Effects of Testosterone on Female Rat Sexual Behavior

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ABSTRACT

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Estrogen and progesterone are ovarian steroid hormones involved in the initiation and maintenance of female sexual behaviors. Recent research suggests that testosterone (T) is also important in sexual behavior, particularly in the restoration of libido in surgically and naturally menopausal women when administered with estrogen (E). The effect of T on the female sexual behavior of aged intact, and young ovariectomized rats has been poorly described in the current literature, despite varying reports of facilitative and inhibitory effects. The goal of the present thesis was to examine the actions of E, T, or their combination, in the restoration of sexual activity at a time when ovarian function is altered either through aging or surgical removal of the ovaries. The effects of both acute and chronic administration of these hormones on sexual behavior were observed. The results of these experiments suggest that the combined action of E with T may facilitate sexual behaviors in young ovariectomized female rats, to levels equivalent to optimal priming with E and progesterone. When varying hormone regimens were administered to aged intact animals, by phasic subcutaneous injections every four days, there was no effect on their sexual behavior acutely, whereas chronic T and E+T resulted in an attenuation of sexual behaviors. When a continuous release of T was administered to aged intact female rat by subcutaneous capsule, sexual behaviors were initially facilitated, but the effect dissipated over time. The mechanism underlying this process is suggested.

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Dedication

I would like to dedicate this thesis to my Mother and Father, the two most important role models in my life, who supported me throughout my life and shared in my successes as well as my hardships. I would like to thank you both for teaching me how to be strong. Without strength, I could not have gotten through all the work presented in this thesis. Thank you and love you both; and miss you deeply Dad....

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Introduction

Sex steroid hormones have traditionally been categorized as either male or female, where androgens are considered male sex steroids since they have masculinization effects, and estrogens as female sex steroids because of their importance in the estrous cycle. Testosterone is an androgen viewed as a male steroid hormone however both the adrenals and ovaries produce it in females (Belanger, Cusan, Caron, Barden, & Dupont, 1981; Hall, Leathard, & Coley, 2001). Testosterone (T) is a precursor to both estradiol (E2) and the androgen dihydrotestosterone (DHT). Testosterone can bind to the androgen receptor either directly, or through its reduction to DHT, and its aromatization to E2 allows binding to estrogen receptors (ERα or ERβ). Steroid hormone binding globulins (SHBG) are proteins that bind steroid hormones and render them inactive, and these proteins have a higher affinity for androgens such as T than they do for E2 (Bachmann et al., 2002).

In recent years, the role of T in female sexual desire stimulated much interest, particularly with respect to menopausal women. As women enter menopause the ovaries, which produce estrogens (E), progesterone (P) and androgens such as T, begin to decline in their functioning. This reduction in functioning leads to diminished E and P levels. In contrast, T declines between the ages of 21 and 40 but remains relatively stable throughout the menopausal transition (Burger, 2008).

Menopause can be surgically induced by the removal of the ovaries and/or uterus, as a treatment for hormone-related cancers or heavy bleeding during menses that can trigger hemorrhages. These women experience an abrupt decline in E, P as well as T (Alexander, Dennerstein, Burger, & Graziottin, 2006; Henderson & Sherwin, 2007;

Kingsberg, 2007; Nathorst-Boos, von Schoulttz, & Carlstrom, 1993). An American based study, WISHeS, found that 9% of naturally postmenopausal women and 26% of surgically menopausal women experience low desire (Alexander et al., 2006; Leiblum et al., 2006).

In both naturally and surgically menopausal women reductions in E and P lead to numerous physiological disruptions and impairments such as fluctuations in mood, loss of skin elasticity, decreased bone density, vaginal dryness, thinning of the vaginal wall as well as psychological deficits such as impaired memory and loss of sexual desire (Dennerstein, Guthrie, Hayes, DeRogatis, & Lehert, 2008; Sherwin, 1994; 1996). The majority of these symptoms can be alleviated with estrogen replacement therapy (ERT) and as such, their occurrence has been attributed to the decline in ovarian E2 production.

Unfortunately, the loss of sexual desire experienced following menopause, is not restored with E alone, or with a regimen of E and P (Sherwin, 1991; 2008). Moreover, the addition of P to ERT can have a negative impact on mood including increased distress, depression, and dementia (Natale, Albertazzi, Zini, & Di Micco, 2001; Nilsen & Brinton, 2002; Sherwin, 1991; 2008). However, the addition of T to ERT can ameliorate symptoms of low libido in both surgically and naturally menopausal women (Burger, Hailes, Menelaus, Nelson, Hudson, & Balazs,1984; Burger, Hailes, Nelson & Menelaus, 1987; Sherwin & Gelfand, 1987; Sherwin, Gelfand, & Brender, 1985). Furthermore, premenopausal women with low androgen levels also report low libido, lack of energy and reduce feelings of well-being, despite adequate E levels, in a syndrome known as Androgen Insufficiency Syndrome (Bachmann et al., 2002). Androgen replacement therapy has been shown to restore libido in these women (Bachmann et al., 2002). The

mechanism underlying how the combined actions of Es and androgens work together to improve desire, however, has yet to be determined.

Studies in nonhuman primates have also shown that androgens can facilitate female appetitive sexual behaviors, defined as female initiated sexual interactions (Beach, 1976). Work in ovariectomized (OVX) rhesus monkeys has found that testosterone propionate (TP) increases appetitive behaviors, both through intramuscular injections (Wallen & Gow, 1977), and by its administration to the preoptic area of the hypothalamus (POA; Everitt & Herbert, 1975).

Testosterone has been shown to effect female sexual function in the rat, both in terms of reproductive organs and sexual behaviors. Studies dating back to the 1930s, show that TP is important in maintaining reproductive organs (Baum, Sodersten, & Vreeburg, 1974; Korenchevsky Dennison, & Hall, 1937; Traish, Kim, Min, Munarriz, & Goldstein, 2002; Traish, Kim, Stankovic, Goldstein, & Kim, 2007; Whalen & Hardy, 1970) and sexual behavior (Baum, Sodersten, & Vreeburg, 1974; Butler, Mills, & Bloch, 2001; de Jonge, Eerland, & van de Poll, 1986; Fernandez-Guasti, Vega-Matuszczyk, & Larsson, 1991; Gladkova & Karpenko,1986; Whalen & Hardy, 1970).

Testosterone as a bisexual hormone

With the emergence of sexual dimorphism in regions of the brain that control sexual behavior and reproduction (e.g., the mPOA; Gorski, Gordon, Shryne, & Southam, 1978; Hines, Davis, Coquelin, Goy, & Gorski, 1985; Jacobson & Gorski, 1981), androgens became popularized as "male sex steroids" and Es as "female sex steroids" (e.g. Machowska, Szlachcic, Pawlik, Brzozowski, Konturek, Pawlik, 2004). This

"gender-specific" role of sex steroids developed, despite work showing that E is important in male sexual behavior (Clancy, Zumpe, & Michael, 2000; Roselli, Cross, Poonyagariyagorn, & Stadelman, 2003) and that androgens are important in the development of female reproductive organs (Korenchevsky, Dennison, & Eldridge, 1937; Korechevsky & Hall, 1937).

Korenchevsky's group published an extensive paper describing differential effects of various hormones on male and female reproductive tissue, classifying certain androgens (i.e. androsterone, TP, androstenediol) as *partial bisexual hormones*, and T as a *true bisexual hormone*, affecting both male and female tissues. The only *pure hormone* that was listed was P, as a *purely 'female' hormone* as it affected only female tissue (Korechevsky & Hall, 1937). When administering TP to OVX females, TP alone and in combination with E increased uterine weight and hypertrophy of the vagina and clitoris (Korenchevsky, Dennison, & Eldridge, 1937).

Androgens have also been shown to stimulate both appetitive and consummatory sexual behaviors such as lordosis (a reflexive arching of the back by the receptive female in response to flank stimulation) in female rats. However the goal of most of these studies was to test their effects in combination with P, or to test the effects of hormones on mounting (a male sexual behavior). And although some studies have found that TP increases appetitive sexual behaviors (Fernandez-Guasti, Vega-Matuszczyk, & Larsson, 1991; Gladkova & Karpenko,1986), these studies have not been followed up. Furthermore, the route and frequency of the androgen administration were inconsistent from one study to the next.

Whalen and Hardy (1970) showed that T can induce lordosis by the daily subcutaneous injection of TP (in doses of 100 μg, 500 μg, or 1000 μg) to OVX female rats, but that this effect was further facilitated by a P injection given 4 hours prior to the test. Butler et al. (2001) administered TP via subcutaneous capsules and primed females with EB for 3 consecutive days, up to 29 hours prior to behavioral testing. Their results showed that TP when administered with estradiol benzoate (EB) induced similar lordosis reflexes as seen in fully primed EB+P treated animals. However the purpose of their study was to investigate the inhibition of lordosis in *male* rats following androgen and P treatment. Consequently, no further investigations into this effect were made.

Satou and Yamanouchi (1998) reported that TP dose-dependently induced lordosis in OVX female rats when administered 48 hours prior to the test, and that P could inhibit TP's effect when administered 4 hours prior to a sexual behavior test. Others have found that TP induced female sexual behaviors when given alone or in combination with P (de Jonge, Eerland, & van de Poll, 1986). In the latter study, TP was administered daily for 14 days prior to the sexual tests and in contexts that had been shown to stimulate aggressive behaviors (de Jonge, Eerland, & van de Poll, 1986; van de Poll, van Zanten, & de Jonge, 1986).

A few studies have examined the effects of androgens (i.e. TP, dihydro-testoterone, dihydrotestosterone propionate) on female rat mounting and receptive behaviors. Baum, Sodersten and Vreeburg (1974) studied the effects of these androgens on mounting behavior in the female to further understand the mechanisms controlling mounting in the male (Baum, Sodersten, & Vreeburg, 1974). The authors used a 200 μg dose of TP, by daily subcutaneous injection (6 hours prior to behavioral testing), and

among various results, they reported low levels of receptivity in all TP-treated females in the presence of a stimulus male. The aim of the study however, was to study mounting behaviors induced by androgens, and consequently, the finding of TP-induced female receptivity was not followed up.

Donahich and Clemens (1983) found that dihydrotestosterone (DHT) antagonized E-activated lordosis in the female rat complementing work by Erskine (1983), which suggested that DHT decreases the duration of female behavioral receptivity. Others have shown that anabolic androgenic steroids inhibit EB-induced sexual receptivity in OVX female rats treated with EB+P (Blasberg & Clark, 1997). However, not all compounds had this effect, for instance, testosterone cypionate did not inhibit lordosis, and the authors failed to investigate the effects of these androgens on appetitive behaviors. Subsequent studies did not investigate the effects of testosterone cypionate, as they were interested in the compounds that inhibit EB-induced sexual behaviors (e.g. Blasberg, Robinson, Henderson, & Clark, 1998).

Interestingly, other groups have shown that certain androgens can facilitate appetitive behaviors in female rats that have been OVX and/or adrenalectomized (ADX). Gladkova and Karpenko (1986) reported that administration of androgens such as DHT and testosterone dipropionate to OVX female rats had no effect on lordosis, but elicited high levels of appetitive behaviors. Others have found that adding TP to OVX+ADX animals treated with EB+P resulted in significantly more appetitive behaviors (i.e. hops/darts and ear wiggling) compared to OVX+ADX animals treated with EB+P alone (Fernandez-Guasti, Vega-Matuszczyk, & Larsson, 1991).

Thus, the effects of T on the full display of female sexual behaviors in the rat have been scarce. The studies that have investigated the effects have been done so in a variety of contexts that do not necessarily favor the study of its direct effects on female sexual behaviors, and have often been designed to study the underlying mechanisms of male sexual behavior.

The ovulatory cycle and sexual behavior in the rat

In the female rat, sexual behaviors coincide in time with the period of high fertility (around the time of ovulation). The full display of appetitive and consummatory sexual behaviors in female rats is traditionally believed to depend on the sequential actions of E and P. These steroids activate protein synthesis in the hypothalamus and limbic system, two regions that underlie the activation of different neurochemical systems that excite sexual behaviors (Pfaff, 1999).

The hormonal profile of the ovarian cycle across species is quite similar, particularly around the time of ovulation (McCarthy & Becker, 2002). The ovulatory cycle refers to the cyclic release of hormones from the ovaries, which are stimulated by the pituitary hormones LH (leutinizing hormone) and FSH (follicle-stimulating hormone), and are under the control of GnRH (gonadotropin releasing hormone) released from the hypothalamus. Varying concentrations of the estrogen estradiol (E2) and P from the ovary feedback onto the hypothalamus, stimulating or inhibiting GnRH release. The cyclical release of steroid hormones over the estrous cycle has been described through steroid concentrations in the blood and ovaries. Butcher, Collins and Fugo (1974), and Nequin, Alvarez, and Schwartz (1979) have described the cyclical variations in plasma hormone and gonadotropin levels throughout the estrous cycle of the rat. In addition,

radioimmunoassays have been used to measure steroid hormones over the estrus cycle both in plasma and the ovary (Belanger, Cusan, Caron, Barden, & Dupont, 1981). Varying steroid hormone concentrations also affect the structure of vaginal epithelial cells.

In the rat, the phases of the estrous cycle (Diestrus, Proestrus, Estrus and Metestrus) can be monitored through the collection of vaginal epithelial cells (Hubscher, Brooks, & Johnson, 2005; Long & Evans 1922; Marcondes, Bianchi, Tanno, 2002). One cycle typically lasts 4-5 days in a young sexually mature animal. Animals on 5-day cycles either have an extra day of Estrus or Diestrus. During Diestrus, which lasts about 55-57 hours (Hubscher, Brooks, & Johnson, 2005), ovarian follicles have FSH but not LH receptors. The pituitary releases small amounts of FSH and LH at this stage, stimulating follicular growth. Low levels of E2 and P are released from the follicles at this stage (McCarthy & Becker, 2002). A Diestrus vaginal smear consists of mainly leukocytes (Marcondes et al, 2002). The Proestrus phase is characterized in a vaginal smear as a predominance of round nucleated epithelial cells, and lasts about 12-14 hours (Hubscher, 2005; Marcondes, 2002). As the follicle grows increasingly large, there is a sudden rapid increase in E2 release, which feeds back on the hypothalamus, stimulating GnRH release leading to elevated levels of FSH and LH from the pituitary. The follicles have LH receptors at this point and can respond to the characteristic LH surge. The increase in LH concentration sets the stage for the final maturation of the follicle. Progesterone also peaks during Proestrus, approximately 4-6 hours after the E2 peak (McCarthy & Becker, 2002). Interestingly, theca and granulosa cells isolated from rat ovarian follicles during Proestrus produce several androgens, including T (Belanger et al.,

1981; Fortune & Armstrong, 1977), and do so under the influence of LH (Fortune & Armstrong, 1977). This suggests that the ovaries secrete androgens during the period of heightened sexual responsivity.

Female rats become receptive in late Proestrus and remain receptive for 12-20 hours, depending on whether or not she is mated (McCarthy & Becker, 2002). This period of receptivity overlaps with the Estrus phase, a phase that lasts about 25-27 hours (Hubscher, Brooks, & Johnson, 2005). Ovulation occurs when the follicle ruptures and releases the secondary oocyte, between 36-48 hours after the increase in E, and 4-6 hours after the increase in P (McCarthy & Becker, 2002). Irregularly shaped, anucleated, cornified epithelial cells characterize Estrus vaginal cell morphology. Elevated levels of E inhibit the release of GnRH from the hypothalamus, keeping LH and FSH levels low.

During Metestrus, which lasts 6-8 hours (Hubscher, Brooks, & Johnson, 2005), and in the absence of fertilization, the corpus luteum disintegrates, resulting in a sharp decline in E2 and P levels. This ceases the inhibitory effects caused by their high concentrations (McCarthy & Becker, 2002). The pituitary begins to release FSH again, stimulating the growth of new follicles in the ovary. At this stage, leukocytes begin to infiltrate the vaginal epithelium and the presence of nucleated cornified and leukocytic cells are present in the vaginal smear.

The typical sexual behaviors of the female rat in behavioral Estrus are comprised of appetitive and consummatory behaviors. Appetitive behaviors are typically distal actions that serve to entice the male to chase or mount her. Ear wiggling (caused by rapid movement of the head), hopping and darting (sudden forward movement with abrupt

stops) and solicitations, defined as a female making a headwise orientation followed by a runaway. Consummatory behaviors refer to lordosis, a reflexive dorsiflexion, which occurs in response to flank stimulation by the male. Appetitive and consummatory behaviors are interspersed across the copulatory bout. Appetitive behaviors begin to decline and rejection responses (e.g. kicking and boxing the male) increase as the number of intromissions (penile insertion into the female's vagina) from the male increase prior to the decline in lordosis magnitude or frequency; this pattern of behaviors is characteristic of Estrus termination (Pfaus, Smith, Byrne, & Stephens, 2000). A decline in occupied P receptors in the hypothalamus has been shown to be involved in Estrus termination (Blaustein & Feder, 1979; Brown & Blaustein, 1984; Brown, Moore, & Blaustein, 1987).

Sensory stimuli can alter the duration of Estrus. Behavioral Estrus persists for approximately 12 to 20 hours however experimentally-applied vaginocervical stimulation (VCS) or paced copulation shortens the duration of this phase (Blandau, Boling, & Young, 1941; Erskine, 1985; Erskine, Kornberg, & Cherry, 1989; Pfaus, Smith, Byrne, & Stephens, 2000). In addition, during the evening of Proestrus, and in OVX rats given steroid priming, paced mating has been showed to accelerate Estrus termination (Coopersmith, Candurra, & Erskine, 1996; Erskine & Baum, 1982). Vaginocervical stimulation that the female receives during multiple pāced intromissions sends signals to the brain that activate reward mechanisms, satiety, analgesia and facilitate sperm transport. This stimulation also activates nightly prolactin surges which serve to maintain the secretion of P from the corpora lutea, ensuring implantation in the case of pregnancy, or inducing a two-week pseudopregnancy (Erskine, 1989; Matthews & Adler, 1978).

Sensory stimulation can also alter ovulatory patterns in aged females. Middle-aged females in persistent Estrus, a time when E concentrations are elevated and the LH-surge is disrupted resulting in anovulation, can begin cycling and ovulating again following caging and mating with a male, though reproductive success is reduced (Day, Morales, & Lu, 1988).

Removing the ovaries from a rat leads to a rapid depletion of ovarian steroid hormones. Other peripheral tissues, such as the adrenals and adipose tissue, produce these hormones; however their levels are relatively low and delivered in a continuous, non-cyclical fashion, blunting estrous cycles (Boling & Blandau, 1939; Hubscher, Brooks, & Johnson, 2005; Stockard & Papanicolaou, 1917). Ovariectomized rats do not display any sexual behavior or sexual interest (Beach, 1942). Behavioral Estrus can be induced in the OVX rat by sequential administration of E2 48 hours and P 4 hours prior to testing (Boling & Blandau, 1939; Beach, 1942; Whalen, 1974).

In the OVX rat, a sufficiently high dose of E2 can induce lordosis and low levels of appetitive behaviors, however E2 followed by P is much more effective, producing the entire pattern of behavior observed in gonadally-intact rats in sexual "heat" (Boling & Blandau, 1939; Beach, 1942). Whalen (1974) administered varying doses of both E2 and P and showed that P mainly affected appetitive behaviors such as solicitations, hops, darts and ear wiggling, and suggested that E2 and P have differential effects on the induction of these behaviors.

Effects of aging on ovarian cyclicity and the sexual behavior of the female rat.

