Estradiol Sensitization of Sexual Behaviors in the Ovariectomized Rat: Mechanisms and Applications

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ABSTRACT

Estradiol sensitization of sexual behaviors in the ovariectomized rat: Mechanisms and applications

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Estradiol sensitization refers to a phenomenon whereby each subsequent injection of estradiol benzoate (EB), when administered to the ovariectomized (OVX) rat to induce sexual behavior, potentiates the occurrence of those behaviors. The goals of the current thesis were to characterize the pattern of estradiol sensitization by varying the EB dose and injection interval, to examine some behavioral, hormonal, and neural factors involved in its induction and maintenance, and to apply it as a diagnostic tool in models that have been shown in the literature to produce sexual inhibition. It was first determined that estradiol sensitization is robustly induced by 10μg EB administered SC every 4 days, and that the effect is further potentiated if EB is administered in the absence of the opportunity to copulate. Furthermore, although adrenal progesterone (P) did not play a role, chronic administration of systemic injections of the P receptor antagonist RU486 revealed that P receptors are important in the maintenance of the sensitization. The next set of experiments determined that vaginocervical stimulation (VCS) received on repeated tests attenuates the sensitization of appetitive sexual behaviors (hops, darts, solicitations), and that inhibitory mechanisms related to estrous termination may be involved, since the attenuation was mimicked by repeated infusions of the glutamate receptor agonist AMPA to the ventrolateral division of the ventromedial hypothalamus in place of copulation. Moreover, the onset of estrous termination was accelerated in estradiol-sensitized animals that were not given the opportunity to copulate. This suggests that estradiol sensitizes mechanisms of sexual excitation and inhibition. The final experimental chapter determined that prenatally androgenized females are not permanently desensitized to the activational effects of EB, since repeated hormone treatments in combination with sexual experience generally restored sexual behaviors and those females also displayed estradiol sensitization. Finally, the inhibitory effect

of corncob bedding, as reported recently in the literature, did not prevent estradiol sensitization. In conclusion, the extent of estradiol sensitization, and the duration of behavioral estrus in estradiol-sensitized animals, interacts with sexual experience, particularly VCS. The data presented herein have implications for all research areas investigating the role of estradiol on physiology and behavior.

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*****

Olivia my love, as I sit here after dropping you at daycare, my heart feels tight. I want to keep you in my arms every second, hold you and never let you go… But I tell you almost everyday that I want you to become all that you can be, just as I am trying to do. Know that it is not always easy, but with support and determination you can do it.

CONTRIBUTION OF AUTHORS

1. Sensitization of sexual behavior in ovariectomized rats by chronic estradiol treatment

Sherri Lee Jones. Conceived and designed Experiment 3. Performed surgeries. Collected data in Experiments 2 and 3. Coded behavior in all studies. Conducted all statistics and interpreted findings. Wrote the manuscript.

Sara Farell. Conceived and designed Experiments 1 and 2. Performed most surgeries in Experiments 1 and 2. Collected the data in Experiments 1 and 2. Contributed to data interpretation.

James Gardner Gregory. Helped with data collection in Experiment 3.

James G. Pfaus. Conceived and designed the experiments. Contributed to data interpretation and writing of the manuscript.

2. RU486 facilitates or disrupts the sensitization of sexual behaviors by estradiol in the ovariectomized Long-Evans rat: Effect of timecourse

Sherri Lee Jones. Conceived and designed the experiment. Performed surgeries; contributed to drug preparation, administration, and data collection. Coded behavior, conducted data analyses, interpreted data, wrote the manuscript.

James Gardner Gregory. Helped with surgeries; did most drug preparations, injections, and data collection. Contributed to interpretation of findings.

James G. Pfaus. Conceived and designed the experiment. Contributed to the interpretation of findings and writing of the manuscript.

3. Sensitization of sexual behaviors in ovariectomized Long-Evans rats is induced by a subthreshold dose of estradiol benzoate and attenuated by repeated copulation

Sherri Lee Jones. Conceived and designed experiment. Performed surgeries, coded behavior, performed data analyses, interpreted data, wrote the manuscript.

James G. Pfaus. Conceived and designed the experiment, contributed to interpretation of findings and writing of the manuscript.

4. Vaginocervical stimulation attenuates sensitized appetitive sexual behaviors in ovariectomized Long-Evans rats

Sherri Lee Jones. Conceived and designed the experiment. Performed surgeries, data collection, coded behavior, data analyses, data interpretation, wrote manuscript.

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Mayte Parada. Performed surgeries. Helped with data collection.

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5. Repeated administration of estradiol sensitizes mechanisms of sexual excitation and inhibition: glutamate signaling in the ventromedial hypothalamus attenuates excitation

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Lucy Farisello. Performed ovariectomies and data collection in Experiment 2.

Nathaniel Mayer-Heft. Contributed to design of Experiment 1. Helped with ovariectomies, data collection and perfusions in Experiment 1. Sliced brains, performed histology and verified placements.

James G. Pfaus. Conceived and designed the experiments. Taught the VCS technique. Contributed to interpretation of findings and writing of the manuscript.

6. The inhibitory effects of corncob bedding on sexual behavior in the ovariectomized Long-Evans rat treated with estradiol benzoate is overcome by male cues

Sherri Lee Jones. Conceived and designed the experiment. Performed surgeries and most data collection. Coded behavior, performed analyses, interpreted findings, wrote the manuscript.

Radu Alexandru Antonie. Performed surgeries and helped with data collection. Performed most cage changes. Helped coordinate housing conditions.

James G. Pfaus. Conceived and designed the experiment. Contributed to interpretation of findings and writing of the manuscript.

7. Behavioral defeminization by prenatal androgen treatment is overcome by sexual experience in adulthood

Sherri Lee Jones. Conceived and designed experiment. Performed surgeries and animal care. Contributed to mating of animals. Data collection. Performed perfusions, slicing, histology. Coded behavior in phase 2, performed data analyses and interpretation. Wrote the manuscript.

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Katuschia Germé. Helped with all surgeries, data collection and animal care. Performed perfusions, slicing, histology.

James G. Pfaus. Conceived and designed the experiments. Contributed to data interpretation and writing of the manuscript.

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ABBREVIATIONS

ACF	animal care facility
ADX	adrenalectomy
AGT	aminoglutethimide
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	analysis of variance
ARC	arcuate nucleus
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
DA	dopamine
E2	17-β-estradiol
EB	estradiol benzoate
ER	estrogen receptor
FSH	follicle stimulating hormone
GABA	gamma-aminobuteric-acid
GnRH	gonadotropin releasing hormone
GPR-30	G-protein coupled receptor 30
GPER1	G-protein coupled estrogen receptor 1
HAT	histone acetyltransferase
HMT	histone methyltransferase
LM	lordosis magnitude
LQ	lordosis quotient
LH	luteinizing hormone
MeA	medial amygdala
mPOA	medial preoptic area
mER	membrane-bound estrogen receptors
mGluR	membrane glutamate receptors
NMDA	N-methyl-D-aspartate
nP	neuroprogesterone
NAcc	nucleus accumbens

OFQ/N	orphanin FQ/ nociceptin
ORL-1	opioid receptor-like receptor 1
OVX	ovariectomy
P	progesterone
PAG	periaqueductal gray
pdMeA	posterodorsal medial amygdala
PR	progesterone receptor
SC	subcutaneous
THF-diols	tetrahydrofurandiols
VCS	vaginocervical stimulation
vlVMH	ventrolateral ventromedial hypothalamus
VMH	ventromedial hypothalamus
VGLUT2	vesicular glutamate transporter type 2

PREFACE

Imagine this happened in your laboratory. You are running a preclinical double-blind study to test whether a compound increases sexual behaviors in female rats. The females are ovariectomized, which abolishes the expression of sexual behavior, treated with the appropriate hormones to reinstate sexual behavior, and given sexual training followed by a hormone washout period. When you test the acute effect of the drug given in combination with estradiol, which is required to prime the circuits that permit the expression of sexual behavior, there is a rock solid facilitation by the drug. However, the company you work with has been made aware that this drug, bremelanotide, has a blood pressure marker issue and aggressive screening is requested. A new set of animals is ordered, ovariectomized, trained, and given a hormone washout, and now the animals are treated repeatedly every four days (mimicking the natural ovarian cycle) for long-term screening of its effects. Once the behavior is scored and the double-blind code is broken, you are astonished to find that the baseline has risen, making it impossible to find any facilitation by the drug. But how could this be? How is it that your female rats that were treated with estradiol alone are displaying greater levels of sexual behavior with repeated administration? Is the female brain responding differently to estradiol when it is repeatedly administered? Has her brain become increasingly sensitive to its effects?

Addressing these questions was the main focus of this thesis.

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CHAPTER 1: GENERAL INTRODUCTION

Ovariectomized (OVX) rats are commonly used in the development of models to study underlying neuroendocrine and neurochemical mechanisms of female sexual behavior, reproductive function, and physiology. Establishing those models relies on strong methodologies and can be hampered if baseline responding is unstable. Such instability occurs with repeated, but not acute, administration of 17-β-estradiol (E2), an ovarian steroid hormone required to prime the neural circuits involved in the display of sexual behavior in the female rat. During the course of a particularly aggressive preclinical screening of pharmaceutical agents that could be useful in the treatment of female sexual arousal and desire/interest disorders, the rising baseline of both appetitive and consummatory sexual behaviors induced by estradiol benzoate (EB), administered every four days to mimic the normal estrous cycle of the female rat, became an obstacle. Although several studies had previously reported enhanced behavioral responding to chronic EB, the dosing and treatment regimens were in no way consistent across studies. Moreover, virtually nothing concrete was known about the mechanisms of this augmentation, nor was it possible to determine a dose or treatment regimen that would delay or prevent it.

The experiments in this thesis first describe the rising baseline that occurs in the OVX rat with repeated administration of EB every four days, such that sexual behaviors become potentiated over time. This behavioral phenomenon is referred to as *estradiol sensitization*. Next, a description of the necessary and sufficient conditions that elicit behavioral sensitization to EB is provided, followed by an examination of important hormonal and behavioral mechanisms that contribute to the effect. The behavioral and neural mechanisms that contribute to an attenuation of estradiol sensitization when the female receives vaginocervical stimulation (VCS) are then investigated. In the final chapter, the estradiol sensitization paradigm is used to examine whether sexual inhibition (by endocrine disruption in adulthood or by prenatal androgen administration) can be overcome. These experiments establish the appropriate dosing regimens that should be used in preclinical models depending on the desired baseline, examine behavioral and neuroendocrine mechanisms that contribute to estradiol sensitization and its attenuation, and illustrate how this phenomenon can be used to assess models of sexual inhibition. The estradiol sensitization data are important for all studies that examine the effects of E2 on physiology, brain, and behavior (e.g., sexual behavior, hypothalamic-ovarian feedback mechanisms; and studies examining the role of E2 in memory and cognition, or in sex differences associated with

psychiatric disorders and drug- taking behaviors). The results of these studies are considered in the global context of the flexibility of female sexual response strategies and neuroendocrine regulatory mechanisms.

Female rat sexual behavior in an historical context

A traditional view of rat sexual behavior describes the male making active copulatory advances toward sexually *receptive* females that passively receive his mounts, intromissions, and ejaculations (Caggiula et al., 1979; Hardy & Debold, 1973). This receptivity posture, called lordosis, is a concave arching of the back that raises the rump and allows males to mount with intromission. In order to study male sexual behavior and to prevent pregnancy and control testing schedules and conditions, females were typically OVX, abolishing the expression of female sexual behavior since ovarian steroid hormones are required for its expression. Early work established systemic (e.g., subcutaneous) dosing regimens that could maximally induce lordosis behavior, and also restore precopulatory or appetitive behaviors such as hops, darts, ear wiggling (Boling & Blandau, 1939). Optimal timing for the full induction of appetitive sexual behaviors and lordosis was also established, with EB administered 48 hours and progesterone (P) administered 4 hours prior to testing, respectively (Boling & Blandau, 1939; Hardy & Debold, 1972; Whalen, 1974). Tests could then be conducted once every four days with exogenous ovarian steroid administration mimicking the rises in plasma hormone levels normally seen with natural ovarian output (Albert et al., 1991), which in turn approximated a normal 4-5 day estrous cycle (Blandau, Boling, & Young, 1941). Thus, most of the early literature relied on priming OVX rats fully with EB and P to ensure that they were maximally *receptive* for the male.

Sexual behavior tests were also traditionally conducted in small testing chambers which permitted the male easy access to receptive females, and which appeared to maximize her display of lordosis at the expense of other sexual behaviors. In part, this focused attention on lordosis as a reliable and consistent sexual response in females, and made it arguably the most thoroughly-studied sexual response after male erection. In fact, Pfaff (1980; 1999) has argued that lordosis represents the first vertebrate behavior for which a complete neural circuit has been identified. However, it became clear thanks to the work of Beach (1976), Madlafousek and Hliňák (1977a), McClintock (1984) and Erskine (1989) that female rats were anything but passive recipients of male sexual behaviors, and in fact females controlled the initiation and rate

of copulation in the wild, or appropriate laboratory situations, using solicitations and runaways to pace or regulate the timing of the sexual stimulation they received from the male. In particular, it became apparent that females controlled the rate and number of vaginocervical stimulations (VCSs) that were induced by penile intromission, which in turn bring about a faster termination of estrus behavior (Lodder & Zeilmaker, 1976), and activate neuroendocrine reflexes that maximize the likelihood of successful pregnancy (Adler, 1969). Indeed, the ability of the female to control sexual stimulation and to display appetitive sexual responses was limited in the small testing chambers used traditionally in the study of male sexual behavior. Such testing environments in fact stimulated the display of defensive responses that many females would use to enforce a longer interval between successive bouts of mounts with intromission. Such behaviors were used to infer decreased sexual motivation in females or that female rats did not "like" the feeling of intromission (Bermant & Westbrook, 1966; Hardy & Debold, 1972; Peirce & Nuttall, 1961). Given the existence of penile spines on a fully erect male rat penis (Sachs, Glater, & O'Hanlon, 1984), such interpretations were often taken at face value.

Female control of the initiation and rate of copulation can be viewed more easily in large open fields (Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005; Kippin, Talianakis, Schattmann, Bartholomew, & Pfaus, 1998; McClintock, 1984), or in bilevel or unilevel pacing chambers that allow females to enforce her preferred temporal intervals by running from level to level, side to side, or crossing a Plexiglas barrier through holes that the male on one side cannot get through (Erskine, 1989; Paredes & Alonso, 1997; Pfaus, Smith, & Coopersmith, 1999). In addition to its effects on estrus duration and pregnancy, pacing is rewarding to the female and induces both conditioned place (Arzate, Portillo, Rodríguez, Corona, & Paredes, 2011; Paredes & Vazquez, 1999) and partner preferences (Coria-Avila et al., 2005; 2006) for contextual or partner-related cues that have been associated with the ability to pace.

Hormonal influences on sexual behavior in the female rat

As mentioned above, in the rat ovulation occurs every 4-5 days and coincides with behavioral estrus, a period whereby she displays stereotypical sexual behavior patterns that allow a male to successfully copulate and impregnate her. It is only during this periovulatory period when she is fertile that sexual behavior is displayed. If a female is not in behavioral estrus, attempted mounts from a male will result in antagonistic escape-related behaviors from the

female. Female sexual behavior is primarily dependent on fluctuating levels of ovarian E2 as well as P. Estradiol begins to rise during the diestrous phase of her cycle as the maturing ovarian follicles that contain the egg (or oocyte) grow under the influence of follicle stimulating hormone (FSH) released from the pituitary in response to hypothalamic gonadotropin releasing hormone (GnRH). During the proestrous phase a sharp rise in E2 from the mature follicles feeds back on the hypothalamus and under a positive feedback mechanism, which involves the stimulation of neuroprogesterone synthesis (nP) (Kuo & Micevych, 2012; Micevych, Soma, & Sinchak, 2008), stimulates a sharp rise in FSH and luteinizing hormone (LH) from the pituitary. This LH surge sets the stage for the final maturation of the follicles. Ovulation occurs when the follicles rupture and release the oocytes, and the follicles (now called the corpora lutea) release large amounts of P and other hormones, preparing the uterus for implantation should the eggs become fertilized.

The gradual rise in E2 primes the hypothalamic circuits necessary for the display of sexual behavior that will occur approximately two days later, and its effects are potentiated by the subsequent rise in P. Sexual behavior begins to appear during late proestrus and carries over into the estrous phase, lasting approximately 12-20 hours depending on whether or not she copulates (Erskine, 1985; Erskine, Kornberg, & Cherry, 1989). In the absence of mating, steroid hormone levels will fall following absorption of the corpora lutea, and sexual behavior becomes inhibited until E2 and subsequently P rise again to induce the LH surge and ovulation four days later. Thus, as with exogenous administration of ovarian hormones, E2 and P act synergistically to activate appetitive and consummatory sexual behaviors. Those behaviors in turn, serve to entice the male to chase and mount her, and to allow successful intromissions, respectively, at optimal time intervals to facilitate fertilization of the ovum following the receipt of ejaculation (McClintock, 1984). Importantly, VCS received from penile stimulation during copulation plays a role in the duration of behavioral estrus, and the activation of neuroendocrine events that are necessary to prepare for and maintain pregnancy.

Sequence of onset and offset of female sexual behavior

As the female transitions into behavioral estrus, lordosis postures are the first to emerge, followed by appetitive behaviors (sometimes referred to as precopulatory (Erskine, 1989), proceptive (Beach, 1976), or paracopulatory (Blaustein & Erskine, 2002) behaviors) which also emerge in a sequential order. When sexually vigorous male and female rats are placed in a

pacing chamber, they begin anogenital investigation (initiated by either the female or the male), followed shortly thereafter by a full solicitation (headwise orientation to the male and a quick runaway) or partial solicitation (hop over and or dart by the male) made by the female (Pfaus, 1996; Pfaus, Jones, Flanagan-Cato, & Blaustein, 2014). This causes the male to chase her. As the female comes to a stop, she initiates a pre-lordosis presentation posture, facilitating the ability of the male to mount her flanks when he arrives, which engages a full lordosis. As mentioned above, lordosis is necessary for penile intromission, and occurs in response to flank stimulation by the male. Its intensity can be measured on a three-point scale primarily according to the magnitude of the concave curvature of the back and elevation of the head and rump (Hardy & Debold, 1972).

The first appetitive behaviors to appear are presenting postures, followed by hops away from the male and then darting fly-bys (Madlafousek & Hlinak, 1977). Hops and darts typically co-occur when the female is fully primed and in a bilevel chamber are displayed when the female is on the same level as the male. In contrast, full solicitations in the bilevel chamber are observed when she runs away from one level to another (Pfaus et al., 1999), or in a unilevel "racetrack" chamber when she runs from side to side (Kippin et al., 1998; Kippin & Pfaus, 2001a; 2001b; Pfaus et al., 2009). Although the expression of full or partial solicitations is somewhat dependent on the amount of space the animals have, if the chambers are large enough (McClintock & Adler, 1978), or constructed in a way that otherwise allows them to be expressed, full solicitations are indicative of greater sexual motivation than hops/darts, since they are more often observed in females fully primed with EB and P than with EB alone (Pfaus et al., 1999). Traditionally it is believed that higher levels of appetitive behaviors are indicative of a female manifesting more intense sexual motivation or desire (Hardy & Debold, 1971b; Madlafousek & Hlinak, 1977; Pfaus et al., 1999). Moreover, it has been proposed that appetitive behaviors in the female rat are used as a means of communication with the male (Madlafousek & Hlinak, 1977), and play an important role in the attractivity of the female (Beach, 1976), since inexperienced male rats will more readily copulate with females displaying greater intensities of those behaviors. For example, approximately 30% of inexperienced males will begin copulating with a female showing present postures, and the proportions increase to 60% and 90% if females are displaying hopping and darting, respectively (Madlafousek & Hlinak, 1977). Solicitations (and the increase in locomotion induced by E2) spread her estrous odors in a wider space, signaling the male that she

is sexually receptive and also enticing the male to search for her and, at least early in the male's sexual experience, to chase her (Erskine, 1989; McClintock & Adler, 1978; Pfaus et al., 2012). Solicitational behaviors are reproductively relevant since they are more likely to elicit intromissions from the male (Erskine, 1989; McClintock & Adler, 1978). For example, McClintock and Adler (1978) reported that 90% of intromissions occurred when the female displayed a full solicitation and 84% of full solicitations were followed by an intromission. Following the receipt of a mount or intromission in a unilevel pacing chamber, a female may run away from the male and return to "her side" of the chamber for a brief time. However, following an ejaculation, the female returns to her side 100% of the time and for a longer time relative to intromissions or mounts (Erskine et al., 1989; Zipse, Brandling-Bennett, & Clark, 2000). In bilevel or racetrack chambers, the female typically responds to an ejaculation by moving immediately to the other level or side from the male (Pfaus et al., 1999; 2009). The receipt of an ejaculation from the male produces a large and sustained amount of VCS, as the ejaculate congeals into a "vaginal plug" acting as a cervical cap to protect and promote sperm transport (Toner, Attas, & Adler, 1987). After a brief period (ranging in minutes) of apparent sexual inhibition, the female returns to the male and shows increasing interest by sniffing his anogenital region or soliciting him to begin a new ejaculatory series initiated by the resumption of mounts with intromission. Sexual exhaustion in the male sets in by approximately 7 to 10 ejaculatory series, and the exhaustion alleviates within four days, coinciding with the females' next bout of sexual receptivity (Beach & Jordan, 1956; Boling & Blandau, 1939; Larsson, 1956).

As the period of sexual receptivity gradually terminates, the behaviors also dissipate in a sequential fashion. First, appetitive behaviors decline, coinciding with an increase in defensive behaviors, before the decrease in lordosis intensity and frequency is observed (Pfaus, Smith, Byrne, & Stephens, 2000). This behavioral pattern defines the transition into "estrous termination". Hence, the period of sexual behavior in the female rat begins and ends with the lordosis posture, and the presence of appetitive solicitations is indicative of a more sexually motivated female.

Factors influencing the activation and termination of sexual behavior

Although E2 is crucial for priming the circuits required for the expression of sexual behavior, their expression per se is influenced by the presence of a male and the sexual

stimulation received. Sexual behavior (lordosis in particular) can be enhanced within an episode of heat by merely exposing the EB-primed OVX rat to a sexually vigorous male without the receipt of intromission (Auger, Moffatt, & Blaustein, 1997; Blaustein, Farrell, Ghavami, Laroche, & Mohan, 2009; Foreman & Moss, 1977; Hardy & Debold, 1973; Rajendren & Moss, 1993; Rajendren, Dudley, & Moss, 1990). This mating-induced enhancement of lordosis is also potentiated by application of crystalline E2 directly to the ventromedial hypothalamus (VMH) of OVX rats (Rajendren, Dudley, & Moss, 1991), is completely prevented by lesions to the VMH (Rajendren et al., 1991), and strongly attenuated by lesions to medial amygdala (MeA) (Rajendren & Moss, 1993). The mating-induced enhancement of lordosis also occurs as a result of experimenter handling (Hardy & Debold, 1973). Although this suggested at first that stress-induced secretion of adrenal P may play a role, the enhancement is not prevented by removal of the adrenal glands (Auger et al., 1997; Blaustein et al., 2009) suggesting that peripheral P is not involved.

VCS itself can induce low levels of lordosis in the OVX rat with or without prior E2 administration if applied manually by an experimenter with a glass rod in combination with manual palpation of the flanks and perineum (Komisaruk, 1972; Komisaruk & Diakow, 1973; Rodriguez-Sierra & Komisaruk, 1983). However E2 administration potentiates lordosis in response to manual VCS with concomitant palpation of the flanks and perineum, even at subthreshold doses (Komisaruk & Diakow, 1973), and induces a faster recovery of lordosis compared to untreated animals, after exhaustion induced by successive stimulations (Rodriguez-Sierra & Komisaruk, 1983). Interestingly, in the study of Komisaruk (1973), lordosis was never observed following initial application of flank-perineal palpation; however after applying manual stimulation that included VCS, lordosis was induced in EB-treated rats by later application of flank-perineal stimuli, and this effect did not occur in untreated rats, suggesting that EB permitted a behavioral facilitation by VCS. By the end of the test procedures, OVX oil-treated rats no longer displayed lordosis in response to manual VCS in combination with palpation of the flanks and perineum, whereas that inhibitory effect did not occur in EB-treated rats. This latter observation was an early indication that EB opposes the inhibitory effect of VCS on the display of lordosis, as proposed later by Pfaus (Pfaus et al., 2000). It was also shown that this mating or VCS-induced enhancement of lordosis (Hardy & Debold, 1972; Komisaruk, 1972; Komisaruk & Diakow, 1973) occurs via progesterone receptor (PR) activation (Auger et al.,

1997). More recently it has been shown that appetitive behaviors are also enhanced within a mating session in OVX rats that were adrenalectomized (ADX) and primed with EB (5μg) (Blaustein et al., 2009); however the exact mechanism of this effect is not known.

Although VCS can facilitate lordosis, it clearly activates mechanisms that are inhibitory to sexual behavior since it increases the probability that the female will withdraw from the male, increases the latency to return to the male, decreases solicitations and increases defensive responses, and accelerates the onset of estrous termination (Lodder & Zeilmaker, 1976; Pfaus et al., 2000). In turn, estrous termination permits the endocrine transition to pseudopregnancy or pregnancy (Erskine, 1985; Pfaus et al., 2000). Experimenter-applied VCS using a glass rod also shortens the period of behavioral estrus (Pfaus et al., 2000), and activates the same neuroendocrine events involved in the maintenance of pregnancy (Lehmann & Erskine, 2004), that occur with repeated mating. The onset of estrous termination by VCS, and the induction of pseudopregnancy is further accelerated if females are given the opportunity to pace the sexual stimulation (Coopersmith, Candurra, & Erskine, 1996; Erskine, 1985; 1989; Erskine et al., 1989). As little as 10-15 paced intromissions are sufficient to induce pseudopregnancy and activate mechanisms that are inhibitory to sexual behavior by accelerating the onset of estrous termination (Erskine et al., 1989), yet females display conditioned place preference for even high levels (approximately 25) of VCS if they are given the opportunity to pace those stimulations (Arzate et al., 2011).

In summary, under the appropriate testing conditions, it has become clear that female rats, and indeed females of a number of disparate species, exert a great deal of control over the sexual stimulation received, which is driven at least in part by the expression of appetitive sexual behaviors, and that she actively controls the rate at which the stimulation is received from the male. Sexual solicitations help signal the male to assume mounting, increase the probability of the receipt of intromissions and ejaculation, which when coupled with lordosis and the ability to pace, increase the probability of successful fertilization. Although mating stimulation can enhance the expression of sexual behaviors, sufficient VCS in particular, especially when paced by the female, activates mechanisms that are inhibitory to sexual behavior (albeit without removing the positive affective state induced by that stimulation (Arzate et al., 2011; Meerts & Clark, 2009a)), but that inhibition is counteracted by E2 administration.

Neural regions implicated in the activation and termination of sexual behavior

The neural circuitry involved in the activation of lordosis by E2 has been carefully studied and described (McGinnis & Pfaff, 2012; Pfaff, 1980; 1999). The circuitry is typically broken down into four modules as shown in Figure 1, although the model has since been expanded upon by numerous research groups, and it is now well known that additional sites are also important in the regulation and facilitation of lordosis (Micevych & Sinchak, 2007). Lordosis requires appropriate hormone priming conditions acting on neurons within the VMH, and occurs in response to flank and perineal stimulation. The spinal cord module receives the majority of the somatosensory information following stimulation of the flanks and pressure placed on the rump, tail base, and perineum, which are necessary and sufficient for lordosis to occur (Pfaff, 1999). That stimulation activates neurons within the dorsal horn of the spinal cord, and those fibers that are relevant for lordosis travel up to the medullary reticular formation and lateral vestibular nucleus in the lower brainstem, and a small number of those fibers also ascend to the midbrain central gray (or periaqueductal gray, PAG). The lower brainstem module integrates the animal's posture across spinal cord segments, including inputs from the vestibular organs to sense head position and movement, and proprioceptors that sense skeletal and muscle movement. The integration of the signals within the spinal cord segment occurs within the midbrain module. However, those ascending signals do not directly control the motor outputs for lordosis to occur, rather they require descending inputs from hypothalamic and surrounding regions which themselves must be primed with E2.

The neurons in and around the ventrolateral region of the VMH (vlVMH) are required for lordosis to occur. Lordosis is abolished by lesioning or pharmacologically blocking those cells, and electrically stimulating those cells facilitates lordosis (Pfaff & Sakuma, 1979b; 1979a). More recently additional hypothalamic and extrahypothalamic circuits have also been extensively studied in relation to their importance in the activation of the lordosis circuit (Micevych & Sinchak, 2007; Mills, Sohn, & Micevych, 2004). Estradiol also binds to membrane-bound estrogen receptors (mER) which form a complex with membrane glutamate receptors (mGluR) within neurons of the arcuate nucleus (ARC) that result in the release of opioids through efferents to the medial preoptic area (mPOA), a subset of which project to the VMH. This effect is initially inhibitory to lordosis, yet the later rise in P deactivates this inhibitory circuit to facilitate lordosis expression (Micevych & Sinchak, 2007; Sinchak & Micevych, 2001).

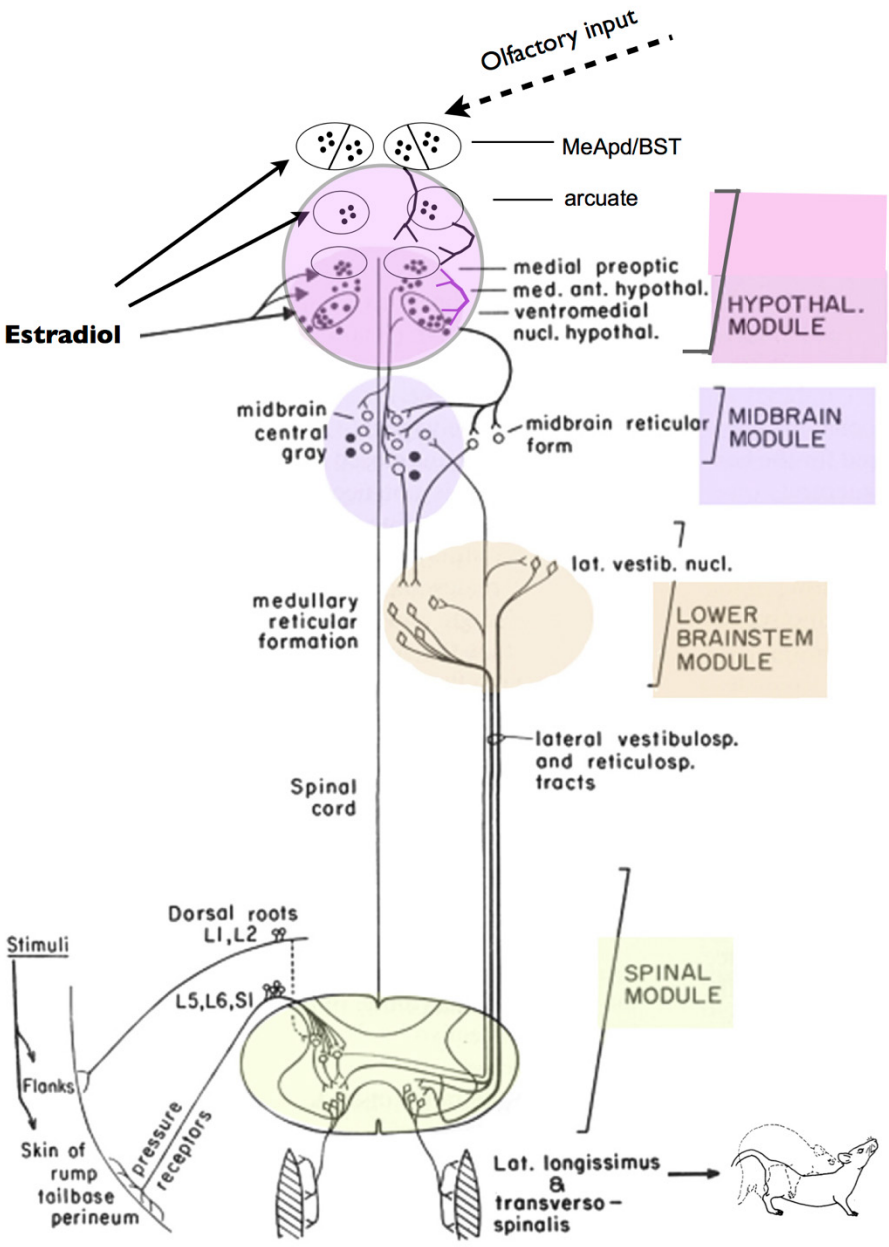

Figure 1.1. Neurocircuitry of lordosis. Lordosis expression is dependent on estradiol binding (black dots) within hypothalamic and extrahypothalamic sites. Olfactory stimulation arriving from the accessory olfactory system and somatosensory cues from perineal and flank stimulation converge their signals in the VMH, which sends descending information to permit the expression of lordosis. The pdMeA, BNST and arcuate signal to the mPOA, which relays information to the VMH. Reproductively relevant olfactory and hormonal information occur in the MeApd, BNST, and PAG (midbrain module) which itself receives ascending somatosensory stimulation from the

spinal module and descending information from the VMH. That descending information signals within the lower brainstem module (via the lateral vestibular nuclei and medullary reticular formation), sending facilitative signals to motor neurons in the spinal module to contract, resulting in a concave arching of the back, called lordosis. The circuitry underlying solicitational behaviors is less understood, but overlap with systems that activate lordosis (such as the VMH, mPOA and pdMeA, see text for details), and are also initiated by estradiol binding. BNST: Bed nucleus of stria terminalis. pdMeA: posterodorsal division of the medial amygdala. mPOA: medial preoptic area. PAG: periaqueductal gray. VMH: ventromedial hypothalamus. (Hardy & Debold, 1972; McGinnis & Pfaff, 2012; Micevych & Sinchak, 2007; Pfaff, 1980; Pfaff, 1999). Adapted from (McGinnis & Pfaff, 2012), reprinted with permission from Elsevier.

Estradiol also acts within the MeA, particularly the posterodorsal division (pdMeA) and bed nucleus of the stria terminalis (BNST) which among other things, are involved in the pathway which receives olfactory signals that facilitate the expression of sexual behaviors (Micevych & Sinchak, 2007). The synergistic actions of E2 and P in the VMH and surrounding hypothalamic regions, through genomic events and electrophysiological effects, send descending efferents to the midbrain central gray to "permit" the lordosis reflex in response to relevant somatosensory inputs. Thus, the hypothalamic module is the primary endocrine control center that is required for lordosis to occur (Kow & Pfaff, 1998; Pfaff, 1999).

The neurons in the midbrain central gray (or PAG), in combination with a small number of neurons within the mesencephalic reticular formation, send descending facilitative signals to the medullary reticular formation in the lower brainstem (Pfaff, 1980; 1999). Thus the midbrain receives incoming hypothalamic peptides and proteins, and translates those signals that are relatively slow to faster-changing electrophysiological signals. Finally, signaling within the lower brainstem module (via the lateral vestibulospinal and reticulospinal tracts) send facilitative signals to motor neurons in the lumbar region that control the deep back muscles. Those motor neurons are attached dorsally to the back muscles such that when the muscles contract in response to stimulation from the motor neurons, they elicit the concave arching of the back, called lordosis.

In the female rat, the potential for sexual behavior depends primarily on the priming actions of E2 within the hypothalamus through classic genomic mechanisms. Estradiol is primarily synthesized in the ovary from its precursor cholesterol, and freely crosses the bi-lipid cell membrane in target tissue due to its lipophilic property, where it binds its receptor, dimerizes and activates hormone response elements on DNA to initiate gene transcription and the upregulation of numerous neurotransmitter, neurosteroid, and receptor systems (Micevych, Eckersell, Holland, & Smith, 1996; Pfaff, 1999; Sinchak & Micevych, 2001). Among its effects is the transcription and synthesis of PR in hypothalamic areas such as the VMH and mPOA which occurs within 48 hours of EB administration (MacLusky & McEwen, 1980). Subsequent P binding to its receptor elicits behavioral effects within four hours of a subcutaneous injection in the OVX rat. Activation of PR in the VMH amplifies the lordosis response whereas PR activation in the mPOA appears to be important in the display of appetitive behaviors (Beyer, Gonzalez-Flores, & González-Mariscal, 1997; Glaser, Rubin, & Barfield, 1983; Hoshina, Takeo,

Nakano, Sato, & Sakuma, 1994; Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994; Rubin & Barfield, 1983; Sakuma, 1994; 2008).

There is a tight interplay between the mPOA and VMH in the control of appetitive and consummatory measures of sexual behavior, which are mutually exclusive. The mPOA is primarily involved in the display of solicitational behaviors which require heightened locomotor activity, and locomotion must be halted in order for the immobile lordosis stance, which itself is dependent on the vlVMH, to occur. Estradiol applied in or around the vlVMH induces lordosis (Davis, Krieger, Barfield, McEwen, & Pfaff, 1982; Davis, McEwen, & Pfaff, 1979; Rubin & Barfield, 1980), whereas its application to the mPOA increases locomotor activity (Fahrbach, Meisel, & Pfaff, 1985; Wade & Zucker, 1970). Neural firing during proestrus, which coincides with the display of sexual behavior, increases in the VMH and the nearby arcuate nucleus of the hypothalamus (Dyer, 1973; Dyer, Pritchett, & Cross, 1972; Moss & Law, 1971). Similarly, neuronal firing rates in the VMH are higher in OVX rats treated with E2, but lower in the mPOA, compared to controls (Bueno & Pfaff, 1976; Hasegawa & Sakuma, 1990; 1993). Moreover, whereas electrical stimulation of the VMH enhances lordosis, stimulation of the mPOA inhibits lordosis, in E2 treated rats (Pfaff & Sakuma, 1979b). Lordosis is abolished following lesions of the vlVMH (Pfaff & Sakuma, 1979a), but facilitated by excitotoxic lesions specific to cell bodies within the mPOA (sparing fibers of passage), yet those mPOA lesions decrease appetitive sexual behaviors (and facilitate defensive behaviors) (Hoshina et al., 1994). Thus, solicitational behaviors and lordosis are mutually exclusive components of the full array of sexual behavior, and would therefore require rapid inhibitory and excitatory bi-directional signals between the vlVMH and mPOA during a copulatory bout.

Ample evidence suggests that glutamate transmission within the VMH is inhibitory to sexual behavior, and that hormone priming opposes this process. Although the application of crystalline E2 to the VMH elicits lordosis (Dörner, Docke, & Moustafa, 1968), infusions of glutamate (an excitatory neurotransmitter) or its ionotrophic receptor agonists, as well as GABA-A receptor antagonists to this region inhibit lordosis (Georgescu & Pfaus, 2006b; Kow, Harlan, Shivers, & Pfaff, 1985; McCarthy, Curran, & Feder, 1991). Conversely, infusions of glutamate receptor antagonists (Georgescu & Pfaus, 2006a) or GABA (an inhibitory neurotransmitter) receptor agonists stimulate lordosis (McCarthy et al., 1991; McCarthy, Kaufman, Brooks, Pfaff, & Schwartz-Giblin, 1995; McCarthy, Malik, & Feder, 1990). Presenting an OVX rat with a

sexually vigorous male elicits the release of glutamate within the VMH, but its release is blunted if females are treated with EB (10µg) 48 hours earlier (Georgescu, Afonso, Graham, & Pfaus, 2014). Moreover, large amounts of VCS that are known to induce estrous termination activate glutamate neurons within the vlVMH but fewer of those neurons are activated if females are pretreated with EB (Georgescu, Sabongui, Del Corpo, Marsan, & Pfaus, 2009). Furthermore, frequent hormone priming with EB and P (for example every 4 days as opposed to every 21 days) reduces the ability of VCS to induce estrous termination (Pfaus et al., 2000). Thus, EB appears to disinhibit neural mechanisms of sexual behavior residing in the vlVMH, and its effects appear to be more robust with frequent administration.

As mentioned above, the amount and pattern of VCS is important in triggering the neuroendocrine events necessary for fertilization and to maintain pregnancy, and the MeA is a critical brain region involved in this process. The MeA has a large number of estrogen receptors (ER) (Pfaff & Keiner, 1973), the application of crystalline E2 to this region induces lordosis, as it does in the VMH (Lisk & Barfield, 1975), and long-term treatment with EB increases the firing rate of its neurons (Schiess, Joëls, & Shinnick-Gallagher, 1988), suggesting that neurons within the MeA sensitize to EB over time (Pfaus, Marcangione, Smith, Manitt, & Abillamaa, 1996). In addition to the activation of glutamatergic cells in the vlVMH that appear to be inhibitory to sexual behavior, the receipt of VCS induces twice daily prolactin surges which is required for the maintenance of pregnancy (or the induction of pseudopregnancy if the egg is not fertilized), and that mechanism occurs, at least in part, via NMDA (N-methyl-D-aspartate) and AMPA (alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor activation within the pdMeA (Lehmann & Erskine, 2005; Lehmann, McKellar, & Erskine, 2005; Oberlander, Lin, Man, & Erskine, 2009; Polston & Erskine, 2001). Thus, the activation of sexual behavior in the female rat requires priming actions of E2 acting on hypothalamic tissue (see Figure 1), and its duration is partly dependent on VCS received during mating. That stimulation in turn activates reproductively relevant neural regions (i.e., such as the pdMeA) that are necessary for the neuroendocrine events required in the maintenance of pregnancy/pseudopregnancy.

What is a physiological dose of estradiol?

Across the ovarian cycle of the rat, plasma E2 levels begin to rise during diestrus, peak in the afternoon of proestrus followed by a sharp decline, resulting in the lowest levels during estrus

and metestrus (Belanger, Cusan, Caron, Barden, & Dupont, 1981; Henderson, Baker, & Fink, 1977). Although the estimates of the precise circulating levels of E2 vary somewhat within the literature (which may due to a variety of factors such as the type of assay used, the age and strain of animal, diet, or the method of blood collection, for example cardiac puncture, jugular or tail vein), plasma levels measured by radioimmunoassay generally range from approximately 6-10pg/mL during estrus into metestrus, begin to rise during diestrus, ranging from approximately 10-50pg/mL, and peak in the afternoon of proestrus reaching approximately 50-70pg/mL (values extracted from (Belanger et al., 1981; Henderson et al., 1977)).

Henderson, Baker and Fink (1977) proposed that physiological doses of EB, when administered acutely in oil to the OVX rat, range from 2.5-10μg. Female rats were OVX on the day of Diestrus and immediately treated with 2.5 or 10μg EB and blood was collected at various times after injection. Following an acute injection of 2.5μg EB, plasma E2 rose slowly, reached a peak of approximately 30pg/mL 24 hours later, and gradually returned to baseline levels approximately 12 hours thereafter. Following the administration of 10μg EB, plasma E2 increased sharply, reached a peak (∼115pg/mL) approximately 24-30 hours later, followed by a sharp decline approaching pretreatment levels approximately 36 hours following the injection. Others have shown that OVX rats receiving doses lower than 10μg EB result in lower, and doses greater than 10-20μg EB resulted in higher, uterine weights compared to controls (Tapper, Greig, & Brown-Grant, 1974). Thus, although a 10μg EB dose may initially induce supraphysiological plasma levels of E2, it is physiological with respect to its actions on target tissue, whereas lower doses (e.g., 2.5μg EB) that do induce physiological plasma levels may not be sufficient to restore normal physiological function in target tissue.

From a behavioral standpoint, a “physiological” dose of EB in the OVX rat should coincide with a reinstatement of the behavior in question. For example, although Silastic© capsule implants may induce plasma E2 levels within the physiological range (15pg/mL) they produce an abnormal pattern of sexual behavior such that females are constantly receptive (despite being approximately a third of the peak plasma E2 level achieved during the estrous cycle) (Albert et al., 1991). The co-administration of 10μg EB with 500μg P, administered 48 and 4 hours prior to testing respectively, fully reinstates sexual behavior in the OVX rat to levels equivalent to that seen in an intact animal (Boling & Blandau, 1939). Thus, as proposed by

Henderson et al., (1977) subcutaneous injections ranging from 2.5 to 10μg EB appear to be both physiologically and behaviorally relevant.

Increased behavioral sensitivity to estradiol

The induction of sexual behavior in the OVX rat is most commonly induced by subcutaneous administration of EB (varying between 2μg to 10μg) followed 18-48 hours later by P, and the behavioral facilitation is seen within four hours of P administration (Boling & Blandau, 1939; Edwards, Whalen, & Nadler, 1968). Sexual behavior is typically tested every four days, mimicking the pattern of fluctuating hormone levels in females, and allowing a four day rest period which is required for alleviation of sexual exhaustion in male rats which takes approximately 72 hours to recover (Romano-Torres, Phillips-Farfan, Chavira, Rodriguez-Manzo, & Fernandez-Guasti, 2007). This four-day behavioral testing pattern has been reliably used in Pavlovian conditioning paradigms to study sexual motivation (Pfaus et al., 1999), place and partner preferences (Coria-Avila et al., 2005; e.g., 2006; Holley, Shalev, Bellevue, & Pfaus, 2014; Ismail, Gelez, Lachapelle, & Pfaus, 2009; Ismail, Jones, Graham, Sylvester, & Pfaus, 2011; Kippin, 2000; Kippin, Samaha, Sotiropoulos, & Pfaus, 2001; Meerts, Schairer, Farry-Thorn, Johnson, & Strnad, 2014; Parada, Abdul-Ahad, Censi, Sparks, & Pfaus, 2011; Parada, Vargas, Kyres, Burnside, & Pfaus, 2012; Paredes & Vazquez, 1999; Pfaus et al., 1999; Pfaus, Erickson, & Talianakis, 2013; Pfaus et al., 2010), as well as in preclinical studies examining both short- and long-term effects of pharmacological agents on sexual behavior (e.g., Pfaus, Shadiack, Van Soest, Tse, & Molinoff, 2004; Rössler et al., 2006). In the case where an inhibition of sexual behavior is predicted by a given manipulation, females are fully-primed with EB and P, yet when a facilitation is expected females are typically primed with EB only, which induces only low levels of lordosis, and (when administered acutely) prevents ceiling effects that occur with subsequent P administration (e.g., Georgescu & Pfaus, 2006a; Graham & Pfaus, 2010; Pfaus, Giuliano, Gelez, 2007; Snoeren et al., 2011).

Another method of E2 administration that has been frequently employed in the literature is called the two-pulse (or discontinuous) paradigm, originally used in the study of uterine cell division (Harris & Górski, 1978), in which free E2 is used to induce sexual behavior. When free estradiol is administered in a single dose, a larger dose dose is required to induce sexual receptivity (between 10-100μg) because free E2 rapidly produces an increase in plasma and brain

levels of E2 which only lasts a brief period (Clark & Roy, 1983; Green, Luttge, & Whalen, 1970; McEwen, Pfaff, Chaptal, & Luine, 1975; Södersten, Eneroth, & Hansen, 1981; Tapper et al., 1974). Such studies have found that P's ability to induce sexual behavior is facilitated following at least two injections of free E2 compared to an equivalent dose given in single injection (Clark & Roy, 1983; Södersten et al., 1981), whereas when E2 was administered in a single injection, the dose had to be at least 10 times higher (Clark & Roy, 1983). These early studies suggested that the first dose of E2 primes subsequent E2 actions to facilitate P's ability to induce sexual behavior.

This increased behavioral sensitivity to subsequent treatment of E2 has also been reported in paradigms that repeatedly administer E2 alone. EB administered acutely, even in very high concentrations, elicits only partial expression of female sexual behaviors, such that lordosis occurs approximately 20-50% of the time that she is mounted by the male, very few appetitive behaviors are displayed, and she will often show aggression or rejection of the male in response to an attempted mount (Boling & Blandau, 1939; Davidson, Rodgers, Smith, & Bloch, 1968a; Davidson, Smith, Rodgers, & Bloch, 1968b; Edwards et al., 1968; Hardy & Debold, 1971b; Meyerson, 1964; Whalen, 1974). However, early studies reported that OVX rats treated repeatedly with EB are behaviorally more sensitive to subsequent treatments (Babcock, Bloch, & Micevych, 1988; Beach & Orndoff, 1974; Blaustein, Finkbohner, & Delville, 1987; Clark & Roy, 1983; Kow & Pfaff, 1975; Parsons, MacLusky, Krieger, McEwen, & Pfaff, 1979; Whalen & Nakayama, 1965). Because those studies used EB dissolved in oil, resulting in a slow release (and more accurately reflecting the gradual rise in E2 that occurs across the ovarian cycle), it was suggested that residual E2 remained in the circulation and as such the concentration of subsequent administrations was additive, resulting in a potentiation of sexual behavior following each treatment (Parsons et al., 1979). One such study reported that following a (supraphysiological) subcutaneous injection of 50μg EB dissolved in 0.1mL sesame oil, E2 plasma concentrations peaked at 1800pg/mL within 10 hours, and remained elevated (100pg/mL) four days later (Legan, Coon, & Karsch, 1975). However Parsons and colleagues (1979) have shown that OVX animals pre-treated with crystalline E2 through a subcutaneous capsule (5mm) for 1 week (which induced relatively constant plasma E2 levels of 40-45pg/mL) were also behaviorally more sensitive to subsequent E2 exposure following a 5-day washout, compared to animals that were not pre-treated with E2. Furthermore, following removal of the capsules,

plasma E2 had returned to control levels within 12 hours. Together those data show that E2 induces long-term changes in how neural tissue responds to subsequent exposure.

Estradiol-induced and estradiol-facilitated paradigms. The enhanced behavioral sensitivity of OVX rats to repeated E2 administration in the absence of P has been studied in two ways, one referred to as E2-induced and the other as E2-facilitated sexual behavior (Blaustein et al., 1987). In the E2-induced paradigm, females are given daily injections of EB (e.g.,, (Blaustein et al., 1987; Davidson et al., 1968a; Tennent, Smith, & Davidson, 1980) or a subcutaneous E2 implant (Gorzalka & Moe, 1994; Parsons et al., 1979), whereas in the E2-facilitated paradigm a bolus injection of free E2 is given in place of P, following a priming injection of EB (Blaustein et al., 1987; Kow & Pfaff, 1975; Parsons, Rainbow, Snyder, & McEwen, 1984). In both paradigms the facilitation of lordosis has been shown to be independent of peripheral sources of P, since ADX does not prevent the increase (Davidson et al., 1968a; Gorzalka & Moe, 1994; Kow & Pfaff, 1975; Tennent et al., 1980); in fact ADX can increase lordosis following E2 administration (Eriksson & Södersten, 1973; Gray & Gorzalka, 1980). In contrast, ADX has been shown to reduce the number of hops, darts and ear wiggles in an E2-induced paradigm that induces constant physiological levels of circulating E2 in OVX animals (15pg/mL) (Tennent et al., 1980). This dose is reportedly lower than the average serum E2 concentration present during the estrous cycle, at about a third of the peak concentration that is reached in the ovulatory cycle. This dose has also been shown to maintain normal body weight and is the minimal effective dose for maintaining lordosis in OVX rats (Albert et al., 1991).

To further examine whether P plays a role in the induction of sexual behaviors by E2 administered alone, PRs themselves were blocked using the competitive antagonist RU486 (mifepristone). It had been shown that E2 has the ability to bind PR, albeit with low binding affinity (approximately 1% that of P) (MacLusky & McEwen, 1980; Parsons et al., 1984), as such Blaustein (1987) asked whether blocking PR would block the facilitation of sexual behaviors in both the E2-induced and E2-facilitated paradigms. Blocking PR with RU486 just prior to P administration in OVX rats primed with EB effectively inhibited the display of sexually appetitive behaviors and attenuated lordosis (Blaustein et al., 1987). Next, OVX rats were implanted with a subcutaneous capsule containing crystalline E2 followed one week later by an injection of RU486 or vehicle and behavioral tests were carried out between 4 and 38 hours later, revealing no effect of PR blockade on E2-induced lordosis. Similarly, OVX rats were

treated with 2μg EB and 44 hours later with a bolus injection of free E2 in place of P (E2-facilitated paradigm) but injected with RU486 or vehicle one hour prior to the second injection of E2, and again no effect was seen on lordosis. Unfortunately, appetitive sexual behaviors were not reported in that study. More recently, the mechanism underlying the increase in lordosis using a two-pulse paradigm has been shown to involve the activation of membrane-bound E2 receptors, following the initial E2-priming effects that occur through classic genomic mechanisms (Kow & Pfaff, 2004).

The effect of PR blockade on appetitive sexual behaviors was more recently evaluated using a modified E2-facilitation paradigm. This paradigm is used to study the mechanisms underlying estrogen-positive feedback on the induction of the LH surge, following the stimulation of nP synthesis by E2 in hypothalamic tissue (Kuo & Micevych, 2012; Micevych et al., 2008). OVX or OVX/ADX rats were treated with 10μg EB every 4 days followed by a bolus injection of free E2 in place of P on the third and fourth injections days, which resulted in an increase in lordosis as previously reported in the literature (and discussed above) as well as appetitive sexual behaviors (hops, darts, ear wiggles) (Micevych et al., 2008). Although blocking PR with RU486 just prior to the bolus injection of free E2 did not inhibit the facilitative effects of E2 on lordosis, in concert with Blaustein et al. (1987), RU486 attenuated the facilitation of sexually appetitive behaviors. Moreover blocking P synthesis (by blocking either P450 side-chain cleavage, or the conversion of pregnenalone to P) mimicked the effect of RU486 in this paradigm. Those results suggest that at least in an E2-facilitation paradigm, nP synthesis within the hypothalamus (particularly the preoptic area (Micevych et al., 2003)) and binding to its receptor, is part of the mechanism that induces the heightened behavioral sensitivity of appetitive sexual behaviors to repeated injections of E2.

In summary, sexual behavior in the OVX rat is dependent on the priming actions of E2 acting on hypothalamic tissue. Although the literature has shown that the OVX rat is behaviorally more sensitive to E2 when treated daily with E2, or with a priming dose of EB following a bolus injection of free E2 in place of P, this phenomenon has never been reported if EB is administered every four days, mimicking the natural ovarian cycle.

Outline of the current thesis

Following initial observations from a number of researchers in our laboratory that sexual behavior in the OVX rat sensitized with repeated injections of 10μg EB every four days, the goal of the next chapter was to systematically characterize appetitive and consummatory patterns of sexual behavior using three doses of EB commonly used in the literature. EB was administered every 4 or 8 days, which established the behavioral pattern of estradiol sensitization by EB and the appropriate baseline dosing regimen for inducing low, steady levels of sexual behavior in the OVX Long-Evans rat (Chapter 2.1). It was found that administration of 10μg EB every four days induced the most robust sensitization, and thus this dose was selected for further examination of the phenomenon. Next, some hormonal and behavioral factors that may contribute to the effect were assessed. The next studies examined whether peripheral P plays a role by examining the phenomenon in ADX/OVX animals (Chapter 2.1, Experiment 2) and whether chronic PR blockade interfered with the induction of estradiol sensitization (Chapter 2.2). The conditions necessary and sufficient for the induction of estradiol sensitization with 10μg EB were examined next, and it was found that repeated copulation attenuated the effect (Chapter 2.3). The experiments of the third chapter discovered that VCS attenuated estradiol sensitization (Chapter 3.1), and addressed whether mechanisms of estrous termination are involved in that attenuation (Chapter 3.2). In Chapter 4 the phenomenon of estradiol sensitization was applied to two paradigms of sexual inhibition to examine whether those inhibitory effects can be overcome. A general discussion is provided in the final chapter (Chapter 5).

