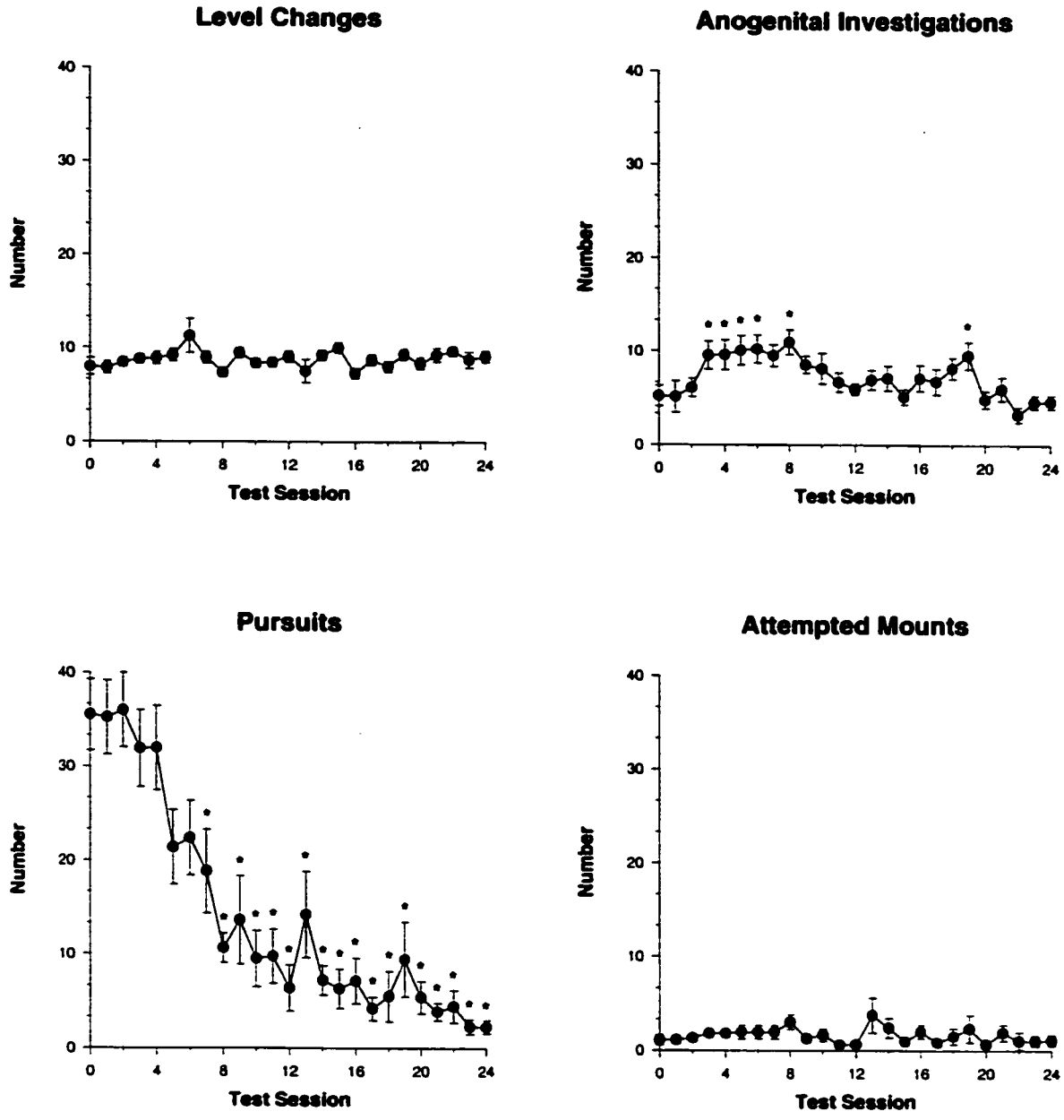


Figure 2. Mean number of appetitive conditioned level changing, and precopulatory behaviors (anogenital investigations, pursuits, and attempted mounts) observed in sexually experienced males following castration. Males were tested with receptive females at 4-day intervals for a period of 96 days (i.e., 24 test sessions). Data are means + SEM. Vertical lines show standard errors. (Day 0: Baseline measure prior to castration). * $p<.05$ significantly different from baseline.

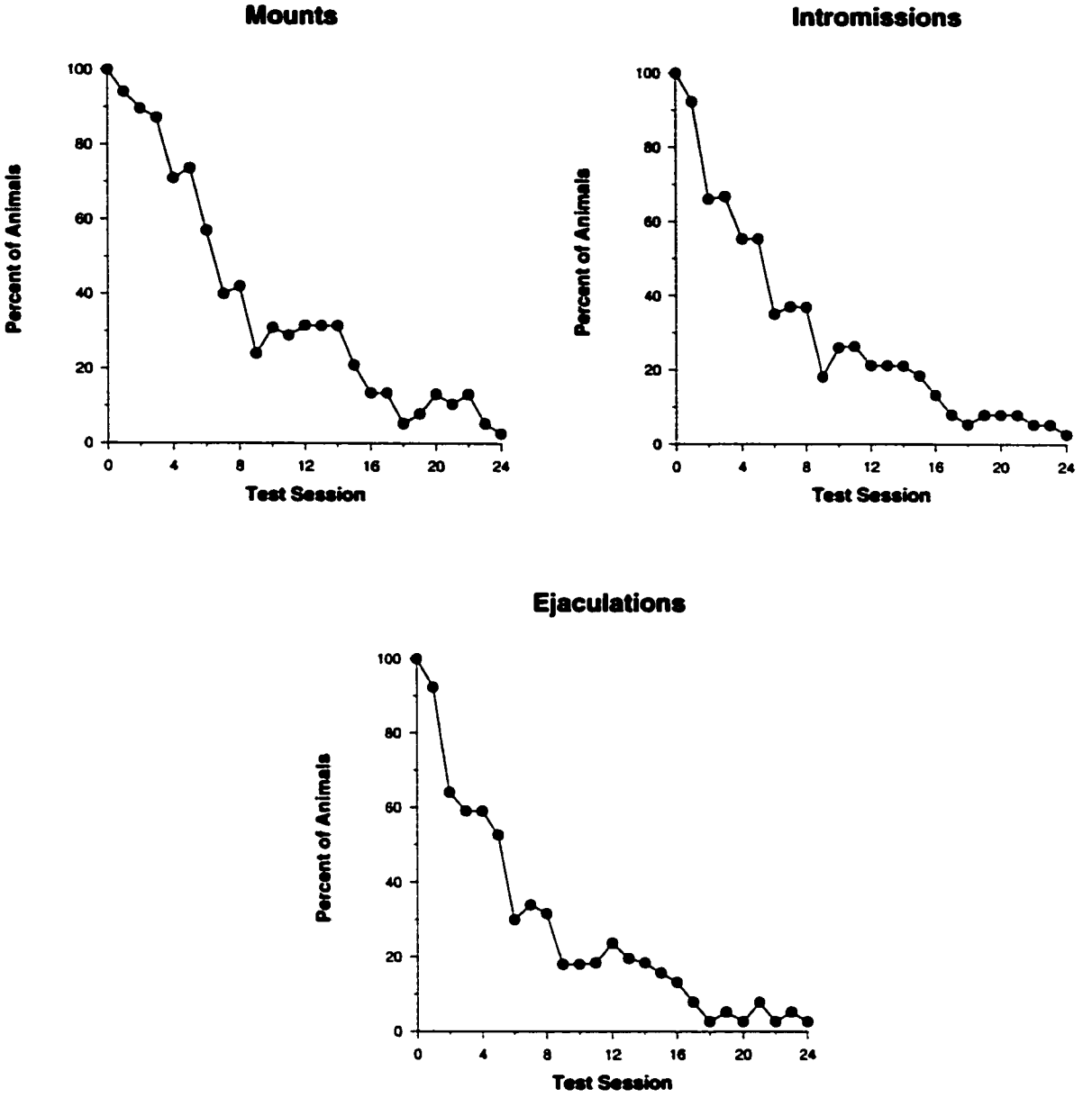


Figure 3. Percentage of sexually experienced males showing mounts, intromissions, and ejaculations following castration. Males were tested with receptive females at 4-day intervals for a period of 96 days (i.e., 24 test sessions). (Day 0: Baseline measure prior to castration).

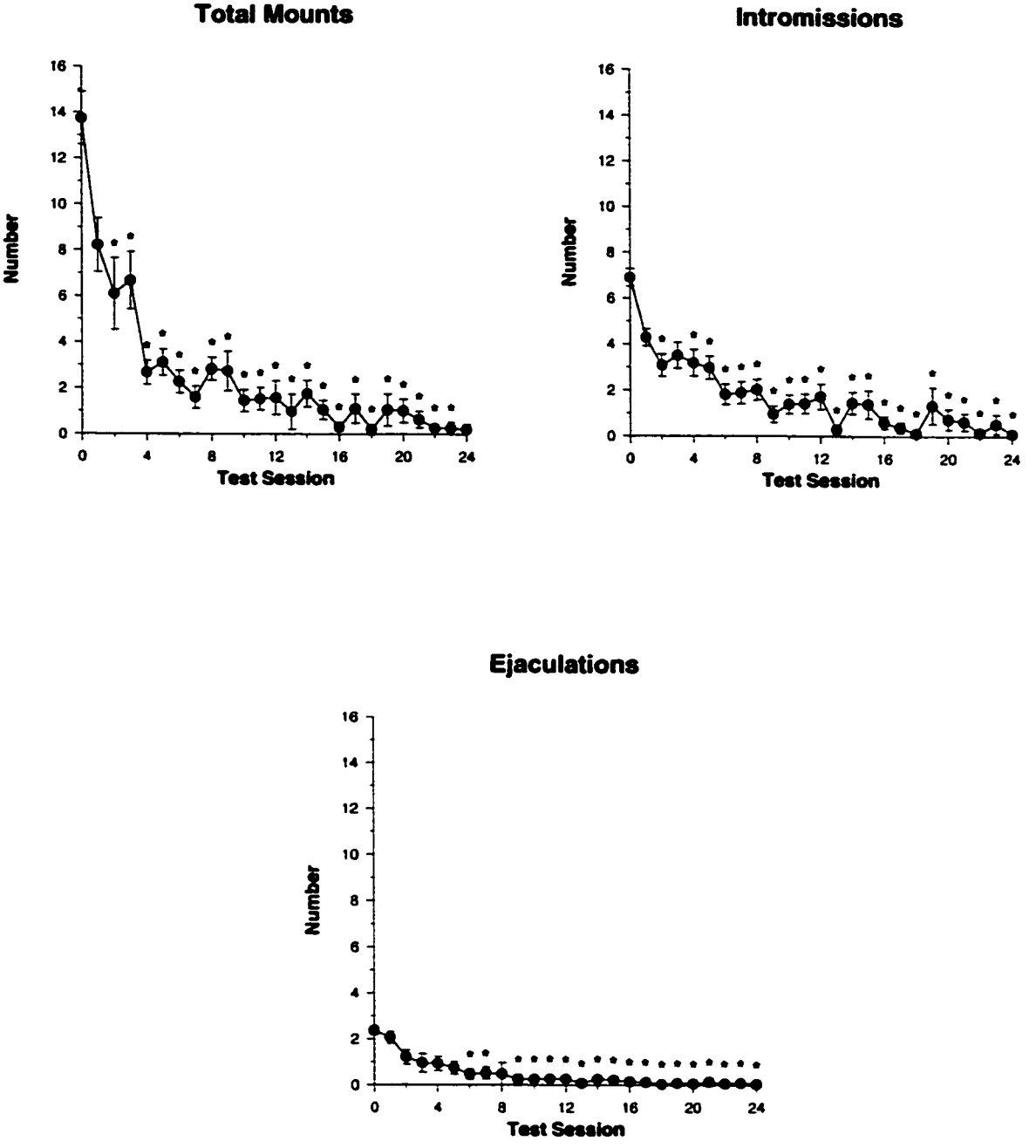


Figure 4. Mean number of mounts, intromissions and ejaculations displayed by sexually experienced males following castration. Males were tested with receptive females at 4-day intervals for a period of 96 days (i.e., 24 test sessions). Data are means + SEM. Vertical lines show standard errors. (Day 0: Baseline measure prior to castration). * $p<.05$ significantly different from baseline.

The mean number of pursuit behaviors significantly increased following TP treatment [$F(13, 507) = 8.67$, $p<.001$]. Post hoc comparisons revealed a significant difference between the post-castration baseline measure and TP treatment days 20-52. The mean number of pursuits observed after 52 days of TP treatment did not return to pre-castration levels (see Figure 2). During the 52 days of TP replacement and starting from Day 0 (i.e., following castration and after 24 test sessions with receptive females), all males showed anogenital investigation of the female. The mean number of anogenital investigations increased significantly after 12 days of TP injections [$F(13, 507) = 4.98$, $p<.05$]. Post hoc comparisons revealed significant differences between the baseline measure and days 16 and 36-52. The mean number of attempted mounts increased and decreased significantly over the 52 days of TP treatment [$F(13, 507) = 3.87$, $p<.05$]. Post hoc analysis showed significant differences between the baseline measure and days 12, 24, 28, 36 and 44. Finally, the mean number of level changes did not change significantly over the 52-day period.

Consummatory Behaviors. The effects of TP replacement on the percentage of animals displaying consummatory behaviors and their mean number are displayed in Figures 7 and 8. Statistically significant differences were found in the proportion of animals displaying mounts, intromissions, and ejaculations following TP-treatment: for mounts [$\chi^2(df = 1) = 71.91$, $p<.001$]; for intromissions [$\chi^2(df = 1) = 53.2$, $p<.001$]; for ejaculations [$\chi^2(df = 1) = 44.64$, $p<.001$]. Figure 8 shows the increase in the mean number of mounts, intromissions and ejaculations following T treatment over a 52-day period. A within group ANOVA detected a significant increase in the mean number

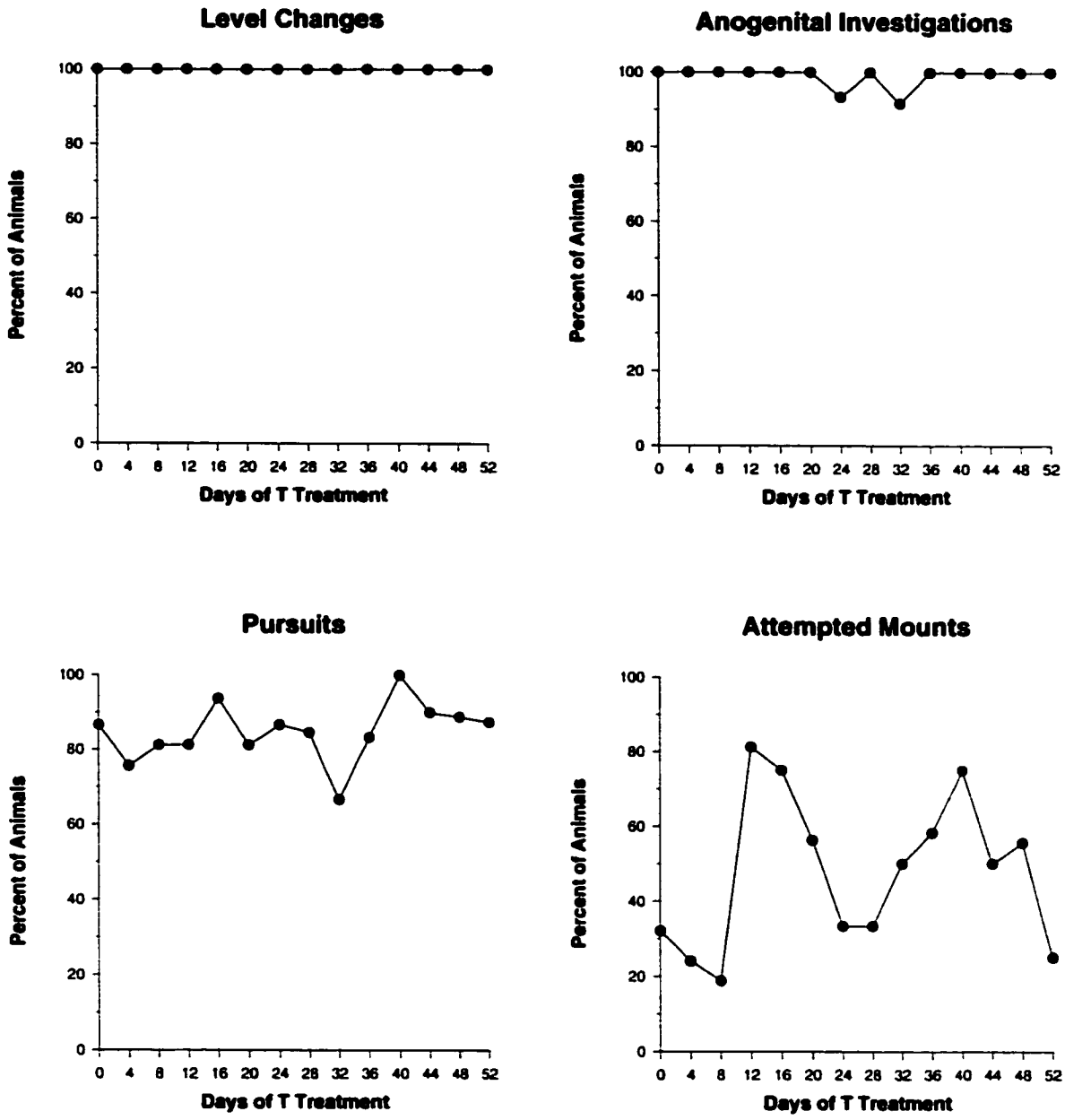


Figure 5. Percentage of castrated sexually experienced males displaying appetitive level changes and pre-copulatory (anogenital investigations, pursuits, and attempted mounts) behaviors following daily TP-treatment. Males were tested with receptive females at 4-day intervals for a period of 52 days. (Day 0: Baseline measure 24 test sessions following castration).

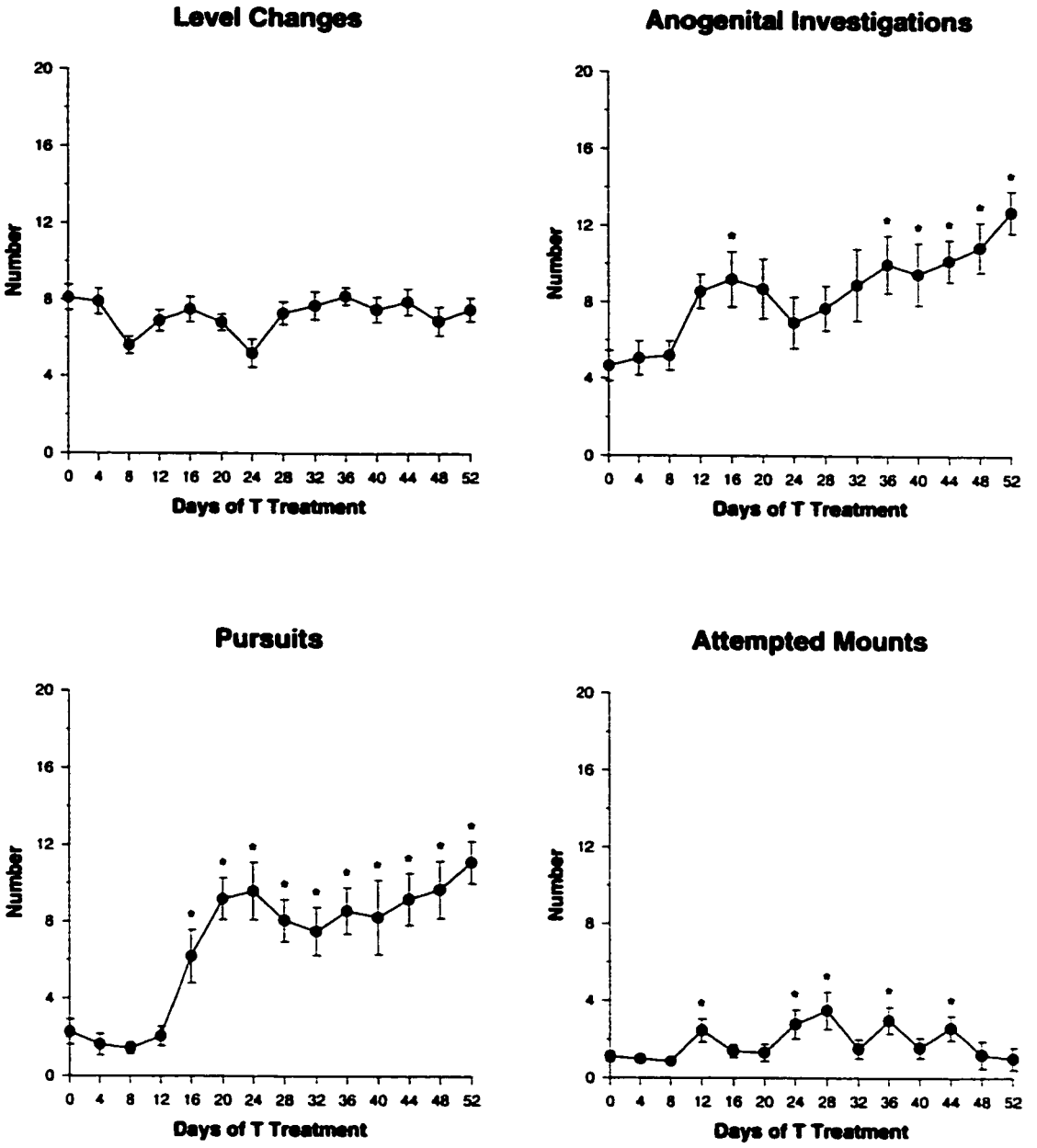


Figure 6. Mean number of appetitive level changes and precopulatory (anogenital investigations, pursuits, and attempted mounts) behaviors observed in castrated sexually experienced males following daily TP-treatment. Males were tested with receptive females at 4-day intervals for a period of 52 days. Data are means + SEM. Vertical lines show standard errors. (Day 0: Baseline measure 24 test sessions following castration).
* $p<.05$ significantly different from baseline.

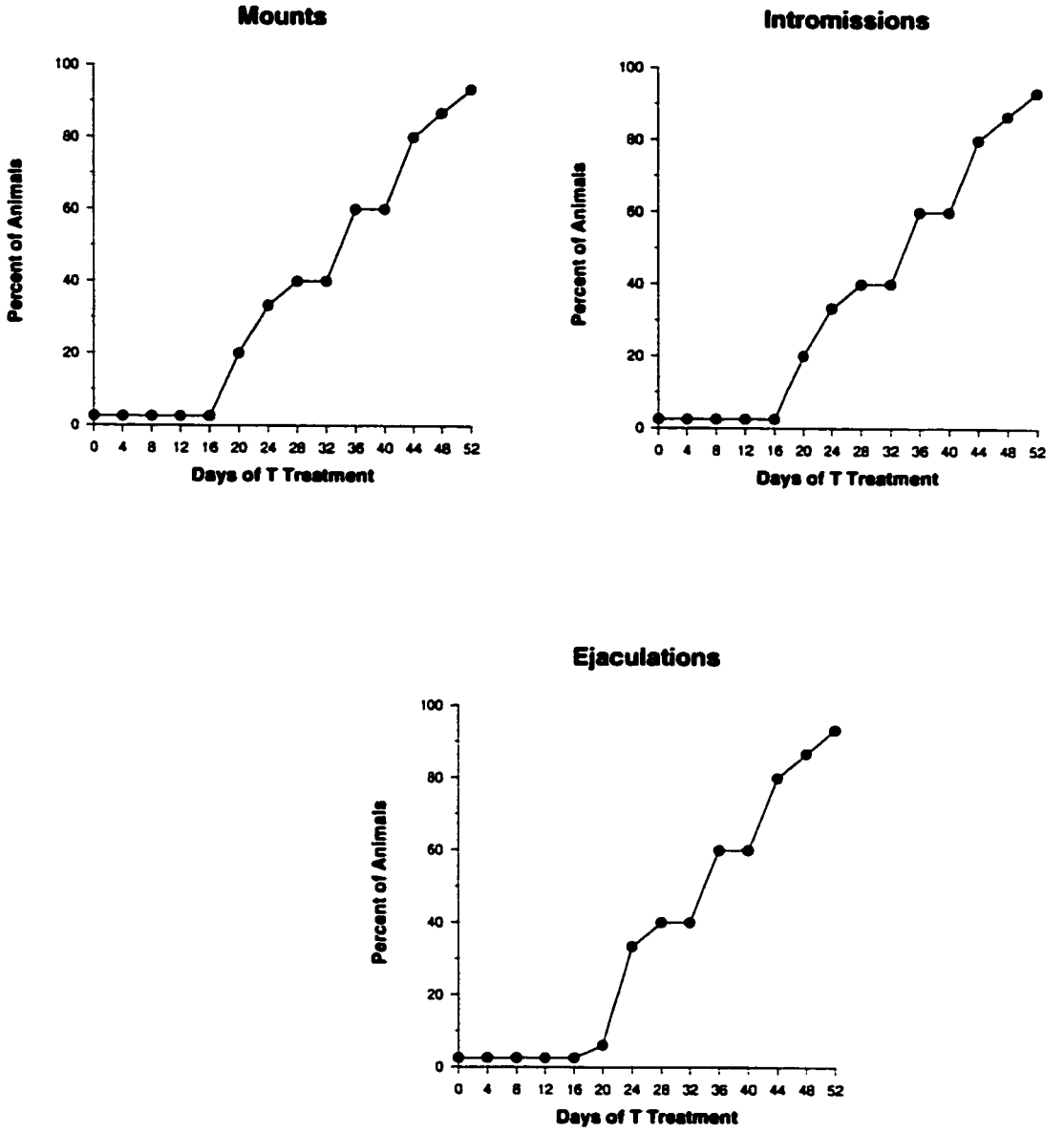


Figure 7. Percentage of castrated sexually experienced males displaying consummatory (mounts, intromissions, and ejaculations) behaviors following daily TP-treatment. Males were tested with receptive females at 4-day intervals for a period of 52 days. (Day 0: Baseline measure 24 test sessions following castration).

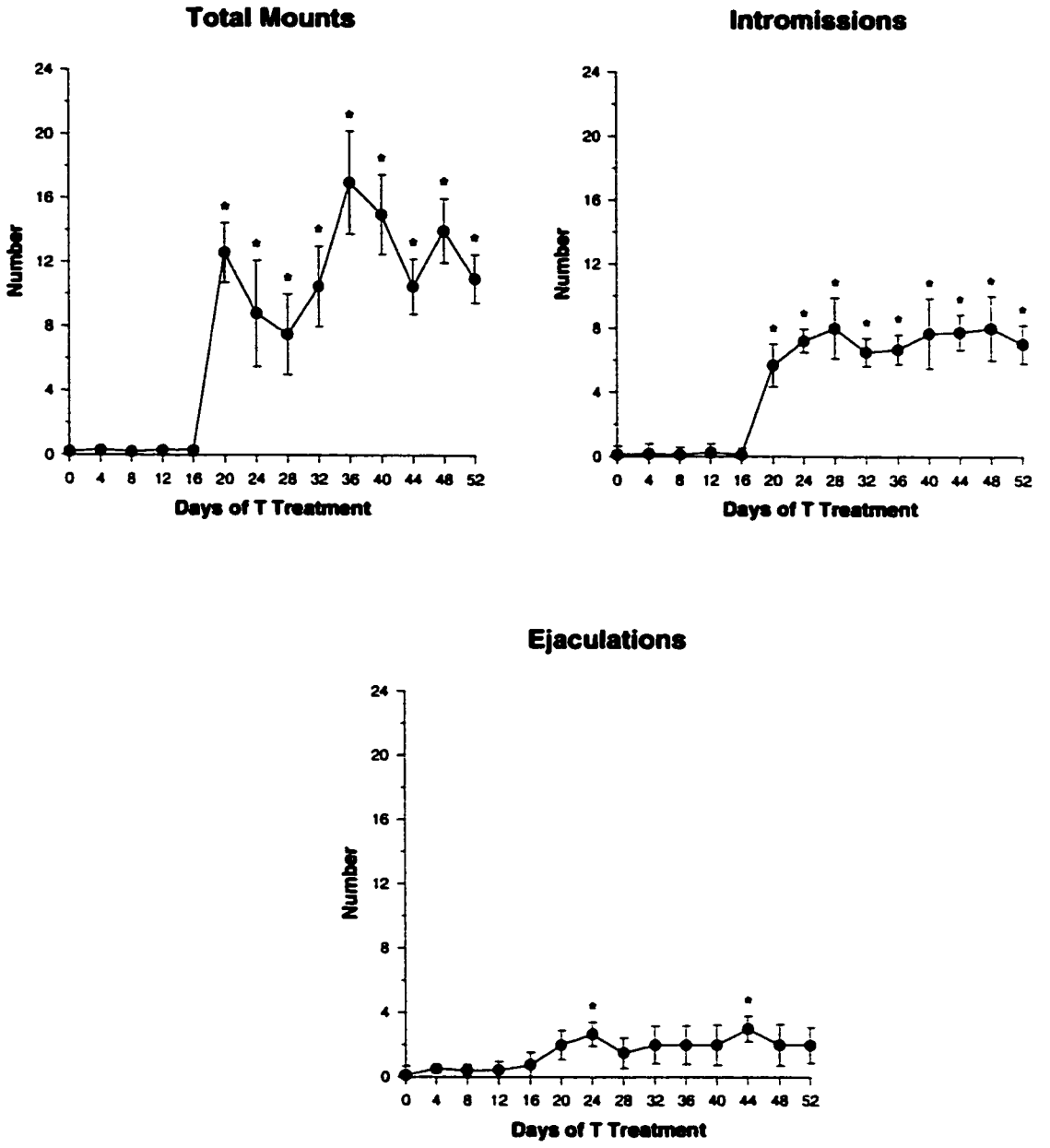


Figure 8. Mean number of consummatory behaviors (mounts, intromissions, and ejaculations) observed in castrated sexually experienced males following daily TP-treatment. Males were tested with receptive females at 4-day intervals for a period of 52 days. Data are means + SEM. Vertical lines show standard errors. (Day 0: Baseline measure 24 test sessions following castration). * $p<.05$ significantly different from baseline.

of mounts, intromissions and ejaculations following 52 days of T treatment: for mounts [$F(13, 507) = 14.06$, $p < .001$]; for intromissions [$F(13, 507) = 18.90$, $p < .001$]; and for ejaculations [$F(13, 507) = 14.19$, $p < .001$]. All consummatory behaviors were reinstated following 20 days of TP treatment. Post hoc comparisons revealed a significant difference in the mean number of mounts and intromissions between the baseline post castration measure and days 20-52. The mean number of ejaculations was found to be significant different from baseline measure on days 24 and 44.

Discussion

The results from this experiment show a progressive decline of male rats' appetitive and consummatory sexual behaviors in bilevel chambers during a 96-day period following castration. These data also show a recovery of the consummatory behaviors following 52 days of TP treatment. Results from the present study were consistent with previous reports showing that consummatory behaviors declined precipitously after castration, following a pattern in which ejaculations and intromissions decline before mounts (Davidson, 1966; 1980; Malmnäs, 1973). In the present study the proportion of animals showing ejaculations and intromissions declined rapidly, whereas the decline in mounts was delayed by two or three test sessions (i.e., 8-12 days).

With respect to appetitive and precopulatory measures, the results of this study were also consistent with those of other studies (Beach & Holz-Tucker, 1949; Madlafousek et al., 1976; Stone, 1939). As mounts declined after castration, an initial increase in the percentage of males displaying attempted mounts was observed, which slowly decreased to baseline levels by the end of the 24 test sessions. On the other hand, the percentage of males showing appetitive level changes and anogenital investigation of

the female, as well as the mean number of those particular aspects, were maintained following castration. These results support the notion that certain appetitive sexual behaviors are more resistant to the effects of castration than consummatory aspects, although these behaviors might have declined had the observation period after castration been extended.

Not all behaviors which are sexually reinforced are extinguished completely by castration, such as partner preference (e.g., Matuszczyk & Larsson, 1994; Merkx, 1984) or bar-pressing for access to second-order sexual rewards (e.g., Everitt & Stacey, 1987). It has been shown that appetitive level changing behavior observed among males is enhanced by unconditioned olfactory stimuli present in the chamber (van Furth & van Ree, 1996). This enhancement could explain the persistence of level-changing 96 days after castration, as the bedding present during test sessions typically contained odours from previous mating tests. A testing period longer than 96 days and/or a chamber free of sexual odours may be necessary for the disruption of this specific appetitive measure.

Augmentation of the males' precopulatory behaviors was observed following 12 to 16 days of daily TP injections (30 μg). Increases were found in the proportion of males showing attempted mounts, and in the mean number of pursuits, anogenital investigations, and attempted mounts. The recovery of the consummatory behaviors following TP treatment was slower compared to the reinstatement of precopulatory behaviors. Following 24 days of treatment, approximately 40% of the males showed mounts, intromissions and ejaculations. This percentage increased to almost 100% after 52 days of TP treatment. These findings suggest that appetitive/precopulatory and consummatory behaviors require different durations of TP treatment (i.e., androgen

priming) in order for complete reinstatement to occur in sexually experienced, long-term castrated males exposed repeatedly to receptive females following castration.

Experiment 2. The Effects of Infrequent Sexual Exposure to Receptive Females on the Copulatory Behavior of Sexually Experienced Male Rats

Reports in the literature with regard to the length of time taken by males to stop copulating following castration are inconsistent. Findings from the previous experiment demonstrated that the length of time taken by castrated, sexually experienced male rats to stop copulating was approximately 13 weeks. As mentioned above, the results from Experiment 1 are consistent with Davidson's findings (1966; 1980), but are inconsistent with those of Beach and Sprague (1971), and Whalen and Luttge (1971) in which none of the males continued to copulate 4 weeks following castration.

Methological differences that might account for the discrepancy between these studies include the amount of sexual experience males had prior to castration (i.e., 1 versus multiple test sessions with receptive females), and the amount of exposure to receptive females received by the males following castration (i.e., for 2, 6, or 11 weeks). Experiment 2 examined the role of infrequent sexual testing on sexually experienced male rats' copulation following castration. Sexually experienced male rats (n=20) were exposed to receptive females only twice, five and ten weeks after castration, prior to the beginning of the 4-day hormone testing cycle which lasted 52 days. The effects of infrequent sexual testing were investigated by examining the decline of copulatory behaviors after castration and the restoration pattern after TP treatment.

Materials and Methods

Animals

Twenty adult-male Long Evans hooded rats from Charles River Canada (St. Constant, Québec, Canada) weighing between 250-280g upon arrival were used as subjects. The stimulus females from Experiment 1 were used in Experiment 2. Artificial estrus was induced as in Experiment 1. All housing conditions were identical to those in Experiment 1.

Procedure

All males were exposed to the same habituation and sex-training procedure as those used in Experiment 1. However, following castration, all sexually experienced male rats were left in their home cages and tested only twice, five and ten weeks after castration. After this period of time, castrated males were given daily injections of TP (30μg) and exposed to receptive females every four days for a period of 52 days.

Behavioral and Statistical Analysis

Copulatory tests were conducted in the same bilevel chambers used in Experiment 1. Tests were videotaped and scored subsequently using the same computer program used in Experiment 1. The same copulatory measures observed following castration and subsequent hormone treatment as in Experiment 1 were analyzed using a within group ANOVA. Significant main effects or interactions were followed by post-hoc analyses of individual means using the Tukey method. Chi-square analysis was used for proportions of rats showing appetitive and consumatory behaviors after castration and after hormone treatment. The level of significance for all comparisons was $p < 0.05$.

Results

Sexual Behaviors Following Castration

Appetitive and Precopulatory Behaviors. Figure 9 (Top) shows the percentage of animals engaged in appetitive and precopulatory behaviors following castration. The proportion of males displaying pursuits of the female declined significantly five weeks following castration when compared to baseline [χ^2 (df = 1) = 22.56, p< .0001]. The proportion of males showing conditioned level changes decreased ten weeks after castration but this decline was not statistically significant. The proportion of male rats showing attempted mounts and anogenital investigations remained unchanged following castration.

The mean number of level changes [F (2, 17) = 8.79, p< .05], the mean number of pursuits of the female [F (2,17) = 9.321, p< .05], and the mean number of anogenital investigations [F (2,17) = 7.251, p< .001], were found to be significantly different 5 and 10 weeks following castration (Figure 9). Post- hoc comparisons revealed significant differences between baseline and the two testing dates for the mean number of level changes, anogenital investigations, pursuit behaviors, but not for the mean number of attempted mounts.

Consummatory Behaviors. The percentage of animals displaying consummatory behaviors is shown in Figure 9 (Bottom). Following castration, the proportion of males showing mounts, intromissions, and ejaculations decreased significantly between the pre-castration baseline measure and the two post-castration testing sessions: for mounts [χ^2 (df = 1) = 16.95, p<.001]; for intromissions [χ^2 (df = 1) = 17.54, p<.001], and ejaculations [χ^2 (df = 1) = 13.89, p<.001]. A within-group ANOVA detected a

significant difference in the mean number of mounts, intromissions and ejaculations: for mounts [F (2,17)=11.24, p<.0001]; for intromissions [F (2,17)=12.01, p< .001]; and for ejaculations [F (2, 17) = 22.16, p< .001]. Post hoc comparisons revealed significant differences for mounts, intromissions, and ejaculations between the baseline and the test session 10 weeks after castration. The mean number of mounts was also found to be significantly lower 5 weeks after castration compared to the pre-castration baseline measure.

Sexual Behaviors Following Hormone Treatment

Appetitive and Precopulatory Behaviors. Changes in the proportion of animals engaging in appetitive and precopulatory behaviors, and in the mean number of those behaviors following TP replacement, are shown in Figures 10 and 11. The percentage of males showing pursuit of the female increased significantly over the 52 days of TP treatment, starting 24 days after TP treatment [χ^2(df = 1) = 45.86, p<.001] (Figure 10). The proportion of animals showing attempted mounts increased significantly after 12 days of TP treatment [χ^2 (df = 1) = 38.75, p<.001], and then gradually decreased to baseline levels by day 40 (Figure 10). The proportions of males displaying appetitive level changes and anogenital investigations did not change significantly across the 52 days of TP treatment (Figure 10).

Following TP administration, the mean number of pursuits increased significantly during the 52 days of treatment [F(13,247)=6.76, p<.05] (Figure 11). Post hoc comparisons of the individual means revealed a significant difference in the mean number of pursuits between the baseline measure and days of treatment 28-52. A significant difference was also observed in the mean number of level changes [F(13,247)=3.89, p<.05]. Post-hoc comparisons of the individual means revealed a

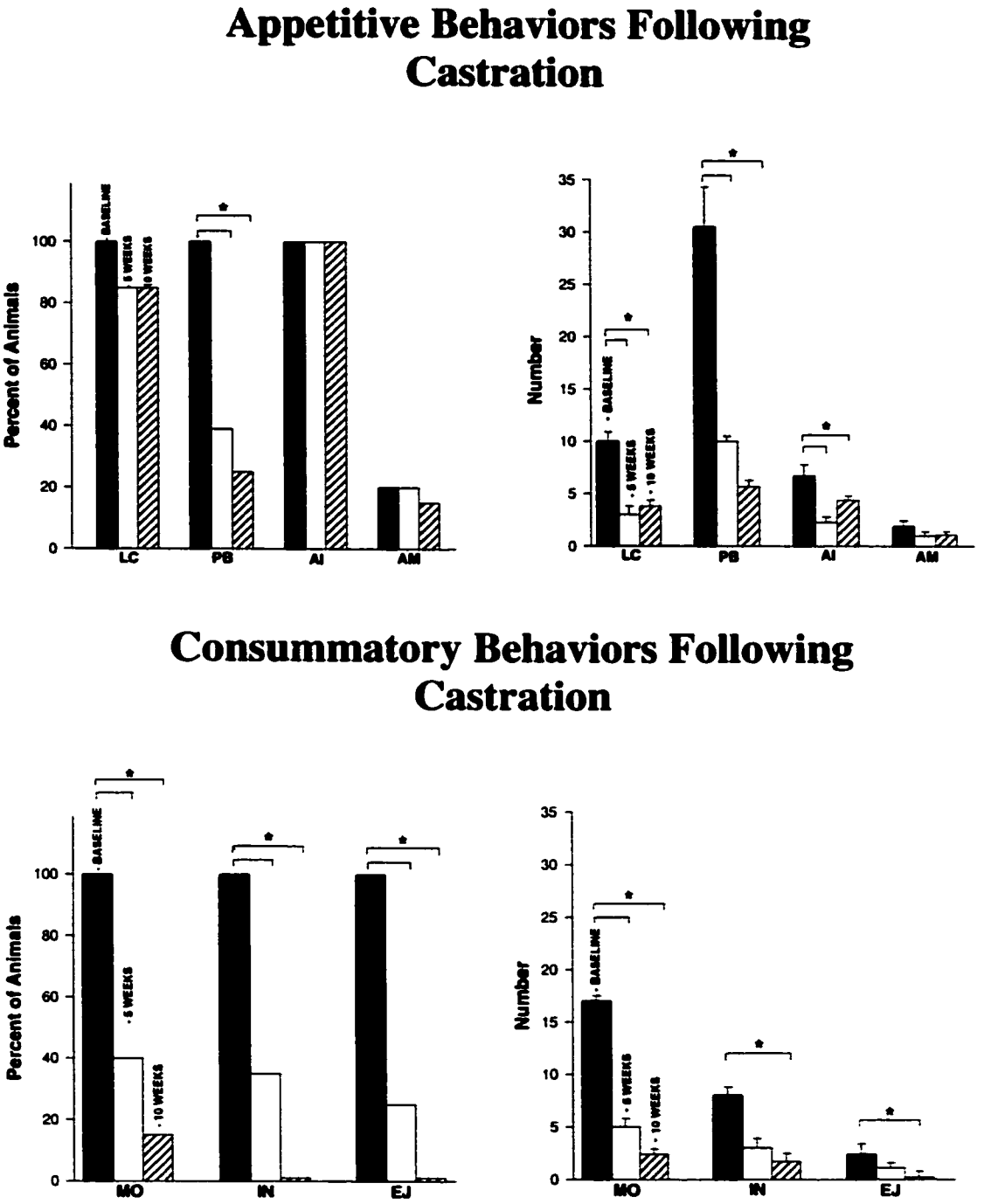


Figure 9. Percentage of animals (N=20) and mean number of appetitive/pre-copulatory behaviors (LC: level changes; PB: pursuits; AI: anogenital investigations; AM: attempted mounts) and consummatory behaviors (MO: mounts; IN: Intromissions; EJ: ejaculations) observed in sexually experienced males tested with receptive females 5 and 10 weeks after castration (Experiment 2). Data are percentages and means + SEM. Vertical lines show standard errors. *p<.001 between baseline and testing days.

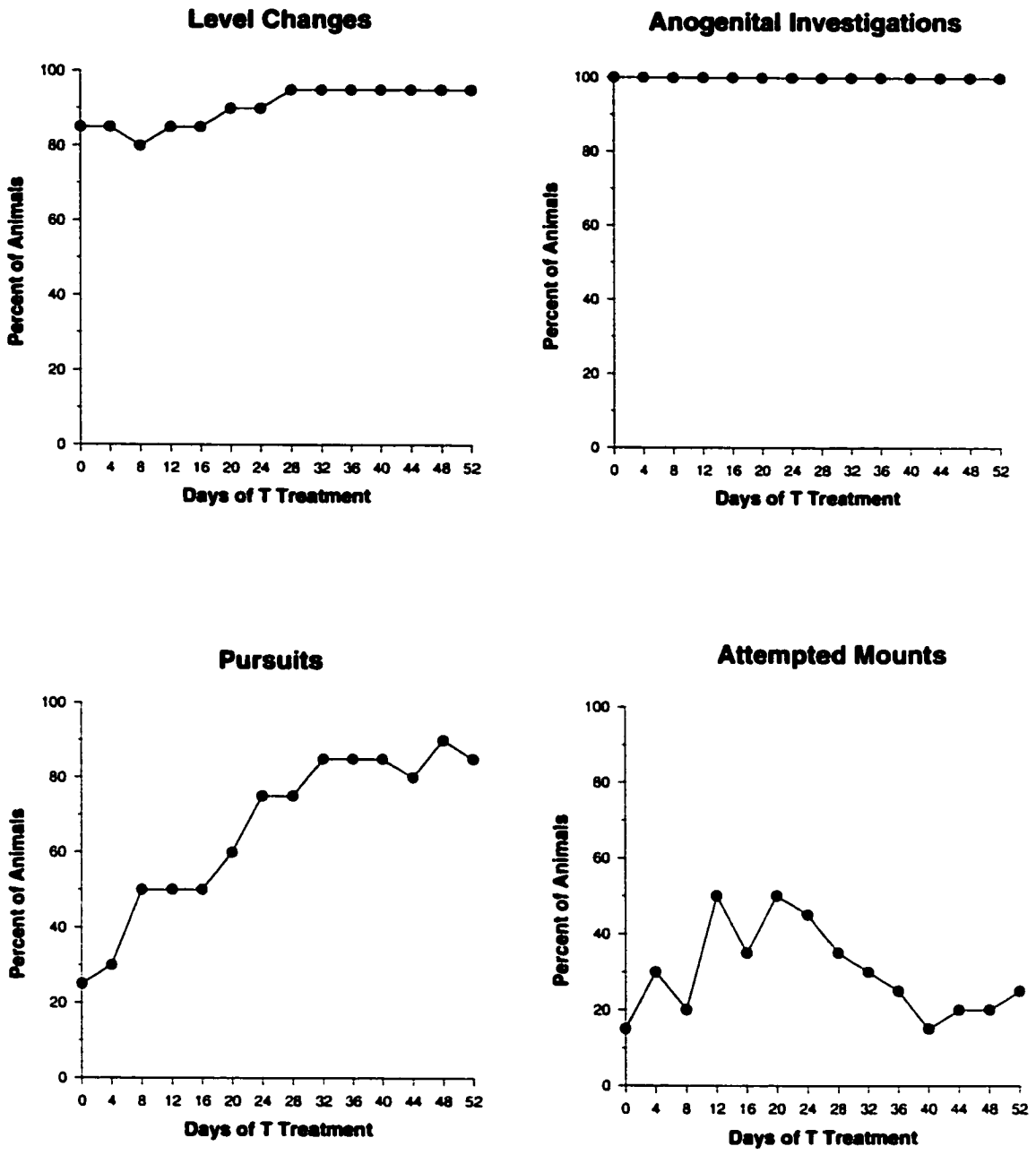


Figure 10. Percentage of castrated sexually experienced males (N=20) displaying appetitive level changes and precopulatory (anogenital investigations, pursuits, and attempted mounts) behaviors following daily TP treatment (i.e., T). Males were tested with receptives females at 4-day intervals for a period of 52 days (Experiment 2). D0: baseline measure following castration.

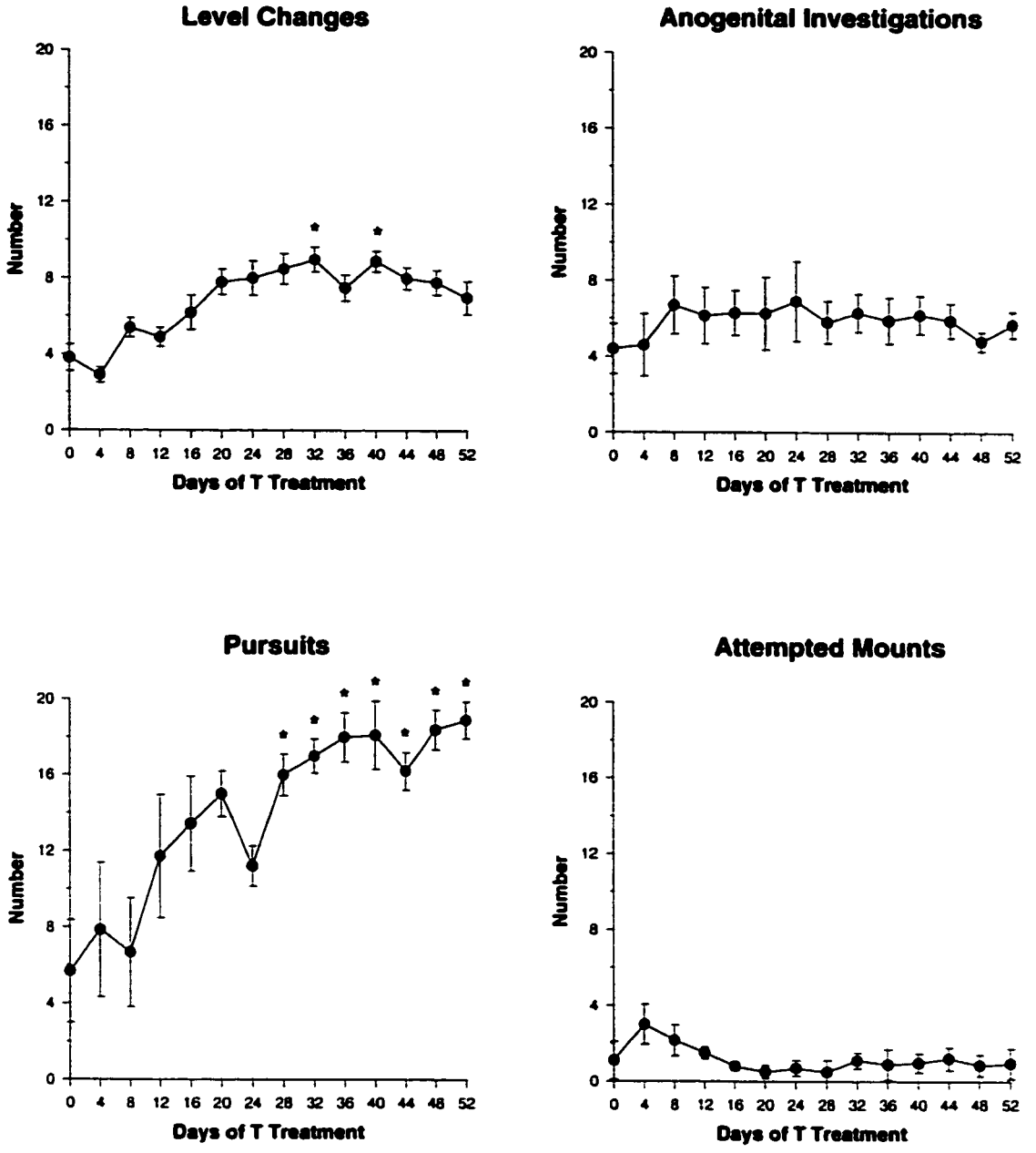


Figure 11. Mean number of appetitive level changes and precopulatory (anogenital investigations, pursuits, and attempted mounts) behaviors observed in castrated sexually experienced males (N=20) following daily TP-treatment (i.e., T). Males were tested with receptive females at 4-day intervals for a period of 52 days (Experiment 2). Data are mean + SEM. Vertical lines show standard errors. * $p<.05$ between baseline and days of TP treatment.

significant difference in the mean number of level changes displayed by males on days 32 and 40 following TP treatment. Although there was an increase in the mean number of anogenital investigations and a decrease in the mean number of attempted mounts, these means did not reach significance by the end of the TP treatment (Figure 11).