Evidence suggests that the changes in ovarian cyclicity associated with age are due to alterations in the function of certain hypothalamic regions. When monitoring vaginal cytology, the female rat begins to show irregular cycles at approximately 8 months of age, persistent Estrus at approximately 10 months of age, and persistent Diestrus at approximately 17 months of age (Lefevre & McClintock, 1988). The ovaries from aged, acyclic females, when transplanted into young OVX females, cause young animals to begin cycling normally again, however the reverse transplant of young ovaries into aged females does not restore cyclicity (Aschheim, 1961; Peng & Huang 1972; Felicio, Nelson, & Finch, 1986).

Sexual behaviors in aging female rats decline with age, as the hormonal profile of the Estrous cycle is disrupted (Borchardt, Lehman, & Hendricks, 1980; Chamber & Phoenix, 1986; Gerall, Dunlap, & Sonntag, 1980; Huang, Steger, Bruni, & Meites, 1978). Females in persistent vaginal estrous reportedly continue to display normal lordosis quotients (Borchardt et al., 1980; Cooper & Linnoila, 1977), whereas appetitive behaviors (e.g. hops and darts) are significantly attenuated (Borchardt et al., 1980). Chambers and Phoenix (1986) investigated the effect of OVX and EB+P priming on aged (13 months old) versus young (4.5 months) female rats. They found no effect of age on consummatory aspects (i.e. lordosis) whereas appetitive behaviors (e.g. hops and darts) were significantly attenuated in these aged animals. A decline in appetitive behaviors in aged animals is consistent with the findings of Borchardt and colleagues (1980) mentioned above, and is reminiscent of the behavioral patterns occurring in females in Estrus termination.

Cooper, Conn and Walker (1980) found that the LH surge in middle-aged (10.5 months) rats is delayed and lower in amplitude compared to young (4.5 months) 4-day cycling rats. As discussed in Cooper et al. (1980), GnRH release may be disrupted in aged females, in turn disrupting LH release. These aged-dependent changes can be attributed to three factors: First off, middle-aged females have lower circulating P levels on the evening of Proestrus, however an injection of P on the morning of Proestrus has been shown to increase the LH surge (Miller & Riegle, 1979). Secondly, OVX of a young female rat leads to high circulating gonadotropin levels, due to a loss in ovarian steroid negative feedback mechanisms. In middle-aged animals, the gonadotropin levels are increased, but not to levels of young females, suggesting a disruption in the gonadotropin feedback mechanisms (Gray et al., 1980). Furthermore, the administration of E to OVX middle-aged animals leads to an LH surge lower in amplitude when compared to young OVX females treated with E. Finally, given that lesions to the suprachiasmatic nucleus of the hypothalamus (SCN) impair gonadotropin secretion and ovarian cyclicity (Brown-Grant & Raisman, 1977), age-related changes in the circadian rhythms from the SCN may be reducing GnRH stimulation, resulting in lowered LH surges (Cooper, Conn, & Walker, 1980; Matt, Coquelin, & Lu, 1987; Rubin, 2000).

Additional support relating the SCN to age related changes in the female rat comes from the work of Cai and Wise (Cai, Scarbrough, Hinkle, Wise, 1997; Cai, Lehman, Lloyd, Wise, 2007), who that show that diurnally-induced Fos expression (a marker of cell activity) in the SCN is disrupted in middle-aged rats. In aged females, baseline Fos expression occurs earlier, during the dark-cycle, and light-induced Fos expression is attenuated and delayed. However, transplantation of fetal SCN tissue into

the third ventricle of middle-aged rats restores the pattern of Fos expression to levels equivalent to that observed in young females. These data raise the possibility that GnRH may be disrupted by changes in the SCN, resulting in disturbances in the pulsatile release of FSH and LH from the pituitary, in turn causing detrimental effects on ovarian follicles and eventually leading to a termination in normal ovarian functioning (Wise, 1999).

Protein synthetic effects of estrogens and androgens

Androgen and E receptors are situated in the cytosol. Upon binding to their ligands, migration of the receptor from the cytosol to the nucleus occurs before binding to the hormone response elements of DNA. This causes a cascade of events that ultimately lead to protein synthesis. Estrogens have been correlated with female reproductive function both spatially and temporally (Pfaff, 1980). Estrogen is required to prepare the brain for sexual behaviors, which takes a minimum of 16-20 hours to take effect (Green, Luttge, Whalen, 1970; McEwen, Pfaff, Chaptal, & Luine, 1975). The ventromedial nucleus of the hypothalamus (VMH), anterior hypothalamus (AH) and medial preoptic area of the hypothalamus (mPOA) are brain regions that have high concentrations of Es, P, and androgens receptors, and in which exogenous E2 applied directly to these areas can facilitate female sexual behaviors (Pfaff, 1980). Lisk (1962) discovered that E2 administered directly into the mPOA of the hypothalamus activated lordosis in OVX females. Subsequently, it was found that the VMH of the hypothalamus was even more sensitive to E2 (Davis, Krieger, Barfield, McEwen, Pfaff, 1982; Dorner, Docke and Mustafa, 1968; Parsons, Rainbow, Pfaff, & McEwen, 1982).

Temporally, the actions of E are a slow process, triggering genomic effects to transcribe proteins and upregulate P receptors, in areas such as the mPOA and VMH

(MacLusky & McEwen, 1978), so that P can bind its receptor and facilitate appetitive aspects of sexual behavior. The eventual decrease in occupied P receptors in the hypothalamus is involved in Estrus termination. Progesterone is also important in the ventral tegmental area (VTA) in the control of lordosis, by acting on dopamine (DA) and/or GABA receptors (Frye, 2001; Frye & Walf, 2008).

In female OVX rats, brought into behavioral Estrus via subcutaneous injection of EB and P, 48 and 4 hours before the test, respectively, the neurotransmitter DA is reportedly increased in the mPOA, at the time that they would normally begin displaying sexual behaviors, in the absence of any sensory stimulation from a male (Matuszewich, Lorrain, Hull, 2000). A further increase in mPOA DA release occurs when a mate is introduced into the cage and copulation begins. In EB-primed OVX rats, DA agonists facilitate sexual behaviors (Melis & Argiolas, 1995). This is evidence that DA in the mPOA is important in both appetitive and consummatory aspects of female sexual behavior. Similarly, DA is increased in the nucleus accumbens (NAcc) of EB+P primed female rats when a male is introduced in the testing chamber, and DA is further increased in conditions where the female is allowed to pace the timing between intromissions (Becker, Rudick, Jenkins, 2001).

Testosterone's effects in the control of male sexual behavior and DA release have been extensively studied in rats (Hull, Du, Lorrain, Matuszewich, 1997; Hull, Lorrain, Du, Matuszewich, Lumley, Putnam, Moses, 1999). The mPOA is a critical brain site involved in male sexual behavior. Stimulating the mPOA increases the rate of copulation and blocking it decreases copulation (Hull, Bitran, Pehek, Warner, Band, & Holmes, 1986; Warner, Thompson, Markowski, Loucks, Bazzett, Eaton, & Hull, 1991). Castration

is the surgical removal of the testicles which are the main source of T synthesis; castration leads to impaired copulatory behaviors in male rats. Testosterone increases nitric oxide synthase (NOS) in the mPOA, in turn stimulating DA release, leading to erection of the penis in castrated male rats. (Du & Hull, 1999; Hull et al., 1999).

Since T can either be reduced to the androgen DHT, or aromatized to E2, researchers have studied the effects of E2 on male sexual behavior. It has been established that aromatase-containing neurons in the mPOA (and elsewhere) are causally related to the effect of androgens on the sexual behavior of male rats (Putnam, Sato, Riolo, & Hull, 2005), as aromatase inhibition disrupts male sexual behaviors, and the application of crystalline E2 to the mPOA can restore mounting in castrated males (Lisk & Bezier, 1980). Thus, T has been shown to be important in the release of DA, a neurotransmitter involved in sexual motivation and function in both males (Bazzett, Lumley, Bitran, Markowski, Warner, & Hull, 1992; Hull, Lorrain, Du, Matuszewich, Lumley, Putnam, & Moses, 1999) and females (Becker, Rudick, & Jenkins, 2001). The role of DA is also particularly important in areas involved in sexual behavior, such as the VMH, mPOA, NAcc and the VTA in both males and females. Given that researchers have investigated the role of E in male sexual behaviors, it is surprising that the study of T on female sexual behaviors has been so limited.

The goal of the present thesis was to study the effects of TP given with or without EB on both appetitive and consummatory sexual behaviors in both the young OVX and aged intact female rat.

Outline of experiments

Very few studies have investigated the effects of TP on the appetitive and consummatory aspects of female sexual behavior in OVX rats. Even fewer are the studies that have looked at sexual behaviors in aging rodents, and how sexual behaviors can be maintained or restored with exogenous hormone treatments. This has perhaps been difficult because institutions often impose a high cost on the care and maintenance of laboratory animals. Retired breeder females from breeding farms are quite expensive, and their sexual history is usually unknown.

Therefore, the present thesis explored the way in which TP treatment either alone or in combination with EB, would affect appetitive and consummatory aspects of female sexual behavior. The overarching hypothesis was that co-administration of EB and TP would facilitate sexual behavior, in particular appetitive behaviors (i.e. solicitations, hops and darts) in young OVX and aged intact animals. The optimal dose and schedule of administration was not clear however, thus dose-responses and varying methods of administration were examined.

Experiment 1 tested whether there is a dose-response relationship to varying TP levels, given with or without EB, on the sexual behaviors of young OVX females. Results from this study would allow us to determine if there is a critical amount of TP required to elicit an effect, to help understand whether high levels of androgens are being aromatized to estrogens, thus restoring some behaviors and whether the addition of EB to a TP regimen would contribute to TP's effect. Experiment 1 also compared the effects of TP treatments to EB+P, a regimen that is known to fully restore female sexual behaviors in

OVX rats. The results of this experiment allow us to understand the magnitude of the effect (if any) of TP on female sexual behavior.

Experiment 2 investigated the acute and chronic effects of TP given with or without EB, on the sexual behaviors of aged female rats. The route of administration was via subcutaneous injection over 4 day intervals. Results from these studies would determine whether or not EB and TP facilitate sexual behaviors in aged animals, and whether or not the effects are maintained over time.

Experiment 3 investigated the behavioral effects of chronic TP administered at a constant rate, by subcutaneous capsules in varying doses, either alone or following chronic EB. The results of these experiments help to determine whether the route of administration, schedule of exposure and dose of TP elicit differential effects (compared to Experiment 1), and whether acute (Experiment 2 *Phase 1*) versus chronic treatment (Experiment 2 *Phase 2*) maintains, facilitates, or inhibits treatment efficacy. In addition, the results help to determine whether either hormone alone is sufficient in maintaining an effect.

All experiments were carried out in accordance with the ethical standards established by the Canadian Council on Animal Care (CCAC), and were approved by Concordia University's Animal Research Ethics Committee.

Experiment 1: Young OVX

Introduction

Studies showing that TP induced lordosis in OVX female rats have used a 200 μg dose of TP injected subcutaneously 6 hours prior to the test (Baum, Sodersten & Vreeburg, 1974). Because we were interested in the restoration of appetitive behaviors as well as consummatory behaviors, a dose-response study of TP both alone and following EB, was performed in OVX Wistars to investigate whether there was an optimal dose of TP in the restoration of female sexual behaviors in OVX rats. In addition, because EB+P restores all aspects of female sexual behaviors, this group was used as a comparison when investigating the effects of varying hormone treatments on female sexual behavior.

Experiment 1 was performed in 3 phases. *Phase 1* investigated the effect of varying TP doses on sexual behaviors in OVX female rats. *Phase 2* took these same animals and investigated varying TP doses when animals were exposed to a common EB dose 48 hours prior. *Phase 3* separated the animals into 4 groups of varying hormone treatments, where a 200 μg dose of TP was used, concurrent with the dose shown to induce lordosis in earlier studies (Baum, Sodersten & Vreeburg, 1974). These experiments were run in sequence, separated by 4 day testing intervals.

Method

Subjects.

Fifty Wistar female rats (200-250 g, approximately 10 weeks old) were purchased from Charles River Canada (St. Constant, Quebec). Animals were caged in groups of five

in stainless-steel cages in a colony room maintained at 21°C and on a 12-hour reverse day-night cycle (lights off at 8AM). Food and water were freely available.

Forty Long-Evans males were used as stimulus animals. Males were approximately 4 months old at the time of the test, were caged in groups of 4 and were sexually experienced in bi-level chambers.

Ovariectomy.

Animals were given one week of acclimatization to the animal colony prior to OVX. Surgeries were done under a mixture of ketamine hydrochloride (50 mg/mL) and xylazine hydrochloride (4 mg/mL) (4:3) injected intraperitoneally (1 mL/kg body weight). When anesthetized animals no longer had a reflex to a foot pinch, they were OVX bilaterally with a single lumbar incision. Post-operative care was given with 0.03 mL each of Banamine and Baytril, followed by 3 mL saline solution, and polysporin was applied to the incision site. Animals were allowed one-week post-surgical recovery prior to sex training. Six animals were lost following surgery prior to the start of the experiment due to unknown reasons, resulting in 44 animals available for the experiment.

Experimental procedure.

Behavioral tests were carried out in the bi-level chamber (Figure 1), which is rectangular in shape (70 cm x 51 cm x 15 cm), with two distinct platforms and stairways on either side, allowing the female to change levels and temporarily escape from the male, pacing the rate of copulation. The advantage of this type of chamber is it orients the animals lengthwise, optimizing the experimenters view (Mendelson & Gorzalka, 1987),

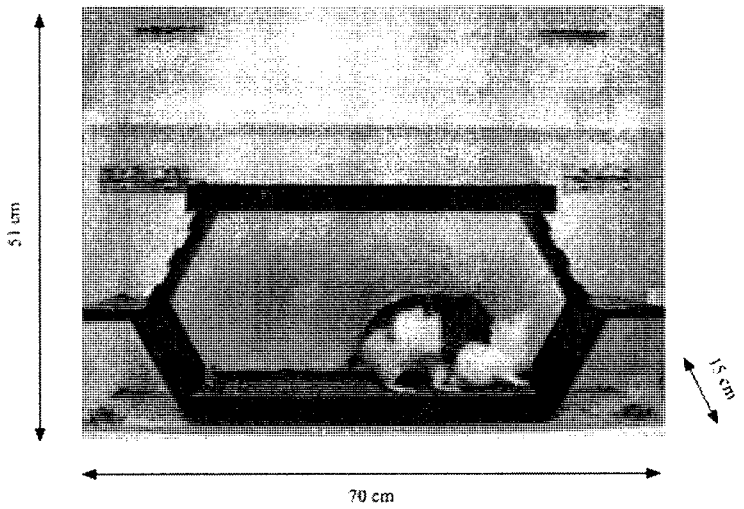

Figure 1. Bi-level chamber. Dimensions of 70 cm (length) x 51 cm (height) x 15 cm (width). The advantage of using this chamber is it orients the animals lengthwise, allowing the observer to clearly see the behaviors.

and permits females to fully express both appetitive and consummatory sexual behaviors (Pfaus, Smith, & Coopersmith, 1999).

Males were always placed first on the bottom level of the bi-level chamber for a five-minute habituation period to the chamber. Females were subsequently placed in the chamber onto the top level, for a 30-minute test session. Testing sessions were recorded by a videocamera onto a DVD-writable disc for subsequent scoring using the Behavioral Observation Program customized for rat sexual behavior (Cabilio, 1996).

Females were hormonally primed with EB (10 μg) 48 hours, and P (500 μg) 4 hours prior to each of 4 training sessions, separated by 4 day intervals. A 12-day washout period was allowed between the final training and first testing sessions. The experimental phases were separated by 4-day intervals. Animals were always tested during the middle-third of their dark cycle.

Behavioral analyses

Female appetitive behaviors include solicitations, defined as a headwise orientation towards the male followed by a runaway, hops which consist of quick hopping motions in proximity of the male, and darts defined as a runaway followed by an abrupt stop. These behaviors were grouped as one measure termed "appetitive behaviors". Level changes were scored when the female changed a complete level (all four paws touched the upper or lower level of the chamber).

Lordosis is a consummatory sexual behavior defined as a reflexive arching of the back in response to flank stimulation (such as mounting by a male). Lordosis magnitudes (LM) were broken down into a three-point scale depending on the magnitude of the

curvature of the back between the head and tail, as described by Hardy and DeBold (1971). A straightening of the spine is scored as the lowest LM of 1, flexion of the back with slight elevation of the head at an approximate angle of 30 degrees, is scored as LM 2, and spinal curvature with the head at a 45 degree angle or more is rated as LM 3. A lordosis quotient (LQ) was calculated for the whole test session by taking the ratio of the sum of all LMs to the sum of mounts, intromissions and ejaculations (LM / mount + intromission + ejaculation). Defensive behaviors (kicks, sideways takedowns, boxing postures, prone positions, as in Barnett, 1967) displayed by the females against the males were scored per incident.

Male behaviors were included in the behavioral analyses, as they provide further insight of the females' responses and can often be taken as an indication of the females' level of receptivity (Pfaus, Smith, Coopersmith, 1999). All male behaviors included mounts (male's front paws on female's flanks), intromissions (the male displays a characteristic "kickback" on the dismount) and ejaculations (the follow-through of the mount results in a strong pelvic thrust and maintaining a grasp on the female's flanks) were scored, to complement the females' behavior, including appetitive and consummatory behaviors.

Phase 1: TP-alone.

Hormonal procedure

Females were taken off all exogenous hormone treatments for a total of 12 days prior to the experimental test. On test day, females were separated into 5 groups of varying TP doses (administered and scored blindly) in a 0.1 mL volume of sesame oil

injected s.c. 4 hours prior to the experiment: 0 μg (n=9), 50 μg (n=9), 100 μg (n=9), 200 μg (n=9) and 400 μg (n=8) and were tested for sexual behavior in bi-level chambers with sexually experienced males. TP doses were administered and scored blindly.

Statistical analyses

All test statistics were run using SPSS 16.0 for Windows, using one-way between subjects ANOVAs, and Tukey's post-hoc test analyzed differences between groups. The level of significance for all comparisons was $p < .05$. Group descriptive statistics are represented as mean (M) +/- standard error of the mean (SEM).

Results

The ANOVA failed to detect significant differences between any of the TP groups, and very few behaviors were recorded overall. Any behavior that occurred during the test sessions is graphed in Figure 2.