CHAPTER 2: CHARACTERIZING BEHAVIORAL SENSITIZATION TO ESTRADIOL

2.1. Sensitization of sexual behavior in ovariectomized rats by chronic estradiol treatment

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Abstract

The ovariectomized (OVX) rat treated with estradiol benzoate (EB) is used to elucidate neuroendocrine mechanisms of sexual behavior. Chronic behavioral and pharmacological manipulations can be confounded by rising baselines, since females are behaviorally more sensitive to repeated EB injections. The literature lacks a systematic examination of chronic effects of EB administered alone to the sexually experienced OVX rat. Long-Evans rats were repeatedly treated (8 tests) with SC injections of 2, 5, or 10μg EB at different time intervals (4 or 8 days). Female sexual behaviors as well as receipt of mounts, intromissions and ejaculations from the male were observed in the unilevel 4-hole pacing chamber. The effects of adrenalectomy (ADX) and strain (Long-Evans vs. Wistar) were also assessed. Long-Evans OVX rats treated with 5μg EB every 8 days showed persistently low levels of sexual behavior. Sensitization was most robust following 10μg EB at 4-day intervals. Very few sexual behaviors were ever induced by 2μg EB. ADX did not affect the development of behavioral sensitization by 10μg EB. Therefore, to achieve a low steady state of sexual behaviors in sexually experienced Long-Evans OVX rats 5μg of EB administered every 8 days is optimal, whereas a persistently high level of sexual behaviors is induced with 10μg EB administered every 4 days. OVX Wistar rats are behaviorally more sensitive to EB. Behavioral sensitization to EB may serve as a mechanism to optimize reproductive success.

Introduction

The ovariectomized (OVX) rat treated with estradiol benzoate (EB) is used to examine neuroendocrine mechanisms underlying sexual behavior, and how pharmacological manipulations might alter these behaviors (e.g., Gelez, Greggain-Mohr, Pfaus, Allers, & Giuliano, 2013; Graham & Pfaus, 2010; Pfaus et al., 2000; Pfaus, Giuliano, & Gelez, 2007; Snoeren et al., 2011). In studies designed to investigate facilitative effects, females are typically primed with EB alone to elicit a low baseline of sexual responding. However, with repeated testing, OVX rats become more responsive to EB (Babcock et al., 1988; Beach & Orndoff, 1974; Blaustein et al., 1987; Clark & Roy, 1983; Gerall & Dunlap, 1973; Kow & Pfaff, 1975; Parsons et al., 1979; Whalen & Nakayama, 1965). This behavioral sensitization to EB confounds and complicates the interpretation of data, especially in studies where the manipulation facilitates sexual behaviors on an acute EB baseline, but appears to lose that effect following repeated testing. The establishment of stable behavioral baselines would be particularly useful in the development of preclinical research models of female sexual function and dysfunction (e.g., Gelez et al., 2013). Ideally, to study excitatory mechanisms, females should express a low but steady rate of sexual behavior at baseline. On the other hand, mechanisms involved in sexual inhibition should be studied in females presenting persistently high levels of sexual behavior. A thorough examination of the development of the sensitization of sexual behaviors following repeated EB can help elucidate underlying mechanisms of this poorly understood phenomenon.

The restoration of sexual behaviors in the OVX rat requires adequate priming with estradiol (E2), since E2-bound to its receptor is necessary for the expression of sexual behavior (Blaustein & Feder, 1979; Blaustein & Wade, 1977; Walker & Feder, 1979). The acute administration of EB to the OVX rat induces, approximately 48 hours later, the partial expression of lordosis, a concave arching of the back that allows the male to intromit and ejaculate during a mount (Hardy & Debold, 1971b; Meyerson, 1964; Whalen, 1974). To maximally induce lordosis and activate sexually appetitive behaviors (such as hops, darts, solicitations, and ear wiggles), additional treatment with progesterone (P) is required 36-48 hours later, and its potentiating effects occur within four hours of treatment (Beach, Etkin, & Rasquin, 1942; Boling & Blandau, 1939; Whalen, 1974). Although the fully-primed OVX rat is useful for testing manipulations that suppress sexual behavior, ceiling effects prevent the examination of dose-responses. Comparing manipulations between fully-primed and partially-primed females can be further complicated by

their different hormonal backgrounds. These issues can be overcome by characterizing behavioral responses to EB administered alone, following varying dosing regimens. Low doses of EB (< 3μg) fail to induce sexual behavior when administered alone, and do not result in behavioral sensitization (Kow & Pfaff, 1975; Micevych et al., 2008); thus, it should be possible to establish a low steady baseline of sexual behavior to allow facilitation of behavior, as well as a higher level of responding to allow for its attenuation.

Although behavioral sensitization to EB is a well-recognized phenomenon, its underlying mechanisms are not well understood. The question is twofold: how does repeated EB-priming alone potentiate lordosis, as well as appetitive sexual behaviors (i.e., independent of subsequent P priming)? In the EB-primed OVX rat, lordosis is dependent on EB dose, yet appetitive sexual behaviors are dose-dependently increased by administration of P, not EB (Whalen, 1974). Moreover, large doses of acute EB administered alone to OVX and adrenalectomized-OVX (ADX/OVX) rats induce hops, darts, and ear wiggles, but those effects are not dose-dependent on EB (Erskine, 1989; Hlinak & Madlafousek, 1983; Zemlan & Adler, 1977), which suggests that EB does not directly mediate those behaviors. Given that P potentiates the priming effects of EB, the sensitization may be a result of adrenal overcompensation (e.g., increased production of P), since adrenal hypertrophy occurs following OVX (Andersen & Kennedy, 1933), and appetitive sexual behaviors are observed more frequently in EB-primed Long-Evans rats that are OVX compared to ADX/OVX (Erskine, 1985; Tennent et al., 1980). ADX has also been reported to decrease the sensitivity of OVX rats to priming with EB and EB+P (Ring, 1945), and it was suggested that although ADX does not prevent the facilitation of sexual behaviors by EB alone, greater doses or extended priming with EB are required to facilitate sexual receptivity (Kow & Pfaff, 1975). However, others have found a facilitation of receptive behaviors by EB following ADX (Davidson et al., 1968a; Eriksson & Södersten, 1973; Gorzalka & Moe, 1994), and have suggested that this effect is dose-dependent, with the facilitation occurring with low doses of EB (Gorzalka & Moe, 1994; Gray & Gorzalka, 1980). It has also been argued that the behavioral facilitation by repeated EB-alone is not dependent on the activation of P receptors (PR), suggesting differential pathways in the activation of sexual behavior by EB+P compared to EB-alone (Blaustein et al., 1987). Some of those studies used pigmented (Long-Evans) whereas others used albino (e.g., Sprague-Dawley or Wistar) rat strains, and both are commonly used throughout the neuroendocrinology literature. However important strain differences have been

reported in sexual behavior, endocrinology, and brain morphology (Garcia-Falgueras et al., 2005; Hurwitz & Riley, 2011; Sachs, 1996; Tohei, Mogi, Kon, Hokao, & Shinoda, 2003; Uphouse et al., 2002), which should be considered in the assessment of the underlying mechanisms.

The primary goals of this study were to establish appropriate dosing regimens of EB to achieve a desired baseline of female sexual behavior, as well as to characterize the development of behavioral sensitization to EB in the sexually-experienced OVX rat. We defined behavioral sensitization as the potentiation of sexual behaviors induced by the same dose of EB administered prior to repeated tests at delayed or intermittent intervals. This is similar to the definition of sensitization that occurs with intermittent administration of psychomotor stimulants, opioids, and stressors (e.g., Kalivas & Stewart, 1991). Accordingly, we examined the effects of acute and repeated treatment with varying doses of EB (8 tests) in sexually-experienced OVX Long-Evans and Wistar rats. The first experiment examined the dose-response and time course effects of repeated EB (2, 5, or 10μg) administered by subcutaneous injections at two dosing intervals (4 or 8 days) in OVX Long-Evans rats. Given that the adrenal gland is an important peripheral source of steroid hormones, the second experiment examined whether ADX would alter the sensitization. The third experiment characterized the sexual behaviors of Wistar OVX rats receiving 2, 5, or 10μg EB every 4 days.

Materials and methods

Animals

Animals were purchased from Charles River Canada (St-Constant, QC), and allowed one week to acclimate to the facilities prior to surgery (females) or sexual training (males). Females (150-200g) were pair-housed, and males (200-250g) were group-housed (4/cage) in Plexiglass cages lined with betachip, with standard laboratory chow (Charles River #5075) and tap water freely available. Colony rooms were maintained at 21°C, on a 12-hour reverse day-night cycle (lights off at 8AM). Males were given at least four sexual training sessions in unilevel 4-hole pacing chambers with stimulus OVX females primed with 10μg EB and 500μg P (48hrs and 4hrs before training respectively), prior to the start of each experiment.

Care was taken to minimize pain and discomfort to all animals throughout the duration of the experiments, which were conducted in accordance with the guidelines of the Canadian

Council on Animal Care, and approved by the Concordia University Animal Research Ethics Committee.

Surgery

Ovariectomy. Bilateral OVX was performed under a 4:3 mixture of ketamine hydrochloride (50 mg/mL; Ketaset©, Wyeth Canada) and xylazine hydrochloride (4 mg/mL; Rompum©, Bayer Healthcare) injected IP (1mL/kg body weight). Animals were identified by ear-punch and post-operative care was given with SC injections of Flunixin meglumine 2.5 mg/kg/mL (Banamine©) and 5 mg/kg/mL Enrofloxacin (Baytril©), and rehydrated with 2 mL of saline administered SC. Animals were given one-week post-operative recovery prior to sexual behavior training.

Ovariectomy and adrenalectomy. Immediately following OVX as described above, the adrenals were located and removed (ADX/OVX group), or were located and left intact (SHAM/OVX). ADX animals were administered corticosterone (4-pregnen-11β,21-diol-3,20-dione, Steraloids; 25 mg/L of 0.9% saline) and cyclodextrin (0.35 mg/L of 0.9% saline) in their drinking water, and a pinch of sea salt pellets was added to the cage daily, until the end of the experiment.

Preparation of steroid hormones

EB and P were dissolved in 0.1mL reagent grade sesame oil and administered by SC injection. EB doses were always diluted down from the 10µg EB solution.

Sexual behavior training and test procedures

At least one week following surgery, females were made sexually receptive by SC injections of 10µg EB 48 hours, and 500µg P 4 hours prior to each of four sexual training sessions occurring at 4-day intervals, to reduce variability in sexual responding (Gerall & Dunlap, 1973). Females were then given a 2-week hormone washout period before the start of testing, to reduce residual effects of prior hormone treatments (Kow & Pfaff, 1975).

Behavioral training and testing were carried out in unilevel pacing chambers (38x60x38cm) lined with betachip, and bisected by a clear Plexiglas divider with 4 square holes cut into the bottom. The holes were adjusted to the size of the female, such that she could easily

traverse to the male's compartment, whereas the male was too large to cross, allowing her to pace the rate of copulation. All tests occurred during the middle-third of their dark cycle.

Males were selected at random and always placed into the chamber for a five-minute habituation period, and only ever used once during a session. Females were subsequently placed into the compartment opposite the male for a 30-minute test session. Sessions were digitally recorded using a Sony Handycam, and subsequently scored using a computerized event recorder customized for rat sexual behavior in a pacing chamber (Cabilio, 1996).

Experiment 1 EB dose response administered at 4- and 8-day intervals in OVX Long-Evans rats. As shown in Figure 1, following sexual training and a washout, Long-Evans OVX female rats received 2, 5, or 10μg EB (n=12/group) and first tested at 8-day intervals for 8 tests (Experiment 1a), followed by a 2-week hormone washout period. Females were next randomly re-assigned (counterbalancing prior hormone treatment) to one of the three doses before testing at 4-day intervals for another 8 tests (Experiment 1b).

Experiment 2 EB dose response administered at 4-day intervals in ADX/OVX Long-Evans rats. As shown in Figure 1, Long-Evans female rats were OVX and either adrenalectomized (ADX/OVX) or sham-ADX (SHAM/OVX) and one week later given 4 sexual training sessions. Following a 2-week hormone washout ADX/OVX females were treated with 5μg EB (n=11) or 10μg EB (n=11), and SHAM/OVX females (n=6) were treated with 10μg EB, at 4-day intervals for a total of 8 tests.

Experiment 3 EB dose response administered at 4-day intervals to OVX Wistar rats. As shown in Figure 1, Wistar female rats (N=26) were OVX, treated with 2μg (n=8), 5μg (n=9), or 10μg EB (n=9), and tested 8 times, at 4-day intervals.

Behavioral Measures

The frequency of female lordosis magnitudes (LM) was scored on a 3-point scale according to Hardy and Debold (1971b) and lordosis quotients (LQ) were calculated as a ratio of the frequency of LM to the number of mounts (including intromissions and ejaculations), multiplied by 100. Frequencies of appetitive behaviors were recorded by taking the sum of solicitations (orients head towards male and runs away), and hops/darts. Defensive behaviors consisted of kicks, sideways takedowns, boxing postures, and prone positions, as in Barnett

(1963). Mounts, intromissions, and ejaculations received from the male were analyzed, since their behavior can provide insight into the female's sexual receptivity (Pfaus et al., 1999).

Statistical analyses

Using SPSS 16.0 for Windows, data were entered as a 2-way ANOVA (8 tests as the repeated measure, and 3 hormonal manipulations as the between subjects factor), and the overall effect of hormone manipulation, as well as the linear and quadratic trends (Keppel & Wickens, 2004), were examined. A significant interaction between test and hormone manipulation prompted an examination of the linear contrasts for each treatment group, with significant contrasts suggesting the development of sensitization. The one-way repeated measures ANOVA was then conducted to determine the development of sensitization, using post-hoc LSD (Least Significant Difference). Hormone manipulation effects (between group measure) were also analyzed using LSD, with the level of significance for all comparisons set at $p<0.05$. Missing data points were replaced with the group means of the respective test day. In Experiment 2, simple linear contrasts were examined on each group to determine whether the patterns of sensitization differed following ADX, based on the findings from Experiment 1. These statistical techniques were selected to maximize power. The pattern of LM data was similar to that of LQ, and therefore not shown for simplification.

Results

Sexual behaviors of females on training 4 when treated with 10μg EB + 500μg P.

Table 1 summarizes the behavior of OVX and OVX-ADX Long-Evans, and OVX Wistar rats on the fourth training session when fully primed with EB+P. An independent t-test verified that ADX did not affect sexual behavior compared to OVX females in Experiment 2 on the fourth sexual training session.

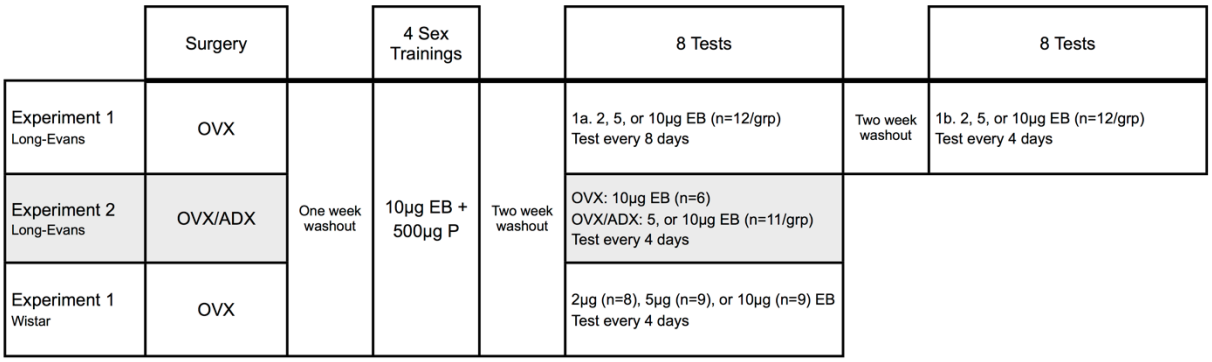

Figure 1. Experimental timelines. One week following ovariectomy (OVX) or adrenalectomy and OVX (ADX/OVX), females were primed with 10µg EB and 500µg P prior to each of 4 sexual training sessions followed by a 2-week hormone washout. In Experiment 1, females were first tested 8 times with varying doses of EB at 8-day intervals followed by a 2-week hormone washout period. Next they were randomly re-assigned to the varying EB doses and tested 8 times at 4-day intervals. In Experiments 2 and 3, females were tested 8 times with varying doses of EB at 4-day intervals. Strain of females used in each experiment is indicated in left-most column.

Table 1.

Average frequency (±SEM) of sexual behaviors on training 4 when fully-primed with 10µg estradiol benzoate and 500µg progesterone.

	Experiment 1a Long-Evans	Experiment 2 Long-Evans		Experiment 3 Wistar
	OVX	OVX	ADX/OVX	OVX
Appetitive	55.60±3.50	53.5±13.12	64.54±6.92	77.73±7.37
LQ	99.81±0.14	100.00±0.00	95.00±0.04	96.15±2.52
Defenses	1.00±0.35	0.50±0.34	1.96±1.38	1.12±0.35
Mounts	15.44±1.93	10±2.52	20.25±2.68	22.31±4.69
Intromissions	20.17±1.09	16.5±2.47	15.5±1.24	18.73±1.60
Ejaculations	2.78±0.11	2.17±0.31	2.04±0.20	1.92±0.18

Note. No differences were detected between OVX and ADX/OVX females in Experiment 2. LQ=lordosis quotient.

Experiment 1a EB dose response administered at 8-day intervals in OVX Long-Evans rats

Figure 2 depicts the effect of repeated administration of 2, 5, or 10μg EB injected SC at 8 day intervals on the expression of appetitive and consummatory sexual behaviors in the sexually experienced OVX rat, across 8 test sessions. An equipment malfunction occurred on Test 2, resulting in the data loss of 4 animals in the 5μg and 10μg groups (2 per group). These data points were replaced with their respective group means.

Appetitive behaviors. As shown in Figure 2A, 10μg EB induced significantly more appetitive behaviors overall, compared to the two lower doses, $F(2,33)=12.380$, $p<0.001$. Following a significant interaction on the linear trend between test and EB group, $F(2, 33)=6.327$, $p=0.005$, it was found that when administered every 8 days, only 10μg EB induced sensitization of sexually appetitive behaviors, $F_{linear}(1,11)=7.315$, $p=0.020$. The development of sensitization was characterized following a significant one-way ANOVA, $F(7, 77)=2.638$, $p=0.017$, as shown in Figure 2A. Sensitization was not detected following 2μg EB, $F(7, 77)=1.149$, $p=0.342$, or 5μg EB, $F(7, 77)=1.960$, $p=0.072$.

Lordosis. Figure 2B summarizes the LQ for animals in each EB group; only 8 females treated with 10μg EB, and 4 with 5μg EB were consistently mounted across all tests (no female treated with 2μg EB was mounted on every test). The linear contrast testing the interaction between EB group and test day was not statistically significant. Although a linear increase was detected across tests when collapsed across EB treatment, $F(1,10)=5.660$, $p=0.039$, the one-way ANOVA failed to detect a difference between any of the test days, $F(7,70)=1.521$, $p=0.174$, suggesting that overall, sensitization of LQ in response to repeated EB did not occur at this dosing interval.

Defensive Behaviors. As shown in Figure 2C, collapsing across EB treatment, the number of defensive behaviors displayed by females generally decreased across tests, $F_{linear}(1,33)=44.971$, $p<0.001$, $F_{quadratic}(1,33)=12.246$, $p=0.001$, such that females were less defensive as of test 3 compared to the two first test, $F(7,231)=10.291$, $p<0.001$, an effect that was generally maintained until the end of the experiment. A main effect of EB dose was also found, $F(2,33)=4.410$. $p=0.020$. Animals that received 2μg EB displayed less defensive behaviors than those treated with 5μg or 10μg EB. The lower expression of defensive behaviors in the 2μg EB group could be explained by fewer mounts received from the male, as described below.

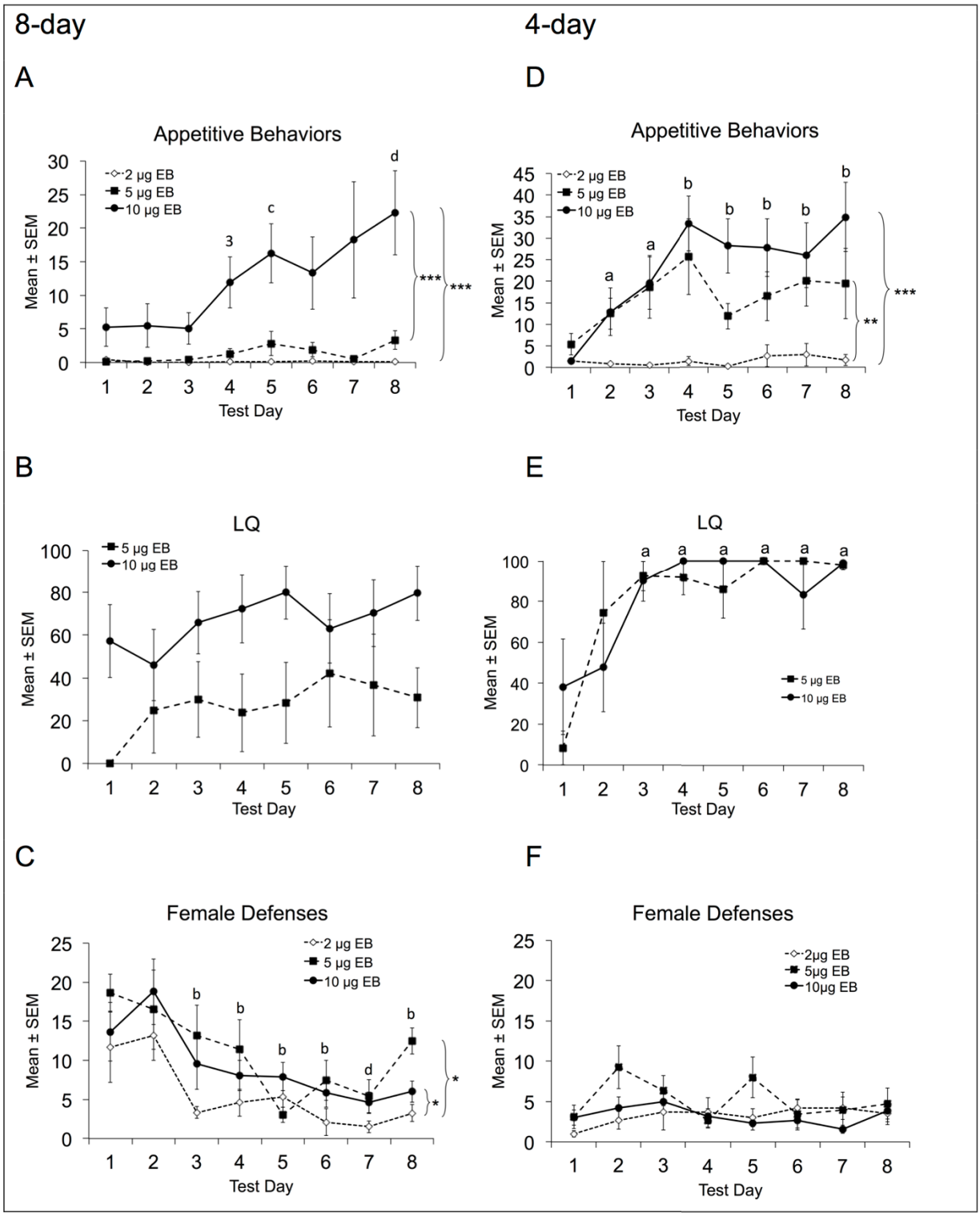

Figure 2. Sexual behaviors of OVX Long-Evans females treated with varying doses of EB at 8-day (left) or 4-day (right) intervals. (A) Appetitive sexual behaviors increased in females treated with 10µg EB, but not 2µg or 5µg when treated at 8-day intervals. (B) LQ did not sensitize when

treated with EB at 8-day intervals. (C) Collapsed across EB treatment group, females were less defensive towards males as of Test 3. (D) When treated at 4-day intervals sexually appetitive behaviors sensitized in females treated with 10µg EB. (E) LQ sensitized in females treated with 5µg and 10µg EB when treated at 4-day intervals. (F) Defensive behaviors were unaffected when treated with EB at 4-day intervals. [a]Different from Test 1; [b]Different from Tests 1 and 2 ; [c]Different from Tests 1, 2, and 3. [d]Different from Tests 1-4. Numerical superscripts are used to indicate differences from specified test day. Brackets represent main effect of EB Group. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Male Behaviors. The number of mounts received was affected by EB dose, $F(1,33)=3.319$, $p=0.049$, such that females treated with 10µg EB received significantly more mounts than those treated with 2µg EB, as shown in Figure 3A. Collapsing across EB treatment, the quadratic trend was significant, $F(1,33)=5.703$, $p=0.023$, and the test differences were analyzed following a significant one-way ANOVA, $F(7,231)=2.122$, $p=0.042$, and are shown in Figure 3A. Collapsing across EB treatment, the number of intromissions generally increased across tests, $F_{linear}(1,33)=5.658$, $p=0.023$, and test differences were analyzed following a significant one-way ANOVA, $F(7,231)=3.092$, $p=0.004$, as shown in Figure 3B. EB dose also affected the number of intromissions, $F(2,33)=9.399$, $p=0.001$, and ejaculations, $F(2,33)=7.097$, $p=0.003$, such that that females treated with 10µg EB received significantly more intromissions and ejaculations than those receiving the two lower doses (Figure 3, panels B and C, respectively).

Experiment 1b EB dose response administered at 4-day intervals in OVX Long-Evans rats

Appetitive Behaviors. The main effect of EB treatment revealed that more appetitive behaviors were induced by 5µg and 10µg EB, compared to 2µg EB. However following up the significant test by EB treatment linear interaction, $F(2, 33)=4.321$, $p=0.022$, only those treated with 10µg EB experienced sensitization of appetitive behaviors across tests, $F(1,11)=10.532$, $p=0.008$; the one-way ANOVA characterized the development, $F(7,77)=4.427$, $p<0.001$, with more expressed on Test 4 compared to Tests 1 and 2, and this effect was maintained until the end of testing, as shown in Figure 2D.

Lordosis and Defensive Behaviors. No female in the 2µg EB group, and only three in the 5µg EB and five in the 10µg EB group were consistently mounted across tests. The linear trend was statistically significant when collapsing across EB treatment, $F(1,6)=13.239$, $p=0.011$. Following a significant one-way ANOVA, $F(7,42)=8.342$, $p<0.001$, a greater LQ was detected on Test 3 compared to Test 1, and this effect was maintained until the end of the experiment. The significant quadratic trend which was also detected, $F(1,6)=24.924$, $p=0.002$, suggests a rapid onset of sensitization followed by a plateau.

As shown in Figure 2F, defensive behaviors were unaffected.

Male Behaviors. As shown in Figure 3D, EB treatment affected the number of mounts, $F(2,33)=20.613$, $p<0.001$. Those treated with 2µg EB received significantly fewer mounts

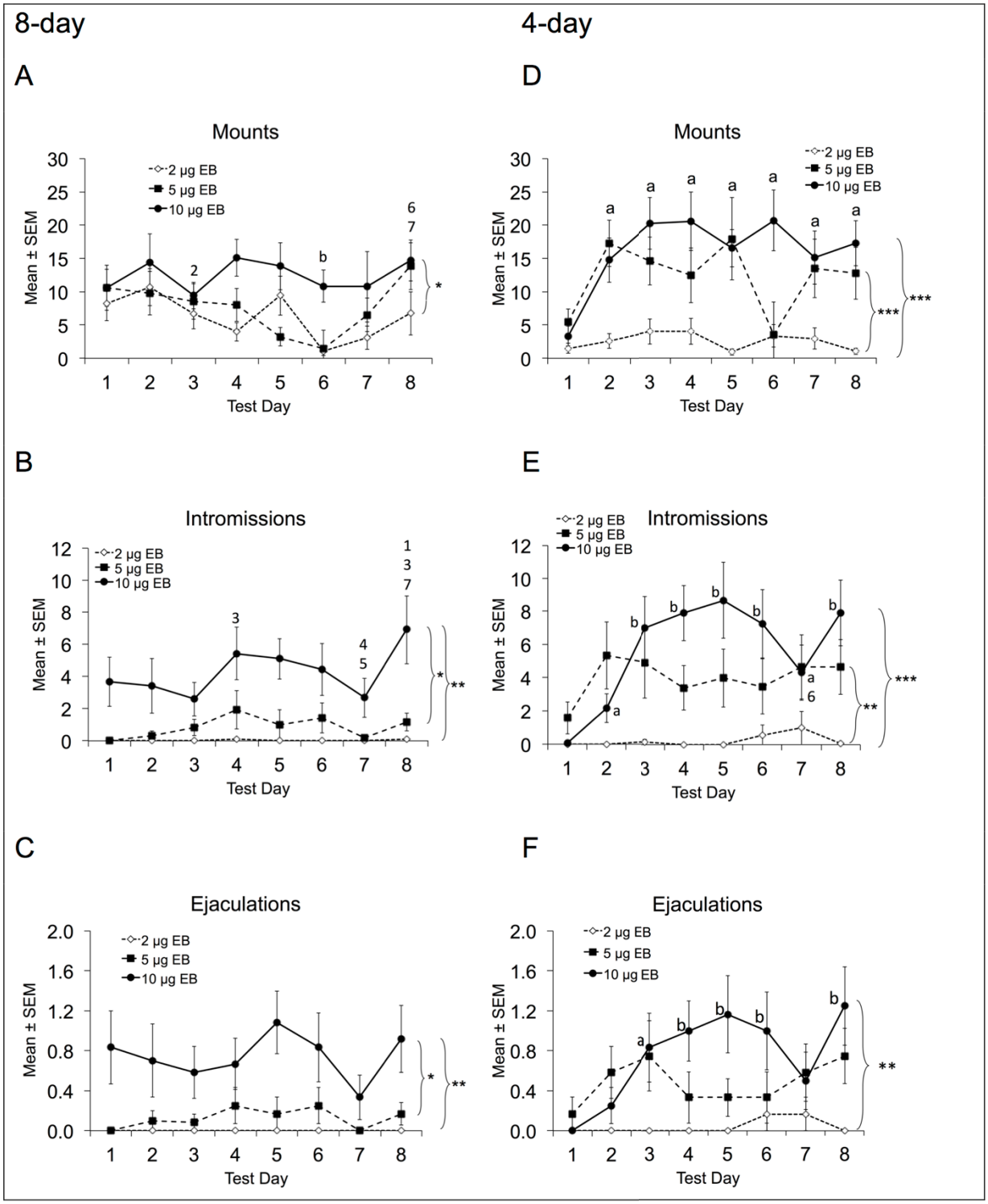

Figure 3. Male sexual behaviors directed towards OVX Long-Evans females treated with varying doses of EB at either 8-day (left) or 4-day (right) intervals. (A-C) Number of mounts, intromissions, and ejaculations received following varying EB doses administered at 8-day

intervals. Test effects are collapsed across EB treatment. (D) Females treated with 2μg EB were mounted less than those treated with 5μg or 10μg EB, and females were mounted less frequently on Test 1 compared to all subsequent tests. (E) Females treated 2μg EB received fewer intromissions than those treated with 5 or 10μg EB, and those treated with 10μg EB received fewer intromissions on test 1 compared to all subsequent tests. (F) Females treated with 10μg EB received more ejaculations across tests, and received more compared to those treated with 2μg EB. [a]Different from Test 1; [b]Different from Tests 1 and 2 ; [c]Different from Tests 1, 2, and 3. Numerical superscripts are used to indicate differences from specified test day. Brackets represent main effect of EB Group. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

compared to the two higher doses of EB. Collapsing across EB treatment, only the quadratic trend was significant, $F(1,33)=14.736$, p=0.001, and the one-way ANOVA $F(7,231)=2.985$, p=0.005, found that fewer mounts were received on Test 1 compared to all subsequent tests.

In the analysis of intromissions (Figure 3E), the linear interaction between Test and EB treatment was statistically significant, $F(2,33)=3.928$, p=0.03. Only females treated with 10µg EB experienced an increase in the number of intromissions received across tests, $F_{linear}(1,12)=8.80$,p=0.013, $F_{quadratic}(1,11)=13.245$, p=0.004. The one-way ANOVA, $F(7,77)=4.777$, p<0.001, detected fewer intromissions on Test 1 compared to all subsequent tests.

The analysis of ejaculations followed a similar pattern. Following a significant linear interaction between Test and EB treatment, $F(2,33)=3.984$, p=0.028, an increase was only detected in females treated with 10µg EB, $F_{linear}(1,11)=10.075$, p=0.009, $F_{quadratic}(1,11)=5.087$, p=0.045, the one-way ANOVA detected differences between tests, $F(7,77)=3.444$, p=0.003, such that fewer ejaculations were received on Tests 1 and 2 compared to all subsequent tests except Test 7, when fewer ejaculations were received.

Experiment 2 EB dose response administered at 4-day intervals in ADX/OVX Long-Evans rats.

Figure 4 shows the effect of repeatedly testing OVX females who were either sham-ADX (SHAM/OVX) and treated with 10µg EB, or ADX/OVX and treated with either 5µg or 10µg EB, at 4-day intervals. Overall, ADX did not prevent the sensitization of sexual behaviors of OVX females when repeatedly tested with 10µg EB.

Appetitive Behaviors. A linear trend analysis tested whether sensitization developed in each group. As expected, SHAM/OVX females treated with 10µg EB sensitized to repeated EB, $F(1,5)=9.228$, p=0.029, and the development, $F(7,35)=4.898$, p=0.001, is depicted in Figure 4A. ADX/OVX females treated with 10µg EB also sensitized, $F(1,11)=7.487$, p=0.019, and the development is shown in Figure 4A, $F(7,77)=2.273$, p=0.037. ADX/OVX females treated with 5µg EB also developed sensitization determined by a significant quadratic trend, $F(1,11)=6.615$, p=0.026; however the ANOVA did not detect overall differences between tests.

Lordosis. Figure 4B shows the LQ for ADX/OVX females treated with 5µg EB (n=10), ADX/OVX females treated with 10µg EB (n=10), and SHAM/OVX animals treated with 10µg EB (n=3) who were consistently mounted across tests. A linear trend analysis tested whether sensitization developed in each group. The analysis failed to detect sensitization of LQ in

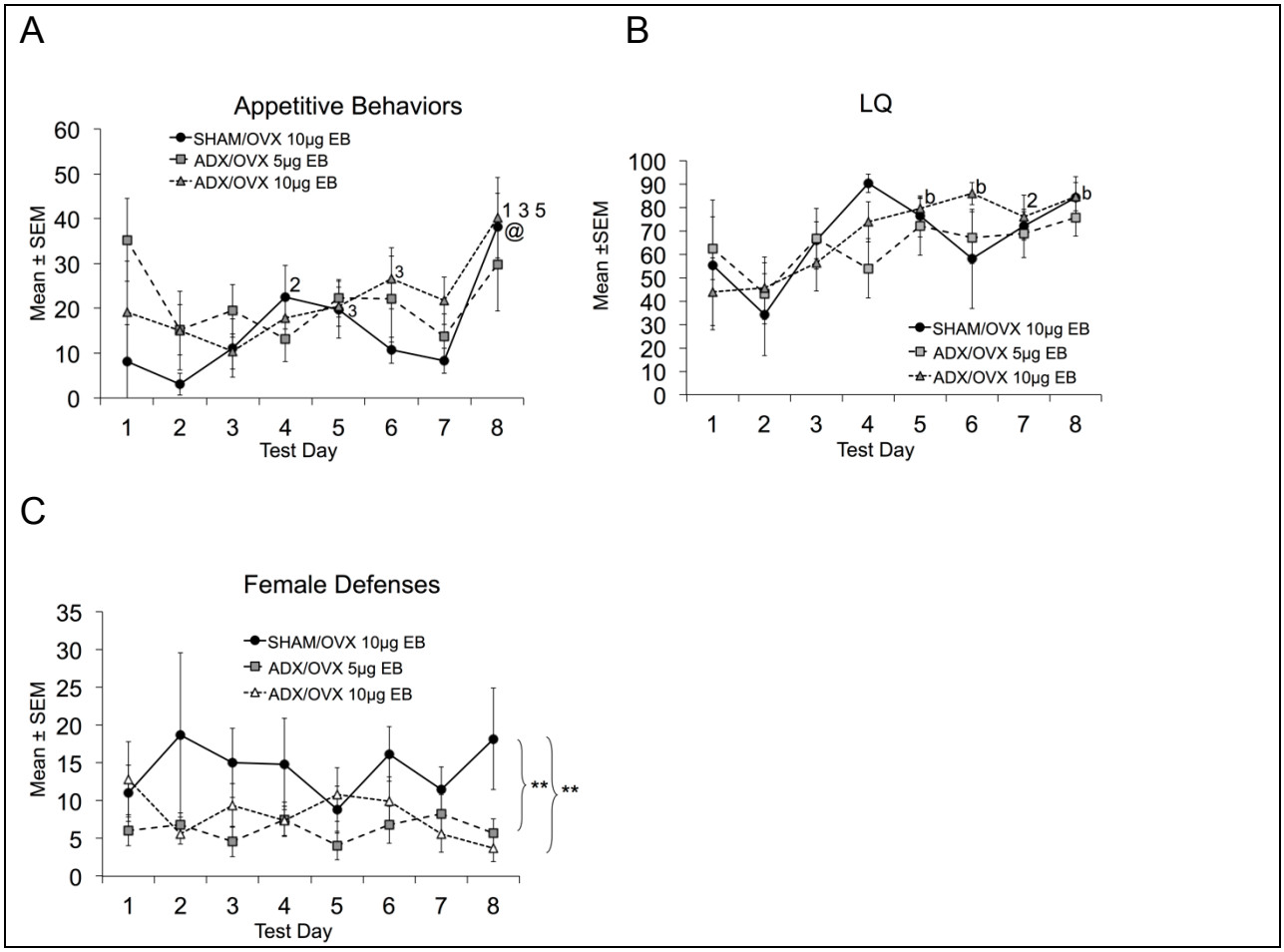

Figure 4. Sexual behaviors of OVX Long-Evans females treated with 10µg EB and ADX/OVX Long-Evans females treated with either 5µg or 10µg EB at 4-day intervals. (A) Appetitive behaviors sensitized following treatment with 10µg EB whether or not OVX females were ADX. (B) Sensitization of LQ occurred in ADX/OVX females treated with 10µg EB. (C) Female defensive behaviors were less frequent in ADX/OVX females overall compared to OVX females who were SHAM-ADX. Brackets represent main effect of EB Group, **$p<0.01$. [a]Different from Test 1, [b]Different from Tests 1 and 2, [@]Different from Tests 1-7. Numbers are used to indicate differences from specified test day.

SHAM/OVX females treated with 10μg EB, $F(1,2)=14.488$, $p=0.063$. This is inconsistent with Experiment 1b, and may be due to a small number of SHAM/OVX females that were consistently mounted by males. However, a linear increase was detected in ADX/OVX females treated with 10μg EB, $F(1,9)=8.425$, $p=0.018$, and test differences are depicted in Figure 4B, $F(7,63)=3.885$, $p=0.001$. LQ did not differ across tests in ADX/OVX females treated with 5μg EB.

Defensive Behaviors. As illustrated in Figure 4C**,** the number of defensive behaviors females made towards males was affected by the hormone manipulation, $F(2,27)=5.87$, $p=0.008$. SHAM/OVX females treated with 10μg EB displayed more defensive behaviors towards males compared to both ADX/OVX groups.

Male Behaviors. As shown in Figure 5A, although mounts were unaffected, when collapsed across hormone manipulation, the number of intromissions, $F(1,27)=5.143$, $p=0.032$, and ejaculations, $F(1,27)=6.361$, $p=0.018$, received generally increased across tests, test differences are depicted in panels B and C, respectively, (intromissions: $F(7,189)=3.047$, $p=0.005$; ejaculations: $F(7,189)=3.076$, $p=0.004$).

Experiment 3 EB dose response administered at 4-day intervals to OVX Wistar rats.

Two females in the 10μg EB group did not cross to the male's compartment on Test 5 because the holes of the barrier were too small, as such their data were replaced with the group means.

Appetitive Behaviors. As shown in Figure 6A, EB dose influenced the number of appetitive behaviors, $F(2,23)=5.827$, $p=0.009$, which were more frequently observed in females treated with 10μg EB compared to 2μg and 5μg EB. In contrast to Experiment 1b (Long-Evans females treated at 4-day intervals), the linear contrast testing the interaction between Test and EB treatment was not statistically significant, $F(2,23)=3.249$, $p=0.057$. The linear contrast collapsing across EB treatment was significant, $F(1,23)=93.405$, $p<0.001$, suggesting that all groups displayed sensitization. To more thoroughly test whether appetitive behaviors sensitized across tests in all EB treatment groups, separate linear trend analyses were conducted for each dose. Similar to Long-Evans, Wistar OVX females treated with 5μg EB, $F_{linear}(1,8)=28.712$, $p=0.001$, and 10μg EB sensitized, $F_{linear}(1,8)=65.639$, $p<0.001$, $F_{\text{quadratic}}(1,8)5.646$, $p=0.045$. However, contrary to Long-Evans females, OVX Wistars treated with 2μg EB also sensitized,

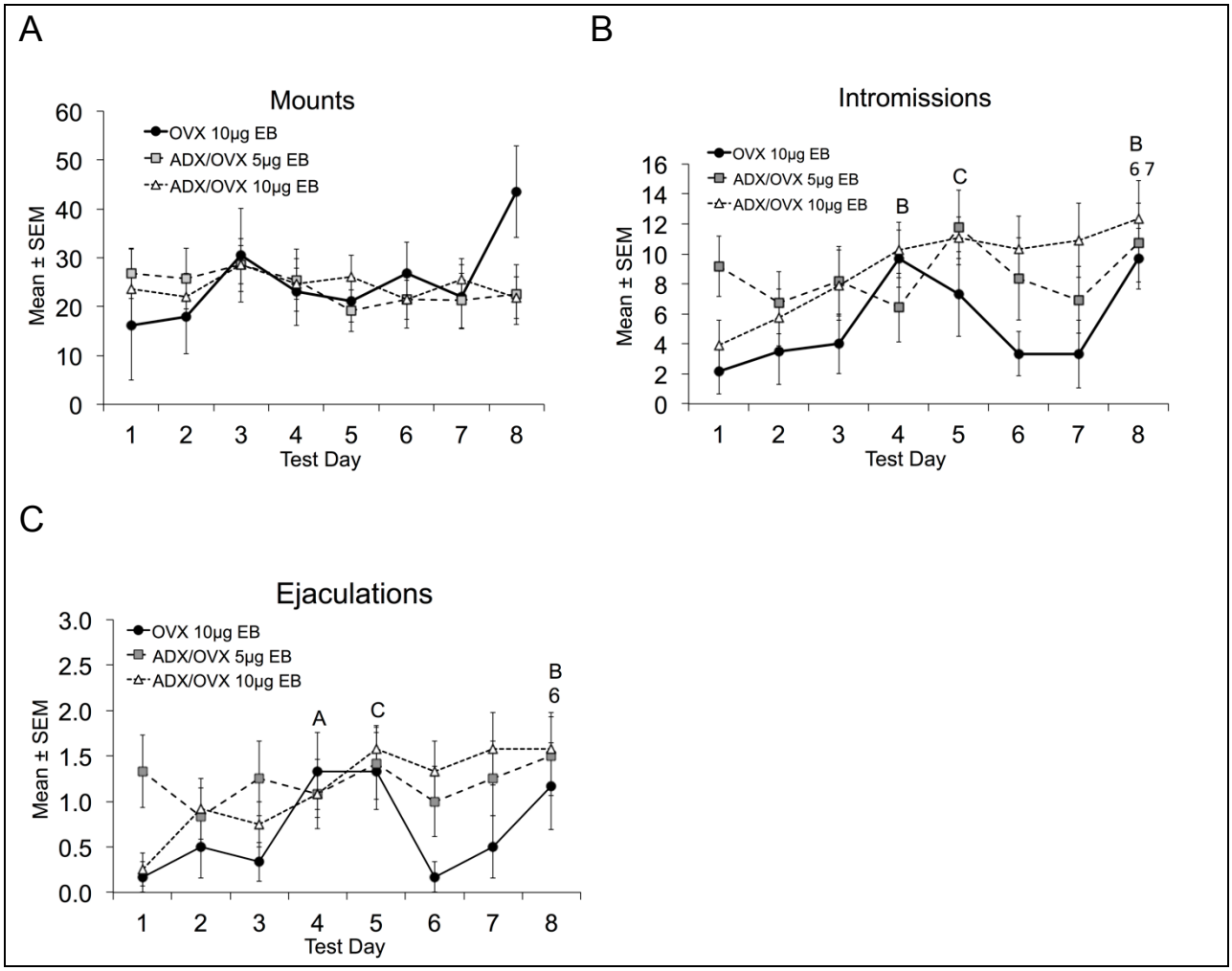

Figure 5. Number of mounts (A), intromissions (B) and ejaculations (C) received from males towards ADX/OVX Long-Evans females treated with either 5µg or 10µg EB, or SHAM/OVX females treated with 10µg EB at 4-day intervals. Test effects are collapsed across hormone manipulation. [A]Different from Test 1; [B]Different from Tests 1 and 2 ; [C]Different from Tests 1, 2, and 3. Numbers are used to indicate differences from specified test day.

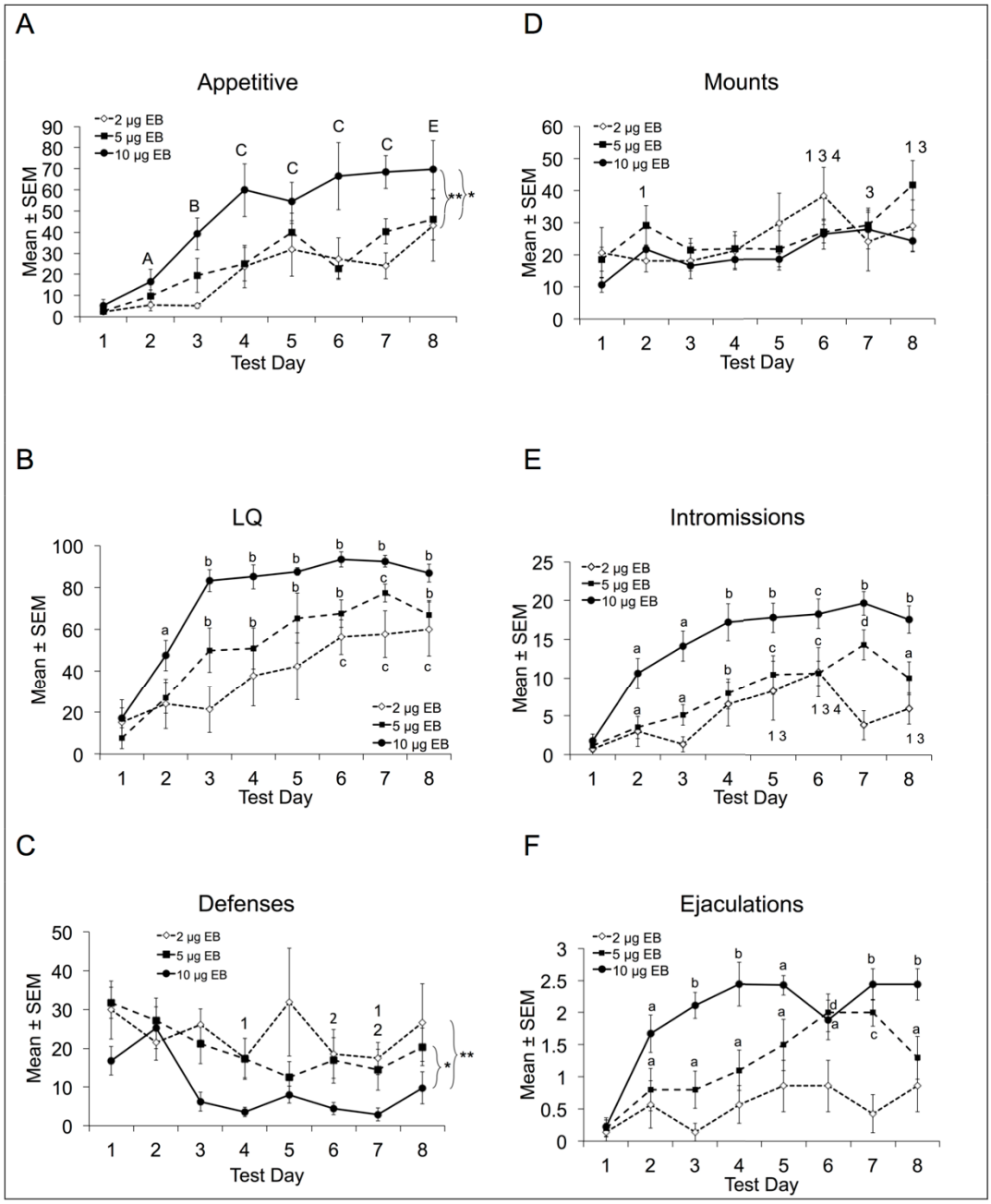

Figure 6. Sexual behaviors of Wistar OVX rats tested at 4-day intervals. [a]Different from Test 1; [b]Different from Tests 1 and 2 ; [c]Different from Tests 1, 2, and 3. [d]Different from Tests 1-4 [e]Different from Tests 1-6. Uppercase letters are used when collapsed across EB treatment, and lowercase are used following a significant interaction. Numbers are used to indicate differences from specified test day.

$F_{linear}(1,7)=13.534$, p=0.008, confirming that all doses of EB induced sensitization of sexually appetitive behaviors in Wistars. The development of sensitization in each group is displayed in Figure 6A (2µg, $F(7,49)=5.336$, p<0.001; 5µg, $F(7,56)=9.403$, p<0.001; 10µg, $F(7,56)=8.275$, p<0.001).

Lordosis. One female from the 2µg EB group, and two from the 5µg EB group were not consistently mounted across tests resulting in a smaller sample size (2µg EB =6; 5µg EB=8). A significant interaction between Test and EB dose was detected on the quadratic trend, $F(2,20)=5.866$, p=0.010. As shown in Figure 6B, 2µg EB induced a significant increase in LQ across tests, $F(1,5)=17.570$, p=0.009, contrary to the Long-Evans data (Experiment 1b; Figure 2E). LQ also increased across tests following treatment with 5µg EB, $F_{linear}(1,7)=80.118$, p<0.001, $F_{quadratic}(1,7)=8.825$, p=0.021, and 10µg EB, $F_{linear}(1,8)=71.353$, p<0.001, $F_{quadratic}(1,8)=52.350$, p<0.001. The development of sensitization in each group is displayed in Figure 6B (2µg EB, $F(7,35)=5.159$, p<0.001; 5µg EB, $F(7,49)=17.169$, p<0.001; 10µg EB, $F(7,56)=36.371$, p<0.001).

Female Defenses. Collapsed across EB treatment, defensive behaviors decreased across tests, $F_{linear}(1,23)=13.874$, p=0.001, $F_{quadratic}(1,23)=4.832$, p=0.038. The differences between tests are shown in Figure 6C, $F(7,161)=3.477$, p=0.002. A main effect of EB dose was also detected, $F(2,23)=6.909$, p=0.004. Females treated with 10µg EB displayed fewer defensive behaviors than those treated with 5µg and 2µg EB.

Male Behaviors. Figure 6, panels D to F, show the number of mounts, intromissions and ejaculations received from the male, respectively. Following a significant linear trend, $F(1,23)=15.428$, p=0.001, it was found that generally, females were mounted more often on later tests compared to earlier tests, $F(7,161)=3.811$, p=0.001. Following up a significant linear interaction on intromissions, $F(1,23)=3.591$, p=0.044, revealed that all doses of EB resulted in increasing numbers of intromissions received across tests, although the effect was less robust in females treated with 2µg EB: $F_{linear}(1,7)=13.740$, p=0.008, compared to 5µg EB $F_{linear}(1,8)=24.983$, p=0.001, $F_{quadratic}(1,8)=6.915$, p=0.030, and 10µg EB, $F_{linear}(1,8)=60.849$, p<0.001, $F_{quadratic}(1,8)=38.824$, p<0.001. Differences between tests are shown in Figure 6E (2µg EB: $F(7,49)=3.925$, p=0.002; 5µg EB: $F(7,56)=8.544$, p<0.001; 10µg EB: $F(7,56)=15.318$, p<0.001).

The analysis of ejaculations detected a main effect of EB treatment, $F(1,23)=16.465$, $p<0.001$, such that as EB dose increased, the number of ejaculations received increased. A significant quadratic interaction between Test and EB treatment was also found, $F(2,23)=2.296$, $p=0.024$. The number of ejaculations received generally increased across tests in females treated with the two higher doses of EB (5µg EB, $F_{\text{linear}}(1,8)=23.745$, $p=0.001$; $F_{quadratic}(1,8)=10.573$, $p=0.012$; 10µg EB, $F_{\text{linear}}(1,8)=71.428$, $p<0.001$, $F_{quadratic}(1,8)=13.004$, $p=0.007$). Differences between tests days are shown in Figure 6F (5µg EB, $F(7,56)=6.120$, $p<0.001$; 10µg EB, $F(7,56)=12.217$, $p<0.001$).

Discussion

This study characterized the pattern of sexual behavior in sexually-experienced Long-Evans OVX rats treated repeatedly (8 SC injections) with either 2, 5, or, 10µg EB at 4- or 8-day intervals. Behavioral sensitization to repeated EB occurred most readily in females treated with 10µg EB at 4-day intervals, since sexually appetitive behaviors and lordosis responses increased across tests, and a greater number of intromissions and ejaculations were elicited by the males, whereas 5µg EB increased LQ but not appetitive behaviors. Females treated with 2µg EB displayed few sexual behaviors overall. When tested at 8-day intervals, sensitization induced by 10µg EB was restricted to appetitive sexual behaviors, and those females received more intromissions and ejaculations by the male; whereas 5µg EB produced a relatively stable, low level of sexual behaviors.