Consummatory Behaviors. The effects of TP treatment on the consummatory components of male rat sexual behavior are summarized in Table 1. Figure 12 shows a gradual increase in the percentage of males showing full mounts, intromissions and ejaculations over the 52-day period of TP administration. A statistically significant difference was found in the proportion of males mounting, $\chi^2(df = 1) = 68.78$, $p < .0001$; intromitting, $\chi^2 (df = 1) = 74.65$, $p < .0001$; and ejaculating, $\chi^2(df = 1) = 84.34$, $p < .0001$. This increase was observed first for mounts (i.e., following 8-12 days of TP treatment), then for intromissions (i.e., following 12-16 days of TP treatment), and finally for ejaculations (i.e., following 16-20 days of TP treatment).

Figure 13 shows an increase in the mean number of mounts, intromissions, and ejaculations following TP treatment over a 52-day period. A within subjects ANOVA showed a significant increase in the mean number of mounts and intromissions following TP treatment [for mounts: $F(13,247) = 11.66$, $p < .0001$; for intromissions: $F(13,247) = 17.75$, $p < .001$]. Post hoc comparisons of the individual mean number of mounts and intromissions revealed significant differences between baseline measures and days 12-52 (for mounts), and 12-52 (for intromissions) of TP treatment.

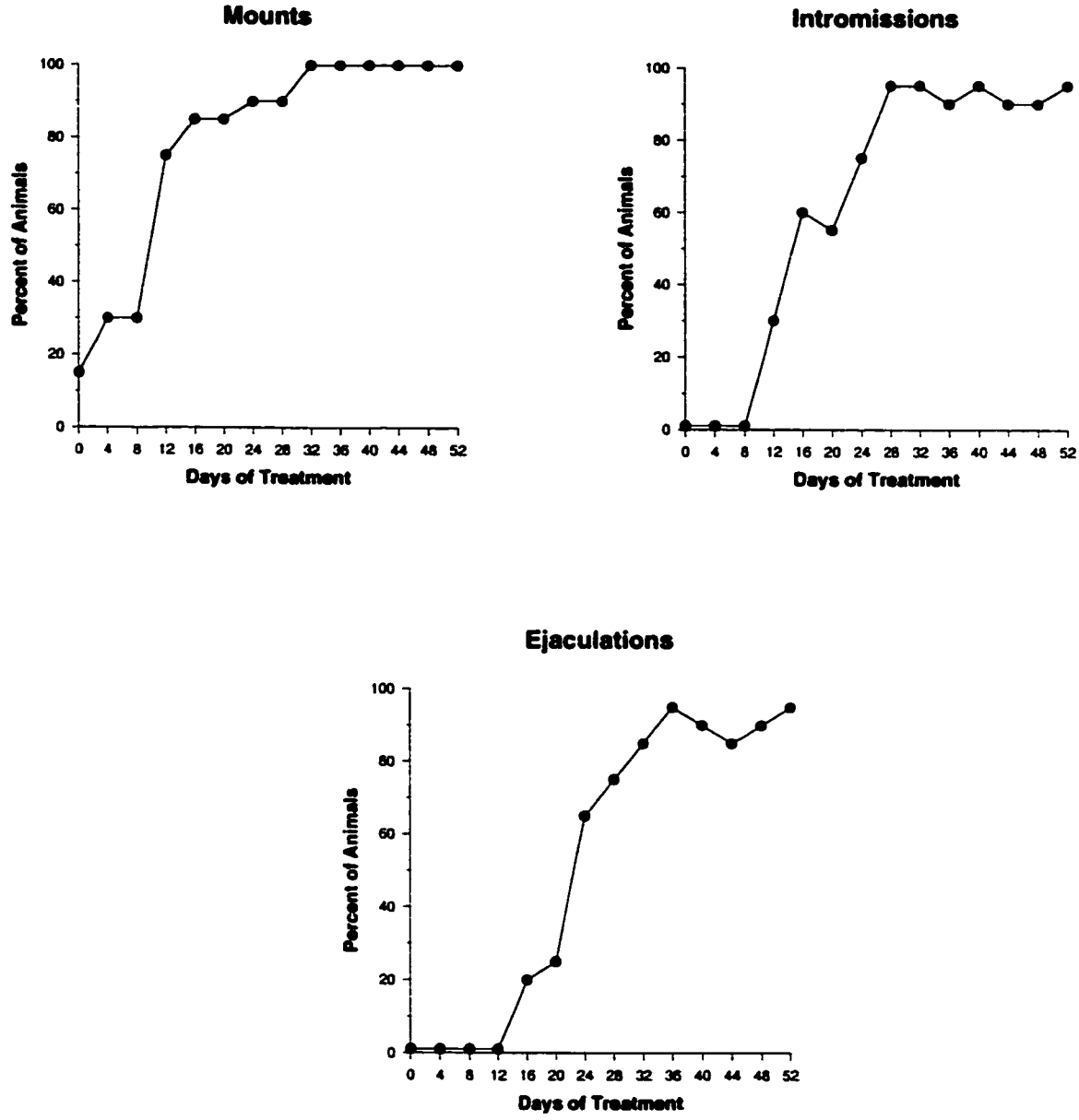


Figure 12. Percentage of sexually experienced castrated males (N=20) showing mounts, intromissions, and ejaculations following daily TP-treatment. Males were tested with receptive females at 4-day intervals for a period of 52 days (Experiment 2). (Day 0: Baseline measure following castration).

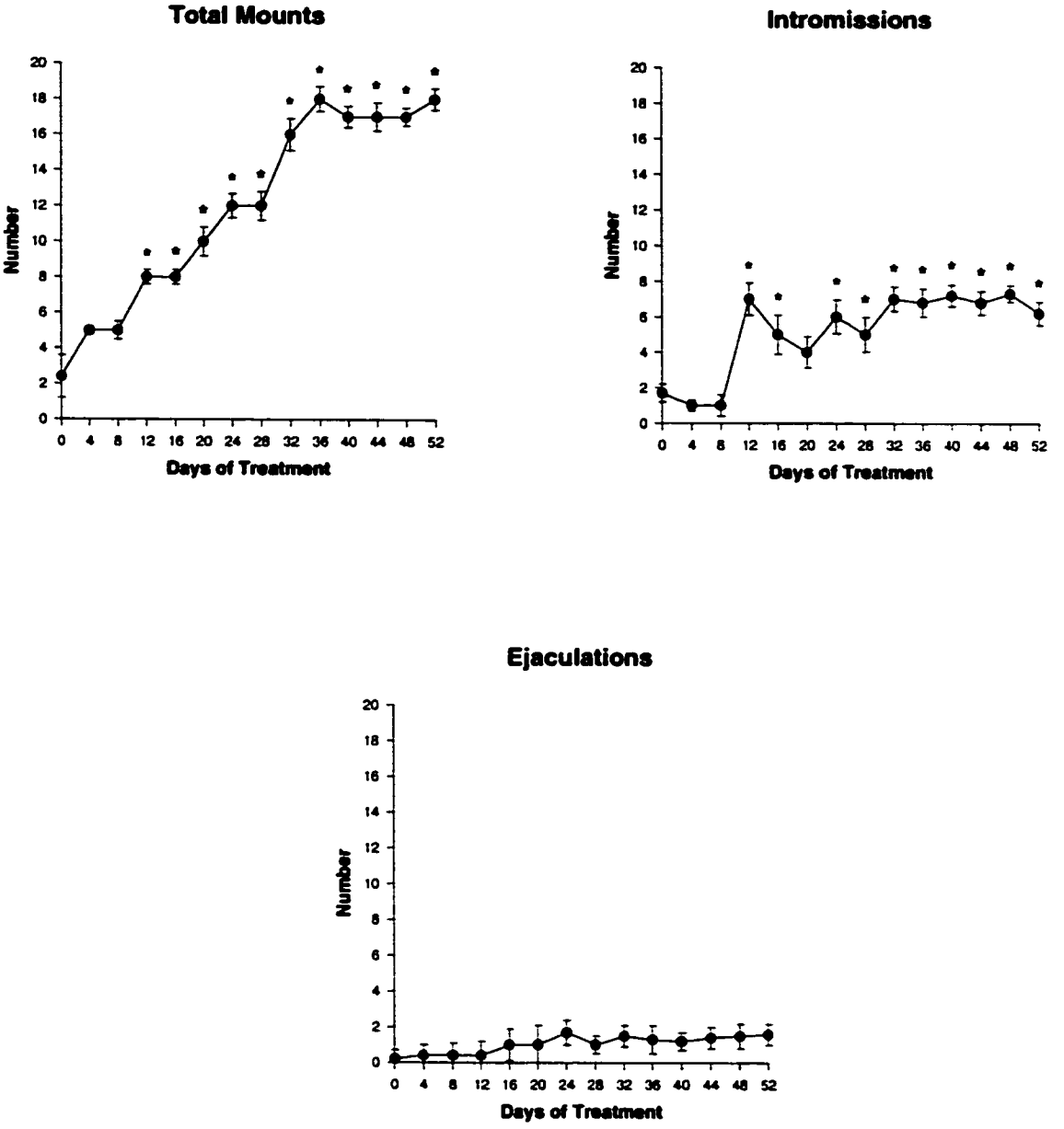


Figure 13. Mean number of mounts, intromissions and ejaculations observed in castrated sexually experienced males (N=20) following daily TP-treatment (i.e., T). Males were tested with receptive females at 4-day intervals for a period of 52 days (Experiment 2). Data shows mean + SEM. Vertical lines show standard errors. (Day 0: Baseline measure following castration). * $p<.05$ between baseline and days of TP treatment.

Discussion

Findings from Experiment 2 showed that 30% to 40% of sexually experienced males displayed consummatory behaviors five weeks following castration when not exposed to repeated testing with receptive females in the five weeks following castration. The percentage of males displaying consummatory behaviors decreased to an average of 5% ten weeks after castration. Furthermore, the mean number of mounts, intromissions, and ejaculations was found to be significantly lower 10 weeks after castration compared to pre-castration levels. These results are consistent with findings from Davidson's (1966, 1980) studies, which reported males intromitting and ejaculating six to eight weeks following castration, suggesting that the animals used in Davidson's studies were most probably sexually experienced prior to castration and tested infrequently with receptive females after castration.

In addition to the decrease in consummatory behaviors, a decrease in the percentage of sexually experienced males showing pursuit behavior was found five weeks following castration. The mean number of level changes, anogenital investigations and pursuits decreased significantly 10 weeks after castration. However, the percentage of males displaying level changes and anogenital investigations was not significantly different 10 weeks after castration, suggesting that 10 weeks are not enough to interfere with the olfactory mechanisms underlying recognition of the female. The partial effects of castration observed on the appetitive measures suggest that males' initiative to copulate might be most influenced by the amount of sexual exposure given to the males prior to castration.

Results from this study demonstrate the importance of frequent sexual exposure to females following castration in buffering the effects of castration on male rat copulation. Ten weeks after castration, the percentages of frequently tested sexually experienced males displaying appetitive and consummatory behaviors was higher in Experiment 1 compared to the percentages of males displaying the same behaviors in Experiment 2. Fifty percent of males exposed to receptive females at 4-day intervals in Experiment 1 showed attempted mounts (i.e., test session 16, see Figure 1), compared to the 15% of males that were exposed only twice to receptive females following castration in Experiment 2 (see Figure 9). The percentage of males showing pursuits was higher (i.e., 90%) in males exposed to receptive females at 4-day intervals, compared to males exposed only twice to receptive females (i.e., 25%). In addition, 10 weeks following castration, 40%, 25%, and 20% of the males exposed to receptive females at 4-day intervals displayed mounts, intromissions, and ejaculations respectively (Experiment 1), compared to 15%, 1%, and 1% of the males exposed twice to receptive females (Experiment 2).

The effects of TP treatment on the reinstatement of appetitive and consummatory behaviors in males also depended on the frequency of behavioral sex tests following castration. Following 24 days of T treatment, approximately 30% of the sexually experienced castrated males exposed to receptive females at 4-day intervals displayed mounts, intromissions, and ejaculations (Experiment 1, see Figure 7); compared to approximately 75% of the sexually experienced males tested only twice (i.e., 5 and 10 weeks) with receptive females after castration (Experiment 2, see Figure 12). Furthermore, the return of all consummatory behaviors in males repeatedly exposed to

receptive females occurred simultaneously after 20 days of TP treatment (see Figure 12), whereas males that were tested twice with receptive females started to display mounts, intromissions, and ejaculations, after 8, 12, and 16 days of TP treatment respectively.

The effects of TP treatment on the reinstatement of pursuits and attempted mounts in males tested repeatedly with receptive females and males tested only twice with receptive females can be seen in Figures 5 and 10. Although there was a significant difference between the two groups' baseline measure recorded after 24 test sessions, by day 24 the percentage of males from each testing group showing pursuits and attempted mounts did not differ significantly. These results suggest that the reinstating effects of TP on appetitive behaviors observed in sexually experienced males does not depend on the amount of sexual experience males have following castration.

Overall findings suggest that the decline in sexual behavior observed in sexually experienced males following repeated behavioral testing with receptive females after castration is determined by the number of sexual encounters males have between castration and initiation of TP treatment, and not by the length of time passed since castration per se. In addition, results from these studies showed that the amount and/or duration of TP treatment required to reinstate the consummatory behaviors in experienced males repeatedly exposed to receptive females is higher than the length of TP required by males tested only twice with receptive females. Furthermore, results from this study suggest that the reinstatement of the appetitive behaviors after TP treatment might be less dependent on the amount of sexual testing males have following castration compared to the experience males have prior to castration.

Experiment 3. The Effects of One Ejaculation Prior to Castration on the Expression of Male Rat Copulatory Behaviors

The discrepancy in the literature regarding the time taken by males to stop copulating following castration (i.e., between 2 and 10 weeks) may be due to the amount of sexual exposure that the males have received following castration (i.e., as investigated in Experiments 1 and 2), but may also depend upon the amount of sexual experience males have received prior to castration (i.e., 1 test versus multiple tests with receptive females). To investigate further the role of sexual experience on the expression of male sexual behavior, Experiment 3 was designed to examine the effect of one ejaculation with a receptive female prior to castration on the length of time taken by the males to stop displaying copulatory behaviors following castration and TP treatment.

Materials and Methods

Animals

Twenty adult male Long Evans hooded rats from Charles River Canada (St. Constant, Québec, Canada) weighing between 250-280g upon arrival were used as subjects. The stimulus females from Experiments 1 and 2 were used in Experiment 3. Artificial estrus was induced as in Experiment 1. All housing conditions were identical to those of Experiment 1.

Procedure

Males were habituated to the bilevel chambers and exposed to receptive females using the same protocol as in Experiments 1 and 2. However, in Experiment 3 males were only allowed a single ejaculation with receptive females prior to castration, instead of the 10 multi-ejaculatory sex training sessions given to males in Experiments 1 and 2.

Following castration, all males were given one week to recuperate from the surgery prior to behavioral testing.

Males were randomly assigned to two groups according to the amount of sexual exposure they would receive following castration. One group of castrated males (n=10) was tested twice, once every 5 weeks, and the other group (n=10) was tested repeatedly on a four day interval for a period of 10 weeks (18 test sessions). The behavioral measures examined in this experiment were identical to those examined in Experiments 1 and 2. The last behavioral test session prior to hormonal treatment was used as a baseline castration measure. As in Experiments 1 and 2, all castrated males were treated daily with TP (30 μg) and exposed to receptive females every four days for an additional period of 52 days. Hormone administration and the procedure used during the testing phase were identical to those in Experiments 1 and 2.

Behavioral and Statistical Analysis

Appetitive, precopulatory and consummatory behaviors were examined following castration and subsequent hormone treatment, and were analyzed using between and within groups ANOVAs. Significant main effects or interactions were followed by post hoc analyses of individual means using the Tukey method. Chi-square analysis was used to assess proportions of rats showing appetitive and consummatory behaviors before and after hormone treatment. The level of significance for all comparisons was $p < 0.05$.

Results

Effects of Infrequent Sexual Behavioral Testing on Male Copulation Following Castration

Appetitive and Precopulatory Behaviors. Figure 14 shows the proportion of males displaying appetitive and consummatory behaviors. All males had 1 ejaculation

with a receptive female prior to castration and were tested with receptive females twice, at five and ten weeks after castration. The mean number of appetitive and consummatory behaviors shown by those males is also displayed in Figure 14. The proportion of males displaying pursuit behaviors decreased significantly five weeks after castration: $\chi^2(df=1)= 11.67$, $p< .001$. Although there was a decrease in the proportion of males showing appetitive level changes, it did not reach the level required for statistical significance [p=.0762]. Also, there were no significant differences in the proportion of males showing anogenital investigations or attempted mounts 5 or 10 weeks after castration. Those results were similar to those observed in Experiment 2 (see Figure 9 and Table 1).

Five weeks following castration, there was a significant decrease in the mean number of pursuits ($F(2,7)= 10.25$, $p< .001$). In addition, there was a significant decrease in the mean number of level changes after 10 weeks $F(2,7)= 9.87$, $p< .001$. The mean number of attempted mounts and anogenital investigations remained the same 5 and 10 weeks after castration (Figure 14). Those results were similar to the ones observed in Experiment 2 (see Figure 9 and Table 1), although no effects on the mean number of anogenital investigations were observed in the less experienced group 5 or 10 weeks after castration (Experiment 3).

Consummatory Behaviors. The proportion of males showing mounts, intromissions, and ejaculations decreased significantly 5 weeks after castration: for mounts [$\chi^2(df=1)= 11.5$, $p< .001$]; for intromissions [χ^2 $(df=1)= 7.9$, $p< .001$]; and for ejaculations [χ^2 $(df=1)= 10.4$, $p<.001$]. Furthermore, the decrease observed in intromissions and ejaculations was more dramatic than observed in Experiment 2 (e.g.,

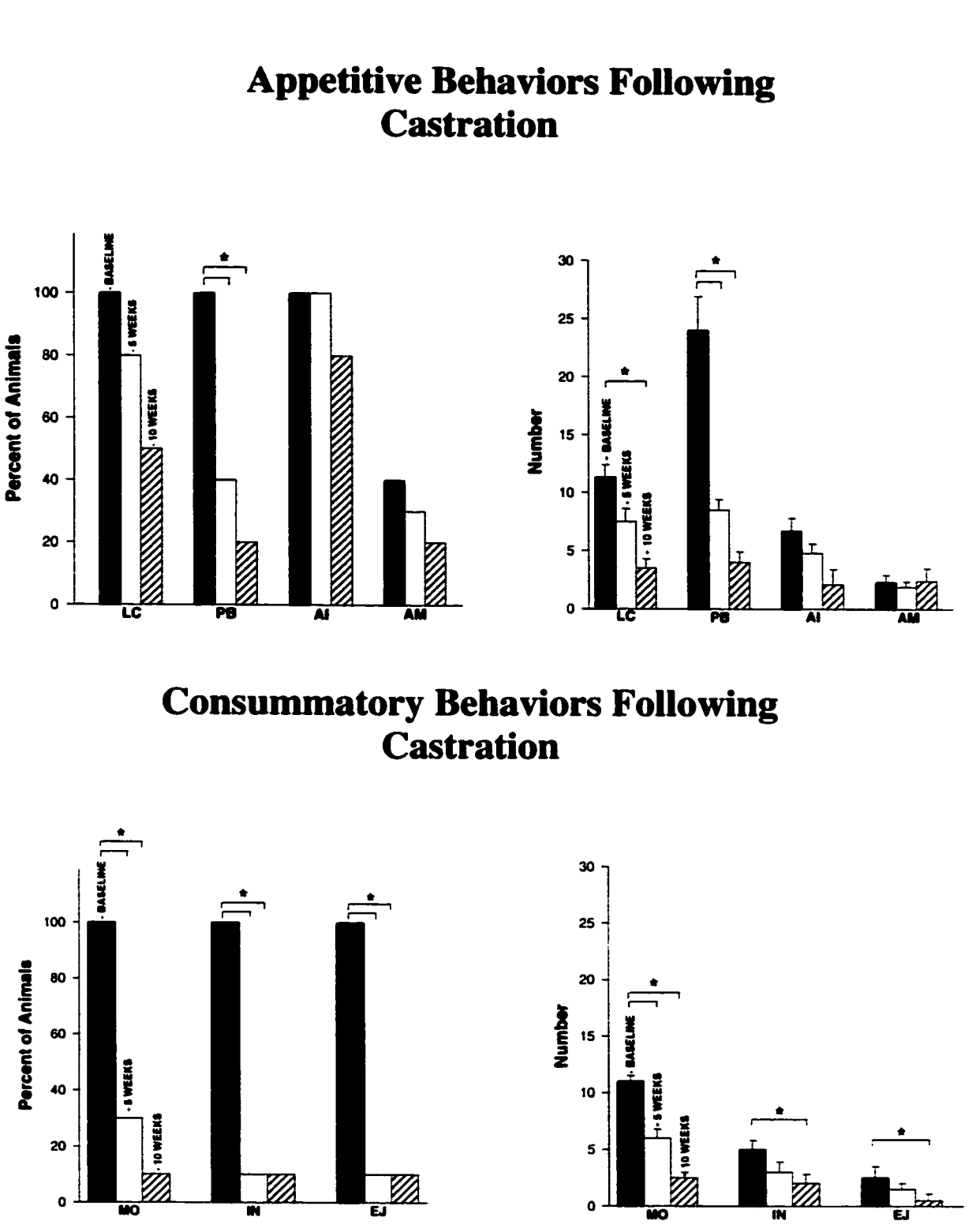


Figure 14. Percentage of animals (N=10) and mean number of appetitive/pre-copulatory and consummatory behaviors in males that had 1 ejaculation with receptive females prior to castration and were tested with receptive females 5 and 10 weeks after castration (Exp. 3). Data are percentages or means +SEM. Vertical lines show standard errors. *p<.001 (i.e, percentages) and *p<.05 (i.e., means) between baseline and testing days.

10% of males intromitting and ejaculating in Experiment 3 versus 35% and 25% in Experiment 2, see Figure 9). In addition, there was a significant reduction in the mean number of mounts, intromissions, and ejaculations 10 weeks after castration: for mounts [$F(2,7)=8.8$, $p< .001$]; for intromissions [$F(2,7)=11.9$, $p<.001$]; and for ejaculations [$F(2,7)=12.7$, $p<.001$]. The mean numbers of mounts, intromissions, and ejaculations was almost identical to those displayed by experienced males tested twice with receptive females (see Experiment 2, Figure 9 versus Experiment 3, Figure 14; Table 1).

Effects of Frequent Sexual Behavioral Testing on Male Copulation Following Castration

Appetitive and Precopulatory Behaviors. Of all the appetitive measures examined, only the proportion of males displaying pursuits decreased significantly [$\chi^2(df=1)= 11.7$, $p<.001$] over the 18 test sessions. The proportion of males showing level changes, anogenital investigations, and attempted mounts remained the same over the 10-week period (18 test sessions) (Figure 15). However, there was a significant decrease in the mean number of level changes and pursuits 10 weeks after castration: for level changes: [$F(18, 162)=4.32$, $p<.05$]; and for pursuits [$F(18, 162)=8.62$, $p<.001$]. Post hoc comparisons of the individual means revealed significant differences between the baseline and test sessions 17-18 for level changes, and the baseline and test sessions 10-18 for pursuits. The mean number of anogenital investigations and attempted mounts did not change over the 10- week period (Figure 16).

Consummatory Behaviors. The proportion of males displaying mounts, intromissions, and ejaculations decreased significantly over a 10 week period: for mounts [$\chi^2(df=1)= 9.7$, $p<.001$]; for intromissions [$\chi^2(df=1)= 10.8$, $p<.001$]; and for ejaculations [$\chi^2(df=1)= 7.4$, $p<.001$]. After 10 weeks, only one of the inexperienced castrated males

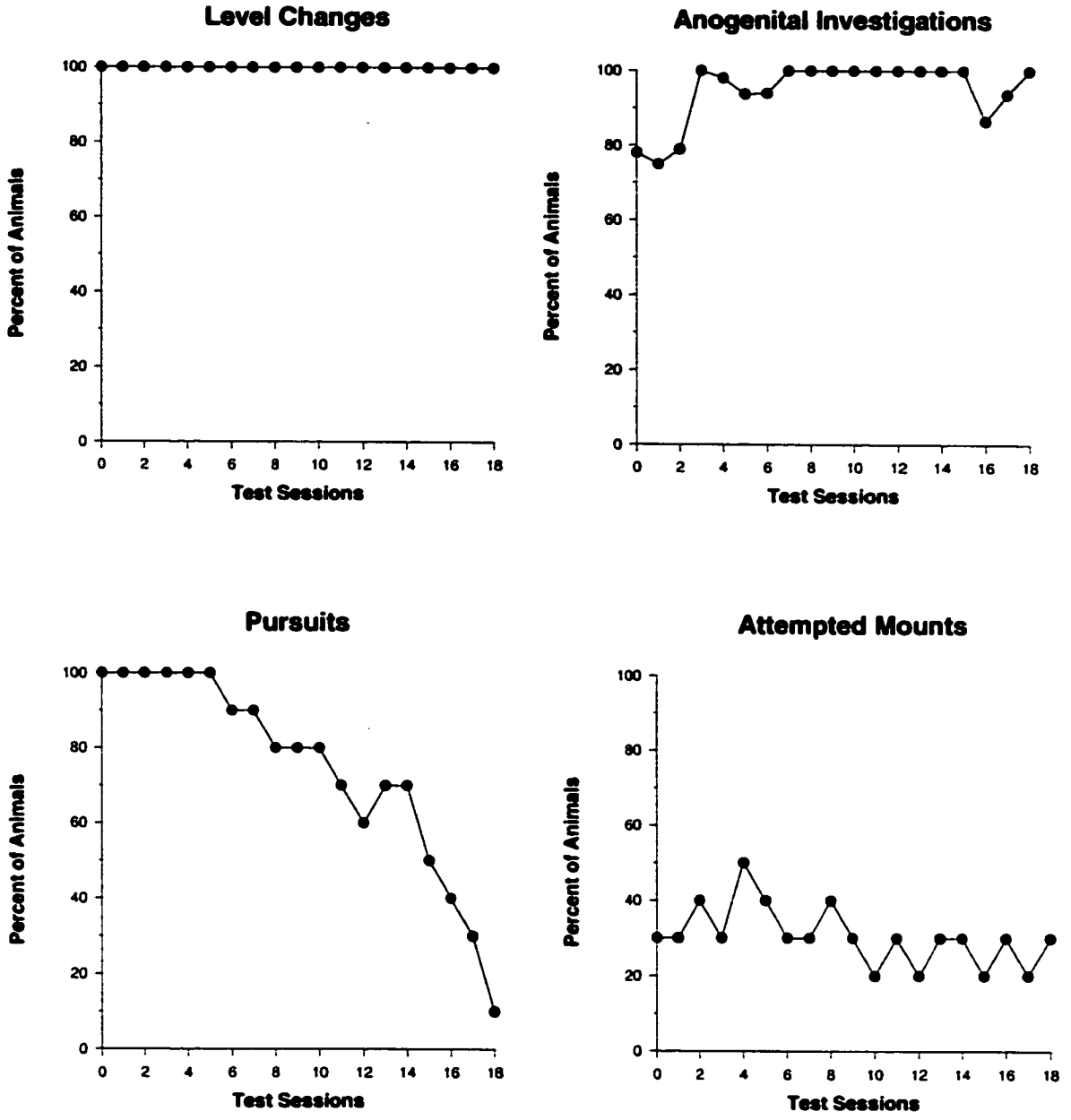


Figure 15. Percentage of males (N=10) showing appetitive/precopulatory (conditioned level changes, anogenital investigations, pursuits, and attempted mounts) behaviors. Males were allowed to have 1 ejaculation with receptive females prior to castration and were tested with receptive females at 4-day intervals following castration for a period of 10 weeks. Data are percentages. (Day 0: Baseline measure prior to castration).

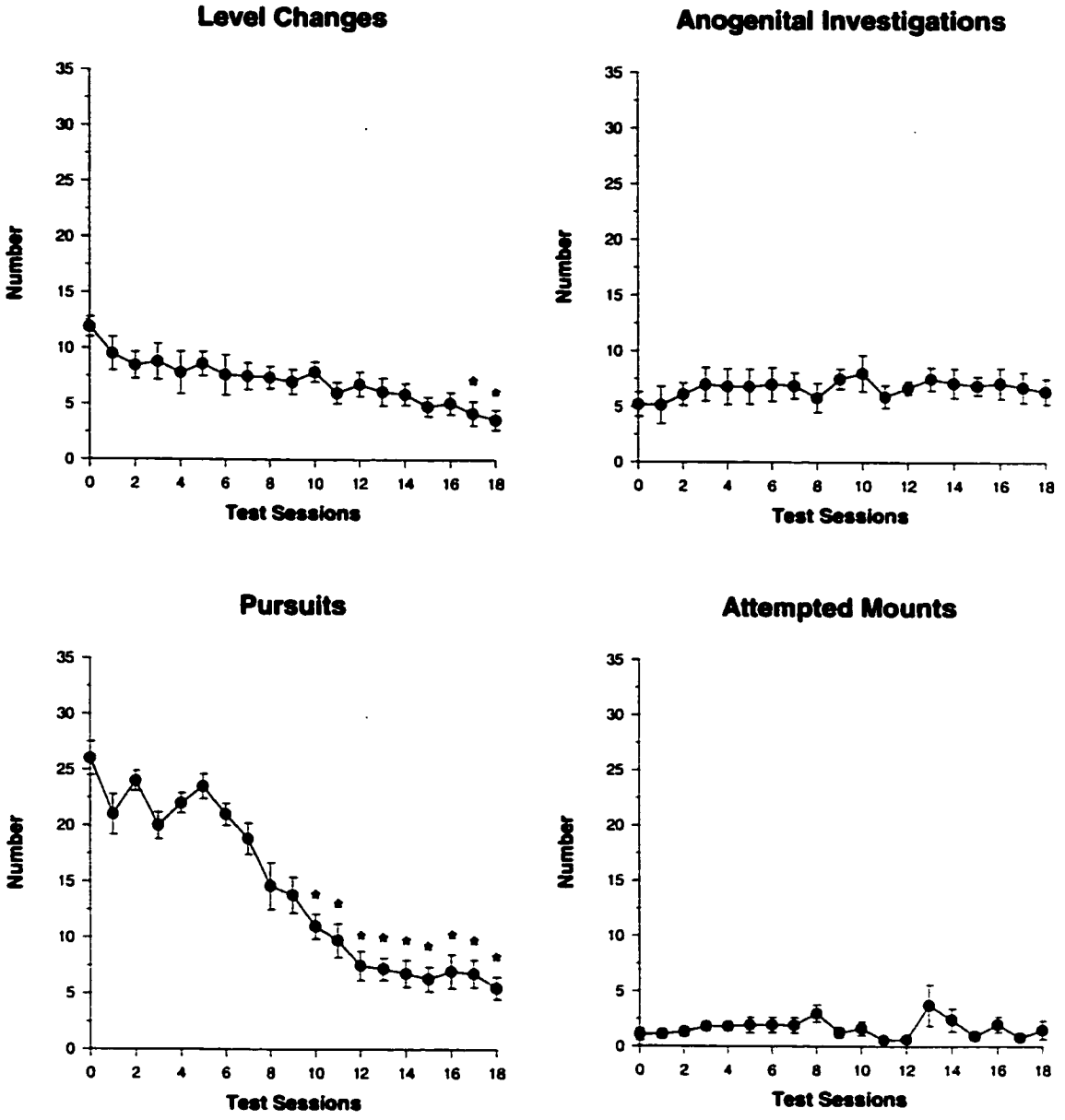


Figure 16. Mean number of appetitive/pre-copulatory behaviors (conditioned level changes, anogenital investigations, pursuits, and attempted mounts) displayed by males allowed to have 1 ejaculation with receptive females prior to castration and were tested with receptive females at 4-day intervals following castration for a period of 10 weeks. Data are means + SEM. Vertical lines show standard errors. (Day 0: Baseline measure prior to castration). *$p<.05$ between baseline and testing sessions.

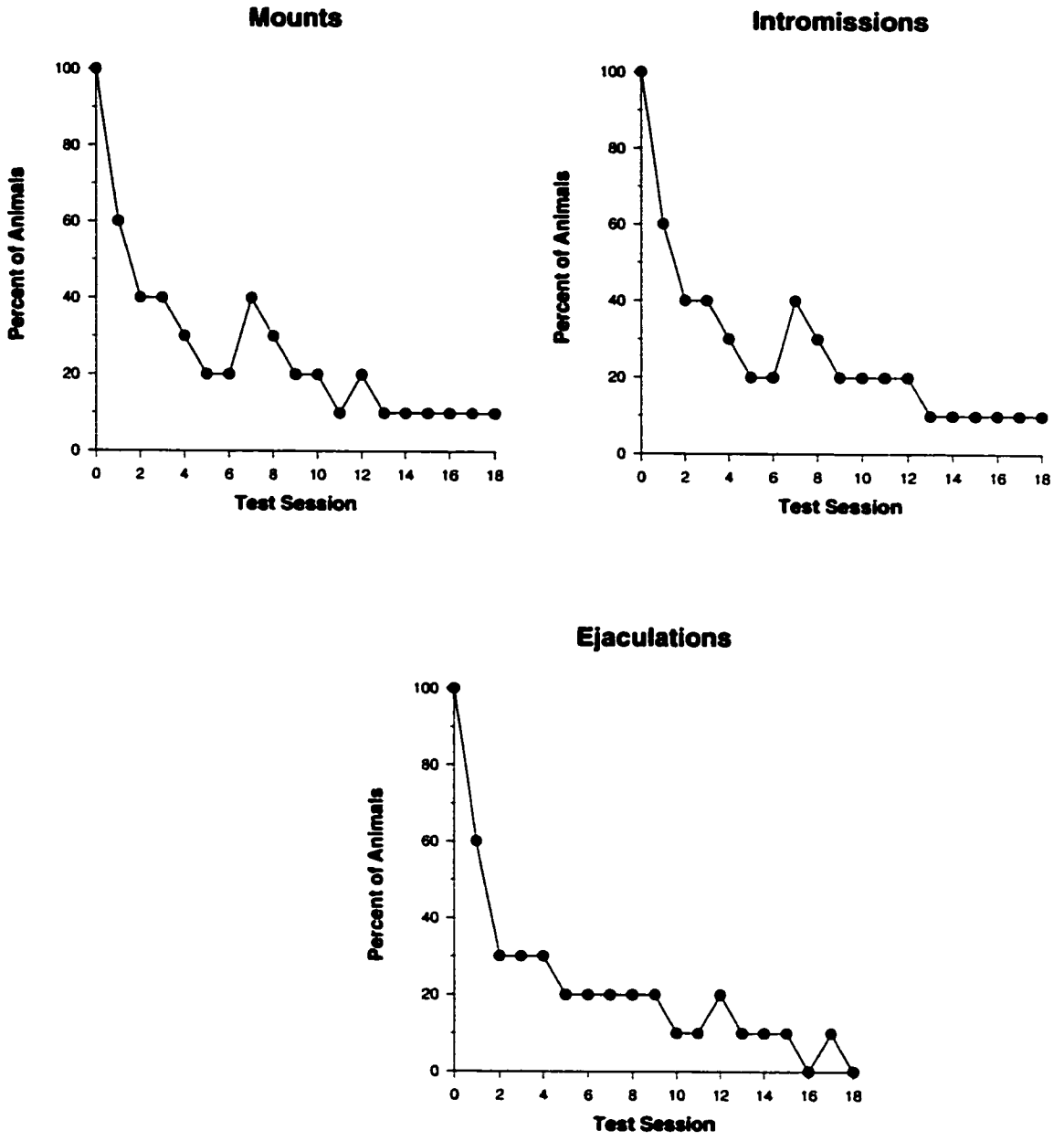


Figure 17. Percentage of males (N=10) showing mounts, intromissions, and ejaculations. Males were allowed to have one ejaculation with receptive females prior to castration and were tested with receptive females at 4-day intervals following castration for of 10 weeks. Data are percentages. (Day 0: Baseline measure following castration).

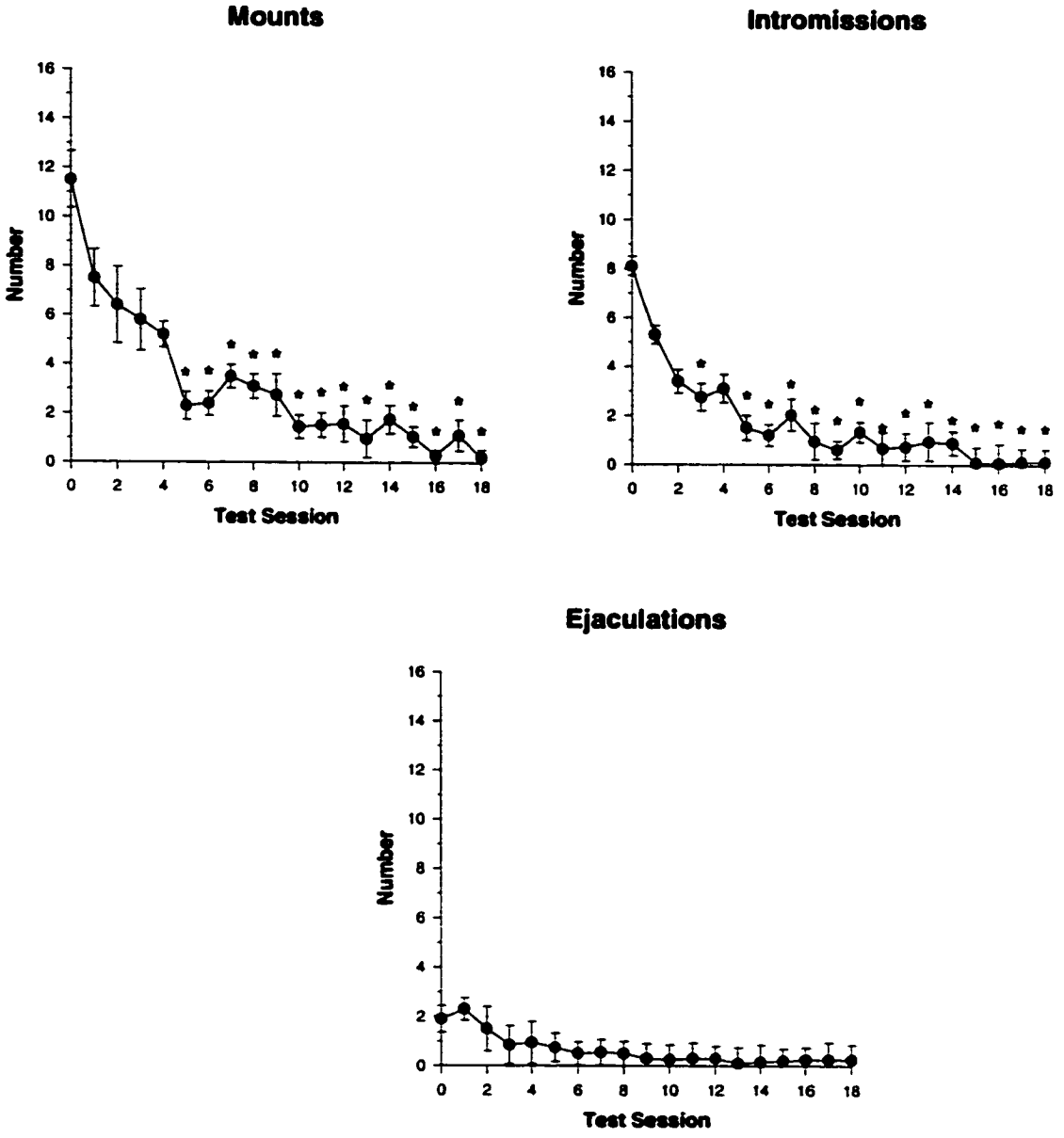


Figure 18. Mean number of mounts, intromissions, and ejaculations displayed by males allowed to have 1 ejaculation with receptive females prior to castration and were repeatedly tested with receptive females at 4-day intervals following castration for a period of 10 weeks. Data are means + SEM. Vertical lines show standard errors. (Day 0: Baseline measure prior to castration). *p<.05 between baseline and testing sessions.

showed mounts and intromissions (Figure 17). The mean number of mounts and intromissions decreased significantly over this 10 week period: for mounts [$F(18, 162)=13.4$, $p<.001$]; for intromissions [$F(18, 162)=12.1$, $p<.001$] (Figure 18). Post hoc comparisons showed significant differences for the mean number of mounts between the pre-castration baseline measure and test sessions 6-18; for intromissions, significant differences were observed between the pre-castration baseline measure and test sessions 3, 5-18. Although a decrease in the mean number of ejaculations was observed over the 10 week period, it was not found to be statistically significant. These overall results are identical to those observed in Experiment 1 (see Figure 1).

Effects of Frequent Sexual Behavioral Testing on Male Copulation Following Castration and Hormone Treatment

Appetitive and Precopulatory Behaviors. Males in the 1 ejaculation condition given repeated behavioral testing with receptive females and administered with TP at 4 day intervals for a period of 52 days showed an increase in the mean number of level changes, pursuits, and anogenital investigations: for level changes [$F(13, 117) =3.12$, $p<.05$]; for pursuits [$F(13, 117) = 5.98$, $p<.05$]; for anogenital investigations [$F(13, 117)= 4.87$, $p<.05$] (Figure 19). Post hoc comparisons revealed significant differences between the post–castration baseline measure and days 32-52 for level changes; days 20 and 28-52 for pursuits; and days 16-52 for anogenital investigations. In contrast, following TP treatment, males with the same sexual experience prior to castration but exposed only twice to receptive females after castration showed an increase in the mean number of pursuits and anogenital investigations, whereas the mean number of level changes and attempted mounts did not change significantly over the 52 days of TP treatment: for pursuits [$F(13, 117)= 6.824$, $p<.05$]; and for anogenital investigations [$F(13, 117) =$

2.712, p<.05] (Figure 19). Post hoc analysis showed significant differences between the post-castration baseline measure and days 40-52 for anogenital investigations, and days 24-52 for pursuits.

A mixed design, between-within groups ANOVA detected a significant increase in the mean number of level changes and anogenital investigations displayed by males exposed to receptive females repeatedly after castration, compared to males exposed to receptive females only twice following castration: for level changes [F(2,7)=7.4, p<.05]; for anogenital investigation [F(2,7)=11.9, p<.05)] (Figure 19). Post hoc comparisons of individual means revealed significant differences on days 32-52 (for level changes) and days 12-52 (for anogenital investigations) of TP treatment. Even though the mean number of pursuits displayed by males exposed to receptive females repeatedly after castration was greater compared to the mean number of pursuits displayed by males exposed only twice with receptive females, this increase did not reach statistical significance. (Figure 19). Although an increase was observed in the percentage of males showing appetitive/precopulatory behaviors when exposed twice or repeatedly to sexually receptive females, it did not reach significance [p=.0683] (data not shown).

Consummatory Behaviors. Males from both groups showed similar patterns of TP-induced restoration for mounts, intromissions and ejaculations. However, the increase in the proportion of males showing mounts, intromissions, and ejaculations was observed earlier in males that had been repeatedly exposed to receptive females after castration compared to males tested only twice with receptive females. For example, by day 12 the proportion of males tested only twice with receptive females following castration was 20%,

Appetitive Behaviors Following T Treatment

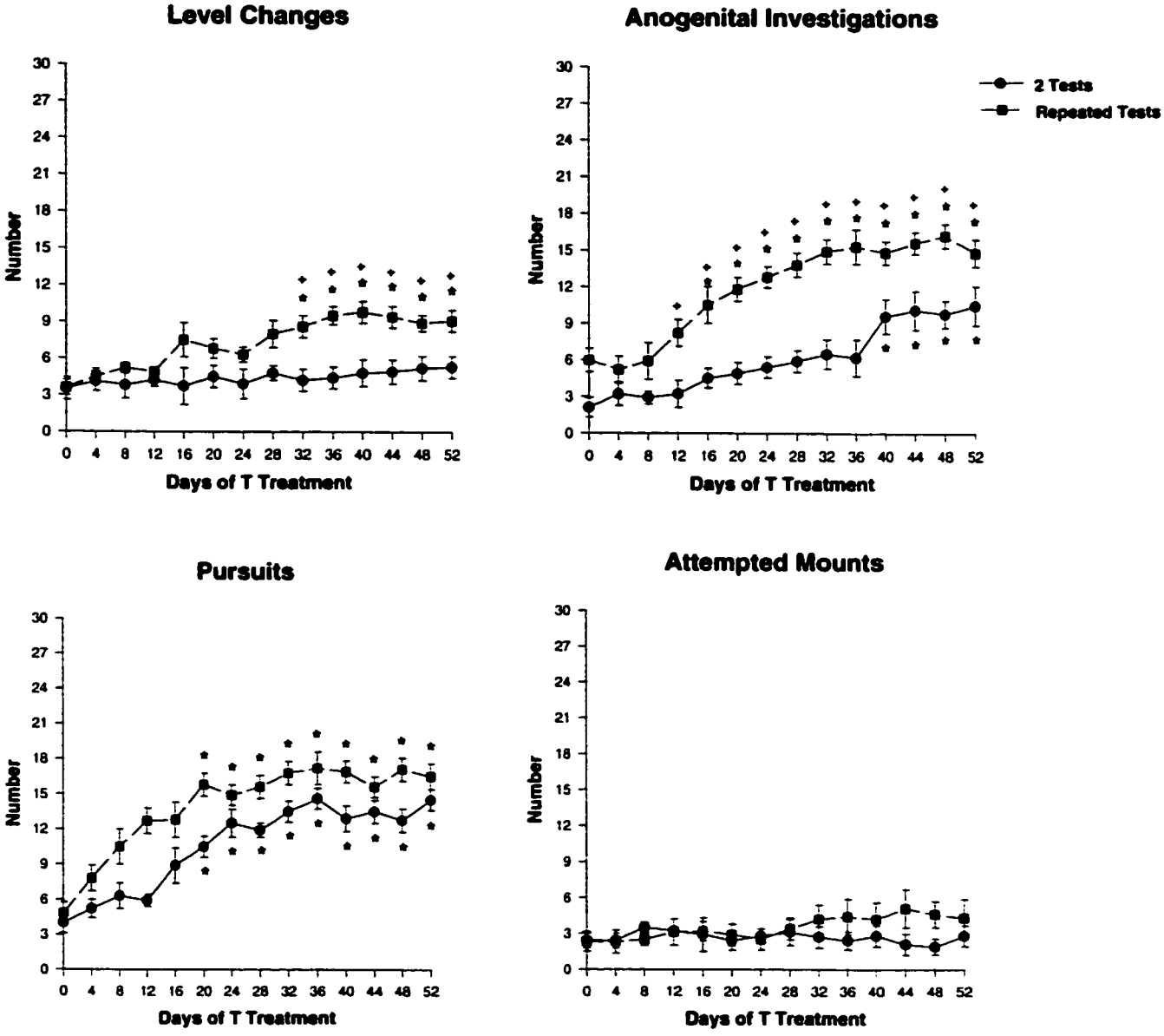


Figure 19. Mean number of appetitive/pre-copulatory (conditioned level changes, anogenital investigations, pursuits, and attempted mounts) behaviors displayed by TP-treated males that had 1 ejaculation with receptive females prior to castration. Males were tested with females either at 5 and 10 weeks or repeatedly, at 4-day intervals, for a period of 10 weeks following castration. Data are means + SEM. Vertical lines show standard errors. (Day 0: Baseline measure following castration). *$p<.05$ between baseline and days of TP treatment. +$p<.05$ between the two test conditions.