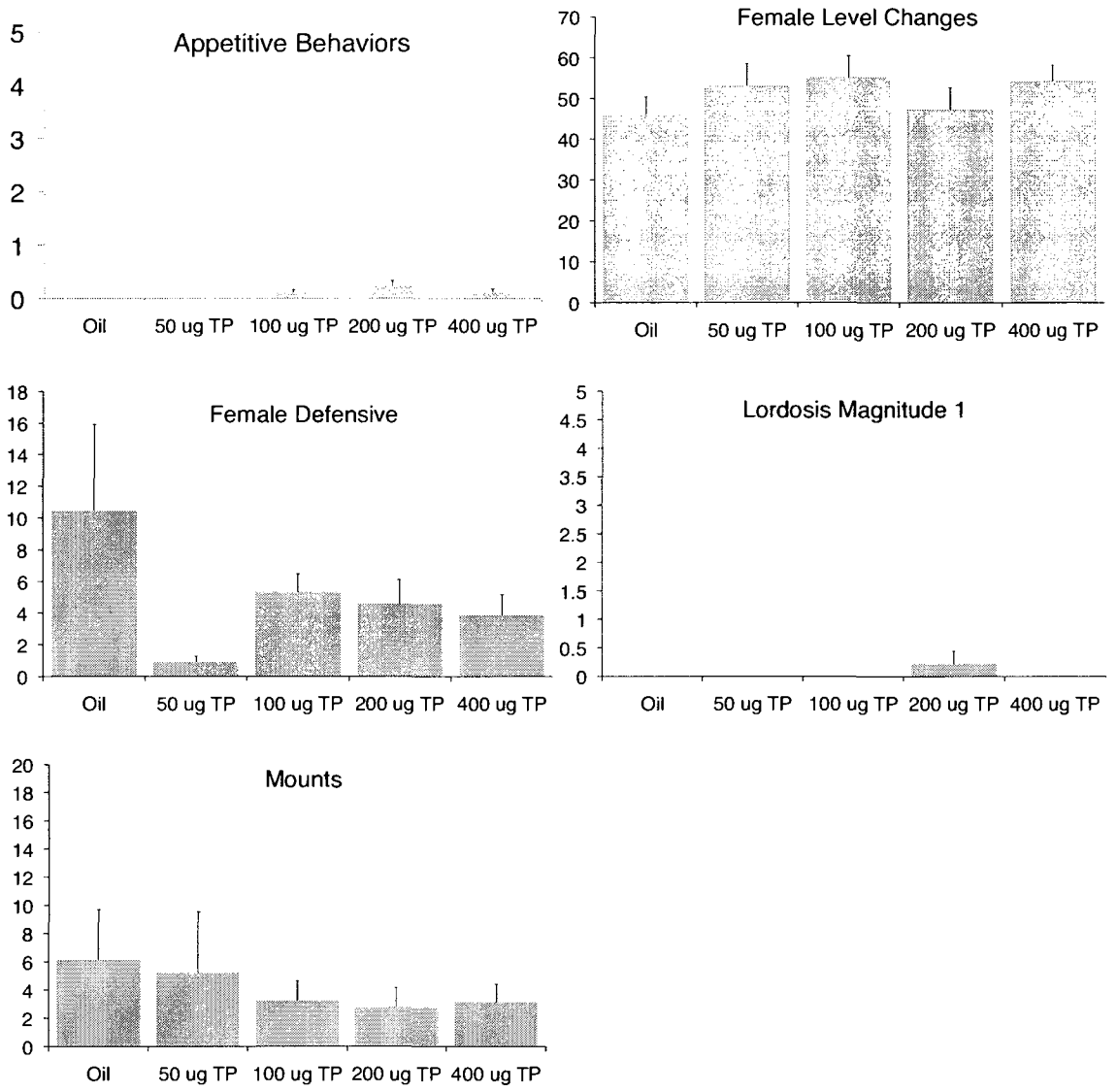

Figure 2. Frequency of sexual Behavior of OVX Wistar rats treated with varying doses of TP. Doses of TP (0, 50, 100, 200, 400 µg) were injected s.c 4 hours prior to the test.

Phase 2: TP with common EB baseline.

Hormonal procedure.

Two days following the TP-alone study, the same females were injected s.c. with EB (10 μg) to be tested 48 hours later for sexual behavior. Animals remained in the same groups, receiving the same TP doses as the TP-alone study (0 μg, 50 μg, 100 μg, 200 μg 400 μg), to which the experimenter was blind. One animal from the 400 μg dose of TP group escaped the chamber, thus was excluded from the analysis, resulting in a group size of 7.

Statistical analyses

One-way analysis of variance (ANOVA) was run on each behavior, and Tukey's post-hoc test determined specific group differences, following a significant overall ANOVA. The level of significance for all comparisons was $p < .05$. Group descriptive statistics are represented as mean (M) +/- standard error of the mean (SEM).

Results.

Although all the animals did show sexual behaviors, there were no significant differences between any of the doses. Figure 3 shows the mean frequency of male behaviors towards the female, female appetitive behaviors are represented in Figure 4, and female consummatory behaviors are depicted in Figure 5.

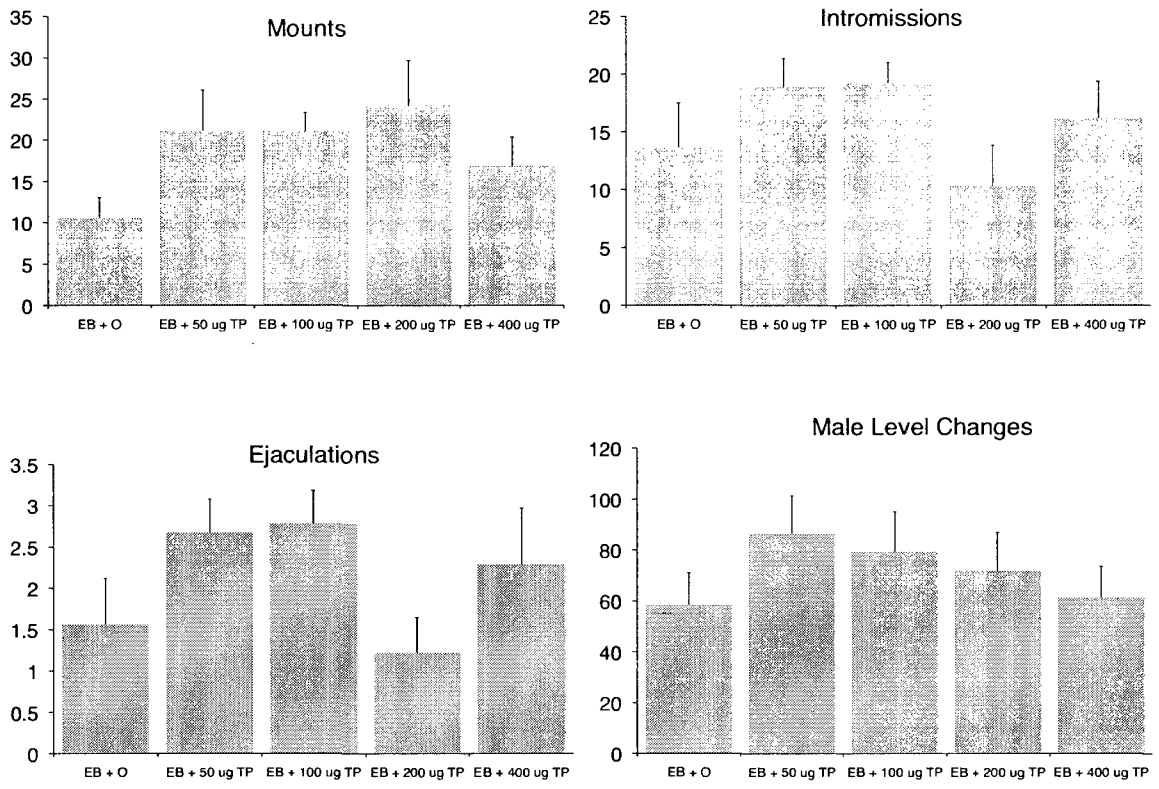

Figure 3. Frequency of male sexual behaviors towards Wistar OVX female rats treated with EB and varying doses of TP. EB (10 μg) injected s.c. 48 hours, and doses of TP (0, 50, 100, 200, 400 μg) injected s.c. 4 hours prior to the test.

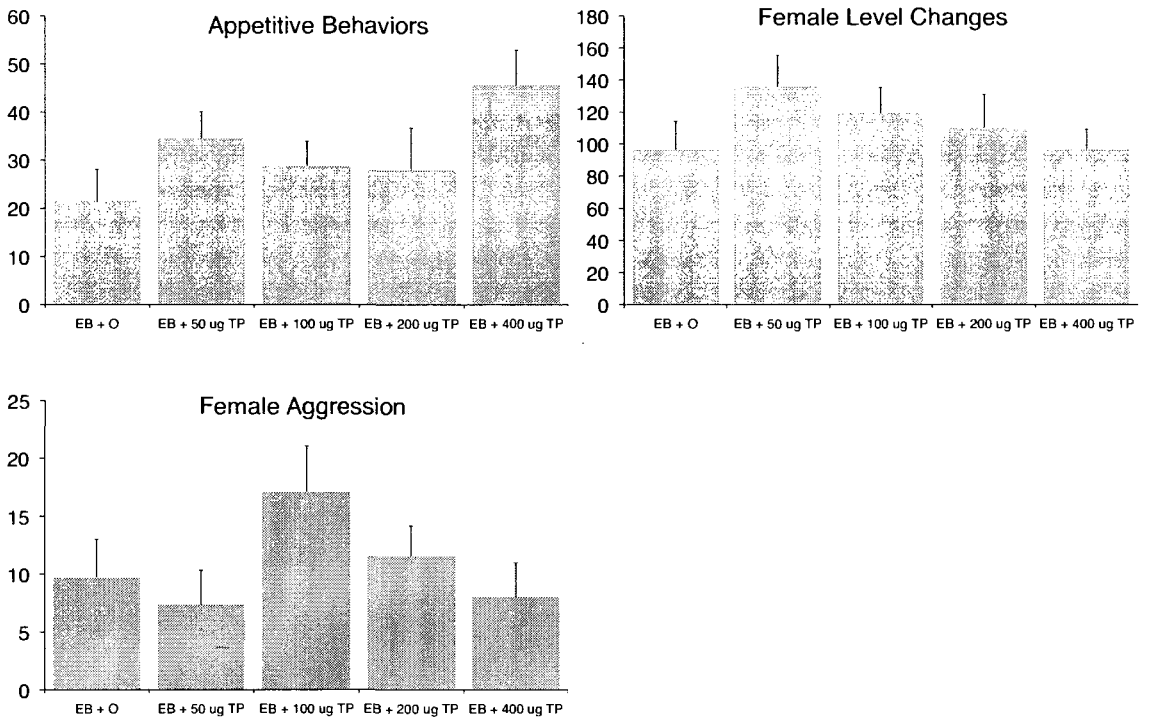

Figure 4. Frequency of appetitive sexual behaviors of Wistar OVX female rats treated with EB and treated with varying doses of TP. EB (10 μg) injected s.c. 48 hours, and doses of TP (0, 50, 100, 200, 400 μg) injected s.c. 4 hours prior to the test.

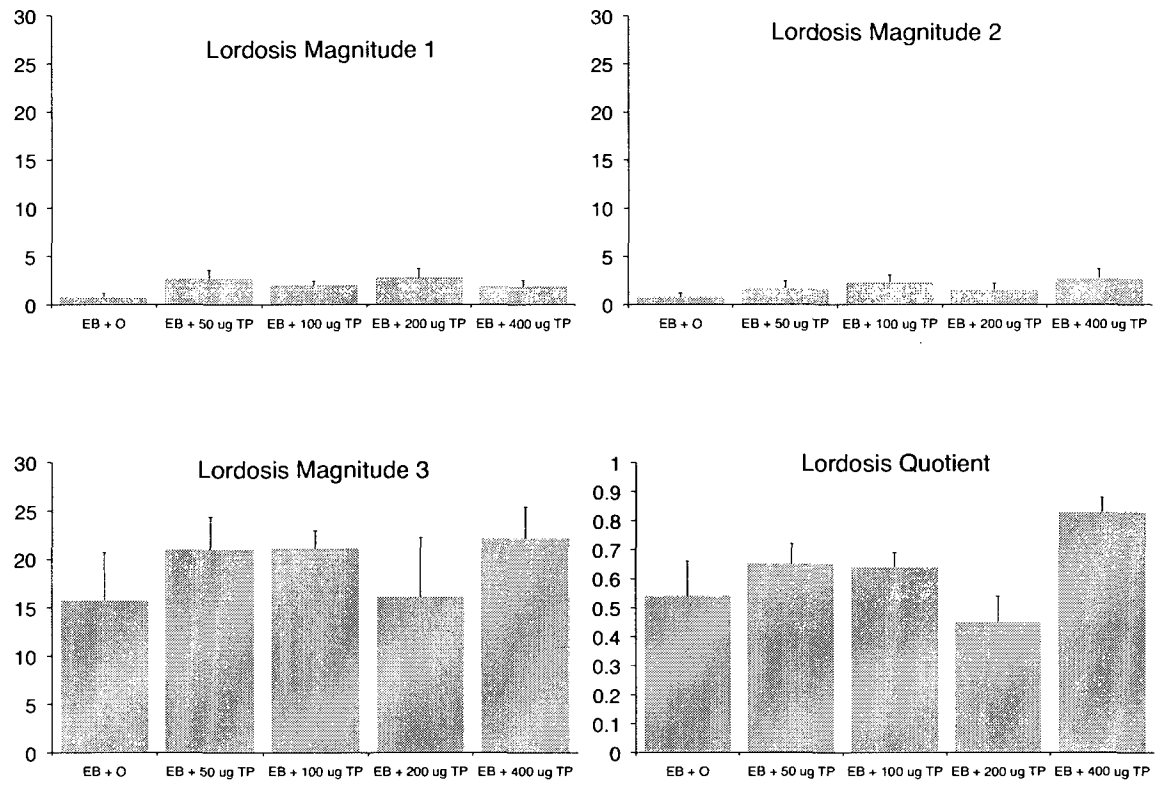

Figure 5. Frequency of consummatory sexual behaviors of Wistar OVX female rats primed with EB and treated with varying doses of TP. EB (10 μg) injected s.c. 48 hours, and doses of TP (0, 50, 100, 200, 400 μg) injected s.c. 4 hours prior to the test.

Phase 3: Varying hormone regimens on sexual behavior of OVX rats

Hormonal Procedure.

Estradiol benzoate (EB) was dissolved in sesame oil and animals were injected s.c. 48 hours prior to the start of the experiment with 10 μg in 0.1 mL sesame oil (O). Progesterone (500 μg) and TP (200 μg) were dissolved in sesame oil, and injected s.c. in a volume of 0.1 mL 4 hours prior to the start of the training sessions. All animals received two injections, the first 48 hours and the second 4 hours prior to behavioral testing with their respective treatment, control animals received 0.1 mL of sesame oil. The groups were administered test hormones as follows: O+O, EB+O, EB+P, O+TP and EB+TP. Each group consisted of 9 animals, except for O+TP which totaled 8.

Results

All behaviors were analyzed using a one-way ANOVA and are represented in Figures 6 (male behaviors toward female), 7 (female appetitive behaviors) and 8 (female consummatory behaviors). Significant effects are discussed in more detail below.

Male behaviors

A main effect of hormone regimen on intromissions was detected, $F(4,39)=8.958$, $p=0.00$. Tukey's post-hoc analyses revealed that females treated with O+O or TP-alone received fewer intromission than females treated with EB-alone, EB+P and EB+TP.

A main effect of hormone regimen on ejaculations was also observed, $F(4,39)= 9.28$, $p=0.00$. Post-hoc Tukey analyses revealed that females treated with O+O or TP-alone received fewer ejaculations than females treated with EB-alone, EB+P or EB+TP.

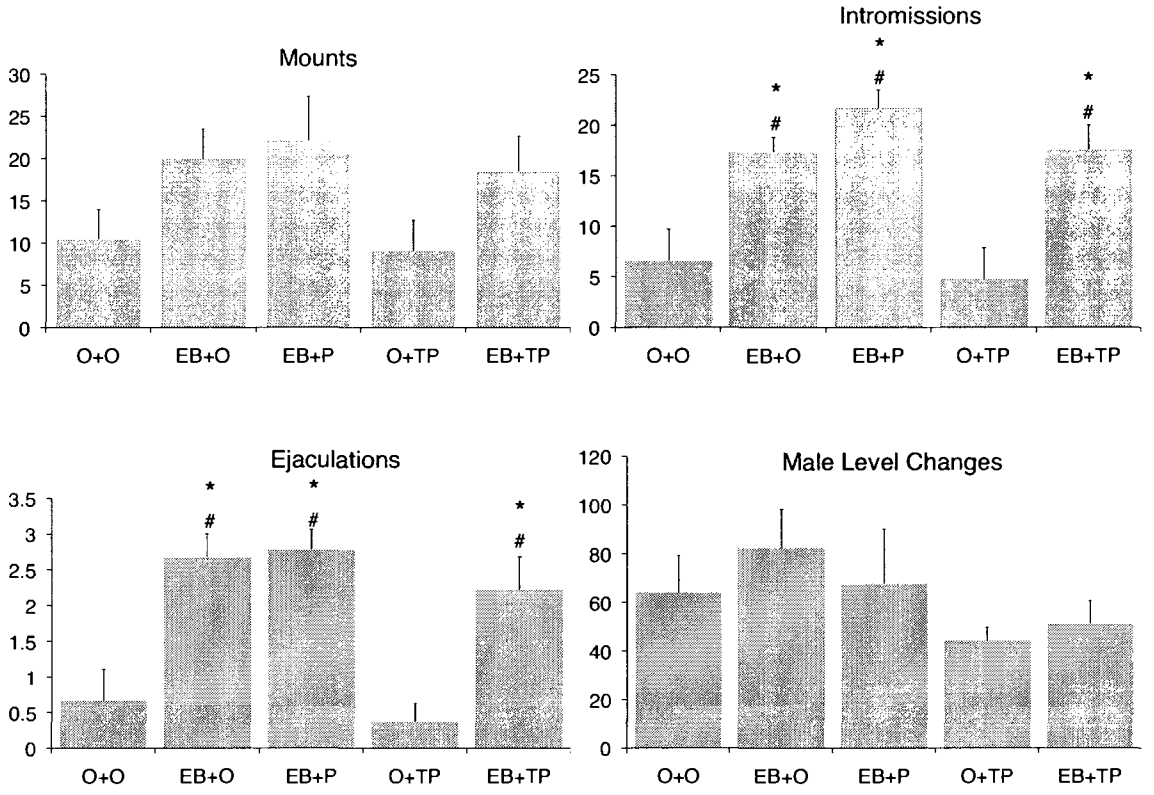

Figure 6. Frequency of sexual behaviors of male rats towards Wistar OVX female rats following acute injections of various hormone treatments. O: sesame oil, EB: estradiol benzoate, 10 µg; P: progesterone, 500 µg; TP: testosterone propionate, 200 µg). Values are mean +/- SEM. * Different from O+O, $p<.05$; # different from O+TP, $p<.05$.