Wistar OVX rats displayed a more robust pattern of sensitization compared to Long-Evans. In contrast to OVX Long-Evans females treated at 4-day intervals, OVX Wistars treated with 2µg EB exhibited sensitization of appetitive behaviors and LQ, and received more mounts and intromissions across tests. The frequency of appetitive behaviors appeared to be approximately double that of Long-Evans rats. Moreover, although Long-Evans rats appeared to display fewer appetitive behaviors following repeated EB-alone compared to EB+P as reported by others (Blaustein et al., 1987), sexual behaviors in sensitized Wistars treated with 10µg EB resemble those following EB+P. We have also observed this strain difference in females tested at weekly intervals in bilevel chambers (unpublished observations). This behavioral sensitization to 2µg EB appears to be specific to Wistar females, since Long-Evans did not sensitize to this dose, and others have also reported the absence of sensitization in both Long-Evans (Micevych et al.,

2008), and Sprague-Dawley females (Kow & Pfaff, 1975) to low doses of EB (<3μg). One possible explanation could relate to greater corticosterone in Long-Evans rats compared to Wistars (Tohei et al., 2003), since corticosterone inhibits sexual behavior (deCatanzaro, 1987; deCatanzaro, Knipping, & Gorzalka, 1981). Interestingly, others have reported that 10μg EB-alone failed to induce LQ or appetitive behaviors in another strain of albino rat, the Sprague-Dawley (Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994), following an even shorter hormone washout period (7 days). Long-Evans and Wistar rats may also use different reproductive strategies based on multi-sensorial vs. predominant olfactory processing, respectively, as proposed by Coria-Avila et al., (2006). This reiterates the importance of considering strain effects in research studies.

This study also determined that the sensitization induced by 10μg EB at 4-day intervals does not involve adrenal steroid hormone release (such as progestins). Although ADX did not prevent the sensitization of sexually appetitive behaviors when treated with 5μg EB, the effect was weak due to a high level of responding on the first test. Moreover, sensitization of LQ was not detected in those females, suggesting dose-dependent differences following ADX and chronic administration of EB, as proposed by Gorzalka and Moe (1994). However, ADX appeared to cause greater variability in responding, which is not surprising since adrenal P may be involved in the fine regulation of the timing of sexual behavior (Barfield & Lisk, 1974).

Has estradiol sensitized sexual behavior or facilitated associative learning?

One possible interpretation of these data is nested within the literature on learning and memory. Fluctuations in circulating levels of estradiol alter learning and memory strategies in female rats (Korol, Malin, Borden, Busby, & Couper-Leo, 2004), and increase synaptic spine density within the hippocampus (Woolley & McEwen, 1992), as well as the hypothalamus (Flanagan-Cato, 2000). As such, it is possible that the potentiation of sexual behaviors induced by EB is the result of enhanced conditioned responding. We attempted to remove confounding learning effects by providing sexual training prior to testing. As such, if behavioral sensitization to EB is highly dependent on learning, we would expect to see similar levels of responding on the first EB-only test following the 2-week hormone washout, or alternately at least some level of responding in the low (2μg) dose of EB, which was not seen in the Long-Evans. As such, the data support a potentiation of sexual behavior to the same dose of EB during the test phase beyond any role of associative learning in the Long-Evans strain. Whether the associative

learning plays a greater role in Wistars is unclear, since strain differences on learning and memory tasks have been reported, albeit often favoring the Long-Evans strain (e.g., Andrews, Jansen, Linders, Princen, & Broekkamp, 1995; Cain, Ko, Chalmers, & Ralph, 2004; Keeley, Wartman, Hausler, & Holahan, 2010; van Goethem et al., 2012). This question warrants a more thorough investigation.

Learning effects during the sexual training phase however, may account for differences reported in ADX/OVX animals across studies. Our data suggest that the sensitization of appetitive sexual behaviors by 10μg EB does not require the presence of adrenal progestin release since ADX animals also sensitized across tests. Although some studies have reported that ADX disrupts the expression of appetitive sexual behaviors in OVX females primed with EB+P or EB-alone compared to adrenal-intact controls (e.g., Erskine, 1985; Gorzalka & Moe, 1994; Tennent et al., 1980), our present findings are in agreement with others (e.g., Davidson et al., 1968a) in which animals were repeatedly tested. As such, a critical level of sexual experience may override any role that the adrenal may play. Although, Tennent et al. (1980) reported that adrenal hormones influenced the expression of appetitive behaviors in sexually-experienced ADX/OVX rats treated with EB-alone and EB+P compared to sham-ADX/OVX, the rats in that study only received two sexual experience tests. This raises the possibility that adrenal steroids may be important in the *learning* of normative sexual behavior in females. In fact, corticosterone is important in the formation of memories associated with other motivated behaviors, such as maternal behavior (Graham, Rees, Steiner, & Fleming, 2006). Unfortunately, inconsistencies in hormone priming regimens across studies make it difficult to draw solid conclusions. For example, in Tennent et al. (1980), daily hormone priming began the day following ADX, and testing occurred 7 days later. Our testing occurred five weeks post-surgery and included hormone washout periods (see Figure 1). Additionally, we replaced corticosterone through drinking water, which was not done in the Tennent et al. (1980) or Erskine (1985) studies. Thus, different behavioral baselines, hormone dose- and time-response regimens, sexual experience, and corticosterone levels likely explain differences in the expression of sexual behaviors of ADX/OVX animals across studies.

What role might a sensitized response to EB play in female reproductive behavior?

With no sexual stimulation, ovulation and sexual receptivity in gonadally intact female rats occur every 4-5 days (Freeman, 1994). With intromissions spaced at optimal intervals,

however, females can become pseudopregnant for approximately 12-14 days, after which they go back into heat (Erskine, Lehmann, Cameron, & Polston, 2004; Frye & Erskine, 1990). Solicitations by the female increase the probability of receipt of intromission (Erskine, 1989), and if intromissions are followed by ejaculation, females are likely to become pregnant and enter a state of lactational diestrus after parturition that would prevent them from going into heat for approximately six weeks. Vaginocervical stimulation (VCS) received during penile intromissions accelerates the onset of estrous termination (characterized by decreased appetitive behaviors and increased rejection responses prior to a reduction in lordosis), which occurs more readily if the intromissions are paced by the female (Erskine, 1985; Erskine et al., 1989; Erskine & Baum, 1982). The present data show that the interval of E2 administration matters greatly in the ability of females to express stable baselines of responding. Indeed, the ability of artificial VCS to induce estrous termination is eliminated if OVX rats are treated with EB and P at 4- or 7-day intervals, and reduced if females receive hormone administration at 14-day intervals (Pfaus et al., 2000). It was suggested that this mechanism of reduced sensitivity to the inhibitory actions of VCS keeps the female sexually active for a longer period of time, thereby increasing the probability of being impregnated. Together with the present data, we suggest that the EB-induced sensitization at 4-day intervals may well reflect a "failsafe" that makes females that did not copulate successfully during one periovulatory period far more likely to solicit and copulate successfully the next time they ovulate.

What neural mechanisms might underlie behavioral sensitization to EB?

The mechanisms underlying the sensitization of lordosis (an estradiol-dependent behavior), as well as sexually appetitive behaviors (that are known to be regulated by P after estradiol administration), by repeated administration of EB-alone are not well understood. Some of the effects on lordosis are likely to be modulated within in the ventromedial hypothalamus (VMH) by membrane-receptor mediated effects and genomic mechanisms (Caldwell, 2002; Flanagan-Cato, 2000; Kow & Pfaff, 2004), as well as epigenetic factors related to sexual differentiation of hypothalamic tissue and subsequent sensitivity to steroid hormones (Gagnidze & Pfaff, 2013; Gagnidze, Weil, & Pfaff, 2010), all of which are induced by EB following receptor binding. Another potential mechanism might involve P receptor (PR) activation.

The role of P in the facilitation of appetitive behaviors is apparent from studies showing that in EB-primed females, systemic P induces dose-dependent increases in appetitive behaviors

(Erskine, 1989; Tennent et al., 1980; Whalen, 1974), and its application directly to the VMH potentiates lordosis and induces appetitive behaviors (Rubin & Barfield, 1983). Furthermore, blocking PR using systemic injections of RU486 (Blaustein et al., 1987), or intracerebroventricular administration of antisense oligonucleotides to PR (Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994), decreases these behaviors in EB+P treated females. It is known that P acts on estrogen-induced PR in both the medial preoptic area (mPOA) to activate appetitive behaviors (Erskine, 1989), and the VMH to potentiate the expression of lordosis (Etgen & Barfield, 1986; Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994; Meisel, Dohanich, McEwen, & Pfaff, 1987; Ogawa, Olazábal, Parhar, & Pfaff, 1994; Rubin & Barfield, 1984). However, our data suggest that the sensitization of appetitive sexual behavior by 10µg EB does not require peripheral P release since ADX/OVX animals also sensitized across tests. Moreover, it has been argued that PR is not involved in the induction of sexual behavior by EB alone (Blaustein et al., 1987), since the PR antagonist RU486 administered at least 4 hours prior to testing does not block sexual behaviors induced by continuous EB (Blaustein et al., 1987), and PR knock-out mice display equivalent levels of lordosis as wild-type mice following EB priming (Mani, Blaustein, & O'Malley, 1997).

However, more recently it was shown that EB induces neuroprogesterone synthesis in hypothalamic tissue of both OVX and ADX/OVX females (Micevych et al., 2003), and although lordosis was not inhibited by RU486 (when treated with 10µg EB every 4 days, and injected with EB+RU486 or EB-alone one hour prior to the fourth test), proceptive behaviors were (Micevych et al., 2008). Therefore it is possible that neuroprogesterone (or some other ligand) activates PR early on to activate lordosis as well as appetitive behaviors at the time of testing in EB-sensitized females. In fact, some of the facilitative effects by dopamine (DA) agonists are known to be mediated through interactions with PR (Mani, Allen, Clark, Blaustein, & O'Malley, 1994). DA increases in the mPOA of female rats during copulation (Matuszewich, Lorrain, & Hull, 2000), and in EB-primed females infusions of DA type 2 receptor agonists facilitate solicitations (Graham & Pfaus, 2010), whereas in fully-primed females DA type 1 receptor activation facilitates solicitations (Graham & Pfaus, 2012). Thus, it is possible that chronic EB increases substances within the mPOA to activate DA receptors and/or PR, or alters DA receptor subpopulations, to facilitate sexual behaviors. Since EB also facilitates the release of DA within the striatum and nucleus accumbens during paced mating (Jenkins & Becker, 2003a;

Mermelstein & Becker, 1995) and increases the behavioral sensitization of locomotor activity and rotational behavior to psychomotor stimulants in female rats, (Forgie & Stewart, 1994; Hu & Becker, 2003; Zhao & Becker, 2010) it would not be surprising to find that DA within these regions is further potentiated in females sensitized to repeated EB.

Part of the underlying mechanisms might also reside in the bidirectional connections between the mPOA and VMH. The role of the VMH in the expression of sexual behavior may involve the inhibition of glutamate by GABA (Georgescu et al., 2009), and repeated EB might sensitize this inhibition. Interestingly, EB treatment increases GAD-65 mRNA in preoptic neurons (Mccarthy, 1995), and the VMH receives a large GABAergic projection from the mPOA (Georgescu, M., and Pfaus, J.G., in preparation). Infusions of $GABA_A$ agonists into the VMH facilitate lordosis (Mccarthy, 1995; McCarthy et al., 1990) whereas infusions of glutamate receptor agonists rapidly and dose-dependently inhibit lordosis and appetitive behaviors (Georgescu & Pfaus, 2006b; Kow et al., 1985; McCarthy et al., 1991). Similar behavioral effects occur following VCS, which accelerates the onset of estrous termination (Hardy & Debold, 1971a; Lodder & Zeilmaker, 1976; Pfaus et al., 2000), and induces Fos in glutamate neurons within the ventrolateral VMH (Georgescu et al., 2009). Since appetitive behaviors are the first to disappear as the female enters estrous termination, glutamatergic efferents might modulate activity within the mPOA. We have recently found that VCS selectively attenuates the sensitization of appetitive behaviors by repeated EB, and the attenuation is mimicked by repeated infusions of AMPA into the ventrolateral VMH (unpublished observations), which strongly supports a role for this system in the behavioral sensitization to repeated EB. Taken together, these findings show that EB plays a critical role in regulating neural activity to optimize reproductive success.

CHAPTER 2.2

Preface

In Chapter 2.1, it was established that the administration of 5 or 10µg EB administered every 4 days sensitizes sexual behaviors in the OVX rat. The next studies addressed hormonal (Chapter 2.2) and behavioral (Chapter 2.3) mechanisms contributing to this effect using the dose that induced the most robust sensitization (10µg EB).

Given that E2 and P act synergistically to activate female sexual behavior, Chapter 2.1 Experiment 2, tested whether behavioral sensitization to EB occurs in the OVX rat following ADX, and this manipulation did not prevent behavioral sensitization to EB, suggesting that adrenal P does not play a role. However, given that hypothalamic PR can be activated through other ligands such as dopamine (Mani, Allen, Clark, Blaustein, & O'Malley, 1994), or E2 induced nP (Kuo & Micevych, 2012; Micevych et al., 2008), and nP synthesis appears to play a role in the potentation of E2-facilitated sexual behaviors (Micevych et al., 2008), the next experiment examined whether chronic blockade of PR would affect the induction and maintenance of estradiol sensitization in the OVX rat.

2.2: RU486 facilitates or disrupts the sensitization of sexual behaviors by estradiol in the ovariectomized Long-Evans rat: Effect of timecourse

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Abstract

An acute injection of estradiol benzoate (EB) to the ovariectomized (OVX) rat activates low levels of lordosis, but subsequent progesterone (P) administration augments lordosis and recruits a complete pattern of sexual behavior including appetitive behaviors (e.g., hops/darts and solicitations). However, repeated injections of 5µg or 10µg EB (but not 2µg EB), administered every 4 days to sexually-experienced OVX rats results in progressively greater LQs and appetitive behaviors, a phenomenon called "estradiol sensitization". We have shown that adrenal P does not play a critical role in this phenomenon because estradiol sensitization is not prevented by adrenalectomy. Here we tested whether P receptors play a role by examining the effects of chronic administration of the P receptor antagonist RU486 at a dose that reliably inhibits sexual behavior in fully primed OVX rats. Females were treated with EB (5 or 10µg), and 5mg RU486 dissolved in 0.4mL vehicle (VEH; 80% sesame oil, 15% benzyl benzoate, 5% benzyl alcohol) 48 and 4 hours prior to each of 7 tests, respectively, occurring at 4-day intervals in unilevel 4-hole pacing chambers. Control animals were treated with 2, 5, or 10µg EB+VEH. As expected, sensitization did not occur in females treated with 2µg EB+VEH, and those females received fewer intromissions and ejaculations than all other groups. RU486 did not prevent the sensitization of LQ (lordosis quotient), lordosis magnitudes (LM2 and LM3) or appetitive sexual behaviors on early tests, and in fact potentiated hops/darts, LM2 and LM3, consistent with its facilitative actions in females treated with EB-alone, as we and others have reported previously. However, despite the initial facilitation, blocking P receptors by chronic administration of RU486 inhibited the maintenance of estradiol sensitization.

Key Words: Estradiol sensitization, sexual behavior, progesterone receptors, RU486

Introduction

Sexual behavior in ovariectomized (OVX) rats can be fully reinstated by administration of estradiol benzoate (EB) followed by progesterone (P), 48hrs and 4hrs prior to testing, respectively (Beach et al., 1942; Boling & Blandau, 1939). Whereas acute doses of EB dose-dependently increase lordosis, appetitive sexual behaviors such as hops, darts, solicitations, and ear wiggles are increased dose dependently by P following EB (Beach et al., 1942; Whalen, 1974). Although the acute administration of EB alone partially reinstates sexual behavior in OVX rats, it is recognized that they become more sensitive behaviorally to EB with repeated administration (Babcock et al., 1988; Beach & Orndoff, 1974; Blaustein et al., 1987; Clark & Roy, 1983; Davidson et al., 1968a; Gerall & Dunlap, 1973; Jones & Pfaus, 2014; Jones, Farrell, Gardner Gregory, & Pfaus, 2013; Kow & Pfaff, 1975; Parsons et al., 1979; Whalen & Nakayama, 1965). We recently characterized the development of this EB-induced sensitization in sexually-experienced OVX Long-Evans rats receiving 2, 5, or 10µg EB at 4- or 8-day intervals, and found that 2µg EB does not induce sensitization, and that the effect is most robust in those treated with 10µg EB every four days (Jones et al., 2013). Although the underlying mechanisms are not well understood, Parsons et al. (1979) determined that increased behavioral sensitivity to EB is not due to the accumulation of EB in plasma. They administered crystalline 17β-estradiol (E2) via subcutaneous capsules and examined sexual behavior for one week followed by their removal. Plasma levels of E2 fell to control levels within 12 hours but the females remained in heat five days after removal, compared to those not pre-treated with E2, indicating that E2 induced long-term changes in neuronal responsivity to subsequent E2 treatment.

Estradiol binding to receptors (ERs) in the ventromedial hypothalamus (VMH) promotes the display of lordosis in response to mounting by the male, and induces P receptor (PR) synthesis in a number of hypothalamic regions, including the VMH and the medial preoptic area (mPOA) (MacLusky & McEwen, 1978; Pfaff, 1980). Subsequent PR activation within the VMH potentiates lordosis, whereas the activation of PR within the mPOA stimulates appetitive sexual behaviors (Beyer et al., 1997; Glaser et al., 1983; Hoshina et al., 1994; Mani, Allen, Clark, Blaustein, & O'Malley, 1994a; Rubin & Barfield, 1983; Sakuma, 1994; 2008). Given the importance of PR in the display of sexual behavior, we examined whether adrenal P contributes to the sensitization of sexual behaviors by EB and found that sexually-experienced OVX rats that had also been adrenalectomized (ADX) and were treated with 5 or 10µg EB every four days

displayed behavioral sensitization (Jones et al., 2013). This suggests that adrenal P does not play a role, as previously shown with repeated daily injections of EB (Davidson et al., 1968a). This is also in agreement with studies showing that the PR antagonist RU486 (administered five hours prior to testing) fails to disrupt the potentiated lordosis response following repeated administration of EB, in estradiol-induced (continuous exposure to estradiol-alone by subcutaneous capsules), and estradiol-facilitated (EB followed 44 hours later by a bolus dose of estradiol administered in place of P, 4 hours prior to testing) paradigms (Blaustein et al., 1987; Micevych et al., 2008). Indeed, mice lacking P receptors (PR knockouts) display similar (albeit slightly lower) levels of lordosis in response to repeated administration of EB (Mani et al., 1996), but the hormonal regulation of sexual behavior may be somewhat different between mice and rats. However, those studies that failed to find an effect of RU486 on lordosis in rats administered it acutely just prior to testing and at least 44 hours after E2 was initially administered.

Although peripheral P is not involved in estradiol sensitization, a role for PR cannot be ruled out. First, PR can be activated by agents other than P, for example by second-messenger effects of neurotransmitters such as dopamine (Mani, Allen, Clark, Blaustein, & O'Malley, 1994a), or neuropeptides such as gonadotropin releasing hormone (Mani, Allen, Rettori, McCann, O'Malley, & Clark, 1994; Moss & McCann, 1973; Waring & Turgeon, 1992). PRs also have a low binding affinity for estradiol (MacLusky & McEwen, 1980; Parsons et al., 1984). Moreover, PR activation is involved in the short-term enhancement of lordosis by mating or vaginocervical stimulation (VCS), as this effect is blocked by RU486 (Auger et al., 1997). PRs may also be activated by neuroprogesterone, particularly with respect to appetitive sexual behaviors. Estradiol induces the production of neuroprogesterone in hypothalamic astrocytes (Micevych et al., 2003; Sinchak et al., 2003), and administration of RU486 disrupts the potentiation of hops, darts, and ear wiggles using an estradiol-facilitated paradigm in OVX-ADX animals (Micevych et al., 2008). This suggests that PRs play a role in the potentiation of appetitive sexual behaviors by repeated administration of EB. It is important to note that in studies that failed to find an effect of RU486 on the facilitation of lordosis, RU486 was administered once, days after the initial exposure to estradiol and after the animals had sensitized. As such, it is unclear whether repeated blockade of PR beginning at the onset of the sensitization

paradigm would interfere with the development of the sensitization of sexual behaviors by 5µg or 10µg EB repeatedly administered SC at 4-day intervals.

The goal of this study was to examine whether chronic administration of RU486, administered at a dosing regimen that is known to block P-activated sexual behaviors, would interfere with the sensitization of sexual behaviors in the OVX, Long-Evans rat treated with 5 or 10µg EB at 4-day intervals. We first confirmed that RU486 inhibits the P facilitation of sexual behavior (Experiment 1) by treating sexually experienced EB-primed OVX Long-Evans rats with either RU486 or vehicle one hour prior to P administration. In Experiment 2, we investigated whether chronic blockade of PR in sexually experienced OVX Long-Evans rats treated with 5µg or 10µg EB every 4 days would disrupt the development of the sensitization of sexual behaviors by chronic administration of EB.

Materials and methods

Animals

Long-Evans rats were purchased from Charles River Canada (St-Constant, QC). Females were pair-housed in shoebox cages lined with a mixture of betachip and corncob bedding, and males were housed in groups of four lined with betachip. All animals had standard laboratory chow (Charles River #5075) and tap water freely available, and were kept in the same colony room which was maintained at 21°C, on a 12-hour reverse day-night cycle (lights off at 8:00 AM). Animals were given one week to acclimate to the animal care facility. All the rats were treated in accordance with the guidelines of the Canadian Council on Animal Care and approval for all experimental procedures was granted by the Concordia University Animal Ethics Committee.

Ovariectomy

Bilateral ovariectomies (OVX) were performed under a 4:3 mixture of ketamine hydrochloride (50 mg/ml; Ketaset©, Wyeth Canada) and xylazine hydrochloride (4mg/ml; Rompum ©, Bayer Healthcare) injected intraperitoneal (1ml/kg body weight), via a single lumbar incision, and identified by ear punch. Polysporin© was applied to the incision site, and post-operative care included 2.5 mg/kg of *Flunixin meglumine* (Banamine©), 0.1 mL/rat of benzylpenicillin (Penicillin G), and 2 mL/rat of 0.9% saline, followed by a one-week post-operative recovery prior to sex training.

Steroid hormones and RU486

EB (10 μg) and P (500 μg) were dissolved in 0.1 mL reagent grade sesame oil. EB doses (2 μg and 5 μg) were always diluted down from 10 μg EB solution, and administered 48 hours prior to testing. Progesterone was administered 4 hours prior to testing. RU486 (mifepristone; 17β-hydroxy-11β-[4-dimethylaminophenyl]-17α-[l-propynyl]-estra-4,9-dien-3-one; 5 mg/0.4 mL; Sigma-Aldrich) was dissolved in 80% reagent grade sesame oil, 15% benzyl benzoate and 5% benzyl alcohol (Blaustein et al., 1987; as in Pleim, Cailliau, Weinstein, Etgen, & Barfield, 1990), 15 hours prior to use, to ensure adequate dissolution. Preparation of this dose of RU486 has been demonstrated to attenuate female sexual behavior when given before a P injection (Blaustein et al., 1987; Brown & Blaustein, 1984). All treatments were administered SC. Throughout the course of the experiment, animals developed subdermal lumps or scabs at the injection sites (regardless of treatment condition); therefore, injection sites were alternated in quadrants to reduce irritation. All animals were closely monitored and scabs were treated with iodine and polysporin as needed. Following any signs of distress or impaired general health animals were immediately removed from the experiment (n=2). Additional details regarding chronic administration of this vehicle solution are reported elsewhere (Jones, S.L., Gardner Gregory, J., Pfaus, J.G., *in preparation*).

Sexual behavior training and testing

Behavioral training and testing were carried out during the middle-third of the dark cycle, in unilevel pacing chambers (38x60x38cm) lined with betachip and bisected by clear Plexiglas® divider with 4 square holes cut into the bottom. The holes were adjusted to allow passage of only the smaller female, restricting the male to one compartment. Males were first placed on one side of the chamber, and allowed a 5-minute habituation period prior to introduction of the female to the empty compartment. All training and test sessions were 30 minutes in duration, were video-recorded using a Sony Handycam Digital camera and subsequently scored using a personal computer and the Behavioral Observation Program customized for sexual behavior in the pacing chamber (Cabilio, 1996) with the experimenter blind to treatment condition.

Experiment 1. RU486 inhibits sexual behavior in females primed fully with EB+P. Sexually experienced females (N=19) used as stimulus animals in unrelated studies in our lab, were primed with 10 μg EB 48 hours, and 500 μg P 4 hours prior to testing. To verify that in our hands RU486 inhibits sexual behavior, half the animals were given RU486 (n=9) or the vehicle

(n=9) 1 hour prior to P. This pattern of RU486 administration has previously been shown to attenuate sexual behavior (Blaustein et al., 1987; Brown, Moore, & Blaustein, 1987).

Experiment 2. Effects of chronic administration of RU486 on EB-sensitization of sexual behavior. Beginning one week post-surgery, females (N=52) were given sexual experience by s.c injections of 10µg EB 48 hours, and 500µg P 4 hours prior to each of four training sessions which occurred at 4-day intervals. Following the 2-week hormonal washout period animals were assigned to one of 5 treatment conditions: 2µg EB+Vehicle (n=8), 5µg EB+Vehicle (n=10), 10µg EB+Vehicle (n=10), 5µg EB+RU486 (n=12), or 10µg EB+RU486 (n=12). The females then received 7 sexual test sessions.

Behavioral measures

The frequency of lordosis magnitudes (LM) was scored on a 3-point scale according to Hardy and Debold (1971b)and the lordosis quotient (LQ) was calculated as a ratio of the number of LM to the number of mounts (including intromissions and ejaculations), multiplied by 100. In Experiment 1, the lordosis rating (total number of points / total number of lordosis postures) was also calculated to compare the effects of RU486 on this measure as reported in the literature. Measures of sexually appetitive behaviors included hops/darts and solicitations, defined as a headwise orientation towards the male, followed by a runaway (Erskine, 1989; McClintock, 1984; Pfaus et al., 1999). Defensive behaviors included kicks, sideways takedowns, boxing postures, and prone positions, as in Barnett (Barnett, 1963). Mounts, intromissions and ejaculation by the male were also analyzed, since their behavior provides insight into the female's sexual receptivity (Pfaus et al., 1999).

Statistical analyses

The results of Experiment 1 were analyzed using independent samples t-tests to investigate whether RU486 disrupts sexual behavior in EB+P primed females. Data from Experiment 2 were analyzed using a (5 groups x 7 tests) mixed ANOVA with group as a between subjects factor and test as a within subjects factor. Significant interactions were investigated using simple main effect analyses. Tukey's post-hoc was used to investigate differences between groups and Sidak-Bonferroni correction was used to investigate differences between tests. Linear trend analyses were also examined (as in Jones et al., 2013), such that a linear increase was interpreted as behavioral sensitization, whereas a quadratic trend on an inverted u-shaped curve was interpreted as sensitization followed by inhibition. In the event of

significant main effects and an interaction, only the interaction is reported. The level of statistical significance for all comparisons was set at $p<0.05$. Eta squared (η^2) and *Cohen's d* are reported as measures of effect size. Data are presented as mean (SEM).

Results

Experiment 1. RU486 inhibits sexual behavior in females primed fully with EB+P.

Two females in the RU486 group were not mounted by the male, and were excluded from all analyses of lordosis behavior. In accordance with other reports, RU486 significantly interfered with the normal display sexual behavior in the OVX rat treated with EB+P. As shown in Figure 1 animals who received RU486 1hr prior to P administration displayed a lower LQ, $t(14)=3.48$, $p<0.001$, $d=1.80$, and a lower lordosis rating, $t(14)=2.19$, $p=0.046$, $d=1.11$.

Treatment with RU486 also resulted in significantly fewer hops/darts, $t(16)=4.587$, $p<0.001$, $d=2.16$ (Figure 2A) and although it tended to decrease solicitations (Figure 2B), this difference did not meet statistical significance, $t(16)=1.84$, $p<0.084$, $d=2.22$, which may be due to lack of power given the large effect size. Females treated with RU486 also received fewer intromissions, $t(16)=2.30$, $p=0.035$, $d=7.11$, and ejaculations, $t(16)=3.96$, $p<0.001$, $d=1.55$, from the male (Figure 2D). There were no significant differences in the number of LMs (Figure 1C), defensive behaviors (Figure 2C) or mounts (Figure 2D) between the RU486 and vehicle conditions.

Experiment 2. Effects of chronic administration of RU486 on EB-sensitization of sexual behavior

One female treated with 2µg EB displayed an abnormally high level of appetitive behaviors on the first test day suggesting abnormal sensitivity to EB ($Z=3.96$), and was excluded from all analyses. Two females were removed from the study over the course of the experiment due to signs of illness (one each from 5µg EB+RU, and 10µg EB+RU). On Test 7, the holes of the barrier were too small for one female in the 10µg EB + VEH group to cross, and her missing data were replaced by her group mean on that day.

Lordosis Quotient. No female treated with 2µg EB was mounted on all tests, indicative of a lack of sensitization as previously reported (Jones et al., 2013), and thus repeated measures and trend analyses could not be conducted. Seven females in the 5µg EB+VEH, 4 in the 5µg EB+RU486, and 3 in the 10µg EB+RU group were not consistently mounted across tests, and were excluded from the repeated measures analyses of LQ data.

Overall, LQ (Figure 3A) was higher on tests 2-6 compared to the first test, but following a decline, on test 7 LQ was no different from the first test, reflected in a significant main effect of test, $F(6,144)=8.02$, $p<0.001$, $\eta^2=0.22$, and significant linear and quadratic trends, $F_{\text{linear}}(1,24)=11.53$, $p=0.002$, $\eta^2=0.30$; $F_{quadratic}(1,24)=21.76$, $p<0.001$, $\eta^2=0.43$. In addition, 2µg EB+VEH elicited a lower LQ compared to all other groups (main effect of group, $F(3,24)=10.20$, $p<0.001$, $\eta^2=0.56$). The interaction was not significant.

LM1. More LM1 (Figure 3B) were observed in females treated with 10ugEB+VEH compared to 2µg EB+VEH and 5µg EB+VEH (main effect of group, $F(4,44)=4.09$, $p=0.007$, $\eta^2=0.27$). Although there was a significant main effect of test, ($F(6, 264)=2.62$, $p=0.018$, $\eta^2=0.05$) Sidak's post-hoc failed to detect significant differences; however there was a significant quadratic trend, $F(1,44)=8.84$, $p=0.005$, $\eta^2=0.16$, suggesting that LM1 initially increased across tests followed by an eventual decline. The group by test interaction was not statistically significant.

LM2. There were no group differences in the display of LM2 on the first test, as expected, yet their expression across tests differed between groups (significant interaction, $F(24, 264)=2.16$, $p=0.002$, $\eta^2=0.13$), as shown in Figure 3C. The frequency of LM2 did not change across tests in 2µg EB+VEH or 5µg EB+VEH groups; however in females treated with 10µg EB+VEH, LM2 was higher on tests 3 ($p=0.001$, $d=1.45$) and 4 ($p=0.005$, $d=1.78$) compared to the first test, and on Test 3 they displayed significantly more LM2 than those females treated with 2µg EB+VEH ($p=0.005$, $d=9.97$) or 5µg EB+VEH ($p=0.029$, $d=7.60$). That increase was followed by a decline on test 5 such that the frequency was no different from the first test and no differences were detected between any of the groups. This was followed by another increase on tests 6 ($p=0.001$, $d=1.07$) and 7 ($p<0.001$, $d=1.82$) compared to the first test, where they displayed more LM2 on test 6 compared to 2µg EB+VEH ($p=0.011$, $d=1.47$), and more on Test 7 compared to 2ugEB+VEH ($p=0.004$, $d=1.43$) and 5µg EB+VEH ($p=0.006$, $d=1.31$). The test effects are

reflected in significant linear ($F(1,9)=5.37$, $p=0.046$, $\eta^2=0.37$), quadratic ($F(1,9)=6.91$, $p=0.027$, $\eta^2=0.43$) and cubic trends ($F(1,9)=6.58$, $p=0.030$, $\eta^2=0.42$) in the 10µg EB+VEH group. Treatment with RU486 initially facilitated LM2s but later led to their inhibition (Figure 3C). When given in combination with 5µg EB there was a significant increase in LM2 on tests on test 2-5 compared to the first test (all $p<0.02$), and on Test 3 females treated with 5µg EB+RU486 displayed more LM2 compared to 2µg EB+VEH ($p=0.011$, $d=2.34$). However this facilitation by RU declined on the last two tests to levels equivalent to the first test, as reflected in the significant quadratic trend, $F(1,10)=28.97$, $p<0.001$, $\eta^2=0.78$, and a lack of group differences on any subsequent test. When RU486 was given in combination with 10µg EB, there was also a significant increase such that they displayed more LM2 on test 2 compared to those

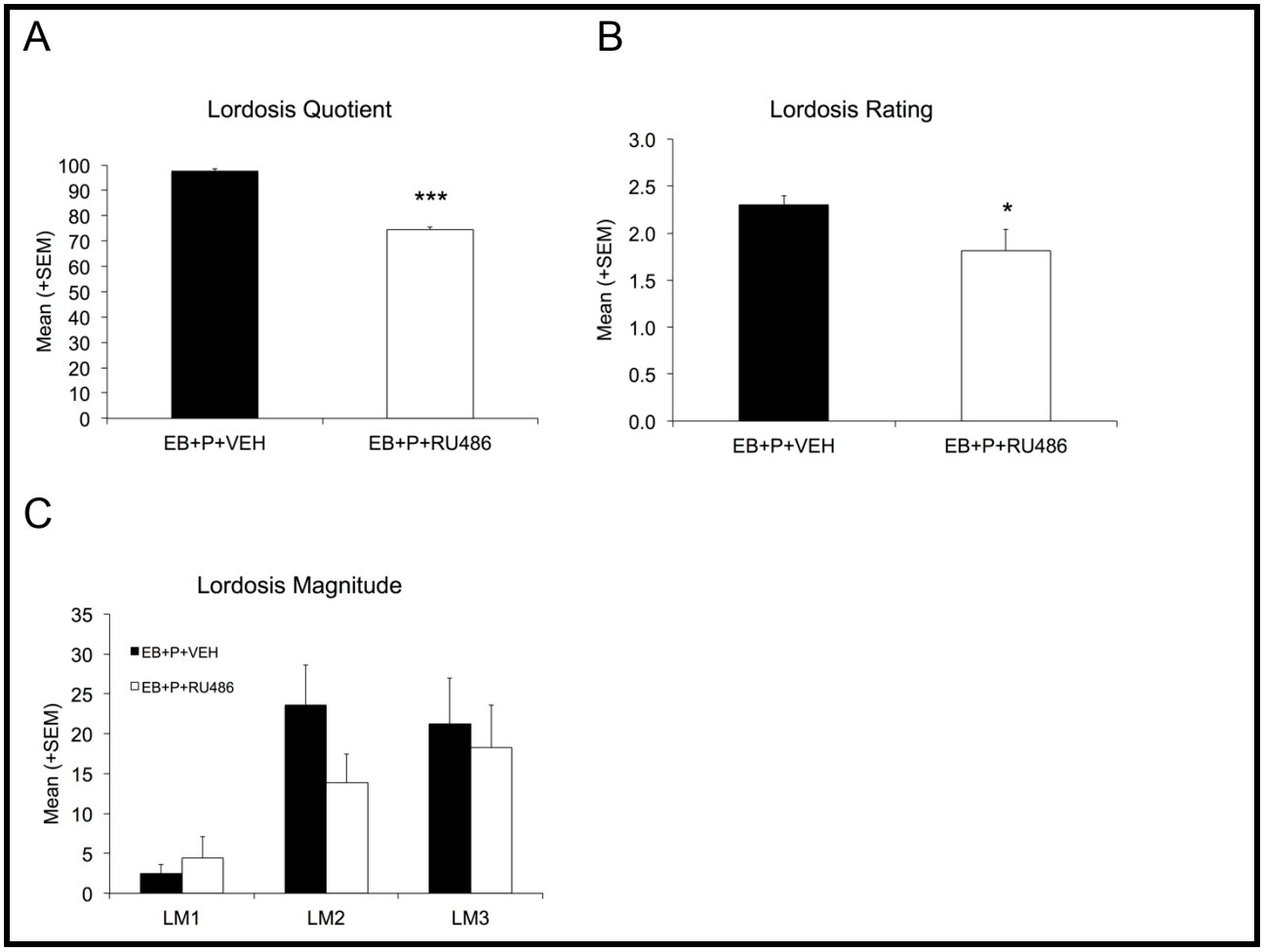

Figure 1. Lordosis measures in ovariectomized rats fully primed with estradiol benzoate (EB) and progesterone (P), and treated with either the progesterone receptor blocker RU486 or vehicle (VEH) one hour prior to P administration. *$p<0.05$, ***$p<0.001$.

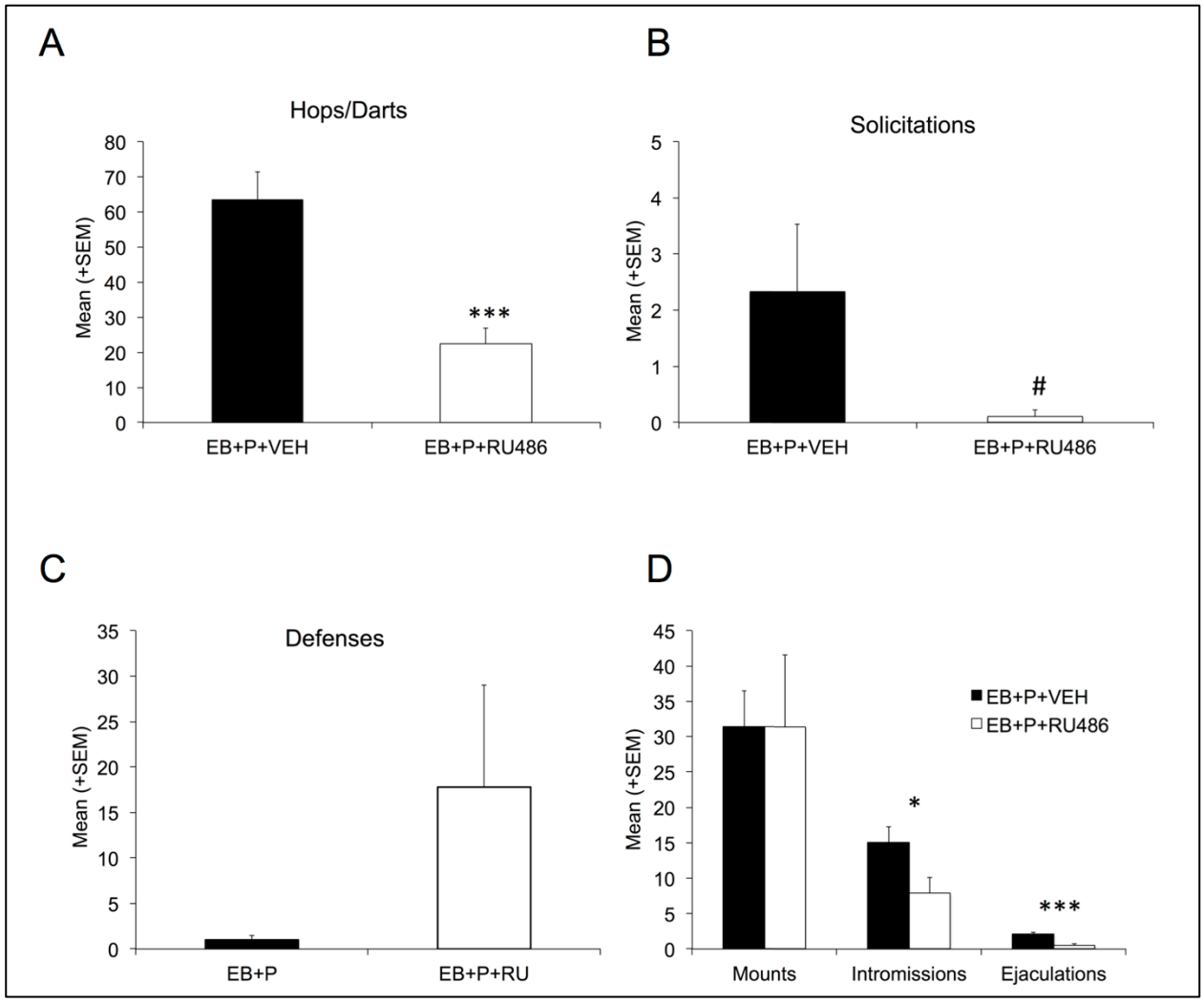

Figure 2. Hops/darts (A), solicitations (B), defensive behaviors (C) and copulatory stimulation received from the male (D) in ovariectomized rats fully primed with estradiol benzoate (EB) and progesterone (P), and treated with either the progesterone receptor blocker RU486 or vehicle (VEH) one hour prior to P administration. *$p<0.05$, ***$p<0.001$, $^{\#}p<0.10$.

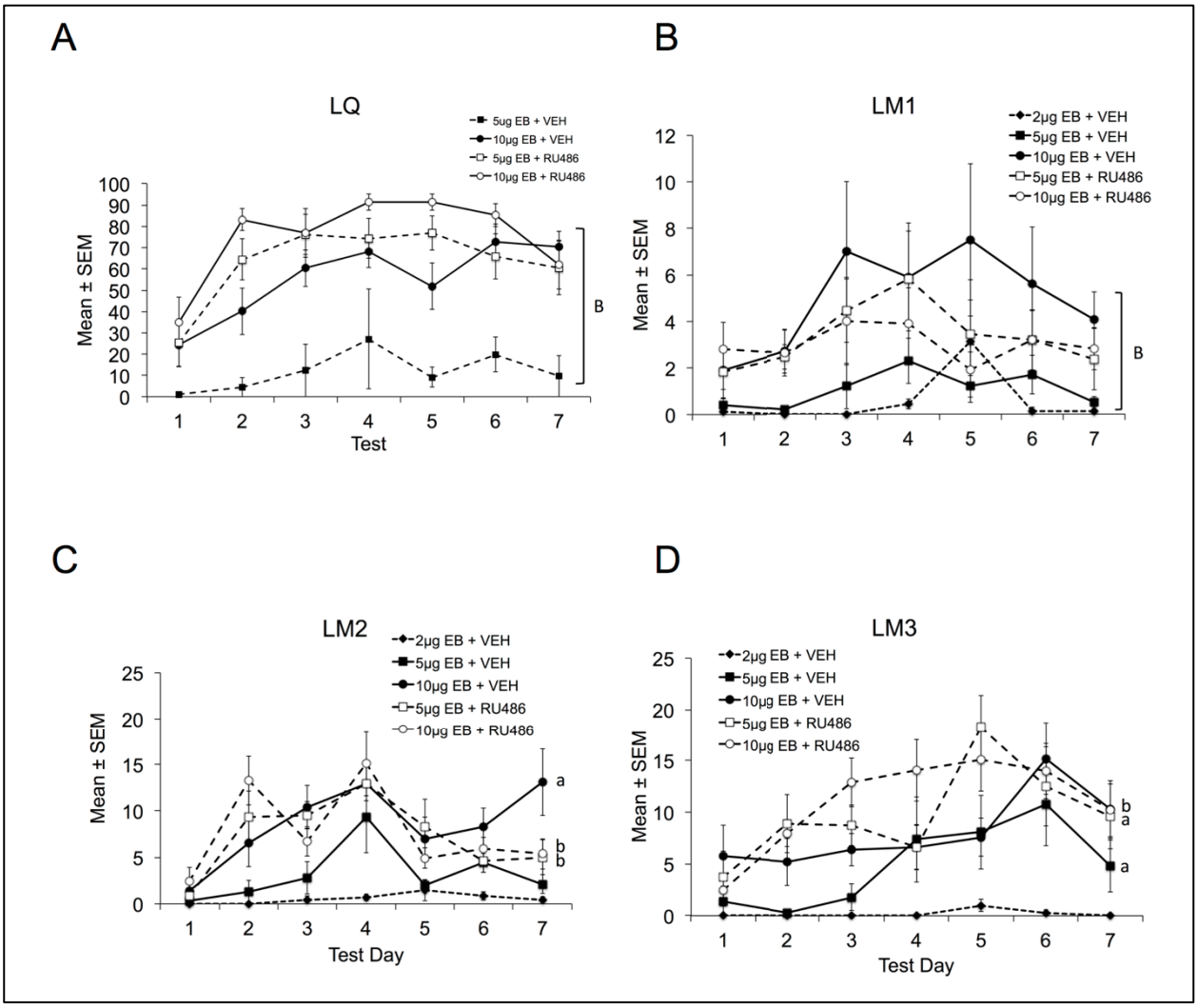

Figure 3. Lordosis measures in ovariectomized rats treated repeatedly with varying doses of EB. Blocking progesterone receptors with RU486 did not prevent the sensitization of any of the measures; however with chronic administration, it led to an inhibition of LM2 in females treated with 5µg and 10µg EB, and an inhibition of LM3 in females treated with 10µg EB. Note that 10µg EB VEH, there was an initial increase in LM2, followed by a decrease and a subsequent increase, reflected in a significant cubic trend, as such only the linear trend is indicated in the figure. A: sensitization as supported by a significant linear trend B: sensitization followed by inhibition, as supported by a significant quadratic trend. Main effects are displayed in capitalized letters, whereas sensitization or inhibition within specific groups following a significant interaction is displayed in minuscule letters. See text for specific test and group effects.

treated with 2µg EB+VEH (p=0.004, d=1.92) and 5µg EB+VEH (p=0.005, d=1.74). The greatest number of LM2 occurred on Test 4, which was greater than that displayed on the first test (p=0.001, d=1.96), and the difference between 10µg EB+RU486 and 2µg EB+VEH was maintained on Test 4 (p=0.013, d=1.58). However this increase was followed by a significant decline on tests 5, 6, and 7, such that there were no longer any group differences, as reflected in the significant quadratic trend, $F(1,10)=7.96$, p=0.018, η^2=0.44.

LM3. There were no differences in the display of LM3 on the first test, as expected, yet their expression across tests differed between groups (significant interaction, $F(24,264)=1.59$, p=0.043, η^2=0.11) as shown in Figure 3D. The display of LM3 did not change in females treated with 2µg EB+VEH, or 10µg EB+VEH (neither the main effects of test, nor the linear trends were significant); however a slight increase occurred in females treated with 5µg EB+VEH on Test 6 compared to Test 2 (p=0.034, d=1.06), which was also reflected in the significant linear trend ($F(1,9)=5.54$, p=0.043, η^2=0.38; note that the quadratic trend was not significant).

RU486 facilitated the display of LM3 (Figure 3D), such that females treated with 5µg EB+RU486 displayed more LM3 on Test 2 compared to 2µg EB+VEH (p=0.031, d=0.58) and 5µg EB+VEH (p=0.017, d=0.57) and on Test 3 compared to 2µg EB+VEH (p=0.025, d=1.70). By Test 5 the frequency was still greater than those treated with 2µg EB+VEH (p=0.002, d=2.16), and they were displaying more LM3 compared to test 1 (p<0.001, d=1.46) and 4 (p=0.004, d=1.23), and tended to display more on Test 5 compared to tests 2 (p=0.055, d=0.96) and 3 (p=0.053, d=0.96), as reflected in a significant linear trend, $F(1,10)=8.64$, p=0.015, η^2=0.464. However, the frequency began to decline on tests 6 and 7, such that there were no longer any group or test differences, but the quadratic trend was not significant.

RU486 also initially facilitated but later inhibited the display of LM3 in females treated with 10µg EB. They displayed more LM3 compared to 5µg EB+VEH (p=0.045, d=2.40) on Test 2, and more than 2µg EB+VEH (p<0.001, d=2.07) and 5µg EB+VEH (p<0.001, d=1.75) treated animals on Test 3. Moreover, they displayed more LM3 on Tests 3-6 compared to Test 1 (all p<0.02), and on Tests 4 and they were still displaying more LM3 than 2µg EB+VEH (p=0.019, d=1.80). However this facilitation was no longer apparent on Tests 6 and 7 as no differences were found between groups, and the frequency on Test 7 was no different than any other test.

This initial facilitatory effect followed by inhibition is also reflected in the significant quadratic trend, $F(1,10)=14.23$, $p=0.004$, $\eta^2=0.59$.

Hops/darts. As shown in Figure 4A, no group differences were detected on the first or second tests; however the groups differed in the expression of hops/darts throughout the course of the experiment (significant interaction, $F(24, 264)=1.66$, $p=0.031$, $\eta^2=0.120$). In contrast to our previous reports, HD did not change across tests in any of the vehicle treated animals, which was also reflected in the lack of significant linear or quadratic trends. This lack of effect may be due to a higher number of HD on the initial tests, compared to that observed in our prior studies (see Jones et al., 2013; Jones & Pfaus, 2014).

Group differences began to emerge on Test 3, as a result of facilitation by RU486, such that 5 and 10µg EB given in combination with RU486 resulted in significantly more HD compared to females treated with 2µg EB+VEH ($p=0.002$, $d=1.56$; $p=0.004$, $d=1.85$, respectively) or 5µg EB+VEH ($p=0.002$, $d=1.54$; $p=0.003$, $d=1.68$). On Test 4, females treated with 5µg EB+RU486 displayed more HD than 2µg EB+VEH animals ($p=0.031$, $d=2.26$), and the increase reached statistical significance on test 5 compared to Test 1 ($p<0.001$, $d=1.43$) when they displayed more HD compared to all EB treated vehicle groups (2µg EB+VEH: $p=0.001$, $d=2.02$; 5µg EB+VEH: $p=0.004$, $d=1.44$; 10µg EB+VEH $p=0.041$, $d=1.24$). However this increase was followed by a gradual decline on tests 6 and 7 ($p=0.006$, $d=1.18$; $p=0.008$, $d=1.66$, respectively), compared to test 5, such that their expression was no different from tests 1-4. The sensitization followed by inhibition is also supported by a significant quadratic trend, $F(1,10)=24.83$, $p=0.001$, $\eta^2=0.26$.

A similar pattern emerged with 10µg EB+RU, such that more HD were displayed on tests 4 ($p=0.018$, $d=1.08$) and 5 ($p=0.031$, $d=1.02$) compared to the first test and compared to 2µg EB+VEH ($p=0.006$, $d=1.48$ on test 4, $p=0.013$, $d=1.81$ on test 5); however following a peak on test 4, a gradual decline in HD was observed such that the drop in the number of HD on tests 6 and 7 were no different from any other test day, and were no different from any other group. The initial sensitization by 10µg EB+RU486 followed by inhibition is reflected in a significant quadratic trend, $F(1,10)=17.31$, $p=0.002$, $\eta^2=0.17$.

Solicitations. As shown in Figure 4B, sexual solicitations were higher on tests 2-6 compared to test 1, (main effect of test: $F(6, 264)=4.66$, $p<0.001$, $\eta^2=0.09$) and the significant linear ($F(1,44)=4.11$, $p=0.049$, $\eta^2=0.08$) and quadratic trends ($F(1,44)=24.73$, $p<0.001$ $\eta^2=0.30$),

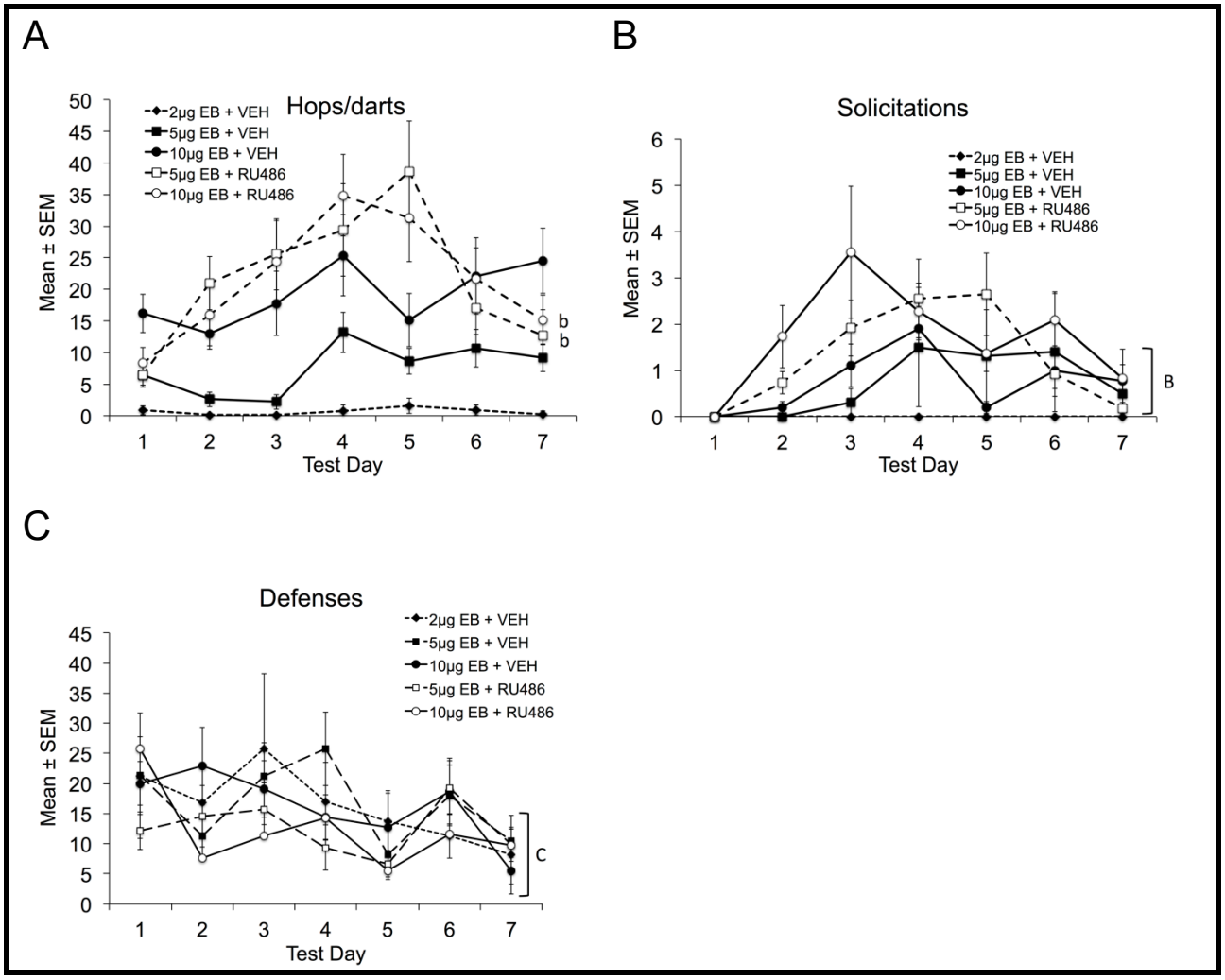

Figure 4. Hops/darts, active solicitations, and defensive behaviors in ovariectomized rats treated repeatedly with varying doses of EB. RU486 did not prevent the sensitization of appetitive measures of sexual behavior, yet its chronic administration led to an inhibition of hops/darts in females treated with 5µg and 10µg EB on later test days. Defensive behaviors declined across tests regardless of hormone treatment. A: sensitization as supported by a significant linear trend B: sensitization followed by inhibition, as supported by a significant quadratic trend. C: significant decline across tests. Main effects are displayed in capitalized letters, whereas sensitization or inhibition within specific groups following a significant interaction is displayed in minuscule letters. See text for specific test and group effects.

suggest that following the increase across tests, they declined on the final test day. A facilitation by RU486 was detected such in females treated with 5µg EB, RU486 induced significantly more solicitations than those treated with 2µg EB+VEH (p=0.004, d=1.07), whereas females treated with 10µg EB+RU486 displayed more solicitations than all vehicle treated females (2µg EB/VEH: p<0.001, d=0.93; 5µg EB/VEH: p=0.013, d=0.46; 10µg EB/VEH: p=0.016, d=0.53) (main effect of group, F(4, 44)=4.84, p=0.003, η^2=0.30). The test by group interaction was not significant.