Consummatory Behaviors Following T Treatment

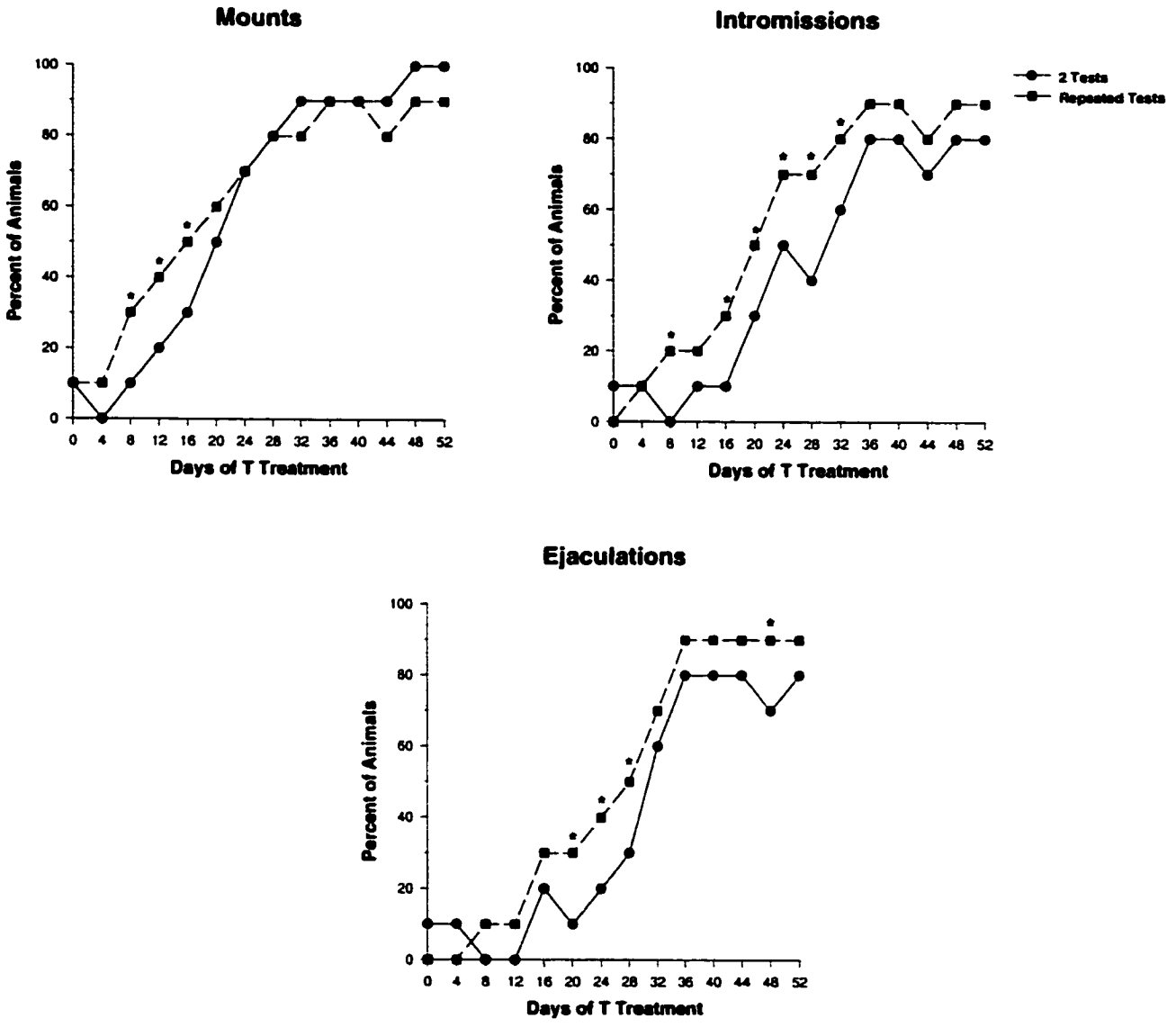


Figure 20. Percentage of castrated males (N=10) displaying mounts, intromissions, and ejaculations following TP-treatment. All males had 1 ejaculation with receptive females prior to castration. Males were tested with females either at 5 and 10 weeks or repeatedly, at 4-day intervals, for a period of 10 weeks following castration. (Day 0: Baseline measure following castration). *p<.05 between the two test conditions.

compared to 40% of males exposed to receptive females at 4-day intervals. Thirty percent of the males which were given exposure twice to receptive females and 50% percent of males given repeated access to receptive females showed a return of mounting behavior 16 days after TP treatment, followed by the same percentages of males within each of the two groups displaying intromissions on day 20, and ejaculations on day 28 (Figure 20). A significant increase in the mean number of mounts, intromissions, and ejaculations displayed by males exposed twice or repeatedly to receptive females following TP treatment was found when comparisons were made between males in the two testing conditions (i.e., for mounts on days 8, 12, 16; for intromissions on days 8, 16, 24-32; and for ejaculations on days 20-28, 48 of TP treatment) (Figure 20).

Discussion

Findings from Experiment 3 suggest that the negative effects of castration on male sexual behavior are attenuated by the amount of sexual experience prior to castration, and less by the frequency of sexual exposure males received following castration. The proportion of sexually inexperienced (i.e., 1 ejaculation prior to castration) males showing mounts, intromissions, and ejaculations decreased to 30%, 10%, and 10% five weeks after castration when tested twice with receptive females. Furthermore, the proportion of males copulating to ejaculation decreased to 10% ten weeks following castration. These results are consistent with findings from Beach and Whalen (1959) and Beach (1949), showing a decrease in all copulatory behaviors two to three weeks after castration.

Given the findings of Experiments 1 and 2, repeated testing with receptive females following castration was expected to increase the amount of time taken for all

sexual measures, and consummatory behaviors in particular, to disappear. However, the data were otherwise: after nine test sessions (i.e., approximately 5 weeks), 20% of sexually inexperienced males were still displaying mounts, intromissions, and ejaculations. This percentage decreased to 10% by test session 18 (i.e., approximately 10 weeks). These results are similar to those observed in inexperienced males (i.e., males that had 1 ejaculation with receptive females prior to castration) tested twice with females, suggesting that the amount of sexual experience males have prior to castration determines the extent of the effects of castration on consummatory behaviors regardless of the amount of sexual testing males have following castration. In contrast to the findings of Experiments 1 and 2, in Experiment 3 castration decreased the percentage of inexperienced males that showed pursuits 5% by the 18th test session in males tested repeatedly, and the mean number of level changes and pursuits was also significantly reduced during the 18 test sessions with receptive females. These findings suggest that the effects of castration on certain appetitive behaviors, such as pursuits and level changes, are also modulated by the amount of sexual experience males have prior to castration.

The effects of TP treatment on the reinstatement of certain appetitive and consummatory behaviors in castrated, inexperienced male rats were influenced by the amount of sex exposure males had following castration. Following 12 days of TP treatment, 20% of castrated males tested twice with receptive females after castration showed mounts, compared to 40% of the castrated males tested repeatedly with receptive females at 4-day intervals. In addition, the mean number of level changes and anogenital investigations were found to be significantly higher in inexperienced males that were

exposed to repeated testing with receptive females compared to the mean number of those behaviors observed in inexperienced males tested only twice. These findings suggest that the reinstatement of consummatory behaviors as well as appetitive behaviors, such as conditioned level changing and anogenital investigations, in inexperienced males following TP treatment is facilitated by the amount of testing males receive following castration.

Summary of Results

The inconsistency in the literature regarding the amount of time required for castrated male rats to cease all consummatory behavior could be due to methodological differences in the amount of sexual experience male rats are allowed to have prior to castration as well as the number of test sessions males are given after castration. Overall findings from Experiments 1-3 are summarized in Table 1. The effects of castration on the expression of appetitive behaviors depended on the amount of sexual exposure males had with receptive females prior to castration. The percentage of males showing level changes and anogenital investigations remained unchanged in sexually experienced males regardless of the amount of testing males had with receptive females after castration. The percentage of inexperienced males showing pursuits and the mean number of pursuits and level changes was reduced as a result of castration. In addition, TP reinstatement of appetitive behaviors (i.e., level changes) was more robust in inexperienced males tested repeatedly with receptive females compared to inexperienced males tested only twice. These findings suggest that the expression of certain appetitive behaviors in inexperienced males after TP treatment depends on the amount of sexual stimulation received following castration, and that these effects are less apparent in males that are

sexually experienced.

The negative effects of castration on the expression of consummatory behaviors were delayed by the number of testing sessions experienced males had following castration. Inexperienced males showed similar decreases regardless of the amount of sexual exposure they received after castration. The reinstatement of consummatory behaviors following TP treatment occurred faster in sexually experienced males tested twice with receptive females following castration (i.e., 80%-90% males after 24 days of TP treatment), compared to the observed percentage of experienced males tested repeatedly or the percentage observed in inexperienced males.

Results from this chapter established the behavioral parameters used in subsequent studies within this thesis. All animals within this thesis referred as being sexually experienced received 10 test sessions with receptive females prior to any testing manipulation (i.e., castration, hormone treatment, hormone implants, lesions, etc.).

TABLE 1. EXPERIMENTS 1-3

			APPETITIVE BEHAVIORS				CONSUMMATORY BEHAVIORS				
FOLLOWING CASTRATION	PERCENTOF ANIMALS		Ex.1	Ex.2	Ex.3-F	Ex3-I		Ex.1	Ex.2	Ex.3-F	Ex3-I
		LC	=	↓	↓	=	MO	↓	↓	↓	↓
		PB	↓	↓	↓	↓	IN	↓	↓	↓	↓
		AI	=	=	=	=	EJ	↓	↓	↓	↓
		AM	↓	=	↓	=					
	MEAN AND SE		Ex.1	Ex.2	Ex.3-F	Ex3-I		Ex.1	Ex.2	Ex.3-F	Ex3-I
		LC	=	↓*	↓*	↓*	MO	↓*	↓*	↓*	↓*
		PB	↓*	↓*	↓*	↓*	IN	↓*	↓*	↓*	↓*
		AI	=	↓*	↓*	=	EJ	↓*	↓*	↓	↓
		AM	=	=	=	=					
FOLLOWING T TREATMENT (52 DAYS)	PERCENT OF ANIMALS		Ex.1	Ex.2	Ex.3-F	Ex3-I		Ex.1	Ex.2	Ex.3-F	Ex3-I
		LC	=	=	=	=	MO	↑	↑	↑	↑
		PB	=	↑	↑	↑	IN	↑	↑	↑	↑
		AI	=	=	=	↑	EJ	↑	↑	↑	↑
		AM	↑ & ↓	↑ & ↓	↑ & ↓	=					
	MEAN AND SE		Ex.1	Ex.2	Ex.3-F	Ex3-I		Ex.1	Ex.2	Ex.3-F	Ex3-I
		LC	=	↑*	↑ns	↑*	MO	↑*	↑*	↑*	↑*
		PB	↑*	↑*	↑*	↑*	IN	↑*	↑*	↑*	↑*
		AI	↑*	↑*	=	↑*	EJ	↑*	↑	↑*	↑*
		AM	↑*	=	=	=					

Table 1. Summary of the percentage of animals and mean number of appetitive and consummatory behaviors displayed by castrated males following castration and T treatment from experiments 1-3. * (statistically significant $p < .05$); = (remained unchanged); N.S. (non significant difference); ↑(increased following castration or T treatment); and ↓ (decreased following castration or T treatment). LC (level changes); PB (pursuit behavior); AI (anogenital investigations); AM (attempted mounts); MO (mounts); IN (intromissions); EJ (ejaculations). Exp.1 (sex experienced, tested 4-day intervals); Exp.2 (sex experienced, tested at 5 & 10 weeks); Exp.3-F (1 ejaculation, tested 4-day intervals); Exp.3-I (1 ejaculation, tested at 5 & 10 weeks).

CHAPTER II

DIFFERENTIAL INDUCTION OF FOS IN CASTRATED MALE RATS FOLLOWING GENITOSENSORY AND OLFACTORY STIMULI

Experiment 1. Differential Induction of Fos in the Castrated Male Rat Brain Following Testosterone Replacement

Brain activation during sexual behavior has been studied extensively using Fos IR as a marker of neuronal activation. In the male rat, copulation to ejaculation increases Fos expression in the BNSTpm, mPOA, MEApd and caudal diencephalon (Baum & Everitt, 1992; Coolen, Peters, & Veening, 1997; Roberston, Pfaus, Atkinson et al., 1991). Sensory input from intromissions and ejaculations activate brain regions such as the NAcc, mPOA, BNSTpm, MEApd, and subparafascicular nucleus (SPF) (Baum & Everitt, 1992; Coolen, Peters & Veening, 1997). Further analysis has revealed that precopulatory behaviors, such as anogenital investigation of a receptive female, result in small increases in Fos IR in the accessory olfactory bulb, BNSTpm, mPOA, and MEApd of male rats and gerbils (Bressler & Baum, 1996; Coolen, Peters & Veening, 1997; Heeb & Yahr, 1996). In all brain regions investigated, the amount of Fos expression is greater following intromissions and ejaculations than following mounts or olfactory stimulation alone (Baum & Everitt, 1992; Bressler & Baum, 1996; Coolen, Peters, & Veening, 1997; Heeb & Yahr, 1996).

Findings from Chapter 1 showed that castrated sexually experienced males exposed infrequently to receptive females took approximately 24 days of daily T administration to show a reinstatement of all consummatory behaviors. In the present study, Fos immunocytochemistry was used to provide a detailed quantitative analysis of neuronal activation in sexually experienced, long-term castrated male rats following 24

days of T-treatment, sexual stimulation, or both. Experiment 1 examined the mean number of Fos cells counted in the BNSTpm, bed nucleus of the accessory olfactory bulb (BAOT), lateral habenula (LH), mPOA, medial preoptic nucleus (MPN), paraventricular hypothalamic nucleus anterior parvicellular part (PaAP), MEApm, MEApd, posterodorsal preoptic nucleus (PdPN), subparafascicular nucleus (SPFp), and VTA of sexually-experienced, long-term castrated male rats following 24 days of T treatment. Experiment 2 investigated the amount of neural activation within the same brain areas in sexually experienced, long-term castrated, T-treated males following intromissions and ejaculations. Experiment 3 examined the neural activation in the same brain areas in long-term castrated, 24 days TP-treated male rats, produced by olfactory stimuli alone.

Materials and Methods

Animals

Eighty-four Long Evans hooded rats from Charles River Canada (St. Constant, Québec, Canada) weighing between 250-280g upon arrival were used as subjects. Twenty adult female Long Evans hooded rats, from the same supplier, weighing 240-250g, were used as stimulus females. All animals were housed two per cage in plastic shoebox cages. All animals were maintained in a reversed 12 hours light-dark cycle with light off at 8:00h. Food (Purina Rat Chow) and water were available ad libidum. The animal colony was maintained at a constant temperature of 21°C. Females were anesthetized and ovariectomized following the same procedures used in Experiment 1 of the previous chapter.

Procedure

Males were pre-exposed to the bilevel chambers and given sexual experience with receptive females using the same procedures as in Experiment 1, Chapter 1. As in those experiments, appetitive, precopulatory, and consummatory measures of males' sexual behaviors were recorded during each session. Animals were given 10 training sessions at 4-day intervals prior to castration.

All male rats were castrated using the same procedure described in Experiment 1, Chapter 1. As in that experiment, a decrease in the males' sexual behaviours was recorded every 4 days, over a 13-week period. The last session prior to treatment was used as a baseline measure. Following the last baseline session, animals were randomly assigned to one of three hormone-partner conditions: testosterone-female (N=28), oil-female (N=28), or testosterone-no female (N=28). Castrated male rats were given daily administration of 0.1 cc TP (30 μg) or oil. All TP and oil injections were administered during the day between 10:00h and 11:00h. Males in the 'female' conditions were exposed to a receptive female once every 4 days for a period of 24 days. Males in the 'no-female' condition were placed alone in the testing chamber at 4-day intervals for a period of 24 days.

The testing procedure was identical to the one described in Experiment 1, Chapter 1. However, in addition to the behavioral data recorded every four days, following each testing session, 4 animals selected at random from each condition were sacrificed and their brains removed and prepared for Fos immunocytochemistry.

Immunocytochemistry

Each male was sacrificed 75 minutes after the start of behavioral testing by an ip injection of 0.9ml sodium pentobarbitol (120mg/kg). They were perfused intracardially with

ice-cold phosphate-buffered saline (400ml), followed by 4% paraformaldehyde in 0.1M phosphate buffer (400ml). To prevent excessive blood clotting, heparin (1ml) was initially added to fill the needle used during the perfusion. Brains were removed, postfixed in 4% paraformaldehyde for 4h, and stored overnight in 30% sucrose at 4°C.

Frozen coronal brain sections (30μm) were cut from each brain on a sliding microtome through the BNSTpm (corresponding to plates 20-21 in Paxinos and Watson [1986]), mPOA (corresponding to plates 21-22 in Paxinos and Watson [1986]), MPN (corresponding to plates 23 in Paxinos and Watson [1986]), BAOT (corresponding to plates 25-26 in Paxinos and Watson [1986]), LHBM (corresponding to plate 27-28 in Paxinos and Watson [1986]), PaAP (corresponding to plates 28-29 in Paxinos and Watson [1986]), MEApv (corresponding to plates 30-31 in Paxinos and Watson [1986]), and MEApd (corresponding to plate 32 in Paxinos and Watson [1986]), and VTA (corresponding to plates 38-42 in Paxinos and Watson [1986]). Sections were washed (3x5 min rinses) in cold 50mM Tris-buffered saline (TBS) and were incubated at 4°C for 30 minutes in TBS and 30% w/w hydrogen peroxide (H_2O_2). Sections were rinsed again (3x5 min TBS washes) and then incubated for 90 minutes at 4°C in 0.05 % Triton TBS and 3% normal goat serum (NGS) to open up plasma membranes and to condition the tissue to the solution, and then washed 3x5 min in cold TBS.

Sections were transferred directly into a solution containing a rabbit polyclonal antibody [raised the N-terminal residues 4-17 of human Fos protein (Oncogene Science; ab#2, diluted 1:75 000)] in 0.05% Triton TBS with 3% NGS at 4°C for 72 h. Next, sections were rinsed in TBS (3x5 min washes) and transferred into biotinylated anti-rabbit IgG made in goat (Vector Laboratories; 1:200) in 0.05% Triton TBS, with 3% NGS for 1 h at 4°C.

Then, sections were rinsed in TBS (3x5 min washes) and were placed into an avidin-biotinylate-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; diluted 1:55.55) for 2 h at 4°C. Following incubation with ABC reagants, sections were rinsed with TBS followed by a 10 minute rinse with 50mM Tris and a 10 min rinse in 3,3'-diaminobenzidine (DAB) in 50mM Tris at room temperature on an orbital shaker. This was followed by another 10 minute incubation of 50mM Tris and DAB [0.1 ml of DAB/Tris buffer, pH 7.8] with 3% H_2O_2 (9ml/100ml) to catalize the DAB and 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer + H_2O_2) to color the DAB chromagen product blue-black. To stop the reaction, sections were rinsed in TBS and then mounted onto gel-coated slides. The sections were then dehydrated in a series of ethanols (30%, 70%, 95% and 100%), cleared in Hemo-D, coverslipped (using Permount), and examined under a microscope.

Behavioral and Statistical Analyses

Tests were videotaped and scored as described in Experiment 1. The mean number of appetitive, precopulatory, and consummatory behaviors observed during each test following castration and subsequent hormonal treatment were analyzed using a between group ANOVA. Significant main effects and interactions were followed by post hoc analyses of individual means using the Tukey method. Chi-square analysis was used to compare the proportions of rats showing appetitive and consummatory behaviors pre- and post-hormone treatment. The levels used to determine statistical significance for all comparisons was $p < 0.05$.

Fos IR cells were counted using a Leitz Laborlux microscope (40 X) connected to a digital image analysis system (MI, Imaging Research, Inc., St. Catherines, ON), with the standard dimension of a single cell nucleus set between 2 and 50 pixels. The mean

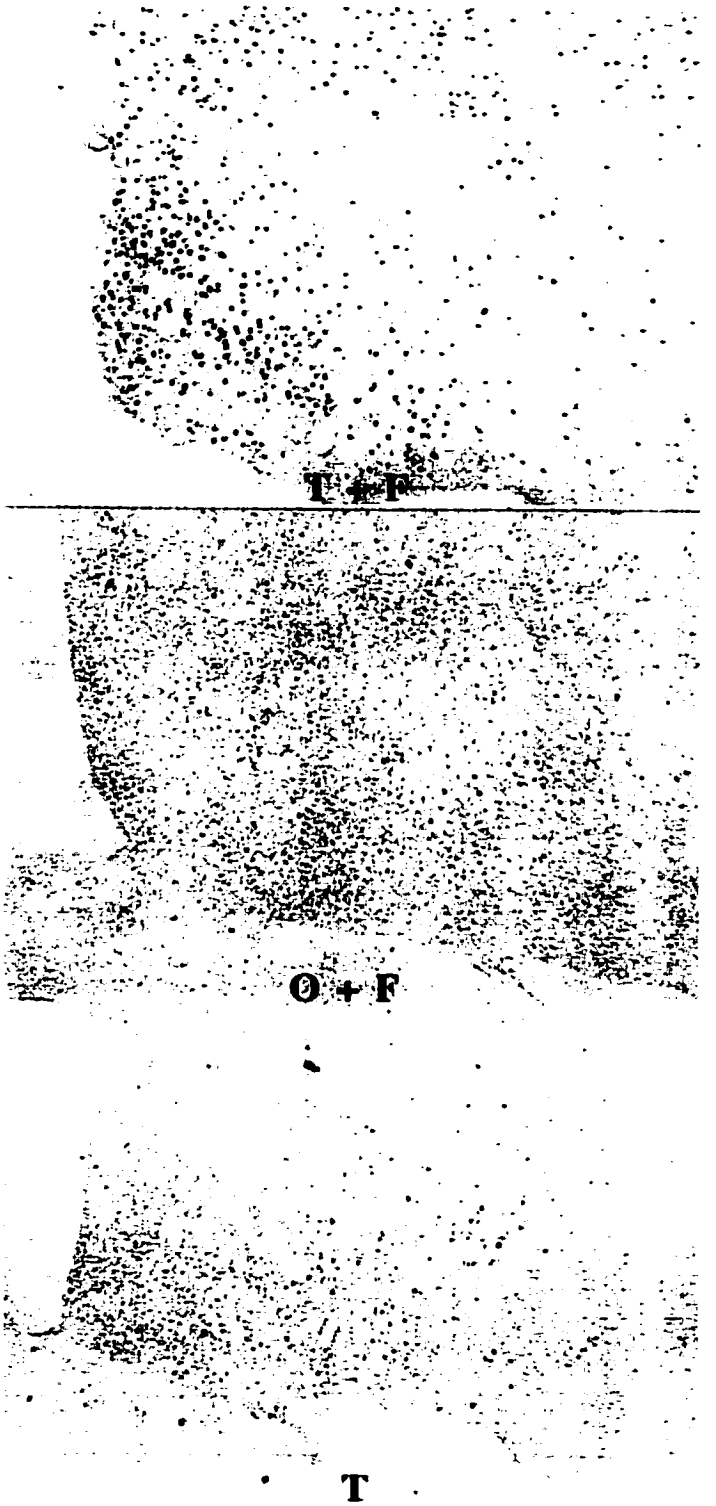
number of Fos IR cells was calculated bilaterally from 5 sections/region/rat which appeared subjectively to contain the largest number of Fos IR cells (as in Pfaus, Kleopoulos, Mobbs, Gibbs, & Pfaff, 1993; Pfaus, Marcangione, Smith, Manitt & Abillamaa, 1996). A between-groups ANOVA was used to determine the effects of copulatory activity on the mean number of Fos IR cells. For each significant ANOVA, post-hoc comparisons of the group mean were conducted using the Tukey method, $p<0.05$.

Results

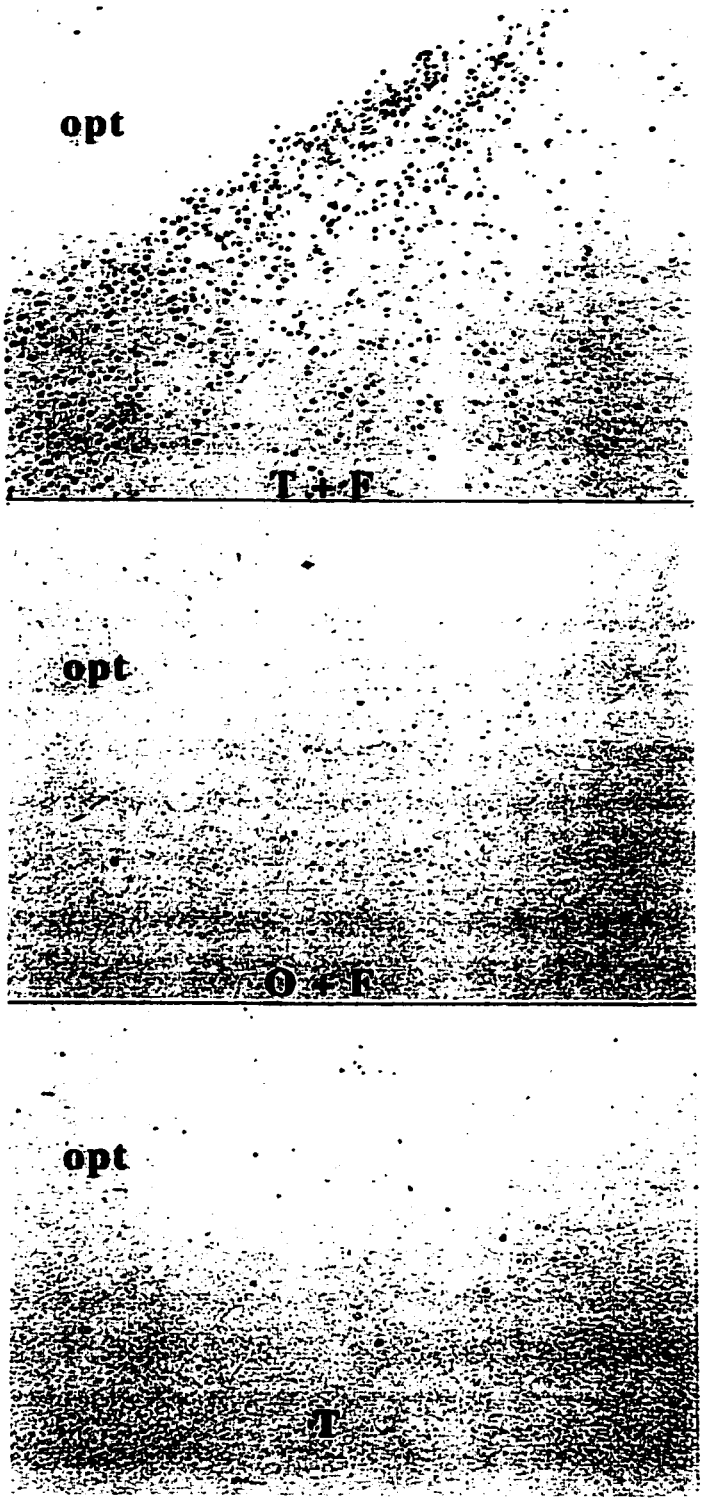
Fos Induction Following 24 Days of T Treatment

During 24 days of subcutaneous injections of T and subsequent reinstatement of copulation, castrated male rats showed an accumulation of Fos-IR in the nuclei of cells in the BNSTpm, mPOA, MEApd, and VTA. Non-copulating control males (i.e., males administered with TP but not exposed to receptive females and males administered with oil instead of TP) showed considerably less Fos-IR in these regions across the 24 days of TP treatment (Photomicrographs 1-4). Figure 21 shows the mean number of Fos cells/section following daily TP administration in the different brain regions examined. A significant increase in Fos-IR was observed in the BNSTpm, mPOA, MEApm and VTA. For the BNSTpm, there was a significant interaction $F(6,12)=54.9$, $P<.0001$, with the T+F group differing from others on days 8, 12, 16, and 24. Within the T+F group, all days differed from day 24: $F(6,12)=79.2$, $p<.0001$. For the mPOA, there was a significant interaction $F(6,12)=169.76$, $p<.0001$, with the T+F group differing from others on days 8, 12, 16, 20, and 24. Within the T+F group all days differed from day 24: $F(6,12)=219.2$, $p<.0001$. For the MEApd, there was a significant interaction $F(6,12)=3467.66$, $p<.0001$,

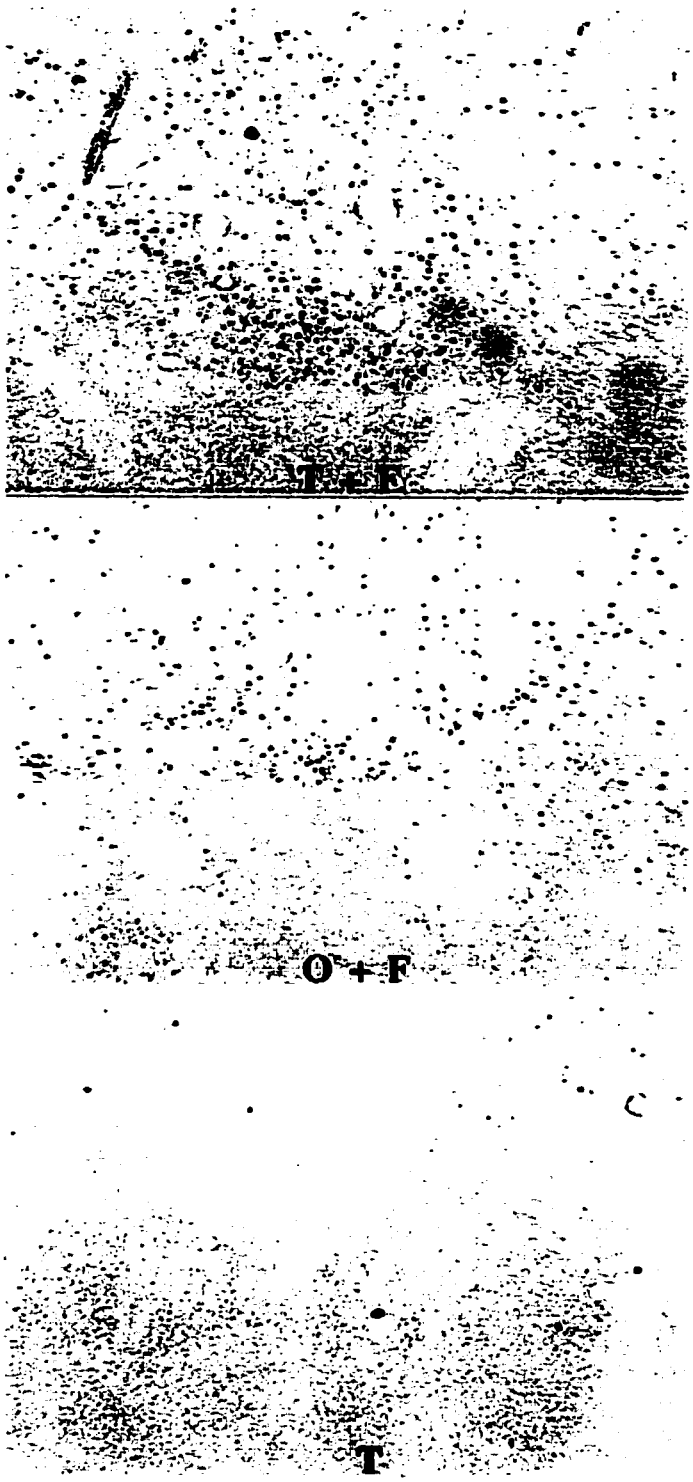
Photomicrograph 1. Fos induction in the BNSTpm of long term castrated rats following 24 days of testosterone (T) or oil (O) treatment. Males were either tested with receptive females or placed alone in the testing chamber.



Photomicrograph 2. Fos induction in the mPOA of long term castrated rats following 24 days of testosterone (T) or oil (O) treatment. Males were either tested with receptive females or placed alone in the testing chamber.



Photomicrograph 3. Fos induction in the MEApd of long term castrated rats following 24 days of testosterone (T) or oil (O) treatment. Males were either tested with receptive females or placed alone in the testing chamber.



Photomicrograph 4. Fos induction in the VTA of long term castrated rats following 24 days of testosterone (T) or oil (O) treatment. Males were either tested with receptive females or placed alone in the testing chamber.

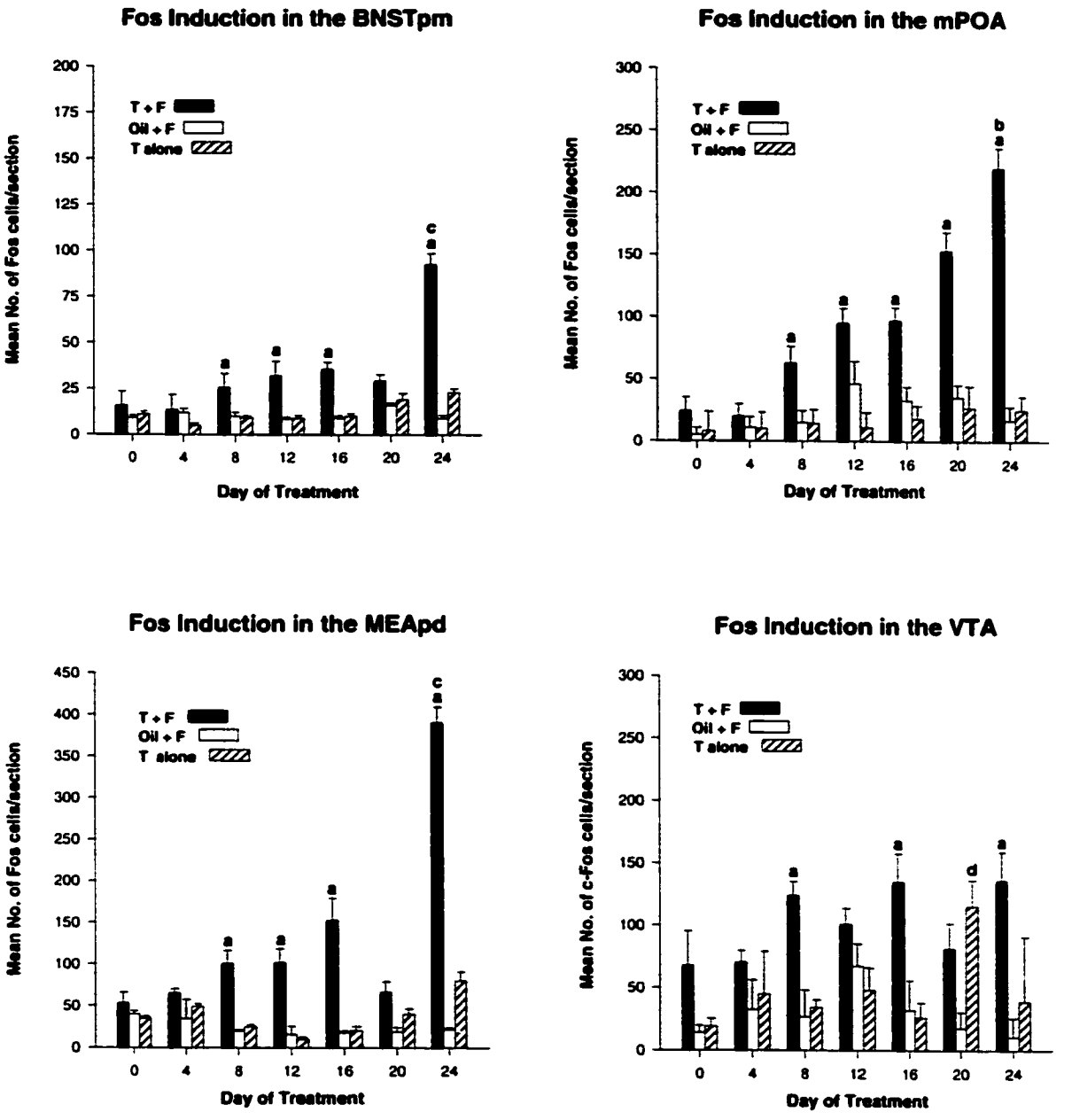


Figure 21. Mean number of Fos cells/section of the BNSTpm, mPOA, MEApd, and VTA in sexually experienced long term castrated male rats following 24 days of T treatment. (T+F: TP-treated males exposed to receptive females; Oil+F: oil-treated males exposed to receptive females; T alone: TP-treated males left alone in testing chamber). Data are means + SEM. Day 0: Baseline measure prior to T treatment. [a]$p<.05$ different from Oil+F and T alone groups. [b]$p<.05$ different from Days 0, 4, 8, 12 and 16. [c]$p<.05$ different from Days 0, 4, 8, 12, 16 and 20. [d]$p<.05$ different from T+F and Oil+F groups.

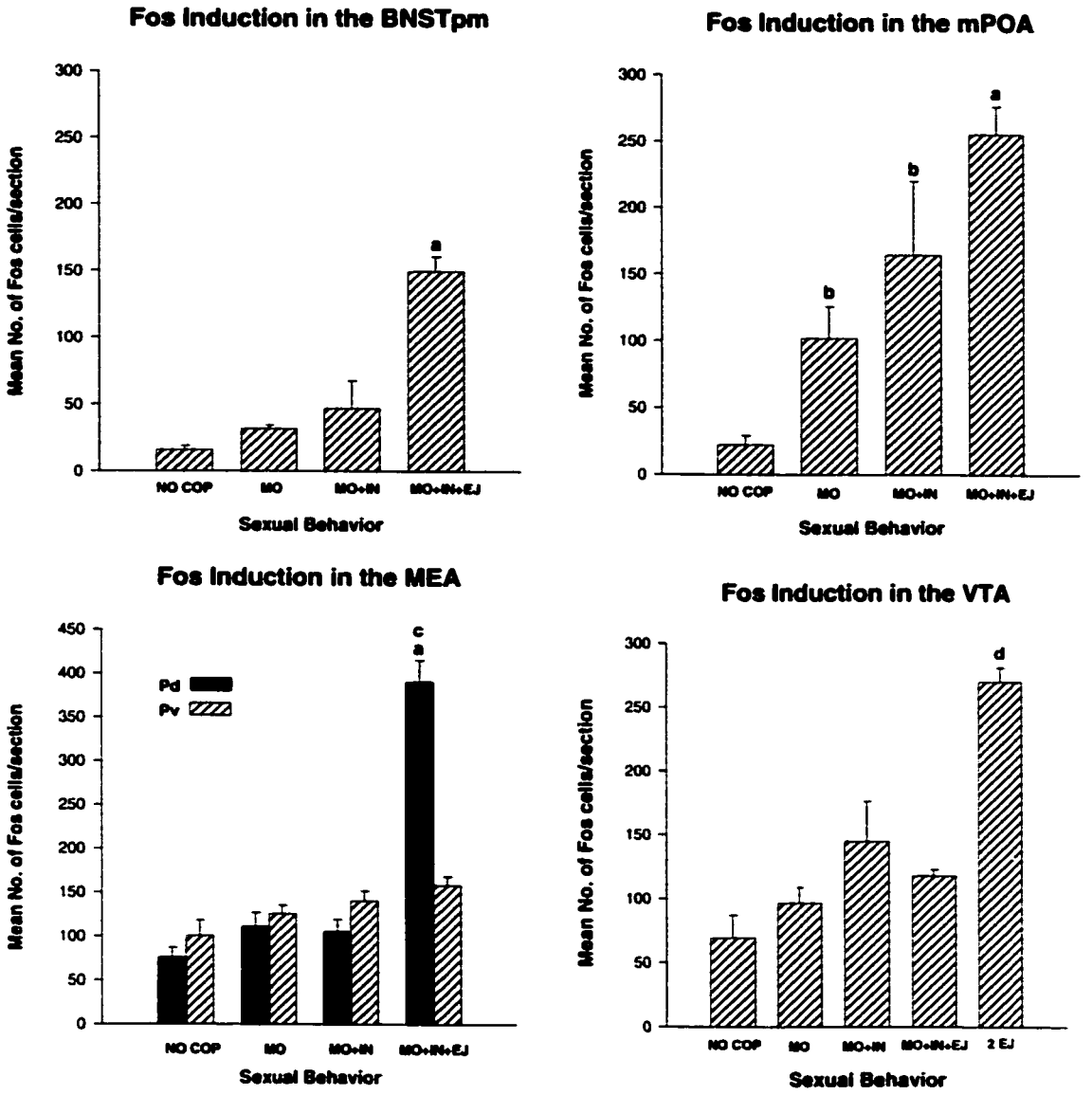
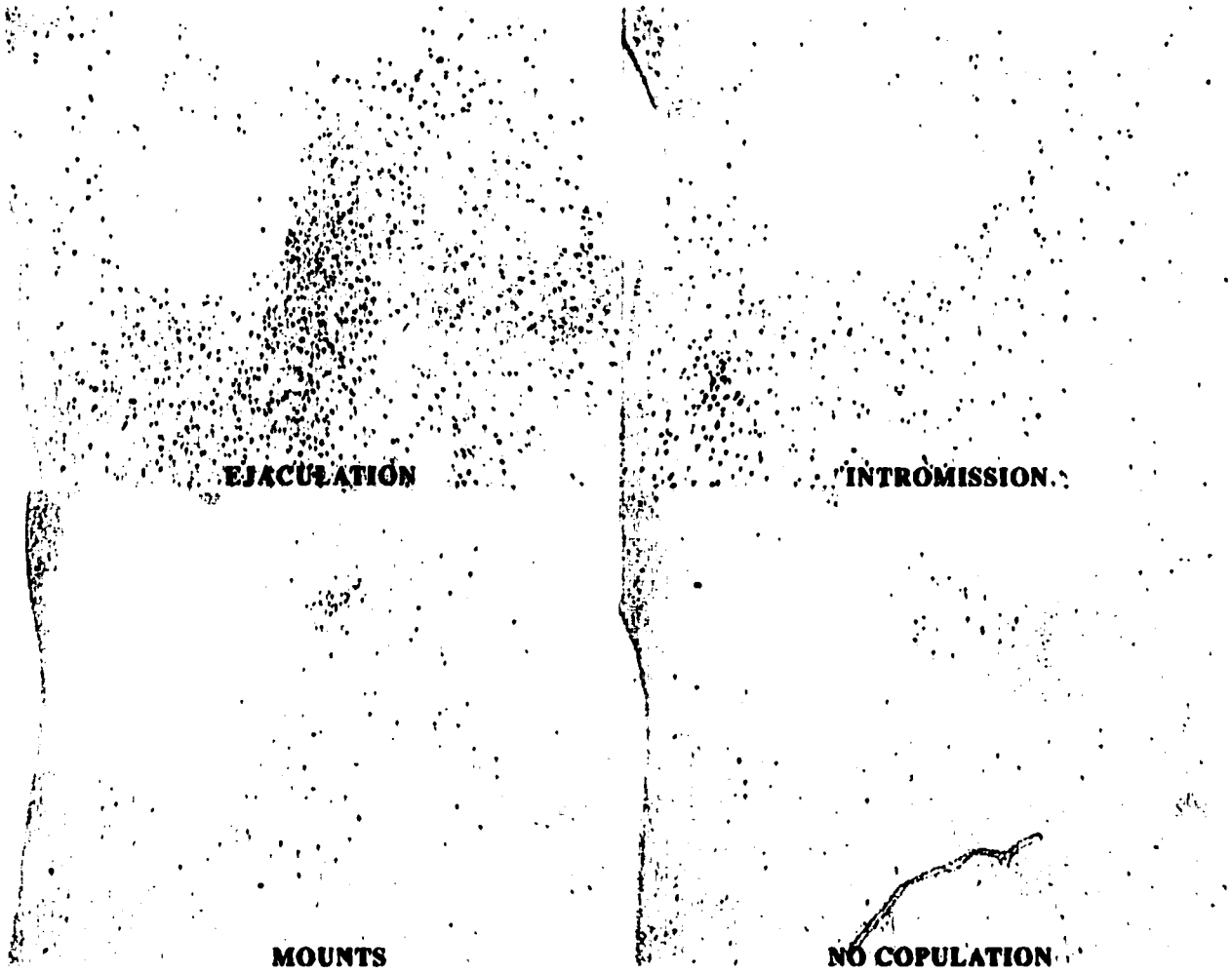
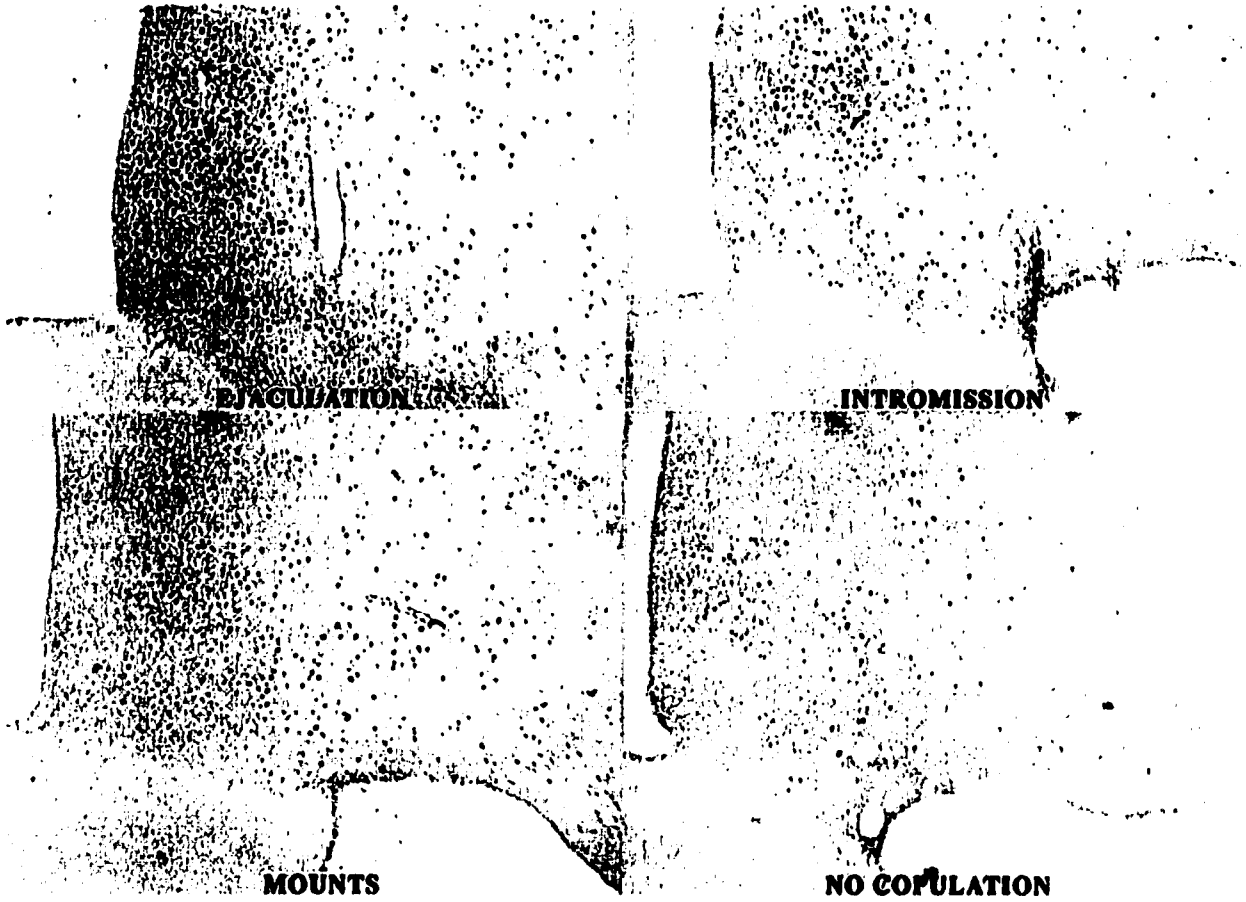


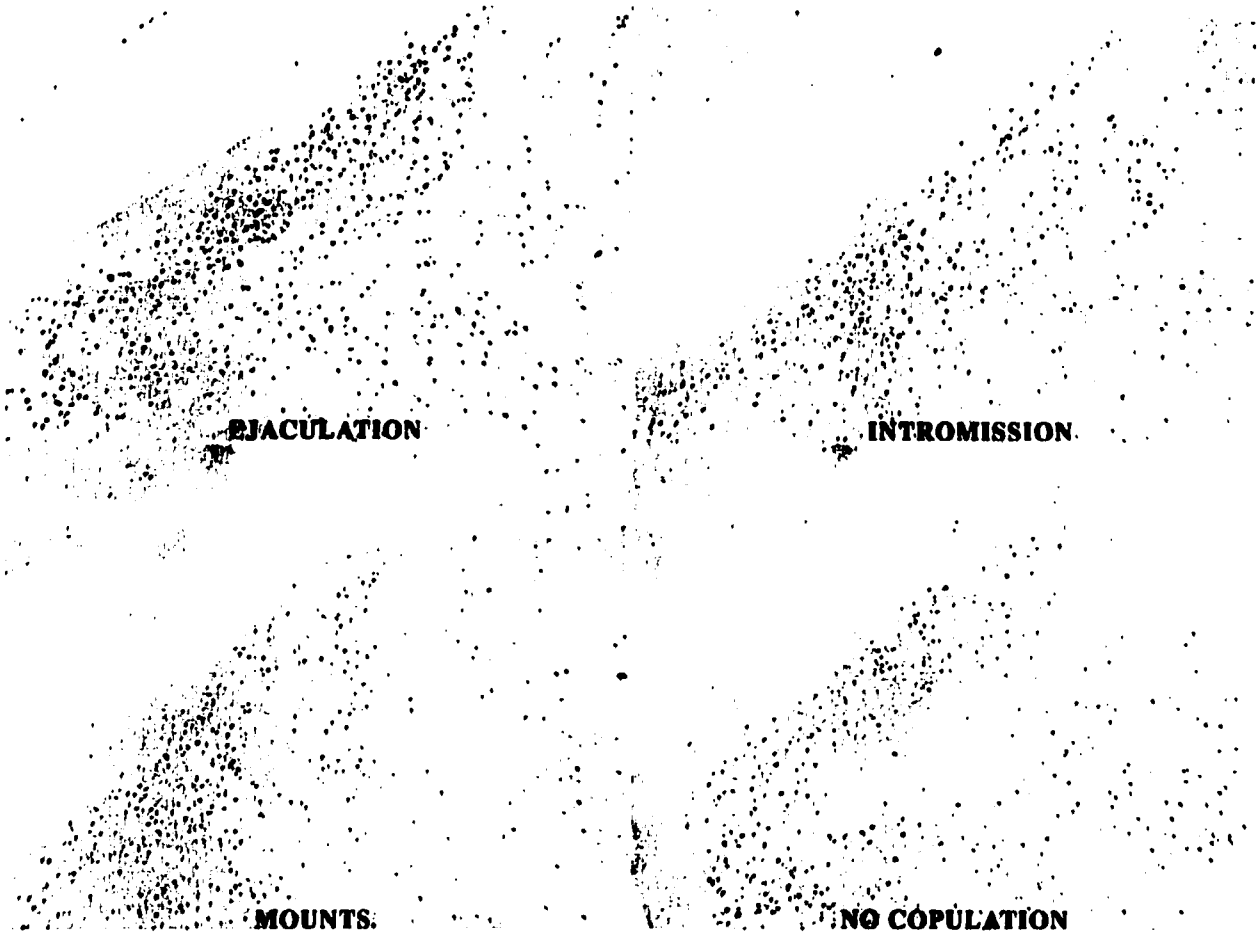
Figure 22. Mean number of Fos cells/section of the BNSTpm, mPOA, MEA (pv and pd), and VTA, in sexually experienced long term castrated male rats following specific aspects of male rat copulation (NO COP: no copulation; MO: mounts, IN: intromissions, EJ: ejaculations). Vertical lines show standard error. Data are means + SEM. [a]$p<.05$ different from NO COP, MO, and MO+IN. [b]$p<.05$ different from NO COP. [c]$p<.05$ different from MEApv. [d]$p<.05$ different from NO COP, MO, MO+IN, and MO+IN+EJ.