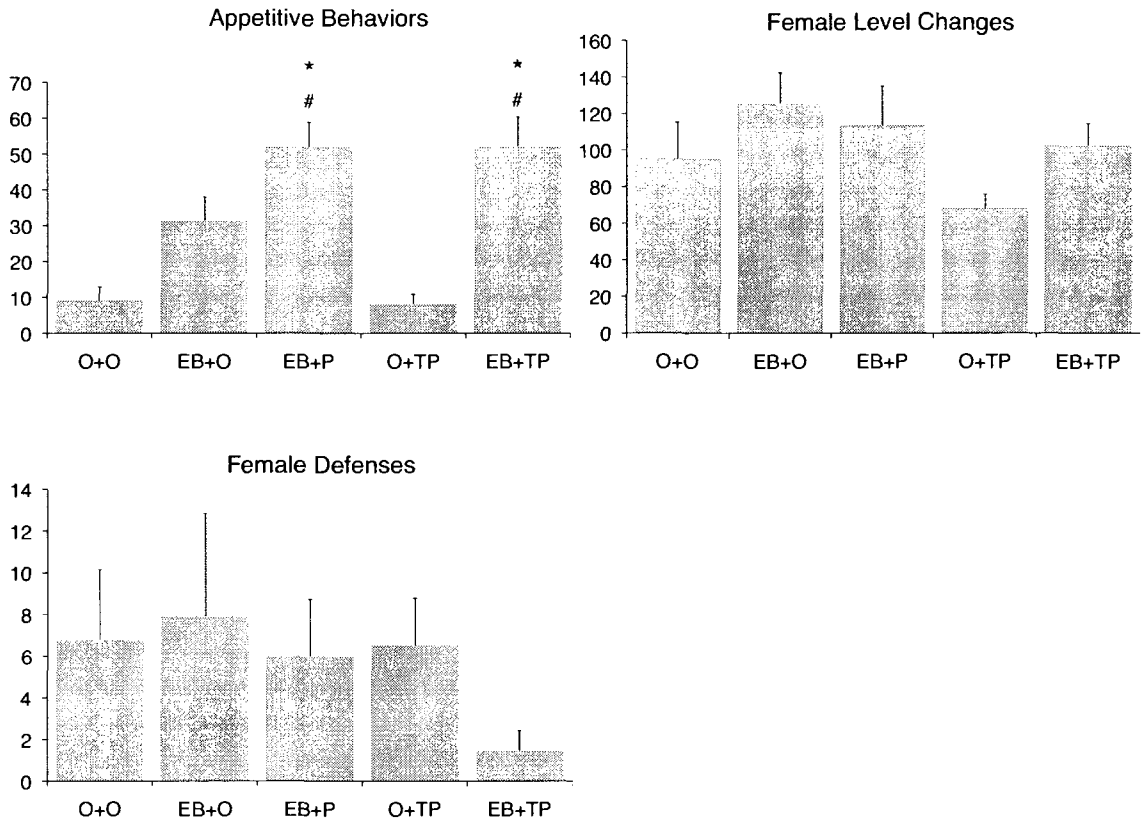

Figure 7. Frequency of appetitive sexual behaviors of Wistar OVX female rats following acute injections of various hormone treatments. O: sesame oil, EB: estradiol benzoate, 10 μg; P: progesterone, 500 μg; TP: testosterone propionate, 200 μg). Values are mean +/- SEM. * Different from O+O, $p<.05$; # different from O+TP, $p<.05$.

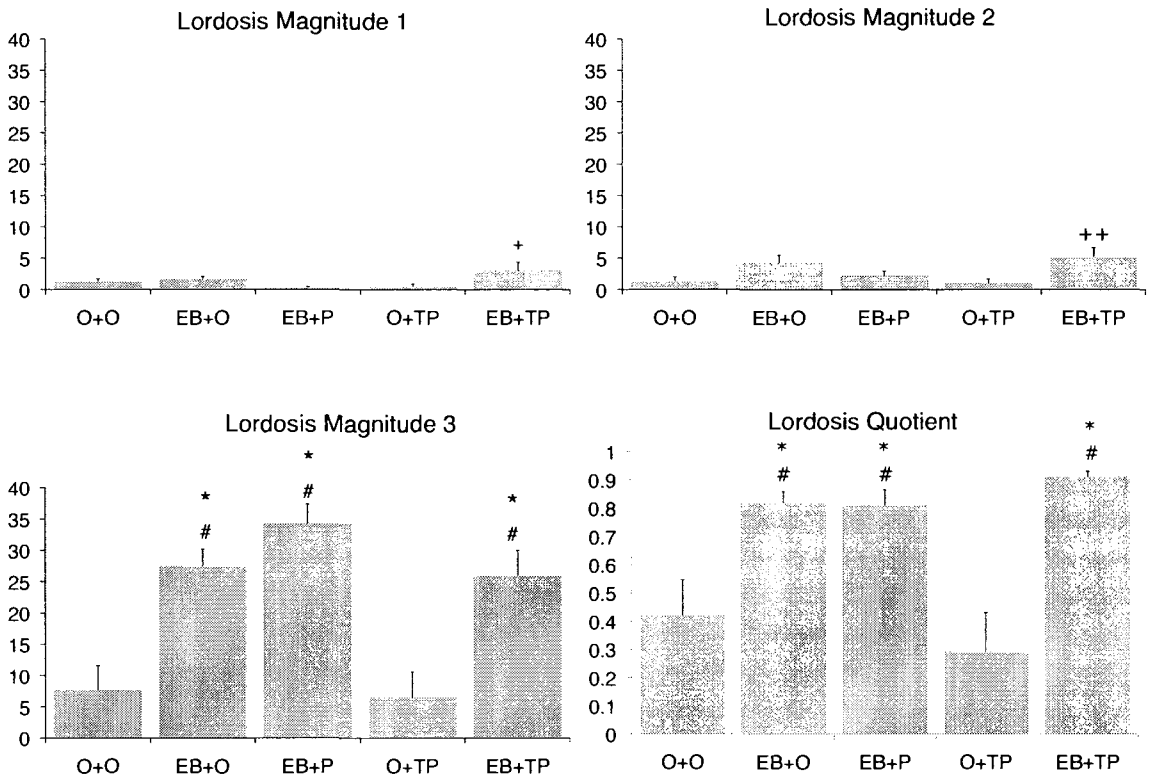

Figure 8. Frequency of consummatory sexual behaviors of Wistar OVX female rats following acute injections of various hormone treatments. O: sesame oil, EB: estradiol benzoate, 10 μg; P: progesterone, 500 μg; TP: testosterone propionate, 200 μg). Values are mean +/- SEM. * Different from O+O, $p<.05$; # different from O+TP, $p<.05$; + trend from EB+P, $p<.10$; ++ trend from O+TP, $p<.10$.

Female Appetitive Behaviors

An overall main effect of hormone regimen was found for appetitive behaviors, $F(4,39)=12.40$. Post-hoc analyses revealed that females treated with O+O or O+TP displayed significantly fewer appetitive behaviors than females treated with EB+P or EB+TP.

Female Consummatory Behaviors

An overall main effect of hormone regimen was found on LM 3, $F(4,39)=11.831$, $p=.00$, with post-hoc tests showing that females treated with O+O or TP-alone displayed significantly fewer LM 3 postures compared to females treated with EB+P or EB+TP.

Discussion

Phase 1 of Experiment 1 confirms that acutely, none of the tested doses of TP have an effect on sexual behaviors of young OVX rats. Males continued to mount the female, however no male successfully intromitted or ejaculated, probably due to the number of defensive responses (which have a similar mean value to the number of mounts, suggesting rejection from the female in response to the mounts). Furthermore, female defensive behaviors (kicks, prone position) were no different than the control group. This contradicts earlier work (Baum et al., 1974; Van de Poll, van Zanten & de Jonge, 1986) however the method of administration (interval of time between androgen administration and test session, route of administration, and dosing regimen) may explain this difference. Routes of administration may have different biological effects through the by-passing, for example, of hepatic metabolism in the case of transdermal routes versus oral routes of administration; the delay between injections and dosing regimen may also

differentially effect biological mechanisms, for example by upregulating or downregulating receptors, leading to changes in behavioral output.

Phase 2 of Experiment 1 tested whether varying doses of TP, when added to a common EB baseline, would affect female sexual behavior. The administration of EB induced both appetitive and consummatory aspects of female sexual behavior, however no dose of TP added to the effect.

Phase 3 of Experiment 1 tested whether varying hormone treatments affect sexual behaviors in young OVX Wistar female rats. These results suggest that EB+TP induces behavioral effects similar in magnitude to EB+P treated animals, though these effects were not statistically different from animals treated with EB-alone. It should also be noted that control animals in Experiment 1 *Phase 3* were oil-treated; however, these animals continued to display low levels of both appetitive and consummatory sexual behaviors. This conflicts with the findings in *Phase 1*, where control animals failed to display any appetitive or consummatory aspects of sexual behavior. Similarly, animals in the TP-alone group of *Phase 3* displayed low levels of sexual behaviors, which again conflicts with the results of *Phase 1*. Furthermore, animals in the EB+TP group were receiving 200 μg of TP, and although they also showed elevated levels of sexual behaviors, to levels similar to EB+P, this increase was only a trend when compared to EB-alone. This conflicts with reports in the literature, showing that the addition of P to an EB-treated animal induces significantly more appetitive behaviors and LQ's (Pfaus, Smith, & Coopersmith, 1999; Whalen, 1974). These results suggest that carry-over effects of TP received 4 and 8 days prior (for *Phase 2* and *Phase 1*, respectively) and/or

EB administered 6 days prior to *Phase 3* affected the behavior of these animals at *Phase 3*.

Experiment 2: Aged Intact Phasic TP

Introduction

The goal of Experiment 2 was to introduce varying hormone treatments to intact, aged female rats to test whether TP, when administered with or without EB, would restore any aspects of female sexual behavior. A 200 μg dose was used, concurrent with the dose shown to induce lordosis in earlier studies (Baum, Sodersten & Vreeburg, 1974).

Experiment 2 was designed in two phases. The first investigated the acute effects of varying hormones on sexual behaviors. The second phase investigated the long-term effects of TP when administered alone, or in combination with EB, on sexual behaviors.

Method

Subjects.

Forty retired-breeders of the Wistar strain (ages ranged from 5-8 months) were obtained from Charles River, St-Constant, QC. Each had bore between 2-5 litters of 8-16 pups. They were kept on a 12-hour reverse day-night cycle (lights off at 8AM), with water and rat chow freely available. Animals were housed in shoebox cages in pairs when possible (i.e. odd numbers of animals resulted in animals caged alone), and identified with markings on the base of the tail. They were housed in shoebox cages for 18 weeks prior to the start of the study.

Cell cytology

The estrus cycles of all animals were monitored for 25 consecutive days prior to the start of the experiment (identification method similar to Marcondes et al., 2002), by

vaginal cell collection with a cotton swab slightly lubricated with distilled water. Cells were taken within the first hour of their dark cycle. All animals were no longer cycling over 4-5 days by the beginning of the experiment, and were approximately 9-12 months of age.

Vasectomy.

To prevent possible impregnation of intact females, 48 sexually experienced Long-Evans males (aged approximately 9 months old) were vasectomized under anesthesia by 1.5 cc per kg body weight with ketamine hydrochloride (50 mg/mL) and xylazine hydrochloride (4 mg/mL) (4:3) injected i.p. A single incision was made to the scrotal sac, followed an incision to the muscular sac of the testes, and by applying slight pressure to the abdomen, the testes were exposed and the vas deferens were located. The vas deferens was separated from the blood vessels, clamped and ligated with 2 ties of surgical thread approximately 5 mm apart, and cut on either side. Testes were left intact and returned to the scrotal sac, the muscle and skin incisions were sutured shut. Animals were allowed two weeks of recovery before being given two sexual experiences, every 4 days with sexually experienced OVX Long-Evans female rats. All males were copulating, as determined by the expression of intromissions with the OVX females 4 days prior to the start of the study.

Experimental procedure

The experimental procedure was identical to Experiment 1, without the EB+P training period, since animals were retired breeders.

Behavioral analyses

The same behavioral measures that were observed in Experiment 1 were analyzed in Experiment 2.

Phase 1: Acute effects of varying hormones on sexual behavior

Hormonal procedures.

Estradiol benzoate (EB) was dissolved in sesame oil and animals were primed 48 hours prior to the start of the experiment with 10 μg in 0.1 mL sesame oil (O). Progesterone (500 μg) and TP (200 μg) were dissolved in sesame oil, and injected s.c. in a volume of 0.1 mL 4 hours prior to the start of the sessions. All animals received two injections, the first 48 hours and the second 4 hours prior to behavioral testing with their respective treatment, control animals received injections of 0.1 mL of sesame oil. The groups were administered test hormones as follows: O+O (n=10), EB+O (n=6), EB+P (n=8), EB+TP (n=8).

Statistical analyses.

A one-way between subjects ANOVA was run for each behavior using SPSS for Windows (version 16.0), and Tukey's test was used for post-hoc comparisons. The level of significance for all comparisons was $p < .05$. Group descriptive statistics are represented as mean (M) +/- standard error of the mean (SEM).

Results.

Of a total of 40 animals, 25 animals were in Proestrus or Estrus on test day, 7 were in Metestrus, and 8 were in Diestrus. Any animal in Diestrus, or who resulted in a division by 0 for the LQ, suggesting the male was not copulating, were excluded from the statistical analyses. Females who were in Metestrus, also displayed appetitive and consummatory behaviors, thus were included in the statistical analyses. Five animals remained in the O+O group, 8 in the EB+O group, 8 in the EB+P group and 9 in the EB+TP group.

Female Defenses

There was a main effect of group on female defense, $F(3,26)=3.227$, $p < .05$. Post hoc analyses showed significantly more defensive behaviors O+O than the EB+O group.

Lordosis Quotient

There was a main effect of group on lordosis quotient $F(3,26)=3.155$, $p<0.05$. Post hoc Tukey analyses showed that O+O displayed significantly less LM in response to flank stimulation compared to EB+O animals.

Although no further behaviors were statistically significant, the graphs of male behaviors towards the females (Figure 9), as well as female appetitive (Figure 10) and consummatory (Figure11) behaviors are provided below.

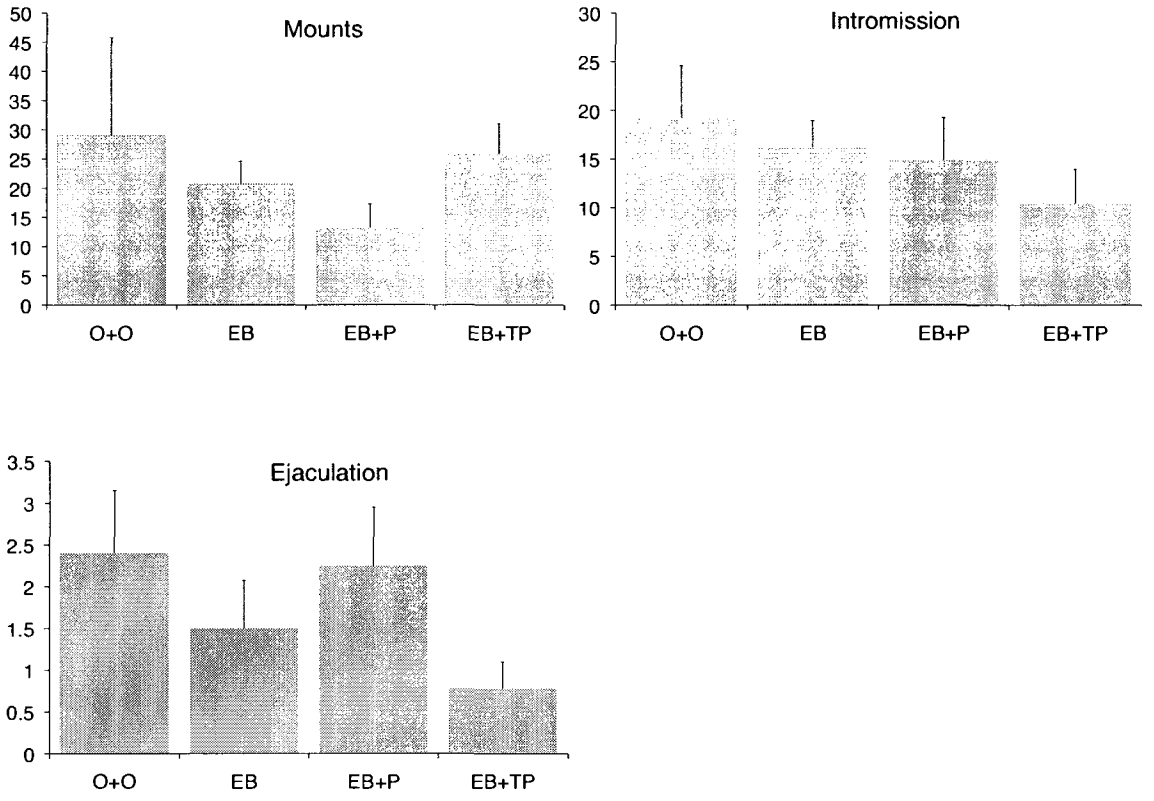

Figure 9. Frequency of male sexual behaviors towards aged female Wistar rats following acute injections of different hormone regimens. (O: sesame oil, E: estradiol benzoate, 10 μg; P: progesterone, 500 μg; TP: testosterone propionate, 200 μg). Values are mean +/- SEM.

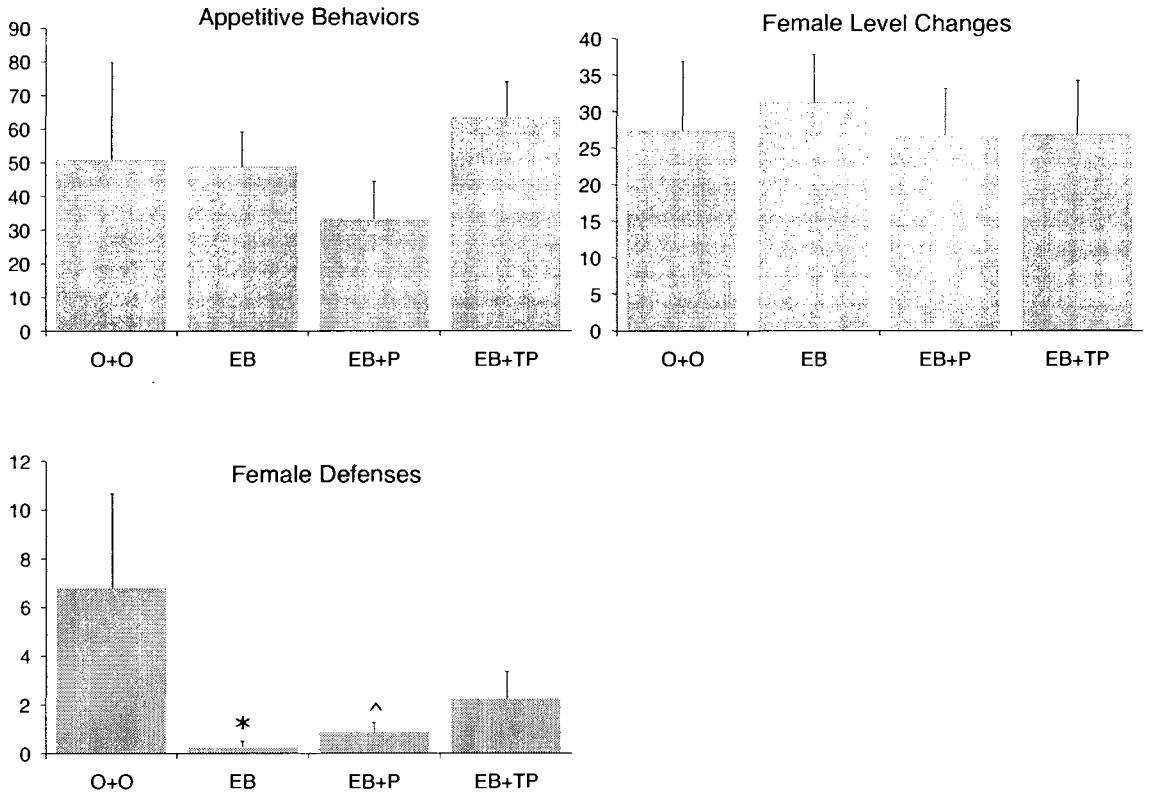

Figure 10. Frequency of defensive and appetitive sexual behaviors of aged female Wistar rats following acute injections of different hormone regimens. (O: sesame oil, EB: estradiol benzoate, 10 μg; P: progesterone, 500 μg; TP: testosterone propionate, 200 μg). Values are mean +/- SEM. * Significantly different from O+O *p<.05, ^p<.10.