Defensive Behaviors. Defensive behaviors declined across tests, as shown in Figure 4C, such that fewer defensive behaviors were displayed on test 5 (p=0.002, d=1.02) and 7 (p<0.001, d=1.40) compared to test 1, and on test 7 compared to test 3 (p=0.002, d=0.97) (main effect of test, F(6, 264)=4.32, p<0.001, η^2=0.082; F_{linear}(1,44)=16.80, p<0.001, η^2=0.256). The group effect and interaction were not statistically significant.

Male behaviors. The number of mounts received initially increased across tests, as shown in Figure 5A, such that significantly more mounts were received on test 4 compared to test 1, before declining on later tests, as reflected in a significant main effect of test (F(6, 264)=2.91, p=0.009, η^2=0.05), and significant quadratic trend, F(1,44)=8.88, p=0.121, η^2=0.16. The group effect and interaction were not significant.

The number of intromissions (Figure 5B) increased across tests (F(6,264)=9.01, p<0.001, η^2=0.15), reflected in statistically significant linear trend, F_{linear}(1,44)=15.13, p<0.001, η^2=0.24. Fewer intromissions were taken on test 1 compared to all other tests (all p<0.001); and despite a significant quadratic trend, $F_{quadratic}$(1,44)=40.68, p<0.001, η_p^2=0.40, no other test differences were detected. Overall, 2µg and 5µg EB+VEH received fewer intromissions than all other groups (all p<0.01), (main effect of group: F(4,44)=18.87, p<0.001, η^2=0.63). The interaction effect was not significant.

More ejaculations (Figure 5C) were received on tests 2-6 compared to the first test, followed by a decline on test 7 such that the number of ejaculations was equivalent to test 1, as reflected in the main effect of test, F(6,264)=5.68, p<0.001, η^2=0.10, and a significant quadratic trend, F(1,44)=29.49, p<0.001, η^2=0.32. Finally, fewer ejaculations were received overall by females treated with 2µg or 5µg EB+VEH compared to all other groups (all p<0.001) (main effect of group, F(1,44)=17.814, p<0.001, η^2=0.62). The interaction was not significant.

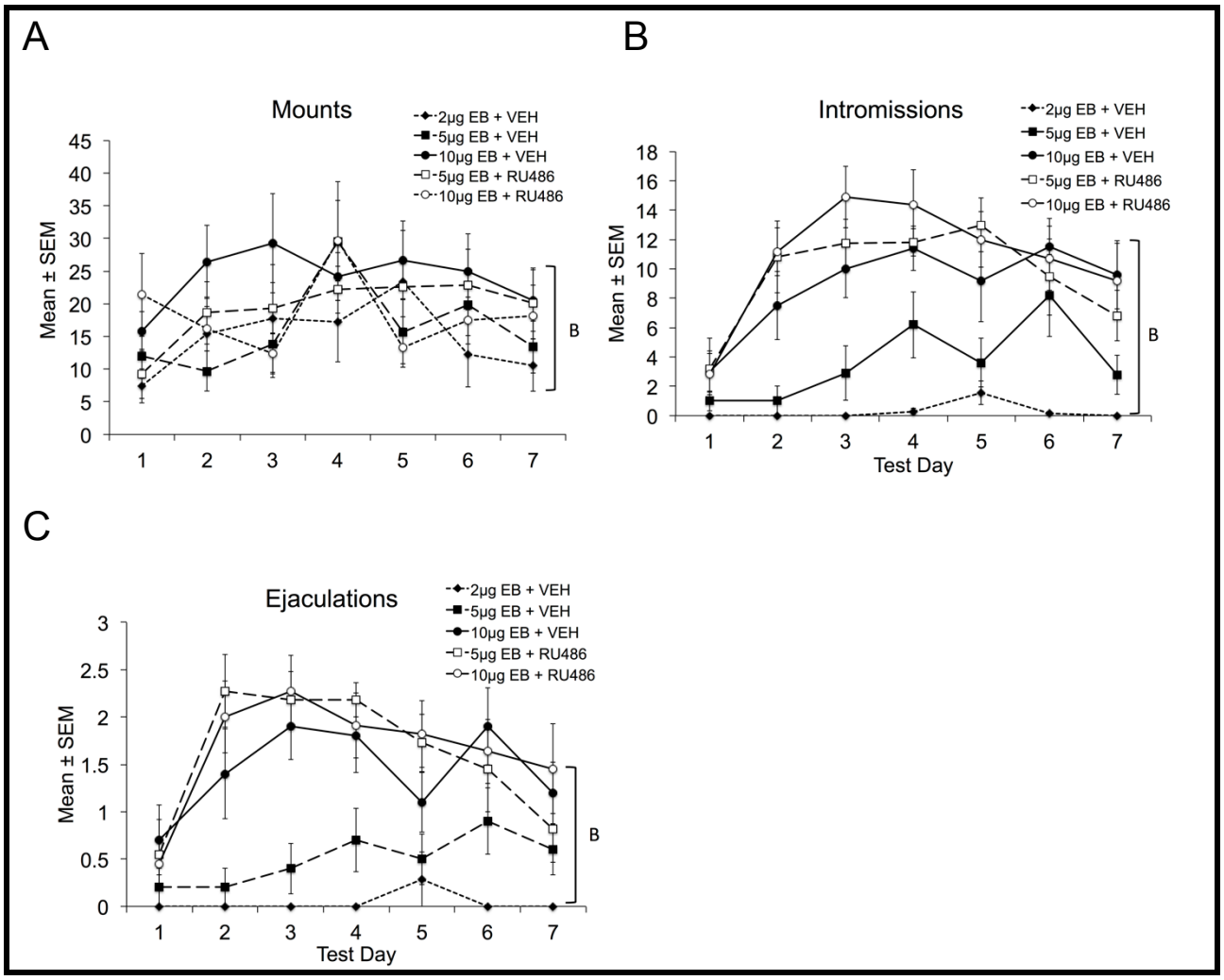

Figure 5. Mounts, intromissions and ejaculations received from males, in ovariectomized rats treated repeatedly with varying doses of EB. RU486 did not prevent the increase in the receipt of sexual stimulations from the male. B: sensitization followed by inhibition, as supported by a significant quadratic trend.

Discussion

The goal of this study was to examine whether repeatedly blocking PR by administration of RU486 just prior to testing would prevent the sensitization of sexual behaviors in the Long-Evans OVX rat treated with 5µg or 10µg EB every four days. Behavioral sensitization to EB occurred in both groups treated with RU486, and had no effect on sexual stimulations received from the male, or the occurrence of defensive behaviors. However hops/darts, LM2 and LM3 were attenuated following its chronic administration. These results suggest that PR activation is required to maintain estradiol sensitization of certain aspects of sexual behavior.

We first established that 5mg RU486 inhibited female sexual behaviors when administered one hour prior to P in EB-treated females, replicating an effect that has been well documented in the literature. However, we note that although RU486 was effective at inhibiting hops/darts, a common measure of appetitive sexual behaviors in the literature, it did not attenuate full solicitations significantly. This is important because it indicates that the mechanism that induces full sexual solicitations is different from that which induces hops/darts (as suggested by Erskine, 1989).

We also established that PR activation is involved in the maintenance of the sensitization of appetitive sexual behaviors by repeated estradiol treatment. This finding extends a report showing that PRs are involved in the display of appetitive sexual behaviors using an E2 facilitated paradigm. Micevych et al. (2008) reported that following repeated administration of E2 (i.e., after the animals had sensitized), an acute injection of RU486 inhibited the facilitation of sexually appetitive behaviors (hops, darts, ear wiggles), an effect that was dependent on estradiol-induced neuroprogesterone synthesis. Thus, our findings are in agreement with those of Micevych et al. (2008) since appetitive sexual behaviors were inhibited by RU486 but only on later tests, after sensitization had occurred. In fact RU486 initially facilitated the sensitization of appetitive behaviors, particularly hops/darts. Together the findings suggest that the initial potentiation may not depend upon PR activation, but that PRs are important in the maintenance of the behavioral sensitization to EB.

The effect of RU486 on the expression of lordosis following administration of EB alone is partly in agreement with previous reports. RU486 failed to block LQ in estradiol-induced (chronic administration of E2 by SC capsules) (Blaustein et al., 1987) and estradiol-facilitated

(E2 given in place of P) (Blaustein et al., 1987; Micevych et al., 2008) paradigms. Our findings parallel those reports, since we did not find a specific effect of RU486 on the frequency of lordosis displayed in response to a mount (LQ) in our paradigm. However, we found that the *quality* of lordosis was affected, particularly the frequency of the two higher magnitudes. In both groups treated with RU486, LM2 and LM3 initially sensitized yet they declined on later tests, suggesting that PR are also involved in the maintenance of the quality of lordosis displayed. These findings illustrate the importance of considering not only LQ, but also the frequency of lordosis magnitudes displayed in the study of female sexual behavior.

There was no effect of PR blockade on the display of defensive behaviors when females were treated acutely with EB+P (Experiment 1), or repeatedly with EB-alone (Experiment 2), suggesting that PRs are not directly involved in their expression. In fact, defensive behaviors appear to be inversely related to estrogen receptor activation in the VMH (e.g., Anchan, Gafur, Sano, Ogawa, & Vasudevan, 2014), possibly through descending pathways to the periaqueductal grey that inhibit defensive behaviors while permitting lordosis (Pfaff, Kow, Loose, & Flanagan-Cato, 2008).

Although we have shown that PRs are important in the maintenance of estradiol sensitization, we cannot yet rule out a role for PR in the induction of sensitization, because it is possible that neuroprogesterone (or some other ligand) had activated PR prior to RU486 administration. This is a possibility, since lordosis is induced as early as 16-24hrs following E2 administration (Green et al., 1970; McEwen et al., 1975), and some of the facilitative effects of subsequent E2 treatment occur rapidly via membrane-bound receptors (Kow & Pfaff, 2004). Moreover, E2 upregulates PR mRNA within six hours in vitro (Lee & Górski, 1996), and induces PR mRNA and protein in hypothalamic sites, particularly the VMH, within 16-24 hours (Moguilewsky & Raynaud, 1979; Parsons, Maclusky, Krey, Pfaff, & Mcewen, 1980; Parsons, Rainbow, Pfaff, & McEwen, 1981; 1982; Romano, Krust, & Pfaff, 1989). Thus estradiol-induced PRs may have been activated by endogenous compounds such as neuroprogesterone (Micevych et al., 2008) or dopamine (Mani et al., 1996; Mani, Allen, Clark, Blaustein, & O'Malley, 1994a) prior to administration of RU486. Because RU486 has a relatively long half-life, ranging from 24-90 hrs (Johanssen & Allolio, 2007), it is possible that with its repeated administration, hypothalamic PRs were increasingly less available for binding by endogenous compounds leading to eventual inhibition. This would suggest a role for PR in the mechanisms underlying

the induction and maintenance of the sensitization of sexual behavior by EB. Thus it is possible that the induction of behavioral sensitization by EB may have been blocked had RU486 been administered earlier or by continuous chronic administration, for example through subcutaneous pellets, which might also prevent the adverse cutaneous effect that we observed.

The initial facilitative effect of RU486 on sexual behavior is also consistent with reports in OVX females primed with EB alone. It has been proposed that RU486 takes on agonistic qualities when co-administered with EB in the absence of P, thereby facilitating lordosis (Pleim et al., 1990). Similarly, agonistic qualities have been reported in breast cancer cell lines (Horwitz, 1985), and when administered alone, RU486 has progestational actions in the endometrium of postmenopausal women yet elicits antagonistic activities when co-administered with progesterone (Gravanis et al., 1985). The administration of RU486 to the VMH of OVX rats primed with 5µg EB facilitates lordosis as effectively as P (Pleim, Lipetz, Steele, & Barfield, 1993), suggesting that the agonistic nature of RU486 may occur through similar mechanisms to P. However we found that the facilitation of RU486 did not occur on the first test; thus if RU486 is acting via a similar mechanism to P, that first exposure must be subthreshold, since a single P injection administered to EB-primed rats maximizes sexual behavior. Moreover, since we found that the facilitation of sexual behaviors only becomes apparent as of the second test (i.e., following the second injection of EB), it suggests that the priming dose of EB increases the cell's ability to respond to subsequent sexual-behavior enhancing treatments (e.g., in this case EB or RU486). One mechanism through which this could occur is through membrane receptor binding, which may open new estrogen (or other ligand) binding sites as proposed by Caldwell (2002).

The agonistic actions of RU486 in the absence of P, but antagonistic actions in the presence of P can explain why certain sexual behaviors eventually became inhibited in our paradigm, if repeated administration of E2 sensitizes the production of neuroprogesterone. RU486 may act as a partial agonist on earlier tests when females display sensitization to EB; however, if neuroprogesterone synthesis is upregulated with chronic EB administration, RU486 may then take on antagonistic qualities due to competitive binding with the newly synthesized progesterone. One way to address this possibility would be to measure hypothalamic neuroprogesterone levels as a function of increasing numbers of EB injections. It is interesting to note that RU486 has agonistic qualities when sexual behavior is in an inhibitory state (i.e., when their expression is typically low or absent), as occurs in lactational Diestrus (Barbosa-Vargas,

Pfaus, & Woodside, 2009) and in OVX rats treated with EB alone (Pleim et al., 1990; 1993) and early tests in the current study). The potential confounding role of the agonistic qualities of RU486 may be overcome using APR19 a novel PR antagonist that prevents the recruitment of co-activators and co-repressors, obliterating the agonistic qualities reported with RU486 (Khan et al., 2013).

The lack of sensitization of appetitive sexual behaviors by EB that occurred in the 5µg EB and 10µg EB+VEH groups suggests that a compound in the vehicle interfered with the mechanism of sensitization, an effect that was overridden (or potentially reversed) by the addition of RU486. The vehicle was composed of 80% sesame oil, 15% benzyl benzoate, and 5% benzyl alcohol, and a literature search in PubMed failed to find any interaction of these compounds with steroid hormone receptors. It is thus unclear why the vehicle treated animals failed to sensitize to 5µg or 10µg EB. However, one plausible reason is that the vehicle facilitated sexual behavior. In our previous studies few solicitations were ever observed, nor did they increase in OVX females repeatedly treated with 10µg EB when given the opportunity to copulate at four day intervals (Jones & Pfaus, 2014), whereas in the current study solicitations did initially sensitize (Figure 2B). We also note that on the initial test hops/darts were higher than that observed in our previous studies, suggesting that the vehicle had a facilitative effect in the 5µg and 10µg EB treated groups, masking any sensitization effect, since that facilitation was maintained throughout the experiment. If that is true, then any facilitation by the vehicle was overridden by chronic administration of RU486, since only in those treatment groups did hops/darts decline on later tests. Together this suggests that despite any facilitation by the vehicle, chronic blockade of PR with RU486 is inhibitory to the sensitization of sexual behaviors.

What neural mechanisms might underlie behavioral sensitization to EB?

Auger and colleagues (1997) found that PRs within the mPOA and VMH are involved in the enhancement of lordosis in OVX and OVX/ADX rats treated acutely with EB when tested in a repeated mating paradigm. In this paradigm, females are given experimenter-applied VCS 48 hours following an injection of EB followed by four 15-minute exposures to a sexually vigorous male at 30-minute intervals. Fifteen minutes after the last mating session, the experimenter applies manual flank stimulation. In control animals, LQ is significantly higher on the third and fourth mating tests compared to the first, but this increase does not occur if PR are blocked with RU486, suggesting that PR activation is involved in that enhancement. Moreover, VCS induced

Fos in OVX and OVX/ADX rats in a number of brain regions including the vlVMH, mPOA and medial amygdala. Blocking PR with RU486 only prevented the increase in Fos expression in the vlVMH and POA, regions that contain PR, suggesting that mating-induced enhancement occurs via PR within those regions (Auger et al., 1997).

Those findings suggest that PR activation by mating stimulation received during repeated testing in our paradigm may also contribute to the sensitization of sexual behaviors by EB. However, if that is true, it could only be part of the mechanism since we recently reported that giving the female access to copulate with a sexually vigorous male attenuates estradiol sensitization of sexually appetitive behaviors (Jones & Pfaus, 2014). Moreover, we found that the attenuation was driven by the receipt of VCS (Jones, S.L., Germé, K., Graham, M.D., Roy, P., Gardner Gregory, J., Rosenbaum, S., Parada, M., Pfaus, J.G., *submitted*), and mimicked by AMPA receptor infusions to the ventrolateral VMH (vlVMH) in place of copulation (Jones, S.L., Farisello, L., Mayer-Heft, N., Pfaus, J.G., *submitted*). Together those findings show that glutamate signaling in the vlVMH opposes the sensitization of appetitive sexual behaviors by EB, and suggests that the induction of sensitization may involve the removal of inhibitory glutamate signaling via an increase in GABA-ergic signaling in the vlVMH (Booth, Wayman, & Jackson, 2010; Georgescu et al., 2009; 2014; McCarthy et al., 1990). Repeated treatments with EB may potentiate the release of GABA within the vlVMH (thereby inhibiting glutamatergic signaling) (Georgescu et al., 2009; 2014), and/or potentiate the synthesis of neuroprogesterone synthesis. Neuroprogesterone may, in turn, not only bind to estrogen-induced PR, but upregulate of GABA-a receptor subunits (Arbo, Andrade, Osterkamp, Gomez, & Ribeiro, 2014), and/or P or its metabolites may also bind to GABA-a receptors directly (Frye & Walf, 2008a; 2008b), leading to a facilitation of sexual behaviors with time. One way a role of GABA can be addressed is by measuring GABA release via microdialysis following repeated treatments with EB, or administering GABA receptor antagonists to test whether estradiol sensitization can be blocked.

In conclusion, the repeated blockade of PR did not disrupt the induction of sensitization by repeated administration of EB, yet hops/darts, LM2, and LM3 were inhibited on later tests, suggesting that PR may play a role in the maintenance of estradiol sensitization. However, given the timing of RU486 administration in this and similar studies, we cannot yet rule-out a role for PR in the induction of estradiol sensitization of sexual behaviors. Part of the underlying

mechanism of estradiol sensitization is likely to be located within the vlVMH, and may involve GABA-ergic signalling.

CHAPTER 2.3

Preface

In Chapter 2.1 it was determined that the repeated administration of EB sensitizes sexual behaviors in the OVX rat. However, characterizing the sensitization required not only the administration of EB but also access to a sexually vigorous male on each test. Thus, the following study tested whether the administration of EB alone, in the absence of the opportunity to copulate, would also induce behavioral sensitization.

2.3: Sensitization of sexual behaviors in ovariectomized Long-Evans rats is induced by a subthreshold dose of estradiol benzoate and attenuated by repeated copulation

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Abstract

Ovariectomy (OVX) abolishes the expression sexual behaviors in the rat, but they can be fully reinstated by sequential administration of estradiol benzoate (EB) followed by progesterone (P). When administered alone, 5 or 10μg EB (but not 2μg) acutely induce only low levels of lordosis, whereas repeated administration potentiates lordosis and induces sexually appetitive behaviors (e.g., hops, darts, solicitations, ear wiggles). The mechanisms mediating this behavioral sensitization are poorly understood, and it is not clear whether stimulation from the male during repeated copulation plays a role. OVX Long-Evans rats were given 4 sexual training sessions with EB (10μg) and P (500μg) 48 and 4 hours prior to testing, respectively, in a unilevel 4-hole pacing chamber followed by a 2-week hormone washout. Females were then treated with 2μg or 10μg EB 48 hours prior to copulation on tests 1 and 8. On tests 2-7, a group of females was treated with 10μg EB and allowed to copulate with a male (10μgEB/Male, n=16), or treated with 2μg or 10μg EB and placed in the chamber alone (2μgEB/Alone, n=6; 10μgEB/Alone, n=18). A negative control group was treated with the oil vehicle and placed in the chamber alone (Oil/Alone, n=6) on tests 2-7, but treated with 2μg EB prior to copulatory tests 1 and 8. All groups, except Oil, displayed behavioral sensitization to EB, suggesting that repeated administration EB is both necessary and sufficient to induce sensitization. Appetitive behaviors were attenuated in those that copulated on every session. Pacing was disrupted in all groups. Together these results suggest that EB activates excitatory mechanisms to promote the expression of sexual behaviors, which are potentiated across time under certain conditions. In contrast, copulatory stimulation attenuates behavioral sensitization to EB.

Key Words: Estradiol sensitization; sexual behavior; female rat; pacing

Introduction

Sexual behavior in the female rat is dependent on the release of ovarian steroid hormones. Ovariectomy (OVX) completely extinguishes the behavior, but it can be reliably reinstated by mimicking ovarian release with the sequential exogenous administration of estradiol benzoate (EB) followed by progesterone (P) (Beach et al., 1942; Boling & Blandau, 1939). The acute administration of EB alone induces low levels of lordosis, the concave arching of the back that permits penile intromission, and subsequent P priming potentiates lordosis and induces sexually appetitive behaviors such as hops, darts, solicitations, and ear wiggles. Repeated administration of EB alone however, induces a behavioral sensitization such that there is an increase in the frequency of lordosis (lordosis quotient, LQ), and the occurrence of sexually appetitive behaviors increases with each subsequent injection until a plateau is reached. We recently characterized this behavioral sensitization to EB when administered in varying doses and intervals, and found that 5 or 10μg EB, but not 2μg EB, administered every 4 days to the sexually experienced OVX Long-Evans rat induces a robust sensitization (Jones et al., 2013). The effect is not dependent on adrenal progestin release, since it was not prevented by adrenalectomy (Jones et al., 2013). Here we examined whether the somatosensory stimulation received from the male on each test contributes to the sensitization of sexual behaviors by repeated administration of EB.

The female rat exerts a great deal of control over the rate of sexual stimulation received from the male, and prefers to mate in pacing chambers which are designed to allow her to regulate copulatory stimulation at her preferred interval (Jenkins & Becker, 2003b; Paredes & Alonso, 1997; Paredes & Vazquez, 1999), an effect that appears to be mediated by the pattern of VCS received (Meerts & Clark, 2007; 2009a). Pacing is reproductively relevant as it decreases the number of intromissions required to induce pregnancy or pseudopregnancy, and results in larger litters (Erskine et al., 1989). The unilevel 4-hole paced mating chamber is bisected with a perforated divider with holes large enough for only the female to pass through, restricting the male to one side, and allowing her to approach and withdraw from the male at her preferred interval. The intensity of sexual stimulation received from the male affects her copulatory pattern. Stimulation progressively increases with mounts, intromissions, and ejaculations, which coincide with increased exit latencies, contact return latencies (CRL), and greater percent exists (Clark, Robinson, Meerts, Quill, & Boisvert, 2011; Erskine, 1989). Pacing is observed in sexually-naïve and experienced females (Erskine, 1985; 1989), and following long-term treatment with EB

alone (5μg for 6 consecutive days) pacing measures are indistinguishable from treatment with EB+P (Brandling-Bennett, Blasberg, & Clark, 1999). It has recently been shown however that sexually-experienced OVX females primed with EB+P return quicker to the male following an intromission and stay away from him longer following an ejaculation (using 30 minute tests), suggesting that they are capable of altering their reproductive strategies to promote reproductive success (Meerts et al., 2014). Whether pacing patterns differ between sexually-experienced females repeatedly treated with EB and not given access to a male compared to those that repeatedly copulate across test sessions, is not known.

The role of somatosensory stimulation received from the male in the enhancement and subsequent termination of sexual behaviors across a single episode has been examined (Bennett, Blasberg, & Blaustein, 2001; Blaustein et al., 2009; Hardy & Debold, 1973). Early in an episode, both lordosis and appetitive sexual behaviors (e.g., solicitations, hops/darts, ear wiggles) progressively increase regardless of whether a female receives intromissive stimulation (Blaustein et al., 2009). However, more ear wiggles are displayed if females do not receive intromissions (by masking the vaginal opening), and a similar pattern emerges for hops/darts (Blaustein et al., 2009), suggesting that intromissive stimulation counteracts the behavioral enhancement of sexually appetitive measures within an episode of heat. As the session progresses, repeated VCS accelerates the onset of estrous termination, defined as a reduction in sexually appetitive behaviors and an increase in antagonistic behaviors prior to the decline in lordosis intensity and magnitude (Erskine, 1985; 1989; Erskine & Baum, 1982; Hardy & Debold, 1972; Lodder & Zeilmaker, 1976; Pfaus et al., 2000). Local anesthesia applied to the vaginal and cervical regions decreases the female's operant responding to gain access to a male (Bermant & Westbrook, 1966), (albeit without interfering with pacing behaviors, see Meerts, Boisvert, Spjut, & Clark, 2010), and removing the sensory stimulation via pelvic nerve transection decreases the latency to return to the male following VCS, increases the time spent with males, and results in more hops, darts, and ear wiggles, and fewer defensive behaviors (Meerts & Clark, 2009b). Together those data suggest that feedback from the vagina and cervix can both facilitate and inhibit aspects of female sexual behavior over an episode of heat. Thus, it is unclear whether copulatory stimulation from the male plays a role in the development of behavioral sensitization following repeated administration of EB, particularly since in our previous study females were administered EB *and* exposed to sexually vigorous males on every test.

The primary goal of this study was to determine whether repeated treatment with EB in the absence of copulation is sufficient to induce behavioral sensitization in the OVX Long-Evans rat. To examine this, females were treated with 10µg EB, a dose which we have previously shown to robustly induce behavioral sensitization, as well as 2µg EB, a dose that has repeatedly been reported in the literature to be subthreshold for inducing sexual behavior when administered alone to the Long-Evans rat. Groups of females were then given access to a sexually vigorous male on each of eight tests, or placed in the chamber alone on all but the first and last test sessions. A secondary goal of this study was to examine the patterns of pacing behavior between those groups.

Materials and methods

Animals

Long-Evans females rats were purchased from Charles River Canada (St-Constant, QC) and housed in pairs in clear Plexiglass shoebox cages lined with beta chip. Male rats were received from the same supplier and housed in groups of four in clear Plexiglass cages lined with beta chip. Males were sexually experienced in the 4-hole pacing chamber. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Concordia University Animal Research Ethics Committee.

Ovariectomy

One week after arrival to the facilities, females were bilaterally OVX under a mixture of ketamine:xylazine, via a single lumbar incision, ear punched, and allowed a week of recovery, as in Jones et al., (2013).

Hormone preparation and administration

EB (17β-estradiol benzoate) and P (progesterone) were dissolved in 0.1mL reagent grade sesame oil (Sigma-Aldrich) and administered by SC injection, 48hrs and 4hrs prior to testing, respectively. Steroid hormones were purchased from Steraloids (Newport, RI). The 2µg EB dose was always diluted down from the 10µg EB solution. An equivalent volume of sesame oil was injected where indicated.

Training and Testing Apparatus

All copulatory sessions occurred in the unilevel 4-hole pacing chamber (60 X 38 X 38cm), lined with beta-chip below a metal grid floor, and bisected with a clear Plexiglass divider with 4 square holes cut into the bottom. The size of the holes were adjusted to the size of the female such that she could easily cross to the male's compartment, while restricting the larger male to his side. This allowed the female to pace the rate of contact with the male.

Training and Testing Procedures

One week following OVX, females were injected SC with 10µg EB followed 44 hours later by 500µg P and given sexual behavior training four hours later, for a total of 4 training sessions. Each training and test session occurred during the middle third of the dark cycle, was 30 minutes in duration, and occurred at 4-day intervals. Following training, females were taken off hormones for a two-week hormone washout period before experimental testing.

To assess the development of behavioral sensitization to EB, female sexual behavior was measured in all four groups on the first and final test day (tests 1 and 8), and manipulations were made on intermediate tests (tests 2-7). To determine whether stimulation received from the male during repeated testing plays a role in the development of behavioral sensitization, two groups of females were treated with 10µg EB prior to each test, but on intermediate tests were either given access to a sexually vigorous male in a four-hole unilevel pacing chamber (10µg EB/Male, n=16), or placed in the chamber alone (10µg EB/Alone, n=18). To assess whether EB treatment is necessary for the development of the sensitization of sexual behaviors, two control groups were used; one was treated with oil (Oil/Alone, n=6) and the other treated with 2µg EB (2µg EB/Alone, n=6) and placed in the chamber alone on intermediate tests. However, since OVX completely abolishes the expression of sexual behavior, both groups were treated with 2µg EB prior to the first and last tests to examine whether sensitization of sexual behaviors occurred. It has been repeatedly shown in the literature that this low EB dose does not induce sexual behavior in the OVX Long-Evans rat without subsequent P priming (Jones et al., 2013; Kow & Pfaff, 1975; Micevych et al., 2008; Sinchak & Wagner, 2012). As such, neither group was expected to display sensitization. EB or the oil vehicle was always administered 48 hours prior to testing.

Behavioral Analyses

Lordosis magnitude (LM) was measured on a 3-point scale according to its intensity as in Hardy and Debold (1971b), and the frequency of each was recorded. Lordosis quotient (LQ) was measured by taking the total number of lordosis postures (LM1+LM2+LM3) and dividing by the total number of mounts (mounts + intromissions + ejaculations) multiplied by 100. Partial solicitations (i.e., hops/darts) and full solicitations (headwise orientation made towards the male followed by a runaway, usually to her side of the chamber) (Gelez et al., 2013; McClintock, 1984) were analyzed separately, as well as combined into a general measure of sexually appetitive behaviors. Kicks, sideways takedowns, boxing postures, and prone positions made by the female were coded as defensive behaviors (Barnett, 1963). Mounts, intromissions, and ejaculations by the male were also analyzed. Pacing behaviors of females included the number of entrances to the male's compartment, contact return latencies (CRL), percent exits from the male's compartment (Erskine, 1989), and the latency to exit the male's chamber following a mount, intromission, or ejaculation (Clark et al., 2011).

Statistical Analyses

Data were analyzed by two-way mixed ANOVA (test X group) followed by Tukey's post-hoc analyses for significant groups effects, and interactions were followed by simple main effect analyses. Since very few male sexual behaviors were coded on the first test day, pacing data were assessed on the final test day only. Group differences were assessed using the one-way ANOVA. The one-way repeated measures ANOVA was used to determine whether females were capable of differentiating between mounts, intromissions, and ejaculations, and significant main effects were investigated using Bonferroni's post-hoc. Significance was considered with $p<0.05$. In the event of significant main effects and an interaction, only the interaction is reported. Data are presented as mean ± SEM. Eta squared (η^2) and Cohen's *d* (correcting for sample size where appropriate) are reported as measures of effect size (Lakens, 2013).

Results

One female in the 10μg EB/male group displayed an abnormally high level of sexually appetitive behaviors on Test 8, (199, Z=5.00) and was removed from all statistical analyses. On

test 7, an equipment failure resulted in the loss of data for two animals, and as such those data were replaced with that of the group mean.

Lordosis. Three of the 15 females in the 10μg EB/Male, 4/6 females in the Oil/Alone, 3/6 in the 2μg EB/Alone, and 5/18 in the 10μg EB/Alone conditions, were not consistently mounted on both tests, and were excluded from the statistical analyses on LQ. Figure 1A illustrates the significant interaction on LQ, $F(1,26)=6.77$, $p=0.002$, $\eta^2=0.30$. LQ was greater on the final test compared to the first in both groups treated with 10μg EB (10μg EB/Male: $p<0.001$, $d=3.10$; 10μg EB/Alone: $p<0.001$, $d=3.34$), whereas there was no change in LQ across tests if females were treated with Oil or 2μg EB and placed in the chamber alone (p's>0.903). On test 1 there were no differences between groups as expected (all $p>0.05$), however on the final test day LQ was significantly greater in females treated with 10μg EB regardless of whether they copulated with a male on every test or not compared to Oil/Alone (10μg EB/Male: $p<0.001$, $d=6.78$; 10μg EB/Alone: $p<0.001$, $d=4.52$) and 2μg EB/Alone females (10μg EB/Male $p<0.001$, $d=3.10$; 10μg EB/Alone $p=0.002$, $d=2.27$), no other differences were detected.

Few LM1s were observed overall (Figure 1B). The frequency of LM1 did not change across tests (main effect of test, $F(1,41)=3.31$, $p=0.076$, $\eta^2=0.07$, and although a main effect of group was detected, $F(1,3)=4.23$, $p=0.046$, $\eta^2=0.18$, Bonferroni's test failed to detect any specific group differences. The interaction was not statistically significant.

More LM2s were displayed on the final test day compared to the first as shown in Figure 1C, (main effect of Test, $F(1,41)=8.50$, $p=0.006$, $\eta^2=0.15$, and the 10μg EB/Male group tended to display more LM2 compared to those treated with Oil ($p=0.057$, $d=0.87$) (main effect of group, $F(3,41)=4.28$, $p=0.010$, $\eta^2=0.24$). The interaction was not statistically significant.

Similarly, more LM3 (Figure 1D) were detected on the final test compared to the first, $F(1,41)=11.55$, $p=0.002$, $\eta^2=0.21$, however no differences were detected between groups, $F(3,41)=1.85$, $p=0.153$, $\eta^2=0.12$. The interaction was not statistically significant.

Sexually appetitive and defensive behaviors. When hops/darts and solicitations were combined into a general measure of sexually appetitive behaviors, an interaction was detected, $F(3,41)=14.89$, $p<0.001$, $\eta^2=0.31$. As expected and shown in Figure 2A, the number of appetitive behaviors did not change across tests in the Oil/Alone group, however they did increase in all groups treated with EB (2μg EB/Alone: $p=0.003$, $d=2.61$; 10μg EB/Alone: $p<0.001$, $d=2.11$; 10μg EB/Male: $p<0.001$, $d=1.45$). There were no group differences in the number of appetitive

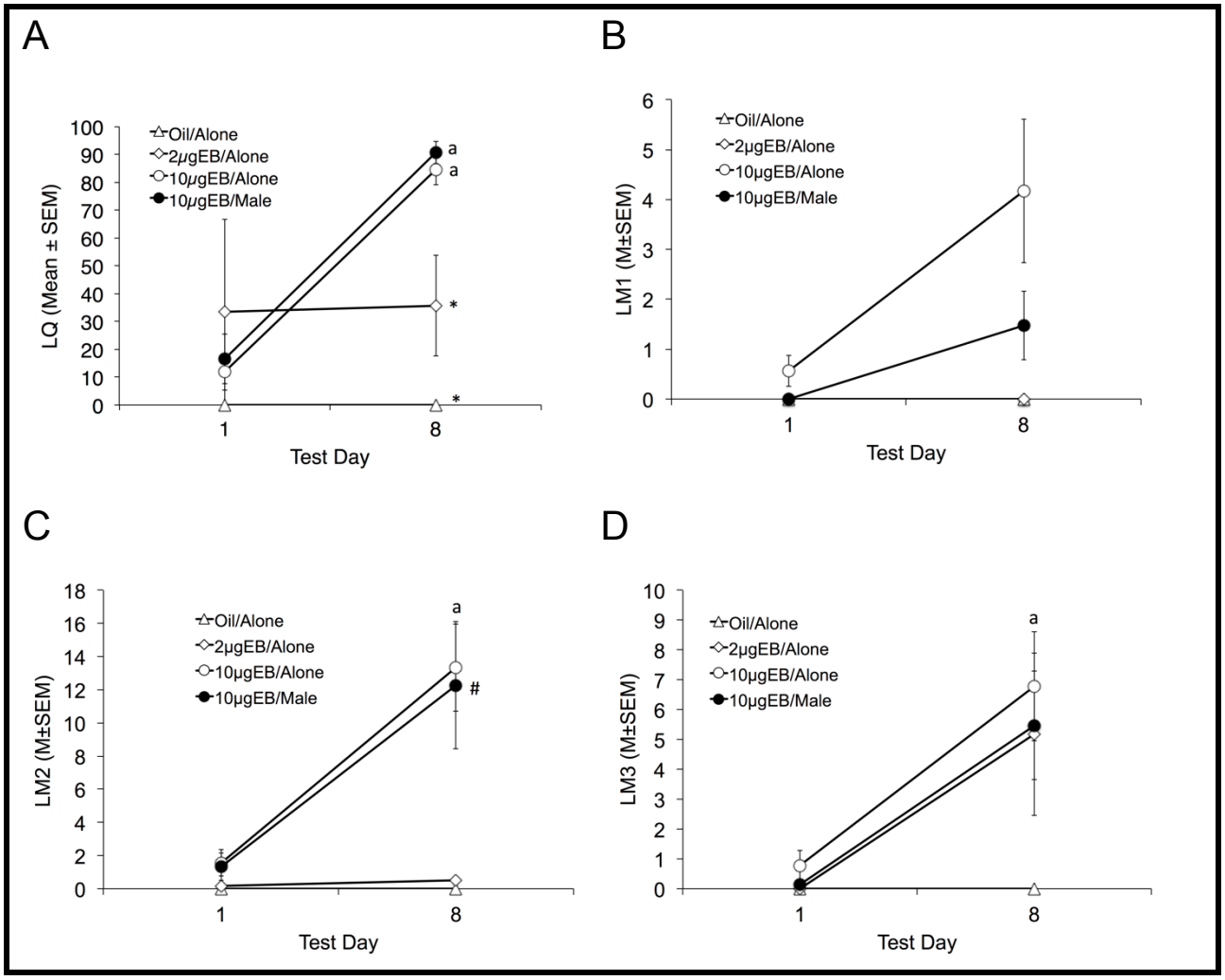

Figure 1. Lordosis Quotient (LQ) and magnitudes (LM) on Tests 1 and 8, of females treated with Oil, 2µg or 10µg EB and placed in the chamber alone, or treated with 10µg EB and repeatedly mated on Tests 2-7. [a]Significantly greater than Test 1. *Different from 10µgEB/Alone and 10µgEB/Male. #Trend, different from Oil, $p<0.06$.

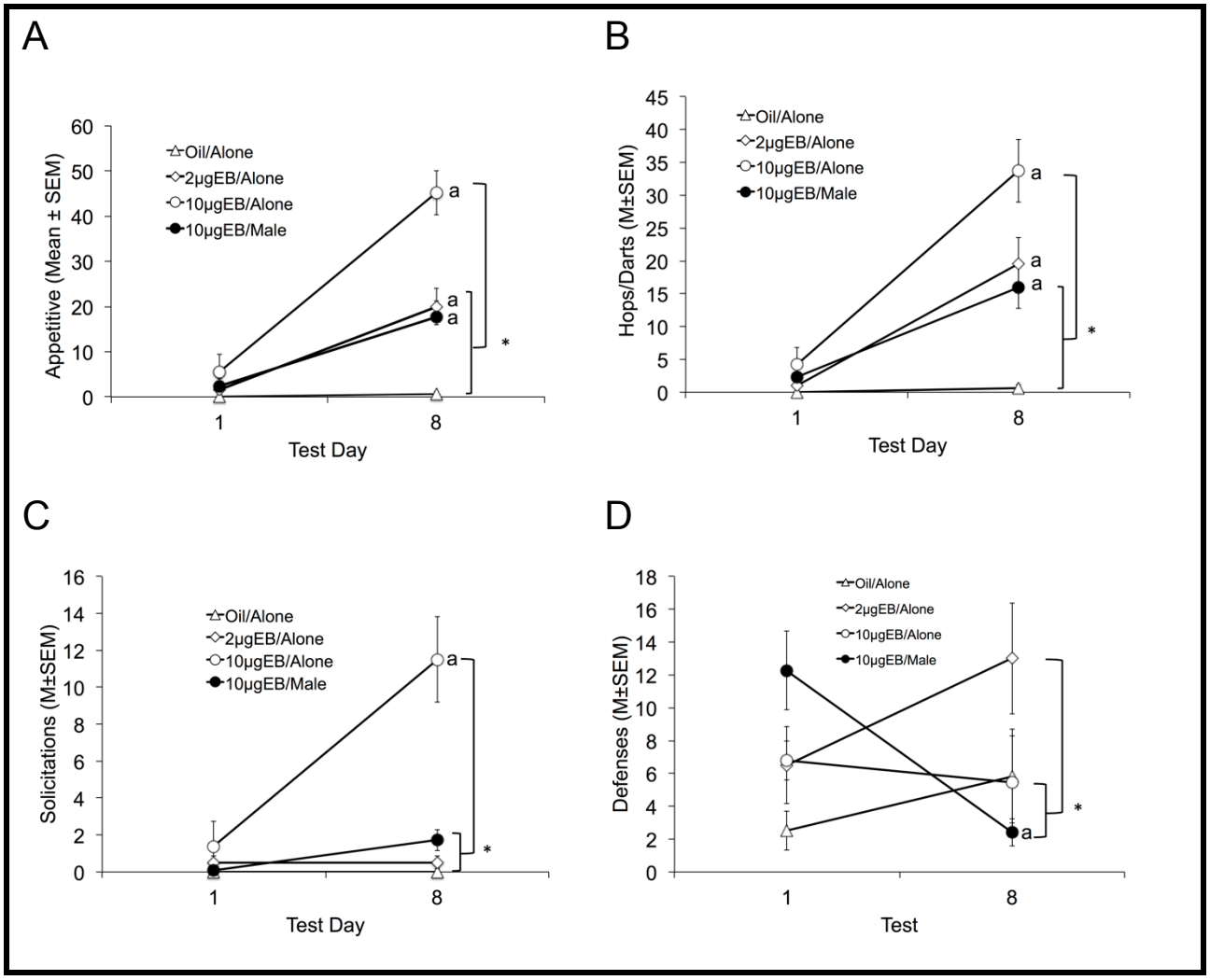

Figure 2. Appetitive and defensive behaviors on Tests 1 and 8, of females treated with Oil, 2µg or 10µg EB and placed in the chamber alone, or treated with 10µg EB and repeatedly mated on Tests 2-7. [a]Significantly different from Test 1, *Group differences.

sexual behaviors displayed on test 1 as expected, however on the final test day, significantly more sexually appetitive behaviors were observed in the 10μg EB-alone group compared to all other groups (Oil/Alone: $p<0.01$, $d=2.44$; 2μg EB/Alone: $p=0.011$, $d=1.33$; 10μg EB/Male: $p<0.001$, $d=1.54$).

When appetitive behaviors were broken down into its components, an interaction was detected on hops/darts, $F(3,41)=9.54$, $p<0.001$, $\eta^2=0.23$. As shown in Figure 2B, hops/darts increased across tests in all groups treated repeatedly with EB (2μg EB: $p=0.001$, $d=2.59$; 10μg EB/Alone: $p<0.001$, $d=1.81$; 10μg EB/Male: $p<0.001$, $d=1.41$), but did not change in the oil controls. No group differences were detected on hops/darts on the first test, however females in the 10μg EB/Alone group displayed significantly more hops/darts compared to the Oil/Alone ($p<0.001$, $d=1.85$), and 10μg EB/Male ($p=0.011$, $d=1.04$) groups on the final test day.

An increase in solicitations was only detected in the 10μg EB/Alone group ($p<0.001$, $d=1.27$), and on the final test day they displayed more solicitations than all other groups (Oil/Alone: $p=0.003$, $d=1.34$; 2μg EB/Male: $p=0.005$, $d=1.28$; 10μg EB/Male: $p=0.001$, $d=1.32$) as shown in Figure 2C (significant interaction, $F(3,41)=4.86$, $p=0.006$, $\eta^2=0.24$).

As shown in Figure 2D, the number of defensive behaviors decreased on the final test day compared to the first in the 10μg EB/Male group (significant interaction, $F(3,41)=6.04$, $p=0.002$, $\eta^2=0.31$). On the final test day, the 2μg EB/Alone group was more defensive towards males than the 10μg EB/Male ($p=0.003$, $d=0.76$) group.

Pacing data. As shown in Figure 3A, females in the 10μg EB/Alone group entered the male's compartment more frequently on the final test day compared to the first ($p<0.001$, $d=2.67$), and on the final test day, entered the male's compartment more frequently than all other groups (Oil/Alone: $p<0.001$, $d=1.82$; 2μg EB/Male, $p<0.001$, $d=1.95$; 10μg EB/Male: $p<0.001$, $d=1.71$) (significant interaction, $F(3,41)=15.56$, $p<0.001$, $\eta^2=0.42$).

As shown in Figure 3B, 10μg EB/Alone females withdrew from the male's compartment more frequently following a mount than the 10μg EB/Male condition ($p=0.034$, $d=1.17$), $F(3,35)=2.91$, $p=0.048$, $\eta^2=0.20$, and more frequently following an intromission, $F(2,24)=8.12$, $p=0.002$, $\eta^2=0.40$, compared to the 2μg EB/Alone ($p=0.006$, $d=2.49$) and 10μg EB/Male ($p=0.023$, $d=1.49$) conditions; since no female in the Oil/Alone condition received an intromission, they were excluded from the analysis. Females in the Oil/Alone and 2μg EB/Alone groups did not receive ejaculations and as such could not be analyzed, and no differences were

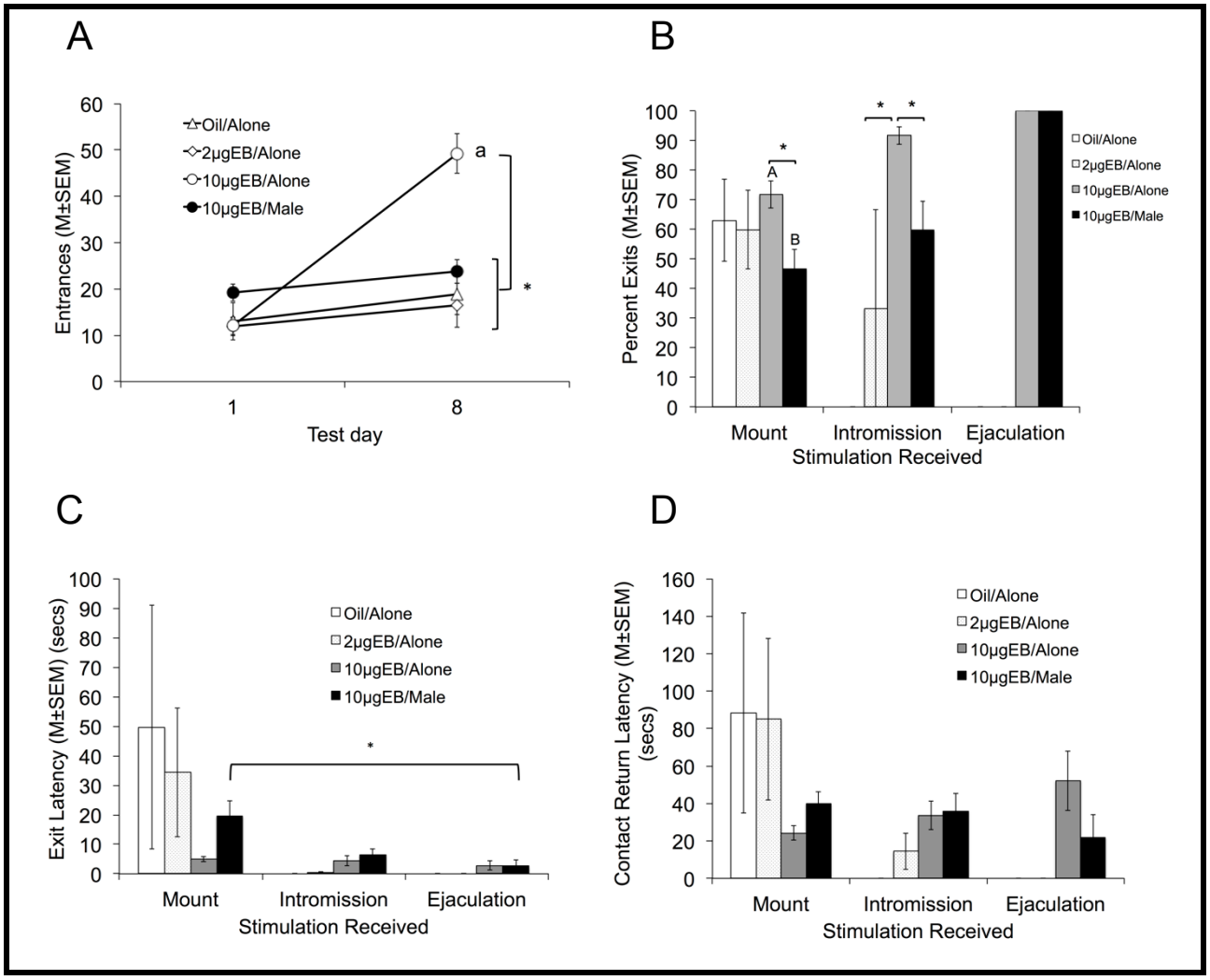

Figure 3. Number of entrances (A) on Tests 1 and 8, of females treated with Oil, 2µg or 10µg EB and placed in the chamber alone, or treated with 10µg EB and repeatedly mated, on Tests 2-7. Percent exits (B), exit latencies (C), and contact return latencies (D) following mounts, intromissions, and ejaculations on Test 8. * Group differences. [a]Significantly greater than Test 1. [A]Different from intromissions and ejaculations, within group. [B]Different from ejaculations, within group.

detected between 10μg EB/Alone or 10μg EB/Male females, as both exited 100% of the time following an ejaculation (Figure 3B), as expected (Erskine, 1989).

Figure 3B also shows that females in the 10μg EB/Alone group were less likely to exit following a mount, compared to an intromission (p=0.032, d=1.26) or ejaculation (p=0.021, d=1.89), $F(2,14)=12.00$, p=0.001, η^2=0.63. And although females in the 10μg EB/Male group exited more frequently as the stimulus increased, $F(2,6)=11.66$, p=0.009, η^2=0.80, significance was only achieved between mounts and ejaculations (p=0.011, d=5.89).

The latency to exit following the different stimulations (Figure 3C) was different in the 10μg EB/Male group only, $F(2,28)=6.59$, p=0.005, η^2=0.32, such that the greater the intensity of stimulation, the quicker she withdrew from the male, however the difference was only significant between mounts and ejaculations (p=0.030, d=1.10). The one-way ANOVA failed to detect group differences in contact return latencies for any group (Figure 3D).

Male behaviors. The number of mounts received (Figure 4A) increased from the first to final test, $F(1,41)=9.23$, p=0.004, η^2=0.16. And a group effect was observed, $F(3,41)=5.68$, p=0.002, η^2=0.50, such that the Oil/Alone group received fewer mounts than the 10μg EB/Alone group (p=0.008, d=1.63) and tended to receive fewer than the 10μg EB/Male group (p=0.052, d=1.34), and the 2μg EB/Alone received fewer mounts than the 10μg EB/Alone group (p=0.042, d=1.34). The interaction was not significant.

The number of intromissions received (Figure 4B) increased across tests $F(1,41)=8.39$, p=0.006, η^2=0.16, and a group effect was found, $F(3,41)=3.68$, p=0.020, η^2=0.79, such that the 10μg EB/Alone females received more intromissions than Oil/Alone females (p=0.047). The interaction was not significant. And no significant effects were detected on ejaculations (for main effects and the interaction $p>0.05$) (Figure 4C).

Discussion

The main purpose of this study was to examine whether or not copulation with the male affects the sensitization of sexual behaviors by repeated administration of EB in the sexually experienced OVX rat. Regardless of whether females had access to a sexually vigorous male on every test, sensitization occurred in females treated with 10μg EB suggesting that repeated administration of 10μg EB is sufficient to induce behavioral sensitization. However, not providing access to the male on intermediate tests resulted in a greater sensitization of sexually

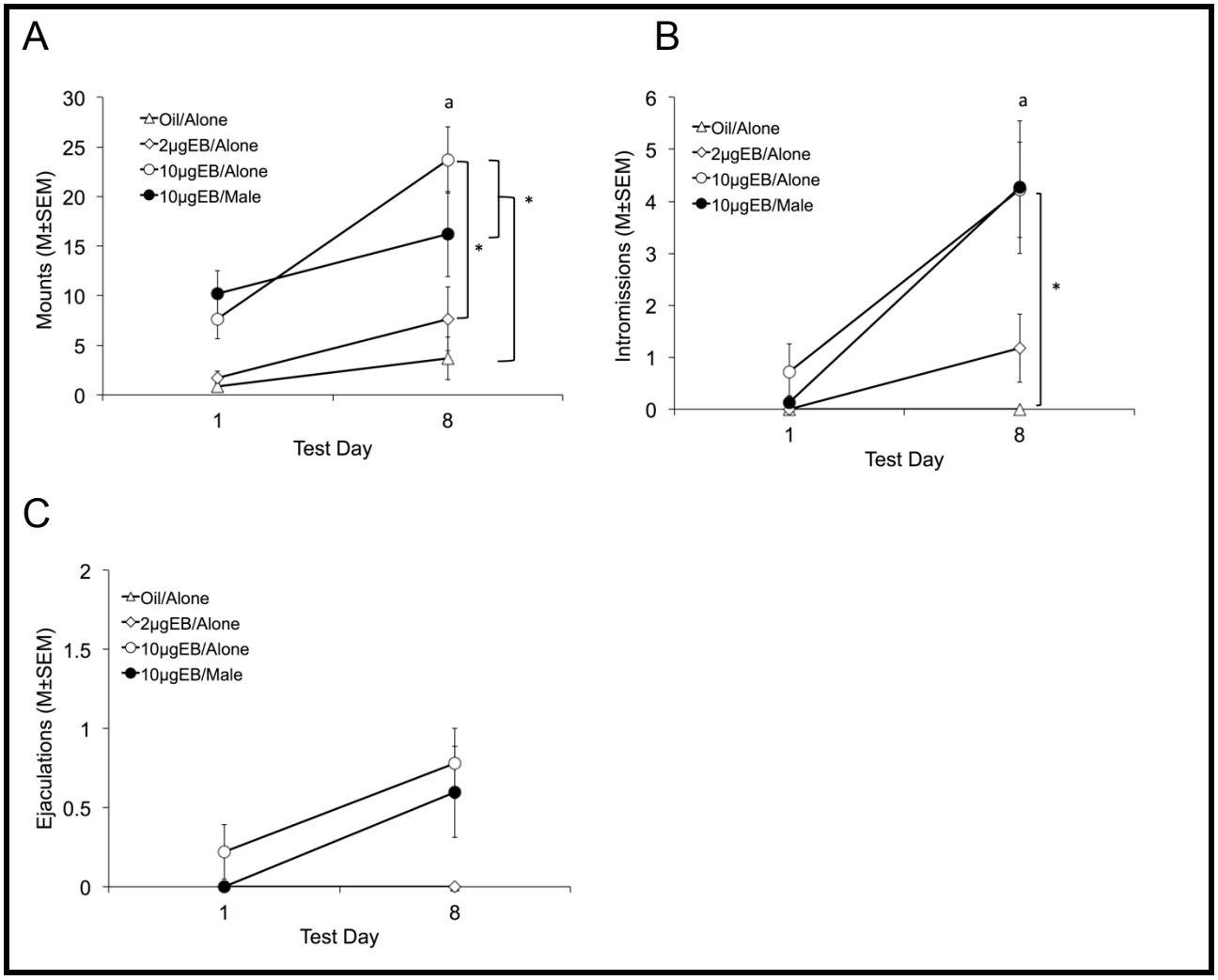

Figure 4. Mounts, intromissions, and ejaculations that females received from the male on Tests 1 and 8, when treated with Oil, 2μg or 10μg EB and placed in the chamber alone, or treated with 10μg EB and repeatedly mated on Tests 2-7. [a]Significantly greater than Test 1, *Group differences on Test 8.

appetitive behaviors (but not lordosis), suggesting that repeated copulation counters the sensitization of sexually appetitive behaviors induced by repeated administration of EB. Surprisingly, sensitization of sexually appetitive behaviors also occurred in females treated repeatedly with 2μg EB and not given access to a male on intermediate tests, an effect driven by hops/darts. This is in stark contrast to females treated with 2μg EB and given access to a male on every test (Jones et al., 2013), and shows that a suboptimal dose of EB alone is sufficient to sensitize some components of appetitive sexual behaviors in the sexually experienced female rat. Moreover, that females treated with oil and not given access to the male on intermediate tests (i.e., Oil/Alone) did not display sensitization, suggests that the repeated administration of EB is necessary for this behavioral sensitization to occur.