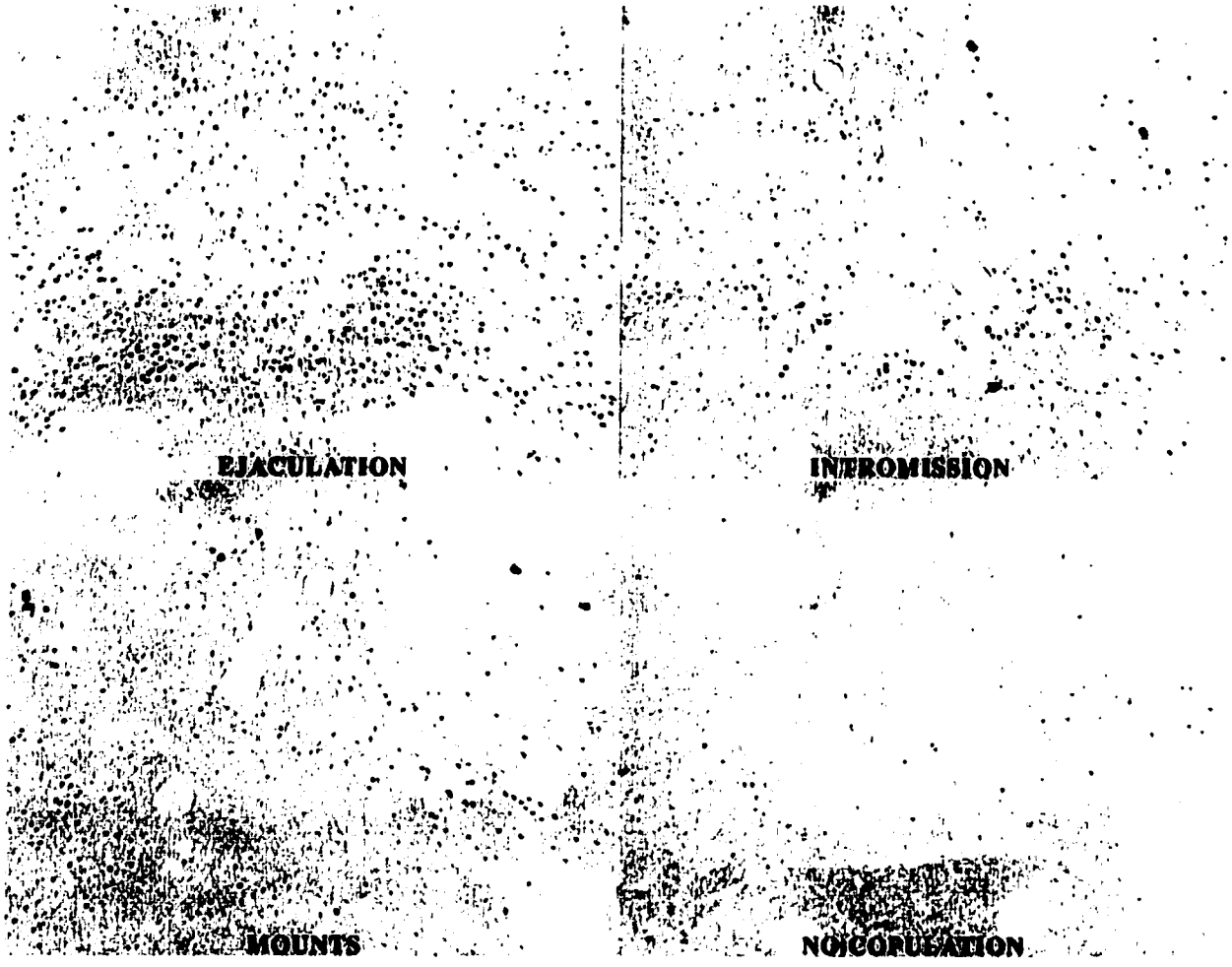
Photomicrograph 5. Fos induction in the BNSTpm of T treated long term castrated rats following ejaculations, intromissions, mounts, or not copulation.



Photomicrograph 6. Fos induction in the mPOA of T treated long term castrated rats following ejaculations, intromissions, mounts, or not copulation.



Photomicrograph 7. Fos induction in the MEApd of T treated long term castrated rats following ejaculations, intromissions, mounts, or not copulation.



Photomicrograph 8. Fos induction in the VTA of T treated long term castrated rats following ejaculations, intromissions, mounts, or not copulation.

with the T+F group differing from others on days 8, 12, 16, and 24. For the VTA, there was a significant interaction F(6,12)=289.25, P<.0001, with the T+F group differing from others on days 8, 16, and 24. Within the T+F a significant difference was found between days 0 and 16, and 24. The T alone group showed an increase on day 20, which was significantly different from the Oil+F group: F(6,12)=81, p<.0001. Finally, no significant differences were found in the MEApm (data not shown).

Fos induction Following Copulatory Activity

The return of the copulatory pattern among castrated male rats following TP replacement was not consistent across animals, differing mainly in the time of the behavioral reinstatement after TP treatment and in the mean number of behaviors reinstated. Because of this, the Fos-IR data were analyzed in males in the T+F group in terms of the behavioral reinstatement following TP treatment (i.e., first incidence of mounts, intromissions, or ejaculations) displayed by the male rats during those 24 days of TP administration (Photomicrographs 5-8).

A summary of the effects of copulatory behavior on the Fos-IR observed in castrated TP treated males is shown in Figure 22. Fos-IR increased as a function of copulatory stimulation. Castrated male rats that copulated to ejaculation with receptive females showed a significant increase in Fos-IR in the BNSTpm [F(3,8)=689.47, p<.00001], mPOA [F(3,8)=586.3, p<.00001], [MEApd F(3,8)= 2562.04, p<.00001], and VTA [F(4,10)=505.68, p<.00001]. Post hoc analysis of the BNSTpm individual means revealed a significant increase in the mean number of Fos cells in the MO+IN+EJ [F(3, 8)=149.23, p<.0001] relative to the means observed in the NO COP, MO, or MO+IN groups. Similar results were observed in the MEApd. Post hoc analysis demonstrated a

significant increase in Fos cells in the MO+IN+EJ [$F(3,8)=392.17$, $p<.0001$] compared to the means seen in NO COP, MO, MO+IN groups. The significant increase in Fos cells found in the VTA was observed in the 2EJ group [$F(4,10)=270.33$, $p<.0001$] relative to the NO COP, MO, MO+IN, MO+IN+EJ groups. The significant increase in Fos cells found in the mPOA was not restricted to ejaculation. Post hoc comparisons revealed a significant increase in Fos cells in the IN group [$F(3,8)=164.9$, $p<.0001$] relative to the means observed in the NO COP or MO groups. In addition, a significant increase in Fos cells in the MO+IN+EJ group [$F(3,8)=250$, $p<.0001$] was found relative to the mean number observed in the NO COP, MO and MO+IN groups.

In summary, the increase in Fos cells observed in the BNSTpm, MEApd, and VTA was linked to ejaculation. It appears that these regions were not stimulated sufficiently to express a significant number of Fos cells in castrated male rats displaying only mounts or intromissions, with the VTA requiring 2 ejaculations. In contrast, a significant, but gradual increase in Fos-IR was found in the mPOA of males that mounted, intromitted or ejaculated, with intromissions resulting in a higher amount of Fos expression than mounts, and with ejaculations resulting in a greater Fos expression than intromissions. Once again, no significant differences were found in the MEApv.

Discussion

Findings from this experiment demonstrate that copulation to ejaculation is necessary to elicit a significant neural activation in the BNSTpm, MEApd, and VTA of castrated male rats treated daily with TP. A gradual increase in Fos cells in the mPOA was observed following mounts, intromissions and ejaculations, with ejaculations having greater effects on Fos-IR expression than intromissions, and with intromissions resulting

in greater Fos-IR than mounts. Ejaculation was critical for Fos-IR in the BNSTpm, MEApm and VTA, with the VTA requiring multiple ejaculations.

Experiment 2. Differential Induction of Fos in the Castrated Male Rat Brain Following Intromissions and Ejaculations

Behavioral observations during the previous experiment revealed an inconsistency in the reinstatement of consummatory behaviors following TP treatment. Castrated male rats displayed different numbers of mounts, intromissions, or ejaculations following TP-treatment. The differences in the mean number of restored behaviors could alter the interpretation of Fos expression, by grouping together males displaying one or more ejaculatory series, or males displaying one single intromission with males displaying 7 intromissions.

Accordingly, Experiment 2 controlled for the amount of sexual stimulation (i.e., number of intromissions and ejaculations) that the castrated male rats received during behavioral testing with receptive females. Given the findings from Experiment 1 and previous literature (Heeb & Yahr, 1996; Coolen, Peters, & Veening, 1997) showing a greater amount of Fos cells in animals displaying ejaculations, a greater increase in Fos expression was expected in the BNSTpm, mPOA, and MEApd in males that ejaculated, compared to males that were only allowed to have multiple intromissions during copulation.

Materials and Methods

Animals

Twenty-eight adult male Long Evans rats from Charles River Canada (St. Constant, Québec, Canada) weighing between 250-280g upon arrival were used as subjects. The stimulus females were the same ones used in Experiment 1. The housing and feeding conditions were identical to those described in Experiment 1 of Chapter 1. Males were castrated under the same conditions described in Experiment 1 of Chapter 1.

Procedure

The initial procedure including habituation to the bilevel chambers and sexual training prior to castration was identical to that described in Experiment 1 of Chapter 1, as was the testing protocol following castration. Males were tested with receptive females for a period of 96 days (i.e., 24 test sessions), at which time animals' consummatory, precopulatory, and appetitive behaviors had almost completely ceased. The copulatory behaviors observed during the last session prior to TP treatment was used as a pre-treatment baseline measure.

Following the pre-treatment baseline session, all animals were administered with daily injections of TP (30 μg) during the day between 10:00h and 11:00h, as in Experiment 1. To hold constant the number of intromissions and ejaculations, males were randomly assigned to one of two groups: one group of males (n=14) were allowed to ejaculate twice with a receptive female, and the other group of males (n=14) were allowed to have a total of 6-7 intromissions during testing. Following the animals' first incidence of displaying either intromissions or ejaculations, the receptive female was removed from the chamber, and the male was left in the chamber for the remaining

testing time (up to 30 min). Four animals from each behavioral group (e.g. allowed to either intromit or ejaculate with the receptive females) were sacrificed 75 minutes from the initiation of behavioral testing, and their brains removed and prepared for Fos immunocytochemistry.

Immunocytochemistry

The immunocytochemical procedure used was identical to the one described in Chapter 2, Experiment 1. Visualization of Fos cells and the preparation of the slides prior to examination under a microscope was performed using the same procedures described in Experiment 1. Fos IR cells were counted in the same areas examined in Experiment 1 where a significant increase in Fos cells was observed.

Statistical Analysis

Between group ANOVAs were used to analyze Fos-IR data from castrated male rats displaying intromissions and ejaculations during the 30 minutes interaction with the receptive females. The level of significance for all comparisons was $p<0.05$.

Results

Relative to males allowed only to intromit, male rats copulating to ejaculation showed a significant increase in Fos-IR (Photomicrographs 9-12). The significance of these findings was confirmed by ANOVA, in the MEApd [$F(1,10)=69.11$, $P<.001$], the PdPN [$F(1,10)=5.95$, $P<.05$], and the anterior VTA [$F(1,8)=6.08$, $P<.05$] (Figure 23). Furthermore, the PdPN showed a Fos cluster among males that ejaculated which was not observed among males that only intromitted. In addition to the PDPN, an accumulation of Fos-IR as a cluster was found in the BNSTpm and in the BNSTst of ejaculating but not intromitting males (Photomicrographs 9). No significant differences were found in the

mean number of Fos cells within the BAOT, LHBM, mPOA, MEApm, PMV, and the SPFp of males that intromitted to ejaculation (data not shown).

Discussion

In Experiment 2, ejaculation produced an increase in Fos-IR in T-treated castrated male rats in the BNSTpm, mPOA, MEApd, PdPN and VTA. These findings are in close agreement with previous studies showing an increase in Fos expression in these brain areas following ejaculation (Baum & Everitt, 1992; Heeb & Yahr, 1996; Coolen, Peters, & Veening, 1997). Relative to those allowed only to intromit, males that ejaculated had more Fos IR neurons in the posterodorsal subregion, but not in the posteroventral subregion, of the MEA. In the MEA, Fos-IR was increased significantly in the lateral posterodorsal subdivision, but not the medial or central posterodorsal subdivisions. In the lateral MEApd, Fos expression followed the pattern seen in the PdPN, i.e., a significant increase following ejaculation. Similar labeling patterns have been observed in male gerbils (Heeb & Yahr, 1996), in which two dense clusters of labelled cells appear laterally and dorsolaterally in the caudal MEApd subsequent to ejaculation. The medial MEApd of castrated male rats that reach ejaculation shows a lower level of Fos expression compared to the lateral MEApd. This would suggest that Fos expression in the lateral MEApd is more dependent upon genital somatosensory input achieved through ejaculation. That is, the genital and olfactory input achieved during intromissions might not be sufficient to trigger Fos expression in the lateral MEApd. An increase in Fos cells was found in the BNSTpm following ejaculation. Findings from this experiment showed that the neurons within a specific subregion of the mPOA, the MPN, were activated by

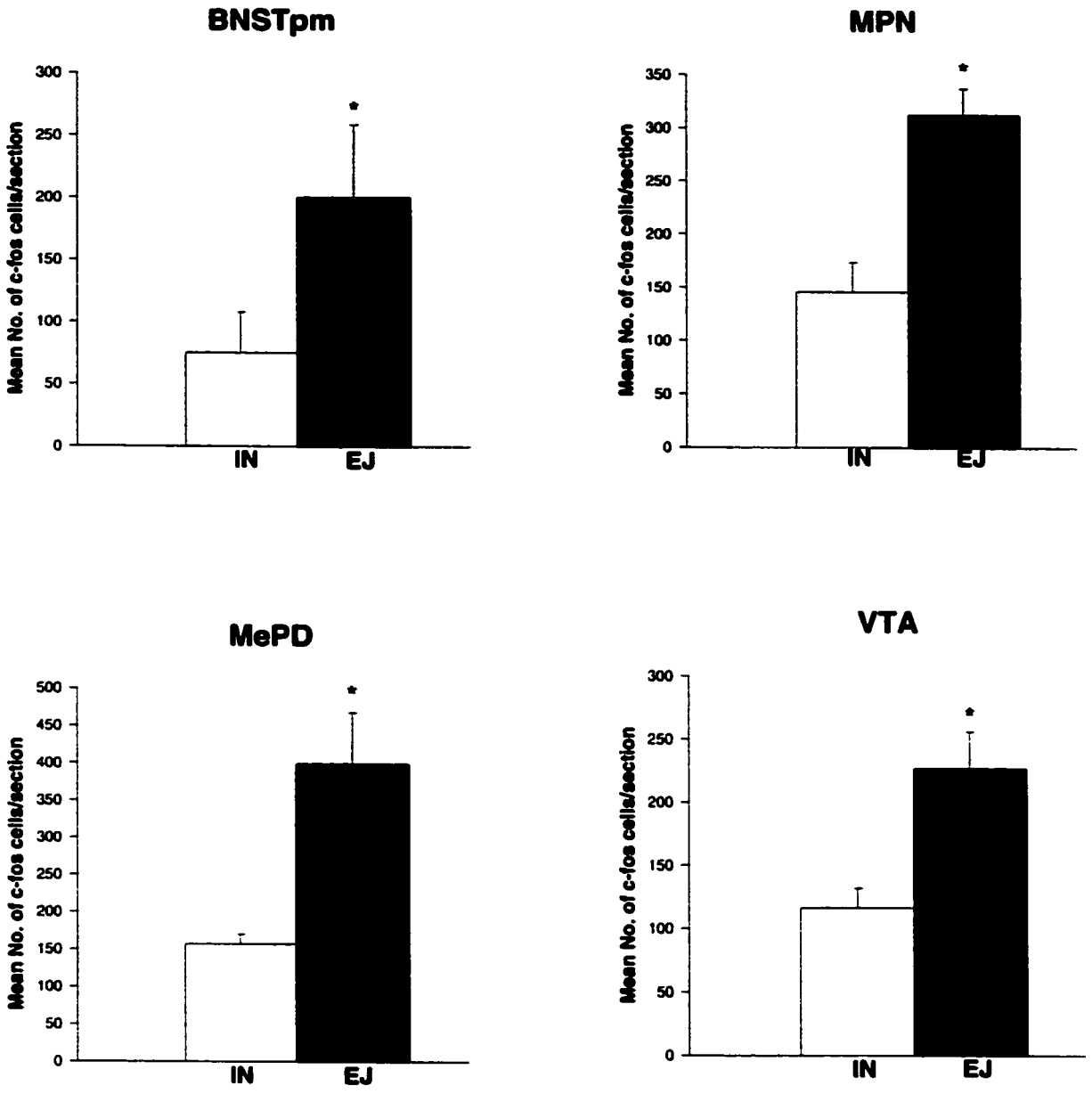
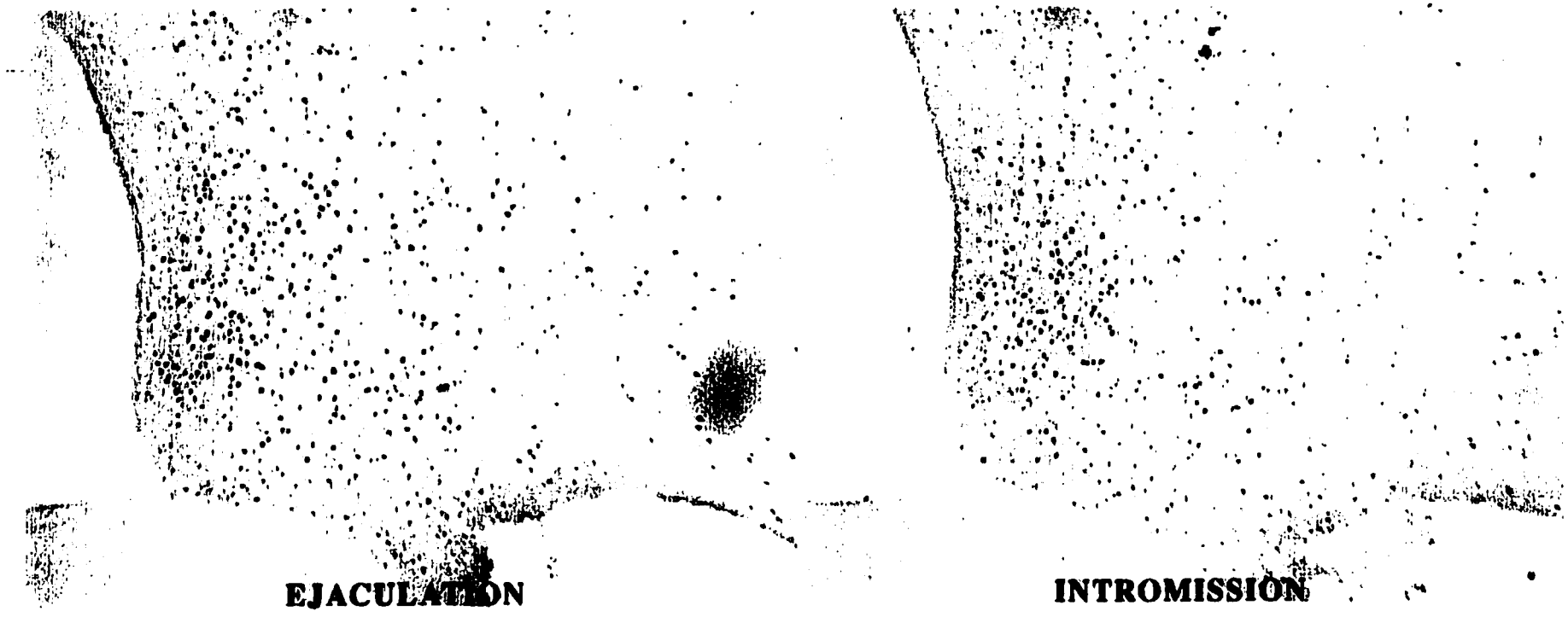
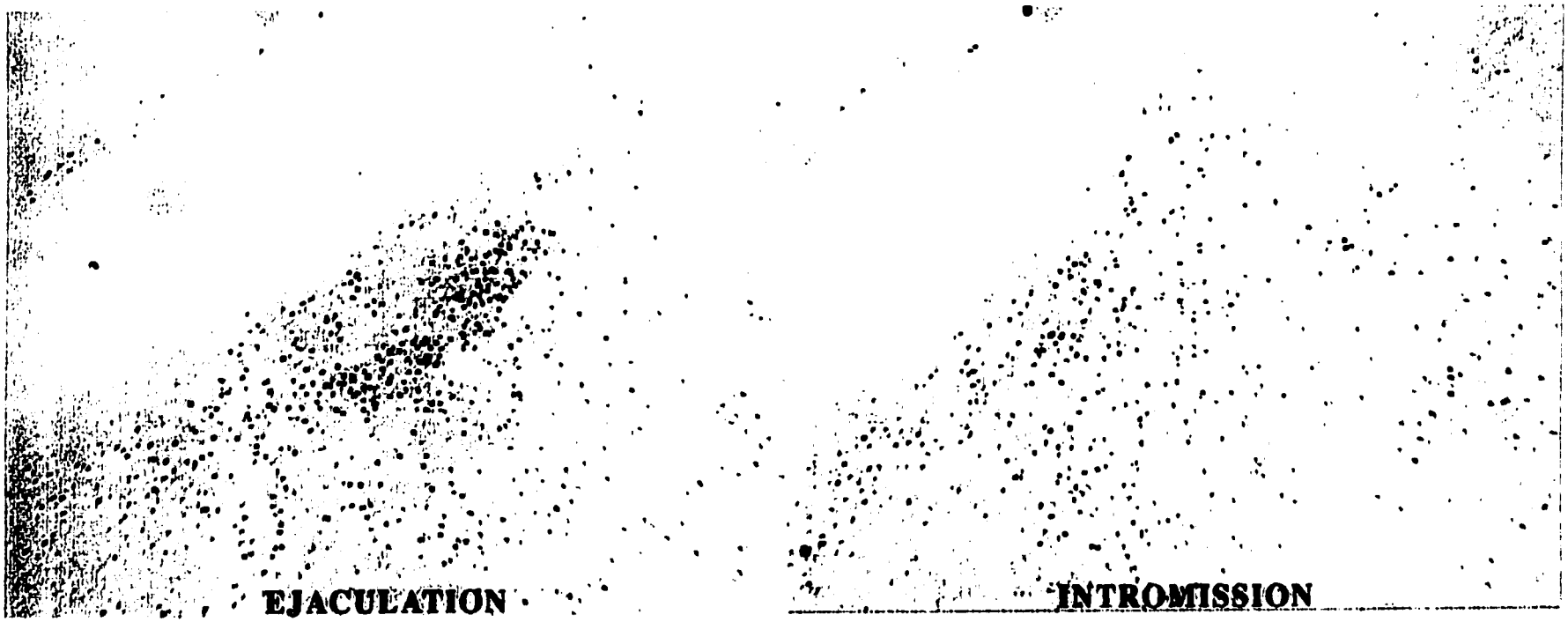


Figure 23. Mean number of Fos cells\section in the BNSTpm, MPN, MEApd and VTA of TP treated castrated male rats displaying intromissions and ejaculations. Vertical lines show standard errors. Data are means +SEM. *p<.05 between behavioral groups.

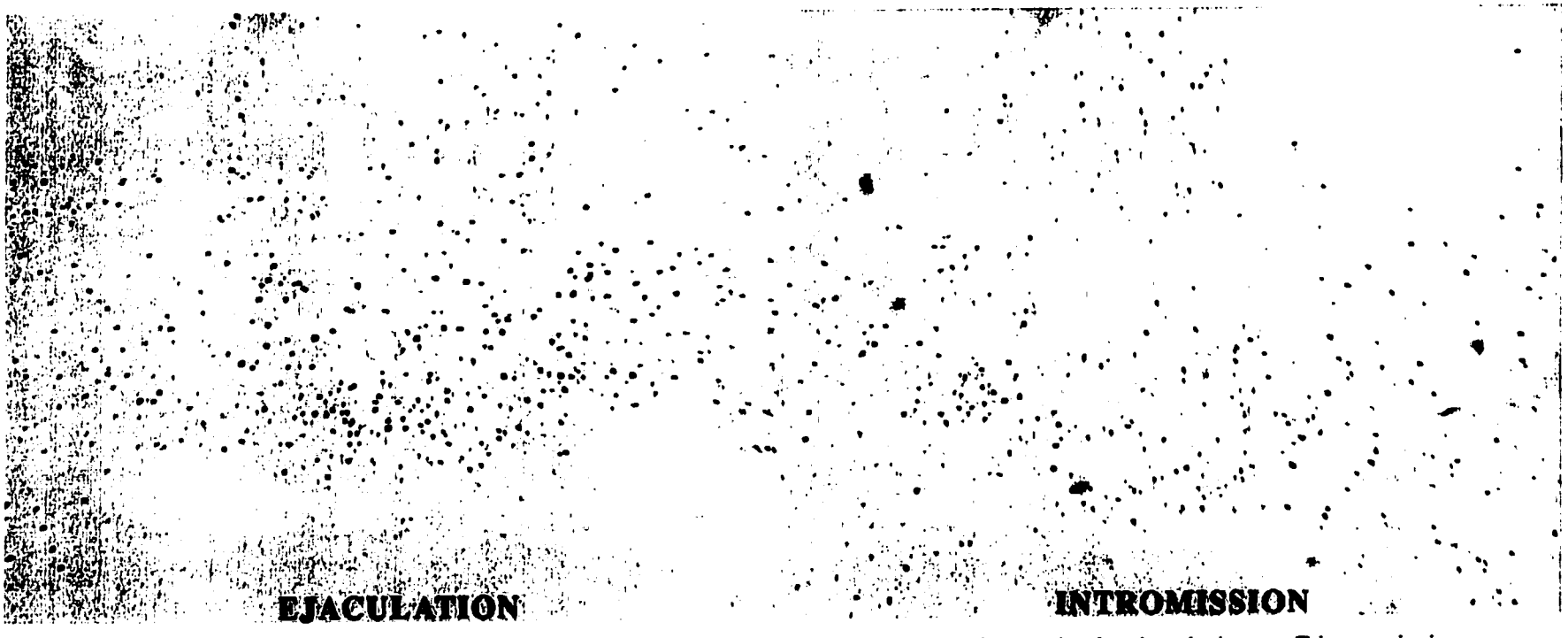
Photomicrograph 9. Fos induction in the BNSTpm of long term castrated rats following a single ejaculation or 7 intromissions.



Photomicrograph 10. Fos induction in the MPN of long term castrated rats following a single ejaculation or 7 intromissions.



Photomicrograph 11. Fos induction in the MEApd of long term castrated rats following a single ejaculation or 7 intromissions.



Photomicrograph 12. Fos induction in the VTA of long term castrated rats following a single ejaculation or 7 intromissions.

ejaculations. An increase in the mean number of Fos cells was observed in the anterior and posterior VTA following ejaculations versus intromissions. These findings demonstrate the critical role of ejaculation in eliciting a brain response within the BNSTpm, MEApd, MPN, and VTA of castrated, TP-treated male rats.

Experiment 3: Differential Induction of Fos in T Treated Male Rats as a Response to Olfactory Stimulation

Findings from Experiment 2 showed brain activation in the BNSTpm, MPN, MEApd, and VTA following somatosensory stimuli from copulatory exposure to receptive females. The third experiment investigated differences in neural activation within those brain areas following visual and olfactory stimulation from non-copulatory exposure to a receptive female. The approach was to examine Fos expression in sexually experienced, long-term castrated, TP-treated male rats exposed to a receptive female placed behind a wire mesh screen for 30 minutes. By holding the visual and olfactory stimulation constant (i.e., by preventing the males from copulating with receptive females), findings from this study determined the brain regions activated by visual and olfactory stimulation alone in castrated TP-treated males. In addition, this study examined the effects of TP on the reinstatement of certain appetitive behaviors (i.e., contacts through the screen, nose-poking, grooming, and climbing) in long-term castrates.

Materials and Methods

Animals

Ninety-six Long Evans hooded rats from Charles River Canada, (St. Constant, Québec, Canada) weighing between 250-280g upon arrival were used as subjects. Thirty

adult female Long Evans hooded rats, from the same supplier, weighing 240-250g, were used as stimulus females. All animals were housed under the same conditions described in Chapter 1. Females were ovariectomized and artificial estrus was induced using the same procedures described in Experiment 1 of Chapter 1.

Apparatus

Prior to castration, all animals were habituated and trained with receptive females in semi-circular mating chambers. These mating chambers were 61cm diameter x 36 cm deep and were constructed of a curved metal back, with a plywood floor covered by 1-2 cm of bedding, and a plexiglass front. A removable wire-mesh partition was placed in the middle of each chamber, dividing each chamber into two halves. Lids were made of a wood frame and wire-mesh to close the chambers during testing in order to prevent animals from leaving the chambers.

Procedure

Males were pre-exposed to the semicircular chambers for 20 minutes on each of five consecutive days. Following this pre-exposure period and prior to castration, males were given 10-multiejaculatory sessions with receptive females. During this training period male rats were placed in the left side of the semicircular chamber for 5 minutes, after which the female was introduced in the right side of the chamber for an additional 10 minutes. After this period of time, the divider was removed and the frequency of mounts, intromissions, and ejaculations during the 30- minute test was recorded. Animals were given ten training sessions every four days prior to castration. Only animals that ejaculated at least twice during the last 5 training sessions were included in the study.

All male rats were castrated using the protocol described in Experiment 1 of Chapter 1. All animals were given a week to recover prior to testing. Males were pre-exposed to the bilevel chambers during 20 minutes on 5 consecutive days. Then, copulatory tests began in the bilevel chambers once a week for a period of 14 weeks, after which time none of the males displayed mounts, intromissions, or ejaculations. As in the pre-exposure phase, male rats were placed in the left side of the chamber for 5 minutes, after which a female was introduced in the right side of the chamber. However, in this session, instead of removing the wire-mesh screen after 10 minutes, males were allowed only to have contact with the females through the screen for an additional 30-minutes. At the end of this period, the females were removed from the right side of the chamber and the male was left in the left side of the chamber for another 30-minutes. This session was recorded and scored as the pre-treatment baseline measure.

The mean number of nose-pokes, climbs, contacts with the receptive female through the screen, and genital grooms were scored. Nose-poking behavior was scored when the male's nose-mouth area was in contact with the metal screen, in an in-out pattern. The climbing of the wire mesh screen was assigned three different scores: climb "1", assigned when the male had its two front paws in the wire mesh at the bottom of the screen; climb "2", assigned when the male had its four paws in the middle section of the metal screen, but did not reach the top of the screen; and climb "3", assigned when the male climbed to the top of the metal screen, touching the top lid that closed the chamber. Contacts were scored when the male had a direct contact with the receptive female through the wire metal screen. Finally, genital gooming was scored every time that the male licked his own genital area.

Following the pre-treatment baseline session, males were assigned based on their ejaculation latency to one of four hormone treatment-partner conditions (N=24/group): Testosterone-female (T+F), testosterone-no female (T-NO F), oil-female (O+F), and oil-no female (O-NO F). Animals in each condition were daily administered subcutaneously with either 0.1cc injections of TP (30 μg) or sesame oil, and exposed to a receptive female at the time of testing depending on their assigned group for a period of 24 days, at 4-day intervals. All injections (T and oil) were administered during the day between 10:00h and 11:00h. Following each behavioral test session, 4 animals from each group were chosen at random and sacrificed and their brains removed and prepared for Fos immunocytochemistry.

Immunocytochemistry

The immunocytochemical protocol used in this study was identical to the one described in Experiment 1 of this chapter.

Behavioral and Statistical Analyses

Tests were videotaped and scored using the same procedure as in Experiment 1 of this chapter. The mean number of nose pokes, climbs, contacts through the mesh, and genital grooming observed in each of the hormone-partner conditions following castration and subsequent hormone treatment, were analyzed using a between-groups ANOVA. Significant main effects and interactions were followed by post-hoc analyses of individual means using the Tukey method. The levels of significance for all comparisons was $p<0.05$.

Fos IR cells were counted as described in Experiment 1 of this chapter. The mean number of Fos cells observed during the 24 days of TP treatment was analyzed using a

between-group ANOVA. Significant main effects and interactions were followed by post-hoc analyses of individual means using the Tukey method.

Results

Appetitive Behaviors

Significant increases in the amount of nose-poking, climbing, contacts, and genital grooming, were observed in males in the T-treated group that had an inaccesible female placed behind the metal screen, compared to oil-treated males regardless of the presence of a receptive female, or T-treated males left alone in the chambers.

Contacts. An increase in the mean number of contacts was displayed by males in the T+F group compared to the males in the O+F group. This significant increase was observed on D12 [$F(3,12)=26.9$, $p<.0001$], D16 [$F(3,12)=14.45$, $p<.0001$], D20 [$F(3,12)=14.35$, $p<.0001$]; and D24 [$F(3,12)=19.62$, $p<.0001$] (Figure 24).

Nose-poking Behavior. The mean number of nose-pokes displayed by males in the T+F and O+F groups was significantly higher compared to the mean number of nose-pokes displayed by males in the O-NO F and T-NO F groups. The increase showed by males in the T+F was observed on D8 [$F(3,12)=30.97$, $p<.0001$], D12 [$F(3,12)=30.05$, $p<.0001$], D20 [$F(3,12)= 27.35$, $p<.0001$, and D24 [$F(3,12)=33.67$, $p<.0001$]. Males in the O+F group displayed significantly more nose-pokes on D8 [$F(3,12)=29.8$, $p<.0001$] and D24 [$F(3,12)=31.56$, $p<.0001$] (Figure 25).

Contacts

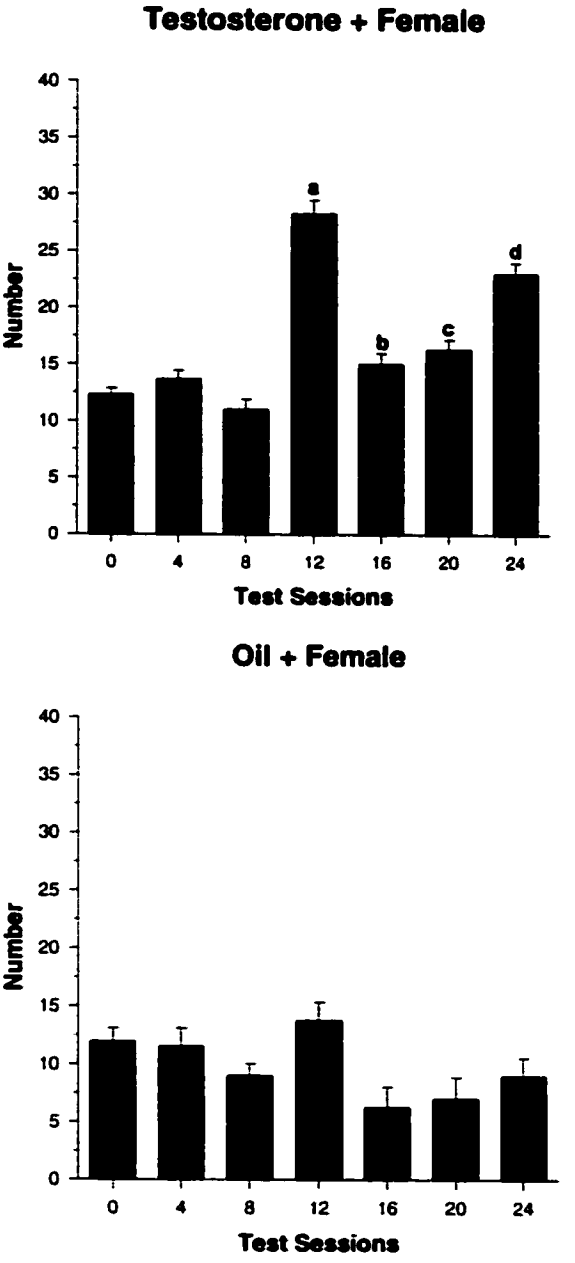


Figure 24. Mean number of sexually experienced long-term castrated males displaying contacts with the receptive female through a wire metal screen following 24 days (D0-D24) of TP or Oil treatment. Data are means +SEM. [a]p< .05 different from D12 Oil+Female group. [b]p<.05 different from D16 Oil+Female group. [c]p<.05 different from D20 Oil+Female group. [d]p<.05 different from D24 Oil+Female group. Vertical lines show standard errors.

Nose Pokes

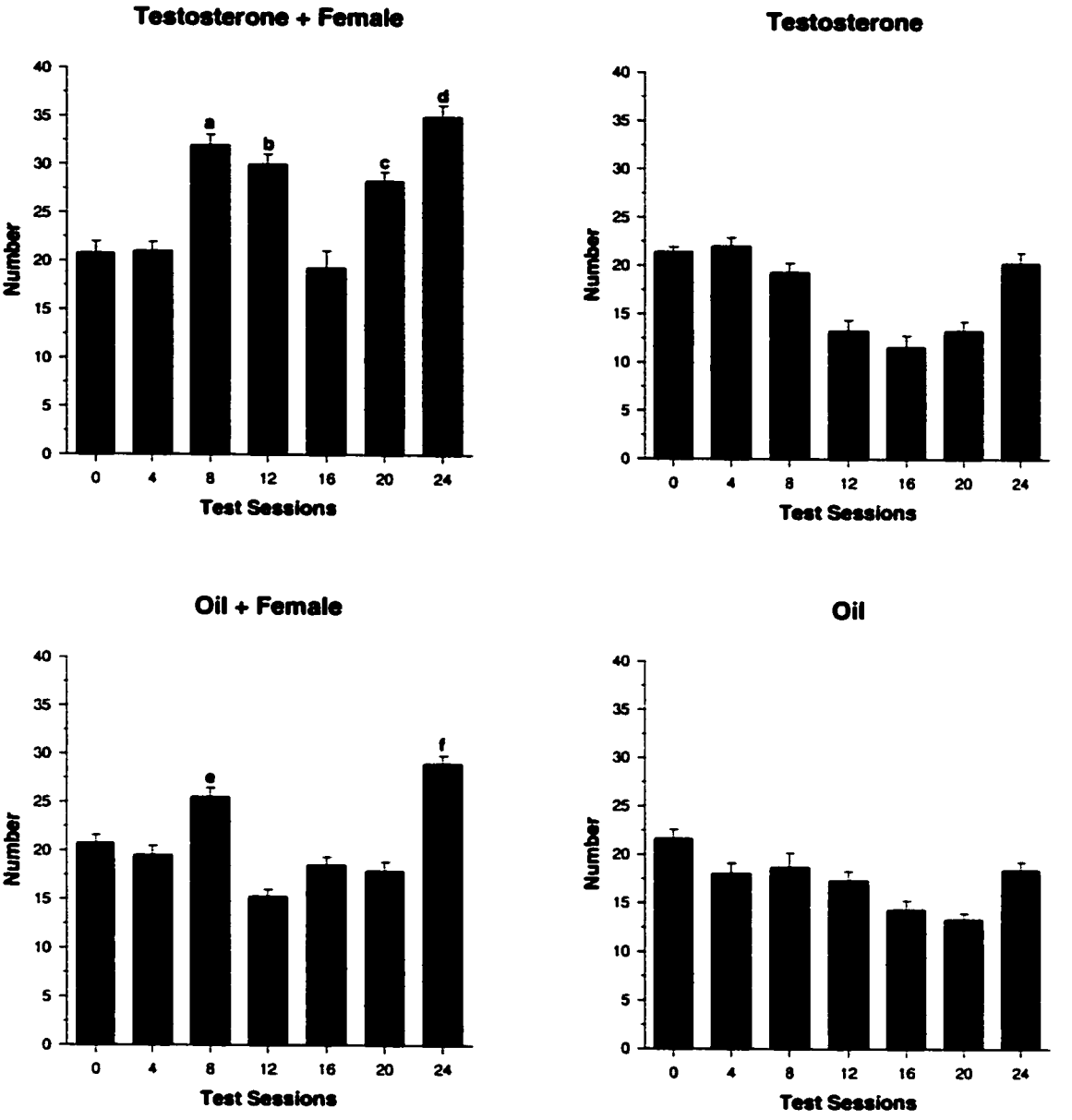


Figure 25. Mean number of sexually experienced long-term castrated males displaying nose pokes through a wire metal screen following 24 days (D0-D24) of TP or Oil treatment. Data are means +SEM. [a]p< .05 different from D8 Oil+Female, Testosterone, and Oil groups. [b]p<.05 different from D12 Oil+Female, Testosterone and Oil groups. [c]p<.05 different from D20 Oil+Female, Testosterone, and Oil groups. [d]p<.05 different from D24 Oil+Female, Testosterone, and Oil groups. [e]p< .05 different from D8 Testosterone and Oil groups. [f]p<.05 different from D24 Testosterone and Oil groups. Vertical lines show standard errors.

Climbs "1"

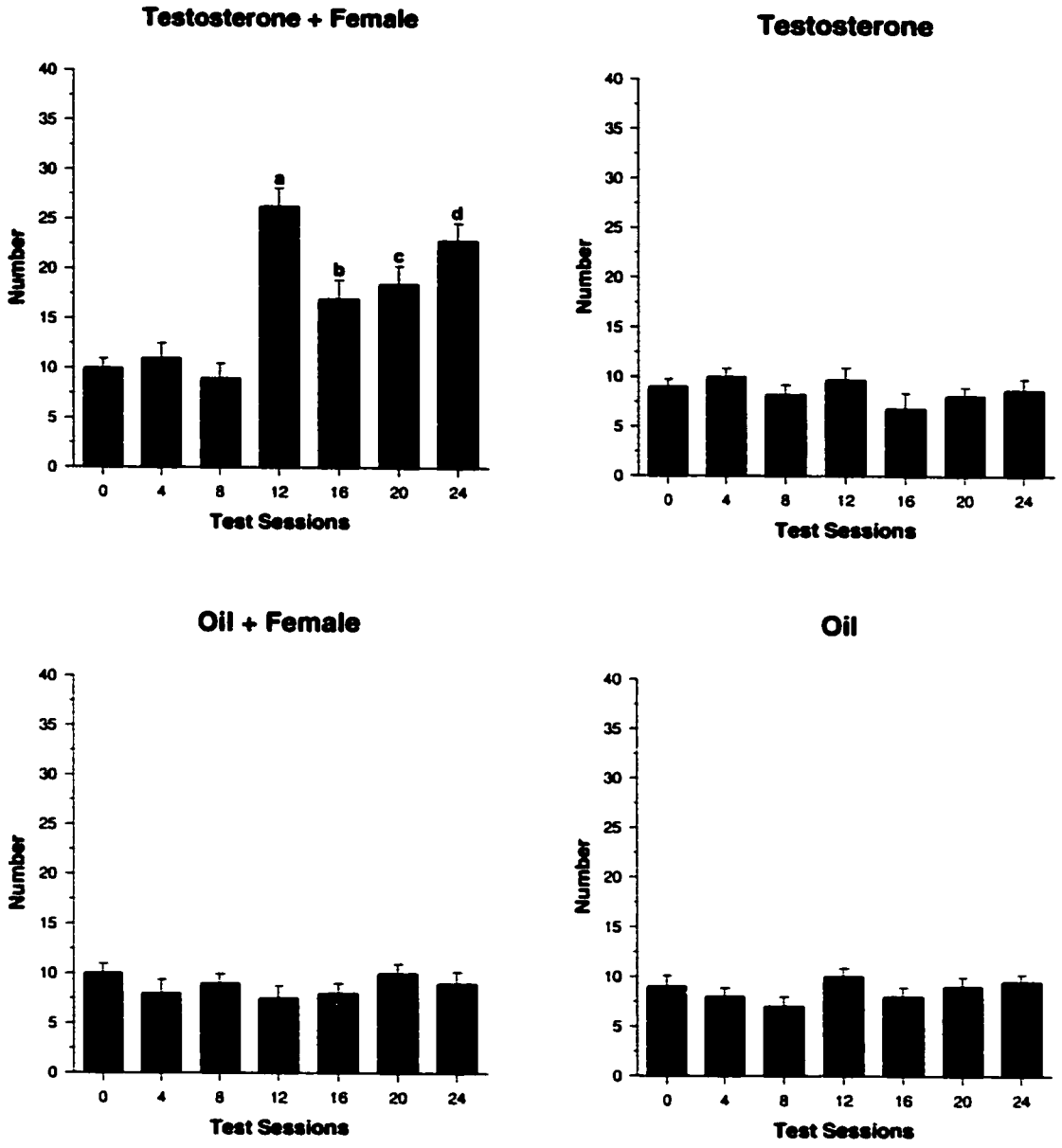


Figure 26. Mean number of sexually experienced long-term castrated males climbing the wire mesh (1: when males's two front paws were in the wire mesh at the bottom of the screen), following 24 days (D0-D24) of TP or Oil treatment. Data are means +SEM. [a]p< .05 different from D12 Oil+Female, Testosterone, and Oil groups. [b]p<.05 different from D16 Oil+Female, Testosterone and Oil groups. [c]p<.05 different from D20 Oil+Female, Testosterone, and Oil groups. [d]p<.05 different from D24 Oil+Female, Testosterone, and Oil groups. Vertical lines show standard errors.