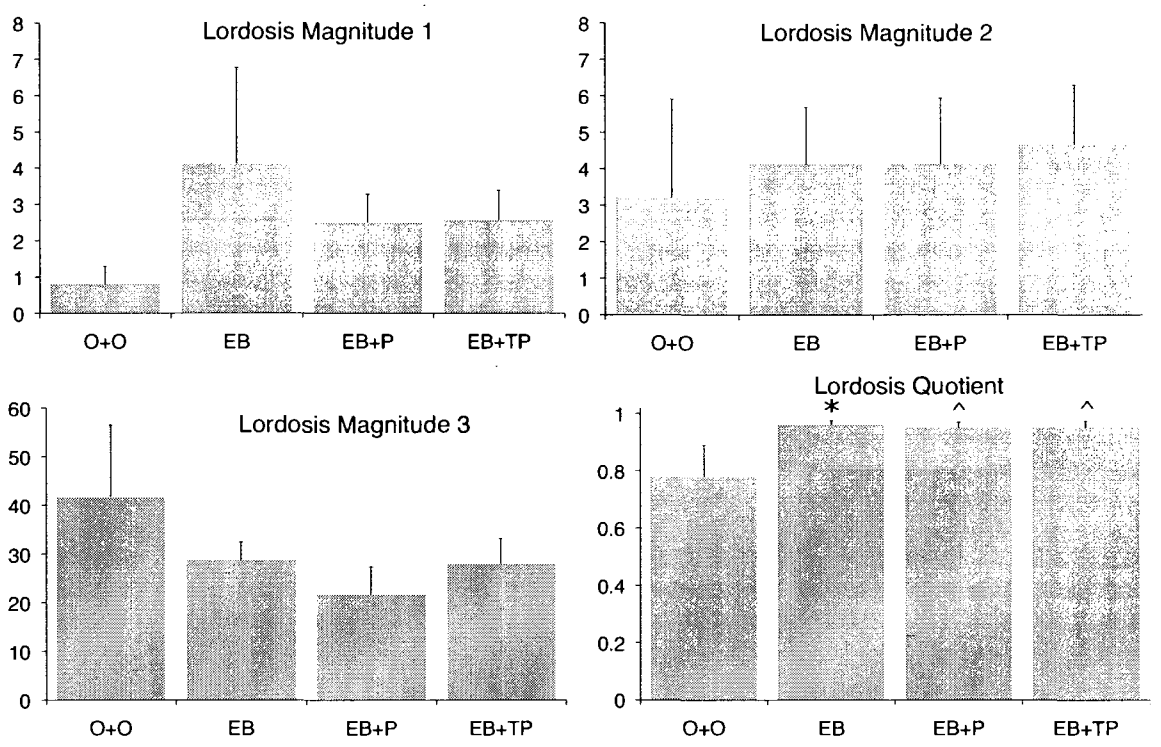

Figure 11. Frequency of consummatory sexual behaviors of aged female Wistar rats following acute injections of different hormone regimens. (O: sesame oil, EB: estradiol benzoate, 10 μg; P: progesterone, 500 μg; TP: testosterone propionate, 200 μg). Values are mean +/- SEM. * Significantly different from O+O. $p < .05$, ^$p < .10$

Phase 2: Chronic administration of TP alone or in combination with EB

Hormonal procedures.

Immediately following *Phase 1*, animals were separated into two groups where half the animals from each of the hormone groups in *Phase 1* were assigned to the O+TP (n=20) and the other half to the EB+TP group (n=20). Testing began 4 days following the end of *Phase 1*, and continued for a total of 5 test sessions.

Both groups received a TP dose of 200 μg dissolved in 0.1 mL sesame oil, and injected s.c. 4 hours prior to the start of the testing sessions. Animals in the EB+TP group also received 10 μg EB dissolved in 0.1 mL sesame oil injected s.c. 48 hours prior to the start of the experiment.

Statistical analyses

Animals who received only one mount or less were removed from the analyses, because it suggested that the male was not copulating normally. Removing an animal from one test resulted in eliminating that animal from all tests, which greatly reduced the group sizes. Consequently, a one-way between subjects ANOVA was run, by separating the animals into four groups, resulting in O+TP at trial 1 (T1; n=14), O+TP at trial 5 (T5; n=11), EB+TP at T1 (n=18) and EB+TP at T5 (n=13). Tukey's post-hoc test was used to compare differences between groups. Significance levels were set at $p<.05$.

Results

The frequencies of sexual behaviors are presented in Figure 12 (male behaviors towards females), Figure 13 (female appetitive behaviors) and Figure 14 (female

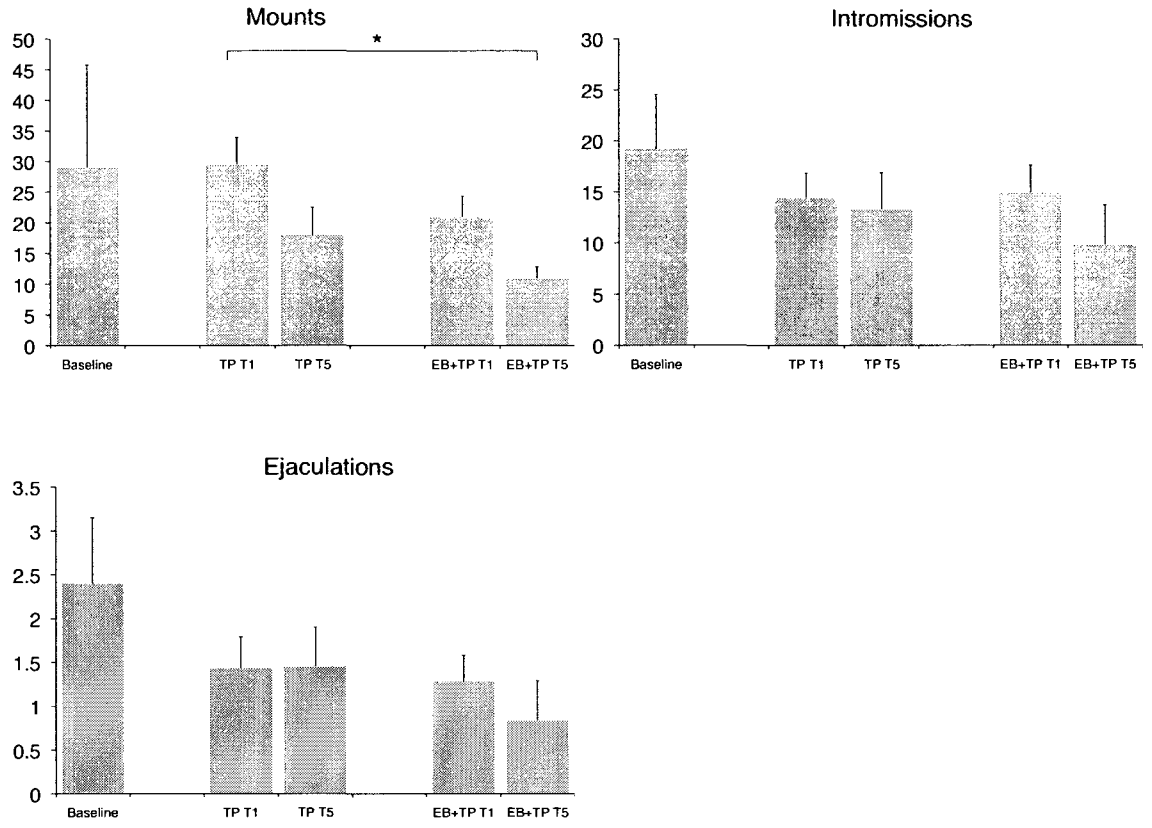

Figure 12. Frequency of male sexual behaviors towards aged female rats following chronic administration of TP-alone or EB+TP injected s.c. over 5 test trials. Bars represent frequency of behavior at baseline, trial 1 (T1) and trial 5 (T5). * $p < .05$.

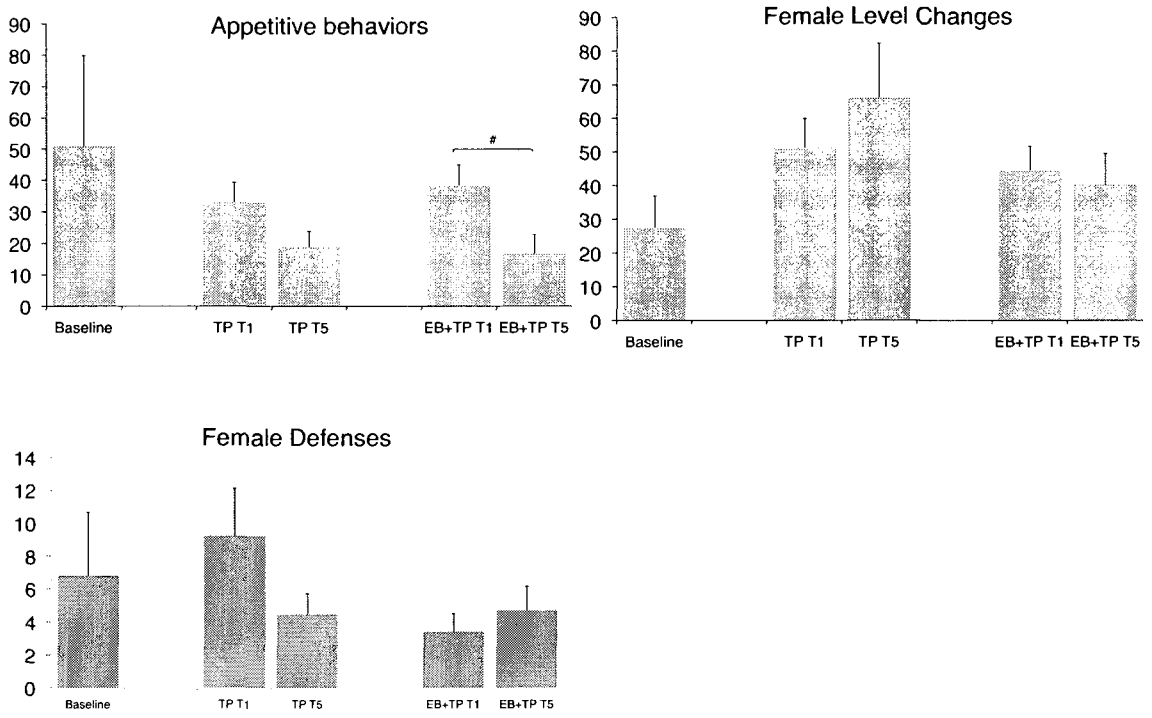

Figure 13. Frequency of appetitive sexual behaviors of aged female rats following chronic administration of TP-alone or EB+TP injected s.c. over 5 test trials. Bars represent frequency of behavior at baseline, trial 1 (T1) and trial 5 (T5). * $p < .05$, # $p > .05 < .1$.

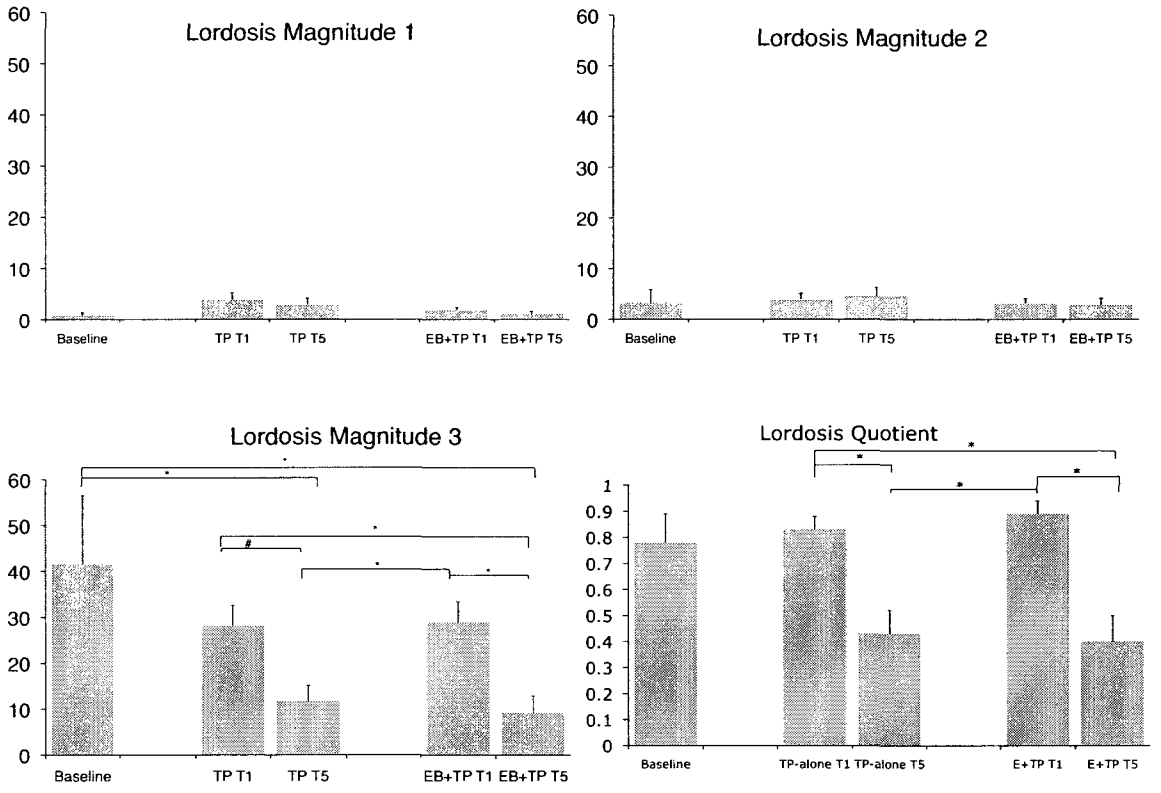

Figure 14. Frequency of consummatory sexual behaviors of aged female rats following chronic administration of TP-alone or EB+TP injected s.c. over 5 test trials. Bars represent frequency of behavior at baseline, trial 1 (T1) and trial 5 (T5). * $p < .05$, # $p > .05 < .1$.

consummatory behaviors). Baseline represents the control animals from the acute test (Experiment 1 *Phase 1*) as a comparison.

Male behaviors.

There was a main effect of hormonal regimen on mounts, $F(3, 52)= 4.129$, $p=.011$. Post-hoc Tukey tests revealed that females that received TP-alone on T1 received significantly more mounts than females that received EB+TP on T5.

Female appetitive behaviors.

There was a main effect of hormone regimen on appetitive behaviors, $F(3,52=2.89$, $p=.044)$ however post-hoc tests detected only a trend between EB+TP at T1, who had higher levels than EB+TP at T5 ($p=.069$).

Female consummatory behaviors.

A main effect was also detected for hormone regimen on LM 3, $F(3,53)=6.085$, $p=.001$. Post-hoc tests revealed that females that received TP-alone at T1 or EB+TP at T1 displayed significantly more LM 3s than females that received EB+TP on T5. Likewise, females that received EB+TP at T1 displayed significantly more LM 3s than females that received TP-alone on T5. There was also a main effect on LQ, showing that LQs at T5, for both females treated with TP-alone or EB+TP were significantly attenuated compared to those same groups at T1.

Discussion

Aged, irregularly cycling female rats, when administered EB acutely, show increased LQs. EB also decreased the frequency of defensive behaviors. These results coincide with data in the current literature suggesting that E controls consummatory aspects of female rat sexual behavior. No other aspect of sexual behavior was affected by acute hormone treatment.

When aged irregularly cycling female rats are administered chronic TP or EB+TP by subcutaneous injection, over time behaviors are attenuated in both groups. There is a time-dependent decrease in LQ regardless of treatment, where both TP-alone and EB+TP at T1 are no different from baseline, however at T5, both treatment groups display significantly fewer LMs in response to flank stimulation from the male. A similar pattern occurs for appetitive behaviors, where there is a time-dependent decrease, irrespective of treatment. These results suggest an activation of inhibitory systems in the display of sexual behaviors. This inhibition may be a result of increased circulating T, or increased bioavailability of E2, by T binding SHBG releasing E2 into the bloodstream, or conversion of T to E2.

There was large variance in behaviors within the groups. Replication of this study with a larger group size, decreased variability in responses by using animals at a fixed age (versus a range of 2 months), and co-varying data by blood hormone levels should help in determining whether or not there is an actual effect of differing hormone regimens on the sexual behavior of aged animals. In addition, the dosage of hormones may have not have been optimal. Therefore, these data should be followed-up with a dose-response study.

An additional source of variance may be due to the males' reaction to the female. Although males were trained prior to the start of the experiment, they were trained with hormonally primed females who were showing normal levels of appetitive and consummatory behaviors. Perhaps the males were responding well to hormonally primed females, and were less inclined to attempt a copulatory bout with an older female who was possibly releasing less pheromonal cues and possibly less attractive, resulting in larger variance. Examining Fos activation in the male rats, when exposed to all but tactile cues could test this hypothesis. The design of such an experiment would place a young female in behavioral Estrus and an aged animal with Estrus cell morphology behind a screen. Male rats typically will show increased Fos activation in brain areas such as the NAcc, in response to estrous odors (Kippin, Cain, Pfaus, 2003). If it is the case that the aged animals are less attractive to the male due to one of these cues, then lower Fos counts would be expected in the animals exposed to the aged female, versus the young female in Estrus. Similarly, we would expect more DA to be released in males in areas such as the mPOA (Hull et al.,1995), when presented with a fully-primed (EB+P) OVX female versus the aged female, given the hypothesis that the OVX female is a more attractive sexual stimulus to the male compared to the aged female, so the males may be more sexually motivated to copulate with the OVX female.

In summary, acute EB in aged animals facilitates sexual behaviors, particularly receptive behaviors (i.e. LQ). Neither the addition of P nor TP had a significant effect on either consummatory or appetitive behaviors. Whereas the chronic administration of both TP-alone or in combination with EB lead to an eventual attenuation of female sexual behaviors.

Experiment 3: Aged Intact Constant TP

Introduction

The results from Experiment 2 *Phase 1* suggest that the 200 μg dose of TP may have been insufficient in the intact animals to see an acute effect, or that the hormonal state of the intact females was leading to large variance within groups to find differences between groups. In addition, chronic phasic administration of 200 μg TP via subcutaneous injection every 4 days did not facilitate sexual behavior, but in fact attenuated both consummatory (e.g. LQ) and appetitive behaviors over time.

Transdermal administration of T in menopausal women has been shown to minimize unpleasant side effects that are often reported with oral doses and is thus the preferred route of administration. This method of administration has been successful in the treatment of sexual dysfunction in Europe. Sommerville and Tartellin (1983) have shown that in rats, subcutaneous silastic capsules are effective for administering prolonged hormone treatments, and that the rate of diffusion is proportional to the capsules' surface area.

Experiment 3 consisted of 2 phases which investigated the effect of constant levels of TP administered subcutaneously via silastic capsules of varying sizes on the sexual behavior of aged female rats when administered alone (*Phase 1*) or with EB (*Phase 2*).

Method

Subjects.

Forty retired breeder female rats of the Wistar strain were received from Charles River Canada (St. Constant, Quebec, Canada). Females were aged approximately 3-4 months. All animals had bore at least one litter, however the supplier did not provide litter counts and sizes. Animal's general health was monitored through examination of behavior, teeth, nails and body weight. One animal died prior to the beginning of the experiment due to unknown reasons.

Cell cytology.

Beginning at approximately 8-9 months of age, vaginal cell cytology was collected in a similar manner to Experiment 2. Cell collection was continued daily during the first quarter of the dark cycle, until the end of the experiment.

Hormonal procedures.

Silastic capsules were made by cutting silastic tubing (Dow Corning Corporation, Midland, MI, USA; ID: 0.058, OD: 0.077) to varying lengths (5 mm, 10 mm) and filled with crystalline TP (Steraloids, Newport, RI) before being sealed with silicone glue. All capsules were incubated overnight in phosphate-buffer saline at room temperature, to release the initial burst of hormone (Mannino, South, Inturrisi, & Quinones-Jenab, 2005).