A secondary goal of this study was to examine whether pacing behavior differed between groups. Meerts et al. (2014) have recently shown that the OVX rat primed with EB+P responds differently to mating stimuli following sexual experience, such that females return to the male quicker following an intromission, and stay away from him longer after ejaculation on the fourth 30-minute sexual behavior test in the pacing chamber compared to the initial test. Our findings extend those data. All the females in our study were sexually experienced in the unilevel four-hole pacing chamber prior to testing. Contact return latencies were disrupted in both groups repeatedly treated with 10μg EB on the final test day, and although the stair-step pattern was pictorially (but not statistically) observed in the 10μg EB/Alone group, it was completely disrupted in the 10μg EB/Male condition, and in fact their latency to exit following sexual stimulation was reversed. Similarly, percent exits were disrupted in the 10μg EB/Male condition. This suggests that repeated sexual contact with a male alters normal pacing behavior to a greater extent than repeated episodes of heat. This further illustrates plasticity in female sexual behavior, and the ability of females to alter their reproductive strategies, an effect that depends on test parameters and sexual stimulation received within a test session (see Meerts et al., 2014) as well as sexual stimulation received across multiple test sessions. Together with the data presented by Meerts et al., (2014), it appears that contact return latencies are sensitive to repeated testing conditions. Moreover, exit latencies were particularly sensitive to the disruptions induced by repeated testing conditions, further illustrating that exit latencies can provide additional information about pacing behavior as proposed by Clark et al., (2011).

Induction of behavioral sensitization by a subthreshold EB dose

Although the literature has repeatedly reported that 2μg EB does not induce sexual behaviors in the OVX Long-Evans female rat without subsequent P priming (Jones et al., 2013; Kow & Pfaff, 1975; Micevych et al., 2008; Sinchak & Wagner, 2012), this appears to only be true when females are given repeated access to a male. When females were primed with 2μg EB and placed in the pacing chamber alone on multiple test days, by the 8th test, appetitive sexual behaviors had increased significantly compared to the first test, though the increase was lower than that induced by 10μg EB/Alone (but equal to that of 10μg EB/Male). This shows that sexual behavior can be expressed with a suboptimal dose of EB given the appropriate context and repeated administration. If Long-Evans females are treated with 2μg EB and given repeated access to a male, they fail to display sexual behavior (Jones et al., 2013) and typically respond to mounts by the display of defensive behaviors. This may condition an aversive state that is inhibitory to sexual behavior. In addition, given that sensitization occurs with 5μg EB given in combination with repeated copulation (Jones et al., 2013), there must be a threshold EB dose required to override that inhibition, which is potentiated in a dose-response manner (since 10μg EB led to a greater increase in appetitive behaviors compared to 2μg EB in those females that were not given the opportunity to copulate).

Sexual reward states do not require the presence of ovarian hormones. Oil treated rats continue to display a place preference for a context that was previously coupled with sexual behavior (Parada et al., 2012). However, the place preference can be partially extinguished by placing the fully primed female (EB+P) in the sexual context, in the absence of copulation (Parada et al., 2012). Although extinction did not occur if females were treated with EB-alone, it is possible that they may require more pairings. Nonetheless, it appears that sexual reward states in the absence of both EB+P persist, and would suggest that 2μg EB/Alone females continued to associate the pacing chamber with sexual reward (as they were sexually experienced in these same chambers), whereas repeatedly placing the sub-primed female in a context where she must fight off the male, overrides the excitatory state induced by EB. Whether this increased sexual motivation in EB/Alone females is driven by, or potentiated by, repeatedly placing the partially EB-primed female in a previously rewarding context cannot be determined by the current study but could be easily tested by training females in a different context (e.g., a bilevel chamber).

Answering this question could potentially lead to a non-pharmacological animal model of hypoactive sexual desire.

Similarities and contrasts can be made between the potentiation of sexual behaviors within a single episode relative to repeated episodes. Similar to the results of Blaustein et al. (Blaustein et al., 2009) preventing copulation led to a greater increase in sexually appetitive behaviors, suggesting that the mechanisms involved in the potentiation of sexual behaviors within a single episode of heat are similar to those across multiple episodes of heat. In contrast, whereas Blaustein et al. (2009) found that preventing intromissive stimulation blocked the increase in defensive (i.e., rejection) responses observed in copulating animals, we found that for females treated with 10μg EB, if they were not given the opportunity to copulate on intermediate tests defensive behaviors were unaltered across test days, whereas those that copulated on every test were less defensive on the final test. This suggests differential mechanisms between the potentiation of sexual behaviors within a single episode, compared to repeated episodes. Changes here could occur within midbrain structures given their role in defensive behaviors and anxiety (Pfaff et al., 2008). Blaustein et al., suggested a dissociation between the structures involved in the display of lordosis and competing behaviors such as aggression, for example, in the periaqueductal gray which may permit the expression of lordosis while suppressing defensive behaviors (Blaustein et al., 2009; Pfaff et al., 2008). It remains to be tested whether deactivation of the periaqueductal gray is involved in the underlying mechanism of behavioral sensitization to 10μg EB. If such deactivation occurs, it must interact with the amount of stimulation received from the male on previous sexual encounters, given that only those females that copulated on every test showed the decrease.

Another brain region that may participate in the sensitization of sexual behaviors by repeated EB is the medial preoptic area (mPOA). The mPOA is critical for appetitive solicitations (Graham & Pfaus, 2010; 2012; Kato & Sakuma, 2000), and lesions to this area reduce solicitations, hops and darts, and ear-wiggling (Guarraci, Megroz, & Clark, 2004; Hoshina et al., 1994; Whitney, 1986). In the present study we found that appetitive sexual behaviors were increased by repeated administration of EB in the absence of repeated copulation on every test (i.e., EB-alone), but this increase was selectively attenuated by repeated copulation. The mPOA appears to normally oppose the inhibitory signal that occurs from vaginocervical stimulation, (i.e., intromissions, ejaculations), since mPOA lesions increase the contact return

latency following intromission or ejaculation (Guarraci et al., 2004; Yang & Clemens, 2000). In the current study, contact return latencies were disrupted in all groups suggesting that the mPOA may play a role, although it is difficult to interpret the Oil/Alone and 2μg EB/Alone pacing data since they received very little stimulation from the male. However, the latency to exit the male's chamber following an ejaculation was significantly shorter than following a mount in females treated with 10μg EB that copulated on every test, whereas this difference was not found in 10μg EB/Alone females. In addition, the percentage of exits following a mount or intromission was increased in the 10μg EB/Alone females compared to those that copulated on every test, suggesting a disruption in the ability to discriminate between the different stimulus intensities. However, despite an increase in the number of exits following mounts and intromissions, 10μg EB/Alone females also entered the male's compartment more frequently, suggesting an increase in sexual motivation. This alteration in pacing behavior coincident with the increase in appetitive sexual behaviors (which serve to increase the male's attention towards the female and stimulate the receipt of mounts and intromissions), suggests that estradiol sensitization serves to enhance reproductive strategies that may facilitate the induction of pregnancy/pseudopregnancy.

Attenuation of behavioral sensitization by repeated copulation

Providing the female repeated access to a sexually vigorous male attenuated the behavioral sensitization of sexually appetitive behaviors to repeated injections of EB. Although it is unclear what induced the attenuation, one likely mechanism is the receipt of VCS. VCS received during mating, or experimenter-applied, accelerates the onset of estrous termination (Erskine, 1985; 1989; Erskine & Baum, 1982; Hardy & Debold, 1972; Lodder & Zeilmaker, 1976; Pfaus et al., 2000), characterized by a reduction in appetitive sexual behaviors and increased defensive behaviors prior to the decline in lordosis intensity and frequency (Pfaus et al., 2000). Fos-IR is generally expressed in higher levels in female rats that receive VCS, compared to no stimulation or flank stimulation alone (Pfaus, Kleopoulos, Mobbs, Gibbs, & Pfaff, 1993). However, steroid hormone priming alters Fos expression in response to VCS differently in different estrogen-concentrating brain regions. For example, EB potentiates the ability of VCS to induce Fos in the mPOA (Pfaus et al., 1996), whereas it decreases the induction of Fos in the ventromedial hypothalamus (VMH) and in particular within glutamate neurons in the ventrolateral portion of the VMH (Georgescu et al., 2009; Pfaus et al., 1996). It is known that

frequent hormone priming counters the onset of estrous termination; as the interval between priming with EB and P prior to manually applied VCS decreases (from 28 to 4 days) the onset of estrous termination is delayed (Pfaus et al., 2000). We have shown that behavioral sensitization to repeated EB is not only dose-dependent but temporally dependent, as increasing the frequency of hormone priming from every 8 days to every 4 days resulted in a more robust sensitization (Jones et al., 2013). Together with the decreased sensitivity to VCS, this finding suggests that frequent hormone priming prevents the activation of systems that are inhibitory to sexual behavior (Jones et al., 2013). As such, the systems activated by steroid hormones appear to act in opposition to inhibitory systems activated by VCS (Pfaus, 2009). Perhaps repeated VCS over multiple testing sessions progressively summates inhibitory signaling (e.g., by altering glutamate receptor densities, or upregulating enzymes involved in glutamate synthesis released into the VMH) to counteract the effect of repeated hormone priming. To answer this question it will be important to first determine whether different somatosensory information from the male (e.g., VCS, clitoral or flank stimulation) differentially affect the development of EB-induced sensitization.

Conclusion

Frequent hormone priming delays the onset of estrous termination following VCS (Pfaus et al., 2000), and increasing the dose and interval of EB administration augments the behavioral sensitization to repeated EB (Jones et al., 2013). Here we have shown that repeated episodes of heat in the absence of repeated sexual stimulation induces a greater sensitization of sexually appetitive behaviors, and altered pacing behaviors, both of which typically serve to enhance reproductive success. Together these results extend the idea that a mechanism of EB-induced behavioral sensitization may reflect a ‘failsafe’ that increases the likelihood of successful copulation (i.e., impregnation) following repeated episodes of heat, given eventual contact with a sexually vigorous male.

CHAPTER 3: ATTENUATION OF ESTRADIOL-SENSITIZATION

The experiments presented in Chapter 2 established that EB is necessary and sufficient to induce behavioral sensitization, but that the sensitization of appetitive aspects of sexual behaviors is attenuated by copulation. The goals of the next chapter were to examine behavioral and neural mechanisms involved in the attenuation.

Chapter 3 Part 1

Preface

The previous study determined that the repeated administration of EB in the absence of the opportunity to copulate facilitated the sensitization of sexual behaviors, whereas giving the female access to a sexually vigorous male on every test attenuated the sensitization of appetitive sexual behaviors. The goal of the next study was to assess the role of varying types of somatosensory stimulation that is received during copulation on behavioral sensitization to EB.

3.1. Vaginocervical stimulation attenuates the sensitization of appetitive sexual behaviors by estradiol benzoate in the ovariectomized rat

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Abstract

The acute administration of estradiol benzoate (EB) to the ovariectomized (OVX) rat induces low levels of lordosis while sexually appetitive behaviors (e.g., hops, darts, solicitations) are absent, yet the repeated administration of EB results in a behavioral sensitization in which lordosis is potentiated and sexually appetitive behaviors are induced. We have shown that repeated copulation attenuates the sensitization of appetitive sexual behaviors. Here, we assessed which component of male stimulation during copulation is involved in the attenuation. On 8 occasions, sexually experienced OVX Long-Evans rats were treated with 10µg EB and 48h later assigned to one of six groups that differed in their experience on intermediates tests (2-7). One was given repeated access to a male (EB/Male), and another was placed in the copulation chamber alone (EB/Alone) on intermediate tests. Three groups were given one of three somatosensory stimuli by the experimenter: manual flank stimulation (FLS), clitoral stimulation (CLS), or vaginocervical stimulation (VCS). Finally, the control group was left undisturbed in the animal care facility (ACF). Sexual behaviors were measured on Tests 1 and 8. VCS received from the experimenter (VCS) or from the male during copulation (EB/Male) attenuated appetitive sexual behaviors on test 8 compared to those that were not brought to the testing rooms (ACF), and the effect was most pronounced on sexual solicitations. These results suggest that VCS received during penile intromission inhibits the sensitization of sexually appetitive behaviors by repeated administration of EB. As such, repeated administration of EB may oppose neural mechanisms that are inhibitory to sexual behavior (e.g., those that induce estrous termination).

Key Words: Estradiol, sexual behavior, vaginocervical stimulation, clitoral stimulation, flank stimulation, sensitization, estrous termination, rat

Introduction

Sensitization of sexual behaviors occurs in the ovariectomized (OVX) rat following the repeated administration of estradiol alone. The acute administration of 5 or 10μg estradiol benzoate (EB), without subsequent progesterone priming, induces low levels of lordosis and very few, if any, sexually appetitive behaviors (e.g., hops, darts, ear wiggles, solicitations). However, its repeated administration induces a behavioral sensitization, potentiating lordosis and activating sexually appetitive behaviors. We have previously shown that the administration of 10μg EB 48 hours prior to copulation every four days results in a robust sensitization (Jones et al., 2013; Jones & Pfaus, 2014), but that the effect on appetitive behaviors is greater when EB is repeatedly administered in the absence of copulation (Jones & Pfaus, 2014). Since repeated copulation attenuates the sensitization of appetitive behaviors, it is possible that somatosensory stimulation received from the male during mating inhibits this sensitization.

During a typical copulatory bout, which begins with anogenital sniffing from either animal, the female will typically entice the male to chase and mount her by displaying partial (i.e., hopping and darting) or full solicitations (defined as a headwise orientation toward the male followed by a runaway, referred to here as solicitations), which occur in proximity or more distal to the male, respectively (McClintock, 1984; Pfaus, 1996). Solicitational behaviors entice the male to mount (Erskine, 1989) which result in palpation of the flanks, and when coupled with thrusting, stimulates the anogenital region including the clitoris (Pfaff, Montgomery, & Lewis, 1977; Pfaus et al., 2014). Solicitations also increase the probability of intromission (Erskine, 1989; McClintock & Adler, 1978), which occurs when the erect penis penetrates the vagina during a mount with a single deep thrust (Bermant, 1965), stimulating the external and internal vagina and presumably the cervix. Ejaculation occurs following approximately 7-9 intromissions and provides strong and sustained stimulation of the vagina and cervix by the deposit of the copulatory plug which congeals and protects sperm transport (Toner, Attas, & Adler, 1987). Behaviorally the female holds the lordosis posture while the male's pelvis is maintained in close contact with the perineal region (Bermant, 1965). The female will then typically engage in a bursting dismount and run away from the male (Pfaus, 1996; Pfaus et al., 2014). A refractory period follows, lasting a few minutes, before the female revisits the male and solicits him to stimulate mounting and a new bout begins. Thus during copulation, the female receives flank, clitoral, and vaginocervical stimulation from the male.

Sensory stimulation of the genitals that is received during mating can potentiate or inhibit sexual behavior. The pattern of clitoral stimulation (CLS) for example, can facilitate or reduce sexually appetitive behaviors, depending on the pattern of stimulation such that solicitations are facilitated when an experimenter applies continuous stimulation prior to copulation, and reduced when applied in a distributed manner (Cibrian-Llanderal et al., 2010). Similarly, vaginocervical stimulation (VCS), induced by penile intromissions, or experimentally applied with a glass rod, potentiates sexual behavior at the beginning of a mating session (Bennett et al., 2001; Foreman & Moss, 1977; Rajendren et al., 1991; Rajendren & Moss, 1993). However, as the amount of VCS increases, sexually appetitive behaviors decline coincident with an increase in defensive behaviors in response to mounts, prior to a decline in lordosis frequency and magnitude, as the female enters estrous termination (Erskine et al., 1989; Erskine & Baum, 1982; Hardy & Debold, 1972; Lodder & Zeilmaker, 1976; Pfaus et al., 2000). The receipt of 10-15 VCS accelerates the onset of estrous termination (Coopersmith et al., 1996; Hardy & Debold, 1971a; Lodder & Zeilmaker, 1976; Pfaus et al., 2000) and pseudopregnancy (Adler, 1969; Erskine et al., 1989; Frye & Erskine, 1990; Lehmann & Erskine, 2004). However, frequent hormone priming offsets the ability of VCS to induce estrous termination (Pfaus et al., 2000) and reduces the activation of glutamatergic signaling in the vlVMH which is associated with inhibition of sexual behavior (Georgescu et al., 2009; 2014; Georgescu & Pfaus, 2006a; 2006b; Georgescu, Cyr, & Pfaus, 2012), suggesting that the mechanisms of repeated hormone administration act in opposition to those that are inhibitory to sexual behavior. Given that VCS is a well-known inhibitory stimulus to sexual behavior, and that frequent hormone priming offsets that inhibition, we selected a stimulation frequency that is known to induce estrous termination and pseudopregnancy following VCS. The stimulation pattern was kept constant in all manual stimulation groups.

Since varying forms of somatosensory stimulation during mating have been shown to both facilitate and inhibit sexual behavior, the aim of the current study was to mimic the stimulation received from the male by experimentally-applying 15 VCSs, CLSs, or flank stimulations (FLSs), to examine whether any of those somatosensory components contribute to the attenuation of the sensitization of sexual behavior by repeated administration of EB, as occurs with repeated copulation (Jones & Pfaus, 2014). Since we also previously reported that repeatedly treating the OVX rat with EB and placing her in the testing chamber alone was more effective at inducing sensitization compared to those that copulated (Jones & Pfaus, 2014), we

also assessed whether sensitization would occur if females were treated with EB but left in their home cages in the animal care facility (ACF). We hypothesized that all groups would display estradiol sensitization, and that a lack of somatosensory stimulation (ACF and EB/Alone groups) would result in higher levels of sexual behavior, whereas the receipt of VCS from the male (EB/Male) or experimenter (VCS) would result in lower levels of sexual behavior on the final test day.

Materials and methods

Animals

Two cohorts of 36 Long-Evans females (200-250g) were purchased from Charles River, St-Constant, QC, Canada) three months apart, and housed in pairs in clear Plexiglas shoebox cages lined with either beta chip or corncob bedding. A group of 36 Long-Evans males (300-350g) were also purchased from the same supplier, housed in groups of four in clear Plexiglas cages lined with Betachip, and used as stimulus males for both cohorts. Prior to the experiment, males were given 4 sexual experience sessions in the 4-hole unilevel pacing chamber with stimulus OVX females primed with estradiol benzoate and progesterone. Each cohort included 6 females per group, for a final sample size of 12 females per group (N=72).

Ovariectomy

All animals were given one week to acclimate to the ACF. Females were then bilaterally ovariectomized (OVX) via a single lumbar incision under a mixture of 4:3 ketamine hydrochloride (50mg/mL; Ketaset ©, Wyeth Canada) and xylazine (4mg/mL; Rompum©, Bayer Healthcare, Canada), injected IP (1mL/kg). They were then identified by ear punch and given SC injections of PenG (0.1mL/rat) and 5 mg/kg/mL Enrofloxacin (Baytril ©) and rehydrated with 2mL of saline. They were given one-week post-operative recovery, as in Jones et al., (2013) prior to sexual training.

Hormone preparation

Estradiol benzoate (EB; 10µg) and progesterone (P; 500µg) were dissolved in 0.1mL reagent grade sesame oil administered SC, 48 hours and 4 hours prior to testing respectively. Steroid hormones were received from Steraloids (Hanover, NH).

Apparatus

All behavioral training and tests occurred in the unilevel 4-hole pacing chamber, lined with beta chip below a metal grid floor elevated approximately 2.54 cm. Both the grid and the 4-hole partition were removed for groups receiving manual stimulation to prevent females escaping the experimenter's grasp. All tests occured in chambers where animals had previously copulated.

Training and testing procedures

All training and tests occurred during the middle third of the dark cycle. Females were injected with EB and P prior to each of four sexual training sessions occurring at 4-day intervals and given a 2-week hormone washout. Females were then treated with EB alone, at 4-day intervals for the remainder of the experiment. For each copulatory session, the male was placed on the right hand side of the chamber, and allowed a 5-minute acclimation period prior to introduction of the female. The animals were then left undisturbed for 30 minutes. All copulatory tests were recorded using a Sony Handycam digital camera and scored using the Behavioral Observation Program (Cabilio, 1996).

All animals copulated on Tests 1 and 8, and groups of animals received one of six experimental manipulations on tests 2 through 7: one group copulated with a sexual experienced Long-Evans male in a 4-hole pacing chamber on all tests (EB+Male), a second was placed in the 4-hole pacing chamber alone (EB-Alone), and a third group was left in their home cage in the ACF. The three remaining groups received experimenter-applied vaginocervical (VCS), clitoral (CLS), or flank (FLS) stimulation as described in detail below. For these groups, the female was first placed in a testing chamber alone, and a 30-minute timer was set. After one minute had lapsed, the first stimulation (described below) was given, and each subsequent stimulation was applied every 2 minutes thereafter, for a total of 15 stimulations over 30 minutes. Following the final stimulation, animals were left in the chamber until the 30 mins had elapsed (i.e., <1min) before returning them to their home cage.

The amount of experimenter-applied stimulation was chosen based on work showing that 10-15 vaginocervical stimulations (VCS) induce estrous termination and pseudopregnancy in a non-paced mating setting (Adler, 1969; Erskine et al., 1989; Frye & Erskine, 1990; Lehmann & Erskine, 2004), whereas 15 paced intromissions or manually applied VCS induce conditioned place preference (Meerts & Clark, 2009a; Paredes & Vazquez, 1999). The experimenter applied artificial VCS using a glass rod with round polished ends lubricated with K-Y® jelly. Females

were lifted by the tail taking care not to stimulate the flanks, and the rod was quickly inserted into the anterior vaginal opening three times at approximately half-second intervals without touching the cervix, followed by a fourth insertion that put pressure against the cervix, held for about two seconds and removed similar to our previous studies (Pfaus et al., 1993; 1996). Those receiving FLS were gently held by the base of the tail and manually palpated three times at approximately half-second intervals followed by a fourth grasp of the flanks lasting approximately two seconds. Finally, clitoral stimulation (CLS) was administered with a number 4 paintbrush lubricated with K-Y® Jelly (as in Parada, Chamas, Censi, Coria-Avila, & Pfaus, 2010). CLS was administered by lifting the tail, taking care not to stimulate the flanks, and three quick strokes of the paintbrush were applied to the clitoris at approximately half-second intervals, followed by a final longer stroke lasting about two seconds that aimed to maximally stimulate the clitoris from the most anterior portion to the most posterior portion of the external structure.

Behavioral measures

Lordosis magnitude (LM) was measured on a 3-point scale (Hardy & Debold, 1972), and the lordosis quotient (LQ) was measured by taking the ratio of the total number of lordosis postures to the total number of mounts (mounts + intromissions + ejaculations) multiplied by 100. Partial (i.e., hops/darts) and full solicitations (headwise orientation made towards the male followed by a runaway) (Gelez et al., 2013; McClintock, 1984) were analyzed separately, as well as combined into a general measure of sexually appetitive behaviors. Kicks, sideways takedowns, boxing postures, and prone positions made by the female were coded as defensive behaviors (Barnett, 1963). Mounts, intromissions, and ejaculations by the male were also analyzed.

Statistical analyses

To assess whether each group displayed behavioral sensitization to EB, separate repeated measures ANOVAs were used to assess whether the frequency of sexual behaviors increased from the first to the final test in each group. Planned comparisons were then used, based on a priori hypotheses, to examine whether females injected with EB but not given any type of somatosensory stimulation on intermediate tests (ACF and EB/Alone) were different from all other stimulus groups on the final test day. Additional analyses using difference scores on each behavior (frequency on Test 8 – frequency on Test 1), which take into account the animal's baseline level of responding, were also used to examine group differences in estradiol sensitization. Despite expected directional effects, two-tailed tests were used. All levels of

significance were set at $p<0.05$. Eta squared (η^2) and *Cohen's d* are reported as measures of effect size.

Results

Two females (one from each of the FLS and EB/Alone groups) displayed abnormally high LQ and the combined measure of appetitive behaviors (Z scores > 2.96 on both measures) on test 1, suggesting heightened sensitivity to EB, and were therefore excluded from all statistical analyses.

Lordosis. As expected, LQ sensitized across tests in all groups as shown in Figure 1A (ACF, $F(1,10)=22.69$, $p=0.001$, $\eta^2=0.69$; EB/Alone, $F(1,9)=18.27$, $p=0.002$, $\eta^2=0.67$; EB/Male, $F(1,10)=26.03$, $p<0.001$, $\eta^2=0.72$; FLS, $F(1,7)=21.85$, $p=0.002$, $\eta^2=0.76$; CLS, $F(1,11)=51.12$, $p<0.001$, $\eta^2=0.82$; VCS, $F(1,10)=21.24$, $p=0.001$, $\eta^2=0.68$). No group differences were detected.

LM1 (Figure 1B) increased across tests in the ACF, and FLS groups only (ACF: $F(1,11)=5.81$, $p=0.035$, $\eta^2=0.35$; FLS: $F(1,10)=5.86$, $p=0.036$, $\eta^2=0.37$), although a trend was detected in females that received VCS from the experimenter, $F(1,11)=4.18$, $p=0.066$, $\eta^2=0.28$. All groups displayed more LM2 (Figure 1C) and LM3 (Figure 1D) on the final test compared to the first (LM2, ACF: $F(1,11)=18.78$, $p=0.001$, $\eta^2=0.63$; EB/Alone: $F(1,10)=7.56$, $p=0.020$, $\eta^2=0.43$; EB/Male: $F(1,11)=9.23$, $p=0.011$, $\eta^2=0.46$; FLS: $F(1,10)=14.23$, $p=0.004$, $\eta^2=0.59$; CLS: $F(1,11)= 11.26$, $p=0.006$, $\eta^2=0.51$; VCS: $F(1,11)= 10.00$, $p=0.009$, $\eta^2=0.48$), (LM3, ACF: $F(1,11)=9.55$, $p=0.010$, $\eta^2=0.46$; EB/Alone: $F(1,10)=13.04$, $p=0.005$, $\eta^2=0.57$; EB/Male: $F(1,11)=6.22$, $p=0.030$, $\eta^2=0.36$; FLS: $F(1,10)= 9.93$, $p=0.010$, $\eta^2=0.50$; CLS: $F(1,11)= 9.60$, $p=0.010$, $\eta^2=0.47$; VCS: $F(1,11)=6.11$, $p=0.031$, $\eta^2=0.36$). No group differences were found.

Appetitive behaviors. As expected appetitive behaviors sensitized across tests in all groups as shown in Figure 2A (ACF: $F(1,11)=66.95$, $p<0.001$, $\eta^2=0.86$; EB/Alone: $F(1,10)=14.25$, $p=0.004$, $\eta^2=0.59$; EB/Male: $F(1,11)=7.72$, $p=0.018$, $\eta^2=0.41$; FLS: $F(1,10)=28.59$, $p<0.001$, $\eta^2=0.74$; CLS: $F(1,11)=22.67$, $p<0.001$, $\eta^2=0.67$; VCS: $F(1,11)=8.92$, $p=0.012$, $\eta^2=0.45$). However females that received VCS from the male (EB/Male, $p=0.010$, $d=1.15$) or experimenter (VCS, $p=0.031$, $d=0.94$) displayed fewer appetitive behaviors compared to those left in the animal care facility (ACF), with correspondingly large effect sizes. This effect

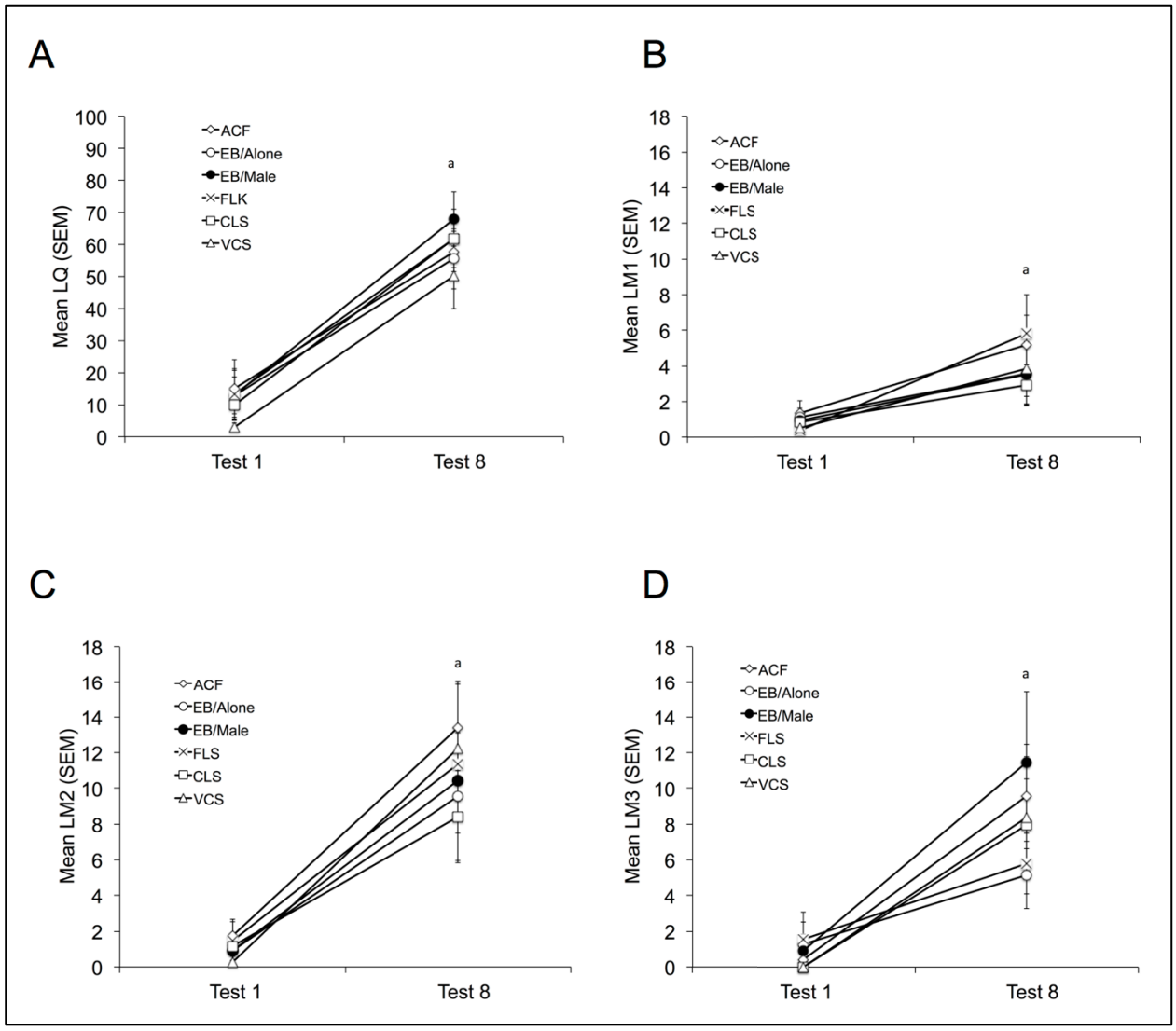

Figure 1. Lordosis measures of OVX rats on the first and final test days following repeated treatment of EB 48 hours prior to each of eight tests. On tests 2-7 females were left in the animal care facility (ACF), placed in the 4-hole pacing chamber with a sexually vigorous male (EB/Male) or alone (EB/Alone), or received 15 manual flank (FLS), clitoral (CLS), or vaginocervical (VCS) stimulations by the experimenter. Sensitization occured on LQ (panel A) and the two higher magnitudes of LM (LM2 and LM3, panels C and D respectively), whereas only ACF and FLS females displayed more LM1 (panel B) on the final test compared to the first. No groups differences in sensitization were found on the final test day. [a] Greater than test 1. [b] Different from ACF on the final test day.

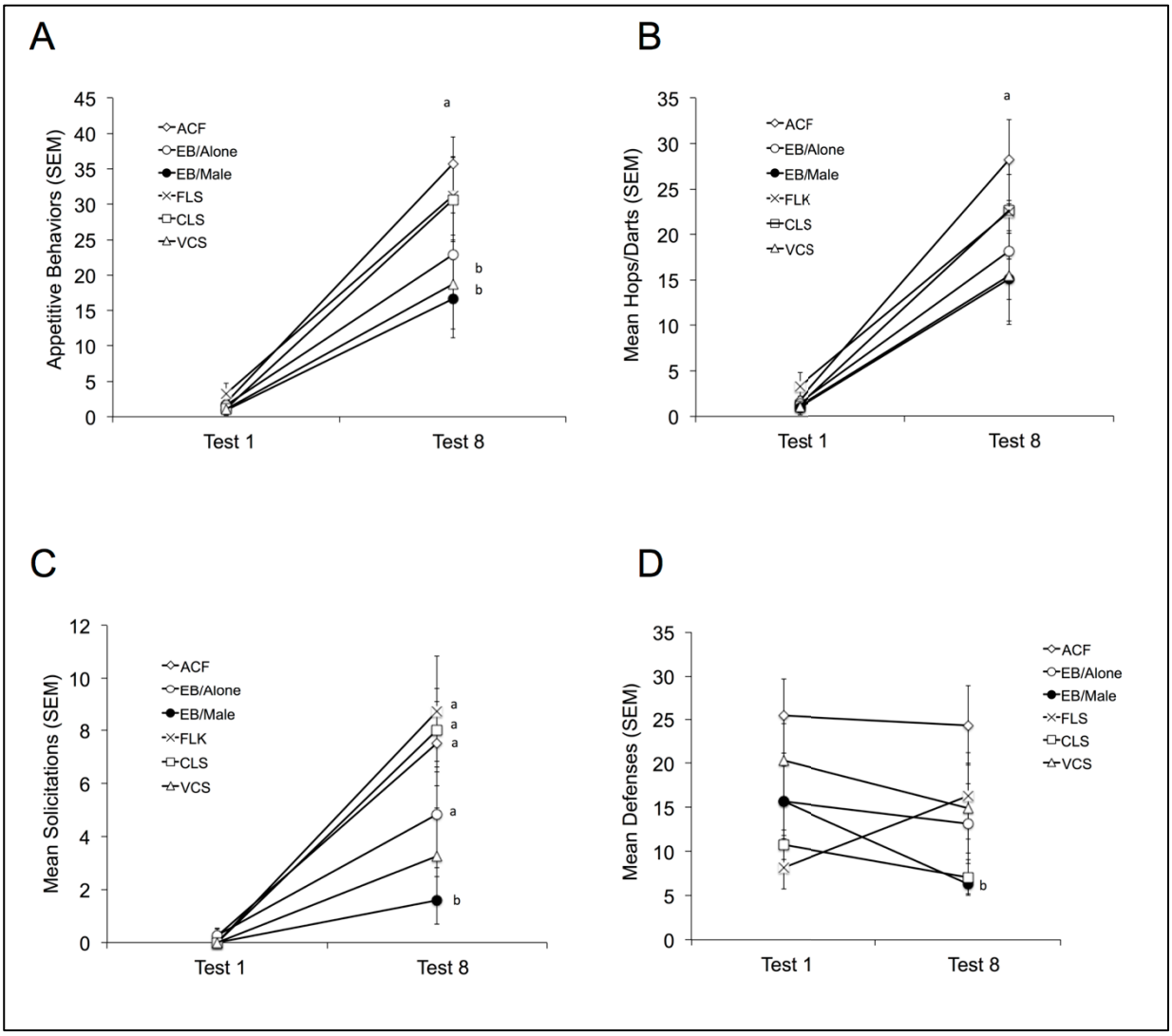

Figure 2. Appetitive sexual behaviors (hops/darts and solicitation) and defensive behaviors of OVX rats on the first and final test days following repeated treatment of EB 48 hours prior to each of eight tests. On tests 2-7 females were left in the animal care facility (ACF), placed in the 4-hole pacing chamber with a sexually vigorous male (EB/Male) or alone (EB/Alone), or received 15 manual flank (FLS), clitoral (CLS), or vaginocervical (VCS) stimulations by the experimenter. Although the combined measure of appetitive behaviors (panel A) suggests that sensitization occured (panel A) the effect was driven by hops/darts (panel B) since all groups sensitized; however solicitations (panel C) failed to sensitize if females were given VCS from the male (EB/Male) or experimenter (VCS). Moreover, on the final test day, females that copulated on every test (EB/Male) displayed fewer solicitations than those left in the ACF. Although defensive behaviors did not change across tests (panel D), those left in the ACF were more

defensive than those that copulated on every test (EB/Male). [a] Greater than test 1. [b] Different from ACF on the final test day.

also occurred when difference scores were analyzed, (ACF vs EB/Male, p=0.018, d=1.04; ACF vs VCS, p=0.036, d=0.91). Although more appetitive behaviors were observed in the EB/Alone group compared to VCS (p=0.628, d=0.20) and EB/Male (p=0.446, d=0.32) on the final test day, the difference did not meet statistical significance and the effect sizes were small. A similar pattern also occurred with the difference scores (EB/alone vs VCS, p=0.657, d=0.18; EB/alone vs EB/Male, p=0.498, d=0.29).

Breaking appetitive behaviors down into its components revealed that hops/darts sensitized in all groups as displayed in Figure 2B (ACF: $F(1,11)=35.61$, $p<0.001$, $\eta^2=0.76$; EB/Alone: $F(1,10)=10.69$, $p=0.008$, $\eta^2=0.52$; EB/Male: $F(1,11)=7.55$, $p=0.019$, $\eta^2=0.41$; FLS: $F(1,10)=27.81$, $p<0.001$, $\eta^2=0.74$; CLS: $F(1,11)=14.27$, $p=0.003$, $\eta^2=0.56$; VCS: $F(1,11)=9.18$, $p=0.011$, $\eta^2=0.45$), all of which were strong effects. Animals that were left in the ACF tended to display more hops/darts on the final test than those that received manual VCS (p=0.069, d=0.78), or VCS from the male (EB/Male, p=0.064, d=0.80), with correspondingly large effect sizes. This pattern was retained when difference scores were analyzed, (ACF vs VCS, p=0.076, d=0.91; ACF vs EB/Male, p=0.085, d=1.04). Solicitations (Figure 2C) also sensitized in all groups (ACF: $F(1,11)=18.34$, $p=0.001$, $\eta^2=0.62$; EB/Alone: $F(1,10)=5.07$, $p=0.048$, $\eta^2=0.34$; FLS: $F(1,10)=16.88$, $p<0.002$, $\eta^2=0.63$; CLS: $F(1,11)=26.07$, $p<0.001$, $\eta^2=0.71$), except those females that received VCS either from the male (EB/Male: $F(1,11)=3.15$, $p=0.103$, $\eta^2=0.22$), or experimenter (VCS: $F(1,11)=3.14$, $p=0.104$, $\eta^2=0.22$), and significantly more solicitations were displayed by ACF compared to EB/Male (p=0.004, d=1.32) on the final test day, and this effect was retained when difference scores were analyzed, (p=0.007, d=1.21).

Defensive behaviors. Defensive behaviors, shown in Figure 2D, did not change across tests in any of the groups, (ACF: $F(1,11)=0.03$, $p=0.874$, $\eta^2=0.02$; EB/Alone: $F(1,10)=9.18$, $p=0.683$, $\eta^2=0.02$; FLS: $F(1,10)=2.42$, $p<0.151$, $\eta^2=0.20$; CLS: $F(1,11)=1.33$, $p=0.273$, $\eta^2=0.11$; VCS: $F(1,11)=0.53$, $p=0.483$, $\eta^2=0.05$), although females that copulated on every test tended to display fewer defensive behaviors on the final test day compared to the first (EB/Male: $F(1,11)=4.43$, $p=0.059$, $\eta^2=0.29$). On the final test day, ACF females displayed more defensive behaviors than EB/Male (p=0.001, d=1.58), yet when difference scores were analyzed, this effect was no longer found, (p=0.314, d=0.42), suggesting that the group differences found on Test 8

were an artifact of higher defensive behaviors overall in the ACF group regardless of the experimental manipulation. No other group differences were detected.

Male behaviors. The number of mounts (Figure 3A) received were higher on test 8 compared to the first test in females that were left in the ACF on tests 2-7 $F(1,11)=6.34$, $p=0.029$, $\eta^2=0.37$, and tended to be higher on test 8 in females that received FLS, $F(1,10)=3.86$, $p=0.078$, $\eta^2=0.28$. On test 8, ACF animals received more mounts than EB/Male ($p=0.019$, d=1.04), and CLS ($p=0.002$, $d=1.42$), yet these differences were attenuated when difference scores were analyzed (ACF vs EB/Male, $p=0.361$, $d=0.38$; ACF vs CLS, $p=0.081$, $d=0.75$. No other group differences were detected.

Females in all groups received more intromissions on the final test day compared to the first, as shown in Figure 3B (ACF: $F(1,11)=23.52$, $p=0.001$, $\eta^2=0.68$; EB/Alone: $F(1,10)=8.65$, $p=0.015$, $\eta^2=0.46$); EB/Male: $F(1,11)=7.08$, $p=0.022$, $\eta^2=0.39$; FLS: $F(1,10)=36.54$, $p<0.001$, $\eta^2=0.78$; CLS: $F(1,11)=20.62$, $p=0.001$, $\eta^2=0.65$; VCS: $F(1,11)=8.21$, $p=0.015$, $\eta^2=0.43$). No group differences were detected in the number of intromissions received on Test 8. However, the analyses on difference scores suggest that EB/Alone females tended to receive fewer intromissions compared to those that received FLS, $p=0.054$, $d=0.87$

As shown in Figure 3D, the number of ejaculations received was higher in all groups on the final test day compared to the first (ACF: $F(1,11)=10.16$, $p=0.009$, $\eta^2=0.48$; EB/Male: $F(1,11)=6.71$, $p=0.025$, $\eta^2=0.38$), FLS: $F(1,10)=5.99$, $p=0.034$, $\eta^2=0.38$; CLS: $F(1,11)=20.12$, $p=0.001$, $\eta^2=0.65$; $F(1,11)=6.06$, $p=0.032$, $\eta^2=0.36$) except for the EB/Alone group, $F(1,10)=3.20$, $p=0.106$, $\eta^2=0.24$. On the final test day, FLS ($p=0.035$, $d=0.96$) and CLS ($p=0.030$, $d=0.97$) received more ejaculations than EB/Alone, yet the statistically significant effect was only maintained between EB/Alone and CLS ($p=0.019$, $d=1.06$) when differences scores were analyzed (EB/Alone vs FLS, $p=0.134$, $d=0.67$). No other group differences were found.

Discussion

This study shows that repeated administration of 10µg EB every four days to OVX rats without any other manipulation (ACF) induces a robust sensitization of both appetitive and consummatory measures of sexual behaviors, and that repeated VCS received from the male (EB/Male) or experimenter (VCS) attenuates the sensitization of sexually appetitive behaviors. The effect was most robust on full solicitations, since this behavior did not sensitize if females

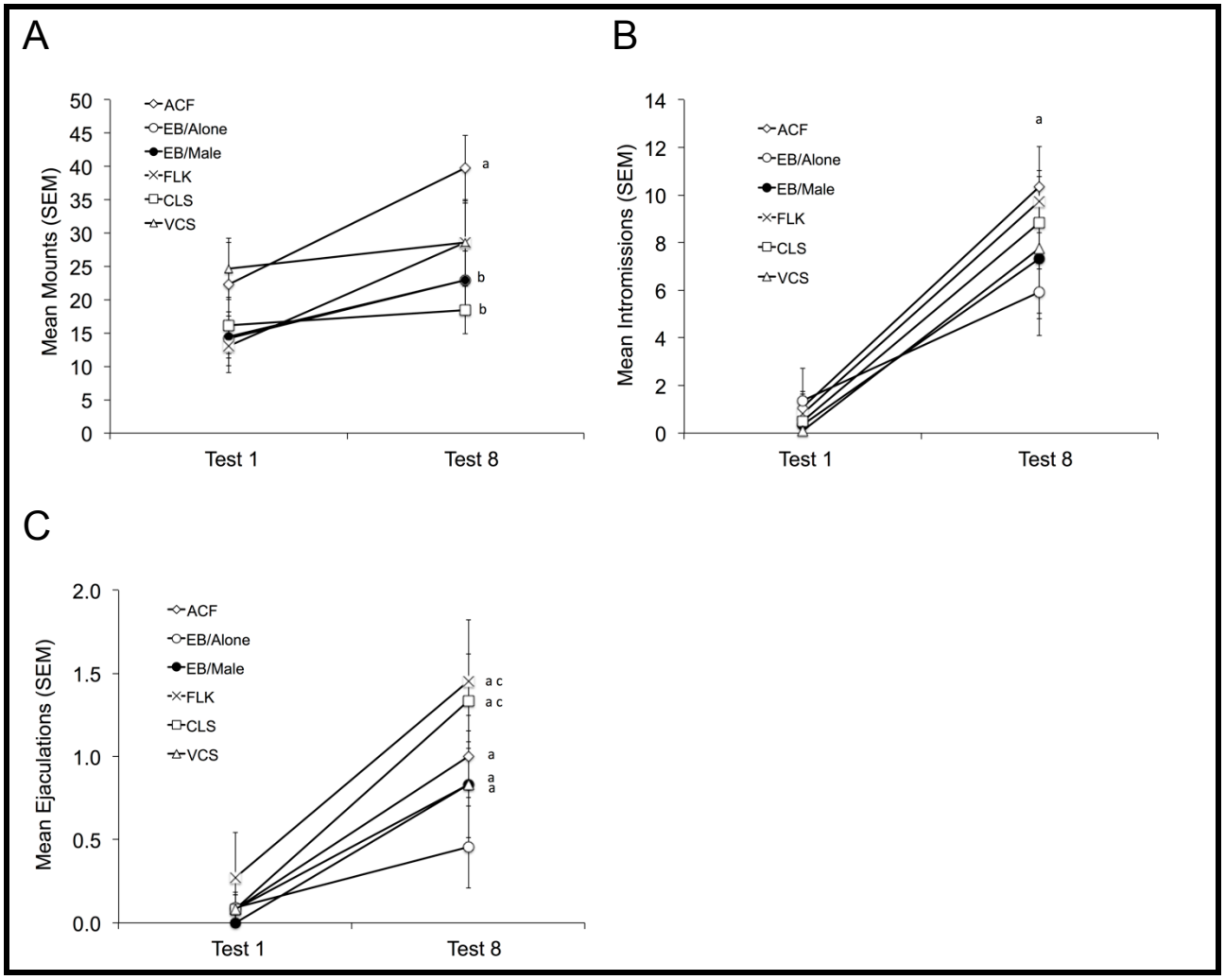

Figure 3. Receipt of mounts (panel A), intromissions (panel B), or ejaculations (panel C) of OVX rats on the first and final test days following repeated treatment of EB 48 hours prior to each of eight tests. On tests 2-7 females were left in the animal care facility (ACF), placed in the 4-hole pacing chamber with a sexually vigorous male (EB/Male) or alone (EB/Alone), or received 15 manual flank (FLS), clitoral (CLS), or vaginocervical (VCS) stimulations by the experimenter. [a] Greater than test 1. [b] Different from ACF on the final test day. [c] Different from EB/Alone.

received VCS (from the male or experimenter), and females that copulated with a male on every test (EB/Male) displayed significantly fewer solicitations than those that received no manipulation (ACF) on the final test day. Together these findings suggest that the systems activated by VCS act in opposition to those that induce sensitization of sexually appetitive behaviors by EB.

Sexually appetitive behaviors sensitized when females were repeatedly treated with EB, yet repeated VCS attenuated the effect. Moreover, when the two subcomponents were analyzed separately, it became apparent that active solicitations did not sensitize in females that received VCS (from the male or experimenter). This is important since active solicitations are commonly interpreted as indicative of a female displaying higher levels of sexual motivation (McClintock & Adler, 1978; Pfaus et al., 1999). VCS also accelerates the onset of estrous termination, which is characterized by a reduction in the number of sexually appetitive behaviors and an increase in antagonistic behaviors, before a decline in the quality and frequency of lordosis. Although female rats tend to show high levels of sexual solicitations in the bi-level chamber (Pfaus et al., 1999), Pfaus et al., (2000) reported observing few solicitations using a paradigm to examine estrous termination following experimenter applied VCS, suggesting that sexual solicitations decline prior to the decrease in other sexually appetitive behaviors such as hop/darts. This is consistent with the idea that full solicitations (i.e., those involving a runaway component) are indicative of greater sexual motivation, as not only are they more readily observed in the fully primed (EB+P) female (Pfaus et al., 1999), they are also first to be inhibited during estrous termination (just as other appetitive measures are inhibited prior to the decline in lordosis). In the current study, females that received VCS from the male on every test (EB/Male) displayed fewer sexual solicitations compared to those left in the ACF, likewise, hops/darts were dampened in those that copulated (EB/Male) or received manual VCS. This suggests that mechanisms that mediate estrous termination may be involved in the attenuation of sensitized sexual behaviors by repeated administration of EB.

Somatosensory stimulation received during copulation alters the expression of female sexual behavior within a test session. This is true for VCS and CLS, and both stimulations have the ability to induce sexual reward and alter reproductive measures. Artificial VCS applied by the experimenter, even in OVX rats that are not hormone primed, can induce lordosis, and this effect is potentiated if females are pretreated with estradiol (Komisaruk & Diakow, 1973). Early

in a copulatory session VCS can facilitate the expression of sexual behavior, and a minimum of six intromissions increases the probability of impregnation following ejaculation (Adler, 1969). On the other hand, the receipt of 10-15 VCS is inhibitory to sexual behavior as it accelerates the onset of estrous termination and pseudopregnancy (Adler, 1969; Erskine et al., 1989; Frye & Erskine, 1990; Lehmann & Erskine, 2004), yet produces a conditioned place preference when received either by male intromissions or when artificially applied by the experimenter (Arzate et al., 2011; Meerts & Clark, 2009a; Paredes & Vazquez, 1999). Similarly, CLS given just prior to a sexual behavior test can either facilitate sexual solicitations when applied continuously (defined as one stimulation every second for a minute, with 1-2 min intervals for five rounds) or inhibit their expression when applied in a distributed manner (defined as one stimulation every five seconds for a minute, with 1-2 min intervals for five rounds) (Cibrian-Llanderal et al., 2010). Distributed CLS also increases the probability of impregnation (Cibrian-Llanderal et al., 2010) and induces conditioned place (Parada et al., 2010) and partner preferences (Parada et al., 2011), supporting the idea that certain patterns of sexual stimulation that are inhibitory to sexual behavior can not only be rewarding, but also increase reproductive success. The data presented here show that sexual stimulation received on prior episodes of heat also have the ability to alter subsequent sexual behavior patterns, but whether they translate into changes in reproductive success is not currently known.

Although repeated stimulation of the clitoris or flanks on intermediate tests did not significantly alter behavioral sensitization of appetitive and consummatory measures of sexual behavior compared to other groups, this does not rule out any role of these stimulations in the sensitization. Here we applied the same stimulation parameters (i.e., 15 stimulations over a 30 minute period) to all conditions based on the large body of work on the neuroendocrine and behavioral effects of VCS, and its role in sexual inhibition. However since the pattern of CLS can either facilitate or inhibit the expression of sexual solicitations depending on the pattern in which it is applied (Cibrian-Llanderal et al., 2010), if a different pattern of stimulations had been given we may have seen further facilitation or an attenuation. In fact, the FLS and CLS manipulations may have had some facilitatory effects given that those females received more intromissions (FLS females) and ejaculations (particularly those that received CLS) compared to EB/Alone. Our CLS manipulation more closely resembled a pattern of distributed stimulation, since the stimulations were applied every two minutes.

The strong sensitization that occurred in females that were left in the ACF on intermediate tests further demonstrates the robustness of the ability of EB to sensitize sexual behavior in the Long-Evans rat, beyond any role of associative learning as we have previously proposed (Jones et al., 2013). Importantly, the animals in our ACF are housed in same-sex groups, but male and females co-exist in the housing rooms. Thus, we cannot rule out a role of auditory or olfactory stimulation in the sensitization. For example auditory stimulation can enhance sexual responding (e.g., McGinnis & Vakulenko, 2003; Thomas, Howard, & Barfield, 1982), and olfactory cues from male rats can increase sexually appetitive behaviors in females (Afonso, Woehrling, & Pfaus, 2006). Moreover, we have recently found that the inhibitory effects of corncob bedding on female sexual behavior (Markaverich, Alejandro, et al., 2002a; Markaverich et al., 2007a; Markaverich, Mani, et al., 2002b) are overcome by housing females and males in the same room suggesting that constant exposure to male cues can overcome that inhibition (Jones, S.L., Antonie, R., Pfaus, J.G., *submitted*). However, the strongest group differences between animals that received VCS and the non-stimulated groups (ACF and EB/Alone) occurred with ACF animals. This suggests that at least some level of sexual inhibition may have occurred by bringing EB/Alone females to the testing rooms, and placing them in the pacing chambers alone, where they presumably were exposed to higher levels of auditory and olfactory (and visual) sexual stimulation than ACF animals. Hormonally priming the females and placing them in a context where they previously experienced sexual reward, and therefore expect to copulate, may be somewhat aversive, particularly when they are hormone primed. In fact, the application of CLS to hormonally primed females (with EB+P) in the presence of an inaccessible almond scented male not only prevents conditioned partner preference to a male bearing the almond odor, it actually produces a partner preference for the male *not* bearing the odor (Parada et al., 2011). Moreover, conditioned placed preference is rapidly extinguished if hormone-primed females are repeatedly placed in a previously sexually-rewarding context in the absence of the opportunity to copulate, whereas this extinction does not occur in oil-treated controls (Parada et al., 2012). Thus, if olfactory and auditory (and potentially visual) signals from sexually vigorous males residing in the same housing rooms as the females contribute to the sensitization, they may interact with the context in which those signals occur.