Climbs "2"

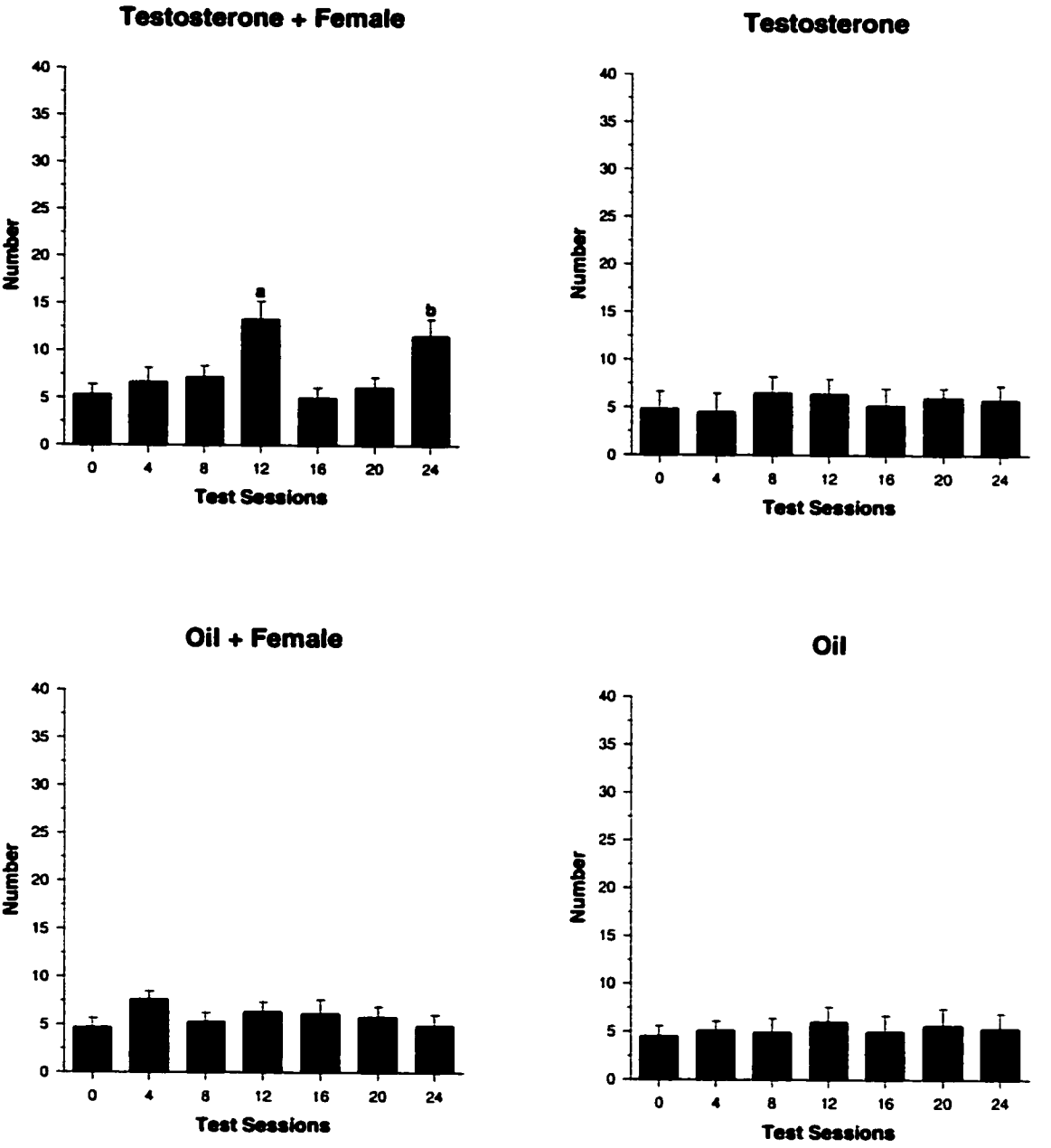


Figure 27. Mean number of sexually experienced long-term castrated males climbing the wire mesh (2: when males had their four paws in the middle section of the wire metal screen), following 24 days (D0-D24) of TP or Oil treatment. D0: Baseline measure prior to TP treatment. Data are means +SEM. [a]$p < .05$ different from D12 Oil+Female, Testosterone, and Oil groups. [b]$p < .05$ different from D24 Oil+Female, Testosterone and Oil groups. Vertical lines show standard errors.

Climbs "3"

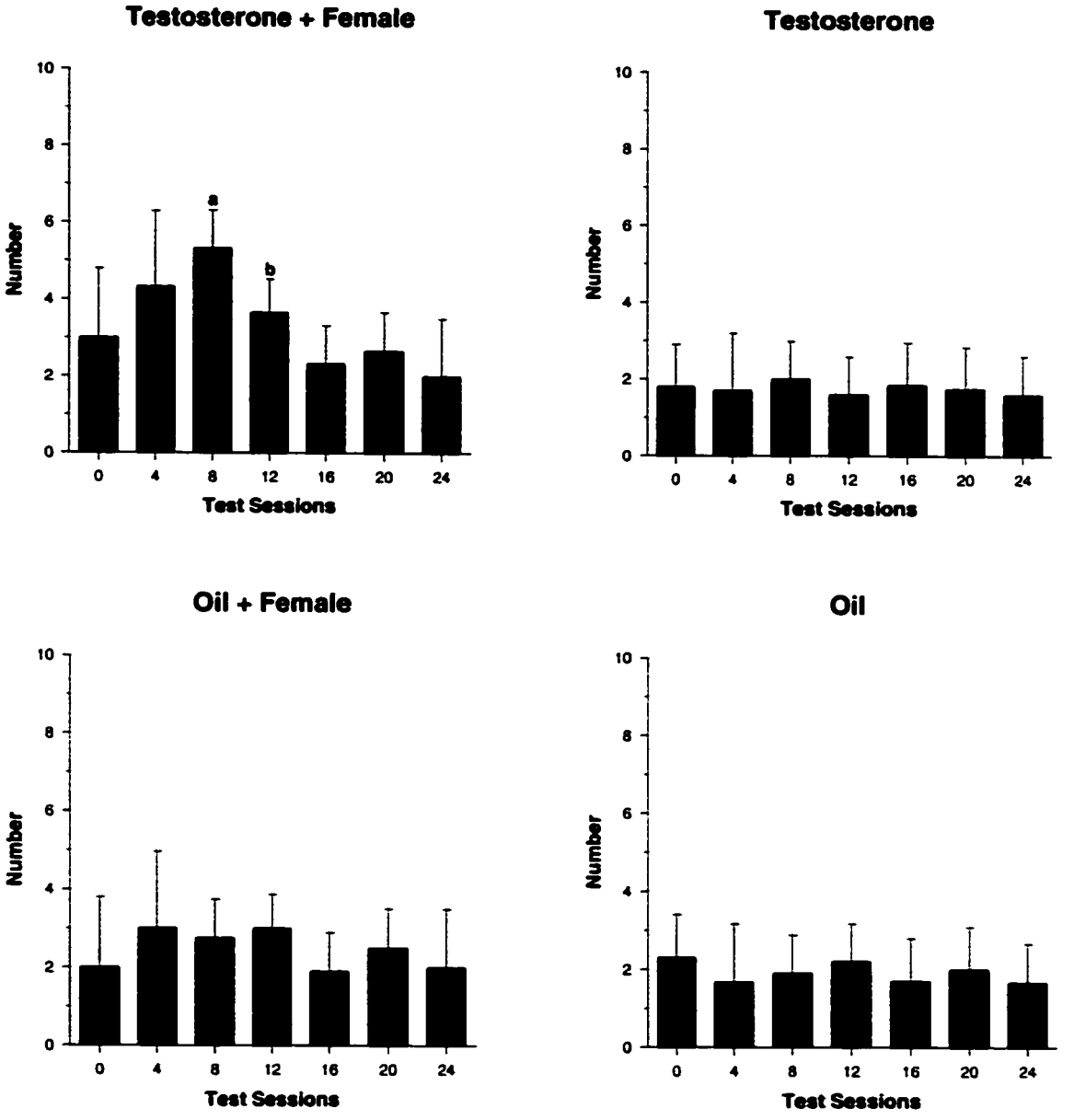


Figure 28. Mean number of sexually experienced long-term castrated males climbing the wire mesh (3: when males climbed to the top of the metal screen and touched the top lid closing the chamber), following 24 days (D0-D24) of TP or Oil treatment. D0: Baseline measure prior to TP treatment. Data are means +SEM. [a]$p < .05$ different from D12 Oil+Female, Testosterone, and Oil groups. [b]$p < .05$ different from D24 Oil+Female, Testosterone and Oil groups. Vertical lines show standard errors.

Genital Grooming

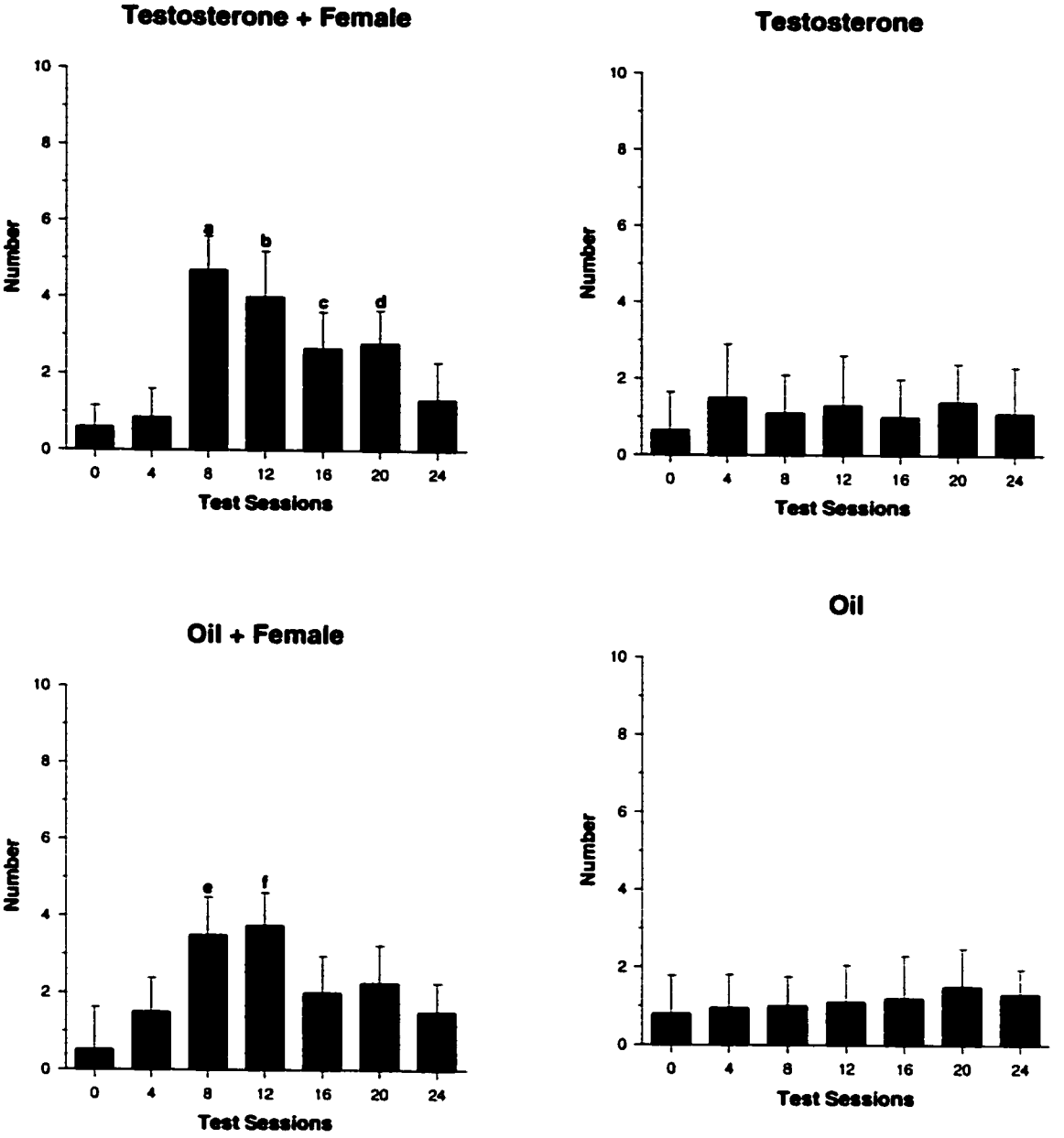


Figure 29. Mean number of sexually experienced long-term castrated males displaying genital grooming following 24 days (D0-D24) of TP or Oil treatment. D0: Baseline measure prior to TP treatment Data are means +SEM. [a]p< .05 different from D8 Oil+Female, Testosterone, and Oil groups. [b]p<.05 different from D12 Testosterone and Oil groups. [c]p<.05 different from D16 Testosterone and Oil groups. [d]p<.05 different from D20 Testosterone and Oil groups. [e]p< .05 different from D8 Testosterone and Oil groups. [f]p<.05 different from D12 Testosterone and Oil groups. Vertical lines show standard errors.

Climbing Behavior. An increase in the mean number of climbs of all types (1, 2, and 3) was observed in males in the T+F group relative to males in the O+F, T-NO F and O-NO F. This significant increase was observed for climb "1" on D12 [F(3,12)= 24.5, p<.0001], D16 [F(3,12)=20.07, p<.0001], D20 [F(3,12)=16.25], and D24 [F(3,12)=22.5, p<.0001]. For climb "2", a significant increase was observed in males in the T+F versus males in O+F, T-NO F and O-NO F on D12 [F(3,12)=11.47] and D24 [F(3,12)=8.1, p<.001]. Males in the T+F group displayed siginificantly more climb "3" than males in either the O+F, T-NO F or O-NO F groups. This significant increase was observed on D8 [(F(3,12)=15.12] and D12 [F(3,12)=6.20, p<.0001 (Figures 26-28).

Grooming Behavior. An overall significant increase in the mean number of genital groomings was observed in males in the T+F and males in the O+F groups compared to the males in the T-NO F and O-NO F. Males in the T+F group showed this significant increase on D8 [F(3,12)=4.65, p<.0001], D12 [F(3,12)=4.075, p<.0001], D16 [F(3,12)=2.80, p<.0001], and D20 [F(3,12)=2.65, p<.0001]. Males in the O+F group showed an increase in the mean number of groomings on D8 [F(3,12)=3.65, p<.0001] and D12 [F(3,12)=3.625, p<.0001]. No significant differences were found between males in the T-NO F and O-NO F groups (Figure 29).

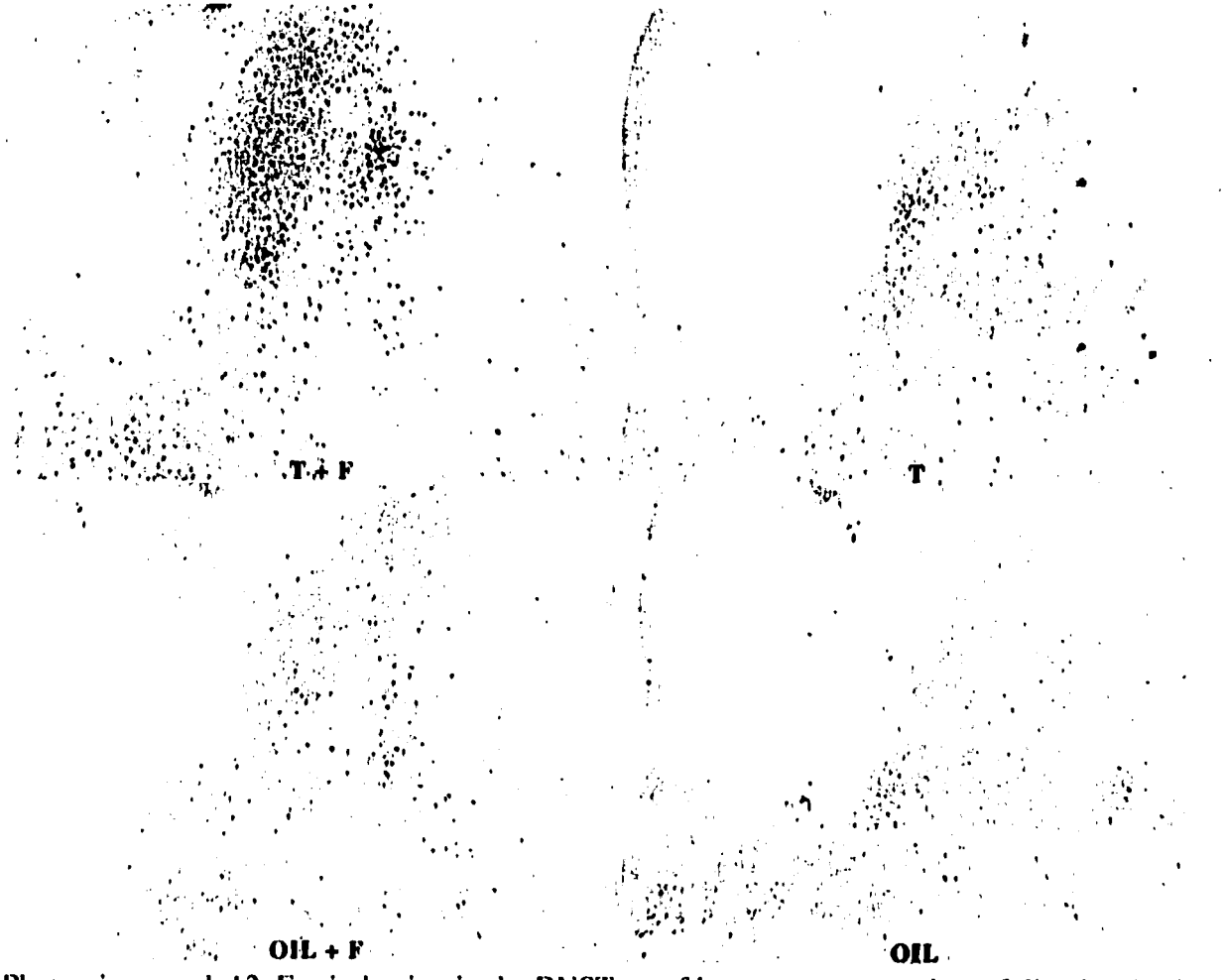
Fos Induction

A significant increase in the mean number of Fos cells was observed in the BNSTpm and MEApd of castrated males in the T+F group, relative to the mean number of Fos cells observed in males from the T-NO F, O+F and O-NO F groups [for BNSTpm: F (6,12)=79.15, p<.0001; for MEApd: F(6,12)=15.36, p<.0001] (Figures 30 and 31) (Photomicrographs 13 and 14). The increase in Fos cells observed within the T+F group

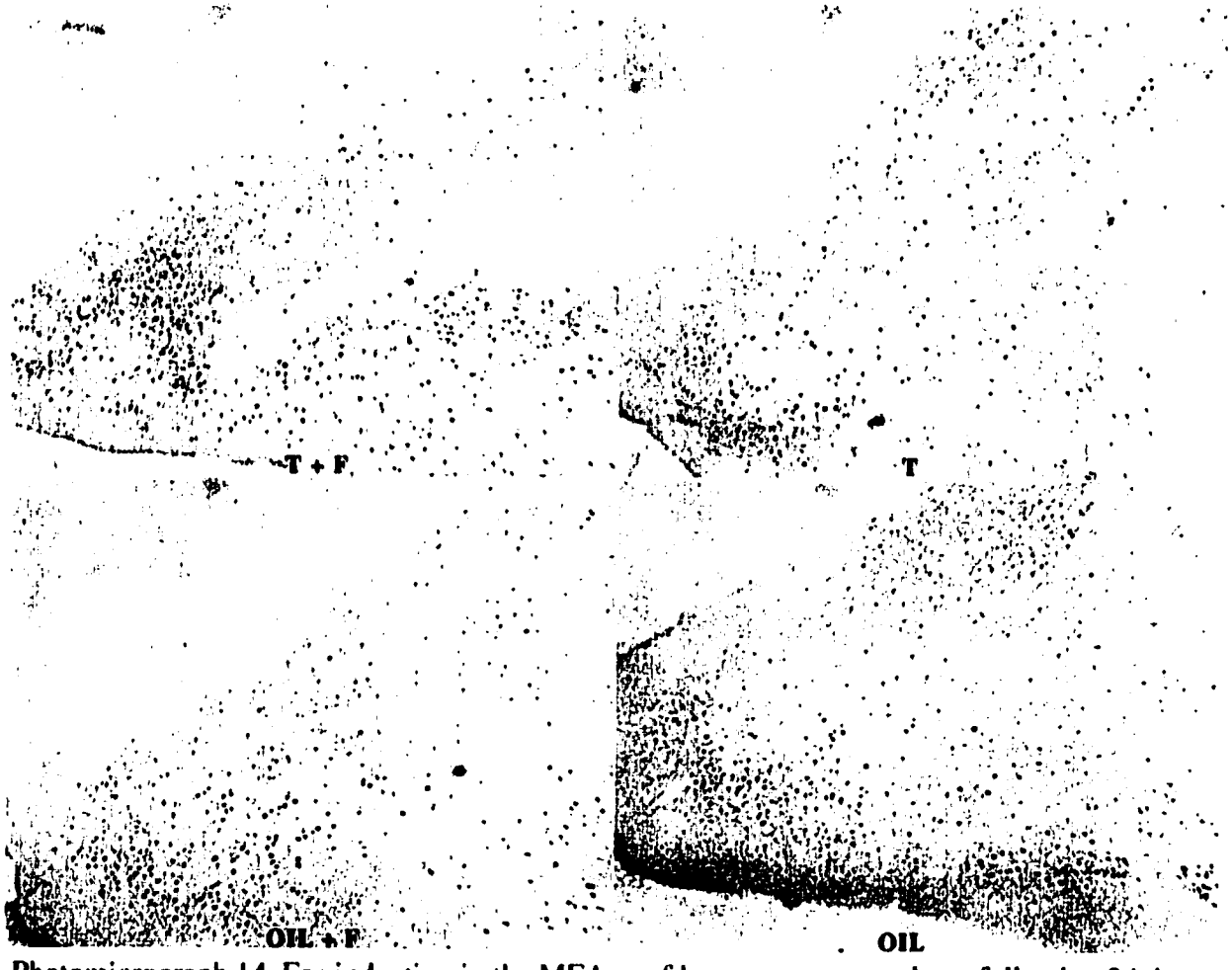
was only seen after 24 days of TP treatment. In addition, the induction of Fos cells observed in the MEApd was located in the ventromedial segment of the MEA close to the optic tract, rather than the more dorsal region associated with ejaculations. Furthermore, a significant decrease in the mean number of Fos cells in the MEApd was found in the T+F group following 16, 20, and 24 days of T administration, relative to the baseline measure. No differences were found between the mean number of Fos cells observed within those brain areas in the O+F and O-NO F groups (data not shown). Furthermore, the induction of Fos-IR seen in the T+F group was not observed in the BAOT, MPOA, MEApv or anterior VTA (data not shown).

Discussion

The present experiment demonstrates the excitatory effects of T on the expression of appetitive behaviors in sexually experienced long-term castrated male rats exposed to an inaccessible receptive female. Higher levels of nose-pokes, climbs, genital groomings, and contacts were observed in TP compared to oil-treated castrated males. This increase was significantly greater than the mean number of those same behaviors found in castrated males treated with TP or Oil and placed alone in the chambers. In addition to the behavioral results, this particular group of males (i.e., TP-treated and exposed to an inaccesible female behind the screen) showed an increase in Fos-IR in the BNSTpm and in the middle region of the MEApd. The medial posterocortical amygdala and BNSTpm are known to receive chemosensory projections from the accessory olfactory bulbs (AOB) (Fernandez-Fewell & Meredith, 1994; Kevetter & Winans, 1981), and their activation is believed to reflect the acquisition of olfactory signals during direct non-copulating contacts with the receptive female (i.e., anogenital investigations) (Coolen,



Photomicrograph 13. Fos induction in the BNSTpm of long term castrated rats following 24 days of testosterone (T) or oil (O) treatment. Males were tested with receptive females or left alone in the testing chamber.



Photomicrograph 14. Fos induction in the MEApv of long term castrated rats following 24 days of testosterone (T) or oil (O) treatment. Males were tested with receptive females or left alone in the testing chamber.

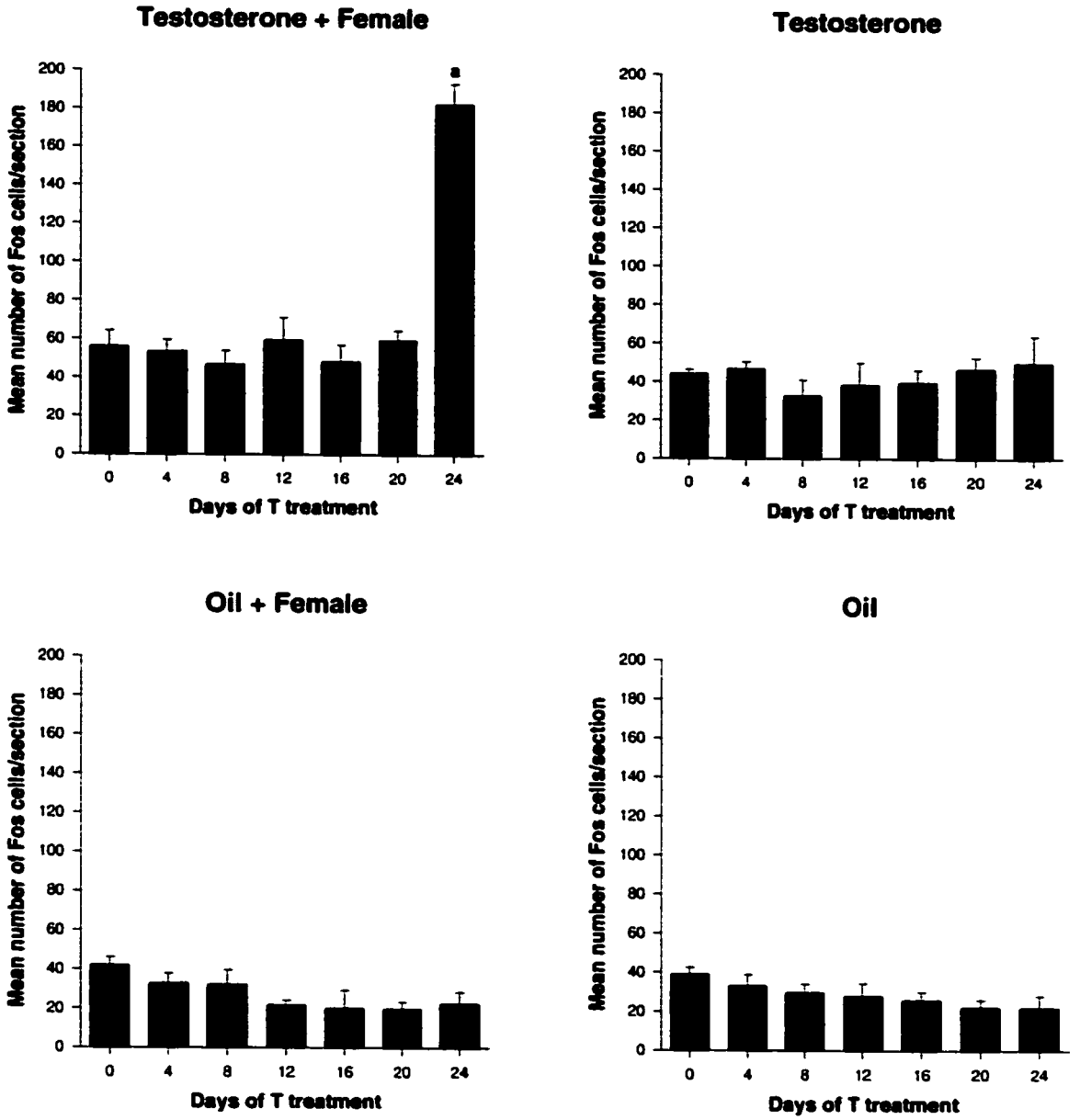


Figure 30. Mean number of Fos cells in the BNSTpm following 24 days (D0-D24) of TP or Oil treatment (N=4/Day of Treatment). D0: Baseline measure prior to TP or Oil treatment. Data are means +SEM. [a]p< .05 different from D0-D20. Vertical lines show standard errors.

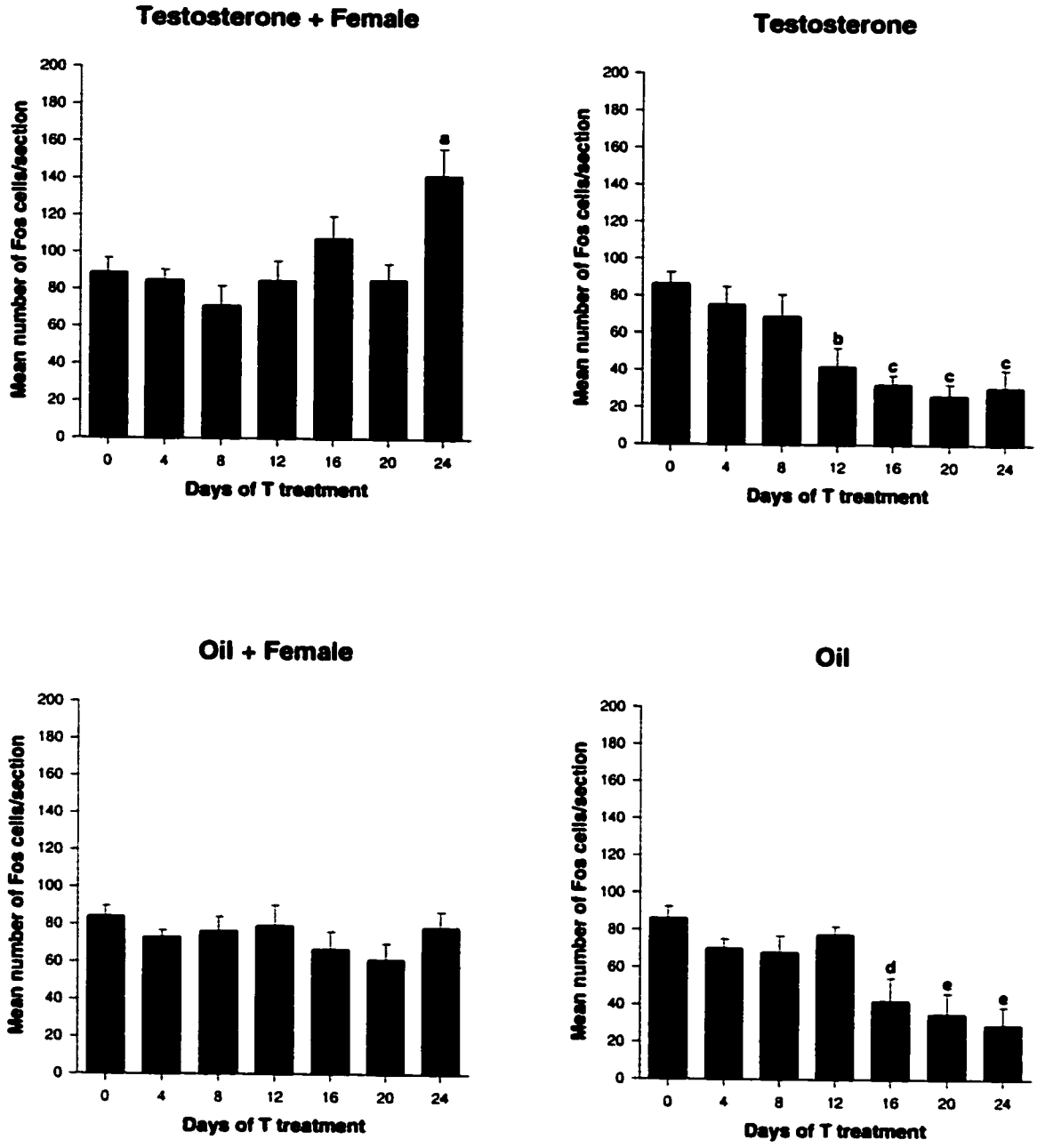


Figure 31. Mean number of Fos cells in the MEApd following 24 days (D0-D24) of TP or Oil treatment (N=4/Day of Treatment). D0: Baseline measure prior to TP or Oil treatment. Data are means +SEM. [a]p< .05 different from D0-D20. [b]p<.05 different from D0. [c]p<.05 different from D0-D8. [d]p<.05 different from D0 and D12. [e]p< .05 different from D0-D12. Vertical lines show standard errors.

Peters, & Veening, 1997). Findings from this experiment demonstrated that visual cues and odors from the receptive females, eminating through the wire mesh, were sufficient to elicit the neural activation observed in the BNSTpm and MEApd.

Summary of Results

Findings from the experiments within this chapter demonstrated that ejaculation is responsible for the neural activation observed in the BNSTpm, mPOA, MEApd, PdPN, and VTA, with the neural activation observed in the mPOA also seen following intromissions. Furthermore, results from this study also demonstrate that olfactory and visual stimuli are sufficient to elicit neural activation in the BNSTpm and MEApd.

An exceptional finding from the experiments within this chapter is the neural activation observed in the VTA following multiple ejaculations. The VTA is the source of dopaminergic projections to the NAcc as well as to the amygdala, lateral septum, and medial prefrontal cortex (Ungerstedt 1971; Moore and Bloom, 1978; Lindvall & Bjorklund, 1978; Thierry, Blanc, Sobel, Stinus, & Glowinsky, 1973). Furthermore, this particular brain area has been shown to contribute to locomotor and motivational aspects of behavior in general (Fibiger & Phillips, 1988; Wise & Bozarth, 1984). The role of VTA and its involvement during male rat copulation was further investigated in the Chapter 3.

CHAPTER III

THE ROLE OF THE VTA IN THE EXPRESSION OF APPETITIVE AND CONSUMMATORY BEHAVIORS IN THE MALE RAT

The VTA regulates the expression of goal-directed behaviors (Livingston & Hornykiewicz, 1978; Mogenson, 1977; Mogenson & Calaresu, 1975; Stevenson, 1969; Yim & Mogenson, 1980) such as attack responses (Bandler, Chi, & Flinn, 1972; Proshansky, Blander, & Flynn, 1974), ingestive behaviors (Huang & Mogenson, 1972), and male copulation (Eibergen & Caggiula, 1973). Electrical stimulation of the VTA facilitates sexual behaviors in males by accelerating the rate of copulation and therefore increasing the number of ejaculations in a 1-hour test (Eibergen & Caggiula, 1973).

The VTA is the source of mesocorticolimbic dopaminergic (A10) neurons projecting to the NAcc, basolateral, and central subnuclei of the amygdala, lateral septum, olfactory tubercle, and medial prefrontal cortex (Lindvall & Bjorklund, 1978; Moore & Bloom, 1978; Thierry, Blanc, Sobel, Stinus, & Glowinsky, 1973; Ungerstedt, 1971), and has been shown to regulate the motivational aspects of behavior through its extensive projections to these forebrain targets (Fibiger & Phillips, 1988; Mogenson & Yang, 1991; Swanson, 1982; Wise & Bozarth, 1984). Male copulation has been shown to increase the firing rate of DA neurons in the VTA (Hull, Bitran, Pehek et al., 1989). DA levels in the NAcc have been shown to increase during copulation and then decrease following each ejaculation (Blackburn, Pfaus, & Phillips, 1992; Pfaus, Damsma, Nomikos et al., 1990). In addition, blocking DA receptors in the NAcc by bilateral infusions of haloperidol decreases conditioned level changing (Pfaus & Phillips, 1991). Furthermore, lesions of the NAcc decrease the proportion of males displaying non-

contact erections, intromissions, and ejaculations (Kippin, Sotiropoulos, Badih, & Pfaus, 2000), suggesting the DA projections from the VTA to the NAcc are involved in the control of both appetitive and consummatory behaviors in the male rat.

Chapter 3 examined further the neural activation observed in the VTA following multiple ejaculations (shown in Chapter 2). Although the effects of DA within the VTA on male copulation have been established (Hull, Bazzet, Warner, Eaton, & Thompson, 1990), the role of GABA neurons within the VTA in male copulation remains unclear at present. Experiment 1 examined the colocalization of Fos in TH or GABA neurons after mounts, intromissions, and ejaculations in intact male rats. Experiment 2 investigated further the role of the VTA in male copulation by examining the effects of bilateral VTA lesions on the expression of appetitive and consummatory behaviors in intact males.

The existence of ARs (Simmerly, Chang, Muramatsu, & Swanson, 1990), the recent discovery of ER-β (Shughrue, Komm, & Merchenthaler, 1996; Shughrue, Lane, & Merchenthaler, 1997; Shughrue & Merchenthaler, 2001) within the VTA, and the effects of dual implants of T in the MPO/VTA in restoring certain reproductive behaviors such as mounting and the preference for receptive females' urine in castrated male mice (Sipos & Nyby, 1996), suggest a hormonal mechanism of action in the VTA underlying male sexual behavior. Experiment 3 investigated the effects of steroid hormones on male copulation when implanted directly in the VTA of castrated males [i.e., TP, estrogen benzoate (EB), progesterone (P) and cholesterol (CH)]. The overall significance of the findings from these studies is reviewed in the General Discussion.

Experiment 1A. Colocalization of Immunoreactivity for Fos and Tyroxine Hydroxylase in the VTA of Male Rats Following Copulation

The present experiment was designed to examine further the findings reported in the preceding chapter showing neural activation in the VTA following ejaculations. The number of dopaminergic cells activated in the VTA following specific copulatory behaviors (i.e., intromissions and ejaculations) was examined using double label immunocytochemistry to colocalize Fos and TH in the VTA of intact sexually experienced male rats allowed to experience different amounts of copulatory behaviors. This study determined the mean number of Fos cells, dopaminergic in nature, that were activated following particular aspects of male copulation (i.e., intromissions and ejaculations) during the first or second ejaculatory series.

Materials and Methods

Animals

Thirty-five intact adult male Long Evans rats weighing between 250-280g upon arrival were used as subjects. Twelve female Long-Evans rats weighing 250g were used as stimulus females. Females had been ovariectomized and subsequent sexual receptivity was induced following procedures identical to those described in Experiment 1 of Chapter 1.

Procedure

Males were pre-exposed to the bilevel chambers and given sexual experience with receptive females using the same protocol described in Experiment 1 of Chapter 1. Following the training phase, males (N=5) were equated according to their ejaculation latency (i.e., most variable measure, see Kippin & Pfaus 2000), and assigned randomly to

one of seven different conditions according to the amount of copulatory activity they were allowed to receive with receptive females during the copulatory test: a) males restricted to 10 mounts, b) males allowed to have 7 intromissions during the first ejaculatory series, c) males allowed to have a single ejaculation, d) males allowed to copulate to the first intromission of the second ejaculatory series, e) males allowed to have 6 intromissions during the second ejaculatory series, and f) males allowed to ejaculate twice during the copulatory test. In all six conditions, the female was removed from the chamber once the male achieved behavioral criteria, and the male remained in the chamber for the duration of the 30 minute test. In the mounting group, males were restricted to mount by taping the females' vaginas. An additional group of males was left in the chamber alone for a period of 30 minutes to serve as controls. All males were sacrificed 75 minutes after behavioral testing and their brains prepared for Fos and TH immunocytochemistry using a protocol for Fos IR similar to that described in Experiment 1 of Chapter 2.

Immunocytochemistry

The immunocytochemical protocol used for the visualization of Fos was identical to that described in Experiment 1 of Chapter 2, with the exception of not using 8% nickel chloride to avoid coloring the DAB chromagen product blue-black, thus making the Fos protein orange-brown in color. Following Fos immunocytochemistry, floating sections were transferred directly into a solution containing a mouse polyclonal TH antibody (Calbiochem; diluted 1:30 000) in 0.2% Triton TBS with 3% normal horse serum (NHS) at 4°C for 72 h. Sections were rinsed in TBS (3x5 min washes) and transferred into biotinylated anti-mouse IgG made in horse (Vector Laboratories; 1:200) in 0.2% Triton TBS, with 3% NHS for 1 h at 4°C. Sections were rinsed in TBS (3x5 min washes) and were transferred into an avidin-

biotinylate-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; diluted 1:55.55) for 2h at 4°C. Following incubation with the ABC reagents, sections were rinsed with TBS. Cells positive for TH were defined by an intense black-dark reaction product in the cytoplasm. Visualization of the Fos-TH cells was performed using a SG substrate kit for Peroxidase (Vector Stain #4700). Following the TBS washes, sections were directly transferred into a solution containing phosphate buffer saline (PBS) ph 7.5, chromagen, and H_2O_2 for a period of 10 min. All section were then rinsed in double distilled water, mounted onto gel-coated slides, dehydrated with ethanols, cleared in Hemo-D, and cover-slipped prior to being examined under a microscope.

Histological and Statistical Analyses

Fos and TH-IR cells were counted manually in sections corresponding to the VTA (corresponding to plates 38-42 in Paxinos and Watson [1986]), using a Leitz Laborlux microscope (40 X). Cells positive for both TH and Fos were defined by an intense black-dark blue reaction product in the cytoplasm and an orange-brown stained nucleus (Photomicrograph 15). The distribution of immunoreactive cells was mapped in relation to cytoarchitectonic landmarks of the rodent midbrain. Average numbers of Fos-TH IR cells were calculated from 5 sections/region/rat which appeared subjectively to contain the largest number of Fos-TH IR cells. A one-way ANOVA was used for each region to examine the main effects of specific sexual behaviors and their interaction with the mean number of Fos-TH IR cells. For each significant ANOVA, post-hoc comparisons of the group mean were made using Tukey's HSD, $p<0.05$.

Photomicrograph 16. GABA-Fos cells in the VTA of castrated male rats following multiple ejaculations. Arrows indicate colocalized cells.

Results

The mean number of Fos-TH cells found in the anterior and posterior VTA in males from the control group (i.e., males left alone in the chambers) was not significantly different from the mean number of double-labelled cells observed in the group of males allowed to mount ($p=0.658$). The anterior VTA showed an overall significant increase in the mean number of co-labelled cells following mounts, intromissions, ejaculations with or without PEI, and 2 ejaculations ($F(5,18)=4.21$, $p<0.01$). The mean number of Fos-TH immunoreactive cells was significantly higher in males that intromitted 7 times during the second ejaculatory series compared to males that had one ejaculation without PEI, or that copulated to the first intromission of the second ejaculatory series [F (5, 18)= 4.21, $p<0.001$] (Figure 32). In addition, the mean number of Fos-TH immunoreactive cells observed following two ejaculations was significantly higher than the mean number found after mounts, intromissions, and single ejaculation with or without PEI [$F(5,18)$ = 4.21, $p<0.01$] (Figure 32). No significant differences were found in the mean number of double Fos-TH IR cells between males that only mounted, only intromitted, or ejaculated once with or without a PEI. Furthermore, no significant differences were found between the group of males that received intromissions during the second ejaculatory series and the group of males that copulated to two ejaculations (Figure 32). The mean number of Fos-TH cells observed following 1 ejaculation or 2 ejaculations corresponded to 30.8% and 45.2% of the counted TH cells in the respective behavioral groups. In the posterior VTA (corresponding to plates 39-40 from Paxinos and Watson, 1986), no significant differences were found following copulation between males from any of the examined conditions (Figure 32).

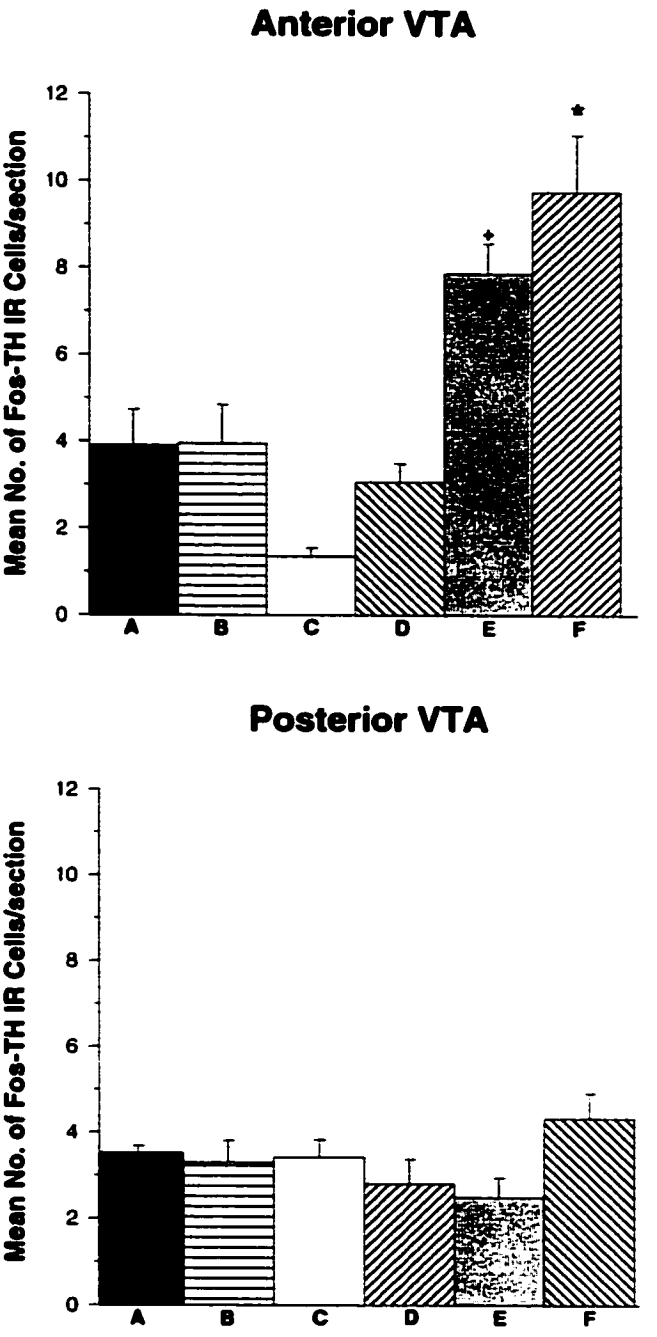


Figure 32. Mean number of double-labeled Fos-TH cells in the anterior VTA of sexually experienced male rats following different aspects of copulatory behaviors (A: males were allowed to mount; B: allowed to have 7 intromissions; C: allowed to copulate to one ejaculation; D: allowed to copulate to the first intromission of the second ejaculatory series; E: allowed to have 7 intromissions of the secondary ejaculatory series; F: allowed to copulate to two ejaculations). Vertical lines represent standard errors. * $p<.05$ different from A,B,C,D and E; +$p<.05$ different from C and D.

Discussion

The results of Experiment 1A show that the number of labeled cells that were immunoreactive for both Fos and TH was significantly greater in the anterior VTA of males that had either 7 intromissions during the second ejaculatory series or copulated to two ejaculations compared to males that were allowed to mount or intromit during the first ejaculatory series and/or have a single ejaculation with or without PEI. Although the temporal resolution of when electrophysiological activation in TH-containing cells within the VTA occurs cannot be determined from the present experiment, findings from this study confirm the activation of dopamine containing cells following intromissions during the secondary ejaculatory series, and following two ejaculations.

Experiment 1B. Colocalization of Fos Within GABA Neurons in the VTA of Male Rats Following Copulation

VTA GABAergic neurons are anatomically positioned to influence the activity of the mesolimbic dopamine system in the VTA (Schwarzer, Berresheim, Pirker et al., 2001). Although many electrophysiological studies have examined the effects of GABA agonists or antagonists on DA neurons within the VTA (see General Introduction), the role of GABA in the control of male copulation remains unknown. The present experiment examined the mean number of GABA neurons within the VTA following different aspects of male copulation. Double immunocytochemistry was used to visualize Fos within GABA neurons in the VTA of intact sexually experienced males following mounts, intromissions, a single ejaculation, or 2 ejaculations. Results from this study identified the particular sexual behaviors facilitating Fos within GABA cells.

Materials and Methods

Animals

Twenty-eight intact sexually experienced adult male Long Evans rats weighing between 250-280g upon arrival were used as subjects. Twelve sexually experienced female Long-Evans rats weighing 240-250g were used as stimulus females. Females were ovariectomized and subsequent artificial estrous was induced using the same procedure described in Experiment 1 of Chapter 1.

Procedure

Males were habituated and pre-exposed to receptive females in the bilevel chambers following the same procedures described in Experiment 1 of Chapter 1. As in Experiment 1 of this chapter, following the training phase males were assigned randomly to one of six different sex-conditions, as described in the previous experiment, according to the copulatory activity they were allowed to have with receptive females. An additional group of males was left in the chamber alone for a period of 30- min to serve as controls. The testing procedures were identical to the ones used in Experiment 1. Following testing, 4 males from each of the sex-conditions were perfused and their brains prepared for Fos and GABA double immunocytochemistry.