Animals were separated into 4 groups receiving varying lengths of TP-filled silastic capsules: 5 mm (n=10), 10 mm (n=10), or two 10 mm (n=10) capsules. Control animals

(n=9) received a blank 5 mm capsule. All capsules were inserted following a baseline sexual behavior session.

Experimental procedure.

Animals were always tested during the middle-third of their dark cycle. Females received a baseline session and 10 sexual behavior tests, each separated by 4-day intervals, in bi-level chambers with vasectomized, sexually experienced males. All experimental tests were conducted with the implanted silastic capsule of TP.

Behavioral analyses

All behaviors that were observed in Experiments 1 and 2 were also analyzed in Experiment 3.

Statistical analyses

A one-way between-subjects ANOVA was run on each behavior to compare groups at baseline, and trial 1 (T1) and trial 5 (T5) of both TP-alone and EB+TP tests. Repeated-measures ANOVAs were not used because the hormonal state of the animals varied from test to test, implying that the animal was actually very different from one test to the next, consequently, a between-subjects analysis was deemed more appropriate.

Phase 1: Chronic administration of varying doses of TP

Results

At baseline, there were no differences between any of the groups on any behavioral measure, M ± SEM are presented in Table 1.

Behavior	Blank (n=7)	5 mm (n=9)	10 mm (n=10)	20 mm (n=10)	p
Appetitive	26.71±14.03	35.00±13.35	21.20±10.40	10.00±6.56	.43
Level Change	15.43±5.34	14.11±2.98	18.40±6.98	14.60±5.32	.94
Defenses	9.71±5.01	5.00±1.99	13.90±5.01	25.10±9.86	.17
LM 1	1.14±0.55	4.56±3.64	1.50±1.08	0.20±0.13	.42
LM 2	5.14±2.38	6.22±3.67	5.30±4.13	0.10±0.10	.47
LM 3	21.29±11.34	15.00±6.09	6.40±3.20	7.30±4.88	.34
LQ	0.46±0.17	0.53±0.15	0.30±0.12	0.20±0.11	.31
Mount	29.00±11.11	26.44±10.27	17.9±4.86	13.60±2.71	.44
Intromission	8.29±3.77	3.44±1.25	3.80±1.88	3.90±2.54	.51
Ejaculation	1.14±0.63	0.22±0.22	0.60±0.34	0.60±0.43	.53

Table 1. Sexual behaviors of intact aged female rats at baseline, prior to insertion of silastic capsules of TP. No differences were found between any of the groups on any behavior.

Figures of the behaviors of TP-alone at the specified trials are summarized in Figures 15 (male sexual behaviors towards female), Figure 16 (appetitive female sexual behaviors) and Figure 17 (consummatory female sexual behaviors). Behaviors at both time periods are presented for comparison purposes.

Male behaviors.

There was a significant effect of TP-alone on mounts at T1, $F(3,32)= 4.238$, $p=.012$. Control animals received fewer mounts ($1.14 \pm .404$) than the 20 mm group (34.4 ± 5.94)

There was a significant effect of TP-alone on intromissions at T1, $F(3,32)=3.857$, $p=.018$. Control animals received significantly fewer intromissions (0 ± 0) than the 20 mm group (9 ± 1.6).

Female appetitive behaviors.

When hops, darts and solicitations were grouped into one measure of total appetitive behaviors, there was a significant effect of TP-alone at T1, $F(3,32)=3.404$, $p=.029$. Controls displayed significantly fewer appetitive behaviors (2.86 ± 2.5) compared to the 20 mm group (47.1 ± 11.5).

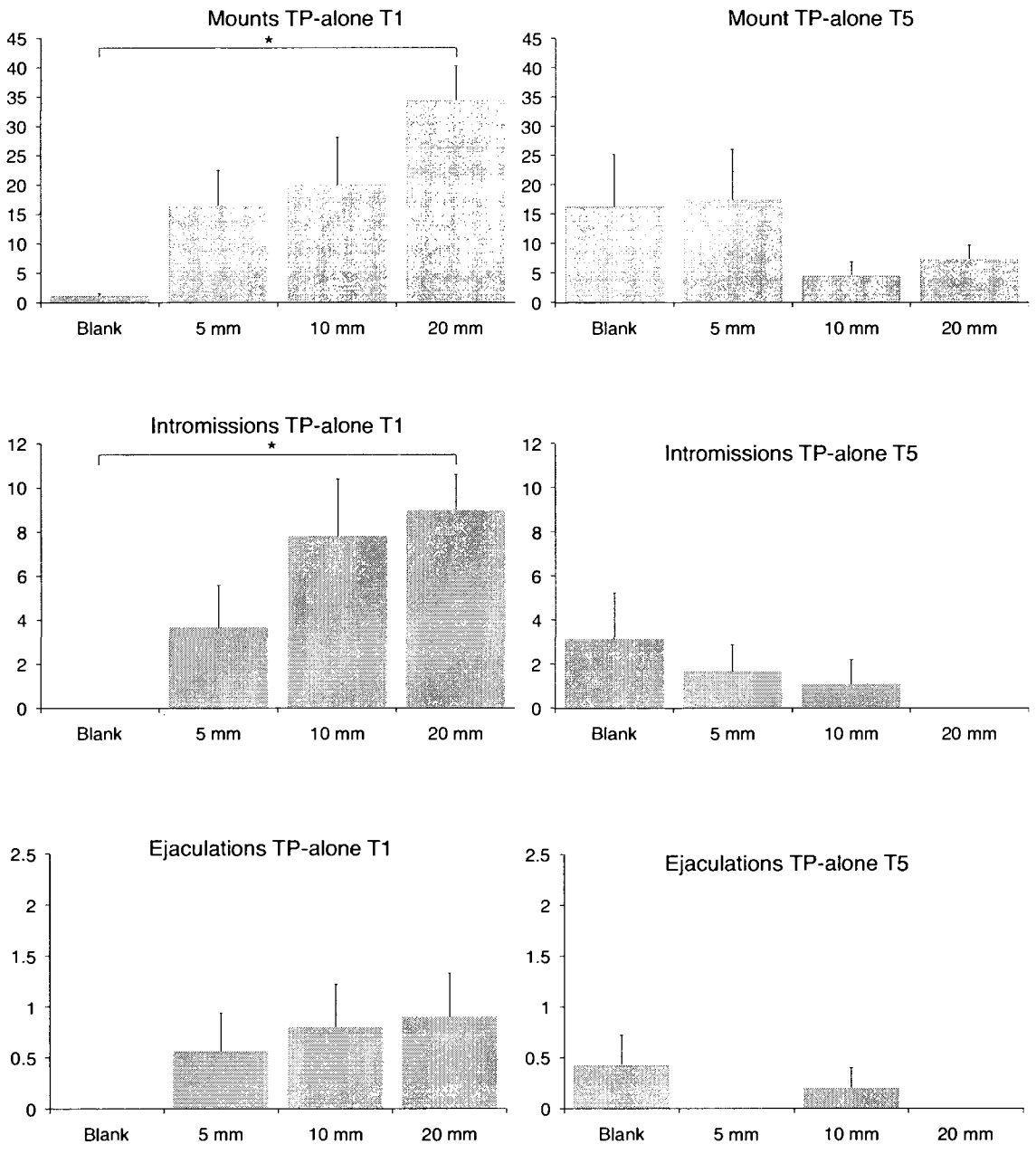

Figure 15. Frequency of male sexual behaviors towards aged female rats following chronic administration of TP via subcutaneous silastic capsules over 5 test trials. Trial 1 (T1) and Trial 5 (T5) significant behaviors are displayed. * $p<.05$.

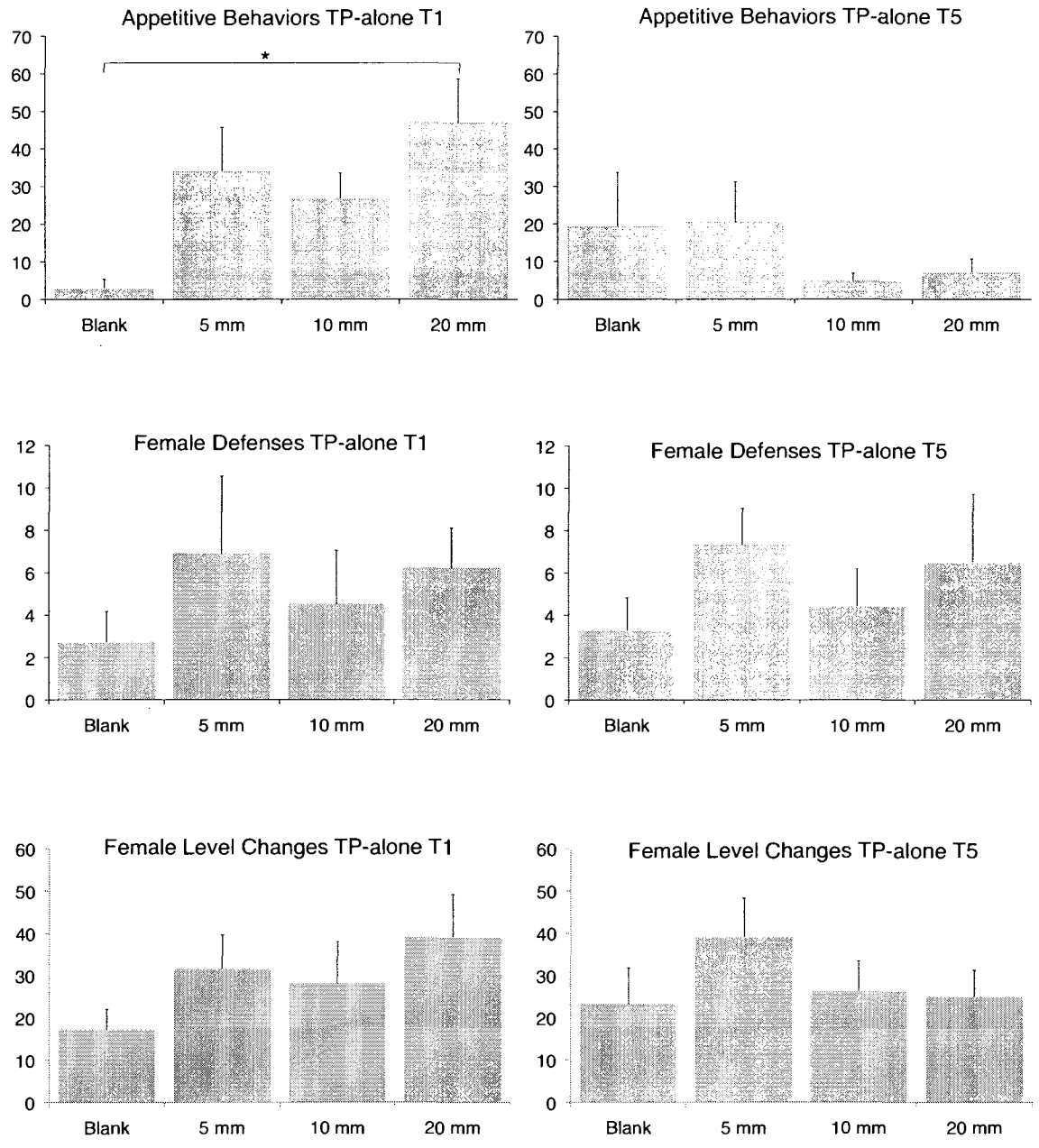

Figure 16. Frequency of defensive and appetitive sexual behaviors of aged female rats following chronic administration of TP via subcutaneous silastic capsules over 5 test trials. Trial 1 (T1) and Trial 5 (T5). * $p<.05$.

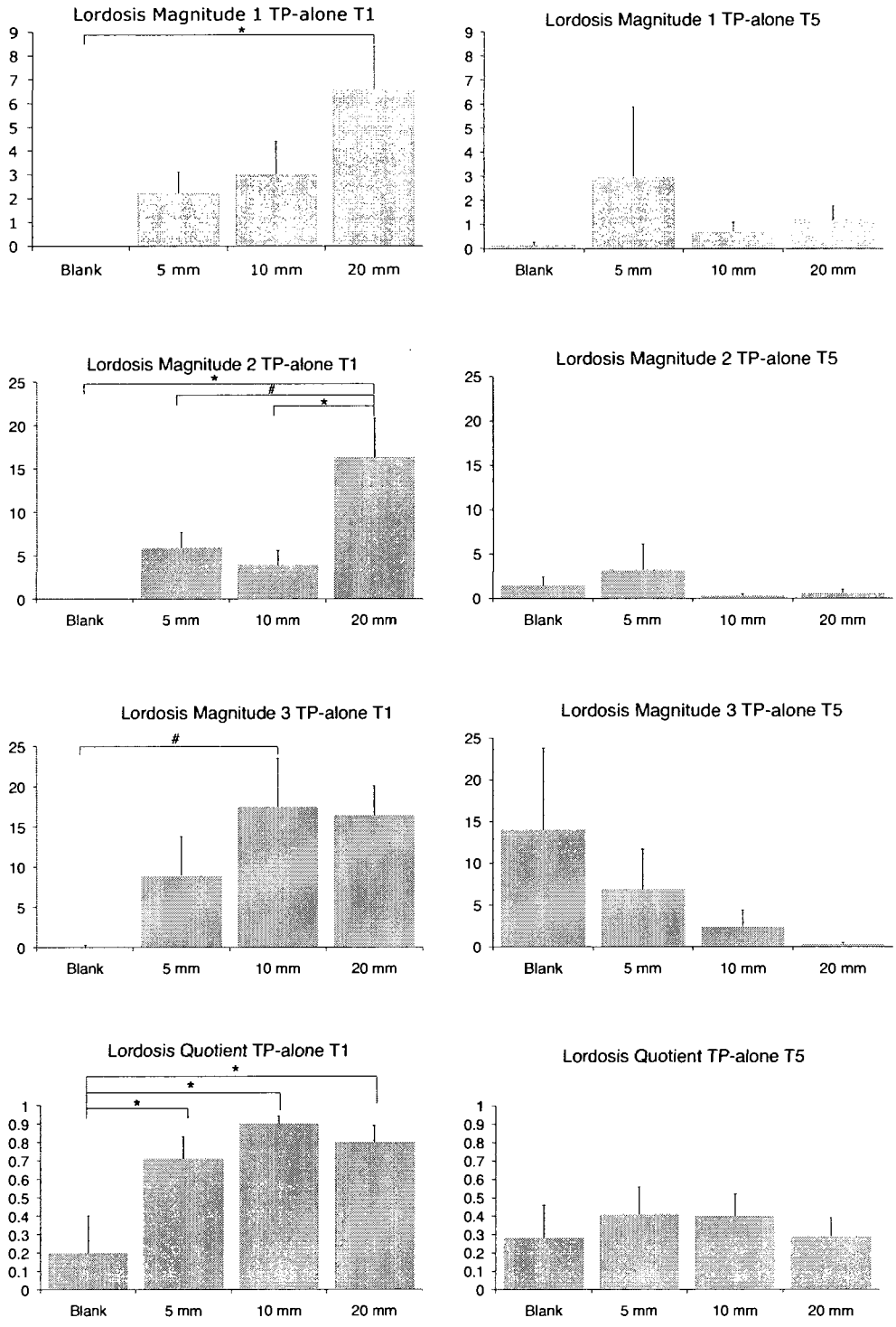

Figure 17. Frequency of consummatory sexual behaviors of aged female rats following chronic administration of TP via subcutaneous silastic capsules over 5 test trials. Trial 1 (T1) and Trial 5 (T5) significant behaviors are displayed. * $p<.05$; # $p>.05<.10$.

Female consummatory behaviors.

There was a significant main effect of TP-alone on LM 1 at T1, $F(3,32)= 3.793$, $p<.05$. Control animals displayed fewer LM 1s (0.00 ± 0.00) compared to the 20 mm group (6.6 ± 1.8).

There was a significant main effect of TP-alone on LM 2 at T1, $F(3,32)= 5.953$, $p<.002$. The 20 mm group displayed significantly more LM 2 (16.3 ± 4.5) compared to controls (0.00 ± 0.00) or 10 mm (3.9 ± 1.7) animals.

There was a significant effect of TP-alone on LQ at T1, $F(3,27)=6.011$, $p=.003$. Controls had a significantly lower LQs (0.20 ± 0.20) than the 5 mm (0.71 ± 0.12), 10 mm (0.90 ± 0.04), or 20 mm (0.80 ± 0.09) groups.

Phase 2: Chronic administration of varying doses of TP in combination with EB

Hormonal procedures

The day following the 5th sexual behavior test of TP-alone, capsules were removed and new ones were inserted (prepared and inserted as previously described). The control group (blank capsules) however, did not undergo any surgery on the second implant day, to reduce the risk of death or infection due to surgery.

Forty-eight hours prior to each test session, animals were administered 10 μg of EB dissolved in 0.1 mL sesame oil via s.c. injection.

Results

Figures of the behaviors of EB+TP at the specified trials are summarized in Figure 18 (male sexual behaviors towards female), Figure 19 (appetitive female sexual behaviors) and Figure 20 (consummatory female sexual behaviors). Behaviors at both time periods are presented for comparison purposes.

Male behaviors.

There was a significant effect of EB+TP on intromissions at T1 $F(3,28)=5.27$, $p=.005$. Control animals received significantly more intromissions than the 20 mm group.

There was a significant effect of EB+TP on ejaculations at T1, $F(3,28)= 3.228$, $p=.037$. Control animals received significantly more ejaculations than the 20 mm group.

Female appetitive behaviors.

There was a significant main effect of EB+TP on level changes at T5, $F(3,32)=4.421$, $p=.010$. The 5 mm group displayed more level changes than the control and 20 mm groups.

There was a significant effect of EB+TP on total appetitive behaviors at T1, $F(3,28)=3.584$, $p=.026$. Controls displayed significantly more appetitive behaviors compared to 20 mm.

Female consummatory behaviors.

There was a significant main effect for EB+TP at T1 $F(3,28)= 5.386$, $p=.005$, where control animals displayed significantly more LM 2s compared to the 5 mm, 10 mm, or 20 mm groups.