Frequent hormone administration potentiates sexual excitation and reduces the ability of VCS to inhibit sexual behavior. Increasing the EB dose and decreasing the dosing interval,

enhances the sensitivity of the OVX rat to the activational effects of EB (in the absence of subsequent P priming) on sexual behaviors (Jones et al., 2013). Moreover, although VCS accelerates the onset of estrous termination, as the frequency of hormone priming with EB+P in the OVX rat is increased from 28 to 14 to every four days, the onset of estrous termination following VCS is delayed (Pfaus et al., 2000). We have shown that although repeated administration of EB alone sensitizes sexual behaviors, the sensitization of appetitive measures of sexual behavior are attenuated in those that are given the opportunity to copulate (Jones & Pfaus, 2014), or given manual VCS 48 hours following each injection (the current study). Together those data set up the hypothesis that repeated episodes of heat in the absence of VCS potentiates the display of sexual behaviors when the female eventually gains access to a male, and that the increase in full sexual solicitations may serve as a mechanism to increase the probability of the receipt of VCS (Erskine, 1989; McClintock & Adler, 1978). They also suggest that inhibitory systems that are activated by VCS act in opposition to those that are involved in the sensitization of sexual behaviors by EB. If that is true, then manipulations that induce behavioral patterns reminiscent of estrous termination should mimic the attenuation of EB induced sensitization by VCS.

Part of the mechanism of estrous termination lies within the ventromedial hypothalamus (VMH), a region that is critical for the display of lordosis and involved in the induction of sexually appetitive behaviors (Pfaff, 1968; 1980). This region contains a high density of estrogen receptors (Pfaff, 1968), and crystalline estradiol applied to the ventrolateral division of the VMH (vlVMH) activates lordosis (R. J. Barfield & CHEN, 1977). VCS accelerates the onset of estrous termination, and induces Fos-immunoreactivity (a marker of neuronal activation) in glutamatergic neurons within the vlVMH (Georgescu et al., 2009). In fact, whereas infusions of glutamate or its selective ionotrophic receptor agonists to this region inhibit female sexual behavior in a behavioral pattern that is reminiscent of estrous termination (Georgescu & Pfaus, 2006b), sexual behavior is activated following infusions of GABA or glutamate antagonists to the vlVMH (McCarthy et al., 1990), suggesting that glutamate signaling in the vlVMH must be inhibited for sexual behavior to occur. Moreover, antagonizing the AMPA/kainate receptor subtype blocks the ability of VCS to induce estrous termination (Georgescu et al., 2012). Experimenter applied VCS induces Fos in the vlVMH to a greater extent in OVX rats that are not given hormone replacement compared to those treated with EB+P, and the activation occurs

in a threshold pattern, with one increase occurring between 1-20 stimulations and a second occurring between 30-50 (Pfaus et al., 1996). This pattern is interesting given that 15 VCS induce pseudopregnancy and generate a conditioned place preference (Adler, 1969; Arzate et al., 2011; Erskine et al., 1989; Frye & Erskine, 1990; Lehmann & Erskine, 2004; Meerts & Clark, 2009a; Paredes & Vazquez, 1999), as well as increases the probability of impregnation in ovary-intact females following an ejaculation (Adler, 1969), suggesting that as the amount of VCS increases, systems that are inhibitory to sexual behavior are increasingly potentiated. However, hormone priming opposes this inhibition, as it reduces the activation of glutamatergic neurons by VCS (Georgescu et al., 2009), and attenuates the release of glutamate in the VMH compared to oil treated controls when presented with a sexually vigorous male (Georgescu et al., 2014).

Together those data illustrate that in the VMH, GABA-ergic signaling activates, whereas glutamate transmission inhibits sexual behavior, and that hormone priming appears to disrupt the ability of VCS to activate glutamatergic systems in the vlVMH that are inhibitory to sexual behavior. Since in the current study we found that VCS attenuated the sensitization of sexually appetitive behaviors, it is possible that glutamate transmission within the vlVMH (possibly acting primarily on the AMPA/kainate receptor subtype) is repeatedly activated by VCS in those females, disrupting the ability of EB to further potentiate appetitive sexual behaviors and this inhibition may be additive with each test. If this is true, then increasing the amount of VCS received (e.g., by allowing a longer test period, or experimentally applying more VCS), or extending the number of intermediate tests would likely lead to an increase in antagonistic behaviors and the inhibition of lordosis (reminiscent of the behavioral pattern of estrous termination). Similarly, we would predict that females that copulate on every test (EB/Male) would enter estrous termination quicker than those that are not given the opportunity to copulate (EB/Alone or ACF), which would suggest that part of the mechanism underlying the sensitization of sexual behaviors by EB involves an increase in GABA-ergic, and/or an attenuation of glutamatergic signaling within the vlVMH, but that repeated VCS opposes those effects.

The vlVMH contains a large number of GABAergic afferent fibers from the medial preoptic area (mPOA) (Georgescu, Graham, & Pfaus, *in preparation*), and estradiol administration upregulates GABA-A receptors on glutamate neurons within the VMH (Georgescu, Del Corpo, & Pfaus, in preparation), and increases GAD-65 mRNA (an enzyme

involved in the synthesis of GABA from glutamate) in the mPOA (McCarthy et al., 1995). Whereas the vlVMH is critical for the display lordosis, it is generally believed that the mPOA is inhibitory to lordosis yet facilitatory to sexually appetitive behaviors and pacing (Guarraci et al., 2004; Hoshina et al., 1994; Pfaus et al., 1996; Whitney, 1986). Estradiol increases the synthesis of progesterone receptors within the vlVMH and mPOA (MacLusky & McEwen, 1978; Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994) and subsequent P priming reliably reinstates lordosis and sexually appetitive behaviors following an acute injection of EB (Etgen & Barfield, 1986; Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994; Rubin & Barfield, 1983). The selective destruction of cell bodies (sparing fibers of passage) within the mPOA by excitotoxic lesions facilitates lordosis and antagonistic behaviors, but impairs sexually appetitive and pacing behaviors (Hoshina et al., 1994), which supports the notion that the mPOA contains systems that are inhibitory to lordosis yet facilitative to sexually appetitive behaviors. Those data suggest that the induction of sexual behavior by estradiol acting within the VMH occurs via a process of disinhibition, whereby GABA transmission (possibly coming from the mPOA) on glutamatergic cells is increased, leading to an inhibition of glutamate release whose normal function is to inhibit sexual behavior. As such, it is possible that repeated administration of EB sensitizes this disinhibitory process.

In summary, the repeated administration of EB in the absence of any other manipulation leads to a sensitization of sexual behaviors in the OVX rat, and repeated VCS attenuates the sensitization of appetitive sexual behaviors. Since VCS accelerates the onset of estrous termination, we suggest that an increase in GABA signaling and/or a decrease in glutamatergic signaling within the vlVMH may be potential mechanisms involved in the sensitization of sexual behaviors by chronic administration of EB.

Chapter 3.2

Preface

In Chapter 3 Part 1 it was found that VCS inhibited the sensitization of sexual behaviors by repeated administration of EB. It is well recognized that VCS is inhibitory in at least one other situation, estrous termination. AMPA receptor activation within the vlVMH is believed to play a role in the induction of estrous termination. Thus, Experiment 1 assessed the role of AMPA receptor activation in estradiol sensitization. Experiment 2 examined whether the pattern of estrous termination differed between females that copulated on every test and those that did not have the opportunity to copulate.

3.2. Repeated administration of estradiol sensitizes mechanisms of sexual excitation and inhibition: glutamate signaling in the ventromedial hypothalamus attenuates excitation

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Abstract

Repeated administration of 10μg of estradiol benzoate (EB) every 4 days to the ovariectomized (OVX) rat induces a behavioral sensitization of sexual behaviors. Repeated copulation or the receipt of vaginocervical stimulation (VCS) attenuates the sensitization of appetitive sexual behaviors, suggesting that VCS acts in opposition to the mechanisms that induce the sensitization. Since it is known that VCS accelerates the onset of estrous termination (characterized by a decrease in appetitive sexual behaviors, and an increase in defensive behaviors prior to the decline in lordosis), and glutamate transmission in the ventromedial hypothalamus (VMH) is an important regulator of this effect, the current studies examined whether mechanisms of estrous termination are involved in the attenuation of sensitization to EB. In the first study, OVX rats received infusions of AMPA to the VMH on Tests 2-4, and sexual behavior was measured on Tests 1 and 5. Appetitive sexual behaviors were lower in females that received AMPA infusions in place of copulation compared to saline, suggesting that AMPA receptor activation by VCS may be playing a role in the attenuation of sensitization. In the second study, females that were not given the opportunity to copulate on tests 2-4 fell out of behavioral estrus faster than those that did, suggesting that both excitatory and inhibitory mechanisms of sexual behavior become sensitized with repeated administration of EB. Together these findings extend our hypothesis that repeated episodes of heat sensitize the activation of sexual behaviors to increase the probability of eventual fertilization.

Keywords: Estradiol sensitization, sexual behavior, estrous termination, vaginocervical stimulation, ventromedial hypothalamus, AMPA

Introduction

The acute administration of estradiol benzoate (EB) to the ovariectomized (OVX) rat induces a low frequency of lordosis in response to mounts from a male, and very few sexually appetitive behaviors such as hops, darts, solicitations, and ear wiggles. Sexually appetitive behaviors are reliably and maximally induced, and lordosis is potentiated, four hours following an acute injection of progesterone (P) administered 36 to 48 hours following the EB injection (Boling & Blandau, 1939). However, chronic administration of 5µg or 10µg EB alone every four days results in a behavioral sensitization such that following the fourth injection, lordosis is maximally induced and sexually appetitive behaviors occur more frequently compared to the initial injection and reach a plateau (Jones et al., 2013). This behavioral sensitization occurs independently of adrenal P (Jones et al., 2013). Interestingly, sensitization of appetitive behaviors is attenuated if females receive vaginocervical stimulation (VCS) during each episode of heat (either from penile intromission or experimenter-applied; Jones, S.L., Germé, K., Roy, P., et al., *submitted*). VCS has also been shown to accelerate the onset of estrous termination, which is characterized by a decrease in sexually appetitive behaviors and an increase in defensive behaviors before a decline in lordosis frequency and magnitude is observed (Pfaus et al., 2000). Thus, here we investigated whether a mechanism underlying estrous termination might be involved in the attenuation of sensitized sexually appetitive behaviors by repeated copulation.

Glutamatergic signaling in the ventrolateral portion of the ventromedial hypothalamus (vlVMH) inhibits sexual behavior yet the inhibition is delayed or reduced if females are primed with EB. Presenting the OVX rat with a sexually vigorous male induces glutamate release into the vlVMH, and the release is attenuated by prior treatment with EB (Georgescu et al., 2014). Moreover, sexual behavior is facilitated in OVX EB-primed rats by infusions of GABA-a agonists (Mccarthy, 1995; McCarthy et al., 1990), or glutamate receptor antagonists (Georgescu & Pfaus, 2006a) into the vlVMH whereas infusions of glutamate or its ionotropic receptor agonists rapidly inhibit lordosis (Georgescu & Pfaus, 2006b; Kow et al., 1985; McCarthy et al., 1991) and hops and darts, while activating defensive behaviors (Georgescu & Pfaus, 2006b), similar to the behavioral pattern characteristic of estrous termination. Importantly, that endogenous glutamate within the VMH is inhibitory to lordosis and is under the control of GABA and steroid hormones has also been supported electrophysiologically in tissue slices examined across the natural estrous cycle (Booth et al., 2010). The onset of estrous termination is

accelerated by the receipt of VCS from penile intromissions or experimenter applied with a glass rod (e.g., Erskine & Baum, 1982; Lodder & Zeilmaker, 1976; Pfaus et al., 2000); however if the frequency of hormone priming is increased from every 28 days to every 14 days to every 4 days, the inhibitory effect of VCS becomes progressively less robust (Pfaus et al., 2000). The administration of VCS also activates Fos, a marker of neuronal activation, within numerous sexually-relevant brain regions (Pfaus et al., 1996), including glutamate neurons of the vlVMH (Georgescu et al., 2009), but the number of Fos-labelled glutamate cells in the vlVMH following VCS is reduced by EB+P priming compared to oil treated controls (Georgescu et al., 2009). Importantly, the ability of VCS to induce estrous termination is prevented by blocking the AMPA/kainate glutamate receptor subtype (Georgescu et al., 2012). Together those data suggest that EB prevents the inhibition of sexual behavior by VCS that appears to be mediated, at least in part, by glutamate transmission and more specifically the activation of AMPA/kainate receptors within the vlVMH.

Because VCS attenuates the sensitization of sexually appetitive behaviors by EB, the first behaviors that decline in frequency as estrous termination sets in (Pfaus et al., 1999), the overarching goal of these studies was to examine whether mechanisms related to estrous termination are involved in the attenuation of appetitive behaviors in females that are repeatedly treated with EB and given the opportunity to copulate on every test. Since glutamate and its ionotrophic receptor agonists infused in the vlVMH inhibit sexual behavior in a pattern reminiscent of estrous termination (Georgescu & Pfaus, 2006b), and antagonizing the AMPA receptor subtype blocks the effect of VCS on estrous termination (Georgescu et al., 2012), in the first experiment we hypothesized that AMPA infusions to the vlVMH would mimic the effect of repeated copulation in females repeatedly treated with EB, resulting in an attenuation of the EB induced sensitization compared to vehicle infused animals. In the second study we hypothesized that estrous termination would be accelerated in females that copulate during each episode of heat (i.e., that repeatedly receive VCS through copulation) compared to those restricted from copulating on intermediate tests.

Materials and methods

Animals

Animals were purchased from Charles River Canada (St-Constant, Québec, Canada), and given one week to acclimate to our facilities. Females were housed in pairs in clear Plexiglas® cages lined with a mixture of corncob and sanichip bedding (females), or in groups of four in large gang cages lined with sanichip (males). Food (Charles River, 5075) and tap water were freely available. Rooms were maintained at 21C, on a 12-hour reverse cycle (lights off at 8AM). Following approximately one-week acclimatization to the animal facility, females were OVX and males were given four sexual training sessions in the pacing chambers with a separate group of OVX stimulus females primed with EB+P. Two cohorts of animals were used for the AMPA study occurring at 3-month intervals. A group of sexually-experienced OVX rats, used in unrelated studies, were used as stimulus females in the estrous termination study.

Animal pain and discomfort was minimized throughout the duration of the animal's stay in our facilities. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Concordia University Animal Research Ethics Committee.

Surgeries

Ovariectomy.

Ovaries were removed bilaterally following a 1 mL/kg IP injection of a 4:3 mixture of ketamine hydrochloride (50mg/mL; Ketaset ©, Wyeth Canada) and xylazine hydrochloride (4mg/mL; Rompum ©, Bayer Healthcare). Animals were also ear punched for identification purposes, and post-operative care was given with SC injections of PenG (0.1mL/rat) and 2.5 mg/kg/mL of flunixin meglumine (Banamine©), and rehydrated with 2 mL of saline administered SC. Animals were given one week post-operative recovery prior to sexual behavior training.

Cannulation.

Cannulations occurred within 3 days of the final sexual training session (described below), during the 2-week hormone washout period. Females were anesthetized using an isofluorane:oxygen gas mixture (2.5-5% isofluorane:0.8L/min O2) and placed into a Kopf stereotaxic instrument. A mixture of 0.05% lidocaine (CDMV-3913) and 0.05% Marcaine

(0.25%, CDMV-95865) in an 8.4% sodium bicarbonate solution (CDMV-93-500-EV) was injected below the scalp prior to incision. Painkiller (0.2mL Anafen) and antibiotic (0.2mL PenG) were then injected SC, and 2mL of saline was administered both before and after surgery for rehydration purposes, and eyes were kept hydrated with an ocular gel (Natural Tears®, Alcon). Using the flat skull technique, bilateral guide cannula (C232G-1.5/SPC 11mm; Plastics One) were then implanted targeting the vlVMH according to the following coordinates: AP 2.6mm, ML ±0.75mm, DV 8.4mm. Guide cannulae were fixed to the skull by three stainless steel screws and dental cement. The dummy cannula (C232DC-1.5; Plastics One, Roanoke, VA) was then inserted, extending 0.5mm beyond the guide, and covered with a dust cap (303DC/1; Plastics One, Roanoke, VA). Finally, Polysporin ® was applied to the incision site. Infusion cannulae (C232i-1.5/SPC; Plastics One, Roanoke, VA) extended 1mm beyond the guide.

Hormone and drug preparation and administration

EB (10μg) and P (500μg) were dissolved in 0.1mL reagent grade sesame oil and administered by SC injection 48 and 4 hours, respectively, prior to behavioral training or testing as indicated. Steroids were obtained from Steraloids (Hanover, NH).

AMPA (2mmol) was dissolved in 0.9% physiological saline and infused into the bilateral cannulae using a Hamilton syringe connected to PE50 tubing connected to an infusion pump (Hamilton Apparatus, Pump 22) at a rate of 1μl/2 minutes on test days 2 through 4. The infuser was left inside for an additional 1.5min in order for the drug to be absorbed by the tissue (Georgescu & Pfaus, 2006b; Kow et al., 1985). Controls were infused with saline vehicle.

Testing apparatus

All training and behavioral testing occurred in unilevel 4-hole pacing chambers (38 x 60 x 38 cm) lined with sanichip below a grid floor (2.54 cm elevation). The chamber is bisected with a clear Plexiglas® divider that has 4 square holes cut in the bottom, which can be adapted to the size of the animals, and is used to restrict the larger male to one compartment while the smaller female is free to traverse to either side, allowing her to pace the sexual interactions with the male.

Training procedures

All training and testing began during the middle third of the rat's dark cycle. One week following OVX, females were primed with EB and P prior to each of four sexual training sessions occurring at 4-day intervals. A male was randomly selected and placed on the right side of the chamber for a 5-minute acclimation period before introducing the female for a 30-minute sexual training session. Each male was only used once on each training or test day. Next females were given a 2-week hormone washout period prior to testing. The training and test procedures for each experiment are summarized in Figure 1.

Test procedures

Experiment 1. AMPA infusions to the vlVMH.

One week following cannulation surgery (i.e., during the hormone washout period), cannulated females were habituated to the test procedures by placing them into the infusion chamber (a clear Plexiglas® cage lined with corncob), removing and immediately replacing the dummy and running the Hamilton Apparatus without any infusion. Next they were placed into the 4-hole pacing chamber for 15-minutes for 3 consecutive days, to habituate movement through the chamber holes with the head caps. In a pilot project we found that females quickly learned to lower their head to facilitate movement through the holes. No hormones or drugs were administered during this habituation period.

Following the hormone washout period, EB was administered SC 48-hours prior to each of 5 test sessions. On the first and fifth test days, males were placed on one side of the 4-hole pacing chamber and given a 5-minute habituation period before introducing the female, and the pair was left undisturbed for a 30-minute sexual behavior test period. On tests 2-4, females were infused with either saline (n=12) or AMPA (n=12), before being placed in the 4-hole pacing chamber alone for 30-minutes.

Experiment 2. Tests of estrous termination.

Females were injected with 10µg EB 48 h prior to each test day, and assigned to one of two copulatory conditions, where they either copulated (EB+Male) or were placed in the chamber alone (EB-Alone) (as in, Jones & Pfaus, 2014) on tests 2-4. All animals copulated on the first test day. To assess the effect of VCS on estrous termination, on test day 5 females were not given behavioral testing, rather at 21:00 h they were given either 50 VCS or SHAM over the

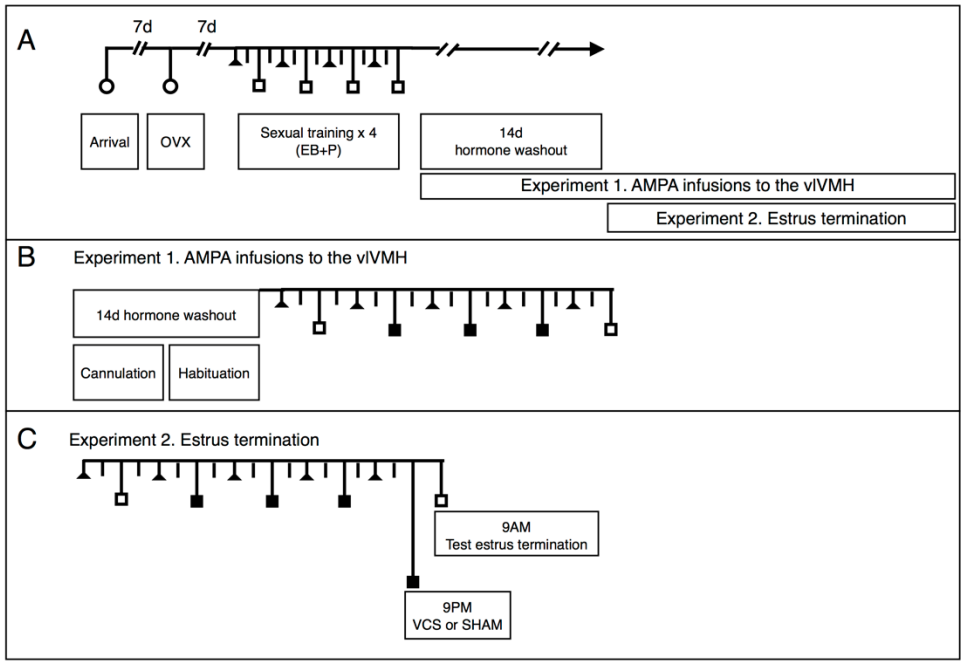

Figure 1. Timeline and procedures. A. In both studies, females were ovariectomized (OVX) one week after arrival to the facilities and given a week to recover. Next they were treated with estradiol benzoate (EB) 48 hours prior, and progesterone (P) 4 hours prior to each of four sexual training tests in the 4-hole pacing chamber, followed by a 2-week hormone washout prior to the start of the experimental phase. B. In Experiment 1, females were cannulated within the first three days of the washout period, given a week to recover, then given three consecutive days of habituation to the experimental procedures (see Test Procedures for details). At the end of the washout period, females were treated with EB prior to each test day. On test 1, females copulated with a sexually vigorous male, and manipulations were made on tests 2-4. Females were then infused with AMPA or Saline to the vlVMH and placed in the pacing chamber alone. Sexual behavior was again measured on Test 5. In Experiment 2, following the washout period, females were treated with EB 48 hours prior to each of four test days. On test 1 they were given access to a sexually vigorous male in the 4-hole pacing chamber. On tests 2-4 they were placed in the 4-hole pacing chamber with a sexually vigorous male, or alone. Two days later they were injected with EB and on Test day 5, they were given VCS or SHAM at 9PM. The following morning at 9AM they were placed in the 4-hole pacing chamber and sexual behavior was measured. Full triangles represent injection of 10μg EB. Open squares indicate that all females had access to a sexually vigorous male in the 4-hole pacing chamber. Full squares indicate experimental manipulations.

course of 1 h, as in Pfaus et al., (Pfaus et al., 1996; 2000), creating a total of four groups (EB+Male/SHAM, EB-Alone/SHAM, EB+Male/VCS, EB-Alone/VCS, n=6/group), and their sexual behavior was tested 12 h later (Pfaus et al., 2000). To administer VCS, females were placed in a clear Plexiglas® cage lined with Sani-chip® bedding, gently held at the base of the tail, and a glass rod with polished ends lubricated with K-Y Jelly® was inserted into the vagina four times at 1 second intervals for approximately 0.5 seconds; the first three insertions only entered the anterior vagina, whereas the rod was pressed firmly against the cervix on the fourth insertion for approximately 2 seconds then removed. VCS was applied in clusters of 5, every 6 minutes, such that they received a total of 50 VCS over the course of an hour (Pfaus et al., 1996; 2000). SHAM animals were handled in the same way for the same amount of time, without insertion of the rod. Rats were returned to their home cage between clusters.

Twelve hours following VCS or SHAM (i.e., beginning at 9AM the following day) females were tested for estrous termination. Male rats were first exposed to fully primed EB+P stimulus females to ensure they were sexually aroused (determined by intromissions with the stimulus female), then placed in the 4-hole pacing chamber for a 5-minute habituation period. Experimental females were then introduced to the chamber until one ejaculation or up to ten minutes, whichever came first (similar to Pfaus et al., (2000)).

Histology

Within seven days of the final behavioral test, cannulated females were bilaterally infused with methylene blue (40 mg/mL distilled water; 0.5µL over 1 min) and were then sacrificed via an overdose of sodium pentobarbital injected IP (120 mg/kg/mL). Intracardial perfusions were then conducted using 120 mL of cold phosphate buffered saline (PBS) followed by 120 mL of 4% paraformaldehyde in 0.1M PBS. Brains were then removed and post-fixed in 4% paraformaldehyde for 4 hours then transferred into a 30% sucrose solution overnight at 4ºC, flash-frozen over dry ice and stored at -80ºC until slicing.

Brains were mounted onto a chuck and sliced using a cryostat set at -21ºC. Coronal sections (40 µm) were taken just before and after cannula tracks and were immediately mounted onto gel-coated microscope slides. Sections were stained in an 8% cresyl violet solution, dehydrated, cleared with xylene and cover-slipped using Permount. Placements were then examined under a light microscope. Cannulation tracks located beyond the anterior or posterior

regions of the VMH or more than 1mm dorsal to it were omitted from all statistical analyses (as in Georgescu & Pfaus, 2006b). Correct cannula placements from all animals included in the statistical analyses are shown in Figure 2.

Behavioral measures

The occurrence of each lordosis magnitude (LM) was coded on a 3-point scale (Hardy & Debold, 1971a), and the lordosis quotient (LQ) was calculated as a ratio of the total number of LM divided by the total number of mounts with or without intromission and expressed as a percentage. Appetitive measures of sexual behavior included partial solicitations (hops/darts) and full solicitations (defined as a headwise orientation followed by a runaway, (Erskine, 1989; McClintock, 1984)). Full solicitations are indicative of greater sexual motivation compared to hops and darts, since they are observed more frequently in females treated with EB+P compared to those treated acutely with EB alone (Pfaus et al., 1999). Kicks, sideways takedowns, boxing postures, and prone positions made by the female were coded as defensive behaviors (Barnett, 1963). Mounts, intromissions, and ejaculations by the male were also analyzed, as they provide a more thorough overview of the female's behavior (Pfaus et al., 1999).

Statistical analyses

Experiment 1. AMPA infusions.

Statistical analyses were conducted using SPSS version 16.0 for Windows. For each cohort, raw data were standardized across tests, as such data are presented as *Z*-scores. Outliers on appetitive behaviors and LQ, the two main outcome variables, were detected using boxplots. Planned comparisons were used to test whether 1) each group would display sensitization of appetitive behaviors and LQ using dependent *t*-tests, and 2) whether behavioral sensitization differed between saline and AMPA infused animals on the final test day using independent *t*-tests. Eta squared (η^2) and *Cohen's d* are reported as measures of effect size.

Experiment 2. Estrous termination.

Tests of estrous termination were analyzed using a two-way ANOVA with male exposure condition (Male or Alone) and VCS (VCS or SHAM) as between subject factors. Chi square (χ^2) was used to examine the proportion of females that received an ejaculation across groups. All significance levels were set at $p<0.05$.

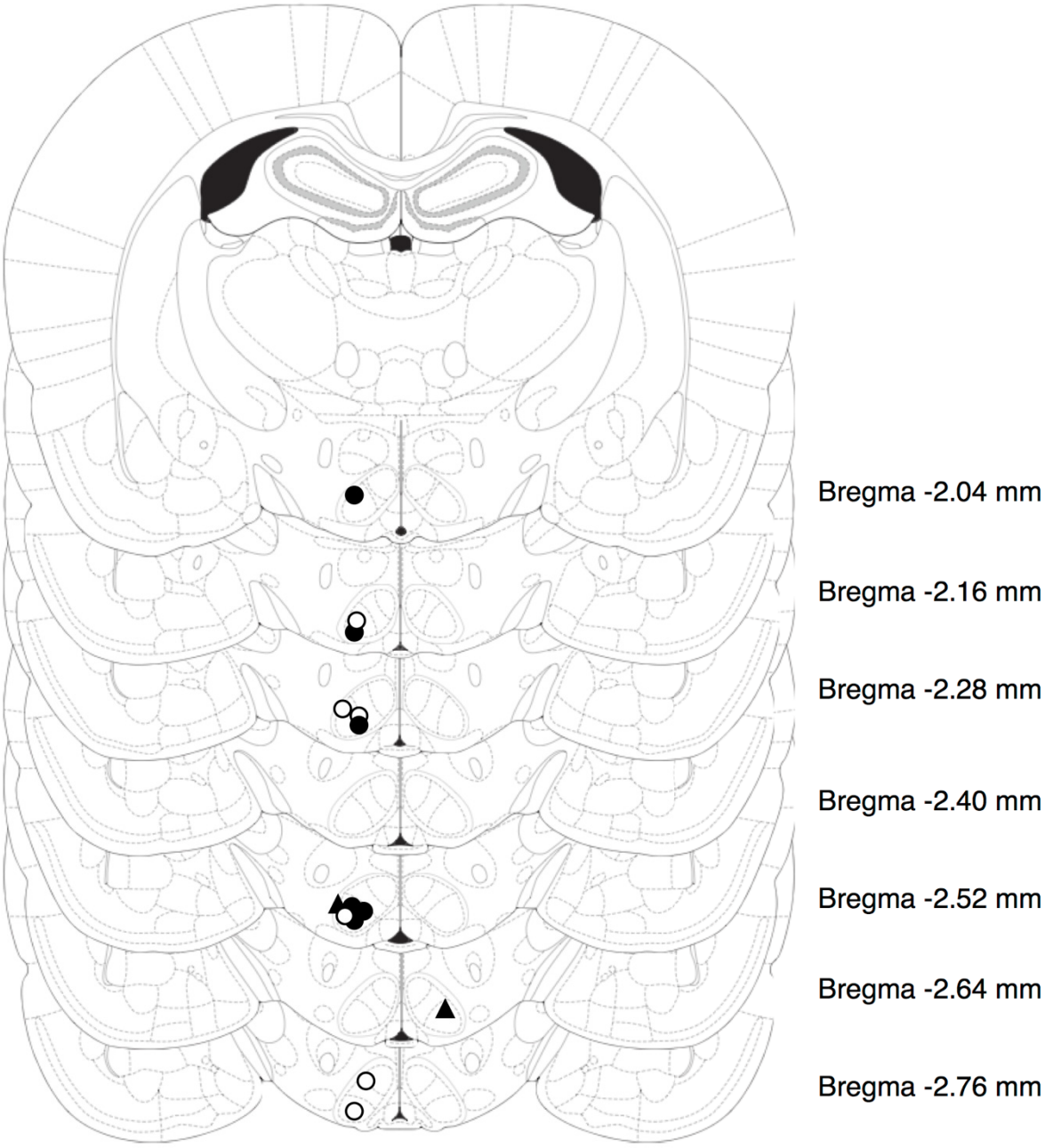

Figure 2. Cannula placements into the vlVMH for all rats included in statistical analyses. Placements represent bilateral coordinates of saline (open circles) and AMPA (full circles) infused animals with correct placements in the same plane. Triangles indicate placements from each hemisphere that were on different planes (i.e., same animal). Figures reproduced from Paxinos and Watson (2004).

Results

Experiment 1. AMPA infusions to the vlVMH

Two saline treated females had placements outside the VMH and were excluded from all analyses. Five animals did not recover well following cannulation surgery and two AMPA treated females did not respond well to the first infusion, as they displayed erratic hyperactivity, or became catatonic, and were immediately excluded from the study. Two females were excluded from the statistical analyses due to a high number of appetitive behaviors on Test 1 (one from each group), or test 5 (one AMPA treated female) as determined by boxplots. Final group sizes were 6 for saline and 7 for AMPA.

Lordosis measures are shown in Figure 3. Females that were not mounted could not be included in the statistical analyses on LQ. As expected and supported by the large effect sizes, LQ sensitized whether females received infusions of saline, $t(3)=2.56$, $p=0.083$, $d=1.42$, or AMPA, $t(6)=2.96$, $p=0.025$, $d=1.10$. Saline treated females displayed more LM3 on the final test day compared to the first, $t(5)=2.51$, $p=0.054$, $d=1.99$, and although not statistically significant, the medium effect sizes suggest that LM1, $t(5)=0.79$, $p=0.465$, $d=0.63$ and LM2, $t(5)=0.84$, $p=0.438$, $d=0.56$, also increased across tests. In AMPA treated females, the frequencies of LM2, $t(6)= 3.57$, $p=0.012$, $d=0.50$, and LM3, $t(6)=3.51$, $p=0.013$, $d=1.29$ also increased, while there was no effect on the frequency of LM1, $t(6)=0.15$, $p=0.887$, $d=0.09$. AMPA infusions did not reduce LQ compared to saline treated animals on the final test day, $t(11)=0.17$, $p=0.865$, $d=0.10$, nor did it affect LM1, $t(11)=0.24$, $p=0.815$, $d=0.14$, although the medium and large effect sizes respectively, suggest that AMPA treated animals displayed more LM2, $t(11)=1.08$, $p=0.302$, $d=0.65$, and LM3, $t(11)=1.83$, $p=0.094$, $d=1.10$ compared to saline treated animals.

As shown in Figure 4, appetitive sexual behaviors sensitized in both groups, (saline: $t(5)=2.53$, $p=0.053$, $d=0.71$; AMPA: $t(6)=5.60$, $p=0.001$, $d=1.97$), which was driven by the effect on hops/darts (saline: $t(5)=2.54$, $p=0.051$, $d=0.71$; AMPA: $t(6)=5.40$, $p=0.002$, $d=1.83$) given that solicitations did not sensitize in either group (saline: $t(5)=1.00$, $p=0.363$, $d=0.45$; AMPA: $t(6)=1.55$, $p=0.173$, $d=0.77$), and in fact, the raw data indicate that very few solicitations were displayed at all (<1 solicitation on average per group in both cohorts, as such data are not shown). On the final test day, the medium effect size suggests that AMPA infused animals displayed fewer appetitive behaviors compared to saline treated animals, $t(11)=0.66$, $p=0.523$, $d=0.40$,

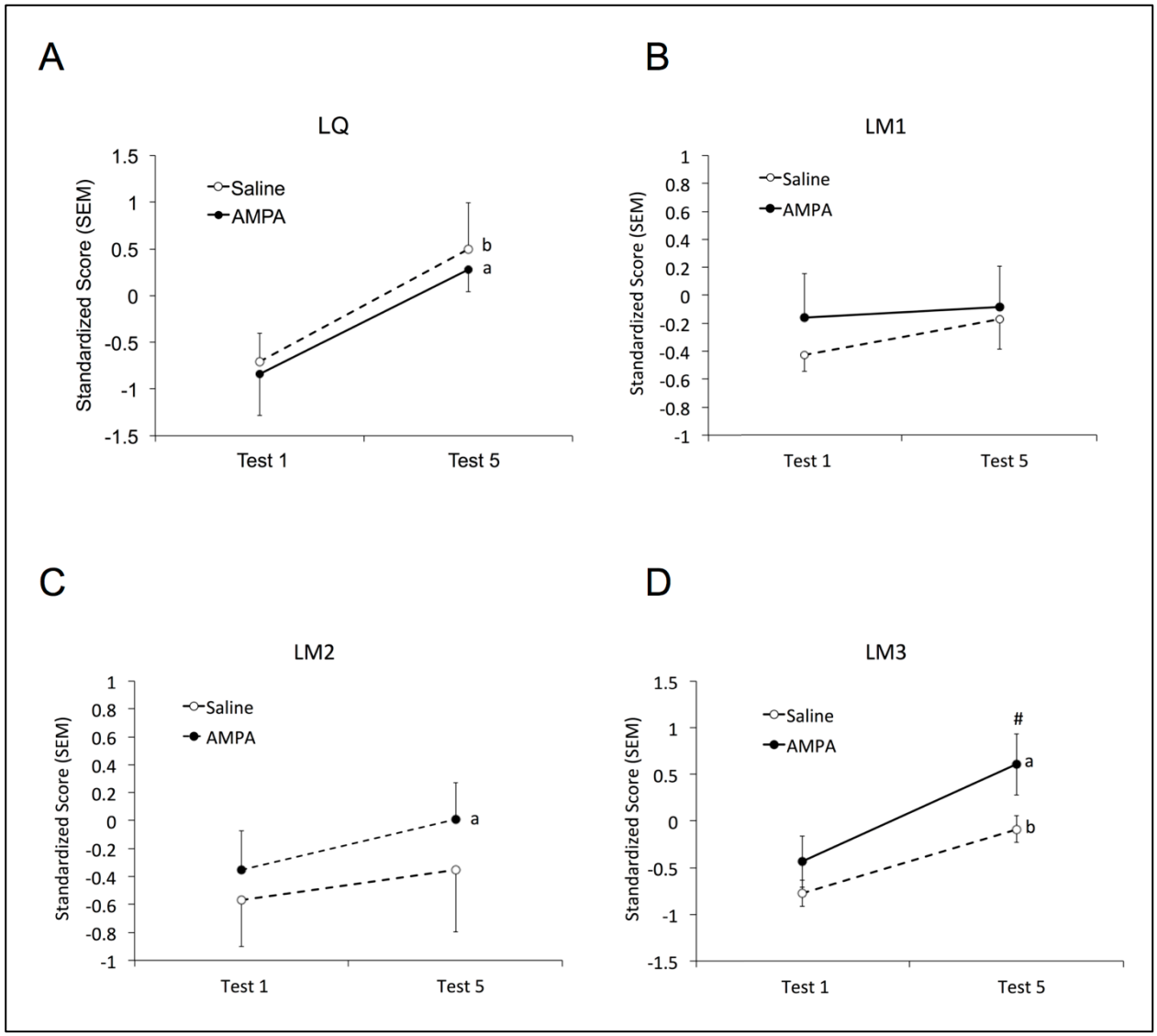

Figure 3. Standardized lordosis measures of OVX rats repeatedly treated with EB and allowed to copulate with a sexually vigorous male on tests 1 and 5. On tests 2-4 females received an infusion of saline, or AMPA into the vlVMH. [a]Different from test 1, $p<0.05$. [b]Different from test 1, $p<0.10$. [#]Between group difference, $p<0.10$. See text for effect sizes.

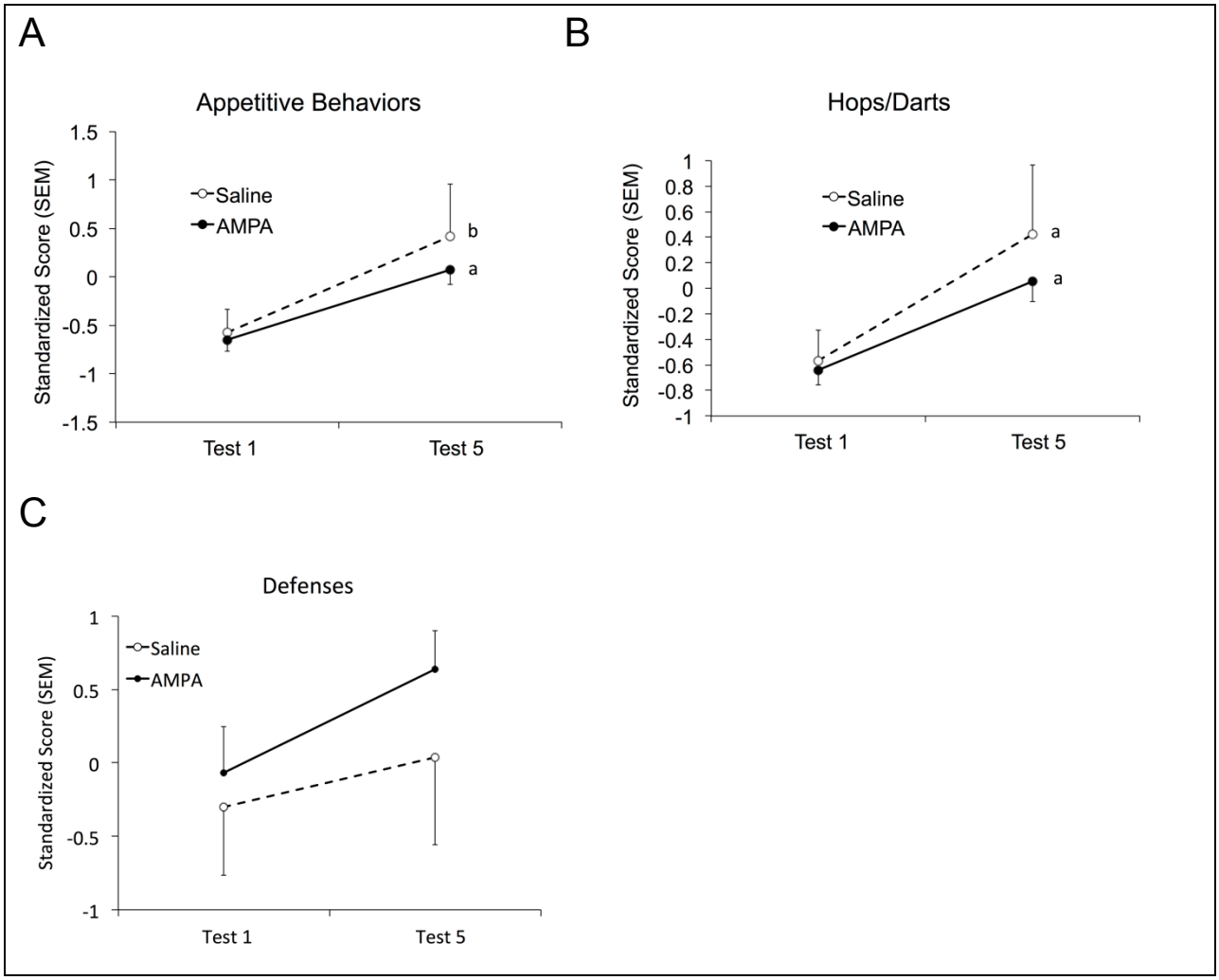

Figure 4. Appetitive behaviors of OVX rats treated repeatedly with EB and allowed to copulate with a sexually vigorous male on tests 1 and 5, standardized across cohorts. On tests 2-4 females received an infusion of saline, or AMPA into the vlVMH. [a]Different from test 1, $p<0.05$. [b]Different from test 1, $p<0.10$. See text for effect sizes.

driven by the effect on hops/darts, $t(11)=0.71$, $p=0.492$, $d=0.428$, the only appetitive component that had sensitized (solicitations: $t(11)=0.95$, $p=0.363$, $d=0.57$, data not shown).

Defensive behaviors, shown in Figure 4, did not change across tests in saline treated animals, (saline: $t(5)=0.578$, $p=0.588$, $d=0.26$), although the large effect size suggests that AMPA infusions increased defensive behaviors on the final test day compared to the first, (AMPA: $t(6)=1.74$, $p=0.133$, $d=0.93$). The medium effect size suggests that on the final test day AMPA treated animals were more defensive towards males than were saline treated animals, $t(11)=0.97$, $p=0.353$, $d=0.58$.

Male behaviors are shown in Figure 5. The large effect sizes suggest that saline treated females received more mounts, $t(5)=0.94$, $p=0.391$, $d=0.66$, intromissions, $t(5)=1.24$, $p=0.271$, $d=0.94$, and ejaculations, $t(5)=1.46$, $p=0.203$, $d=0.74$ on the final test day compared to the first, whereas in AMPA treated females, the large and medium effect sizes, respectively, suggest that mounts, $t(6)=2.44$, $p=0.05$, $d=1.20$, and intromissions, $t(6)=1.19$, $p=0.278$, $d=0.60$ increased across tests, while the number of ejaculations received did not, $t(6)=0.19$, $p=0.858$, $d=0.09$. Large effect sizes suggest that on the final test day AMPA treated animals received more mounts, $t(11)=2.00$, $p=0.070$, $d=1.20$, and intromissions, $t(11)=1.63$, $p=0.131$, $d=0.98$, than saline treated animals, but did not differ in the number of ejaculations received, $t(11)=0.16$, $p=0.880$, $d=0.09$.

Experiment 2. Estrous termination

Lordosis. Two females in the Male/SHAM group were not mounted and as such were excluded from the statistical analyses on LQ. As expected, those that were given VCS the evening prior had a lower LQ the following morning as shown in Figure 6 (main effect of VCS condition, $F(1,18)=7.86$, $p=0.012$, $\eta^2=0.17$). In contrast to our prediction, those that copulated on every test (EB/Male) had a higher LQ on the following morning compared to those that did not (EB/Alone) (main effect of male exposure condition, $F(1, 18)=21.00$, $p<0.001$, $\eta^2=0.45$). The interaction was not statistically significant, $F(1,18)=0.03$, $p=0.857$, $\eta^2=0.000$.

Those females that copulated on every test (EB/Male) displayed more LM3 (Figure 6) compared to those that were not given the opportunity to copulate (EB/Alone) (main effect of male exposure condition: $F(1,20)=4.86$, $p=0.039$, $\eta^2=0.19$). The effect of VCS, $F(1,20)=0.36$, $p=0.555$, $\eta^2=0.01$, and the interaction, $F(1,20)=0.36$, $p=0.555$, $\eta^2=0.01$, were not significant. LM1 and LM2, shown in Figure 6, did not differ between VCS (LM1: $F(1,20)=0.08$, $p=0.774$,

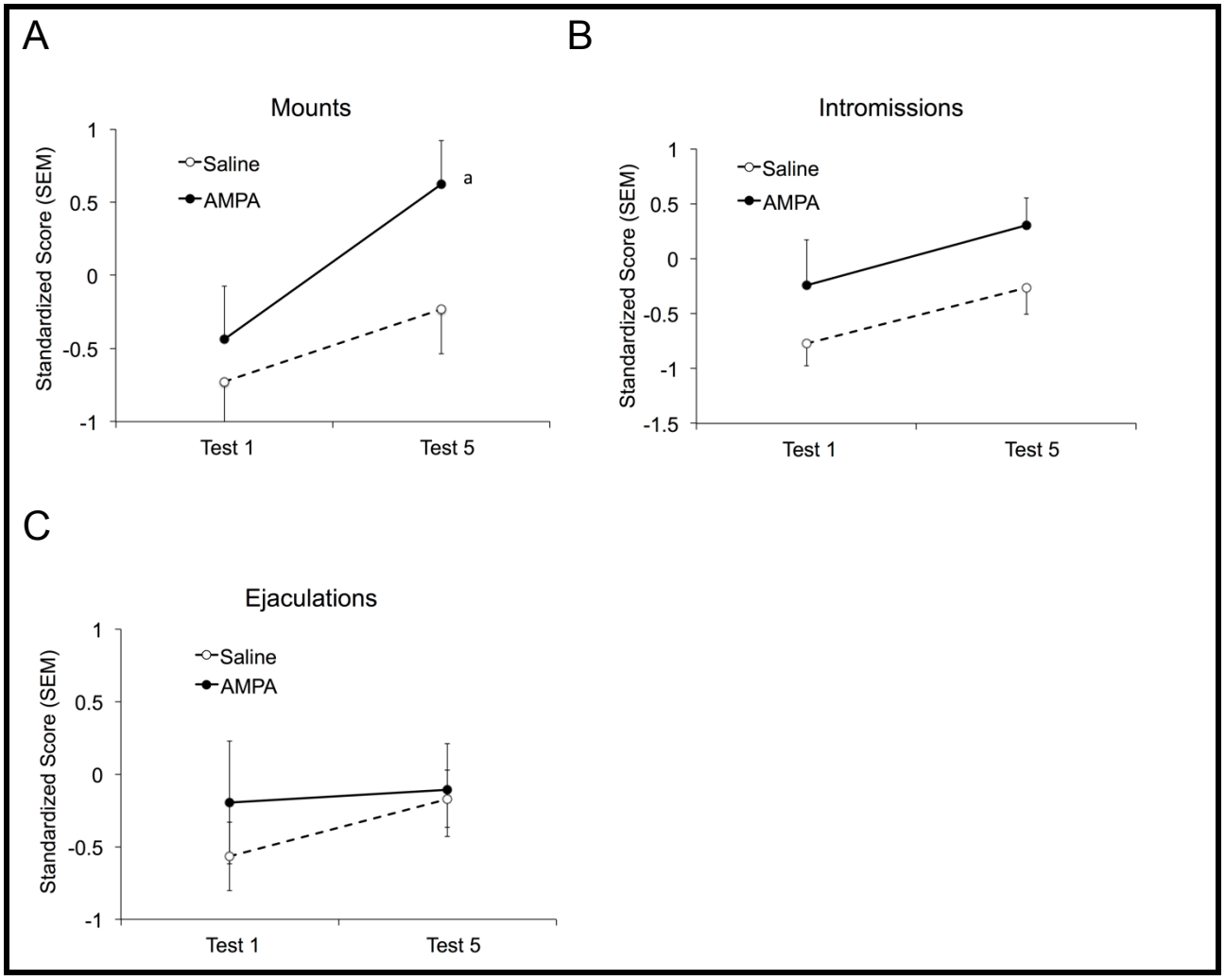

Figure 5. Mounts, intromissions and ejaculations that females received from the male on tests 1 and 5, standardized across cohorts. On tests 2-4 females received an infusion of saline, or AMPA into the vlVMH. [a]Different from test 1, $p<0.05$. See text for effect sizes.

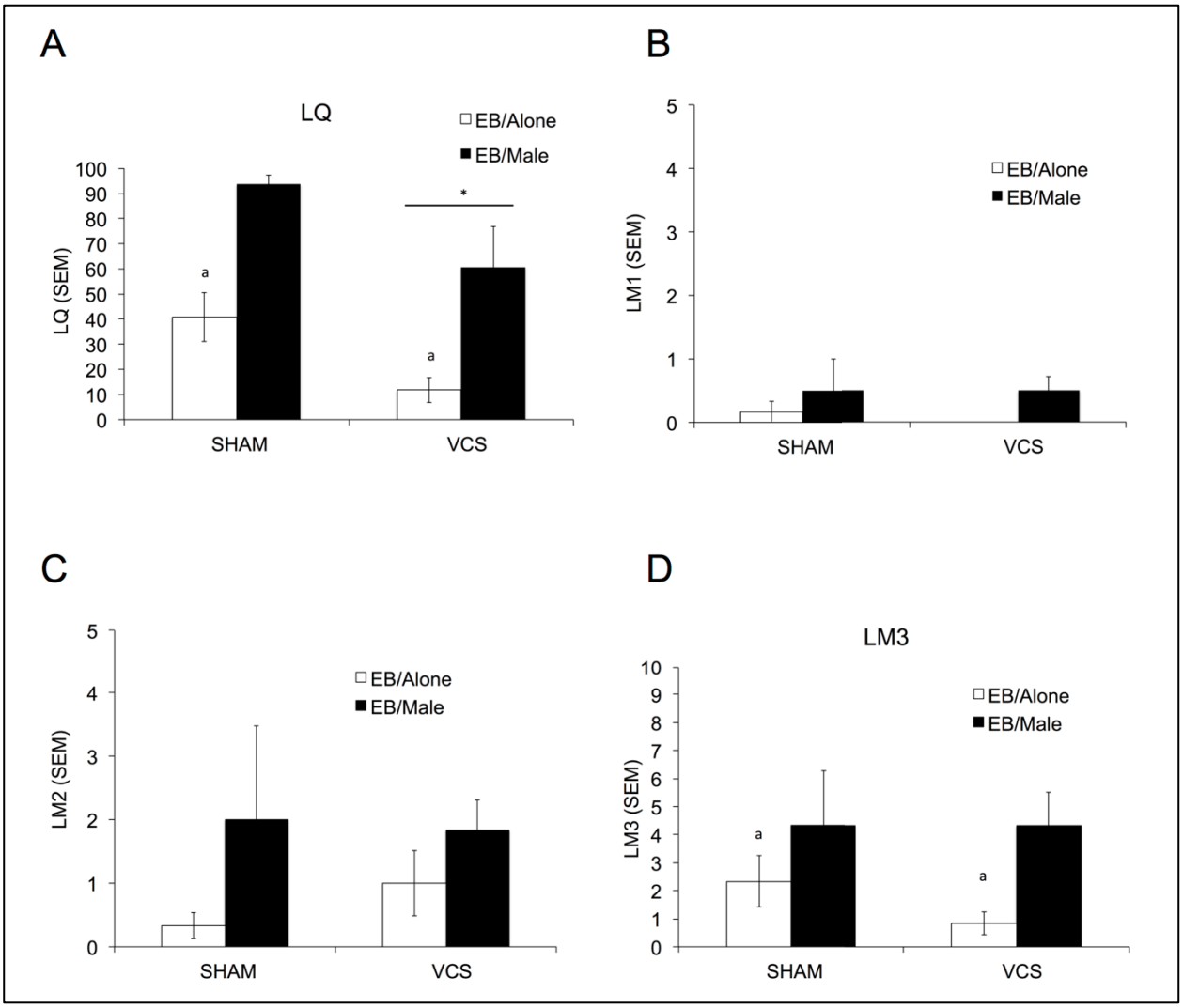

Figure 6. LQ of OVX rats repeatedly treated with EB and allowed to copulate with a sexually vigorous male (EB/Male) on every test, or placed in the chamber alone (EB/Alone) on intermediate tests (tests 2-4). Females were given VCS or SHAM on test day 5 and sexual behavior was examined the following morning, to test estrous termination. [a]Significantly lower than EB/Male condition. *Significantly different from SHAM. See text for effect sizes.

η^2=0.00; LM2: F(1,20)=0.09, p=0.766, η^2=0.10) or male exposure conditions (LM1: F(1,20)=2.12, p=0.161, η^2=0.10; LM2: F(1,20)= 2.28, p=0.147, η^2=0.00), and the interactions between conditions were not significant, LM1: F(1,20)=0.08, p=0.774, η^2=0.00; LM2: F(1,20)=0.25, p=0.620, η^2=0.01).

Appetitive behaviors. The breakdown of appetitive behaviors is shown in Figure 7. Very few solicitations were observed overall (all group means <1), as such, no effect of VCS condition, F(1,20)=1.45, p=0.242, η^2=0.06, or male exposure condition, F(1,20)=1.45, p=0.242, η^2=0.06, was found, and their interaction was not significant, F(1,20)=0.16, p=0.692, η^2=0.01. There were no effects on hops/darts, regardless of male exposure, F(1,20)=0.89, p=0.357, η^2=0.04, or VCS conditions, F(1,20)=0.52, p=0.479, η^2=0.02, and the interaction was not statistically significant, F(1,20)=0.03, p=0.870, η^2=0.00. Given that nearly no solicitations were coded, the sum of appetitive behaviors is not shown, as they closely resemble hops/darts.

Defenses. As expected, females that received VCS the night before testing were significantly more defensive compared to those that received SHAM, F(1,20)=8.76, p=0.008, η^2=0.28, as shown in Figure 7. However whether or not they copulated on every test had no effect, F(1,20)=2.49, p=0.131, η^2=0.08, and the interaction was not statistically significant, F(1,20)=0.40, p=0.532, η^2=0.01.