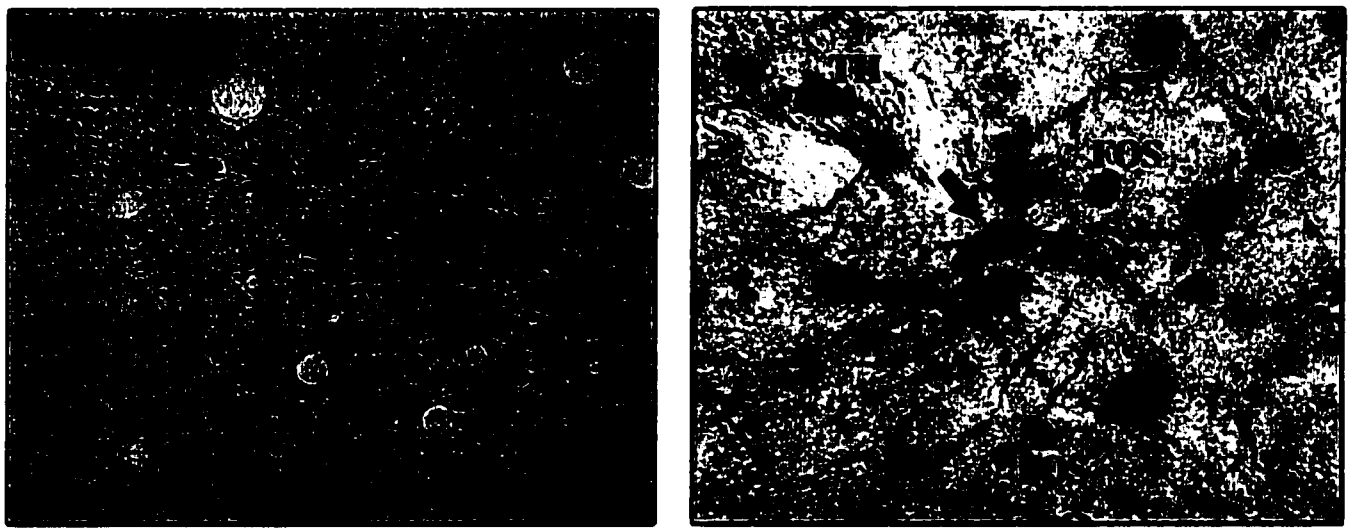
Immunocytochemistry

Males were sacrificed and perfused using protocols identical to the ones described in Experiment 1. All brains were processed for Fos first, followed by GABA immunocytochemistry. Frozen coronal brain sections (30μm) were cut from each brain on a sliding microtome and Fos immunocytochemistry was performed using the same protocol described in Experiment 1. Following Fos immunocytochemistry, floated sections were

transferred directly into a solution containing a rabbit polyclonal GABA antibody (Calbiochem; diluted 1:1,000) in 0.05% Triton TBS with 3% normal goat serum (NGS) at 4°C for 72 h. Sections were rinsed in TBS (3x5 min washes) and transferred into biotinylated anti-rabbit IgG made in goat (Vector Laboratories; 1:200) in 0.05% Triton TBS, with 3% NGS for 1 h at 4°C. Sections were rinsed in TBS (3x5 min washes) and were transferred into an avidin-biotinylate-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; diluted 1:55.55) for 2h at 4°C. Following incubation with the ABC reagents, sections were rinsed with TBS. Visualization of the GABA neurons was performed using a SG substrate kit for Peroxidase (Vector Stain#4700) and following the protocol described in Experiment 1. All section were then rinsed, mounted, dehydrated, cleared in Hemo-D, and cover-slipped as described in Experiment 1 of Chapter 2.

Histological and Statistical Analyses

As described in Experiment 1, Fos and GABA neurons were counted in sections corresponding to the VTA. Fos-GABA immunopositive cells were defined by an intense black-dark blue reaction product in the cytoplasm and an orange-brown stained nucleus (Photomicrograph 16). The distribution of immunoreactive cells was mapped in relation to cytoarchitectonic landmarks of the rodent midbrain. As in Experiment 1, average numbers of Fos-GABA IR cells were calculated from 5 sections/region/rat. A one way ANOVA was used for each region to examine the main effects of specific sexual behaviors and their interaction on the mean number of Fos-GABA IR cells. For each significant ANOVA, post-hoc comparisons of the group mean were calculated using Tukey's HSD, $p<0.05$.



Photomicrograph 15. TH-Fos cells in the VTA of castrated male rats following multiple ejaculations. Arrow indicates colocalized cell.

Results

The mean number of Fos-GABA cells found in the anterior and posterior VTA in males from the control group (i.e., males left alone in the chambers) was not significantly different from the mean number of double cells observed in males mounting (data not shown). In the anterior VTA (corresponding to plates 36-38 from Paxinos and Watson), the mean number of Fos-GABA immunoreactive cells as significantly different between experienced males following different copulatory behaviors [$F(5,18) = 6.65$, $p < 0.001$ (Figure 33). Post-hoc comparisons revealed a significant decrease in the mean number of co-labeled Fos-GABA cells in males that were allowed to achieve two ejaculations compared to males that had one ejaculation with or without a PEI or intromitted during the second ejaculatory series (Figure 33). The mean number of Fos-GABA observed following 2 ejaculations corresponded to 6% of the counted GABA cells. The mean number of co-labeled cells following one ejaculation and following 7 intromissions during the second ejaculatory series did not reach statistical significance (Figure 33).

Following different aspects of copulation, the posterior VTA (corresponding to plates 39-40 from Paxinos & Watson, 1986), showed an overall significant difference in the mean number of Fos-GABA immunoreactive cells [$F(5,18) = 5.23$, $p<0.01$]. Post-hoc comparisons revealed a significant increase in the mean number of co-labeled cells observed in males that were allowed to have two ejaculations and males allowed to mount, which corresponded to 32% of the counted GABA cells. No significant differences were observed between the mean number of co-labeled cells following mounts, intromissions, ejaculation with or without PEI, or intromissions during the second ejaculatory series.

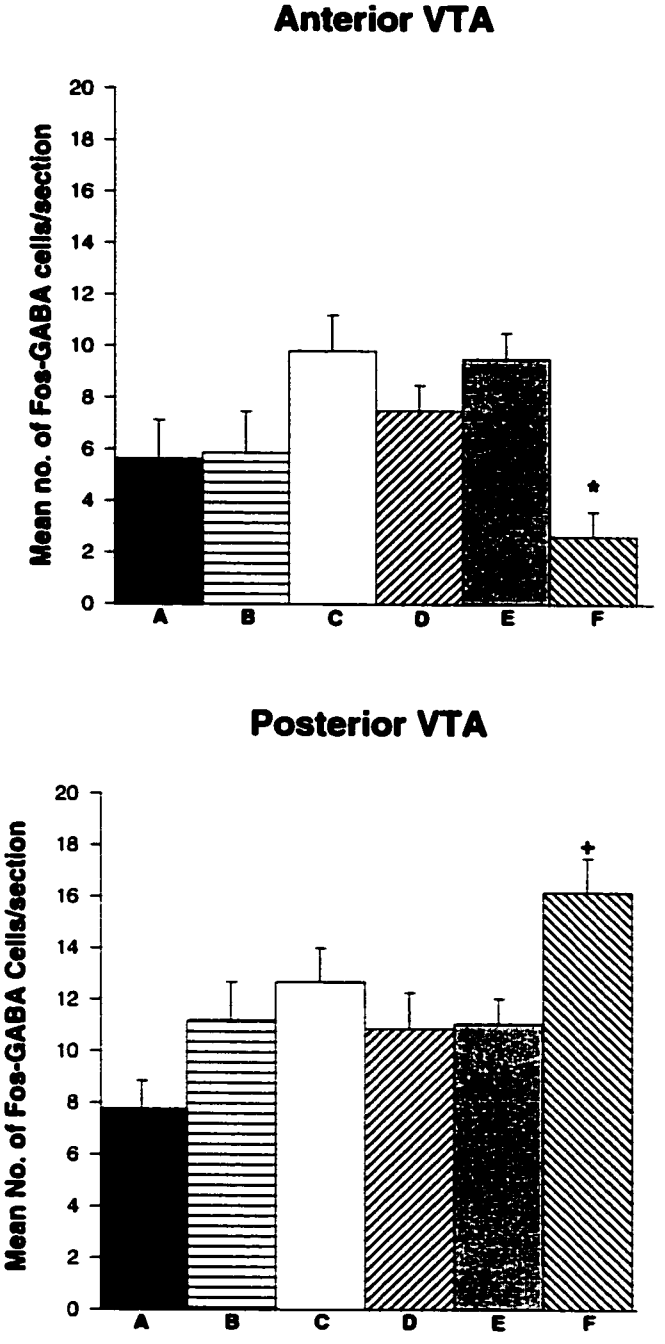


Figure 33. Mean number of double-labeled Fos-GABA cells in the posterior VTA of sexually experienced male rats following different aspects of copulatory behaviors. (A: males were allowed to mount; B: allowed to have 7 intromissions; C: allowed to copulate to one ejaculation; D: allowed to copulate to the first intromission of the second ejaculatory series; E: allowed to have 7 intromissions of the secondary ejaculatory series; F: allowed to copulate to two ejaculations). Vertical lines represent standard errors. *$p<.05$ different from C, D, and E; +$p<.05$ different from A, B, C, D, and E.

Discussion

Results from Experiment 1B showed a significant decrease in the mean number of Fos-GABA cells in the anterior VTA of intact, sexually experienced males following two ejaculations. In contrast, an increase in the mean number of co-labelled Fos-GABA cells was observed in the posterior VTA following 2 ejaculations. This increase was significantly different from the mean number of co-labelled cells observed following mounts. Although the temporal resolution of when the electrophysiological activation in GABA neurons occurs within the VTA cannot be determined from the present study, the different pattern of activation for Fos within GABA cells observed in the anterior and posterior VTA following 2 ejaculations suggests the existence of two sub-populations of GABA neurons within the VTA that are activated during male copulation.

Experiment 2. Effects of Lesions of the Ventral Tegmental Area on Appetitive and Consummatory Aspects of Male Rat Sexual Behaviors: Modulation by Sexual Experience

The purpose of Experiment 2 was to determine the effects of bilateral N-methyl-D aspartate (NMDA) lesions of the VTA on the expression of appetitive and consummatory male sexual behaviors. An additional factor investigated in this experiment was the effect of sexual experience prior to bilateral VTA lesions. Comparisons were made between sexually experienced males that had ten 30-min tests with receptive females (allowing multiple ejaculations within each test), and males that had only 1 ejaculation with receptive females, prior to the lesions.

Materials and Methods

Animals

Thirty-six adult male Long Evans rats (Charles River, Canada) weighing between 250-280g upon arrival were used as subjects. Twenty sexually experienced female Long Evans hooded rats (Charles River, Canada) weighing 240-250g were used as stimulus females. All animals were housed under conditional identical to the ones described in Experiment 1 of Chapter 1. Females were ovariectomized and subsequent artificial estrous was induced using the same protocols described in Experiment 1 of Chapter 1.

Procedure

All behavioral tests were conducted using the same bilevel chambers described in Experiment 1 of Chapter 1. Males were divided randomly into two groups: 10-experiences (n=20) or 1-experience (n=16). Within each group half of the males underwent bilateral VTA lesions and the other half were given sham treatment, and used as controls. Males in the 10-experience group (n=20) were habituated to the bilevel chambers (i.e, protocol described in Experiment 1 of Chapter 1) and exposed to receptive females 10 times prior to bilateral NMDA lesions (n=10) or saline injections (n=10). Following the habituation procedure, males received bilateral NMDA or saline injections into the VTA. One week after the NMDA or saline infusion, males in the 10-experience group were tested two more times with receptive females at 4-day intervals.

Males assigned to the 1-experience group (n=16) were allowed to have a single ejaculation with receptive females prior to receiving bilateral NMDA or saline infusions into the VTA. Following this, all males received bilateral NMDA or saline infusions. One week after the bilateral infusions, males were tested with receptive females two more

times at 4-day intervals. During behavioral testing appetitive, precopulatory, and consummatory sexual behaviors were scored for males in either: 10-experience or 1-experience groups.

VTA Lesions

Bilateral lesions were made in the VTA using stereotaxic procedures with bregma and lambda in a horizontal plane and using the following VTA coordinates: AP= -3.6mm, ML= ±3.6mm, DV= -8.3mm from skull. The lesions were made by intracerebral injections of 80 nmol of NMDA (or saline), infused through a guide cannula placed at 16 degrees to the vertical to avoid the periventricular gray (PVG) and penetration of the cerebral aqueduct. Animals were lesioned under sodium pentobarbital anesthesia (60mg/kg) supplemented with methoxyflurane inhalant (Metofane) in a Kopf stereotaxic apparatus. Males recovered for 7 days before behavioral testing resumed, and were tested twice with receptive females at 4-day intervals. The same procedure was used for control animals except that saline was injected instead of NMDA.

Histology

After behavioral testing, all males were sacrificed as before using sodium pentobarbitol (120mg/kg). Animals were perfused transcardially with cold physiological saline, followed by 10% formalin. Brains were stored in 10% formalin for 24 hours. Frozen 30 µm coronal sections were taken using a cryostat through the extent of brain damage produce by the NMDA injection (Photomicrograph 17). Collected sections were mounted, stained with cresyl violet, and cover-slipped using Permount. Lesion damage was assessed by microscopic analysis of all stained sections. Only animals that showed

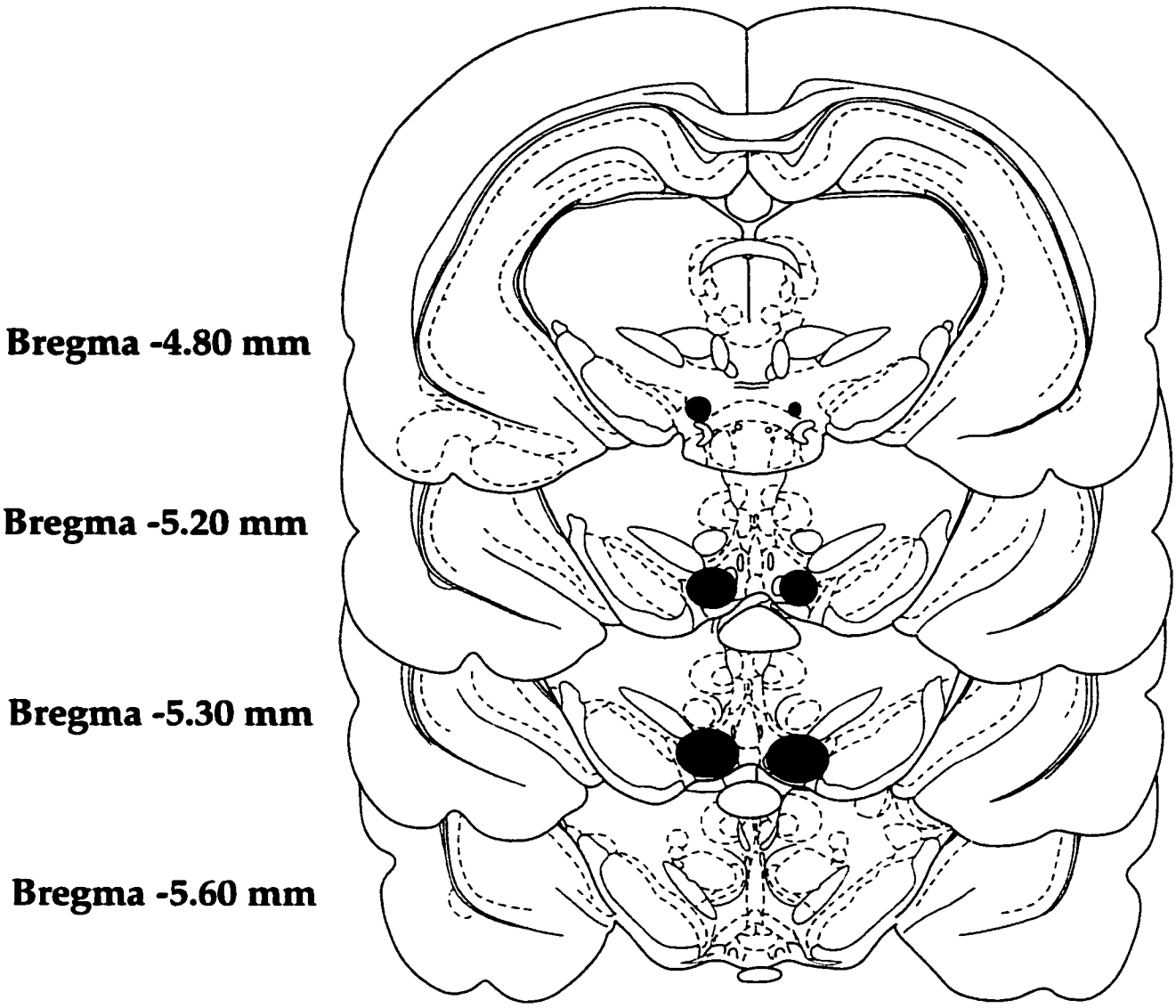
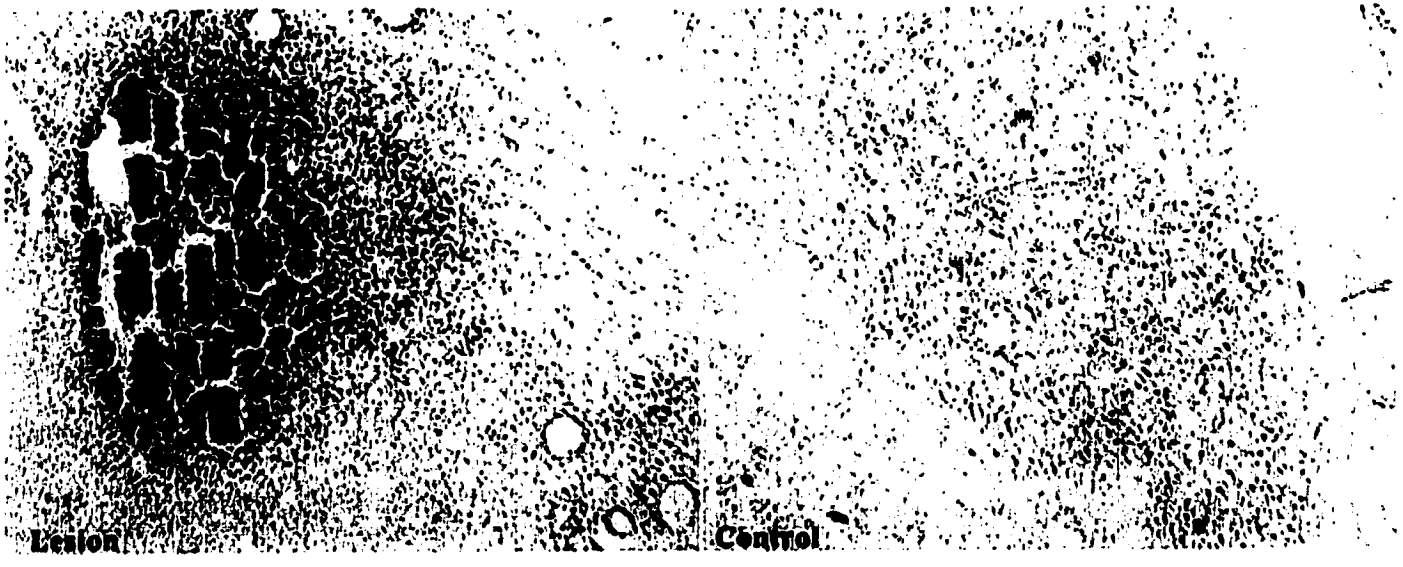


Figure 34. Schematic representation of the tissue damage observed following bilateral NMDA lesions of the VTA.



Photomicrograph 17. Effects of 80nm NMDA or saline injections on the VTA of intact male rats.

an amount of VTA damage ranging between 50 and 100% were included in the study (Photomicrograph 17).

Statistical analysis

A 2-way ANOVA was used to compare behavioral differences between lesioned and control animals in the 10-experience and 1-experience groups. Significant main effects and interactions were followed by post-hoc analysis of individual means using the Tukey method. The level of significance for all comparisons was $p<0.05$.

Results

Of the 10 males in the 10-experience group undergoing VTA lesions, two were eliminated due to the fact that the lesions extended into the dorsal mesencephalic tegmentum. In the remaining 16 animals, 8 in the 10-experience and 8 in the 1-experience conditions, the amount of VTA damage ranged between 50 and 100%. A schematic representation showing the extent of the VTA NMDA lesions is shown in Figure 34.

The effects of VTA lesions on appetitive and consummatory sexual behaviors in the 10-experience and 1-experience groups are shown in Figures 35 and 36. Comparisons between males in the 10-experience versus 1-experience groups revealed the following: A significantly higher mean number of level changes was found in males in the 10-experience than those in the 1-experience groups [$F(3,22)=16.61$, $p<0.001$] (Figure 35). The difference between the mean number of anogenital investigations, pursuits and attempted mounts displayed by males in the 10-experience and that displayed by males in the 1-experience condition was not found to be statistically significant.

Results from the comparison made between lesioned and control males were the following: The mean number of pursuits was significantly lower in lesioned males

compared to control males in the 10-experience [F (3,22)=4.65, p<0.01] and 1-experience [F(3,22)=3.98, p<0.01] groups. In addition, the mean number of anogenital investigations was significantly lower in lesioned males compared to controls in both the 10-experience [F(3,22)=26.43, p<0.0001] and 1-experience [F (3,22)=19.56, p<0.0001] groups (Figure 35). Finally, the mean number of level changes was not significantly different between lesioned and control males assigned to either the 10- or 1-experience groups. No significant differences were found in the mean number of attempted mounts between lesioned and control males in either condition (Figure 35).

The mean number of mounts, intromissions, and ejaculations between groups was significantly lower in lesioned compared to control males in the 1-experience group [F(3,22)=14.7, p<0.001]. No significant differences were found between lesioned and control males in the 10-experience group. However, an interaction effect was found in lesioned males in the 1-experience versus the 10-experience groups (Figure 36). The mount and ejaculation latencies displayed by lesioned males in the 10-experience group was significantly lower than the latencies displayed by lesioned males in the 1-experience group. However, the mean mount and ejaculation latencies displayed by the lesioned males in the 1-experience group was significantly higher than the latency observed in control males within the same group [for mount latency: F(3,22)=11.2, p<0.0001; for ejaculation latency: F(3,22)=7.53, p<0.001] (Figure 37). The difference between mount and ejaculation latencies displayed by lesioned and control males in the 10-experience group was not statistically significant. Furthermore, no significant differences were found in the intromission latency displayed by lesioned and control males in either condition [In the 10-experiences group: p=0.126; in the 1-experience group: p=0.214].

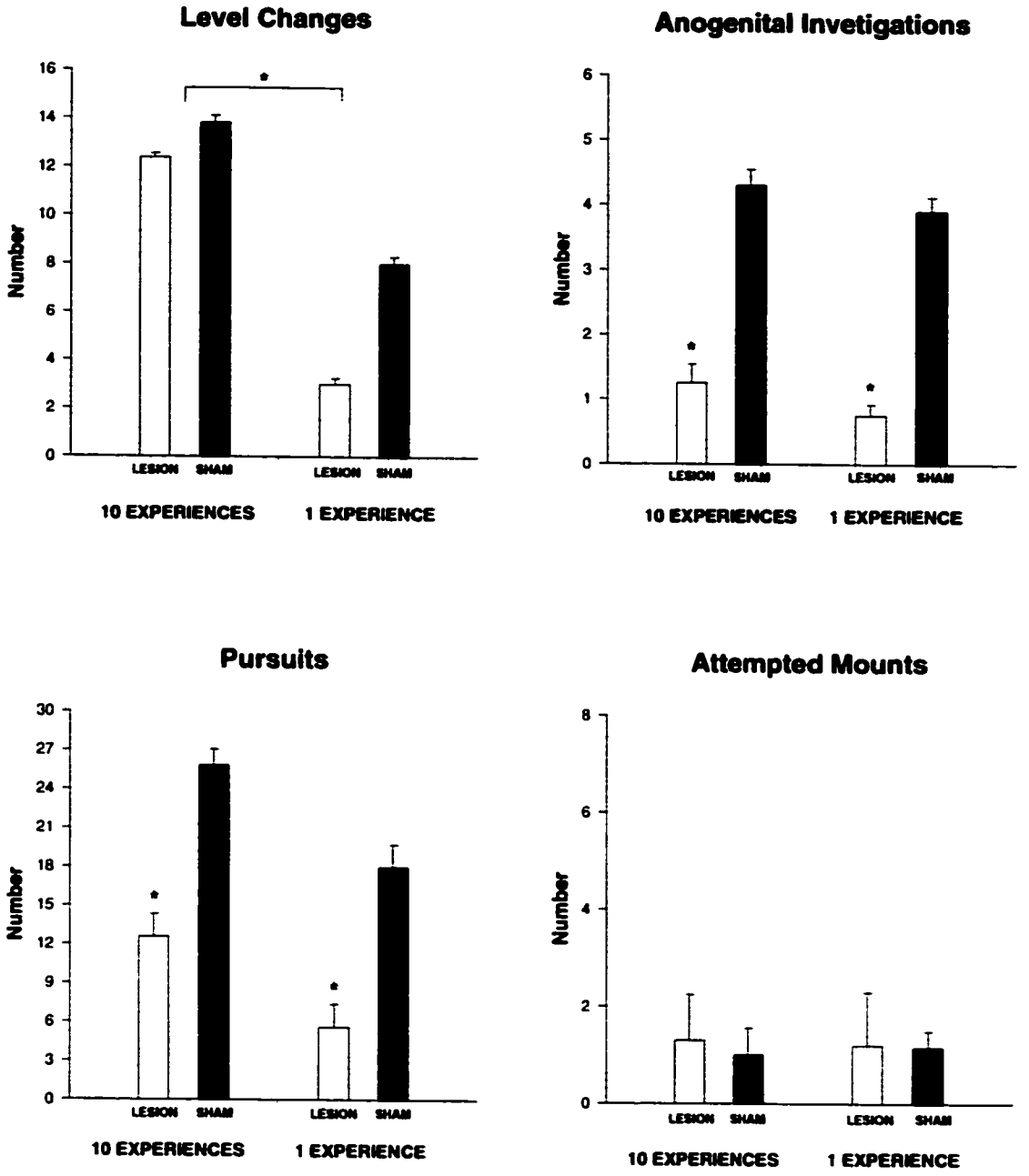


Figure 35. Mean number of level changes, anogenital investigations, pursuits, and attempted mounts displayed by males in the 10-experiences (e.g. 10 sex-training sessions with receptive females prior to the lesion) and 1-experience (e.g., 1 sex-test with a receptive female prior to the lesion) condition groups following bilateral NMDA lesions of the VTA. Data are means + SEM. * $p<.05$ between lesioned and sham-lesioned males either in the 10-experiences or in the 1-experience group.

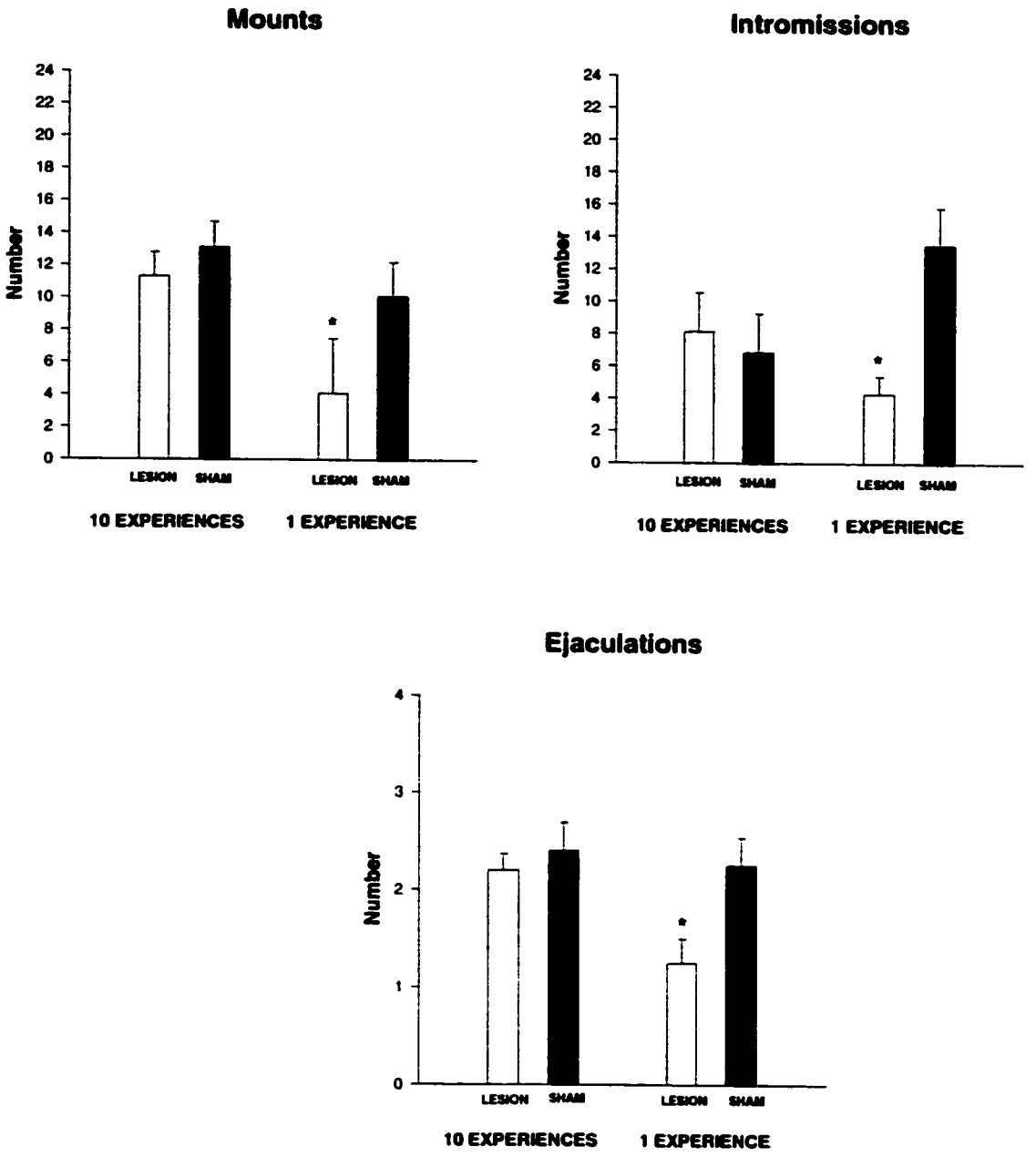


Figure 36. Mean number of mounts, intromissions and ejaculations displayed by males in the 10-experiences (e.g. 10 sex-training sessions with receptive females prior to the lesion) and 1-experience (e.g., 1 sex-test with a receptive female prior to the lesion) condition groups following bilateral NMDA lesions of the VTA. Data are means + SEM. * $p<.05$ between lesioned and sham-lesioned males either in the 10-experiences or in the 1-experience group.

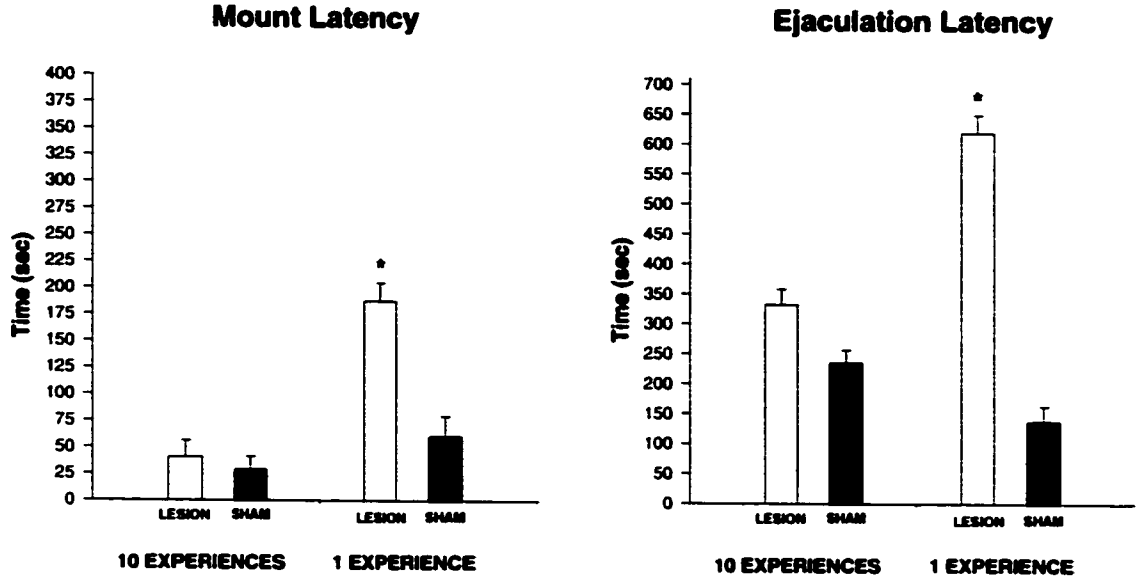


Figure 37. Mount and ejaculation latencies among males in the 10-experiences (e.g. 10 sex-training sessions with receptive females prior to the lesion) and 1-experience (e.g., 1 sex-test with a receptive female prior to the lesion) condition groups following bilateral NMDA lesions of the VTA. Data are means + SEM. * $p<.05$ between lesioned and sham-lesioned males either in the 10-experiences or in the 1-experience group.

Discussion

Bilateral NMDA lesions of the VTA decreased the mean number of anogenital investigations and pursuits of receptive females in both the 10- and 1-experience groups. Even though the mean number of level changes was not significantly different between lesioned and control males assigned to either experience group, a significant difference in the mean number of level changes was observed between males in the 10- versus 1-experience groups overall, as has been observed previously (Mendelson & Pfaus, 1989). In addition, findings from this study showed that the mean number of mounts, intromissions, and ejaculations was significantly lower, and the ejaculation and mount latencies significantly higher, in lesioned compared to control males in the 1-experience group. No significant differences were found for these measures in males in the 10-experience groups. Results from this study suggest an excitatory role of the VTA on the expression of certain appetitive and consummatory behaviors.

As in Chapter 1, the results of this study also demonstrated a role of sexual experience in buffering the disruptive effects of bilateral VTA lesions on male copulatory behaviors. Males with 1 sexual experience to ejaculation showed a more severe effect of the bilateral VTA lesions compared to males in the 10-experience group. There are many studies demonstrating the facilitative role of experience in behavioral outcomes (e.g., partner preference) (Carr, Krames & Constanzo, 1970; De Jonge, Burger, van Haaren, Overdijk, & van de Poll, 1987). Previous studies have found that intact, sexually experienced males compared to sexually naïve males prefer the odors of receptive females over those of non-receptive females (Carr, Krames, & Constanzo, 1970; Carr,

Loeb, & Wylie, 1966; Carr, Wylie & Loeb, 1970). Furthermore, intact, sexually experienced males are less susceptible to the disruptive effects of anosmia, castration, and novelty stress on copulation, than intact sexually naïve males (Pfaus & Wilkins, 1995; Thorn & Flanelly, 1977). The present study indicates that lesioning of the VTA prior to a full acquisition of mating experience prevents an increase in sexual arousal as measured by the shorter mount latency and increased mount and intromission frequency observed in control animals.

Experiment 3. Hormonal Stimulation of the Ventral Tegmental Area of Sexually Experienced Long Term Castrated Male Rats

Intracranial implants of crystalline steroid hormones have been used in a variety of species to examine the role of different steroid actions in brain areas that underlie male- or female-typical reproductive behaviors. For example, implants of T to the mPOA of males facilitate mounts, intromissions, and ejaculations in intact rats, and mounting behavior in castrated males (Christensen & Clemens, 1975; Davidson, 1966; Lisk, 1968; Wood & Newman, 1995). Implants of estradiol (E) have been shown to be more effective than T in facilitating mounts in long-term castrated male rats (Christensen & Clemens, 1975). Testosterone is converted into estrogen by the enzyme aromatase in neurons of the mPOA (Roselli & Klosterman, 1998), and implantation of the aromatase inhibitor fadrozole into the mPOA abolished copulation in castrated T-treated male rats (Bonsall, Clancy, & Michael, 1992; Vagell & McGinnis, 1997).

Recent studies have investigated the effect of intracranial androgen implants to multiple brain sites, including the VTA. Wood and Newman (1995) investigated the effects of T in long term castrated male hamsters when implanted directly into the MEA

or into the BNST and the mPOA simultaneously (BNST/mPOA). Implantation of T in either MEA or BNST/mPOA resulted in an increase in the number of anogenital investigations and mounts, and in a decrease in the latency to the first mount displayed by long-term castrated hamsters (Wood & Newman, 1995). To examine the role of chemosensory and hormonal cues in male copulation, the combined effects of unilateral bulbectomy and T implants in the BNST/mPOA have been also investigated (Wood & Newman, 1995). An increase in the number of mounts was observed in castrated males with T implants in BNST/mPOA and contralateral bulbectomy. However, copulation was not restored in males with ipsilateral bulbectomy despite equivalent implant placement, demonstrating that communication between neurons receiving hormonal and chemosensory cues is required for the expression of male copulatory behavior (Wood & Newman, 1995). An additional study investigating concurrent androgenic stimulation of the mPOA and the MEA in the control of long-term castrated male hamster sexual behavior demonstrated that dual T implantation in these areas stimulated mounts, intromissions, and ejaculations above levels seen in castrates. These results, however, were not significantly different from those copulatory behaviors of castrated males with unilateral implants in either brain site (Coolen & Wood, 1999), suggesting both the mPOA and the MEA are equally important in the expression of consummatory male sexual behaviors. Finally, a recent study has shown evidence supporting an interaction between the MPO and the VTA in the expression of male-typical behaviors (i.e. courtship vocalizations, urine marking, urine preference, and mounting) in castrated mice using concurrent androgenic stimulation of both areas (i.e., MPO/VTA) (Sipos & Nyby, 1996). An increase in mounting and urine preference in castrated house mice was shown

following concurrent bilateral implantation of T in the MPO/VTA (Sipos & Nyby, 1996). Unfortunately, this study failed to examine the effects of the unilateral implants on male copulation in either brain site.

The present study investigated the effects of gonadal steroid implants in the VTA on male copulatory behaviors, given the existence of AR, ER, and PR within this brain area (Don Carlos, Monroy, & Morrell, 1991; Heritage, Stumpf, Sar, & Grant, 1981; Kritzer, 1997; Pfaff & Keiner, 1973; Simmerly, Chang, Muramatsu, & Swanson, 1990). Cannulae containing testosterone propionate (TP), estradiol (E), progesterone (P), and cholesterol (CH) were implanted into the VTA of sexually experienced, castrated males that had either 10 sessions with multiple ejaculations prior to the steroid implant or 1 ejaculation prior to castration.

Material and Methods

Animals

Fifty-two adult male Long Evans hooded rats from Charles River Canada (St. Constant, Québec, Canada) weighing between 250-280g upon arrival were used as subjects. Twenty adult sexually experienced female Long Evans hooded rats weighing 240-250g, from the same supplier, were used as stimulus females. Housing conditions were identical to those used in Experiment 1. Females were ovariectomized and subsequent artificial estrus was induced following procedures identical to those described in Experiment 1 of Chapter 1.

Stereotaxic Surgery

Methods for implant preparation and placement were similar to those of previous studies (Wood & Newman, 1995; and Coolen & Wood, 1999). Castrated male rats were

implanted stereotaxically with 22-gauge stainless-steel cannulae (Plastics One Products, Roanoke, VA) aimed bilaterally at the VTA using the following coordinates:AP=-3.6mm, ML= ±3.6mm, DV= -8.5mm from skull. The guide cannulae were implanted at 16 degrees to vertical to avoid the paraventricular gray (PVG) and penetration of the cerebral aqueduct. Crystalline TP, EB, P or CH, was dissolved in ethanol, and introduced in one end of the guide cannula by tapping the tip of the cannula several times until approximately 2 mm of the steroid was inside the cannula. To ensure that the amount of hormone placed within each cannula was constant (2 mm), a cannula blocker, cut 2 mm shorter than the guide cannula, was inserted each time a cannula was filled by tapping in a small amount of the hormone. The ethanol solute evaporated leaving the steroid inside the cannula. Any hormone remaining on the outside of the cannula was removed with a tissue and ethanol prior to implantation. Animals were implanted under sodium pentobarbital anesthesia (60mg/kg) supplemented with methoxyflurane inhalant (Metofane) in a Kopf stereotaxic apparatus. Cannulae were secured to the skull with acrylic cement molded around 4-5 stainless steel screws embedded in the skull, and once the cement had hardened the skin was sutured. Animals recovered for 7 days before behavioral testing resumed.

Procedure

Males were habituated to the bilevel chambers and pre-exposed to receptive females 1 or 10 times prior to castration, using protocols similar to those described in Experiment 1 of Chapter 1. All male rats were castrated as described in Experiment 1 of Chapter 1. Following castration, all males were tested with receptive females for a period of three months to ensure a decrease in the number of appetitive and consummatory

behaviors. The copulatory testing procedure used was identical to that described in Experiment 1 of Chapter 1. Following this, male rats received bilateral cannula steroid hormone implantations to the VTA. One week after cannula implants, males were randomly assigned to one of three groups according to the hormone that filled their cannulae and tested with receptive females for a period of 52 days at 4-day intervals. An additional group of sexually experienced intact male rats (n=6) was tested for sexual behavior following cannulae implantation to ensure that the implantation itself did not disrupt copulatory behaviors. The percentage of animals showing mounts, intromissions and ejaculations, and the mean number of mounts, intromissions, and ejaculations achieved by the implanted males during the 30-minute test with the receptive female, was examined for a period of 52 days. Males were tested with receptive females at 4-day intervals.

After the thirteenth post-implantation test session, all castrated male rats were overdosed with sodium pentobarbital (120mg/kg) and perfused intracardially with phosphate buffered saline followed by 4% paraformaldehyde. The brains were removed, placed in 10% formalin for 1-3 days, and coronal sections taken at 40 μm through the extent of the cannula tracks. The sections were then placed on glass slides and stained with cresyl violet to verify the site of cannula implantation. Cannula placements were defined in terms of Paxinos and Watson's (1986) stereotaxic coordinates and plotted on coronal sections for graphical presentation (Figures 38-41). In addition, the seminal vesicles of each male were then dissected out, cleaned of adipose tissue, and weighed to the nearest .01mg. The additional group of intact males (N=6) and a group of castrated

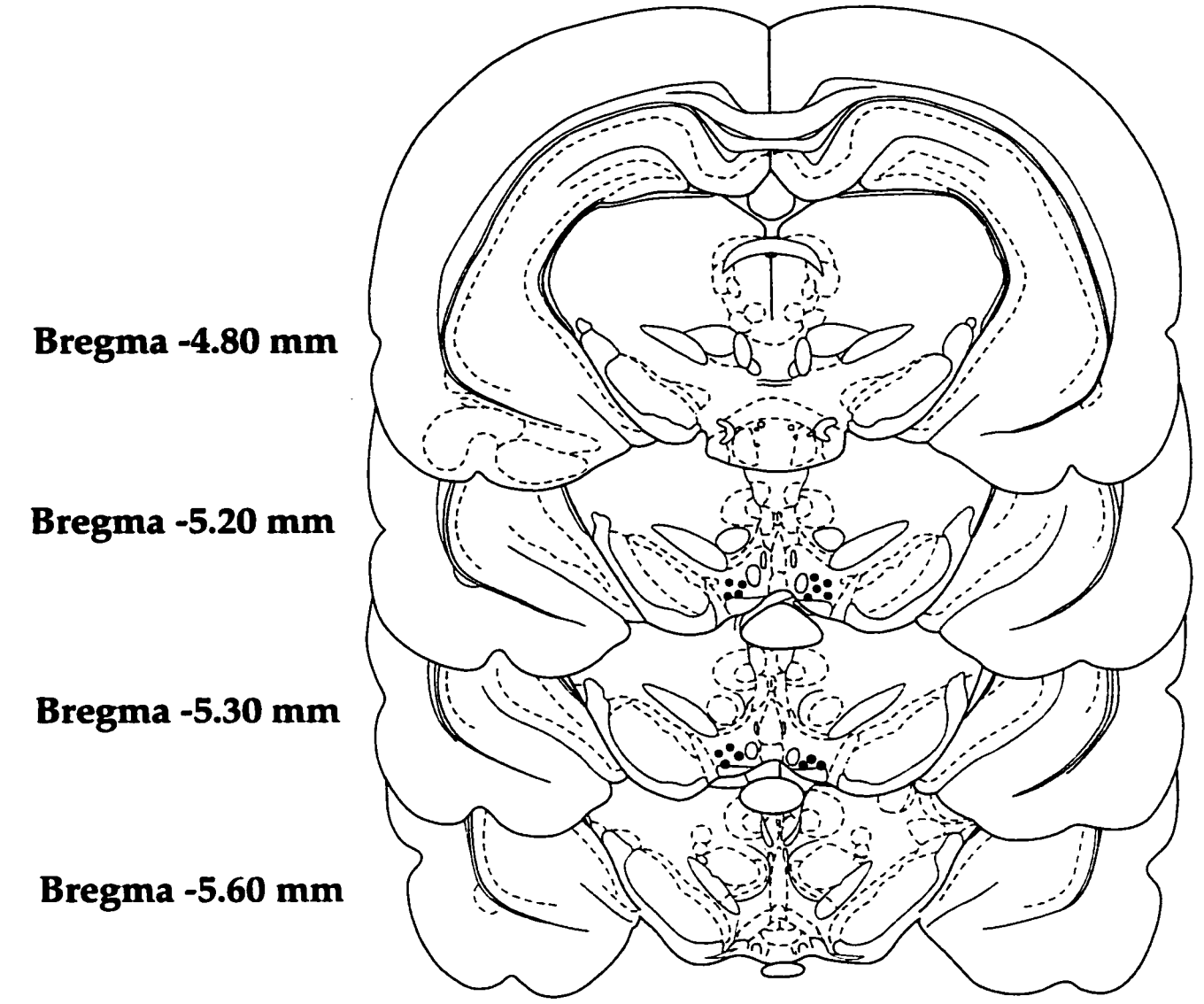


Figure 38. Bilateral cannula T implants in the VTA of sexually experienced long-term castrated male rats.

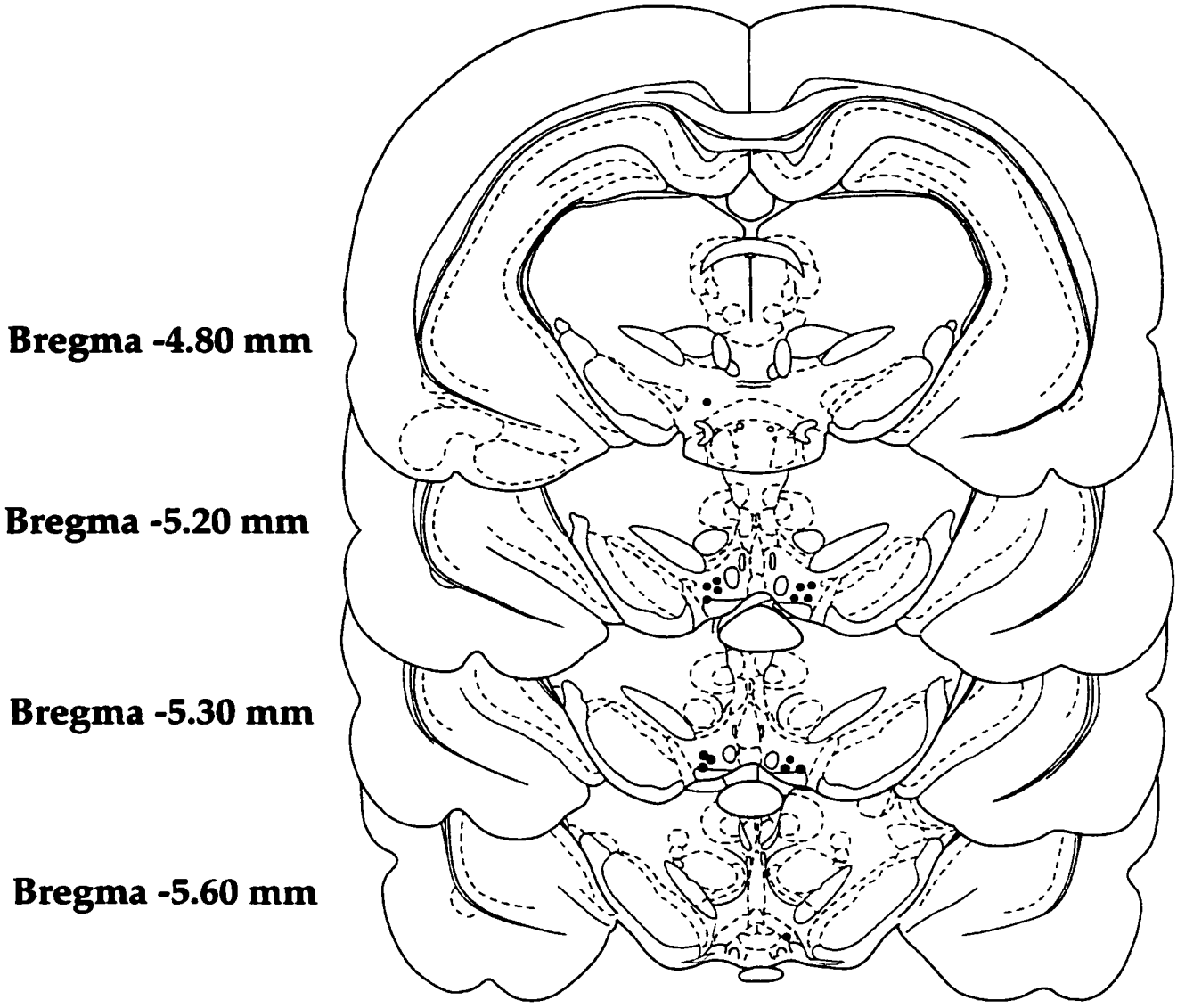


Figure 39. Bilateral cannula E implants in the VTA of sexually experienced long term castrated male rats.