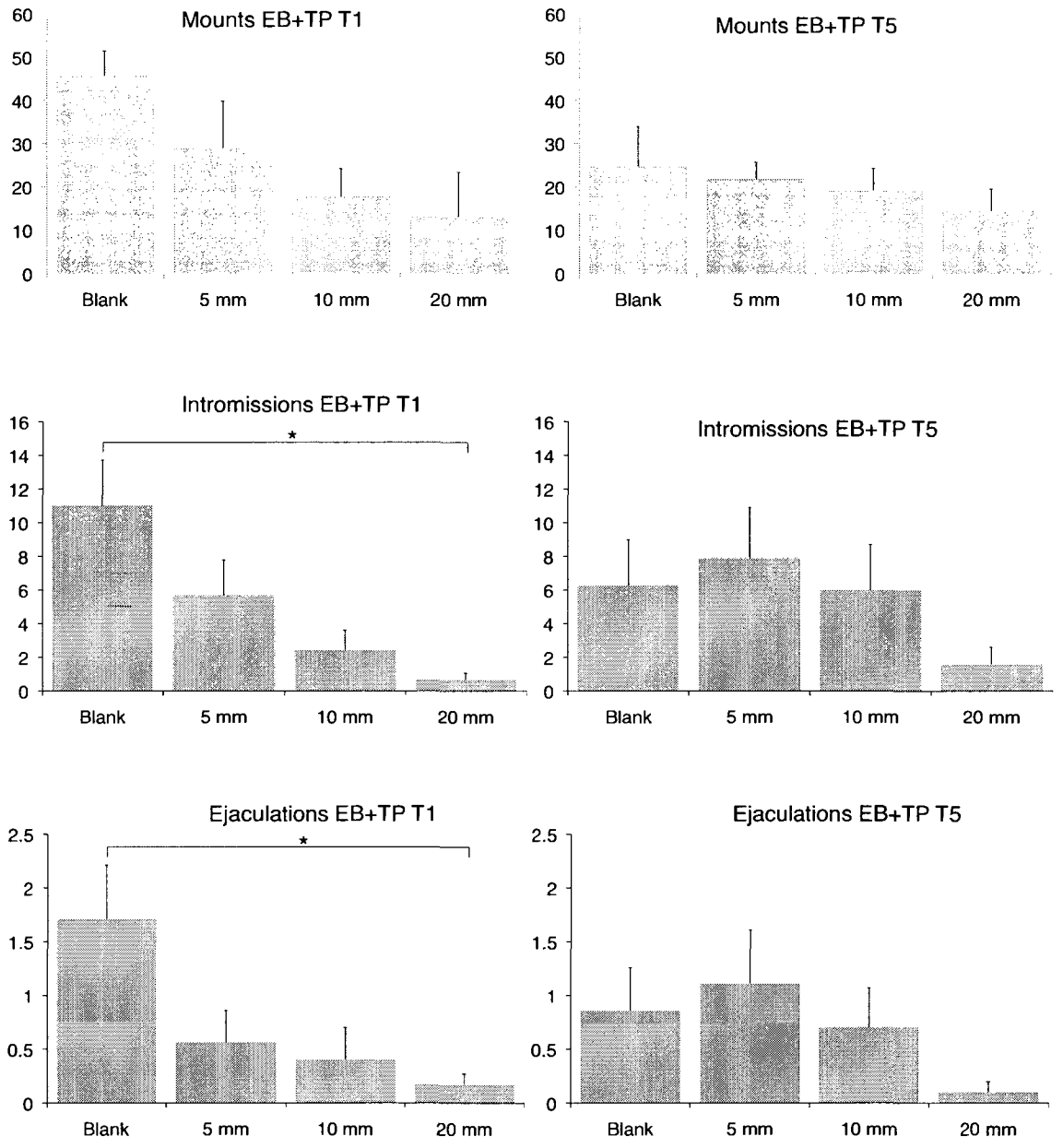

Figure 18. Frequency of male sexual behaviors towards aged female rats treated with chronic TP via subcutaneous silastic capsules over 5 trials, when administered EB 48 hours prior to testing (EB+TP). Test 1 (T1) is represented on the left and Test 5 (T5) is represented on the right; * $p<.05$.

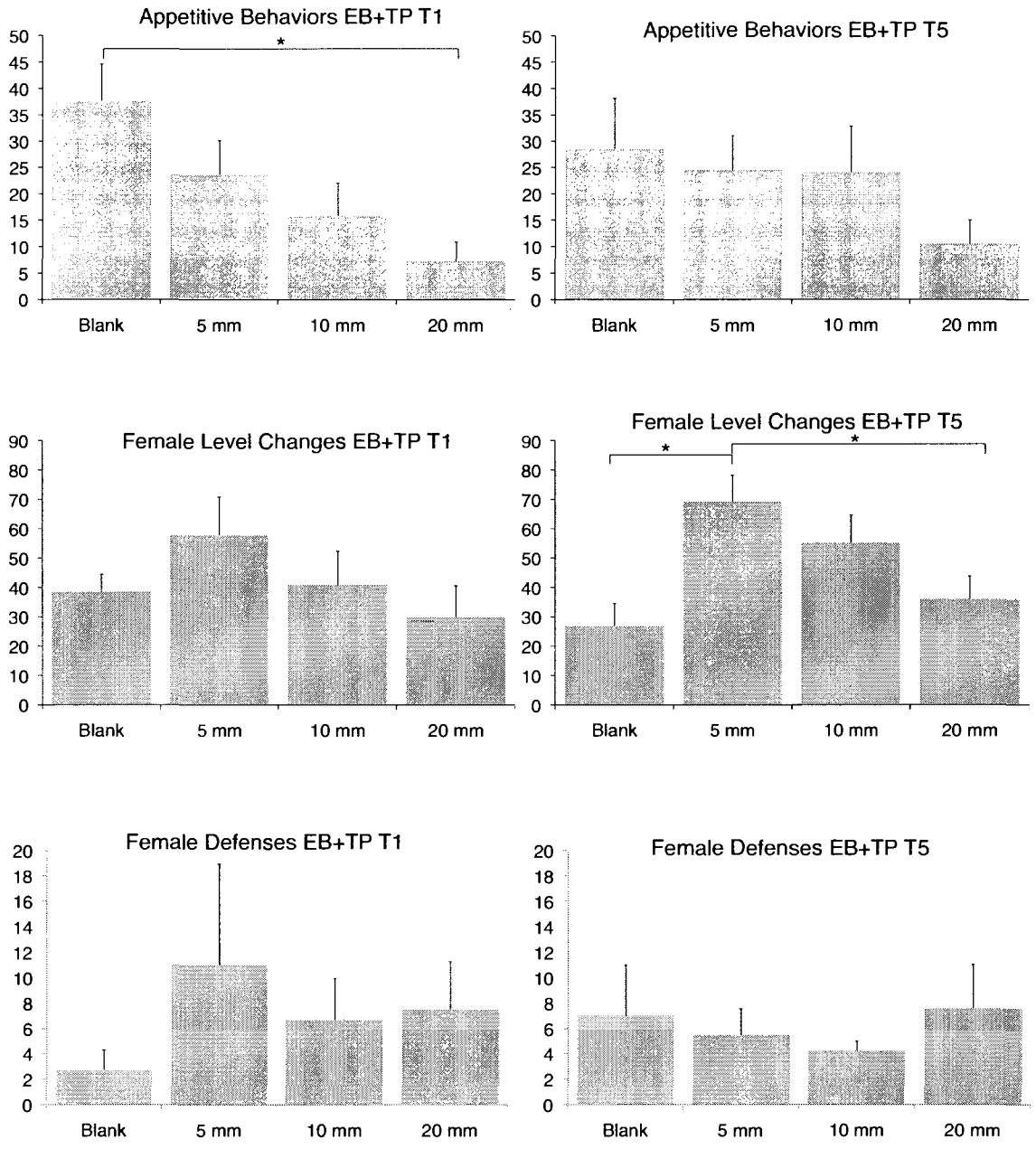

Figure 19. Frequency of appetitive and defensive behaviors of aged female rats following chronic administration of TP via s.c. silastic capsules over 5 trials, when treated with EB 48 hours prior to testing (EB+TP). Test 1 (T1) is represented on the left and Test 5 (T5) is represented on the right; * $p<.05$. # $p<.10$.

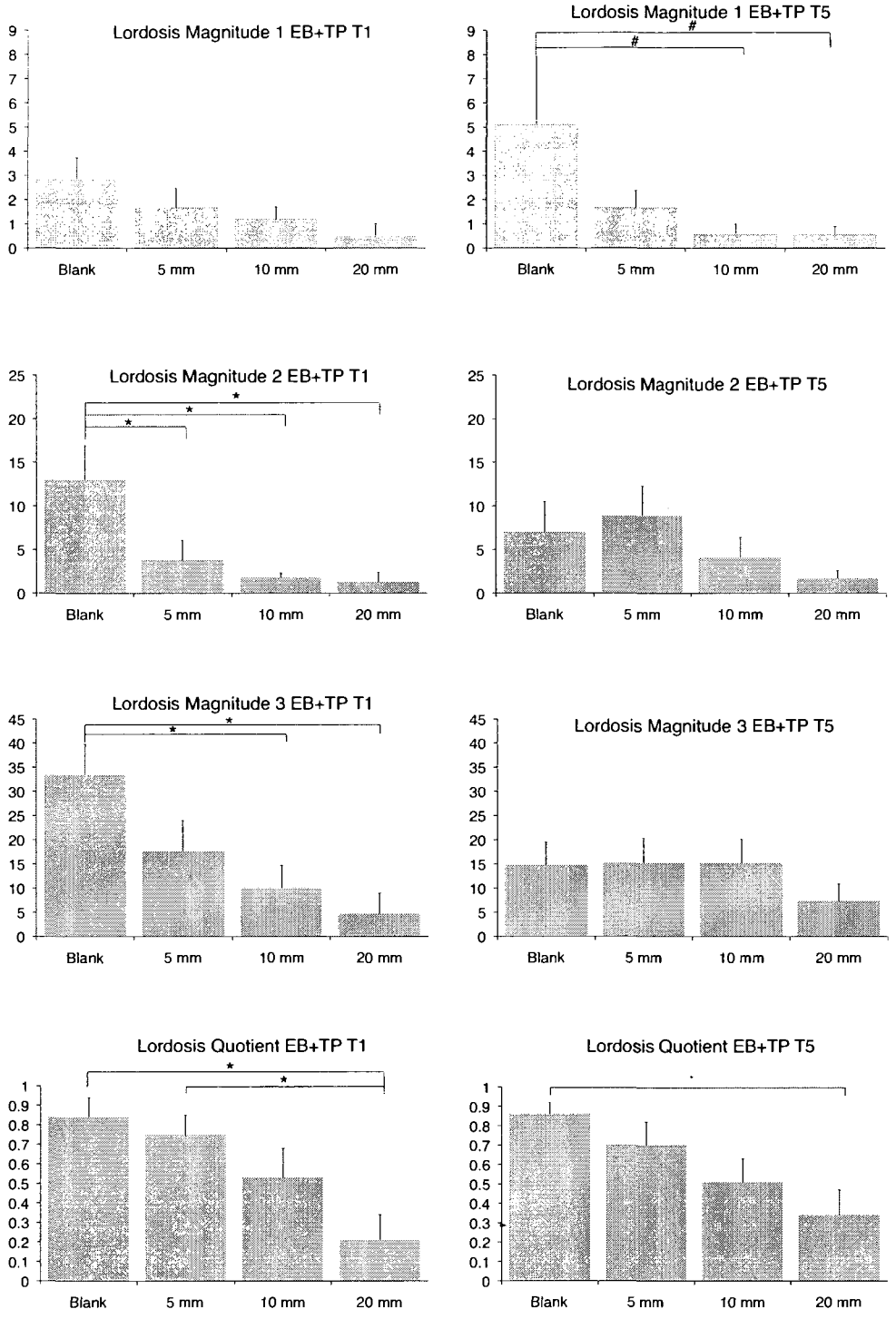

Figure 20. Frequency of consummatory sexual behaviors of aged female rats following chronic administration of TP via subcutaneous silastic capsules over 5 trials, when treated with EB 48 hours prior to testing (EB+TP). Test 1 (T1) is represented on the left and Test 5 (T5) is represented on the right; * $p<.05$.

There was a significant main effect of EB+TP on LM 3s at T1, $F(3,28)= 4.357$, $p=0.012$. Control animals displayed significantly more LM 3s than the 10 mm or 20 mm groups.

There was a significant effect of EB+TP on LQs at T1, $F(3,25)=4.022$, $p=.018$. There was a dose-dependent decrease in LQ, where control animals displayed significantly more LQs than the 5 mm, 10 mm, or 20 mm groups.

There was also a significant effect of EB+TP on LQs at T5, $F(3,31)=3.218$, $p=.036$. Here, there was also a dose-dependent decrease in LQ, where control animals had a significantly higher LQ than the 5 mm, 10 mm, or 20 mm groups.

Discussion

In Experiment 3 aged female rats were chronically exposed to varying doses of TP via subcutaneous capsules, either alone, or in combination with EB. When TP was administered alone, males initially responded with increased mounts and intromissions and females displayed more lordosis reflexes compared to control animals, in a dose-dependent manner. Over time, however, the differences between groups were generally lost where animals receiving TP showed behaviors similar to control animals.

When EB was administered 48 hours prior to the test, there was a dose-dependent decrease in all aspects of sexual behaviors that were observed, and this effect remained relatively stable over time. In this experiment, control animals were exposed to EB-alone. Thus, comparing control animals at T1 and T5 shows that chronic EB maintains a stable level of sexual behaviors. This suggests that when EB is administered with TP, there are changes affecting release of pheromonal cues (less mounts, intromissions and

ejaculations from the male), as well as activation of negative feedback onto the hypothalamus, inhibiting sexual behaviors, since LQs and appetitive behaviors were also dose-dependently decreased. This inhibition may be the result of increased levels of E2 into the CNS by T binding SHBG, allowing more bioavailable E2 into the bloodstream, and via aromatization of T to E2. In this scenario, bioavailable E2 would increase as a function of TP dose. Increased E2 could, therefore, lead to the inhibition of sexual behaviors in aged females.

General Discussion

The role of T in female sexual function and behavior has become more and more prominent in the literature, particularly with respect to its effects on sexual desire. Although there are reports in the literature pertaining to the effects of T on female sexual function in the OVX rat, few have been conducted in contexts that favor the full display of female sexual behavior. The present experiments studied the effect of TP, either alone or in combination with EB, on the sexual behavior of both young OVX and aged intact female rats. These experiments were run in bi-level chambers, to allow the full expression of female sexual behaviors (Pfaus, Smith, Coopersmith, 1999).

The results from Experiment 1 *Phase 1* showed that TP alone had no effect on any aspect of female sexual behavior in young OVX rats. When females were treated with EB, they displayed both appetitive and consummatory behaviors, however the addition of TP did not further affect sexual behaviors at any dose (*Phase 2*). *Phase 3* investigated varying hormone treatments on the sexual behavior or these same rats. The results showed that when animals were administered EB+TP, their combined effect was greater than the control groups receiving either oil or TP alone, and the effect was similar in magnitude to EB+P, particularly with respect to appetitive behaviors.

Experiment 2 *Phase 1* found that none of the tested hormone treatments, when administered acutely, had any effect on the sexual behavior of aged intact female rats. However, chronic subcutaneous injections of TP, either alone or in combination with EB, resulted in a progressive decline in sexual behaviors over time (Experiment 2, *Phase 2*).

Experiment 3 showed that initially, the steady release of TP via subcutaneous capsules, dose-dependently served to facilitate all aspects of sexual behaviors in aged females, however over time the effect was dose-dependently reversed. When animals were administered EB 48 hours prior to the test, there was a dose-dependent decrease in all aspects of sexual behavior initially, which was generally maintained over time. This suggests that steady levels of exogenous TP initially serve to facilitate sexual behaviors in aged female rats, but this effect is reversed with EB priming and with chronic TP administration. A viable explanation is that TP increases bioavailable E2 in the blood stream to exert negative feedback on the hypothalamus, inhibiting sexual behaviors in aged animals. In both Experiments 2 and 3, a decline in sexual behaviors occurred over time, suggesting that chronic administration of EB and TP activate negative feedback pathways that inhibit female sexual behavior.

Effects of TP on sexual behaviors of young OVX females

The results of Experiment 1 suggest that acute TP alone, when administered 4 hours prior to the test, has no effect on female sexual behavior. However, when TP was administered with a prior injection of EB, this combined treatment did not increase sexual behaviors to any degree greater than treatment with EB alone. In addition, when comparing varying hormone treatments, the combined effect of EB+TP increased appetitive and consummatory behaviors similar in a treatment of EB+P and greater than EB-alone, though this latter effect was not significant.

The fact that the oil and TP-alone groups displayed low levels of sexual behaviors in *Phase 3*, suggests that carry-over effects from EB and/or TP from *Phase 2* and possibly TP doses from *Phase 1* may have masked the effects. Consequently, this study

should be replicated with proper controls. An important factor contributing to these results is that the doses of EB and P used in these experiments are based on studies used in the literature to study female sexual behavior when they are showing maximal levels of receptivity. The EB dose, in particular, is supraphysiological and the behavioral effects are not necessarily representative of the levels that would be observed in intact females in behavioral Estrus. The high levels of hormones may also be resulting in ceiling effects, obscuring the effects of varying hormone treatments of TP when given in combination with EB. Consequently, future studies should ensure that the behavioral baseline is low, perhaps by decreasing the EB dose or allowing a sufficient time lapse between experiments. The proper dosing regimen and schedule to prevent a high baseline, is not yet known.

The addition of P to an EB-treated female typically results in a significant increase in sexual behaviors (Pfaus, Smith, Coopersmith, 1999; Whalen, 1974). Experiment 1, *Phase 3* did not find any significant differences between these groups, which may be due to carry-over effects of TP and/or EB from *Phases 1* and *2* of the same experiment. Consequently, this experiment should be replicated with a washout period to prevent carry-over effects. The duration of this washout period however, is not known, thus it would first be important to sequentially test EB over varying time intervals (e.g. 4, 8, 12, 16 days). Furthermore, the EB dose used in these experiments (10 μg) are supraphysiological and consequently, varying doses of EB (e.g. 0, 2.5, 5, 10 μg) should also be tested over varying time intervals, where an ideal dosing regimen would induce consistent levels of sexual behaviors at four day intervals, mimicking the estrous cycle.

Optimal levels of estrogen facilitate sexual behaviors in aged animals

When administered acutely to aged animals, EB significantly increased LQ (Experiment 2). The administration of TP-alone had no effect on sexual behaviors, and when administered in combination with EB, did not add to EB's effect. When EB or EB+TP were administered chronically, no initial effects on sexual behavior were observed, however, these behaviors decreased over time (Experiment 2 *Phase 2*). In contrast, when TP was administered alone via subcutaneous capsules, releasing steady levels of TP (Experiment 3 *Phase 1*), sexual behaviors were at first facilitated in aged animals but then attenuated over time. A similar reduction in sexual behaviors occurred when moderate or high doses of TP were added to the EB regimen in Experiment 3 *Phase 2*. These results suggest that low levels of EB in aged animals due to decreased ovarian steroid production blunt the expression of sexual behavior and the addition of exogenous EB facilitates sexual behavior in those animals, but only when administered at an optimal dose. However, when EB reaches high levels (increasing bioavailable E2 by either the chronic administration of 10 mm and 20 mm TP capsules [TP-alone on T5] or when these TP doses are added to an EB regimen [EB+TP on T1 and EB+TP on T5]) sexual behaviors are dose-dependently attenuated. This relationship is depicted in Figure 21, where varying bioavailable E2 levels lead to an inverted U-shaped curve with respect to sexual behaviors in aged animals, such that low and high bioavailable E2 levels prevent the expression of sexual behaviors, whereas moderate levels facilitate them.