Mounts and intromissions. Those females that received VCS were mounted more frequently compared to those that received SHAM stimulation (F(1,20)=7.212, p=0.014, η^2=0.26) (Figure 8). The main effect of male exposure condition, F(1,20)=0.01, p=0.924, η^2=0.00 and the interaction, F(1,20)=0.06, p=0.804, η^2=0.00, were not significant. As shown in Figure 8, no differences were detected on the number of intromissions received, (VCS condition: F(1,20)=0.35, p=0.559, η^2=0.02, male exposure condition: F(1,20)=0.63, p=0.438, η^2=0.03, interaction: F(1,20)=0.04, p=0.845, η^2=0.00).

Ejaculations. As expected, there tended to be fewer females that received an ejaculation from the male if they received VCS (1/12) the prior evening compared to those that received SHAM (5/12) (collapsed across copulatory condition), χ^2=3.556, p=0.059.

When examining whether copulatory condition played a role, we found that of those females that copulated on every test (EB/Male), there were no differences between SHAM (2/6) and VCS (1/6) conditions in the number of females that received an ejaculation within the first 10 minutes χ^2=0.44, p=0.505. However, in those females that were not given the opportunity to

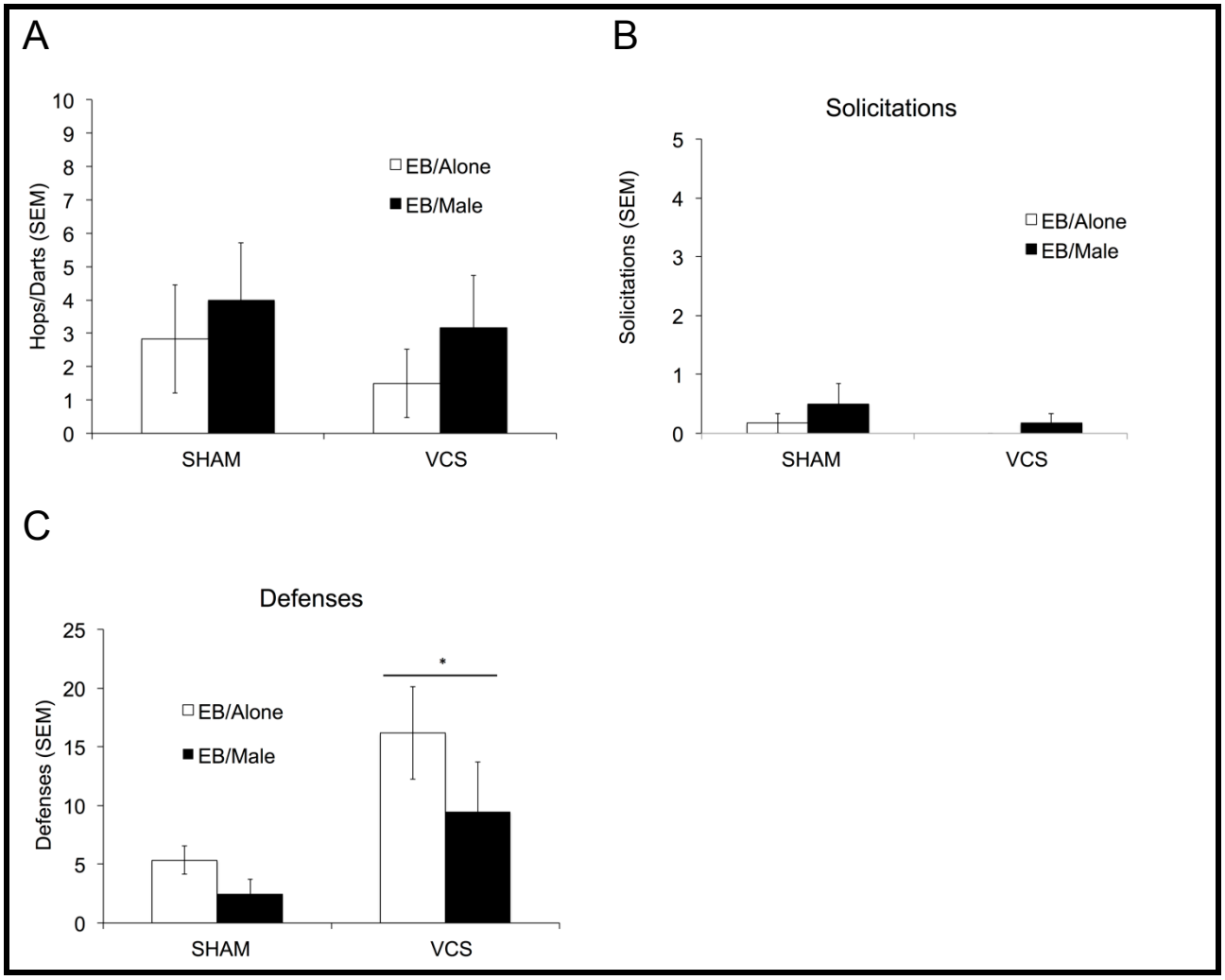

Figure 7. Solicitations and hops/darts displayed by OVX rats repeatedly treated with EB and allowed to copulate with a sexually vigorous male (EB/Male) on every test, or placed in the chamber alone (EB/Alone) on intermediate tests (tests 2-4). Females were given VCS or SHAM on test day 5 sexual behavior was examined the following morning, to test estrous termination. [a]Significantly lower than EB/Male condition. *Significantly different from SHAM. See text for effect sizes.

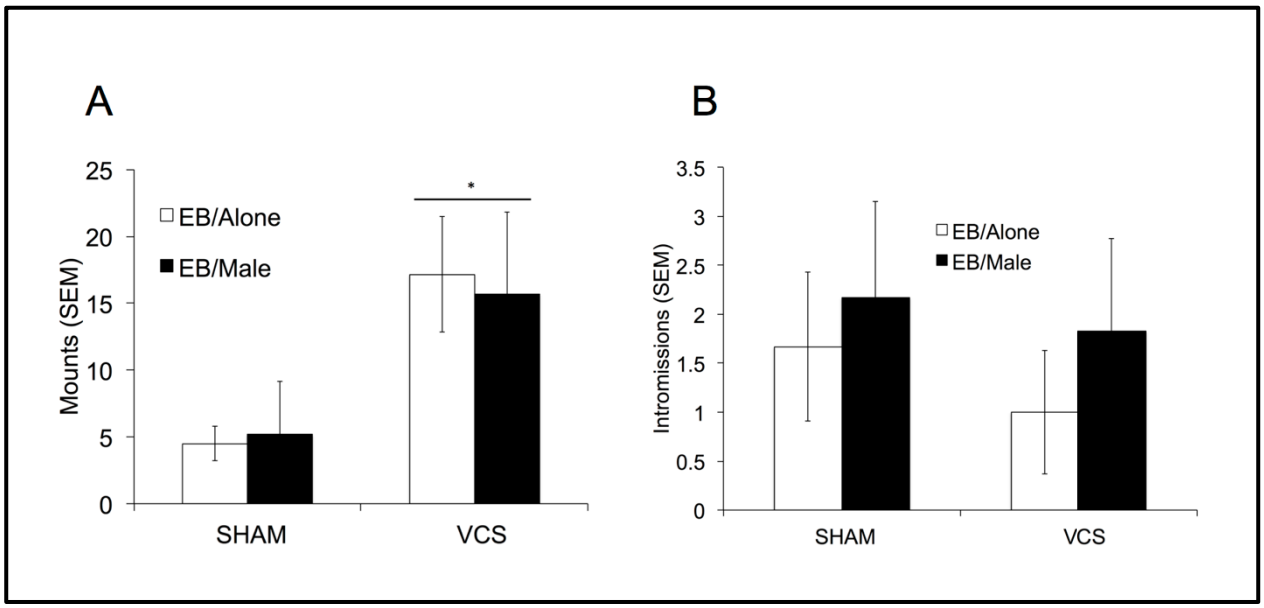

Figure 8. Mounts and intromissions received from males by OVX females repeatedly treated with EB and allowed to copulate with a sexually vigorous male (EB/Male) on every test, or placed in the chamber alone (EB/Alone) on intermediate tests (tests 2-4). Females were given VCS or SHAM on test day 5 sexual behavior was examined the following morning, to test estrous termination. [a]Significantly lower than EB/Male condition. *Significantly different from SHAM. See text for effect sizes.

copulate on intermediate tests (EB/Alone), whereas half the animals received an ejaculation if they received SHAM the prior evening (3/6) no female received an ejaculation if they were given VCS the prior evening (0/6), χ^2=4.00, p=0.046.

Discussion

The purpose of these studies was to examine whether mechanisms related to estrous termination are involved in the attenuation of the EB-induced sensitization of appetitive sexual behaviors in OVX females that receive repeated VCS. In the first study we confirmed that following repeated administration of EB, lordosis measures and hops and darts sensitized whether females received infusions of saline or AMPA into the vlVMH on intermediate tests, further demonstrating that estradiol sensitization is a robust effect (Jones et al., 2013; Jones & Pfaus, 2014). As predicted and supported by the large effect sizes, we found that repeated AMPA infusions to the vlVMH mimicked the behavioral pattern of attenuated sensitization of appetitive sexual behaviors observed in females that receive VCS from repeated mating, and increased defensive behaviors, a behavioral pattern reminiscent of estrous termination. These data suggest that glutamate transmission in the vlVMH induced by VCS plays a role in the attenuation of sensitized appetitive sexual behaviors by repeated EB, as predicted. In the second study we examined the patterns of estrous termination following VCS between EB-sensitized females that copulated on every test (EB/Male) versus those that were not given that opportunity (EB/Alone). As expected, females that received VCS the prior evening had a lower LQ, expressed more antagonistic behaviors towards the male, and tended to take fewer ejaculations compared to those that received SHAM. We also found that females that were not given the opportunity to copulate on every test (EB/Alone) had a lower LQ and displayed fewer LM3 compared to those that copulated on every test (EB/Male), and none of the EB/Alone females that received VCS the prior evening received an ejaculation during the estrous termination test, suggesting that the onset of estrous termination was accelerated in that group, contrary to our expectation. Together these findings suggest that mechanisms underlying estrous termination act in opposition to those activated by repeated administration of EB.

The sensitization of lordosis measures following AMPA infusions was somewhat surprising. Whether females were infused with AMPA or saline on intermediate tests, both LQ and the quality of the lordosis sensitized, and in saline treated animals the sensitization effect

was strongest on LM3. Interestingly, in AMPA treated animals, both LM2 and LM3 sensitized and the effect was also strongest on LM3; however the quality of their lordosis magnitudes appeared to be greater on the final test day compared to saline, reflected in the large between groups effect size on LM2 and LM3. This is intriguing since AMPA infusions to the vlVMH just prior to testing decreases LQ and lordosis magnitudes (Georgescu & Pfaus, 2006b), and antagonizing AMPA receptors just prior to experimenter applied VCS completely blocks the decrease in LQ and lordosis magnitude (Georgescu et al., 2012). Although in a previous study we did not see any group differences in the quality of LM3 between females that were given the opportunity to copulate on every test compared to those that did not (Jones & Pfaus, 2014), a similar pattern was observed following experimenter-applied VCS on intermediate tests (in place of copulation) (Jones, S.L., Germé, K., Graham, M.G., et al., submitted), and more LM3 were also observed in EB+Male compared to EB/Alone during the estrous termination test. Thus, repeated AMPA receptor activation (which may occur with repeated VCS) on the three episodes of heat prior to the final test day in the current study did not attenuate the sensitization of LQ, and in fact may have potentiated the quality of the lordosis magnitude, which suggests that a component of the consummatory aspect of sexual behavior (i.e., lordosis magnitude) may sensitize following repeated activation of the AMPA receptor subtype in the vlVMH during previous episodes of heat. Together with the literature showing that glutamate or its ionotropic agonists infused into the vlVMH rapidly inhibit lordosis (Georgescu & Pfaus, 2006b; Kow et al., 1985; McCarthy et al., 1991) our findings that repeated AMPA receptor activation in the vlVMH led to an attenuation of appetitive behaviors coincident with a *facilitation* of the lordosis quality support the idea first proposed by Kow et al., (1985) that the vlVMH contains lordosis inhibiting and excitatory subsystems.

A mechanism whereby the lordosis quality becomes potentiated with repeated AMPA receptor activation may make it more likely for the female to become pregnant/pseudopregnant. Using experimenter-applied VCS in ovary-intact females in proestrus, Lehmann and Erskine (2004) found that females that displayed at least one LM3 became pseudopregnant (indicating that the neuroendocrine events necessary for the maintenance of pregnancy had been activated) following just two VCS, and this effect did not occur with the two lower magnitudes. To contrast the mechanisms through which EB induced sensitization can differentially optimize reproductive success, it makes intuitive sense that repeated episodes of heat would sensitize appetitive and

consummatory aspects of sexual behaviors. An increase in appetitive solicitational behaviors is adaptive, as they make it more likely she will receive an intromission upon the eventual encounter with a male (Erskine, 1989). On the other hand, if on multiple episodes of heat she receives intromissions (and thus AMPA receptor activation in the vlVMH), an alternate strategy to optimize reproductive success may be to further sensitize the consummatory component, thus increasing the occurrence of LM3, and therefore the probability of activating the neuroendocrine events necessary for the maintenance of pregnancy. However, it is not clear from the current study under which contexts such a mechanism might become activated.

Repeated AMPA infusions in place of copulation resulted in a behavioral pattern on the final test day that is consistent with estrous termination. As predicted, AMPA infusions on intermediate tests attenuated the display of sexually appetitive behaviors; thus it appears that glutamatergic signaling (particularly via AMPA receptors) in the vlVMH, which is involved in the induction of estrous termination, is inhibitory to the sensitization of appetitive sexual behaviors induced by repeated administration of EB. We also replicated our previous finding that repeated treatment of EB in the absence of copulation on intermediate tests (the saline group in the current study) had no effect on defensive behaviors (Jones & Pfaus, 2014), yet we found that repeated AMPA infusions on intermediate tests increased the incidence of defensive behaviors towards males on the final test day compared to saline treated animals. This is consistent with previous findings that agonizing AMPA receptors in the vlVMH activates defensive behaviors (Georgescu & Pfaus, 2006b); however it is in contrast to our previous findings that defensive behaviors generally decline in females repeatedly treated with EB and given the opportunity to copulate (Jones et al., 2013; Jones & Pfaus, 2014), which suggests that repeated copulation opposes the process that facilitates the onset of defensive behaviors by AMPA receptor activation. Although the mechanism is unclear, we have previously suggested a role for the periaqueductal gray (PAG) (Jones & Pfaus, 2014), which has bi-directional connections with the VMH (Calizo & Flanagan-Cato, 2002), based on a model proposed by Blaustein et al. (2009) and Pfaff et al. (2008).

Blaustein et al. (2009) examined the potentiation of sexual behaviors by mating stimulation within a single episode of heat in females treated with a low dose of EB and found that preventing VCS from intromissions (by masking the vaginal opening) prevented the increase in defensive behaviors found in copulating animals. We reported that repeatedly treating OVX

rats with EB (every 4 days) but not allowing them to copulate sensitized sexual behaviors when tested on the 8th test, yet defensive behaviors were unchanged compared to their initial behavioral test (similar to that reported by Blaustein et al. 2009). On the other hand, defensive behaviors were significantly lower on the final test day if females were given the opportunity to copulate on each test (in contrast to the effect of mating stimulation on defensive behaviors within an episode of heat). Together those data suggest that although intromissive stimulation can initially activate defensive behaviors, presumably through activation of AMPA receptors as shown by Georgescu and Pfaus (2006b), repeated mating across episodes of heat overrides the activation of defensive behaviors (Jones & Pfaus, 2014). Since repeatedly treating females with AMPA (in place of copulation) in the current study resulted in an increase in defensive behaviors, it is possible that AMPA receptor activation in the vlVMH, (or some downstream mechanism), may be reduced in the EB/Male condition. To extend our prior discussion related to a potential role of the PAG (Jones and Pfaus, 2014), it is hypothesized that if AMPA receptor activation within the vlVMH (following VCS) interacts with the PAG to inhibit lordosis and facilitate defensive behaviors, this pathway must be overridden in those females that are repeatedly treated with EB and allowed to copulate. Overall, these findings are important as they further demonstrate that glutamate signaling in the vlVMH via AMPA receptors, is inhibitory to sexual behavior, and induces a behavioral pattern reminiscent of estrous termination (Booth et al., 2010; Georgescu et al., 2009; 2012; 2014; Georgescu & Pfaus, 2006a; 2006b).

This is the first study to examine estrous termination using an estradiol sensitization paradigm. We have shown that VCS accelerated the onset of defensive behaviors and the offset of lordosis, and nearly no solicitations were observed as previously reported in estrous termination studies using fully primed females in bilevel chambers (Pfaus et al., 2000). However, no effect of VCS was observed on hops/darts, which is likely a floor effect since few hops/darts were observed overall, (although the patterns suggest that those that received VCS displayed fewer hops/darts compared to SHAM). Therefore it is likely that estrous termination was already well underway. For future studies to capture the gradual onset of estrous termination following repeated EB treatment some minor changes should be made to the methodology. Our method was based on a well-established estrous termination paradigm in OVX rats fully-primed with EB+P, which induce maximal levels of sexual behavior. Thus, perhaps earlier administration of VCS (such as 48 hours following EB administration as opposed to a 56 hours as is customary

when using fully EB+P primed females), and running estrous termination tests 12 hours later would be a better method to assess the offset of the full array of sexual behaviors (i.e., including appetitive sexual behaviors). Nonetheless, it is clear that VCS accelerates the onset of estrous termination in EB sensitized females, as reported in EB+P primed females, albeit the onset latency appears to be shorter.

Although we predicted that estrous termination would be accelerated in the EB/Male group, in fact the EB/Alone females were at a later stage of estrous termination since their LQ was lower, and of those that received VCS, none received an ejaculation. This suggests that the sexual behavior system as a whole (i.e., those that activate sexual behavior and those that inhibit) sensitizes with repeated administration of EB. Thus it appears that the system is set up such that repeated administration of EB (or repeated episodes of heat) sensitizes the activation as well as the termination of sexual behavior. Moreover, the system appears to be more sensitive to the receipt of VCS if prior episodes of heat occurred without copulation, such that once it does occur, mechanisms that are inhibitory to sexual behavior are more effective, resulting in an earlier termination of behavioral estrus and presumably in eliciting the neuroendocrine events required for the maintenance of pregnancy. If the latter is true, then the medial amygdala (MeA) must be an important brain region involved in these effects.

What neural mechanisms might underlie behavioral sensitization to EB?

We have previously suggested that neural regions underlying the sensitization of sexual behaviors by repeated administration of EB may involve the mPOA and VMH (Jones et al., 2013; Jones & Pfaus, 2014) given their roles in the expression of solicitational behaviors and lordosis respectively, which is further supported by the current data. We now propose that the MeA must also play an important role given that EB/Alone females were more sensitive to the induction of estrous termination by VCS, and the heightened sensitivity on lordosis intensity that appears to have occurred in those females repeatedly infused with AMPA. We previously proposed that GABA within the vlVMH may inhibit glutamate signaling to allow the expression of sexual behavior and that repeated administration of EB might sensitize this inhibition (Jones et al., 2013). GABAergic projections to the vlVMH arise largely from the mPOA, a region that is critical in the display of sexually appetitive behaviors, but a smaller projection also arises from the MeA (Georgescu, Graham, & Pfaus, in preparation). The posterodorsal MeA (pdMeA) is

implicated in the neuroendocrine changes necessary for the maintenance of pregnancy (or pseudopregnancy) following VCS, via activation of the ionotropic glutamatergic receptors AMPA (Oberlander et al., 2009) and NMDA (Lehmann et al., 2005; Polston & Erskine, 2001). The MeA contains a large number of estrogen receptors (Pfaff & Keiner, 1973), and crystalline estradiol applied to this region facilitates lordosis as it does in the VMH (Lisk & Barfield, 1975). Moreover, neurons within the MeA appear to sensitize to EB, since long-term exposure to EB increases their firing rate (Pfaus et al., 1996; Schiess et al., 1988). We propose that glutamatergic signaling through NMDA receptors in the pdMeA may be sensitized, given their role in the induction of neuroendocrine signals necessary for pregnancy/pseudopregnancy following VCS. How this mechanism would interact with activity within the vlVMH (and potentially the mPOA) is not clear, but one potential pathway may involve glutamatergic and/or GABAergic signaling between these regions.

VCS accelerates the onset of estrous termination (Hardy & Debold, 1971a; Lodder & Zeilmaker, 1976; Pfaus et al., 2000), particularly if intromissions are paced by the female (Coopersmith et al., 1996; Erskine, 1985; Erskine et al., 1989; Erskine & Baum, 1982), and it induces Fos within the mPOA, vlVMH and pdMeA (Erskine, 1993; Pfaus et al., 1993; 1996; Polston & Erskine, 1995; Tetel, Getzinger, & Blaustein, 1993). Mating stimulation leads to the release of glutamate within the VMH (Georgescu et al., 2014) and presumably the pdMeA, since NMDA receptor activation within the pdMeA induces twice daily prolactin surges, required for the maintenance of pregnancy, mimicking the effect of mating, and VCS in particular (Lehmann et al., 2005; Lehmann & Erskine, 2005; Polston & Erskine, 2001), and AMPA receptor activation in this region is critical for the induction of pregnancy/pseudopregnancy. Bilateral pelvic nerve transections (which innervate the vagina and cervix) prevents the induction of Fos within the pdMeA (Rowe & Erskine, 1993), while stimulation of NMDA glutamate receptors within the pdMeA induces Fos expression in a number of sexually relevant brain regions including the mPOA and vlVMH (Lehmann & Erskine, 2005), indicating that the pdMeA sends efferents to these regions following VCS.

Thus glutamate release within the vlVMH could be modulated by glutamatergic activity in the pdMeA, for example if those NMDA (and possibly AMPA) receptors within the pdMeA are themselves located on glutamate neurons that project to the vlVMH. Importantly, the induction of pseudopregnancy (which requires twice daily prolactin surges) depends on repeated

and temporally distinct activation of NMDA receptors within the pdMeA, indicating that it plays an important role in the intromission mnemonic within a mating session required for initiating pseudopregnancy/pregnancy (Lehmann et al., 2005), and our data suggest that this mnemonic may summate across mating sessions. We know that VCS activates glutamate neurons within the vlVMH (Georgescu et al., 2009), and that the vlVMH sends efferents to the pdMeA (Akesson, Ulibarri, & Truitt, 1994); however it is not known whether the glutamatergic neurons of the vlVMH are the same that project to the pdMeA, but if so, they may play a role in that mnemonic. Thus, an important pathway implicated in the sensitization of sexual behaviors by EB likely occurs through complex interactions between the vlVMH, mPOA and pdMeA, which is possible given the bilateral connections between all these regions (Akesson et al., 1994; Behbehani, 1995; Flanagan-Cato, 2000; Georgescu, Graham, & Pfaus, in preparation; Lehmann & Erskine, 2005). Finally, the integration of afferents within the vlVMH would subsequently be relayed down to the PAG to influence the occurrence of defensive behaviors and lordosis (Pfaff et al., 2008).

Conclusion

Together the findings from these studies suggest that repeated administration of EB sensitizes mechanisms that are not only permissive to the display of sexual behavior, but also those that are inhibitory to its duration. Repeated administration of EB increases both sexual solicitations, which increase the probability of receipt of mounts and intromissions from the male, as well as consummatory behaviors (lordosis), which is required for successful intromission and thus VCS that is necessary for the induction of twice daily prolactin surges required for the maintenance of pregnancy. Moreover, the data suggest that a neural mechanism exists whereby repeated activation of AMPA receptors within the vlVMH, presumably from the receipt of VCS, attenuates the display of appetitive sexual behaviors while facilitating maximal lordosis intensity, which has been shown to trigger pseudopregnancy. Therefore it appears that the system has the potential to switch reproductive strategies not merely to maximize the probability of receiving VCS (by potentiating solicitational behaviors) necessary for fertilization, but to enhance behaviors (LM3) that trigger the neuroendocrine events necessary to maintain successful pregnancy.

CHAPTER 4. APPLICATIONS OF THE ESTRADIOL SENSITIZATION PARADIGM

The primary findings of the data presented to date have shown that behavioral sensitization to EB is a robust effect, as it can be reliably induced in different strains of animals, by varying doses of EB. Given this robust behavioral effect, the aim of the next chapter was to use the paradigm as a diagnostic tool to test the female's behavioral sensitivity to E2 in two models of female sexual inhibition. Animal care facilities are moving towards the use of corncob bedding in their housing procedures in order to improve environmental conditions for the animals and staff. However there is some evidence in the literature that corncob bedding interferes with E2 signaling, and disrupts female ovarian cyclicity as well as rodent sexual and aggressive behaviors, which are dependent on ER signaling. The primary goal of the first study (Chapter 4.1) was to determine whether any deficits in sexual behavior due to corncob bedding could be overcome by the repeated administration of EB. In a second model (Chapter 4.2) of sexual inhibition we asked whether females that were prenatally androgenized, and thus show sexual behavior deficits in response to hormone priming when OVX and tested in adulthood, would display behavioral sensitization to EB.

4.1. The inhibitory effects of corncob bedding on sexual behavior in the ovariectomized Long-Evans rat treated with estradiol benzoate is overcome by male cues

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Abstract

The mechanisms underlying the sensitization of sexual behaviors by repeated administration of estradiol benzoate (EB) to ovariectomized (OVX) rats are not well understood. Here we tested whether two housing conditions play a role. Sexual behavior in the female rat is dependent on the activation of ERα (estrogen receptor alpha) by estradiol. Corncob (CC) bedding has been reported to have adverse effects on the reproductive behavior and physiology of rats, and to disrupt ERα signaling in mice. In addition, some rodent behaviors are stimulated by olfactory stimuli and enhanced in the presence of estradiol. Upon arrival to the facilities OVX Long-Evans rats were housed on either Sani-Chips (SC) or CC in a room that housed only females (F) or males and females (M). Females were first given four sexual training sessions with 10μg EB+500μg progesterone (P; administered 48hrs and 4hrs prior to training, respectively), followed by a 2-week hormone washout period. Next, 10μg EB was administered sc every 4-days, 48hrs prior to each of 8 test sessions in a unilevel 4-hole pacing chamber. On the final training day (i.e., when primed with EB+P), no inhibitory effects of corncob bedding were found, but a facilitation of the lordosis quality occurred in SC/F. Although all groups appear to have sensitized to the repeated administration of EB, CC/F animals displayed fewer high quality lordosis magnitudes and hop/darts, and received fewer mounts and intromissions overall. They also had a lower lordosis quotient (LQ) on tests 2-4 although this effect disappeared by test 5. These results suggest that although CC may inhibit some components of female sexual behavior when primed with EB alone, cues from sexually vigorous males can overcome that inhibition. Moreover, they suggest that male cues can facilitate mechanisms of estradiol sensitization. We recommend that quality control studies be conducted at individual institutions to assess any impact of corncob bedding on animal physiology and behavior.

Keywords: Corncob; Housing; Estradiol Sensitization; Sexual Behavior; Rat

Introduction

Ovariectomized (OVX) rats are behaviorally more sensitive to subsequent injections of estradiol benzoate (EB) administered alone compared to the initial injection (Babcock et al., 1988; Beach & Orndoff, 1974; Blaustein et al., 1987; Clark & Roy, 1983; Gerall & Dunlap, 1973; Jones et al., 2013; Kow & Pfaff, 1975; Parsons et al., 1979; Whalen & Nakayama, 1965). Whereas the acute administration of EB alone partially activates lordosis, repeated administration potentiates lordosis and activates sexually appetitive behaviors (such as solicitations, hops/darts and ear wiggles). Recently we examined the time-course of this behavioral sensitization using three doses of EB that are commonly used to induce sexual behavior in the OVX rat. We found that in two strains of rats (Long-Evans and Wistars), sensitization is robust following administration of 5 or 10µg EB every four days (Jones et al., 2013), particularly if females are not given the opportunity to copulate on every test (Jones & Pfaus, 2014). Moreover, in the absence of the opportunity to copulate, sensitization also occurs using 2µg EB, a dose that had previously been reported to be subthreshold at inducing sexual behavior without subsequent priming with progesterone (Jones & Pfaus, 2014). We are currently investigating the mechanisms that underlie this effect, using the dose that induced the most robust sensitization (10µg). In the present study we examined whether two housing factors might interfere with the effect.

Recently our animal care facility considered implementing corncob bedding in home cages. The primary advantage of this bedding is greater absorbency, which reduces bacterial growth and ammonia levels (Burn & Mason, 2005). The bedding is also beneficial to staff, since dust and allergens are reduced, and due to its increased absorbency, requires less frequent cage changes compared to wood chip bedding. However some groups have reported adverse effects of housing animals on corncob. Within two weeks, male and female (ovary-intact and OVX) rat sexual behavior was inhibited and ovarian cyclicity was disrupted (Mani, Reyna, Alejandro, Crowley, & Markaverich, 2005; Markaverich et al., 2007a; Markaverich, Alejandro, et al., 2002a; Markaverich, Mani, et al., 2002b; Romero, Beltramino, & Carrer, 1990), effects that were reversed when returned to woodchip bedding or wire-mesh cages (Markaverich, Mani, et al., 2002b). Although corncob is plant-based and may act as a phytoestrogen, the compound THF-diols (tetradydrofuran diols), which appear to be the inhibitory agents in corncob bedding, do not compete for estrogen receptor (ER) binding sites in uterine tissue (Markaverich, Alejandro, et al.,

2002a; Markaverich, Mani, et al., 2002b). However, Villalon Landeros et al. (2012) recently reported that male Californian mice housed on corncob exhibited differences in estrogen receptor alpha (ERα) signaling pathways in a number of brain regions associated with aggression, many of which are also implicated in rat sexual behavior. For example, they found fewer ERα-positive cells in the ventromedial hypothalamus (a region critical in the expression of lordosis, the concave arching of the back that permits penile intromission) and reduced numbers of phosphorylated ERK in the medial preoptic area and medial amygdala (regions that are implicated in sexually appetitive behaviors). Together these data suggest that compounds within corncob bedding do not act through ER, but merge and disrupt downstream ER signaling pathways. This is problematic for studies investigating endocrine control of behavior. Given the implications this could have on the underlying mechanisms mediating the sensitization of sexual behaviors by chronic EB, we tested whether bedding type would alter this sensitization.

Olfactory cues from males might also affect the sensitization of female sexual behavior by repeated EB. Many rodent behaviors can be strongly influenced by olfactory cues, and enhanced in the presence of estradiol. For example the exposure of nulliparous and/or OVX female rats to pup odors can stimulate maternal behavior (Mayer & Rosenblatt, 1980), and the facilitation of maternal behaviors in OVX pregnancy-terminated (gestational day 15) dams is accelerated in the presence of estradiol (Stolzenberg et al., 2009). Olfactory stimuli from a sexually vigorous male may also be involved in mating-induced enhancement of sexual behaviors in estradiol-primed OVX rats repeatedly mated within an episode of heat (Rajendren et al., 1990). Moreover, female rats in estrus have an unconditioned preference for a sexually vigorous male (Agmo, 1999; Sakuma, 2008), an effect dependent on the presence of estradiol, and an intact medial preoptic nucleus (Sakuma, 2008; K. Xiao, Kondo, & Sakuma, 2005). Thus, if reducing exposure to cues (e.g., olfactory, auditory, visual) from the male (by housing the sexes in separate rooms), influences the development of the behavioral sensitization to EB, it may help pinpoint the underlying neural pathways involved.

The current study examined whether housing females on corncob versus Sani-Chips bedding, or whether housing males and females in separate colony rooms, would alter sexual behavior in fully primed (EB+P) females, or the sensitization of sexual behaviors following repeated EB injections alone (10μg).

Materials and Methods

This experiment was conducted in accordance with the ethical standards established by the Canadian Council on Animal Care (CCAC), and approved by Concordia University's Animal Care Committee.

Animals

Long-Evans female rats weighing approximately 150-200g were purchased from Charles River Laboratories (St-Constant, Quebec) where they were housed on Beta Chip® until weaning, then group housed in stainless steel cages with mesh bottoms. Upon arrival to our facilities, they were housed in pairs in clear Plexiglass chambers lined with either Sani-chips (category 7090A, Harlan, Montreal, Quebec) or corncob bedding (1/4" category 7097, Harlan, Montreal, Quebec) until the end of the experiment (total of 11 weeks). All colony rooms were maintained on a 12-hour reversed light cycle (lights on at 8PM) at approximately 21ºC. Animals had ad lib access to standard laboratory chow (Charles River #5075) and tap water. They were given a week of acclimatization to the animal facilities and handled daily from days 4 to 7, when they were OVX.

Sexually experienced Long-Evans males (housed 3-4/cage) obtained from the same supplier, were housed on Sani-chips bedding, and used as stimulus animals for training and test sessions. Each male was only used once on each training or test day, and assigned randomly to the female. Whereas females were strictly returned to their assigned colony room, males were returned at random (but never to a "female-only" room). Thus, they were always housed in a room that contained females.

Ovariectomy

Ovariectomies (OVX) were performed following an i.p. injection (1mL/kg) of ketamine (50mg/mL; 50 mg/mL; Ketaset©, Wyeth Canada) and xylazine hydrochloride (4mg/mL; Rompum©, Bayer Healthcare) mixed at ratio of 4:3 respectively. When animals were no longer responsive to a foot pinch, the eyes were lubricated with drops (Natural Tears, Alcon), and ears were punched for identification purposes. They were then bilaterally OVX via a single lumbar incision. Polysporin was applied to the incision site, and Flunixin Meglumine 2.5 mg/kg/mL (Banamine©) and 5 mg/kg/mL Enrofloxacin (Baytril©) were given by SC injection. Animals were next hydrated with 1mL 0.9% saline injected s.c, and given one week to recover.

Hormones

Estradiol benzoate (10μg) was dissolved in 0.1mL of sesame oil, and injected SC 48 hours prior to each training and test session. Progesterone (500μg) was dissolved in 0.1mL sesame oil and injected SC 4 hours prior to each training session.

Treatment Conditions

One of the more practical advantages of using corncob bedding is it requires less frequent cage changes compared to woodchip beddings. One potential explanation for disrupted sexual behavior reported in animals housed on corncob bedding is the frequency of cage changes, and subsequently less frequent handling (Castelhano-Carlos & Baumans, 2009). For example, locomotor activity and grooming behaviors are increased on cage cleaning days (Saibaba, Sales, Stodulski, & Hau, 1996), and increased heart rate, blood pressure (Duke, Zammit, & Lawson, 2001), and corticosterone (De Boer, Koopmans, Slangen, & Van der Gugten, 1990) are observed following handling procedures. Good laboratory practices include handling of animals to minimize stress, by reducing the novelty of the experimental conditions. Novelty and stress disrupt sexual behavior in rodents (Pfaus & Wilkins, 1995; Yoon, Chung, Park, & Cho, 2004), and cage cleaning induces a stress response (Castelhano-Carlos & Baumans, 2009). Since corncob bedding requires less frequent cage changes, variations in handling and stress responses may account for these differences. Therefore, to control for the potential confound, cage change frequency was experimentally controlled. Sani-chips bedding was changed on a bi-weekly schedule, whereas corncob bedding was changed on a weekly schedule. However females housed on corncob also received a "sham-cage change", such that they were simply lifted out of their cage, and placed back in, on days coinciding with the second cage change of Sani-chips animals. This ensured that handling was consistent between all groups. All cage changes were done in the afternoon (i.e., never prior to training or testing) to minimize the potential impact of cage changes on test days.

To determine whether the presence of males housed in the same room as females would affect the sensitization of female sexual behavior, females on each bedding type were housed in either a female-only room or a room housing both males and females. Hence, this experiment consisted of a total of four groups according to housing condition: corncob in a room with males

(CC/M; n=8), corncob in a female-only room (CC/F; n=8), Sani-chips in a room with males (SC/M; n=9), or Sani-chips in a female-only room (SC/F; n=8).

Apparatus and Sexual Behaviors

All training and test sessions took place in unilevel pacing chambers (46x39x37cm), bisected by a clear Plexiglas® divider with 4 square holes cut in the bottom (5x4cm), with the size adjusted such that the smaller female could pass but the larger male could not. The dustpan of the chamber contained Sani-chips, which lied below an elevated (1") metal grid floor.

Sexually appetitive behaviors included hops/darts, and full solicitations (a headwise orientation towards the male followed by a runaway) (Erskine, 1989; McClintock, 1984; Pfaus et al., 1999). Lordosis magnitudes were coded on a 3-point scale according to the intensity of dorsiflexion, and lordosis quotient (LQ) was calculated by taking the sum of lordosis postures divided by the sum of mounts, intromissions and ejaculations x 100 (Hardy & Debold, 1971b). Kicks and prone positions were coded as female defensive behaviors (Barnett, 1963). Male mounts, intromissions and ejaculations were also coded.

Training and Testing Procedures

Following a one-week recovery period from surgery, females were put into heat with EB (10µg) and P (500µg) prior to each of 4 training sessions at 4-day intervals, followed by a 2-week hormone washout period. Next, females were tested with 10µg EB-alone at 4-day intervals for 8 test sessions. Training and test sessions always began by placing a male in the 4-hole pacing chamber for a 5-minute period prior to introduction of the female. Females were placed on the empty side of the chamber and the pair was left to copulate for 30 minutes. All training and test sessions were video-recorded using a Sony Handycam Camcorder. Videos were uploaded to a personal computer, and behaviors were scored blindly using the Behavioral Observation Program (Cabilio, 1996), customized for rat sexual behavior.

Statistical Analyses

A preliminary analysis first tested whether bedding type, or housing females in a room with or without males, would affect sexual behavior on the final training day when primed fully with 10µg EB + 500µg P, using a two-way ANOVA with bedding type and presence of males as

between subject factors. Next, to test the primary hypotheses, a 3-way mixed ANOVA was used by entering test as the within subject's factor, and housing condition (bedding type, male housing condition) as between subject factors. Significant interactions were further analyzed with simple effect analyses using Sidak's post-hoc test. A significant linear contrast using linear trend analysis was used to determine whether sensitization occurred (Jones et al., 2013; Keppel & Wickens, 2004). Eta squared (η^2) and *Cohen's d* are reported as measures of effect size. To overcome a statistical limitation on the analysis of LQ (given that no female in the CC/F group was consistently mounted across tests), a one-way ANOVA was conducted on each test day, to assess differences between groups. Chi-square analyses were used to test the proportion of females mounted on every test across groups, and *Phi* is presented as a measure of effect size. The level of significance for all analyses was set at $p<0.05$. All data are presented as mean (±SEM) unless otherwise indicated.

Results

Sexual Training with EB+P

As shown in Table 1, housing females on Sani-chips in a room that only housed females (SC/F) induced more LM3 compared to females housed on corncob in a female-only room (CC/F; $p<0.001$, $d=2.97$) or on Sani-Chips in a room with males (SC/M; $p=0.031$, $d=2.27$) (significant bedding*male interaction, $F(1,29)=5.74$, $p=0.023$, $\eta^2=0.15$). In addition, those females (SC/F) received more intromissions compared to those housed on Sani-Chips in a room that also housed males (SC/M; $p=0.028$, $d=2.31$) and tended to receive more than those housed on corncob in a room that only contained females (CC/F; $p=0.06$, $d=1.95$) (significant bedding*male interaction, $F(1,29)=6.28$, $p=0.018$, $\eta^2=0.18$). Although the interaction between bedding and male condition on hops/darts approached statistical significance, the effect size was rather small, $F(1,29)=4.15$, $p=0.051$, $\eta^2=0.12$, and simple effects analyses failed to detect any specific group differences (all $p>0.09$). No effects were detected on any other measure.

Sensitization of sexual behaviors by EB-alone

Lordosis. Some females were not mounted on every test limiting the statistical analyses on LQ (SC/F, n=6; SC/M, n=1; CC/F, n=0; CC/M, n=5). As shown in Figure 1A, fewer females were consistently mounted across tests in the CC/F group compared to the CC/M ($X^2=7.28$,

p=0.007 Phi=0.67) and SC/F (X^2=9.60, p=0.002, Phi=0.78), and fewer were consistently mounted in the SC/M compared to the CC/M (X^2=4.90, p=0.027, Phi=0.54) and SC/F groups (X^2=7.14, p=0.008, Phi=0.65) the lack of females consistently mounted in the CC/F group resulted in their exclusion from the repeated measures analyses, and as such the bedding*male and test*bedding*male interactions could not be properly assessed. Nevertheless, sensitization of LQ did occur in the remaining groups as depicted in Figure 1B (F_{linear}(1,9)=57.35, p<0.001, η^2=0.84). Significantly higher LQ occurred on tests 5 (p=0.038, d=1.32), 6 (p=0.046, d=1.28), and 8 (p=0.033, d=1.34) compared to the first test (main effect of test, F(7,63)=12.77, p<0.001, η^2=0.57). The main effects of bedding type, presence of males in the room, nor any of the interactions met statistical significance.

Table 1

Mean (±SEM) of sexual behaviors of Long-Evans female rats on training day 4 (primed fully with 10µg EB + 500µg P) and housed on either sanichip or corncob bedding in a room that housed females only, or males and females.

	Sanichip		Corncob	
	Female-Only	Male/Female	Female-Only	Male/Female
LQ	93.75±6.25 (8)	100±0.00 (7)	95.96±2.63 (7)	100±0.00 (8)
LM1	2.62±1.24	1.67±0.99	5.75±2.16	3.12±1.01
LM2	13.00±2.82	9.00±4.22	13.25±4.07	12±2.33
LM3	26.88±7.02[a,b]	12.56±3.16[a]	7.62±3.89[b]	15.00±3.17
Hops/Darts	71.50±11.20	43.78±10.53	45.12±10.94	64.12±13.13
Solicitations	4.50±1.44	1.89±1.19	3.38±1.43	4.38±2.13
Defenses	0.12±0.12	0.89±0.68	0.88±0.52	0.75±0.41
Mounts	24.5±6.49	13.22±4.59	16.12±4.05	14.75±2.29
Intromissions	15.75±3.34[a]	8.11±1.70[a]	9.12±2.12	13.38±2.15
Ejaculations	2.38±0.38	1.89±0.39	1.88±0.44	2.00±0.42

Note. LQ: Lordosis Quotient, LM: Lordosis Magnitude. Matching letters indicate group differences.

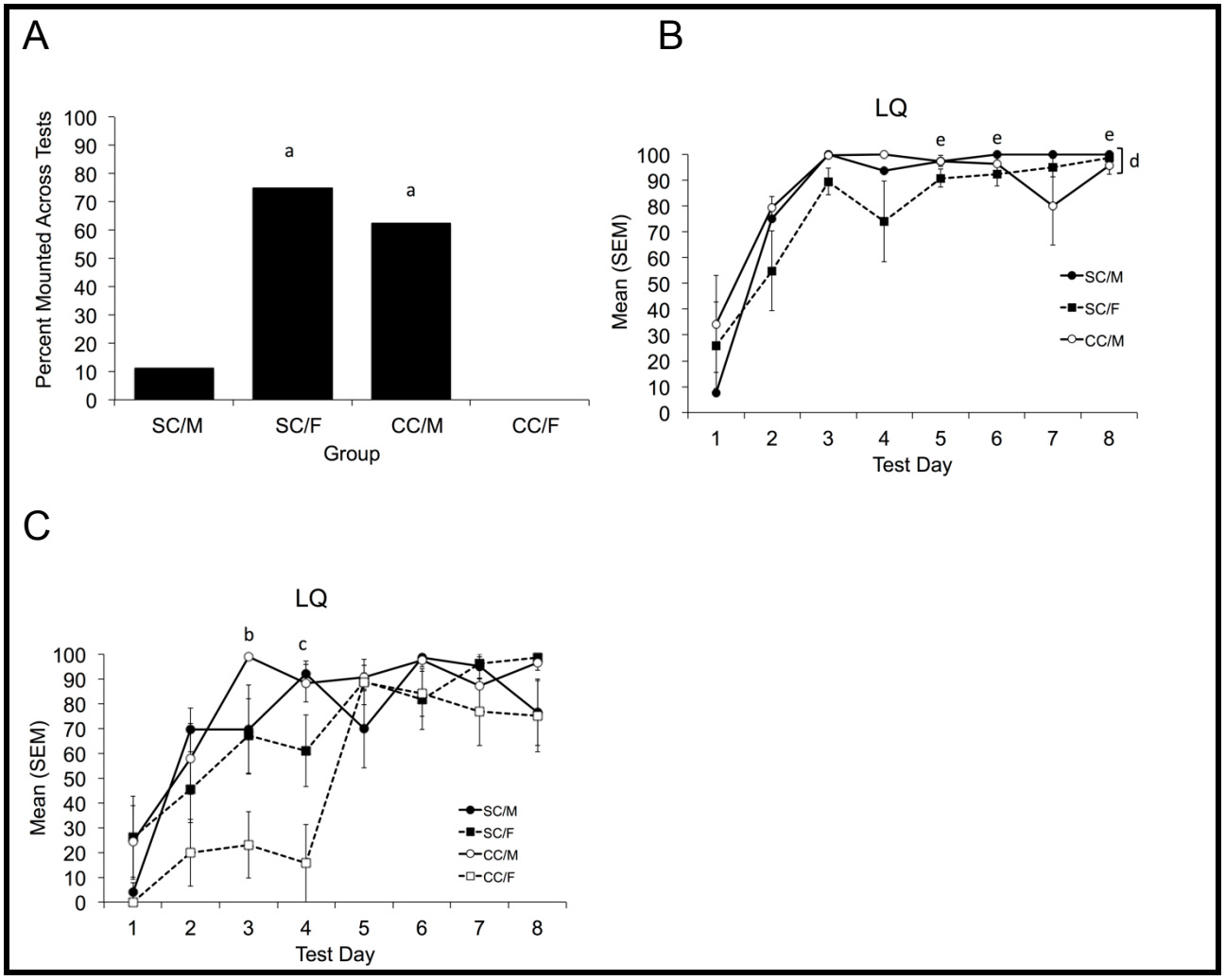

Figure 1. Females were housed either on Sani-Chips (SC) or corncob (CC) bedding in a room that only housed females (F) or also housed males (M). A. Percentage of females that were consistently mounted across the 8 estradiol sensitization tests following different housing conditions. Fewer CC/F (0/8) and SC/M (1/9) were mounted on every test compared to SC/F (6/8) and CC/M (5/8). B. Mean (SEM) LQ of females that were consistently mounted across all tests. C. Mean (SEM) LQ of females that were mounted on each given test day (see Table 2 for number of animals included on each test). [a]Significantly greater than CC/F and SC/M groups. [b]Difference between CC/F and CC/M. [c]Lower in CC/F compared to CC/M and SC/M. [d]Significant linear trend. [e]Different from test 1. Significance set at $p<0.05$. See text for effect sizes.

To partly overcome the statistical limitation of the mixed ANOVA caused by no female in the CC/F and only one female in the SC/M group being consistently mounted, a one-way ANOVA was conducted on each test day to assess differences between groups, which revealed that housing females on corncob in a female only room may have had a short-term inhibitory effect on the display of LQ. Figure 1C displays the average LQ of all females in each group that were mounted on a given test day. The pattern of LQ of the CC/F females was generally similar to that of the other groups on later tests, suggesting that sensitization did occur; however group differences were detected on early tests during the development of sensitization. There were no differences on test 1, but on Test 2 CC/F tended to have a lower LQ than SC/M (p=0.047, d=2.86) (trend toward a significant main effect of group, $F(3,27)=2.92$, p=0.052, η^2=0.24). Group differences emerged on Test 3 such that CC/F had a lower LQ than CC/M (p=0.006, d=3.74) (main effect of group, $F(3,24)=4.70$, p=0.10, η^2=0.370) and on Test 4 CC/F had a lower LQ than both groups housed with males (CC/M, p=0.001, d=4.38; SC/M, p=0.001, d=4.46) and tended to have a lower LQ than SC/F (p=0.066, d=2.73) (main effect of group, $F(3,25)=8.52$, $p<0.001$, η^2=0.51). However despite these strong effects, the group differences disappeared by Test 5 and no further differences were detected on LQ throughout the remainder of the experiment.

LM1 sensitized across tests as shown in Figure 2A ($F_{linear}(1,29)=4.37$, p=0.045, η^2=0.12). Fewer LM1 were observed on test 4 compared to tests 6 (p=0.047, d=0.60) and 7 (p=0.034, d=0.62) (main effect of test, $F(7,203)=3.00$, p=0.005, η^2=0.08). In addition, females that were housed in a room with males (i.e., SC/M and CC/M) displayed more LM1 on test 2 compared to test 4 (p=0.008, d=0.99), and displayed more LM1 on test 2 (p=0.031, d=2.27) and tended to display more on test 3 (p=0.051, d=2.03) compared to females that were housed in a female-only room (significant test*male presence interaction, $F(7,203)=2.74$, p=0.010, η^2=0.08).

LM2 also sensitized across tests as shown in Figure 2B, $F_{linear}(1,29)=10.38$, p=0.003, η^2=0.26. Fewer LM2 were observed on Test 1 compared to all subsequent tests (all $p<0.02$ and $d>0.65$) except test 4 (main effect of test, $F(7,203)=4.77$, $p<0.001$, η^2=0.13). No other effects were found.

LM3 also sensitized across tests as shown in Figure 2C ($F_{linear}(1,29)=42.99$, $p<0.001$, η^2=0.58). Fewer LM3 were observed on test 1 compared to all subsequent tests (all $p<0.02$, $d>0.65$), and fewer were seen on Test 2 compared to tests 4-8 (all $p<0.05$. $d>0.60$) (main effect

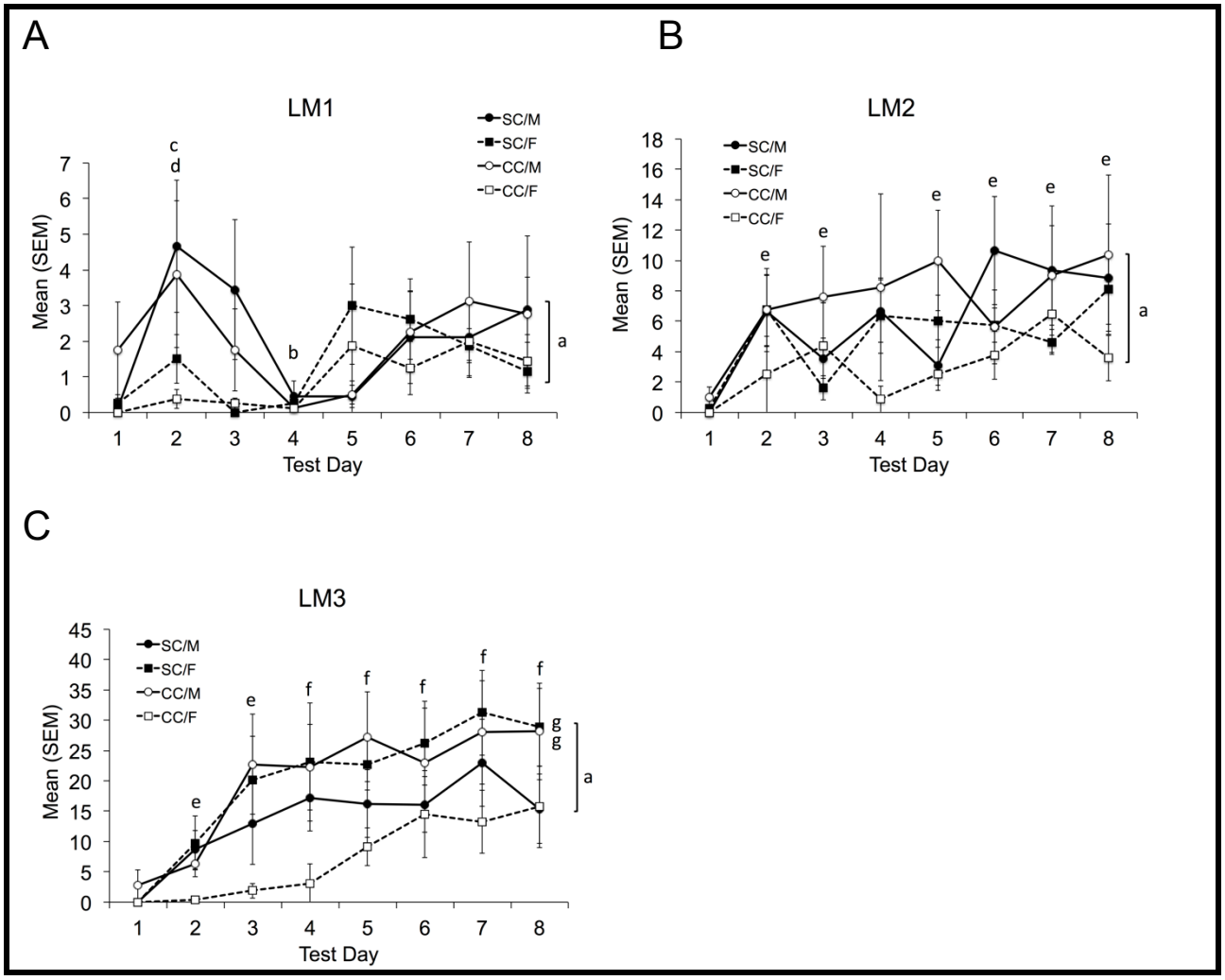

Figure 2. Lordosis magnitudes of females were housed either on Sani-Chips (SC) or corncob (CC) bedding in a room that only housed females (F) or also housed males (M). [a]Significant linear trend. [b]Different from tests 6 and 7 [c] Different from test 4 in females housed with males (SC/M and CC/M groups). [d]Difference between male housing conditions. [e]Different from test 1. [f]Different from tests 1 and 2. [g]Different from CC/F. Significance set at $p<0.05$. See text for effect sizes.

of test $F(7,203)=12.28$, $p<0.001$, $\eta^2=0.28$). However, housing females on corncob without the presence of males in the room appears to inhibit the display of LM3. CC/F females displayed fewer LM3 overall compared to CC/M ($M_{diff}=12.80\pm5.66$, $p=0.031$, $d=2.26$), and SC/F ($M_{diff}=13.02\pm5.66$, $p=0.029$, $d=2.30$) (significant bedding*male presence interaction, $F(1,29)=6.04$, $p=0.20$, $\eta^2=0.16$). No other effects were detected.

Appetitive Behaviors. Hops/darts sensitized across tests ($F_{linear}(1,29)=47.82$, $p<0.001$, $\eta^2=0.61$) as shown in Figure 3A. Fewer hops/darts were displayed on test 1 compared to all subsequent tests (all $p<0.03$, $d>0.63$) and fewer occurred on test 2 compared to tests 5-8 (all $p<0.007$, $d>0.73$), and the most hops/darts occurred on test 7, such that it was significantly greater than tests 1-4 (all $p<0.038$, $d>0.62$) and test 6 ($p=0.012$, $d=0.82$) (main effect of test, $F(1,29)=47.82$, $p<0.001$, $\eta^2=0.34$). Moreover, CC/F displayed significantly fewer hops/darts than CC/M, ($M_{diff}=20.31\pm8.64$, $p=0.026$, $d=2.35$) and fewer than SC/F, ($M_{diff}=19.87\pm8.64$, $p=0.029$, $d=2.30$) (significant bedding*male presence interaction, $F(1,29)=4.21$, $p=0.049$, $\eta^2=0.11$). No other effects were detected.