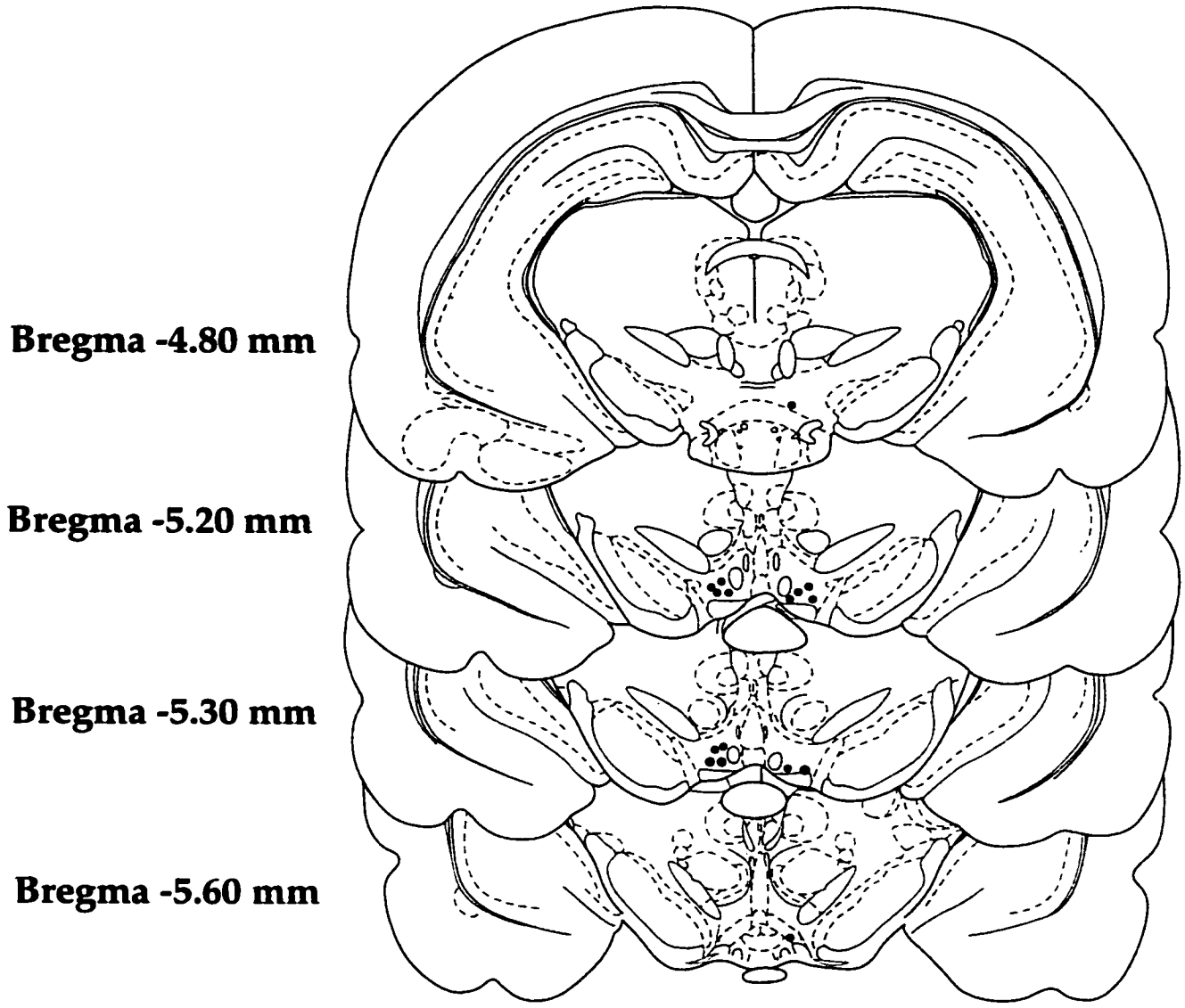


Figure 40. Bilateral cannula P implants in the VTA of sexually experienced long-term castrated male rats.

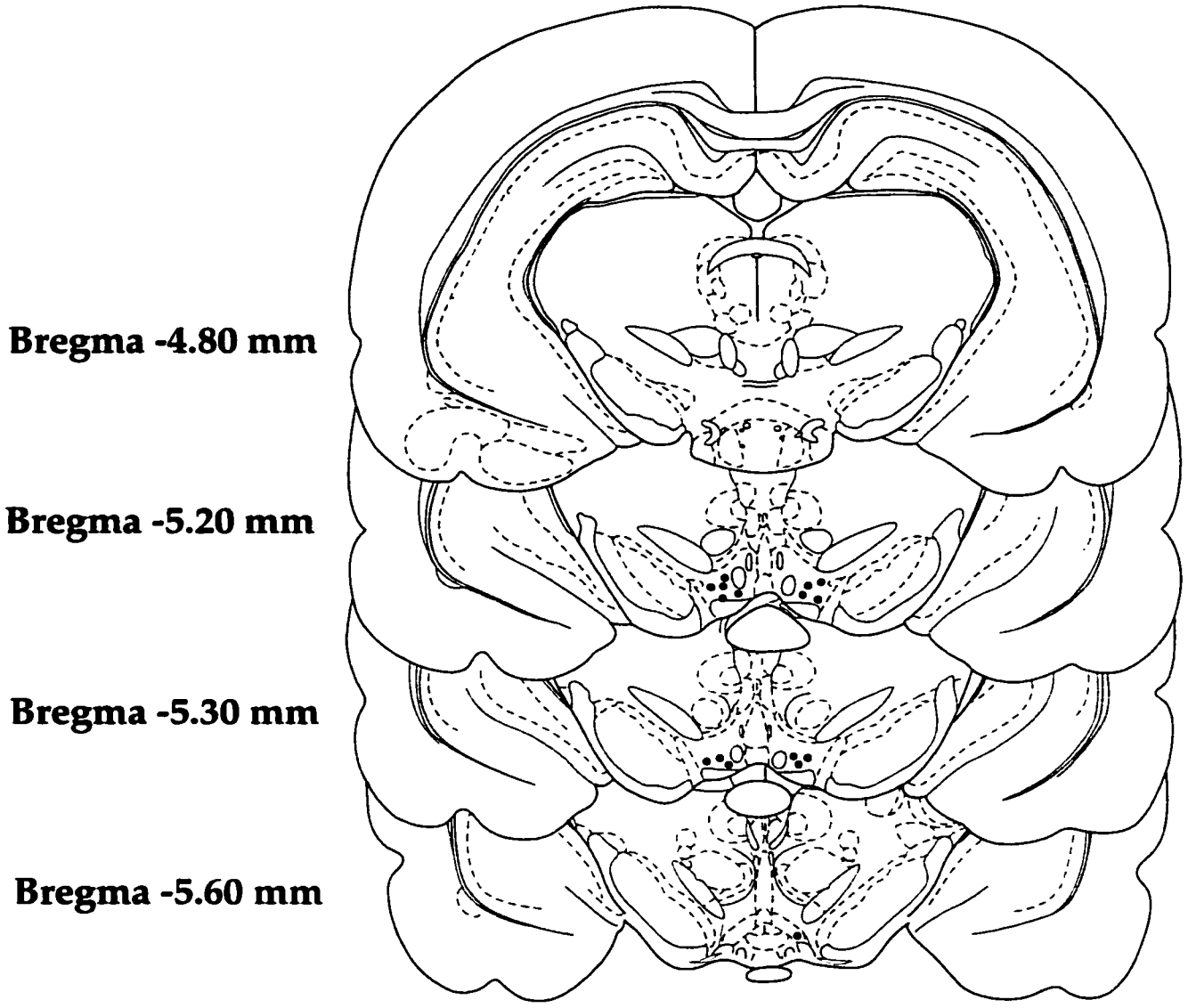


Figure 41. Bilateral cannula CH implants in the VTA of sexually experienced long-term castrated male rats.

untreated males (N=6) were also sacrificed at this time so that the reproductive tissue weights of intact animals and castrated untreated males could be determined.

Behavioral and Statistical Analysis

Tests were videotaped and scored as in Experiment 1 of Chapter 1. The mean number of appetitive and precopulatory behaviors observed following castration and subsequent hormonal implantation (i.e., in T, E, P, and CH groups) were analyzed using a between group ANOVA. A within group ANOVA was used to analyze the effects of each hormone on appetitive and precopulatory behaviors across the 13 test sessions. Significant main effects and interactions were followed by post hoc analyses of individual means using the Tukey method. The mean number of consummatory behaviors was also analyzed using between-within groups ANOVAs. The proportion of rats showing consumatory behaviors before and after hormone implants were analyzed using Fisher's Exact Probability tests. The pre-determined level of statistical significance for all comparisons was $p < 0.05$.

Results

The seminal vesicles of the brain-implanted males were weighed and compared to the weight of the vesicles of intact and castrated male rats. There were no significant differences in the seminal vesicle weights among males that were implanted with, either T, E, P or CH. All the weights were similar to those observed in long-term castrated male rats not treated with hormones, and all were significantly less than the weights from intact males (Table 2). The similarity in the weights in T implanted males when compared to the weights from untreated castrated male rats ensured that the T from the brain-implanted animals did not leak out of the brain into circulation. Therefore, the

Table 2.

Hormone Treatment	N	Body Weight (g)	Seminal Vesicles (mg)
T	8	582 3.4	120.5 8.0*
E	8	534 7.9	115.7 4.2*
P	8	511 13.3	127.6 3.6*
CH	8	603 7.8	119.5 5.2*
Intact	6	498 8.5	950.2 12.8
Untreated	6	595 9.5	122.7 9.1*

* Statistically significant compared to intact group

Table 2. Comparison of the seminal vesicle weights (Mean and SE) between sexually experience intact males, hormone untreated long term castrated males, and long term castrated male rats following bilateral implantation of T, E, P or CH into the VTA.

behavioral effects found in this study could only be attributed to the effects of the hormone implanted inside the brain.

Appetitive Behaviors

A mixed ANOVA was used to analyze differences in the mean number of appetitive behaviors displayed by males implanted with T, E, P, or CH across the 13 test sessions. A significant main interaction was observed for the mean number of level changes, anogenital investigations, and pursuits [for level changes: (13, 416)=65.21, $p<0.001$; anogenital investigations: $F(13, 416)= 91.35$, $p<0.001$; and for pursuits: $F(13, 416)= 88.95$, $p<0.001$]. The between effect revealed a significant increase in the mean number of anogenital investigations among males implanted with E compared to males implanted with T, P, or CH [$F(3, 28)=2.10$, $p<0.005$]. The mean number of level changes differed in the E and CH groups on T6 and T7. The significant interaction for the mean number of anogenital investigations was observed between the T and E groups on T7-9, and between the E and the CH groups on T8. The interaction for the mean number of pursuits was observed between the E and P groups on T8-13. Further analyses of mean number of level changes and anogenital investigations displayed by males in the E group across the 13 test sessions revealed a significant difference between baseline and T6-T7, and baseline and T7, respectively (Figures 42 and 43). The mean number of attempted mounts was not significantly different between any of the hormonal conditions (Figures 44). Finally, an increase in the percentage of males showing anogenital investigations and pursuits was found in those implanted with E compared to P or CH.

Consummatory Behaviors

None of the males implanted with P or CH displayed mounts, intromissions, or

ejaculations (Figures 46-48). A mixed ANOVA was used to analyze the statistical differences in the behavioral output displayed by males implanted with T or E across the 13 test sessions. A significant main interaction was observed for the mean number of mounts and intromissions [for mounts: $F(13, 182) = 80.40$, $p<0.001$; and for intromissions: $F(13, 182) = 63.64$, $p<0.001$]. Furthermore, the between group effects revealed an overall significant difference between the two hormone groups [for mounts: $F(1,14)=30.27$, $p<0.0001$ and for intromissions: $F(1, 14)=403.9$, $p<0.001$]. Post hoc comparisons of the individual means revealed significant differences in the mean number of mounts and intromissions displayed by males implanted with T or E across each of the following testing days [for mounts: T5, T11-13; for intromissions: T7-8, T11]. Additional comparisons revealed that the mean number of mounts displayed by males implanted with T on T5-T8 was significantly different from the pre-implantation baseline measure (Figure 46). Similarly, post hoc comparisons revealed that the mean number of mounts displayed by males implanted with E on T7-9 was significantly different from the pre-implantation baseline measure (Figure 46). No significant differences were observed in the mean number of ejaculations displayed by males in the T or E groups across the 13 test sessions.

The percentage of males displaying mounts, intromissions, and ejaculations, is shown in Figure 49. The behavioral effects observed were not seen prior to test session 7 (28 days after cannulae implantation) and were followed by a rapid decline of all consummatory behaviors by test sessions 12/13. None of the males in the P or CH

Level Changes

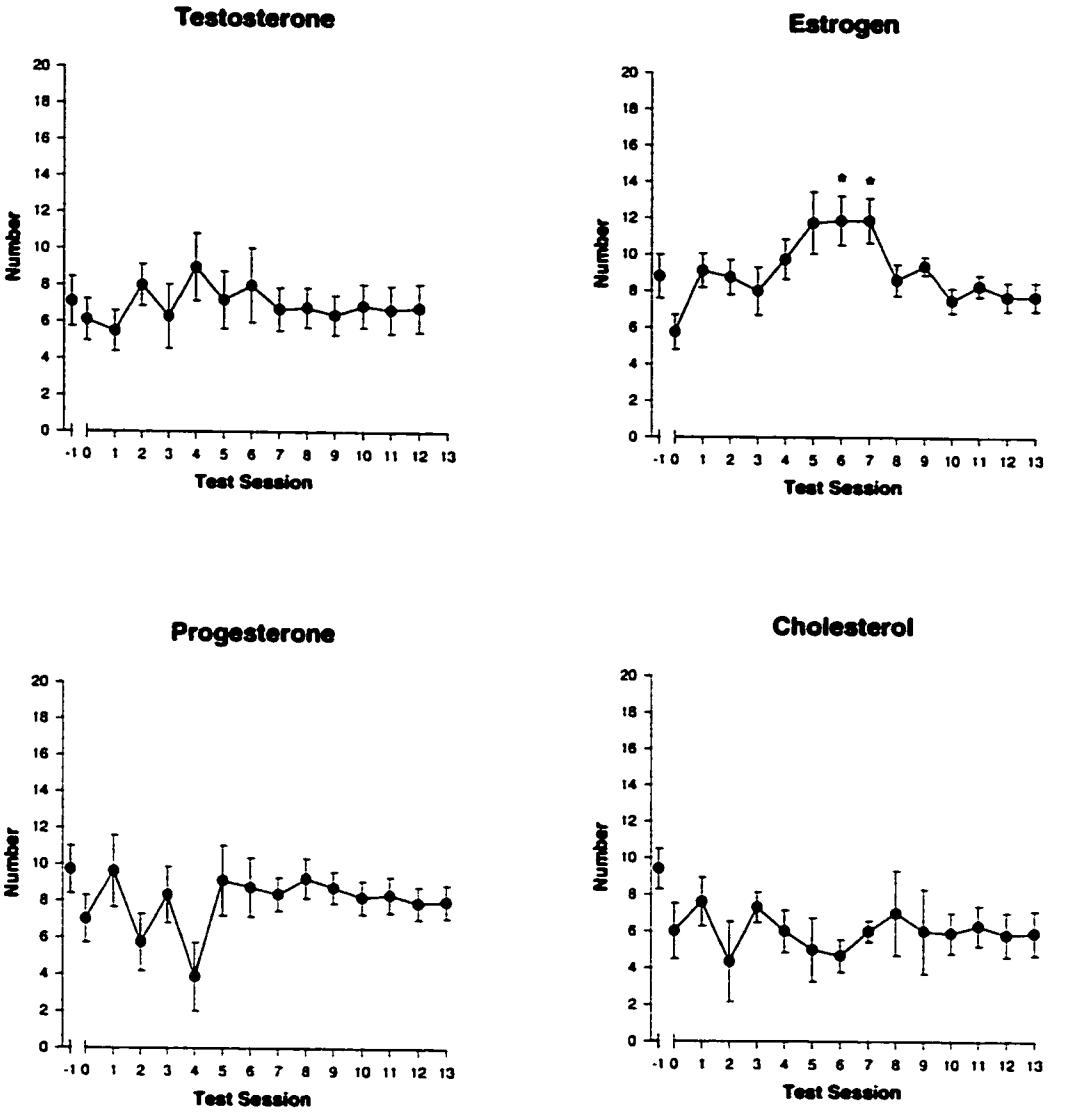


Figure 42. Mean number of level changes displayed by long term castrated male rats following T, E, P, and CH bilateral cannulae implants into the VTA. Behavioral effects are shown across 13 test sessions (52 days) post-implantation. Vertical lines represent standard errors. Test session –1: pre-castration baseline measure. Test session 0: baseline measure prior to steroid implant. *$p<.05$ significantly different from Test session 0.

Anogenital Investigations

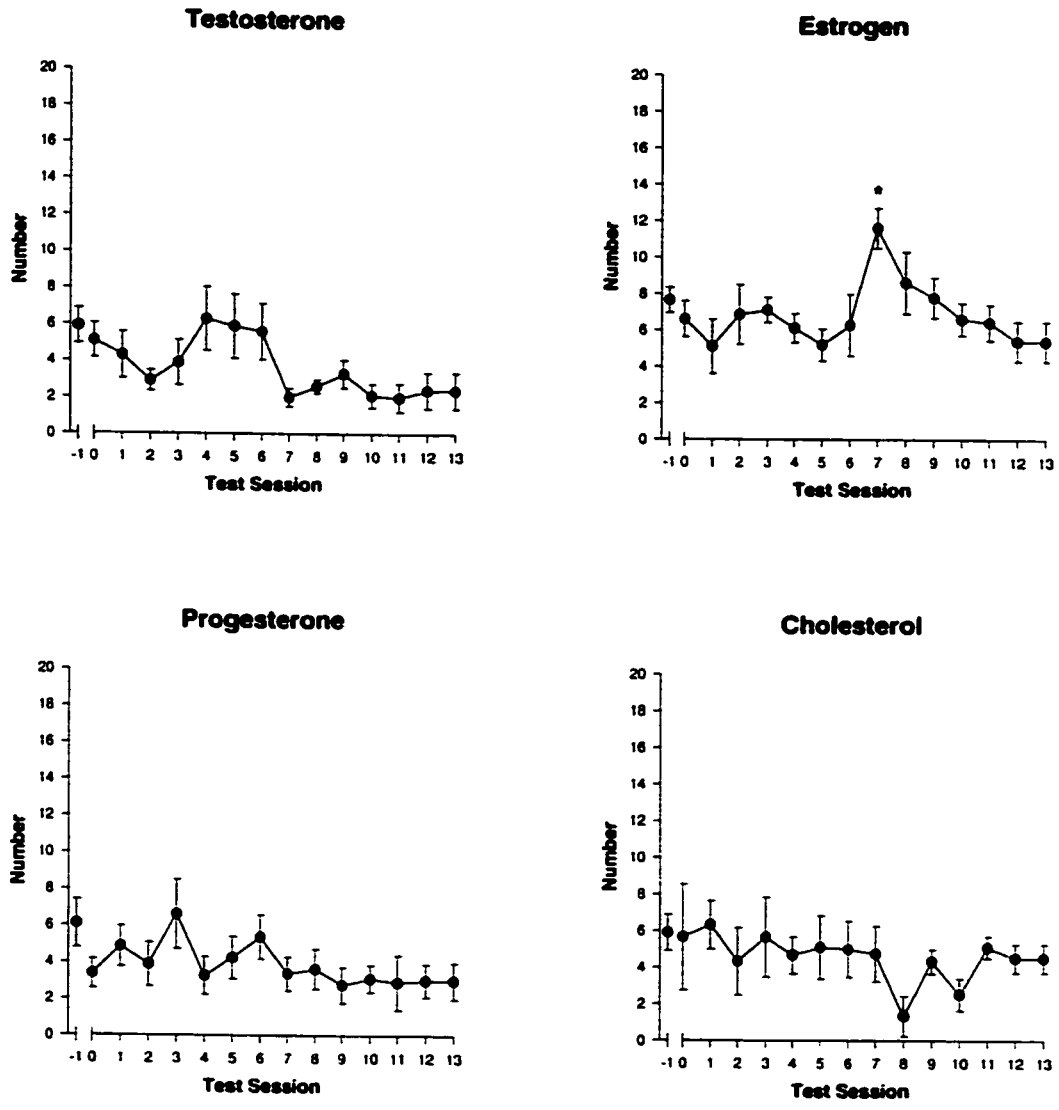


Figure 43. Mean number of anogenital investigations displayed by the long term castrated male rats towards the female genital area following T, E, P, and CH bilateral cannulae implants into the VTA. Behavioral effects are shown across 13 test sessions (52 days) post-implantation. Vertical lines represent standard errors. Test session –1: Pre-castration baseline measure. Test session 0: baseline measure prior to steroid implant. *$p<.05$ significantly different from Test session 0.

Attempted Mounts

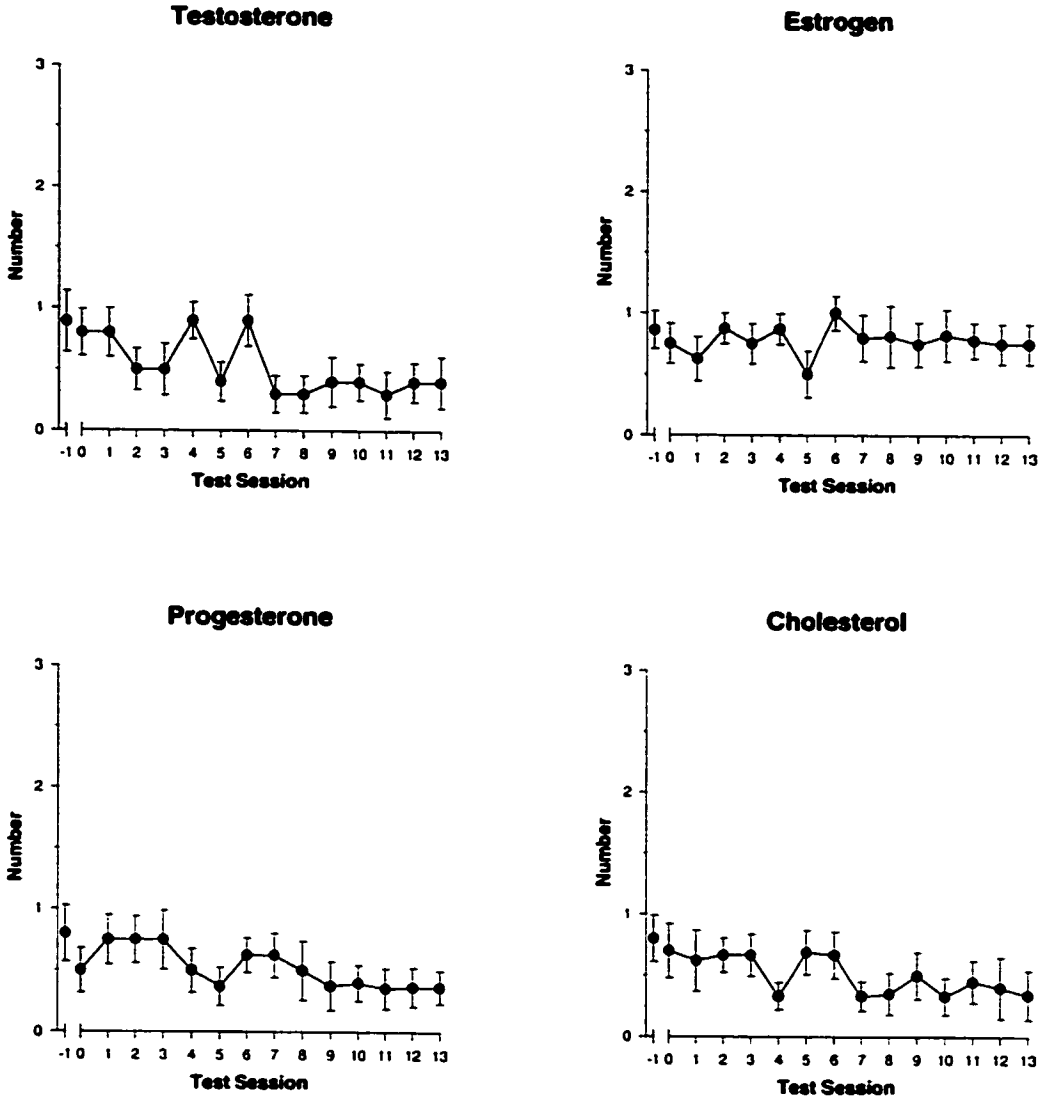


Figure 44. Mean number of attempted mounts displayed by the long-term castrated male rats following T, E, P, and CH bilateral cannulae implants into the VTA. Behavioral effects are shown across 13 test sessions (52 days) post-implantation. Vertical lines represent standard errors. Test session –1: Pre-castration baseline measure. Test session 0: baseline measure prior to steroid implant.

Pursuits

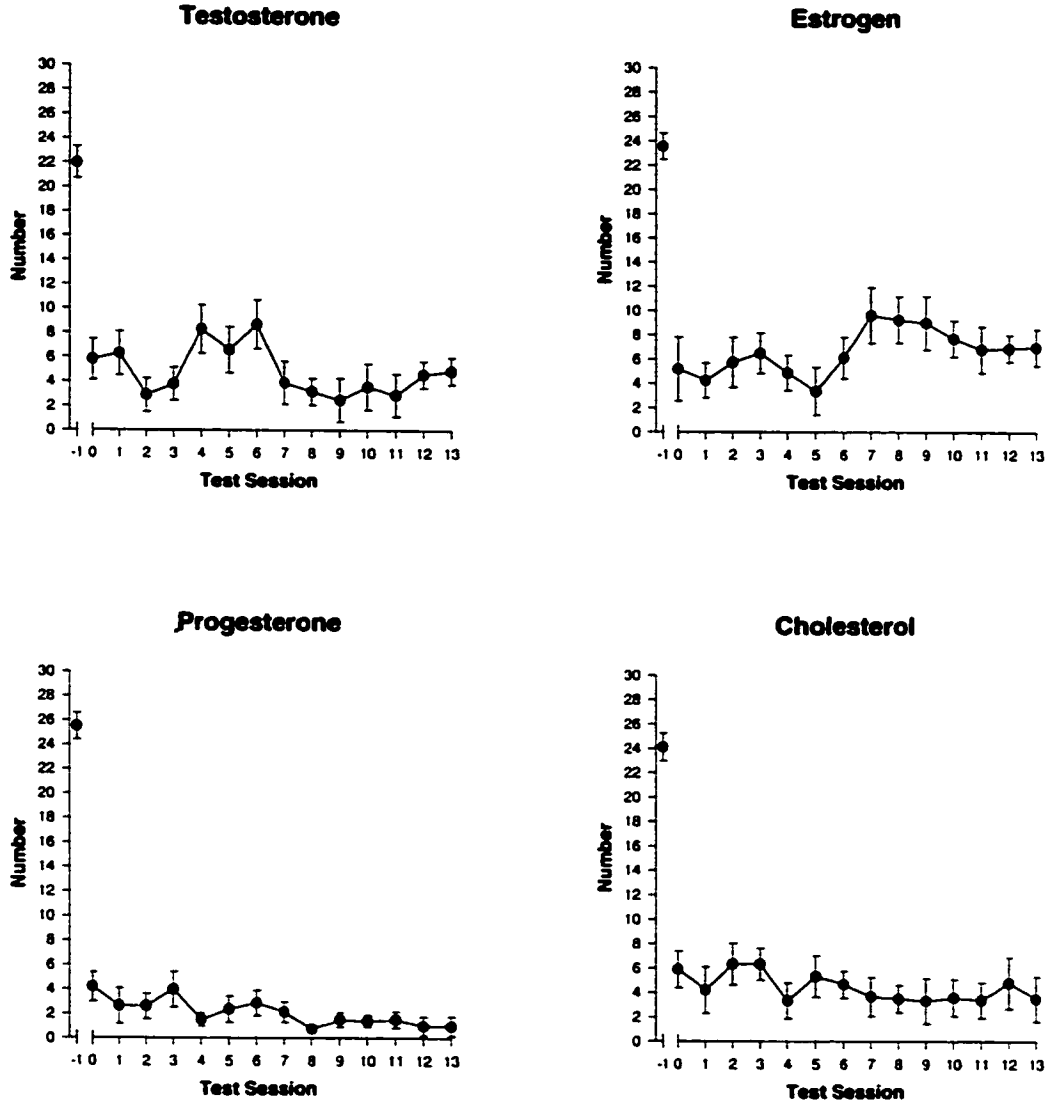


Figure 45. Mean number of pursuits displayed by the long-term castrated male rats following T, E, P, and CH bilateral cannulae implants into the VTA. Behavioral effects are shown across 13 test sessions (52 days) post-implantation. Vertical lines represent standard errors. Test session –1: Pre-castration baseline measure. Test session 0: baseline measure prior to steroid implant.

Total Mounts

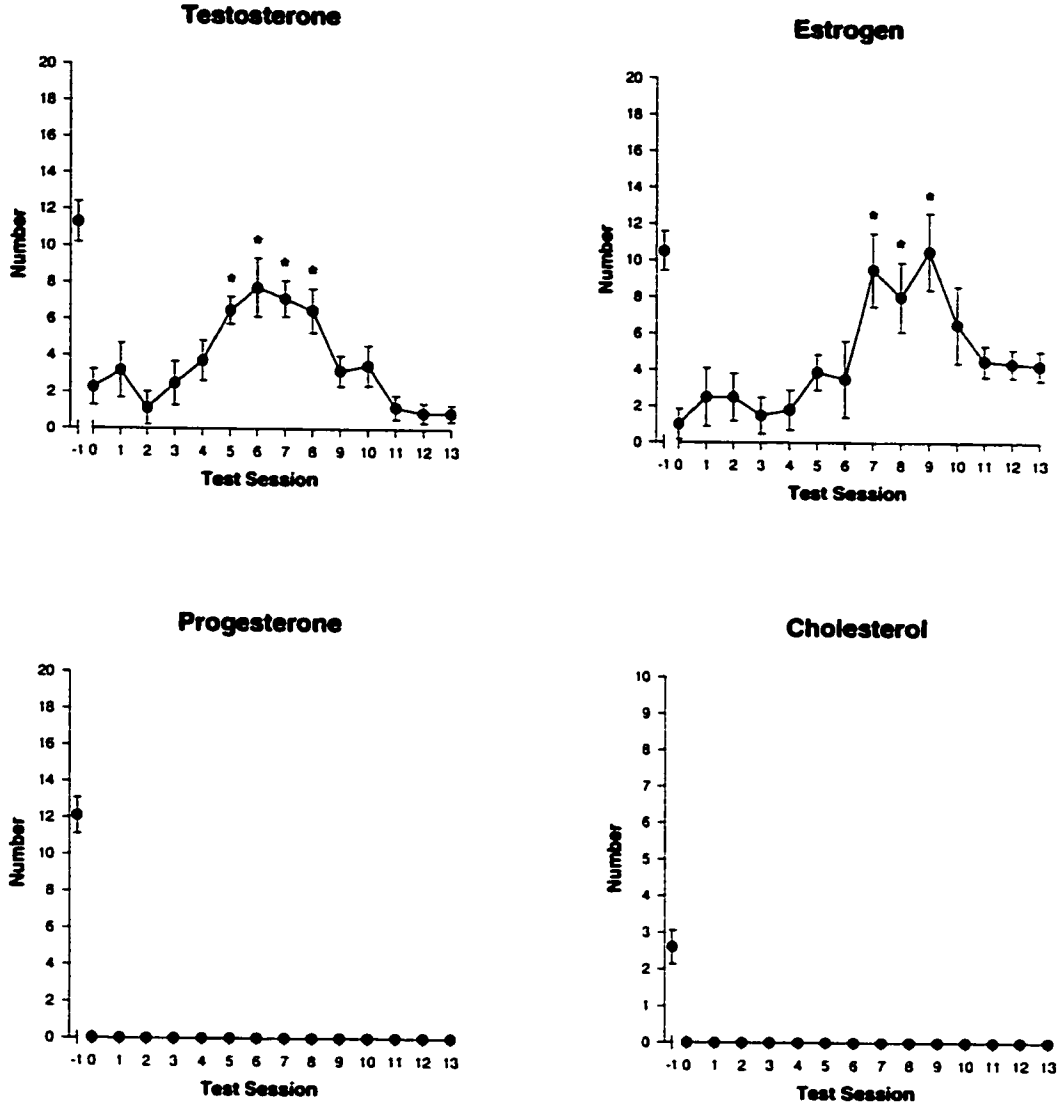


Figure 46. Mean number of mounts displayed by the long-term castrated male rats following T, E, P, and CH bilateral cannulae implants into the VTA. Behavioral effects are shown across 13 test sessions (52 days) post-implantation. Vertical lines represent standard errors. Test session –1: Pre-castration baseline measure. Test session 0: baseline measure prior to steroid implant. *$p<.05$ significantly different from Test session 0.

Intromissions

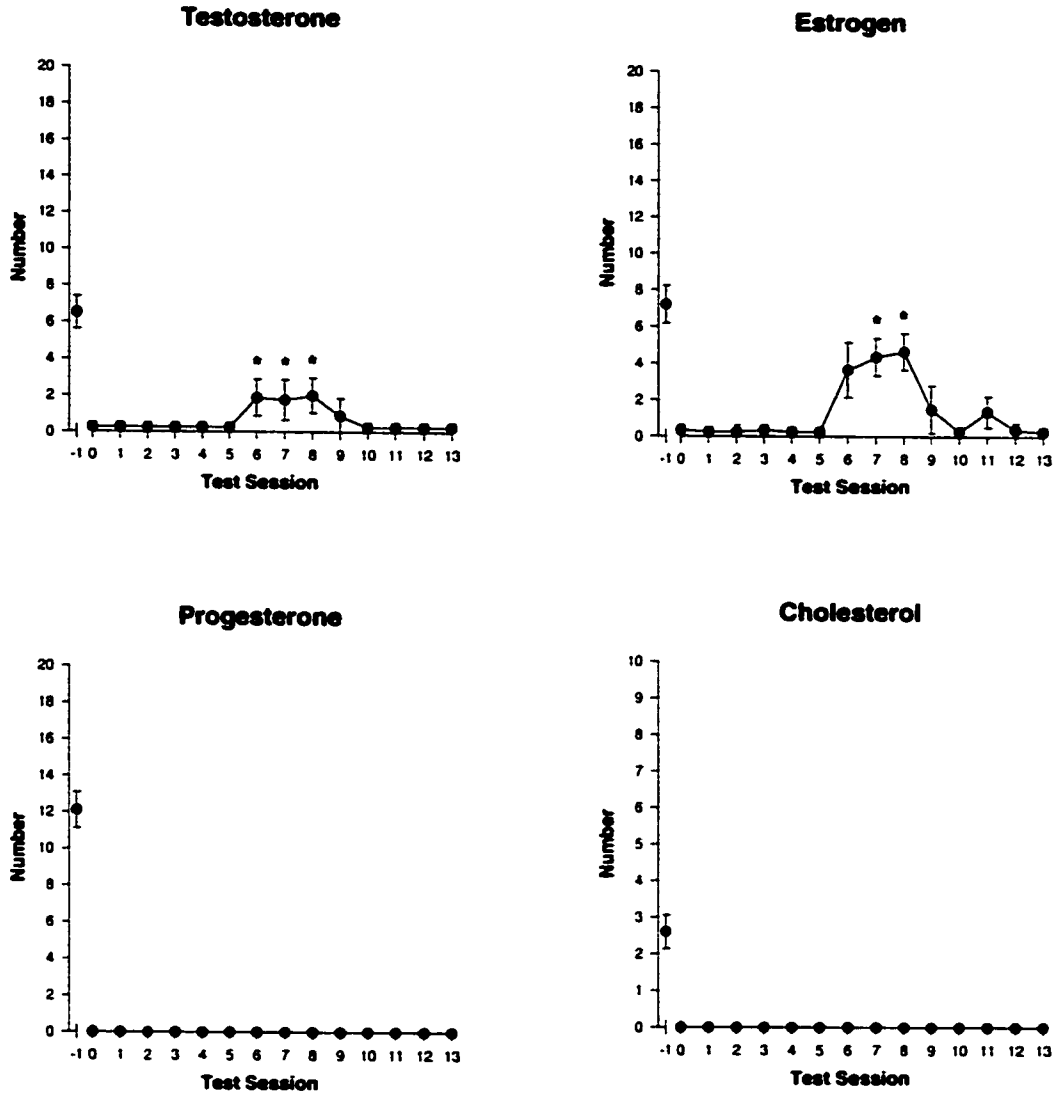


Figure 47. Mean number of intromissions displayed by the long-term castrated male rats following T, E, P, and CH bilateral cannulae implants into the VTA. Behavioral effects are shown across 13 test sessions (52 days) post-implantation. Vertical lines represent standard errors. Test session –1: Pre-castration baseline measure. Test session 0: baseline measure prior to steroid implant. *p<.05 significantly different from Test session 0.

Ejaculations

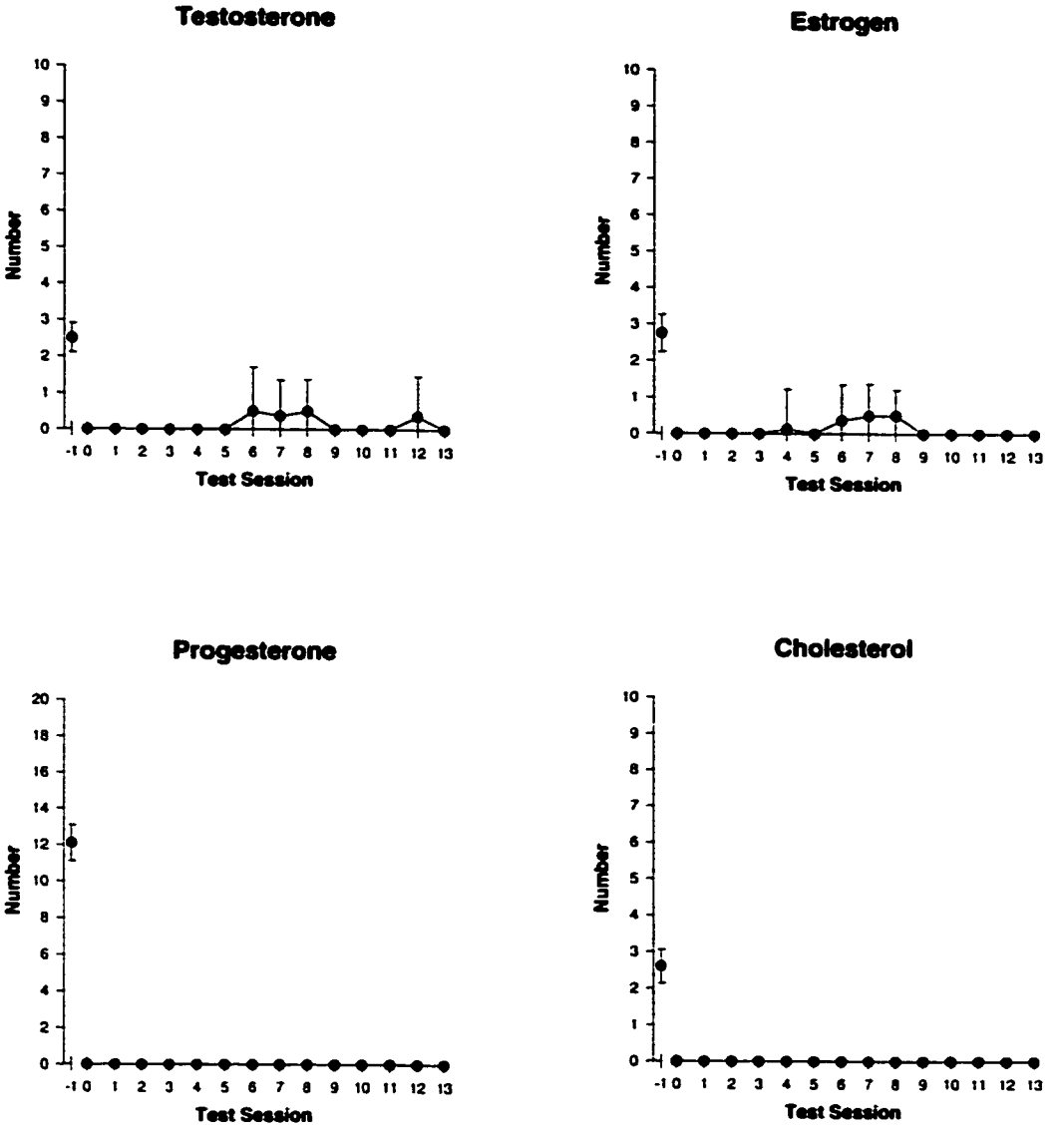


Figure 48. Mean number of ejaculations displayed by the long-term castrated male rats following T, E, P, and CH bilateral cannulae implants into the VTA. Behavioral effects are shown across 13 test sessions (52 days) post-implantation. Vertical lines represent standard errors. Test session –1: Pre-castration baseline measure. Test session 0: baseline measure prior to steroid implant.

implanted groups showed mounts, intromissions, or ejaculations (Figure 49). During test sessions 7-9, seventy-five percent (i.e., 6/8) of males implanted with E showed mounting compared to twenty-five percent (i.e., 2/8) of males in the T-group, and zero percent of males in the P or CH groups. Fisher exact probability tests were used to analyze the differences observed in the percentage of males displaying mounts, intromissions, and ejaculations following either T, E, P, or CH implants (Figure 49). The percent of males implanted with E displaying mounts was significantly different from males implanted with either P or CH [$p<0.01$], but was not significantly different from the percentage observed amongst males implanted with T. The percentage of males implanted with T displaying mounts was not significantly different from the percentage observed amongst males in the P or CH groups. The proportion of males in the E group showing intromissions (i.e., 50%) was significantly greater than the proportion of males implanted with either P or CH [$p<0.05$], but was not significant different from the percentage of males displaying mounts in the T group. The proportion of males in the E and T-groups (25%) showing ejaculations was not significantly higher than that observed among males implanted with P or CH. Intact males showed no alterations in the mean number of appetitive behaviors across the 13 test sessions (Figures 50-51).

Discussion

Findings from this study indicate that either T or E in the VTA can facilitate copulatory behavior in long-term castrated males, although E is more potent in this regard. After implantation of the cannulae in the VTA, mounts and intromissions were enhanced above castrated levels in 6/8 and 4/8 of the males from the E-group respectively and in 2/8 of the males (e.g., same percentage observed for males displaying mounts and

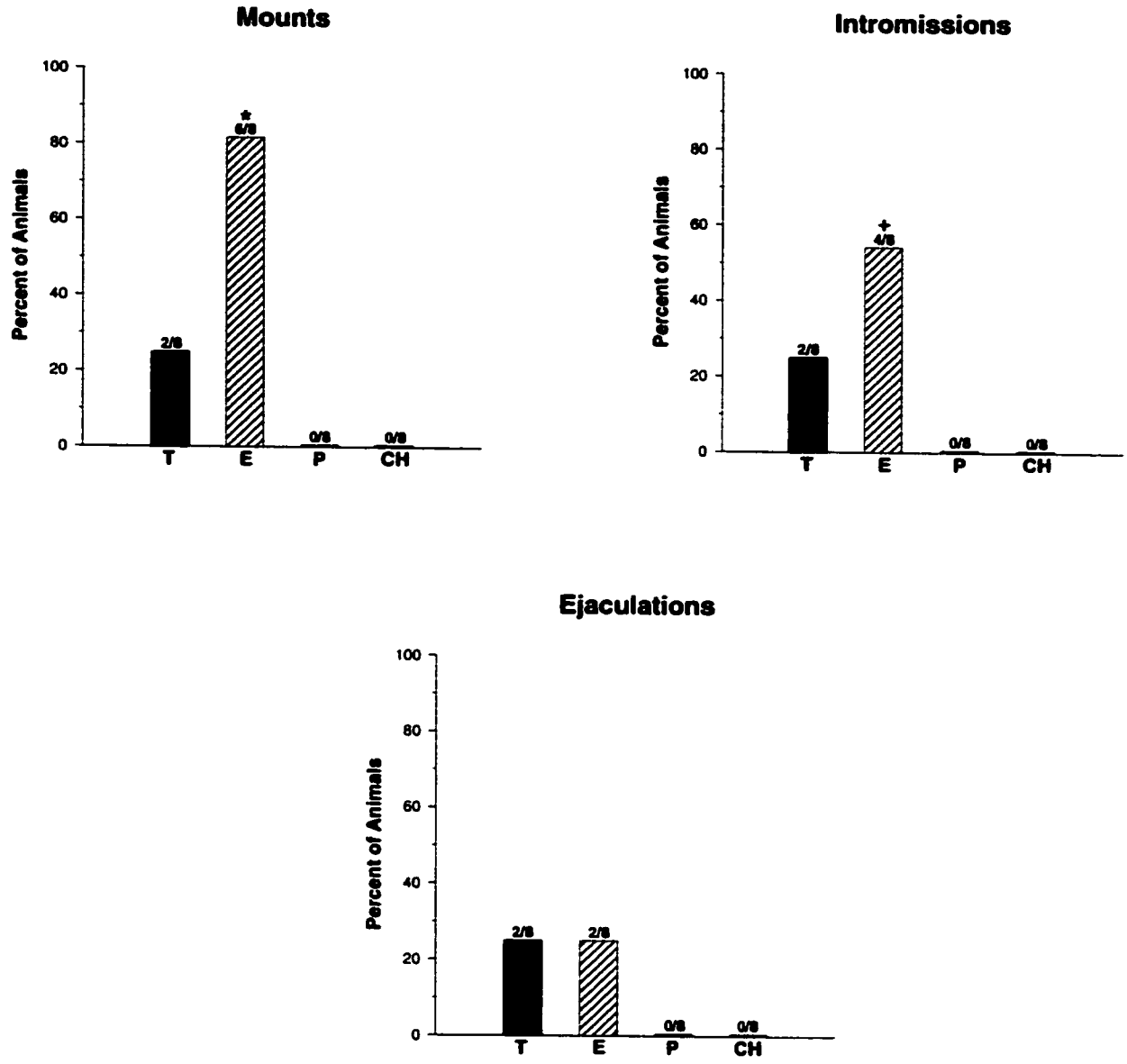


Figure 49. Percent of sexually experienced long-term castrated males showing mounts, intromissions, and ejaculations, following intracranial T and E implants directly into the VTA. None of the males implanted with either P or CH displayed mounts, intromissions, or ejaculations. Data are the average percentage between test sessions 7-9 following VTA implants. *p<.01 between hormone implanted groups. +p<.05 between hormone implanted groups

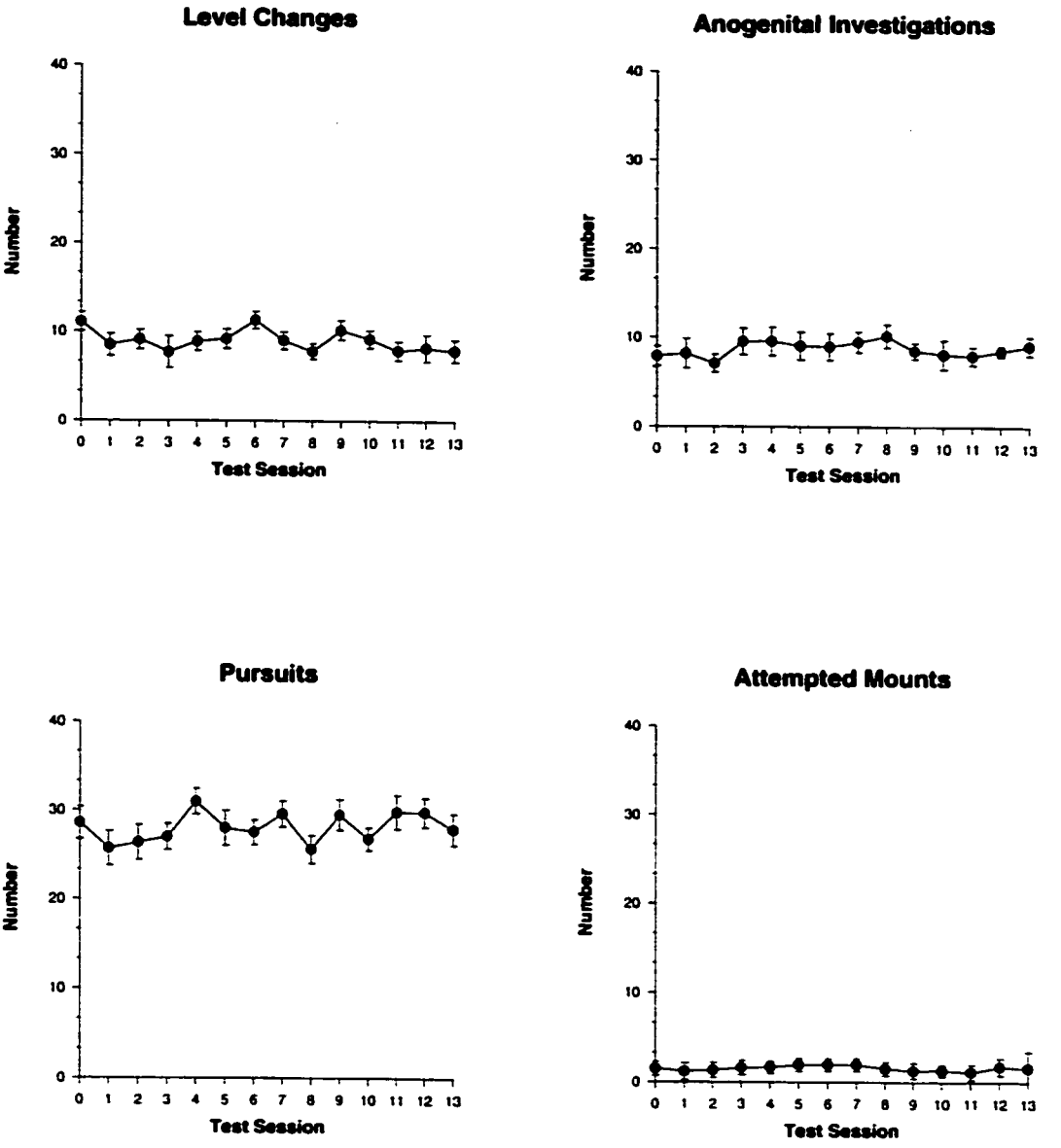


Figure 50. Mean number of appetitive conditioned level changing, and precopulatory behaviors (anogenital investigations, pursuits, and attempted mounts) observed in sexually experienced intact males. Males were tested with receptive females at 4-day intervals for a period of 13 weeks. Data are means + SEM. Vertical lines show standard errors. Test session 0: baseline measure following sexual habituation (i.e., 10 sessions with receptive females) prior to the 13 copulatory test sessions.