Dow, Hart and Forrest (1983) reported no differences in the improvement of sexual symptoms between postmenopausal women treated with E2 or EB+T. However, Burger, Hailes, Nelson and Menelaus (1987) attributed this lack of effect to the selection

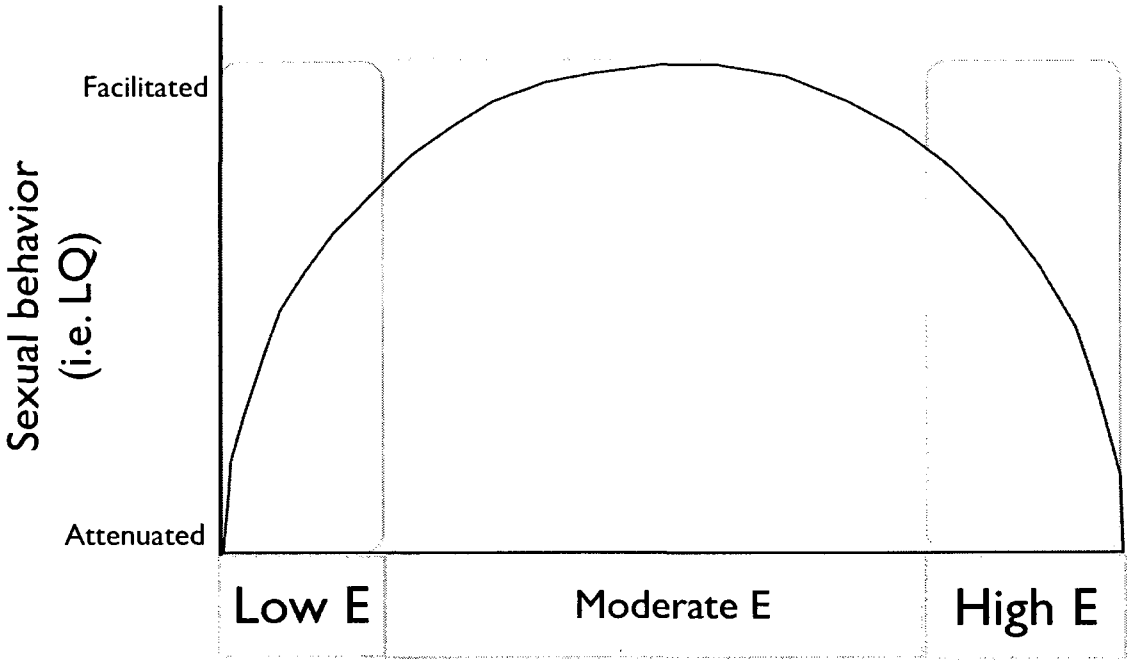

Figure 21. Proposed effect of bioavailable estrogen levels on sexual behavior in aged female rats. Low levels of estrogen (i.e. due to a decline in ovarian steroid hormone production with age) as well as high levels of E (secondary to chronic administration of TP, or the addition of TP to an EB-regimen) attenuate sexual behaviors. Moderate levels of EB (by the steady release of TP administered alone via subcutaneous capsules in the short-term, or by subcutaneous administration of EB-alone) serve to facilitate sexual behaviors in aged animals.

criteria of the subjects, and subsequently showed that a factor determining the successful treatment effect of E2+T pertains to prior treatment with E2. The authors selected women who had reported low libido despite an E2 and P hormone regimen. Subjects were randomly assigned to either an E2-alone regimen or an E2+T regimen. Libido and sexual enjoyment self-report scores were measured prior to treatment and every 6 weeks following treatment. Women in the E2+T group reported significant improvement on both libido and sexual enjoyment scales, whereas those in the E2-alone group did not show any improvement. In addition, when T was added to the E2-alone group, the self-reports 6 weeks later showed a significant improvement on both libido and sexual enjoyment scales. The authors conclude that E2+T may be effective for a sub-group of women who are unresponsive to E. Interestingly, the effect was maintained for up to 18 weeks, however at week 24, both reports of libido and sexual enjoyment returned to baseline levels. The eventual attenuation of sexual behavior in aged animals treated chronically with TP or EB+TP (Experiment 2 *Phase 2*) or high doses of steady levels of TP (Experiment 3 *Phase 1*) parallels well with the study reported by Burger et al. (1987), which suggests similar underlying inhibitory mechanisms of hormone actions on sexual behavior in the female human and rat.

Korenchevsky and Dennison (1937) found that in OVX female rats, chronic TP administration (21 days), both alone and in combination with E2, led to increased uterine weight, and hypertrophy of the vagina and clitoris. The results of the chronic experiments presented in this thesis, would suggest that regrowth of reproductive tissue does not translate into improved sexual responding. This is similar to the actions of sildenafil citrate (Viagra) which increases blood flow to reproductive tissues in women, but does

not ameliorate disorders of sexual arousal or desire (Basson, McInnes, Smith, Hodgson & Koppiker, 2002; Laan, van Lunsen, Everaerd, Riley, Scott & Boolell, 2002). Therefore, restoring reproductive tissue in both women and female rats does not imply restoration of reproductive behaviors. This further reinforces the idea of a "disconnect" between central and peripheral aspects of sexual function in females.

E+T in the treatment of HSDD in menopausal women

Work in humans has provided strong evidence to suggest that reduced levels of T are important in the disruption of certain psychological aspects in women, including those with reduced androgen levels caused by ovarian, adrenal, hypothalamic, drug-related or idiopathic factors (Bachmann et al., 2002; Braunstein, 2002). Testosterone in menopausal women has been shown to increase positive feelings of well-being, energy and libido, symptoms that are not restored with ERT alone (Burger, Hailles, Menelaus , Nelson, Hudson & Balasz, 1984; Burger, Hailes, Nelson & Menelaus, 1987; Davis, McCloud & Sherwin, 2002; Sherwin & Gelfand, 1987; Davis, McCloud, Strauss & Burger, 1995). Although some studies have found no correlation between androgen levels and increased libido (Dennerstein, Dudley, Hopper, & Burger, 1977), many clinical studies have reported significant increases in sexual functioning in particular with regard to the psychological aspects such as desire, sexual fantasies, and sexual reward or enjoyment (e.g. Burger et al., 1995; Burger et al., 1997; Sherwin & Gelfand 1985).

Europe and Australia have an approved transdermal T patch as a treatment of libido disorder for women receiving ERT. The US FDA reviewed the transdermal patch and its approval was rejected (FDA Inrinsa Advisory Committee Background Document

Overview, 2004). The addition of T to ERT in naturally and surgically menopausal women is controversial, namely because the long-term effects are not well known, and because little is known regarding the mechanism of action of androgens on female sexual function.

The results from Experiments 2 and 3, examining chronic administration of TP suggest that long-term treatment of TP diminishes the initial increase in sexual desire, at least in aged animal. This is preliminary evidence that long-term use of E-A regimens attenuates the initial beneficial effect on libido, and suggests that treatment may require "on" and "off" periods to thwart or delay the reduced effectiveness.

Localization of E-A mechanisms

Subcutaneous administration of E+T has profound effects on sexual function. Some of these effects are peripheral, while others are central. The present experiments investigate downstream central effects on sexual behavior. But where in the central nervous system are these effects taking place? Some of the most obvious areas to begin looking would be the substructures of the hypothalamus, particularly the SCN, since it is involved in the control of circadian rhythms (estrous cycles), and the mPOA and VMN which are critical in the control of sexual behaviors; as well as brain areas involved in motivated and rewarding behaviors such as the VTA and NAcc.

Suprachiasmatic nucleus of the hypothalamus.

Ovarian transplants of aged acyclic females into young females cause young animals to begin cycling again, whereas young ovaries into aged females does not (Aschheim, 1961; Felicio, Nelson, & Finch, 1986; Peng & Huang, 1972), suggesting that

the brain plays a major role in reproductive aging and acyclicity. Lesions to the SCN have been shown to disrupt ovarian cyclicity, and light-induced Fos expression in the SCN is attenuated and delayed in middle-aged females, but can be restored to levels equivalent to young animals following transplantation of fetal SCN tissue into the third ventricle (Cai, Lehman, Lloyd & Wise, 1997). The SCN is involved in the cyclicity of the estrus cycle and lesions to the SCN result in persistent estrus (Wiegand & Terasawa, 1982). The SCN presumably does not have steroid hormone input, supported by autoradiography studies finding no ER in the SCN (Pfaff, 1980). However, the SCN has projections to GnRH neurons and neurons involved in the LH surge (Watson, Langub, Engle & Maley, 1995). Consequently, although it may have downstream actions in the control, cessation and reinstatement of sexual behaviors, it is unlikely that E and T are having direct effects on this structure.

Medial preoptic and ventromedial hypothalamus.

Other areas of the hypothalamus such as the mPOA and VMH, however, are critical for female sexual behaviors. Estrogen and androgen receptors are located in these areas, where E2 and P act in the VMH to induce lordosis in females, and P is known to act in the mPOA to facilitate appetitive behaviors, in particular, solicitations. Estrogens and androgens also act in the mPOA of males to restore copulatory behaviors.

Progesterone binding to its receptor first activates sexual behaviors but additional P binding results in deactivation of the receptor, terminating sexual receptivity (McCarthy and Becker, 2002; Reading, Blaustein, 1984). In OVX middle-aged female rats, E2 nuclear receptor and P cytosol receptor densities have been shown to be significantly lower in the POA and medial basal hypothalamus after 2 days of E2

exposure compared to young OVX rats. This difference was alleviated on day 4 of E2 exposure, suggesting that middle-aged rats require greater or longer exposure to E2 for protein synthesis of P receptors (Wise and Parsons, 1984). When comparing nuclear E binding in the POA of middle-aged rats versus young females, it was found that decreased nuclear ER binding was correlated with increased ER in the cytosol, suggesting that the interaction between the ER complex and the nucleus is what is disrupted in aged females and not the receptors themselves (Rubin, Fox, Bridges, 1986). This may explain both the results of EB+P treated intact females in Experiment 2 *Phase 1*, as well as the results of Chambers and Phoenix (1986) who found that EB+P restored lordosis, but only low levels of appetitive behaviors in OVX aged animals. Aging animals may require longer exposure or higher doses of EB for protein synthetic effects to occur. For instance, if P receptors were not yet synthesized, the administration of P would result in only a low level of P-mediated appetitive sexual behaviors.

The VMH and mPOA are important regions for E2-induced consummatory sexual behaviors (Pfaff, 1980), where lesions of these areas reduce or abolish the expression of lordosis. (Whitney, 1986; Yang & Clemens, 2000). Administration of crystalline E2 into these brain areas facilitates sexual behaviors (Pfaff, 1980). Androgens have been shown to be important in non-human primates with respect to appetitive behaviors, which were increased in OVX rhesus monkeys following TP administration into the POA (Everitt & Herbert, 1975).

It has been shown that cystolic androgen receptors are present in many brain areas involved in sexual behavior, including the mPOA and VMH, in both males and females, although the density is higher in males than in females (McGinnis & Katz, 1996). This

raises the possibility that androgens may contribute to the acute effect of facilitation of sexual behaviors in EB+TP primed animals, through androgen receptors in the mPOA.

It is known that E2 potentiates lordosis behaviors through actions in the VMH, and P further facilitates appetitive behaviors by acting on the mPOA (Blaustein & Erskine, 2002; Pfaff, Schwartz-Giblin, McCarthy & Kow, 1994; Blaustein & Mani, 2007). Because T restored appetitive behaviors equivalent to EB+P in OVX rats, and because androgen receptors are present in the mPOA, it is reasonable to hypothesize that T may be acting on androgen receptors in the mPOA to restore sexual behaviors in young OVX females. If this were true, then perhaps androgen receptor densities or binding is attenuated or delayed in the aged female brain given that the effect of EB+TP in these animals was no different from EB-alone (Experiment 1). Future studies could use autoradiography to compare androgen receptor binding in hypothalamic regions such as the mPOA and VMH at varying time periods in both aged intact and OVX rats given hormone replacement.

Alternatively, given that crystalline E2 administered directly into the VMH and mPOA facilitates sexual behaviors (Pfaff, 1980), the aromatization of T to E2 in these brain areas may also explain the facilitation of sexual behaviors in these animals. Co-administering T and aromatase inhibitors directly into the brain would test this hypothesis. If aromatase inhibitors prevented an increase in sexual behaviors, it could be suggested that E2 is responsible for the effect.

It is known that the LH surge is attenuated and delayed in middle-aged rats (Cooper, Conn & Walker, 1980), that it can be restored in OVX middle-aged rats with

physiological levels of E, but that this restoration necessitates longer exposure to E2 compared to young OVX rats (Wise & Parsons, 1984). Also, as previously discussed, synthesis of P receptors requires greater or longer exposure to E2 in middle-aged OVX females compared to young OVX animals (Wise & Parsons, 1984). To test whether receptor synthesis or binding is delayed in aged animals, future experiments can study the effects of TP and P following a longer exposure to E2, which should facilitate sexual behaviors to greater degree than E2-alone if receptor synthesis is delayed in these animals.

Testosterone has been shown to act directly on the androgen receptor to affect female reproductive tissue (Traish et al., 2002; Traish et al., 2007) as well as to affect motivational aspects of sexual behavior in primates following TP implants to the AH and mPOA (Everitt & Herbert, 1975). Cystolic androgen receptors are present in many brain areas involved in sexual behavior (McGinnis & Katz, 1996) including the mPOA and VMH. The results of the TP-alone dose response study in young OVX females suggest that T may be acting synergistically with EB and directly acting on androgen receptors to restore sexual behavior. In experiment 1 *Phase 1*, young OVX females were administered varying doses of TP, including the highest dose of 400 μg. If aromatization was responsible for the restoration of sexual behaviors, the animals receiving the highest dose should have displayed some level of sexual behaviors, which was not the case. This lends support to the hypothesis that TP must be interacting with EB in some way, in order to facilitate sexual behaviors. Androgen receptor blockers such as flutamide, administered with EB+TP treatment would test whether or not T is binding its receptor to activate

sexual behaviors. If the effect were to be blocked following flutamide administration, the results would support direct actions of T on its receptor.

Mesolimbic dopamine system.

The mesolimbic DA system projects from the VTA to limbic structures such as the NAcc, amygdala, and septum, and is involved in motivation and attention to reward-related stimuli. Progesterone in the VTA has been shown to be important for the induction of lordosis behaviors via binding to GABAα receptors or interacting with second-messenger systems activated by dopamine binding to the DA receptor 1 subtype (Frye, 2001; Frye and Walf, 2008). Estrogen binding in the NAcc and striatum decreases inhibitory actions of GABA or DA autoreceptors, promoting DA release (Becker, 1999). Dopamine in the NAcc increases in female rats when the context is rewarding for the female, e.g., when copulation occurs at the females' preferred intervals (Becker, Rudick & Jenkins, 2001). These mechanisms of steroid hormone actions in aged females are likely to be involved in the disruption of sexual behavior as well as the restoration of sexual behaviors in both OVX and aged animals treated with EB+TP.

Mechanism of estrogen/androgen treatment

Estrogenic actions.

The effect of EB+TP observed in the present experiments may due to an estrogenic effect, through the aromatization of T to E2, or T binding to SHBGs displacing E2 which releases more of it into the blood stream to elicit its effects, as proposed by Wallen (2001). Both EB and EB+TP elicited a similar effect on lordosis in the aged intact animals receiving acute administration, suggesting that TP does not add to EB's effect.

Similarly, in the young OVX animals, EB+TP treated animals displayed more appetitive behaviors, which may have occurred due to increased levels of bioavailable E2 into the hypothalamus (possibly the mPOA or VMH) to further facilitate sexual behavior. This idea can be tested by the addition of a group of OVX animals receiving EB 48 hours before, followed by EB 4 hours prior to testing, and comparing this effect to EB+TP animals. If the effect is due to increased circulating E2, then administering additional EB 4 hours prior to the test should result in sexual behaviors equivalent to EB+TP.

In aged animals, although TP-alone via subcutaneous capsules facilitates the induction of sexual behaviors, chronic TP-alone eventually leads to a reduction in these behaviors, and EB+TP reduces sexual behaviors as of the first test trial. It seems probable therefore, that TP is either being aromatized, or binding SHBG, ultimately increasing bioavailable E2, activating negative feedback in the CNS and attenuating sexual behaviors. Replicating this study but with the addition of an aromatase inhibitor, androgen receptor blocker, or administering E2 directly into the brain (e.g., to mPOA or VMH), may help to determine whether this hypothesis is tenable. If the effect is not apparent following aromatase inhibition then it can be assumed that E2 is responsible for the inhibitory effect of sexual behaviors following long-term use. This can be further supported with E2 infusions into areas such as the VMH or mPOA, which should yield similar results.

An additional hypothesis to explain the acute effects observed in the aged females is the possibility that the female brain may become masculinized as hormonal cyclicity is disrupted, such that E2 cells in the hypothalamus become dormant, until moderate amounts of E2 activate them. It may also be that aged cycling females synthesize higher

concentrations of aromatase in the CNS, which would explain the facilitatory effect of subcutaneous capsules of TP-alone in increasing sexual behaviors. To test this, aromatase activity in the hypothalamic structures involved in sexual behavior can be measured in young receptive, middle-aged and old-aged females, with the hypothesis that aromatase concentration increases with age.

Summary of mechanisms.

Estrogen and T, when administered together, restore sexual behavior in OVX female rats, similar to the actions of EB+P, which should typically restore all aspects of sexual behavior including appetitive and consummatory behaviors. Testosterone may be directly involved in the induction of sexual behaviors in females, in particular with respect to appetitive behaviors in young OVX rats, as well as in aged animals exposed to a steady dose of TP through subcutaneous silastic capsules. The biological mechanisms involved in this effect are unclear, however the aromatization hypothesis, whether androgens lead to increased bioavailable E2 which would then elicit the effects, or whether what we are seeing is a direct effect of androgens on its receptor may be reasonable explanations, but the available evidence in the literature at this time, fails to support one clear hypothesis. The results of these experiments, however, raise the possibility that androgens may have facilitatory effects in young OVX females as well as in aged animals receiving constant TP, but that E2 activates negative feedback mechanisms, preventing long-term restoration of sexual behaviors, in older intact aged females, and possibly in young intact animals, though this experiment has not yet been conducted.

Overall, these studies suggest that E2 and perhaps other steroid hormone effects are attenuated and delayed in the aging female, necessitating longer exposure to elicit the desired effects. They also suggest a potential for the restoration of function provided adequate treatment is administered, since many of these negative effects (e.g., light-induced Fos expression, delays in P receptor synthesis, decreases in sexual behaviors) can be reversed with transplantation, or exposure to a particular hormone either acutely or chronically.

Conclusion

The present experiments have investigated varying doses of TP on the sexual behavior of young OVX and aged intact female rats. The results of these experiments have shown that TP-alone has no effect on the sexual behavior of young OVX female rats, and the administration of EB facilitates both appetitive and consummatory behaviors but the addition of TP does not affect EB's effect at any of the doses tested. When the effects of varying hormone regimens were compared, the administration of EB+TP reinstates sexual behaviors to levels equivalent to EB+P, however this was not significantly different from EB-alone treated animals, which suggestes carry-over effects due to the methodology. The aged female rat, when treated acutely by subcutaneous injection with either TP-alone or in combination with EB does not initially affect sexual behaviors, however over time the behaviors are reduced. The administration of TP at a constant dose through subcutaneous capsules, dose-dependently facilitates sexual behaviors, however chronically the effects are lost. Similarly, when EB is added to the TP regimen, sexual behaviors are dose-dependently decreased. The initial facilitation and subsequent attenuation of these behaviors following EB and TP treatment in aged animals enables the study of these hormone interactions both centrally and peripherally, in the aging brain. The experiments on chronic administration allude to the idea that the initially beneficial effects of these treatments are abolished following chronic HRT.

The results of the present experiments prompt additional research questions regarding the underlying mechanisms of E-A interactions. The brain areas that are activated or deactivated, and neurotransmitter effects in these regions following these different treatments can be investigated with Fos immunocytochemistry and micro-

dialysis respectively, allowing us to further understand how these hormone treatments differentially effect these brain areas.

The receptors that are involved in the effects can be studied via androgen and E receptor blockers, and receptor subtypes in the case of ERs can be further explored by selectively blocking ERα or ERβ. Subsequent research in the mesolimbic DA system can explore how DA synthesis or release act to ameliorate symptoms initially, and what leads to the disruption chronically. It is hoped that the present experiments have opened the door to myriad questions that can now be addressed regarding the hormonal mechanisms associated with the decline of sexual function when ovarian function has been altered, and how it can be restored by exogenous hormone replacement therapy.

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