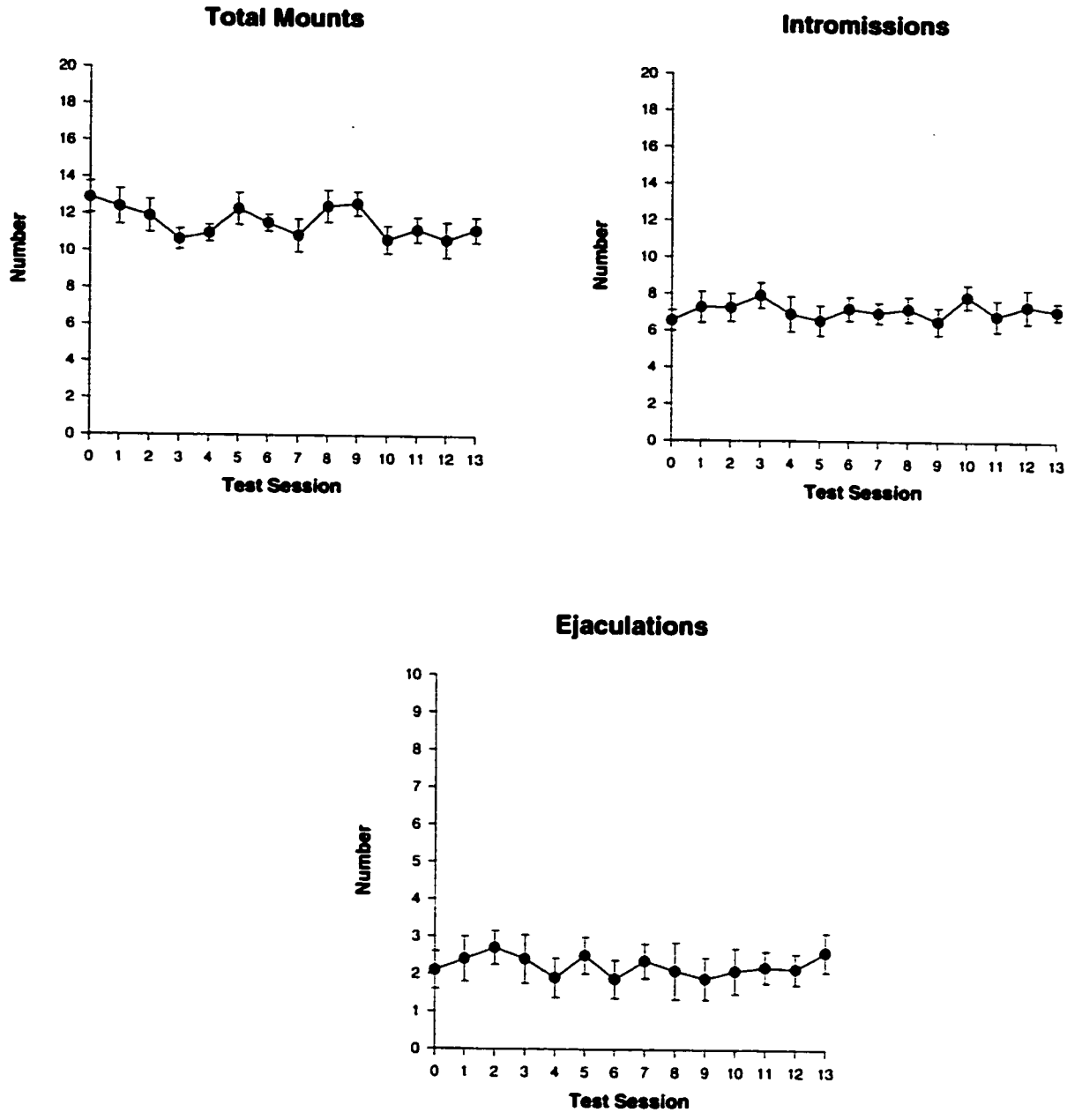


Figure 51. Mean number of mounts, intromisions, and ejaculations observed in sexually experienced intact males. Males were tested with receptive females at 4-day intervals for a period of 13 weeks. Data are means + SEM. Vertical lines show standard errors. Test session 0: baseline measure following sexual habituation (i.e., 10 sessions with receptive females) prior to the 13 copulatory test sessions.

intromissions) in the T-group. Effects found among males implanted with E included an increase in the mean number of level changes, pursuits, anogenital investigations of the female genital area, and the reinstatement of mounts and intromissions. Proportionately fewer T-implanted males displayed mounts and intromissions compared to those implanted with E. In contrast, none of the males implanted with P or CH displayed any of the above behaviors.

An important issue raised by this study is the mechanism of action of T or E in the VTA during male copulation. Androgens can exert non-classical actions, which are characterized by rapid effects of short duration such as a change in membrane fluidity and/or the regulation of $GABA_A$ receptors on plasma membranes (Brann, Hendry & Mahesh, 1995; Lieberherr & Grosse, 1994). Findings from other studies have shown that some T metabolites, such as dianabol (1,4-androstradien-17alpha-methyl-17beta-ol-3-one) synthesized by glial cells, can act as $GABA_A$ agonists, increasing GABA-stimulated chloride (Cl-) influx 2 to 4 weeks after dianabol administration (Bitran, Kellogg, & Hilvers, 1993; Bitran, Hilvers, Frye, & Erskine, 1996). However, the effects of T or E on male copulation in the VTA were observed 20-36 days after the steroid implants, which argues against an immediate non-genomic mechanism. Furthermore, microinjection of GABA agonists hyperpolarize DAergic neurons in the VTA (Johnson & North, 1992; Olpe, Koella, Wolf, & Hass, 1977; Seabrook, Howson, & Lacey, 1990). If T metabolites within the VTA were acting as a GABA agonist, facilitation of male copulation would not be predicted.

Immunocytochemical studies have mapped ARs within the parabrachial and paranigral VTA, and ERs in the paranigral VTA, of male and female rats (Kritzer, 1997),

in a 3:1 ratio. It is possible that the effects of T and E implanted in the VTA took place through a genomic mechanism of action via ARs and ERs. Although previous studies have reported ER binding activity within the VTA, the recent discovery of estrogen receptor–beta (ER-β) and its localization within the male rat's central nervous system, including the VTA (Shughrue, Komm, & Merchenthaler, 1996; Shughrue, Lane, & Merchenthaler, 1997; Shughrue & Merchenthaler, 2001), raises the possibility of a mechanism of action for E involving ER-β in addition to estrogen receptor-alpha (ER-α).

Aromatase plays a crucial role in the mechanism of action of T in the mPOA. Nevertheless, the exact cellular localization of this enzymatic process within the different cell populations within the VTA is uncertain. *In vitro* studies have evaluated aromatase activity in neurons, astrocytes, and glial cells in the mPOA, showing a higher aromatase activity within neurons, compared to lower activity in glial cells (Canick, Vaccaro, Livingston, Leeman, Ryan, & Fox, 1986; Celotti, Melcangi, Negri-Cesi, & Poletti, 1991; Cesi, Melcangi, Celotti, & Martini, 1993; Negri-Cesi, Melcangi, Celotti, & Martini, 1992). However, the aromatase presence within neurons or glial cells *in vivo* in the VTA remains unknown. The relative importance of the aromatization process on the observed effects of E versus T in the VTA on male copulation could be tested by implanting T in combination with an aromatase inhibitor, such as fadrozole and examining its effects of male's appetitive and consummatory behaviors.

Summary of Results

The role of the VTA in male copulation was examined in a series of experiments within this chapter. A significant increase in the number of colabelled Fos-TH IR cells was found in the anterior VTA of rats that copulated to two ejaculations compared to rats

that only mounted or intromitted. In contrast, in the anterior VTA the mean number of Fos-GABA cells was found to be significantly lower following two ejaculations, compared to the mean number of double cells observed following a single ejaculation, suggesting a different time of activation for dopaminergic and GABAergic neurons in this brain region during male rat copulation.

Lesions of the VTA with NMDA decreased the mean number of pursuits and anogenital investigations of receptive females regardless of the amount of sexual experience males had prior to the lesion. In contrast, the inhibitory effects of the NMDA VTA lesions on the expression of consummatory behaviors was only observed among males in the 1-experience condition. Results from this experiment suggest that the VTA controls both appetitive and consummatory sexual behaviors, and add to evidence from Chapter 1 that sexual experience "buffers" male rats from the effects of treatments that disrupt copulatory behavior.

An increase in the percentage of males displaying mounts, intromissions and ejaculations was found following implants of E to the VTA of long-term castrated males. Smaller effects were observed with T. None of the males implanted with P or CH displayed any consummatory behaviors. In addition, the mean number of level changes and anogenital investigations was significantly higher for males that were implanted with E compared with males from any of the other hormone-implanted groups. These results indicate that the actions of either T or E in the VTA can facilitate copulatory behavior in long-term castrated males, although E is more potent in this regard. Although the potential difference between E or T within the VTA is not clearly understood at present,

these findings raise the possibility of a mechanism of action for T in the VTA involving glial aromatase.

GENERAL DISCUSSION

Overall, the findings from this thesis show that the effects of castration and TP treatment on the decline and reinstatement of appetitive and consummatory sexual behaviors in male rats depend on the amount of sexual experience males have before castration and the amount of sexual exposure they receive following castration. Results from the Fos studies revealed the activation of the VTA, along with limbic and hypothalamic regions following multiple ejaculations, demonstrating the importance of neural activity in this region for the expression of male ejaculation. Lesions of the VTA led to a decrease in the mean number of pursuits and anogenital investigations of receptive females regardless of the amount of sexual experience males had prior to the lesion. Interestingly, however, the effects of the NMDA VTA lesions on copulatory behaviors were only observed among males in the 1-experience condition. Finally, cannulae containing T, E, P, and CH were implanted bilaterally into the VTA of long-term castrated male rats that received extensive sexual experience prior to castration. Findings from this experiment revealed a higher percentage of males implanted with E displaying mounts, intromissions and ejaculations, compared to the percentage of males implanted with T displaying the same behaviors. In addition, the mean number of level changes and anogenital investigations was significantly higher in males that were implanted with E than in males from any of the other hormone-implanted groups. Thus, the behavioral effects of castration and VTA lesions on male copulation are modulated by the amount of sexual experience males received prior to testing. The findings from the

studies following E and T implants demonstrated the existence of an androgen/estrogen mechanism of action in this region that contributes to male copualtion.

Role of Sexual Experience and Male Sexual Behavior

Sexual experience has pronounced effects in promoting consistent and automated female-oriented behavior (Carr, Loeb, & Dissinger, 1965; Carr, Loeb, & Willie, 1966; Dewsbury, 1969; Larsson, 1956). It is possible that associations between the odors or visual cues given by receptive females and intromissions or ejaculations need to be established for rapid recognition of the hormonal state of the receptive female. Experienced males when in contact with receptive females do not need to spend time making sense of a stimulus that is already recognized (i.e. they spend less time investigating the anogenital area or making attempted mounts), and engage almost automatically in consummatory behaviors (i.e., mounts, intromissions and ejaculations). Indeed, sexually experienced males are not sensitive to the disruptive effects of novel environments on copulatory behavior, despite freezing in such environments prior to the introduction of a receptive female (Pfaus & Wilkins, 1995). In contrast, inexperienced males that had only a single ejaculation with receptive females may have fewer associations between the different stimuli involved in copulation, and might need to investigate the receptive females longer before displaying any consummatory behaviors. Such males are also correspondingly more sensitive to the disruptive effects of novel environments on copulation (Pfaus & Wilkins, 1995).

Differences in levels of arousability might explain the different behavioral output observed between experienced and inexperienced males following castration. Beach (1942) suggested the existence of a sexual arousal continuum state within the rat, in

which steroid hormones induce sexual motivation by facilitating the ability to be aroused by external sexual stimuli (i. e., receptive females). Subsequently, Whalen (1966) suggested that steroid hormones serve to increase sexual arousability by augmenting neural systems that either recognize a sexual incentive or that translate such recognition into a copulatory response. In a study on the induction of mating behavior through conditioned arousal, Crowley, Popolow, & Ward (1973), suggested that inexperienced rats at the lower end of the continuum, once having gained copulatory experience, do not return to the lower end as they have developed a higher level of arousability. Thus, experience may sensitize the same systems augmented by steroid hormones. Examples of this were observed in the present studies. The amount of time taken by sexually experienced, castrated males to stop copulating when exposed every 4 days to receptive females was three months (Experiment 1 of Chapter 1), compared to 1 month when experienced, castrated males were tested with females only twice (Experiment 2 of Chapter 1). The stimulation obtained with repeated testing seems to delay the effects of castration, perhaps by maintaining a greater strength of the associations between different stimuli, that leads sexual experienced males to expectations of normal performance that might compensate for decreased hormone actions.

Sexual experience prior to castration likely plays a critical role in strengthening the associations between stimuli (i.e., the odors/sight of receptive females and mounts, and/or odors/sight and intromissions) and hence strengthening the likelihood of behavioral output. For example, sexually inexperienced male rats take longer to initiate copulation in the presence of a sexually receptive female, display more mounts without intromission, and have longer ejaculation latencies, compared to sexually experienced

rats (Beach, 1942; 1956; Dewsbury, 1969; Larsson, 1956; Stone, 1922). Similar results have been found in male cats (Michael, 1978; Rosenblatt & Aronson, 1958) and hamsters (Bunnel & Kimmel, 1965). McGill (1967) found that the number of mounts directed by male mice at a female's head decreased with sexual experience. These effects are likely due to instrumental learning, which appears to "fix" motor patterns into stable, although individual, baseline of copulatory responding. Beach (1956) noted that once male rats gained vaginal intromission, the display of subsequent intromissions became relatively fixed or stereotyped. Indeed, over the course of the first few sexual experiences, male rats initiate sexual contact faster, and their first mount usually coincides with their first intromission, making these two behaviors nearly identical, and hence statistically redundant, despite the fact that they may be controlled by different, but converging, brain regions. For example, some drugs that disrupt the ability of male rats to gain erection (e.g., haloperidol) can increase the intromission latency at doses that do not alter the mount latency (Pfaus & Phillips, 1991). This demonstrates that these measures are controlled independently but occur together in the normal display of male copulatory behavior. Similarly, sexual experience has also been shown to diminish the importance of estrous odors in the initiation of copulation (Beach, 1942; Carr, Wylie, & Loeb, 1970). In those studies, only sexually naïve male rats made anosmic by olfactory bulb lesions, or by application of $ZnSO_4$, had severe deficits in the initiation of copulation; sexually experienced males were not affected significalntly by these treatments.

It appears that sexual experience sensitizes the ability of sexual stimulation to activate different neural systems related to sexual behavior. For example, copulation to ejaculation activates Fos mRNA within sensory cortical neurons of the parietal lobe of

male rats, and the amount of activation increases from the first to fifth sexual experience (Bialy, Nikolaev, Beck, & Kaczmarek, 1992). Exposure to a test chamber that contained female hamster vaginal secretions induced Fos protein in more neurons within the MEApd, and medial preoptic nucleus, of sexually experienced male hamsters compared to sexually naïve males (Kollack-Walker & Newman, 1997). Similarly, mesolimbic dopamine release in the nucleus accumbens, an important component of appetitive and consummatory sexual responses (Blackburn, Pfaus, & Phillips, 1992; Everitt, 1990; Pfaus & Phillips, 1991), has been shown to sensitize in male rats with repeated exposure to female estrous odors (Mitchell & Gratton, 1991). Conversely, a larger proportion of sexually naive male rats sensitized to amphetamine copulate to ejaculation during their first sexual experience (Fiorino & Phillips, 1999a; 1999b). An increase in incertohypothalamic DA release in the mPOA in anticipation of sexual activity has been shown in 1 week castrated male rats that display copulatory behaviors compared to the lack of release in those that do not (Hull, Du, Lorrain, & Matuszawich, 1995; Hull, Du, Lorrain, & Matuszawich, 1997). In this regard, it is interesting to note that male rats with lesions of the subthalamic or mesencephalic locomotor regions (Edwards & Maillard, 1988), which include the zona incerta, the region that gives rise to DA projections to the mPOA, are less likely to mount sexually receptive female rats treated with haloperidol (which facilitates lordosis but abolishes proceptive behaviors) relative to undrugged females. These data suggest that incertohypothalamic DA release is sensitive to the incentive value of the female, as is mesolimbic DA release.

Consistent with many previous studies, castrated male rats in the present experiments showed a reinstatement of all consummatory components following TP

treatment (Davidson, 1966; Malmnas, 1973; Smith, Damassa, & Davidson, 1977). In Experiment 1 of Chapter 1, TP treated, sexually experienced castrated males tested with receptive females showed a gradual return of full mounts, intromissions, and ejaculations starting at session 5 (i.e., after 20 days of TP). Furthermore, following TP treatment, all consummatory behaviors returned simultaneously rather than in the opposite order of their disappearance. In contrast, Experiment 3 showed that males allowed to have a single ejaculation with receptive females prior to castration and tested with receptive females at 4-day intervals had a gradual return of mounts, intromissions and ejaculations starting at Day 8 of TP treatment. In addition, the consummatory behaviors returned in the opposite order of disappearance, with mounts the first behavior to reappear followed by intromissions and then ejaculations. The differences in the patterns of decline and return following castration and TP treatment are thus affected by the amount of sexual experience the males receive prior to and following castration.

Neural Activation of Male Copulation

The coordination of different copulatory responses into different sequences of behavior, and their dependence on sexual experience may occur because the brain regions that control them are largely interconnected. For example, following copulation, Fos protein has been observed in the accessory olfactory bulbs, NAcc, ventrolateral sepum, mPOA, PdPN, BNST, paraventricular nucleus of the hypothalamus (PVN), ventromedial nucleus of the hypothalamus (VMH), ventral premammillary nucleus, MEApd, amygdalohippocampal area, subparafascicular thalamic nucleus (SPFp), VTA, and central tegmental field (CTF) (Baum & Everitt, 1990; Coolen, Peters, & Veening, 1997; Coolen, Peters, & Veening, 1996; Pfaus & Heeb, 1997). Many of these regions

concentrate steroid hormones and receive a variety of sex-related sensory inputs, including olfactory and genitosensory (Baum & Everitt, 1991; Coolen, Peters, & Veening, 1997; Coolen, Peters, & Veening, 1996; Kollack & Newman, 1992). These regions are also interconnected either directly or through diffuse pathways, such as the stria terminalis (Bracket & Edwards, 1984; Coolen & Wood, 1999; Edwards, & Einhorn, 1986; Meisel & Sachs, 1994). Some of these sites are activated together by specific sensory stimulation (e.g., activation of the lateral region of the MEApd, posteromedial nucleus of the BNST, PdPN, and SPFp by ejaculation; Coolen, Peters, & Veening, 1997; Heeb & Yahr, 1996). However, adjacent regions are activated by olfactory stimuli (e.g., activation of the MEApd by anogenital investigations; Lehman, Winans, & Powers, 1980), suggesting that sensory information converges in these regions. Such convergence may be the root of the interdependence of behavioral outputs controlled by olfactory or genitosensory inputs, including mounts, intromissions, and ejaculations. Some of these regions are known from lesion studies to facilitate copulation. The role of gonadal hormones in their activation may be to lower the threshold for activation by sensory inputs. Such an effect was observed electrophysiologically in the mPOA by Pfaff and Pfaffmann (1969), and is consistent with Whalen's (1966) idea that hormones "set the stage" for sexual responding by augmenting arousability (Pfaus, Kippin, & Centeno, 2001).

Findings from Chapter 2 demonstrated an increase in the number of Fos cells in the BNSTpm, PDPN, MEApd, and anterior VTA of TP-treated, long term castrated male rats following multiple ejaculations. An increase in Fos cells was also observed in the BNSTpm and MEApd of TP-treated, long term castrated male rats following visual and

olfactory stimuli alone. Anatomical studies in rats have shown the projections of the BNST to the mPOA (Chiba & Murata, 1985; Paxinos & Watson, 1986; Saper, Swanson & Cowan, 1979; Simerly & Swanson, 1986). In hamsters, many studies have demonstrated that the caudal MEA projects via the stria terminalis to both the mPOA and BNST (Wood & Newman, 1995). Subsequent experiments have shown that the BNST, but not the mPOA, also receives input from the rostral MEA via a non-striatal, ventral amygdala-fugal pathway (Kevetter & Winans, 1981; Lehman & Winans, 1983). The importance of the mPOA, BNST and MEA on male copulation has been demonstrated previously by lesion studies. Lesions of the MEA abolish male hamster copulatory behavior and severely reduce chemosensory investigation of females and their odors, by diminishing the male's sniffing and licking of the female hamster's anogenital region (Lehman, Winans, & Powers, 1980). In male rats, lesions of the corticomedial amygdala reduce their ability to ejaculate, except after many intromissions (Harris & Sachs, 1975). On the other hand, male hamsters with BNST lesions displayed significant reductions in their chemoinvestigatory responding even though the majority of them continued to copulate normally (Powers, Newman, & Bergondy, 1987). In addition, lesions of the BNSTpm of inexperienced male rats results in a decrease in anogenital investigations of a female, and a slight increase in the first mount and intromission latency (Claro, Segovia, Gillamon, & Del Abril, 1995). The most drastic effects on male copulation are observed following mPOA lesions. Bilateral destruction of the mPOA eliminates copulation in males of many species, but does not alter the male's chemoinvestigation of female odors (Brackett, Iuvone, & Edwards, 1986; Everitt & Stacey, 1987; Powers, Newman, & Bergondy, 1987). The neural activation observed in the NAcc and mPOA following

mounts, intromissions, and ejaculations, and in the MEA and VTA following ejaculations, suggests that multiple brain regions coordinate the inputs from olfactory, visual, and genitosensory stimuli during male copulation.

The immunocytochemical results observed in this thesis following chemosensory stimuli were consistent with earlier reports by Baum and Everitt (1992), and Coolen et al. (1997); however findings form Chapter 2 provide additional information regarding the facilitative role of TP in the expression of appetitive behaviors. Following 12-24 days of TP administration, a significant increase in certain appetitive behaviors (i.e., nosepokes, climbs, contacts, and grooming) in sexually experienced long term castrated males was observed. These findings suggest that some amount of circulating T is neccessary for the reinstatement of these particular behaviors, indicating that the mechanism of action underlying the statement of these specific effects depends on T and/or its metabolites. Furthermore, these findings suggest an interaction between learning (i.e., sexual experience) and gonadal hormones during male copulation. Repeated exposure to receptive females prior to castration buffered the males partially from the disruptive effects of castration, allowing them to get aroused or excited by receptive females, and resulting in the expression of certain sexual behaviors for a longer period of time.

Following visual and olfactory stimulation, males in Experiment 3 of Chapter 2 showed a distribution of activated neurons in the medial region of the MEApd close to the optic tract. This MEApd subdivision is known to correspond to the distribution of afferent fibers relaying vomeronasal input from the AOB (Kevetter & Winans, 1981; Lehman & Winans, 1982; Scalia & Winans, 1975). An accumulation of Fos within the lateral part of the MEApd in TP-treated long-term castrated males displaying

consummatory behaviors was also observed (Baum & Everitt, 1992; Centeno, Jaques, & Pfaus, 1996; Coolen, Peters, & Veening, 1997). Fos activation in the 'medial' region of the MEApd has been shown previously in male hamsters exposed to female hamster vaginal secretions (Fiber, Adams, & Swann, 1993), and in male rats allowed to display anogenital investigations of an estrous female (Coolen, Peters, & Veeing, 1997). In addition, a decrease in such neural activation been shown following the removal of the vomeronasal organ in male golden hamsters (Fernandez-Fewell & Meredith, 1994), and in male rats following unilateral lesions of the olfactory pudencle (Baum & Everitt, 1992). Thus, findings from Chapter 2 contribute to the hypothesis that the 'medial' part of the MEA is involved in the processing of chemosensory signals, whereas the 'lateral' part of the MEA is involved in the processing of the genital sensory inputs.

The increase in Fos cells observed in the BNSTpm of TP treated castrated male rats exposed to odors or that had direct contacts through the screen with an inaccessible receptive female, is also consistent with previous studies (Baum & Everitt, 1992; Coolen, Peters, & Veeing, 1997). Fos activation in BNSTpm by odors or contacts with an inaccessible receptive female remained the same following removal of the vomeronasal organs or following lesions of the olfactory pudencle (Fernandez-Fewell & Meredith, 1994; Baum & Everitt, 1992). The BNSTpm has reciprocal connections with both the MEApd and the MPN, the latter of which is known to receive olfactory and genital sensory input (Canteras, Simmerly, & Swanson, 1992; Canteras, Simmerly, & Swanson, 1995; Turner & Herkenham, 1991), suggesting the involvement of the BNSTpm in the processing of chemosensory and genital sensory inputs coming from the MEApd and MPN.

Role of the VTA

The role of the VTA in male copulation was examined in Experiment 2 of Chapter 3. The role of sexual experience on male copulation following VTA lesions were also considered in this chapter. Lesions of the VTA decreased the mean number of anogenital investigations and pursuits in both sexually experienced (i.e., 10 tests with receptive females prior to lesions) and less experienced (i.e., 1 test with receptive females prior to lesions) male rats, whereas the mean number of intromissions and ejaculations was only decreased in males with 1 prior experience. This decrease in sexually inexperienced, relative to experienced males suggests that sexual experience strengthens the neural projections from the VTA to its cortical, limbic, and hypothalamic targets to produce a full complement of appetitive and consummatory sexual behaviors.

Neural connections with the VTA. The neural systems that might activate the VTA in response to sensory cues of receptive female are presently unknown. Figure 52 shows a schematic representation of possible neural mechanisms within the VTA during male copulation. These neural mechanisms are discussed in the following sections. Recent retrograde studies using Fluoro-Gold injections into the VTA have shown retrograde labeling in the mPOA, NAcc, lateral preoptic area, BNST, and rostral lateral hypothalamus (Zahm, Grosu, Williams, Qin, & Berod, 2001; Zahm, Williams, Latimer, & Winn, 2001). These findings have shown the direct connection between brain areas involved in the expression of male sexual behaviors and the VTA, suggesting the existence of a mechanism of action underlying the act of male copulation in the VTA.

The mPOA is essential for male copulatory behavior in all vertebrate species that have been studied, from fish to primates (reviewed in Meisel & Sachs, 1994). As with the

NAcc, extracellular DA is increased in the mPOA of male rats during copulation (Hull, Eaton, Moses, & Lorrain, 1993) and falls precipitously after each ejaculation (Blackburn, Pfaus, & Phillips, 1992). Given that mesolimbic and incertohypothalamic DA systems are neuroanatomically distinct, it has never been clear why DA release in these two terminal regions follows a nearly identical pattern. However, stimulation of DA receptors in the two regions contributes differentially to male sexual behavior. In the NAcc, DA appears to facilitate appetitive sexual behaviors, such as bar pressing for a sexual incentive (Everitt, 1990), or conditioned level changing (Pfaus & Phillips, 1991), and the initiation of copulatory behavior (Everitt, 1990; Pfaus & Phillips, 1991). Stimulation of mPOA DA receptors facilitates genital reflexes required for mounts, intromissions, and ejaculations (Hull, Lorrain, & Matuszewich, 1986; Pehek, Warner, Bazzett et al., 1989), and certain aspects of sexual motivation (Moses, Loucks, Watson, Matuszewich, & Hull, 1995; Warner, Thompson, Markowski et al., 1991). Although lesions of the mPOA abolish copulatory behavior and decrease certain appetitive behaviors such as maze running, they do not affect the proportion of males showing anogenital investigation and pursuit or attraction to female odors (Edwards & Einhorn, 1986; Hansen, Kohler, Goldstein, & Steinbusch, 1982; Powers, Newman, & Bergondy, 1987). In contrast, lesions of the NAcc reduce non-contact erections and the proportion of males that copulate to ejaculation (Kippin, Sotiropoulos, Badih, & Pfaus, 2000). Thus, the combined action of DA in these two regions appears to be critical for the activation and coordination of both appetitive and consummatory sexual behaviors (Hull, Lorrain, Matuszewich et al., 1998; Pfaus, 1996; 1999).

The mPOA receives chemosensory-related inputs from the BNST, which has been

recently shown to receive projections from the VTA (Komisaruk, Rosenblatt, Barona et al., 2000), and where an accumulation of Fos was observed following exposure to odors from a receptive female placed behind a screen (see Chapter 2). In addition, the mPOA projects directly to the VTA (Mogenson & Yang, 1994; Swanson, 1982), although the nature of these projections and their reciprocity remain unknown at present. If such projections were GABAergic, they could have two possible effects on DA output from the VTA (Figure 52). First, they could terminate directly in DA cell bodies or dendrites, and inhibit DA cell firing, leading to a decrease in DA release in terminal regions such as the NAcc. Second, they could terminate on GABA interneurons and disinhibit DA cell firing. A similar mechanism for opioid control of DA release was proposed by Devine (1993) and features opioid axons that make contact with GABA terminals. Activation of μ-receptors inhibits GABA release and thereby disinhibits DA cell firing. Both of these mechanims would activate DAergic neurons in the VTA during copulation, and might underlie the release of DA in the NAcc. The opioid mechanims, in particular, could explain the ability of intra-VTA infusions of morphine to faciliate mounting in sexually sluggish males (Mitchell & Stewart, 1989), or intra-VTA infusions of naloxone to inhibit conditioned level changing (van Furth & van Ree, 1996).

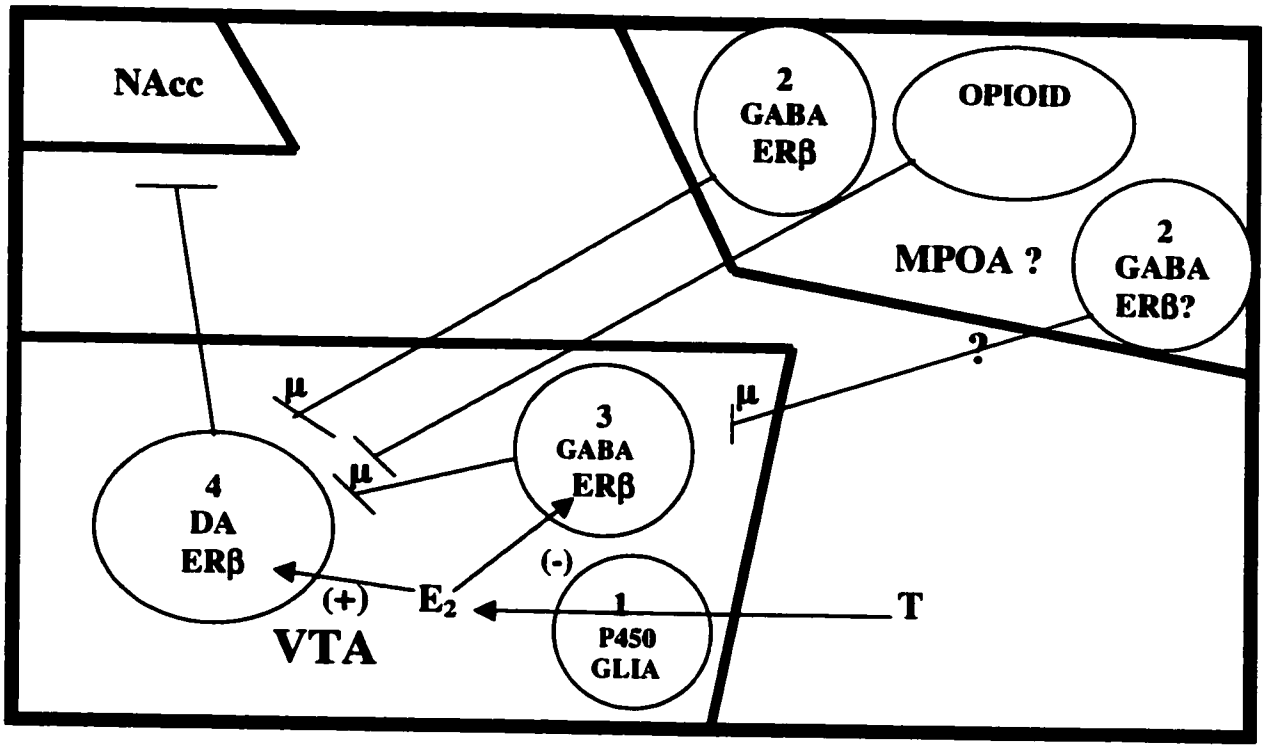


Figure 52. Schematic representation of the VTA. Proposed mechanism of action underlying the effects of E or T on male copulation involving GABAergic and DAergic neurons, opioids, and ER-β. In the VTA, 1) the effects of T on male copulation might involved its conversion to E by the aromatization process within glial cells. E might act at the ER-β within the cell bodies of GABAergic and DAergic neurons: 2) activation of ER-β in GABAergic neurons could decrease the GABA-mediated inhibition of long axon GABAergic afferents presynaptic to the DAergic neurons, increasing the activity of mesolimbic DA neurons; or decrease GABA-GABA inhibiton on GABA interneurons, resulting in a net disinhibition of mesolimbic DAergic neurons; 3) E might act on ER-β within the cell bodies of GABA interneurons decreasing the inhibition on DA neurons, also resulting in a net disihibition of mesolimbic DAergic neurons; 4) E might also act on the cell bodies of DA neurons increasing DA levels in brain areas such as the NAcc. Location of μ-receptors on GABAergic terminals presynaptic to GABA interneurons, on GABA interneurons, and on long axon GABAergic afferents presynaptic to the DAergic neurons.

Role of DA and GABA within the VTA in Male Copulation. The increase in Fos-stained cells observed within the BNSTpm, mPOA, and the MEApd, and the VTA following different sexual behaviors raised questions regarding the identity of the cells being activated (e.g., DA or GABA) and the possible sensory mechanisms underlying their statement. Results from Experiments 1A and 1B of Chapter 3 showed an increase in the number of Fos-TH cells but a decrease in the number of Fos-GABA cells in the anterior VTA following two ejaculations. The increase observed in Fos-TH cells was also found after multiple intromissions during the second ejaculatory series. Although the temporal resolution of electrophysiological activation of TH-containing cells within anterior VTA cannot be determined from these studies, findings from these experiments confirm the activation of DA containing cells following intromissions during the second ejaculatory series and following 2 ejaculations. Similarly, these studies cannot determine the temporal resolution of electrophysiological activation in GABA neurons within the posterior VTA, but can confirm the decrease in activation of GABA neurons following 2 ejaculations.

The neural inhibition exerted synaptically by GABAergic neurons at all levels of the brain is a critical regulatory process in the organization and control of behavior. Roberts (1986) argues that structures possessing GABAergic projection neurons, such as the cerebellar cortex, globus pallidus, substantia nigra pars reticulata, and reticular nucleus of the thalamus, act as coordinative command centers restraining excitatory output systems. A second source of inhibition comes from local GABAergic interneurons that are found in the cerebral cortex and hippocampus. Therefore, within GABA-GABA connections, behavioral activation occurs as a result of disinhibition of excitatory

pathways (Roberts, 1986). The underlying mechanism of action of GABA neurons and behavioral responses can be explained by the undergoing process when a cat encounters a mouse described in Roberts' study (1986): "When a mouse is first seen, processing of sensory input in the cat's corpus striatum leads to a suppression of inhibitory GABAergic activity from the globus pallidus and substantia nigra to the superious collicullus, thereby permitting collicular neurons to help direct visual tracking of the prey. At the same time neurons in the thalamic motor nuclei and their projection areas are disinhibited so as to allow the movements required for stalking, killing, and ultimately consuming the mouse. The cerebellum also participates in this process by modulating fine motor activity" (p.105).

GABA neurons have also been shown to act as inhibitors in the regulation of copulatory behavior (Fernandez-Guasti, Larsson, & Beyer, 1986; Paredes & Agmo, 1992). Bicuculline, a GABA antagonist, when injected into the mPOA of male rats shortens the animals' ejaculation latency and PEI (Fernandez-Guasti, Larsson, & Beyer, 1986). In addition, injections of muscimol, a GABA agonist, to the mPOA decreases the number of intromissions and ejaculations (Fernandez-Guasti, Larsson, & Beyer, 1986). These data suggest that activation of GABA transmission in the mPOA inhibits copulation. A similar mechanism might exist within the VTA.

The increase observed in Fos-TH cells and the decrease in Fos-GABA cells in the anterior VTA following two ejaculations, is consistent with a potential dishinbition of DA neurons in the VTA when GABA neurons are not active. In the posterior VTA, a decreased activation was observed in the number of Fos-TH cells and an increase in Fos-GABA cells following one ejaculation. These findings suggest that within the anterior

and posterior VTA intromissions and ejaculations activate DAergic and GABAergic systems in opposite ways. However, these results do not determine which brain areas are responsible for the activation of GABA neurons during male copulation. Retrograde techniques in combination with Fos could be used to determine the brain areas that are activated during specific aspects of male copulation and the nature of the cells projecting back to the VTA. Fluorescence microscopy is used to show extensive filling of perikarya and distal dendrites following injections of Fluoro-Gold (FG) into their terminal fields. FG injections in combination with immunocytochemical labeling using silver-intensified 1nm colloidal gold (immunogold-silver labeling method) has been used to detect the projections from the VTA to the NAcc, and to identify GABA labeled neurons within these projections (Van Bockstaele, Wright, Cestari, & Pickel, 1994). Such techniques could be used to determine the specific brain regions that activate TH or GABA neurons in the VTA.

Effects of Hormones in the VTA on Male Copulation

An increase in the proportion of sexually experienced long term castrated males displaying mounts, intromissions and ejaculations was observed following bilateral VTA implants of E or T. These results were observed four weeks (test sessions 7-8) following E cannula implants (i.e., 28-32 days after hormone implant) and three weeks (test sessions 5-8) following T cannula implants. A rapid decline of all consummatory behaviors was subsequently observed by test sessions 13-14. The behavioral effects on copulatory behaviors in castrated male mice following T implants in the VTA were observed 2 weeks after the implantation procedure (Sipos & Nyby, 1996). The time difference observed for the reinstatement of behaviors might be explained by the time

that passed after castration prior to the implantation of the hormone. That is, in previous studies, males were castrated three weeks prior to receiving hormone implants, whereas males in Experiment 2 of Chapter 3 were castrated and tested with receptive females for a period of 13-15 weeks prior to undergoing hormone implantation. Findings from Chapter 1 demonstrated that experienced castrated male rats can take up to 13 weeks to show a complete decline of consummatory behaviors.

There was no evidence of hormone leakage from the brain into circulation as measured by seminal vesicle weights. Furthermore, previous studies using radiolabelling and receptor-immunocytochemistry techniques have shown the radiolabelled hormone binding 1 mm from the cannula tip (Palka, Ramirez, & Sawyer, 1966; Sipos & Nyby, 1996; Smith, Damassa, & Davidson, 1977; Wood & Newman, 1995), which also suggests that hormones did not leak to brain areas near the VTA. Overall, these findings suggest that the behavioral effects of the intracranial hormone implants observed within this thesis must be accounted for by their effects inside the VTA

The present results clearly indicate that E implants to the VTA are sufficient to induce mounts in 75%, intromissions in 50%, and ejaculations in 20% of the males without significant stimulation of penile size or morphology. In contrast, T implants facilitated mounts, intromissions, and ejaculations in 20% of the males. Daily systemic injections of E (100µg/day) have been shown to restore ejaculation in 30% of castrated males (Hart, 1974; Paup, Mennin, & Gorski, 1975). The proportion of castrated males displaying ejaculations following administration of E increases to 100% when E is combined with DHT (Baum & Everitt, 1992; Larsson, Sodersten, & Beyer, 1973) (for details see General Introduction). Therefore, the lack of penile growth could account for

the lack number of intromissions and ejaculations observed only after test sessions 7-8, and also for the lower percentage of males ejaculating (i.e., less than 100%). It is possible, that bilateral implants of T or E in the VTA in combination with daily injections of DHT could accelerate the return of copulatory behaviors in long-term castrates. However, these results might also suggest that for the reinstatement of male copulatory behaviors activation of AR or ER in the VTA needs to be complemented by the activation of steroid hormones receptors in other brain sites (i.e. BNST or MPOA). Sipos and Nyby (1996) implanted T in the MPO and the VTA of castrated male house mice and found that this combined activation was necessary for the restoration of male behaviors, such as mounting and urine preference. It is possible that combined hormone implants to the VTA and the mPOA are neccesary for the complete restoration of male copulation in long-term castrated rats.

Mechanism of Action of T and/or E in the VTA on Male Copulation

The facilitative role of T in the VTA during male copulation was demonstrated in this thesis. Sipos and Nyby (1996) suggested possible mechanisms by which androgen action in the VTA could maintain male copulatory behavior. Most of these mechanisms related to the possibility that T maintains the functioning of DA cells in the mesolimbic pathway necessary to support sexual performance. Several studies have investigated the effects of castration on DA concentrations within mesolimbic terminals, showing a decrease in DA levels at the presynaptic terminals in the NAcc and a return to pre-castration DA levels following T treatment (Alderson, & Baum, 1981; Baum, Melamed, & Globus, 1986; Mitchell & Stewart, 1989). The facilitative effects of T on DA release have also been examined in the mPOA of castrated and T treated male rats (Hull, Lorrain,

& Matuszewich, 1995). It has been suggested that T may facilitate copulation by allowing the release of DA in the mPOA of males in response to cues (odors?) from receptive females. More specifically, it has been suggested that the role of T is to up-regulate nitric oxide (NO), which then enhances basal DA concentrations presynaptically (Hull, Du, Lorrain, & Matuszewich, 1994) and DA release in response to pheromonal or copulatory stimulation. A similar mechanism of action of T could occur within the VTA. Given the effectiveness of E in the present study, an additional mechanism for the effects of T observed within the VTA might be its conversion into E by the enzyme aromatase. In the mPOA T gets converted into E by aromatization, and E is the critical hormone that stimulates copulation in this region (Luttge, 1975; Luttge, Hall, Wallis, & Campbell, 1975). It is known that the VTA contains aromatase within glia cells in the form of P-450 (Garcia-Segura, Wozniak, Azcoitia et al., 1999; Poletti, Negri-Cesi, Melcangi et al., 1997). Therefore, it is possible that once T is converted into E, it could bind to ER (in the form of ER-β) in cells of the VTA (Figure 52).

A final explanation for the action of gonadal steroids within the VTA is via a nongenomic mechanism; that is, T or E might act through membrane receptors in the VTA. Evidence has shown that progesterone acting on $GABA_A$ benzodiazepine receptor complex in the VTA facilitates females' hamster receptivity (Frye & Debold, 1993; Frye & Leadbetter, 1994; Frye, Mermelstein, & Debold, 1992). It is also known that some T metabolites, synthesized by glia cells, are $GABA_A$ agonists (e.g., dianabol) (Bitran, Kellog, & Hilvers, 1993; Bitran, Hilvers, Frye, & Erskine, 1996). A similar nongenomic mechanism for T could exist in the VTA, making T metabollites operate as agonists of $GABA_A$ receptors. However, our findings suggest a genomic mechanim of action given

that the increase on the number of male sexual behaviors following E or T implants was not observed immediately but required at least 28 days of implantation. In addition, if T metabolites were acting as GABA agonists, then a decrease in sexual behaviors should have been observed.

The effects of E on male copulation could occur by acting on ER-β located on GABA or DA neurons, with E possibly having oppossite effects within these two cell populations. Recent evidence has identified ER-β within the VTA, demonstrating that ER-β mRNA is translated into the immunoreactive protein within this brain region (Shughrue & Merchenthaler, 2001). These data suggest that the effects of E on male copulation could take place within the cell nucleus of neurons in the VTA. The disinhibition of mesolimbic DAergic neurons by E, following the activation of ER-β within the cell bodies of the GABAergic neurons in the VTA, would result in increased DA tone in areas such as the NAcc. The excitatory effects of E on DA release in the striatum and NAcc have already been shown (Becker, 1990; Becker & Beer, 1986; Mermelstein & Becker, 1995; Xiao & Becker, 1998). In females, ovariectomy decreases striatal DA release and turnover, and E systemic replacement restores the response to that of the intact female in estrus (Becker, 1990; Becker & Rudick, 1999; Mermelstein & Becker, 1995). An enhanced increase in NAcc DA is observed in ovariectomized E-treated females, compared to oil-treated animals (Mermelstein & Becker, 1995). In addition, castrated male rats showed a significant increase in striatal D2 DA receptor-binding in rostral striatum 4 hours after an injection of E (Bazzet & Becker, 1994).

A second possibility is that E modulates the observed DAergic responses by binding to ER-β within DA cells and upregulating enzymes involved in DA release, such

as nitric oxide synthase (NOS) or TH, the rate-limiting enzyme for DA synthesis. As mentioned above, evidence for a mechanism of action involving T and DA synthesis within the mPOA has been proposed, suggesting the role of T in increasing NO levels, which been shown to promote DA release (Du & Hull, 1999). Ovariectomized female rats have shown an increase in neuronal NOS mRNA in the VMN, which is known to contain ERs, following E treatment (Ceccatelli, Grandison, Scott, Pfaff, & Kow, 1996), which might also occur in E treated castrated males. Finally, recent evidence from single unit recordings of DA neurons in the substantia nigra performed concurrently with striatal microdialysis techniques, has demonstrated that striatal NOS signaling regulates the neuronal activity of midbrain DA neurons by increasing DA cell population firing rate (West & Grace, 2000). E has been shown to act via ER-β within serotonin neurons in the raphe to increase the statement of tryptophan hydroxylase, leading to increased-serotonin sysnthesis (Lu, Shlaes, Gundlah et al., 1999). Findings from a double label immunocytochemistry study have identified ER in TH cells within the VTA (Kritzer, 1997). Therefore, it is possible that E might act within DAergic neurons in the VTA by, increasing the statement of TH and thereby increasing DA synthesis. Future studies need to be done to examine the effects of E on mesolimbic DA systems release and turnover during male copulation.

Although the existence of ER-β within the GABAergic neurons of the VTA is unknown at present, a recent study using combined application of cell culture and double-label immunocytochemistry has demonstrated the existence of ER-β within cell bodies of GABAergic neurons in the suprachiasmatic nucleus of neonatal rats (Su, Qiu, Zhong, & Chen, 2001). These recent findings demonstrated the existence of ER-β within cell bodies

of GABAergic neurons in the suprachiasmatic nucleus and raised the possibility of finding ER-β within the cell bodies of GABAergic interneurons in the VTA. It is also possible for ER-β to be found in the cell bodies of the GABA interneurons and DA neurons in the VTA, where E might disinhibit GABA input on DA neurons, or might facilitate DA release depending on the location of the receptors. Additional studies should used double-label immunocytochemistry to investigate the existence of ER-β within the cell bodies of GABAergic and DAergic neurons in the VTA.

Conclusions

For successful copulation to occur males need to respond to hormonal and neurochemical signals that determine their own sexual desire and arousal, to recognize external stimuli, to identify chemosensory cues or behavioral patterns of potential sex partners, and to pursue sex partners once sexual contact has been solicited. The facilitative effects of sexual experience on male copulation have been shown previously (Larsson, 1956; Dewsbury, 1969). The recognition of potential sex partners and the initiation of certain mating strategies becomes automated following repeated exposure to sexual stimuli (i.e., sexual experience) and especially if such exposure leads to sexual reward (produced by intromissions and/or ejaculation; Whalen & Beach, 1961; Kippin & Pfaus, 2001).

Gonadal hormones appear to facilitate males' levels of arousability, allowing males to become aroused or motivated by sexual incentives, and acting as the initial "switch-on" mechanism for males to initiate contact with receptive females. The behavioral differences observed between experienced and inexperienced males following disruptive treatments such as castration or brain lesions can be explained by a proposed

interaction between sexual experience and gonadal hormones (Pfaus, Kippin & Centeno, 2001). It is known that sexual experience facilitates male copulation, and to some extent buffers sexual behavior from the effects of disruptive treatments such as castration. Following repeated exposure to receptive females, the acquired behavioral patterns become automated and males' expectations of normal performance during copulation might compensate for the initial loss of hormonal stimulation.

In summary, sexual experience facilitates the overall performance of appetitive and consummatory sexual behaviors (i.e., "what males can do"), whereas gonadal hormones facilitate males' arousability (i.e., "what males want to do"). The findings presented in this thesis provide evidence for a facilitative role of the VTA in the expression of appetitive and consummatory male sexual behaviors. Although speculative, the VTA may integrate hormonal signals necessary for sexual arousability, and neural signals from other sensory relays, to facilitate DA release crucial for incentive sexual arousal.

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