The number of full solicitations also sensitized across tests $F_{linear}(1,29)=6.92$, $p=0.014$, $\eta^2=0.18$, as shown in Figure 3B. More solicitations were observed on test 3 compared to tests 1 ($p=0.034$, $d=0.62$) and 4 ($p=0.035$, $d=0.62$), and more were observed on test 7 compared to tests 1 ($p=0.002$, $d=0.81$), 4 ($p=0.002$, $d=0.79$), 6 ($p=0.038$, $d=0.62$) and 8 ($p=0.048$, $d=0.60$) (main effect of test, $F(7,203)=7.64$, $p<0.001$, $\eta^2=0.19$). Moreover, females housed on corncob bedding tended to display fewer solicitations overall compared to those on Sani-Chips (trend toward a main effect of bedding: $F(1,29)=4.09$, $p=0.053$, $\eta^2=0.12$). No other effects were detected.

Female defenses. The number of defensive behaviors generally decreased across tests ($F_{linear}(1,29)=17.05$, $p<0.001$, $\eta^2=0.33$) as displayed in Figure 3C. More defensive behaviors occurred on test 2 compared to tests 4 ($p=0.031$, $d=0.63$), 7 ($p=0.022$, $d=0.65$), and 8 ($p=0.043$, $d=0.61$) (main effect of test, ($F(7,203)=5.02$, $p<0.001$, $\eta^2=0.13$). No other effects were detected.

Male Behaviors. As shown in Figure 4A, the number of mounts received from males increased across tests ($F_{linear}(1,29)=18.59$, $p<0.001$, $\eta^2=0.39$). Fewer mounts were received on test 1 compared to all subsequent tests (all $p<0.03$, $d>0.63$) except test 3 (main effect of test, $F(7,203)=5.72$, $p<0.001$, $\eta^2=0.16$). In addition, CC/F received fewer mounts than CC/M ($M_{diff}=10.18\pm4.68$, $p=0.038$, $d=2.18$) (significant interaction between bedding and male presence, $F(1,29)=4.77$, $p=0.037$, $\eta^2=0.14$). No other effects were detected.

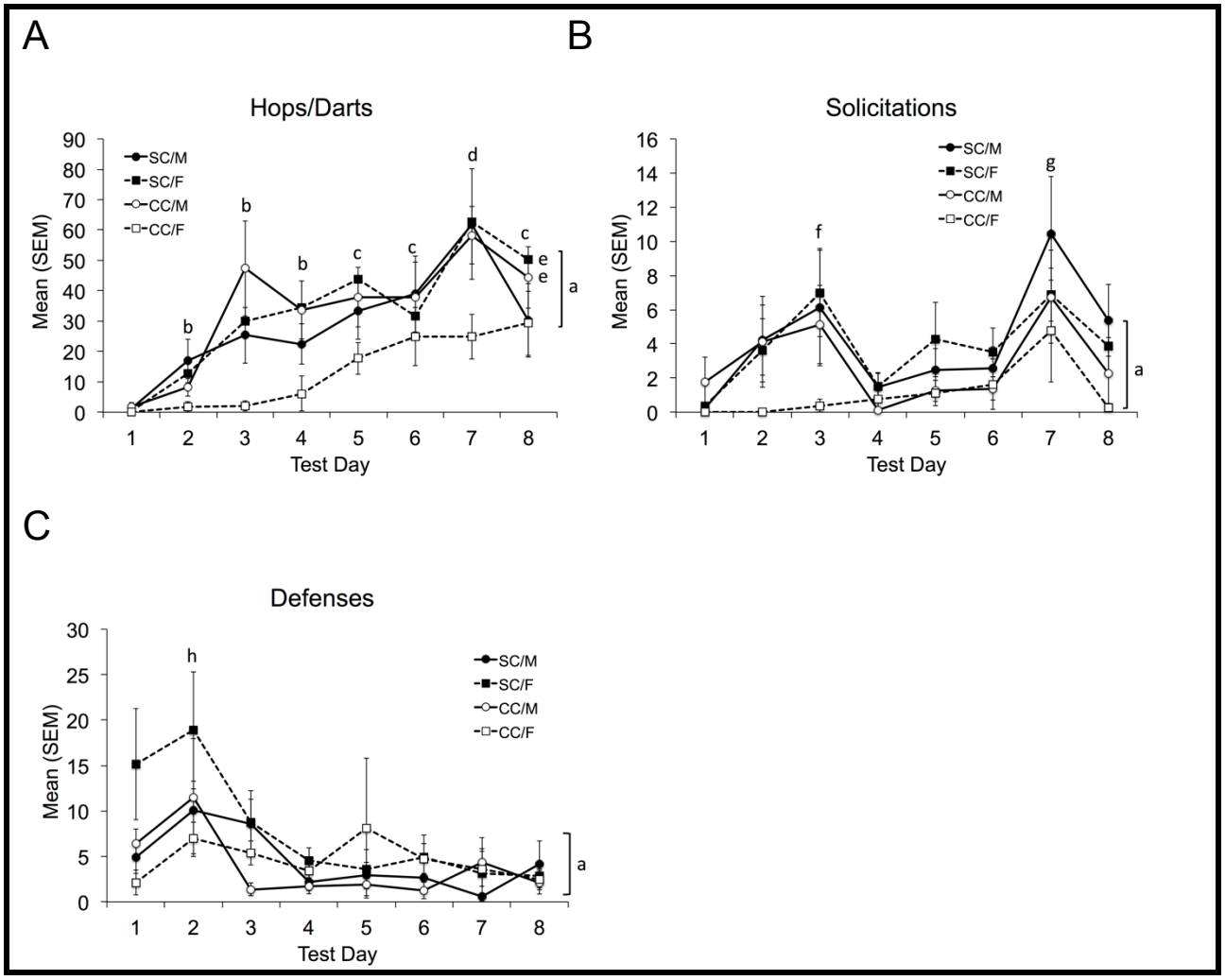

Figure 3. Appetitive and defensive behaviors displayed by females that were housed either on Sani-Chips (SC) or corncob (CC) bedding in a room that only housed females (F) or also housed males (M). [a]Significant linear trend. [b]Different from test 1. [c]Different from tests 1 and 2. [d]Different from tests 1, 2 and 6. [e]Different from CC/F. [f]Different from test 1 and 4. [g]Different from tests 1, 4, 6. [h]Different from tests 4, 7, 8. Significance set at $p<0.05$. See text for effect sizes.

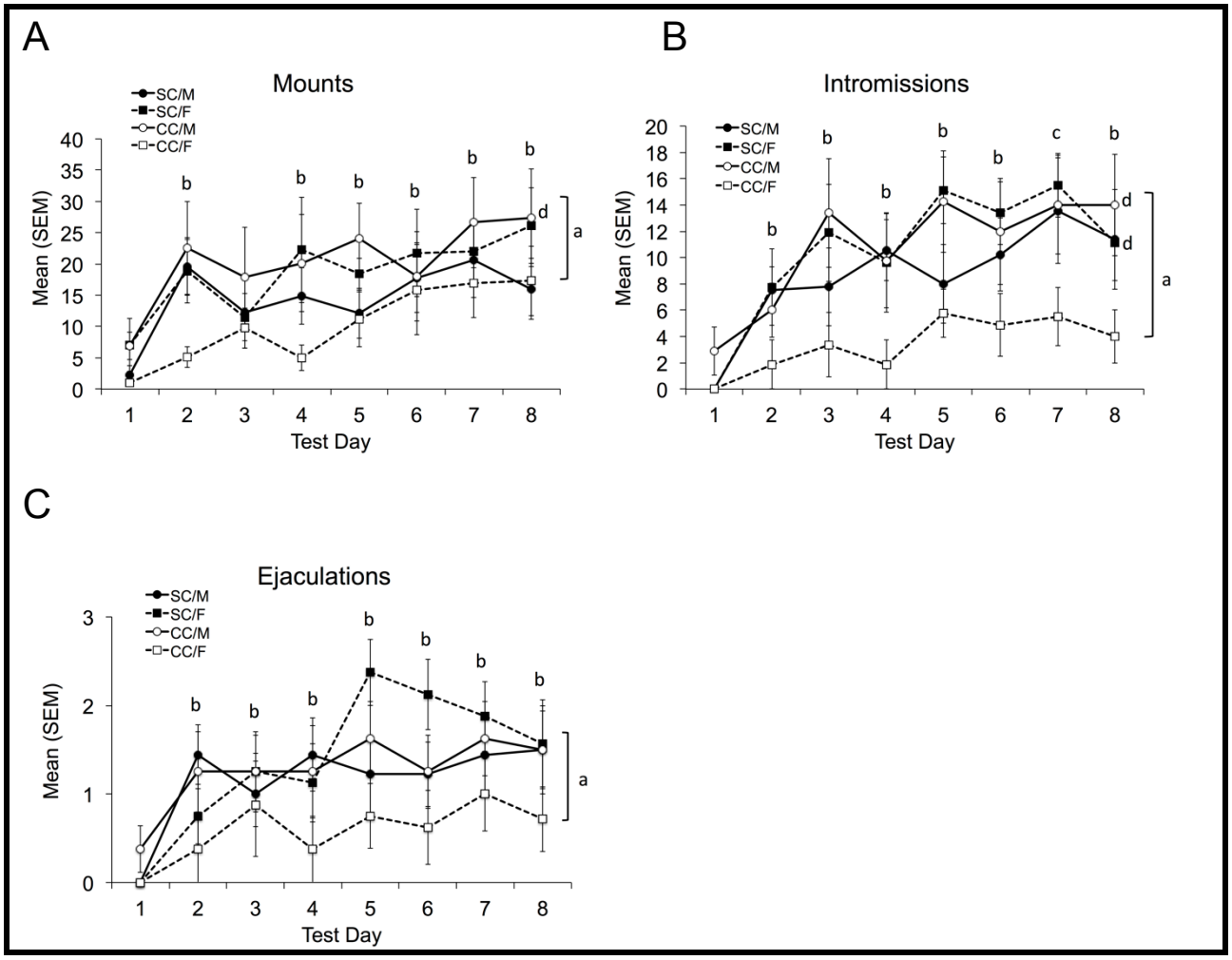

Figure 4. Number of mounts, intromissions and ejaculations received by the male in females that were housed either on Sani-Chips (SC) or corncob (CC) bedding in a room that only housed females (F) or also housed males (M). [a]Significant linear trend. [b]Different from test 1. [c]Different from tests 1 and 2. [d]Different from CC/F. Significance set at $p<0.05$. See text for effect sizes.

The number of intromissions (Figure 4B) received also increased across tests ($F_{linear}(1,29)=30.04$, $p<0.001$, $\eta^2=0.49$). Fewer intromissions were received on test 1 compared to all subsequent tests (all $p<0.001$, $d>0.85$), and fewer were received on test 2 compared to test 7 ($p=0.017$, $d=0.67$) (main effect of test, $F(7,203)=11.17$, $p<0.001$, $\eta^2=0.26$). In addition, CC/F received fewer intromissions than CC/M ($M_{diff}=7.38\pm2.84$, $p=0.015$, $d=2.60$) and SC/F ($M_{diff}=7.14\pm2.84$, $p=0.018$, $d=2.51$) (significant interaction between bedding and male presence, $F(1,29)=5.49$, $p=0.026$, $\eta^2=0.14$).

The number of ejaculations (Figure 4C) received increased across tests ($F_{linear}(1,29)=24.63$, $p<0.001$, $\eta^2=0.43$). Significantly fewer ejaculations were received on test 1 compared to all subsequent tests (all $p<0.003$, $d>0.78$) (main effect of test, $F(7,203)=7.37$, $p<0.001$, $\eta^2=0.19$). Although CC/F animals tended to receive fewer ejaculations than SC/F ($M_{diff}=0.80\pm0.35$, $p=0.031$, $d=2.29$) and CC/M ($M_{diff}=0.68\pm0.35$, $p=0.06$, $d=1.94$), the interaction between housing conditions only approached statistical significance with a relatively small effect size, $F(1,29)=3.38$, $p=0.076$, $\eta^2=0.09$.

Discussion

The main goal of this study was to assess whether OVX Long-Evans rats would display behavioral sensitization of sexual behaviors following the repeated administration of EB while manipulations were made to their housing conditions, either by housing them on corncob bedding or in a room separate from males. Our main finding is that the sensitization of sexual behaviors by repeated administration of EB occurs regardless of housing conditions. However housing females on corncob bedding in a room separate from males inhibits sexual behavior when primed with EB alone, but this inhibition is overcome by exposure to male cues. The data also show that estradiol sensitization is a robust effect, since it occurs in a paradigm where sexual behavior is inhibited and the magnitude of the sensitization was robust (as reflected in the effect sizes). A secondary finding is that in females primed with EB+P, sexual behavior was not inhibited in any condition on the final training day. Thus, despite research suggesting that corncob bedding may disrupt estrous cyclicity and attenuate sexual behavior in male and female Sprague-Dawley rats, this study demonstrates that corncob bedding supplied from Harlan (Montreal, Canada) does not inhibit sexual behavior of OVX Long-Evans rats when primed with

EB+P, nor does it inhibit sexual behavior when females are primed with EB alone if those females are continuously exposed to male cues by housing them in the same room. As such, the disruption of endocrine-dependent behaviors by corncob bedding may interact with other housing conditions, and has the potential to be overcome.

Sexual behavior was inhibited in females housed on corncob in a room separate from males when treated with EB alone. CC/F animals had a lower LQ on earlier tests, and displayed lower levels of the highest quality of lordosis (LM3), fewer H/D, and received fewer mounts and intromissions overall. They also tended to display fewer full solicitations and to receive fewer ejaculations from the male overall. The inhibitory effect on LM3 may be reproductively relevant, since the occurrence of at least one LM3 is more likely to induce the neuroendocrine changes associated with pregnancy/pseudopregnancy (Lehmann & Erskine, 2004). These results suggest that sexual behavior is inhibited in females housed on corncob bedding but only if they are housed in a room separate from males, and that the inhibitory effect on LQ can be overcome with repeated EB treatments. Moreover, cues from sexually vigorous males can buffer against any inhibitory effect of corncob bedding on female sexual behavior since no inhibitory effect was seen in females housed on corncob bedding if they were housed in a room that also housed males. Although from the current study it is not clear whether olfactory, auditory, or visual cues (or any combination of those cues) contribute to the effect, the data suggest that continuous exposure to male cues can facilitate the sensitization of sexual behaviors that occurs with repeated administration of estradiol.

We found that corncob bedding was not inhibitory to sexual behavior on the final training day when females were primed with EB+ P, regardless of housing condition. This contrasts with other reports in the literature (Markaverich et al., 2007a; Markaverich, Alejandro, et al., 2002a; Markaverich, Mani, et al., 2002b; Romero et al., 1990). Although the details of housing conditions, such as bedding type and whether animals are housed in rooms that contain both sexes, are not frequently reported, informal discussions with colleagues revealed that a number of investigators use corncob bedding without any negative effects on sexual behavior. Our data suggest that since those groups also study sexual behavior, males and females may have shared housing rooms (as is customary in our lab) and therefore any inhibitory effect of corncob bedding was overcome. Whether sexes were housed in the same rooms in those studies that reported an inhibitory effect of corncob bedding (Mani et al., 2005; Markaverich et al., 2007a;

Markaverich, Alejandro, et al., 2002a; Markaverich, Mani, et al., 2002b; Romero et al., 1990; Villalon Landeros et al., 2012) is unclear.

One other possible explanation for lack of inhibition in our study (when females were primed with EB+P) compared to those of Markaverich's group (2005; 2007a; 2002a; 2002b; 1990) may be handling. We attempted to control for amount of handling associated with the differences in cage change frequency, and it is unclear whether this factor could account for the inhibitory effects found in the Markaverich studies. Animals housed on woodchip beddings (such as Beta Chip or Sani-Chips) compared to corncob bedding have their cage changed more frequently, since the increased absorbency of corncob bedding allows for less frequent changing (Burn & Mason, 2005). Cage changes are stressful for animals (Castelhano-Carlos & Baumans, 2009; De Boer et al., 1990; Duke et al., 2001), and stress and handling are known to affect sexual behavior (Pfaus & Wilkins, 1995; Yoon et al., 2004). The increased frequency of cage changing with woodchips may allow the animal to learn more quickly that this procedure is not threatening, and subsequently lead to an attenuated stress response and normal levels of sexual behavior.

Alternate explanations may also include strain and species effects, and differences in steroid hormone administration or sensitivity. The disruptive effects of corncob bedding on sexual behaviors and reproductive physiology of male and female rats were performed in the Sprague-Dawley strain (Mani et al., 2005; Markaverich et al., 2007a; Markaverich, Alejandro, et al., 2002a; Markaverich, Mani, et al., 2002b; Romero et al., 1990), and those females were treated with lower levels of steroid hormones (2µg EB + 100µg P). Numerous reports exist within the literature outlining strain effects on various physiological and behavioral measures (e.g., Brand & Slob, 1991; Maswood, Sarkar, & Uphouse, 2008; Miryala, Hiegel, & Uphouse, 2013; Sachs, 1996; Sarkar, Hiegel, Maswood, & Uphouse, 2008; Uphouse et al., 2002). We have found that albino Wistar rats are behaviorally more sensitive to EB compared to the pigmented Long-Evans strain (Jones et al., 2013), whereas others have reported that 10µg EB administered alone failed to induce both lordosis and appetitive behaviors in albino Sprague-Dawley rats (Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994), suggesting strain differences in sensitivity to EB. Additionally, species differences regarding the effects of corncob bedding on neuroendocrine signaling may interact with differential sensitivities to steroid hormones. Impaired ERα signaling was reported in California mice (Villalon Landeros et al., 2012), and it is well known that ERα activation is important in the induction of female rat sexual behavior,

which suggests that ERα signaling was not disrupted in our rats when primed with EB+P or housed with males. This is consistent with reports showing that the active compound THF-diol in corncob bedding (which appears to be the compound involved in the inhibition of sexual behavior and cyclicity) does not compete for ER binding sites in the rat (Markaverich, Alejandro, et al., 2002a), which may reflect species differences in signaling pathways associated with corncob bedding. Relatedly, plasma levels of THF-diols were not increased in mice housed on corncob bedding when treated with the aromatase inhibitor fadrozole, as opposed to those treated with saline (Villalon Landeros et al., 2012); it was thus proposed that fadrozole may decrease fatty acid clearance, subsequently decreasing plasma levels of THF-diols (Villalon Landeros et al., 2012). Although speculative, strain and species differences in fatty acid metabolism may therefore explain differential responses to corncob bedding when primed with EB+P.

It is also possible that some of the discrepancy in findings can be accounted for by variations in bedding composition (for example, variations in phytoestrogenic compounds from the variety of corn used, concentration of THF-diols, or other compounds, between lot numbers, or suppliers). In fact, the corncob bedding was produced by different suppliers throughout the research groups described here (Andersons, USA; Green Products company, USA; Harlan, Canada), and although THF-diols have been detected in corncob beddings by a variety of manufacturers (Markaverich, Mani, et al., 2002b), it is unclear whether beddings of Canadian manufacturers were assessed.

Housing conditions between animal supplier facilities (i.e., during the perinatal period) may also account for differences in physiology and behavior. For example, the early perinatal environment, particularly by exposure to sex steroid hormones and endocrine disruptors, can have substantial effects on factors such as reproductive physiology and behaviors, brain morphology, and protein expression (Frye et al., 2012; Khurana, 2000; MacLusky, Bowlby, Brown, Peterson, & Hochberg, 1997; Patisaul & Polston, 2008; Pereira, Curvalho, & Cdos, 1997; Phoenix, Goy, Gerall, & YOUNG, 1959). Aromatizable androgens during the perinatal period masculinize and defeminize sexually dimorphic brain areas and behaviors, and there is evidence that THF-diols present in corncob bedding may regulate aromatase activity (Markaverich, Crowley, Rodriquez, Shoulars, & Thompson, 2007b). As such, it would be important to determine whether THF-diols act as, or influence the synthesis or activity of, aromatizable androgens particularly in sexually dimorphic brain areas. If so, this would explain

why THF-diols do not bind ER (Markaverich, Alejandro, et al., 2002a; Markaverich, Mani, et al., 2002b), but do affect downstream ERα signaling pathways (Villalon Landeros et al., 2012). If THF-diols increase aromatization of androgens to estrogens, then early perinatal exposure to corncob (and other critical periods, such as puberty) may disrupt activational effects of hormones. The animals in our study were housed on woodchip bedding during the perinatal period, and exposed to corncob only upon arrival to our facilities, whereas in the studies reported by Trainor's group (2013; 2012), animals were continuously housed on corncob bedding. Trainor et al., (2013) recently suggested that housing on corncob bedding during development may masculinize brain circuits controlling behavioral responses to social defeat, since females housed on corncob (rather than Sani-Chips) behave more male-like. Although perinatal exposure to aromatizable androgens on sexually differentiated behaviors are typically considered permanent, we have recently found that the decrease in sensitivity to the activational effects of sex steroid hormones in prenatally androgenized female rats can be overcome with sexual experience (Jones, S.L., Cordeaux, E., Germé, K., Pfaus, J.G., *submitted*), suggesting that any potential endocrine disrupting effects on sexual behavior by perinatal housing on corncob bedding may also be reversible. Importantly, Markaverich et al. (2002b) using a within-subjects design, were able to reverse the detrimental effects of corncob bedding by returning the animals to woodchips bedding or wire-mesh cages. Thus, although corncob bedding may be inhibitory to sexual behavior through alterations in steroid hormone signaling perinatally or in adulthood, there is evidence to suggest that that inhibition may be reversible.

Housing females on Sani-chips separately from males (SC/F) facilitated sexual behaviors. During the training phase when those females were fully primed with EB+P, they displayed more high quality lordoses (LM3) and received more intromissions than females housed on Sani-chips in a room with males. During the estradiol sensitization paradigm, SC/F animals were more likely to be consistently mounted across tests, but there were no beneficial effects of housing females alone on LM during the tests of estradiol sensitization. Together those results suggest that male sexual motivation was greater towards the "novel" SC/F females (novel since males were only ever exposed to them during test days) and that female sexual arousal was facilitated. Since a higher quality LM (LM3) was facilitated when SC/F females were primed with EB+P (but not when treated with EB alone), it is possible that progesterone signaling facilitates an

increase in female sexual arousal when those females are not given continuous exposure to male cues.

The greater number of males mounting SC/F compared to SC/M animals is reminiscent of the Coolidge effect, a phenomenon whereby sexual behavior is reinstated in a sexually satiated male, or maintained for a longer period of sexual activity in sexually active males, by presentation of a novel female (Beach & Jordan, 1956). The data presented here suggest that the cues (e.g., estrous odors, or ultrasonic vocalizations) of a novel female (i.e., housed in a separate room) primed with EB can stimulate sexual activity in males when those females are housed on woodchips (i.e., Sani-chips). The cues associated with a novel female might trigger an increase in mesolimbic dopamine, stimulating the initiation of sexual behavior (Fiorino, Coury, & PHILLIPS, 1997), since neural firing increases in the nucleus accumbens, (a brain region implicated in appetitive sexual behaviors) in sexually-naïve males presented with estrous odors from a novel colony compared to females from a familiar colony (Wood, Kosobud, & Rebec, 2004). The pathway mediating an increase in sexual motivation may be through the vomeronasal organ, whose activation is reduced (as measured by Fos-IR) in non-copulating males compared to sexually active males in response to estrous odors (Portillo & Paredes, 2004). Males are capable of attending to cues associated with a familiar female that predicts sexual reward (Ismail et al., 2009; 2011), and quickly learn to inhibit mounting sexually non-receptive females (Pfaus & PINEL, 1989). Thus, it is also possible that housing males and females in the same room might elicit a *frustration* phenomenon in males towards those females, such that all attempts to escape their cage and reach the sexual partner were unsuccessful. As such, when the animals are presented in the testing chambers, the motivation to mount the female may be reduced. A similar *frustration* phenomenon was proposed in female rats receiving clitoral stimulation in the presence of an inaccessible male restricted behind a screen (Parada et al., 2011). This inhibitory effect may have been overcome by housing the females on a "novel" bedding since CC/M females were also more likely to be mounted than SC/M and CC/F females, but it is unclear why this occurred.

Conclusion

Housing conditions are known to have an important influence of experimental outcomes, and as such it is important to consider how the conditions potentially impact study parameters.

Here we have shown that corncob bedding supplied in Canada from Harlan does not disrupt the sexual behavior of OVX Long-Evans rats treated with EB+P regardless of whether they are housed with males or not, nor does it disrupt the sensitization of female sexual behaviors by repeated treatment with EB-alone. However, if females were housed on corncob in a room separate from males, their sexual behavior was inhibited when treated with EB-alone. We have also shown that housing females on woodchip bedding (Sani-Chips) in a separate room from males may increase male sexual motivation towards those females and female sexual arousal when primed with EB-alone. In summary, the broad implications of these findings are that the beneficial effects of corncob bedding in animal husbandry can be applied to groups studying sexual behavior as long as males and females are housed in the same room (which is not an inconvenience since it is commonplace in the study of sexual behavior). Nevertheless, we recommend that quality control studies be conducted at individual institutions and research groups to assess any negative impact of corncob bedding on endocrine-dependent physiology and behavior.

4.2. Behavioral defeminization by prenatal androgen treatment in rats is overcome by sexual experience in adulthood

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Abstract

Exposure to testosterone during a critical period of prenatal development prevents the normal display of sexual behaviors in adult ovariectomized (OVX) rats treated with estradiol benzoate (EB) and progesterone (P). The organizational hypothesis posits that prenatally androgenized females (PNAFs) are desensitized to EB. We tested this hypothesis by first treating PNAFs with varying doses of EB (2.5, 5, 10, 20µg) and P (500µg), and second by subjecting females to an established estradiol sensitization paradigm where females are first given sexual experience with EB (10µg)+P prior to repeated testing with EB alone. Long-Evans females were androgenized in utero by a SC injection of 500µg testosterone propionate or the oil control to pregnant dams on gestational day 18. Female offspring were OVX on postnatal day 80 and tested one week later in the unilevel 4-hole pacing chamber. Genital tissue was defeminized in PNAFs, and the lordosis quotient (LQ) and partial (i.e., hops/darts) and full solicitations were significantly lower, while defensive behaviors were higher, in PNAF females, relative to non-PNAF females regardless of acute EB priming. However, repeated testing with EB alone (10µg), or EB+P eliminated the differences between groups on LQ and hops/darts, indicating that the behavioral deficit can be overcome by sexual experience. These results suggest that PNAFs are not desensitized to EB, and despite morphological differences in genital development, the deficiency in sexual behavior in response to acute EB+P can be experientially overcome. However PNAFs appear to have a deficit in the expression of full solicitations.

Key Words: organizational hypothesis, estradiol sensitization, testosterone, sexual behavior

Introduction

The hormonal environment during the perinatal period organizes reproductive and neural tissue in such a way that steroid hormone administration in adulthood differentially activates sexually dimorphic sexual behaviors (Janis L Dunlap, Gerall, & Carlton, 1978; Gerall & Ward, 1966; Phoenix et al., 1959; Rhees, Kirk, Sephton, & Lephart, 1997). The presence of biologically active aromatizable androgens during the critical perinatal period in rodents induces masculinization and defeminization, whereas the absence of androgens, results in feminization. Behaviorally, perinatally androgenized rodents gonadectomized in adulthood display male-typical sexual behaviors in response to androgens, whereas priming with estradiol benzoate (EB) and progesterone (P) fails to elicit female-typical sexual behaviors, regardless of chromosomal sex. If, on the other hand, androgens are not present during this critical period, animals fail to display male-typical sexual behaviors in response to androgens in adulthood, and instead will display female-typical sexual behaviors in response to EB and P. Similar effects of early hormone exposure are seen anatomically. For example, males and prenatally androgenized females (PNAF)s have a larger sexually dimorphic nucleus of the preoptic area (SDN-POA) compared to untreated females (Döhler et al., 1982; Gorski, Gordon, Shryne, & Southam, 1978; Gorski, Harlan, Jacobson, Shryne, & Southam, 1980), and genital morphology is masculinized by prenatal androgen treatment in females, resulting in a longer anogenital distance (Rhees et al., 1997).

Although it is commonly believed that the behavioral effects of perinatal hormonal disruption are permanent, earlier studies suggest that the deficits can be at least partially overcome by altering the hormonal priming conditions. Following gonadectomy in adulthood, both male and PNAF rats treated with estradiol and P display rates of lordosis similar to that observed in ovariectomized (OVX) rats in adulthood, provided estradiol is administered in a pulsed fashion (Olster & Blaustein, 1988; Södersten, Petterson, & Eneroth, 1983). In some cases, such males are also capable of displaying P-facilitated appetitive sexual behaviors such as ear-wiggling (Södersten, 1976); but not more active forms such as hops and darts (Olster & Blaustein, 1988). Whether full solicitations (McClintock, 1984) can be restored in PNAF has never been examined. Thus, it is possible that the behavioral deficits can be restored in PNAF using higher hormonal priming EB doses given acutely in combination with P.

Two critical hypothalamic regions that control female sexual behavior in a variety of species are the medial preoptic area (mPOA) and ventromedial hypothalamus (VMH). In adult female rats, estradiol binds to its receptors (ER) within the VMH to induce lordosis, as well as to promote P receptor synthesis in both these regions (MacLusky & McEwen, 1978). Progesterone binding to its receptor in the VMH potentiates lordosis, and binding within the mPOA induces sexually appetitive behaviors (such as hops/darts, solicitations, and ear wiggles) (Beyer et al., 1997; Hoshina et al., 1994; Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994; Rubin & Barfield, 1983). Within these regions ER densities are sexually dimorphic in favor of females (Brown, MacLusky, Shanabrough, & Naftolin, 1990; Lauber, Mobbs, Muramatsu, & Pfaff, 1991a; Lauber, Romano, & Pfaff, 1991b). This is mediated, at least in part, by aromatization of testosterone to estradiol during the perinatal period which organizes sexual differentiation of the brain and subsequent behavior (McCarthy, 2008; McCarthy, Schwarz, Wright, & Dean, 2008). Perinatal exposure to estradiol methylates the ERα promoter region, associated with a down-regulation of ERα mRNA (DonCarlos, McAbee, Ramer-Quinn, & Stancik, 1995; Kurian, Olesen, & Auger, 2010). As such, early androgen exposure permanently reduces ERα expression, and it has been suggested that females are permanently desensitized to the activational effects of estrogens (MacLusky et al., 1997). In support of this, the expression of estradiol-induced P receptors is attenuated in PNAFs, suggesting that neurochemical events triggered by estradiol are disrupted (Foecking, Szabo, Schwartz, & Levine, 2005).

Although these data suggest a reduced responsivity of the androgenized female rat to estradiol, the degree of this insensitivity and whether it can be fully or partially rescued by subsequent hormonal treatment has not been fully explored. Thus in the first study described here we asked whether increasing the dose of EB when given acutely in combination with P, would facilitate sexual behavior. Next we tested whether prenatally TP-treated females would display behavioral sensitization to chronic EB. Although, acute administration of EB alone only partially activates female sexual behaviors repeated administration induces higher levels of sexual behaviors than the initial dose (Babcock et al., 1988; Beach & Orndoff, 1974; Blaustein et al., 1987; Clark & Roy, 1983; Gerall, Dunlap, & Hendricks, 1973; Jones et al., 2013; Kow & Pfaff, 1975; Parsons et al., 1979; Whalen & Nakayama, 1965). For example, we recently reported that when treated every four days, both OVX and OVX-adrenalectomized sexually-experienced Long-Evans rats administered 5 and 10μg EB and given access to a sexually

vigorous male in a uni-level four-hole pacing chamber, display increasingly greater lordosis quotients (LQ) and sexually appetitive (hops/darts, full solicitations) behaviors with each injection, reaching a plateau by the fourth test (Jones et al., 2013). However EB doses below 3µg, when repeatedly administered alone, are insufficient to induce sexual behavior if the female has access to the male on every test (Jones et al., 2013; Jones & Pfaus, 2014; Kow & Pfaff, 1975; Micevych et al., 2008). Because PNAFs are less sensitive to the activational effects of EB on sexual behavior, in the second study described below, we investigated whether those females would express behavioral sensitization to repeated injections of EB, following sexual experience with EB and P. We hypothesized that if PNAFs were permanently desensitized to EB, as proposed by the organizational hypothesis, they would remain impaired to the activational effects of steroid hormone priming on the full array of sexual behaviors regardless of the EB dose administered, and fail to display behavioral sensitization to repeated EB treatments.

Materials and Methods

This experiment was conducted in accordance with the ethical standards established by the Canadian Council on Animal Care (CCAC), and approved by Concordia University's Animal Care Committee.

Animals, Mating, and Prenatal Treatment Procedures

All animals in this study were housed in colony rooms maintained at approximately 21°C on a 12:12 light:dark schedule (lights off at 0800). Animals were given ad libitum access to food (Charles River, 5075) and tap water at all times, and environmental enrichment in the form of shredded paper was added to the homecage at each bi-weekly cage change. Home cages as well as the testing apparatus were lined with Betachip®. Adult males and females were housed in pairs in Plexiglass shoebox cages (48 X 25 X 20 cm) unless otherwise indicated.

Dams. Twelve females of the Long-Evans strain were used. Sexual receptivity was verified daily by placing the female in a unilevel 4-hole pacing chamber with a sexually vigorous male. Copulation in these chambers allows the smaller female to pace the rate of copulation by entering the males' side of the chamber, whereas the larger male is restrained to his side. Pacing the rate of sexual contact at her preferred interval is rewarding to the female, and increases the probability of impregnation (Erskine et al., 1989; Jenkins & Becker, 2003b; Paredes & Vazquez,

1999). Those displaying proceptive hopping, darting and solicitations as well as lordosis in response to a mount were left to copulate for one hour. The experimenter ensured that females received at least one ejaculation, and this day was considered GD0.

Impregnation was successful in 7 of the 12 time-mated females, verified by an increase in body weight accompanied by abdominal distention in the weeks following copulation. Each female was randomly given a SC injection of either sesame oil (Oil, n=3) or 500µg of TP dissolved in 0.5mL of sesame oil (TP, n=4) on GD18, were housed individually, and provided with shredded paper for nest building. This TP dose disrupts genital morphology and the expression of female-typical sexual behaviors in adulthood (Rhees et al., 1997). As of GD20, twice a day (approximately 8AM and 5PM) a researcher verified whether the pregnant females had given birth. The day of birth was identified as postnatal day zero (PN0). Between PN21-23 the pups were weaned, sexed and housed in same-sex groups of 3 or 4 until they required additional space (approximately 2 months of age), when they were finally housed in pairs until the end of the experiment. Since males were not experimental subjects in the study, those from oil-treated mothers were distributed as stimulus animals in other studies, whereas those from TP-treated mothers were sacrificed under CO_2 gas following weaning.

Stimulus Males. A group of Long-Evans male rats (~6 months of age) that had previously been used in unrelated studies in our laboratory were used for mating, and behavioral training and tests. They were sexually experienced in the unilevel 4-hole pacing chambers.

Experimental Females. Females born from dams treated on GD18 were used as experimental animals. On GD80 females were OVX. On PN95 the anogenital region was photographed and anogenital distance was measured with a caliper.

Ovariectomy

Anesthesia was induced with a 4:3 mixture of ketamine hydrochloride (50 mg/mL; Ketaset®, Wyeth Canada) and xylazine hydrochloride (4 mg/mL; Rompum®, Bayer Healthcare) injected i.p (1mL/kg). An ocular ointment was applied (Natural Tears®, Alcon), and when unresponsive to a foot pinch, bilateral OVX was performed via a single lumbar incision. Animals were numbered by ear-punch and post-operative care was given with SC injections of Flunixin meglumine 2.5mg/kg (Banamine®, an anti-inflammatory, analgesic, and antipyretic) and 5mg/kg Enrofloxacin (Baytril®, an antibacterial), followed by 3mL of saline administered SC, and

polysporin was applied to the incision site. Animals were given one-week post-operative recovery prior to testing.

Hormone treatments in adulthood

17β-estradiol benzoate (2.5, 5, 10, or 20μg), and P (500μg) were dissolved in 0.1mL reagent grade sesame oil. EB was always injected SC 48 hours, and P 4 hours, prior to training or testing. Hormones and sesame oil were supplied by Steraloids (Newport, RI).

Experimental procedures

Experiment I. Increasing doses of EB given in combination with P on female sexual behavior. The week prior to OVX, females were acclimated to the 4-hole unilevel pacing chamber for 5 consecutive days, for 15 minutes each. One week following OVX females were primed with 2.5, 5, 10 or 20μg EB (n=6-7/group - see sample sizes in Table 1), followed by P.

Table 1.
Sample sizes of prenatally oil or testosterone propionate (TP) treated females used in Experiment 1. Females were treated with varying EB doses in combination with 500μg progesterone.

	Prenatal Treatment	
	Oil	TP (500μg)
EB Dose		
2.5μg	6	7
5μg	6	9
10μg	6	6
20μg	6	7
Total	24	29

Experiment 2a. Estradiol sensitization: Sexual training with EB+P. Rats treated with 2.5 or 10µg EB in Experiment 1 were selected to assess behavioral sensitization to EB. Following a two-week hormone washout period, female rats were treated with 10µg EB and 500µg P every 4 days and given four 30 minute sexual training sessions in the unilevel four-hole pacing chamber, followed by a second 2-week hormone washout period (as in Jones et al., 2013; Jones & Pfaus, 2014).

Experiment 2b. Estradiol sensitization: Repeated administration of 2.5µg or 10µg EB alone. Following training, half the females previously treated in Experiment 1 with 2.5µg EB were reassigned to receive 10µg EB, and half the females previously treated with 10µg EB were reassigned to receive 2.5µg EB, to balance prior EB-treatment history between groups. To test for behavioral sensitization to repeated EB over 6 test days, females were administered their respective EB dose, and tested 48 hours later in the unilevel 4-hole pacing chamber. After completion of all behavioral testing brains were collected from all rats in this study to assess the effect of prenatal androgen administration on the size of the sexual dimorphic nucleus using standard histological techniques.

Behavioral Measures

Lordosis magnitude (LM) was coded on a 3-point scale as in Hardy and Debold (1971b), and lordosis quotient (LQ) was calculated as a ratio of the number of lordosis postures displayed to the total number of mounts, intromissions, and ejaculations received (x100). Hops/darts, and full solicitations (headwise orientation towards the male followed by a runaway) were analyzed both separately, and summed together as a general measure of appetitive sexual behaviors (as in Jones et al., 2013; Jones & Pfaus, 2014). Full solicitations are indicative of greater sexual motivation compared to hops and darts, since they are observed more frequently in fully EB+P primed females compared to those treated with EB alone (Pfaus et al., 1999). Kicks, sideways takedowns, boxing postures, and prone position were coded as defensive behaviors (Barnett, 1963). Mounts, intromissions, and ejaculations from the male were also measured, as they provide insight into the female's sexual receptivity and attractivity (Pfaus et al., 1999).

Volume of SDN-POA

Two days following their last test day, rats in Experiment 2 were once again treated with their appropriate EB dose. Forty eight hours later rats were given an overdose of sodium pentobarbital (120mg/kg, ip), the descending aorta was clamped and animals were perfused intracardially with 250mL of ice-cold phosphate-buffer saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed, postfixed in 4% paraformaldehyde for 4 hours and dehydrated in 30% sucrose solution overnight. The following day they were flash-frozen on dry ice and stored at -80C until anatomical staining with cresyl violet (<8 months).

Coronal sections (30μm) of the preoptic area were collected onto gel-coated slides, dried overnight, and stained with cresyl violet the following day. Slides were submerged sequentially in wheaton dishes containing dH_2O, followed by 70%, 95% and 100% alcohol (3 mins in each). Next they were agitated in xylene for 2 mins, and rehydrated in 100%, 95%, 70% alcohol (3 mins each) before rinsing in dH_2O. Slices were then submerged for 5-7 mins in a 0.1% aqueous cresyl violet acetate solution (1% cresyl violet stock solution diluted in dH2O + 4 drops of glacial acetic acid per 100mL solution), and rinsed in dH_2O. Next, slices were dehydrated in 70, 95, and 100% ethyl alcohol (3 mins each) before rinsing in a 1% acid alcohol decolorizing solution (10% acetic acid dissolved in ethanol). Slices were then submerged for 3 mins each in 100% alcohol, xylene:alcohol (mixed at 1:1), and finally xylene before coverslipping.

The sexually dimorphic nucleus of the preoptic area was located by first identifying the anterior commissure, suprachiasmatic nucleus and the optic chiasm. Serial pictures were taken with QCapture Pro 7 software (2010), with a 4x objective, between plates 33 and 44 of Paxinos and Watson (Paxinos & Watson, 2013) using the third ventricle, anterior commissure, and optic chiasm as landmarks. An investigator blind to the prenatal treatment independently selected the section containing the largest diameter (LD) of the SDN-POA for each brain, and calculated the area using ImageJ v.1.46 (Schneider, Rasband, & Eliceiri, 2012). Images were converted to 8-bit, brightness/contrast was automatically adjusted, the scale was set using a metric ruler and using the irregular tool the nucleus was carefully outlined. The average area of the left and right hemispheres was taken.

Statistical analyses

Anogenital distance. Independent t-tests were used to compare anogenital distance, and area of the largest section of the SDN-POA between prenatal treatment groups. *Cohen's d* is reported as a measure of effect size.

Experiment I. Increasing doses of EB given in combination with P on female sexual behavior. A 2 way between subjects ANOVA was used to assess the effects of prenatal treatment and EB dose on sexual behavior. Where appropriate *t* tests with Bonferroni adjustment were used to assess differences between EB groups.

Experiment 2a. Estradiol sensitization: Sexual training with EB+P. The effect of prenatal treatment on sexual behavior displayed during training was assessed using a two way mixed ANOVA with prenatal treatment (TP vs oil) as the between groups factor and training day (Day1 vs Day 4) as the within subjects factor.

Experiment 2b. Estradiol sensitization: Repeated administration of 2.5µg or 10µg EB alone. To evaluate any effects of prenatal treatment on the ability of repeated EB treatment to increase appetitive sexual behaviors a 3 way mixed ANOVA was used with prenatal treatment (TP vs oil) and EB treatment (2.5 vs 10µg EB) as between group factors and test day (1-6) as the within subjects factor. Eta squared ($\eta 2$) is reported as a measure of effect size. Simple main effect analyses were used to investigate interactions. Because the number of females receiving mounts varied across days, repeated measures ANOVA could not be used to assess differences in LQ. Thus, A 2 way ANOVA (prenatal treatment by EB dose) was used to assess differences in LQ on each test day. All alpha levels were set at p=0.05. Data are reported as mean ± SEM, unless otherwise noted.

Results

Subjective observations during ovariectomy

Although the morphology of ovaries and uterus appeared normal in oil-treated animals, the uterus of most prenatally TP-treated females was distended and filled with fluid, similar to that reported in Wolf (2002). Three prenatally TP-treated females died from surgical complications, and final sample sizes are reported in Table 1.

Anogenital distance

The anogenital distance was significantly longer in females treated prenatally with TP (M=2.18 cm, SD=0.23) than with Oil (M=1.76cm, SD=0.15), $t(51)=7.55$, $p<0.001$, $d=2.08$. Figure 1 illustrates representative photographs of the anogenital region of Oil (left) and TP-treated females (right) at 95 days of age. Few PNAFs had a vaginal opening at this time, although no systematic measurements were taken.

Sexually dimorphic nucleus of the preoptic area

Figure 2 panels A and B depict representative images of the largest section (30µm thick) through the SDN-POA of prenatally Oil and TP treated females. Although the area of a section of the SDN-POA was larger in prenatally TP treated females (Figure 2 panel C), the independent t-test failed to meet statistical significance, but the large effect size suggests that the lack of significance may be due to low power, $t(10)=1.67$, $p=0.126$, $d=0.96$.

Experiment I. Increasing doses of EB given in combination with P on female sexual behavior

Lordosis. Fewer prenatally TP-treated females were mounted compared to controls, $\chi^2=4.12$, $p=0.043$, such that two females from the Oil group (2.5µg EB, n=1; 20µg EB, n=1) and nine in the TP group (2.5µg EB, n=4; 5µg EB, n=1; 10µg EB, n=2; 20µg EB, n=2) were excluded from the analysis of LQ. LQ was lower in females treated prenatally with TP (Figure 3A) (main effect of prenatal treatment on LQ, $F(1,34)=5.68$, $p=0.023$, $\eta^2=0.14$). Neither the main effect of EB dose, nor the interaction effect reached statistical significance. Similarly LM did not differ with either prenatal treatment, dose of estrogen or their interaction (data not shown).

Appetitive and Defensive Behaviors. As shown in Figure 3, females treated prenatally with TP displayed fewer solicitations (panel B) and hops/darts (panel C) than Oil treated animals reflected in a significant main effect of prenatal treatment on both parameters (solicitations, $F(1,45)=4.54$, $p=0.039$, $\eta^2=0.09$; hops and darts, $F(1,45)=35.10$, $p<0.001$, $\eta^2=0.44$). Neither the main effect of EB dose nor the interaction reached statistical significance. The same pattern was maintained when summed into a general measure of sexually appetitive behaviors, (main effect of prenatal treatment, $F(1,45)=36.17$, $p<0.001$, $\eta^2=0.45$; data not shown). The main effect of EB dose and the interaction were not statistically significant.

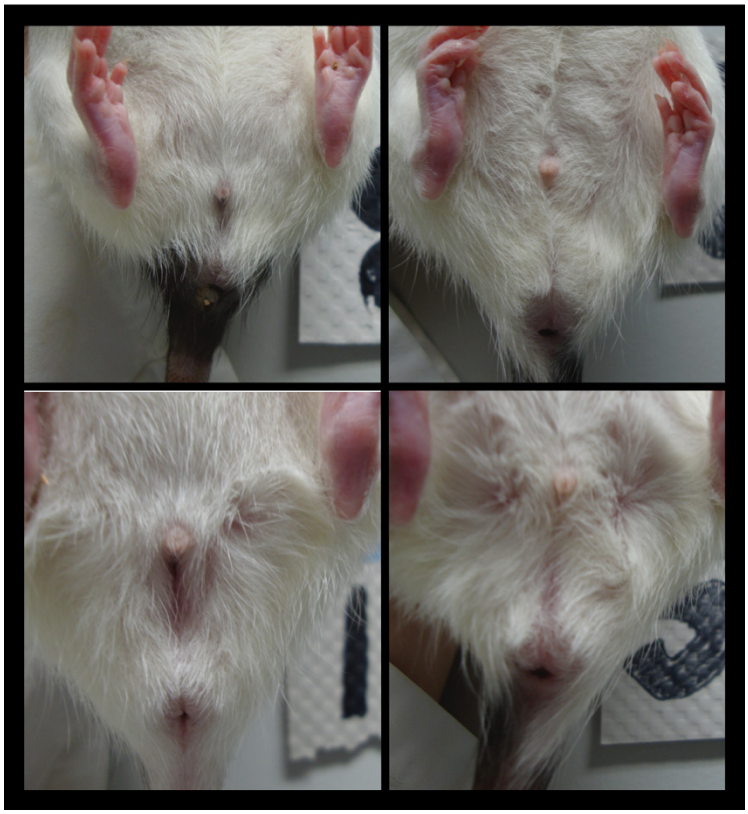

Figure 1. Representative photographs of the anogenital region of Oil (Left) and TP-treated (Right) females. The anogenital distance was longer in prenatally TP-treated females, and most did not have a vaginal opening (see text for details).

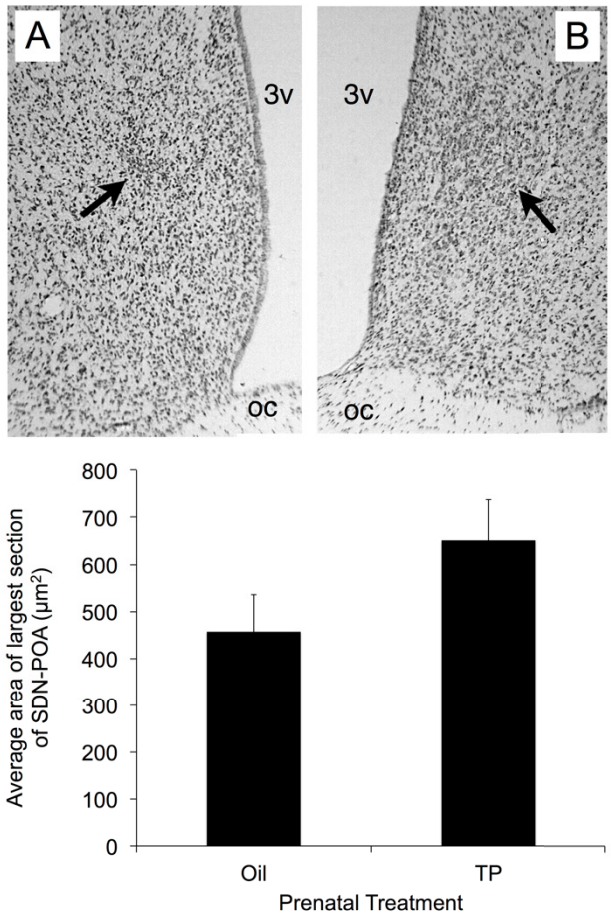

Figure 2. The section (30µm) with the largest diameter of the SDN-POA was selected for each rat, captured at 40x, and the average area of the left and right hemispheres were calculated. Prenatally oil treated females (A) generally had a smaller SDN-POA than TP treated females (B), as illustrated in the photographs. Group means and SEM are depicted in panel C.

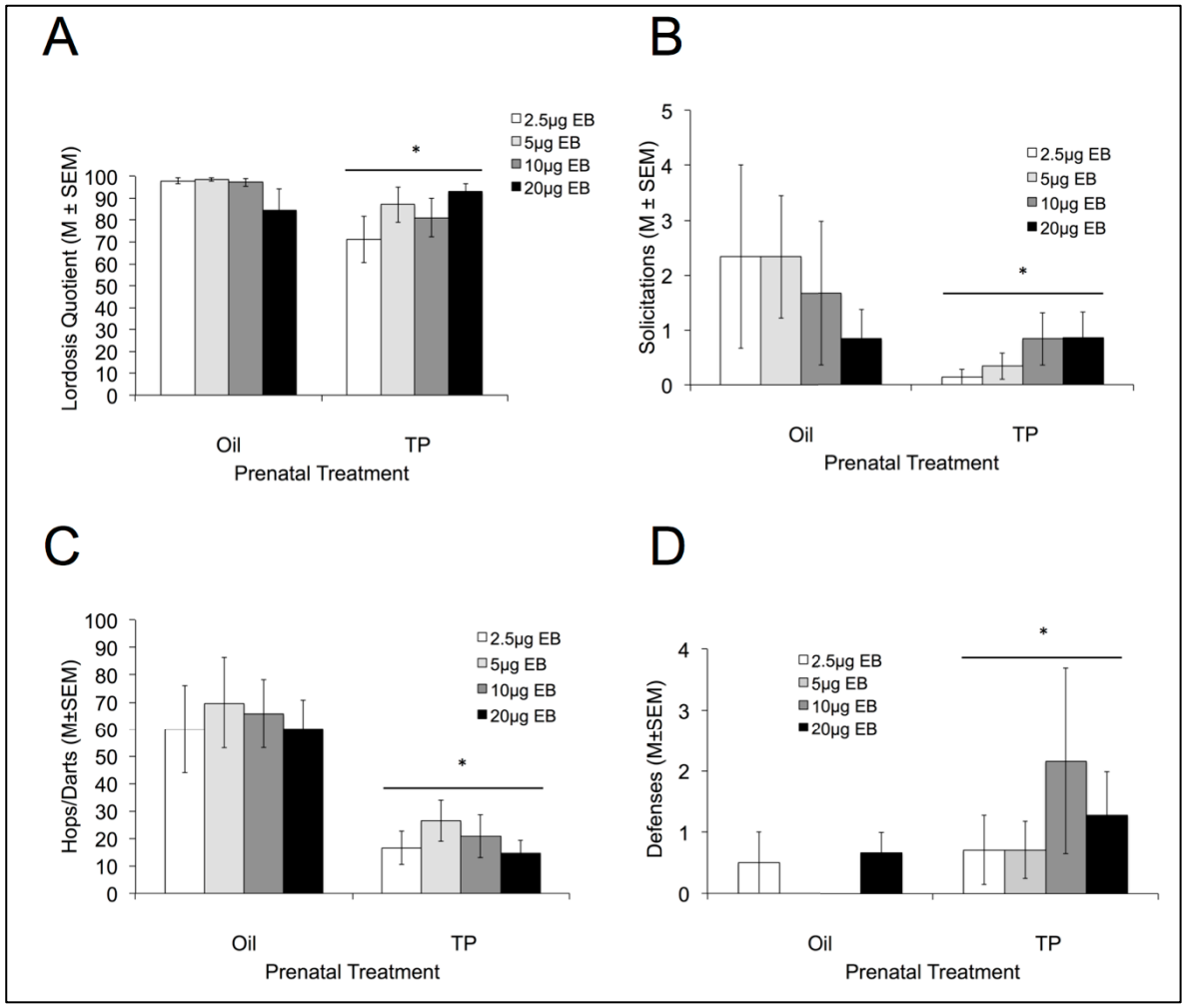

Figure 3. Varying doses of EB given in combination with 500µg P in prenatally Oil-treated or TP-treated females. Lordosis Quotient (A), Solicitations (B), and Hops/darts (C) were significantly attenuated, whereas Defensive behaviors (D) were significantly higher in prenatally TP-treated females. *Significantly different from controls.

Females treated prenatally with TP displayed more defensive behaviors than control animals (Figure 3D) (significant main effect of prenatal treatment $F(1,45)=4.01$, $p=0.051$, $\eta^2=0.08$). Neither the main effect of EB nor the interaction was statistically significant.

Male Behaviors. Number of mounts (Figure 4A) and intromissions (Figure 4B) received did not vary significantly as a function of prenatal treatment or dose of EB. A significant main effect of prenatal treatment was detected on ejaculations, ($F(1,45)=15.93$, $p<0.001$, $\eta^2=0.26$, Figure 4C) which is probably attributable to the lack of vaginal openings in the TP treated group. Neither the main effect of EB nor the interaction met statistical significance.

Experiment 2a. Estradiol sensitization: Sexual training with EB+P.

Lordosis. LQ was higher on the final training day compared to the first (Figure 5A) (significant main effect of training day, $F(1,15)=5.77$, $p=0.030$, $\eta^2=0.28$) but there was no effect of prenatal treatment on this parameter. LM neither changed across days nor differed between groups (data not shown).

Appetitive and Defensive Behaviors. Significantly more full solicitations were observed overall in oil-treated animals than in TP-treated animals (Figure 5B; main effect of prenatal treatment, $F(1,23)=10.43$, $p=0.004$, $\eta^2=0.31$). Neither the main effect of training day nor the interaction was significant. No differences were detected in the number of hops/darts (Figure 5C), on the combined measure of appetitive behaviors (data not shown but closely resembles hops/darts), or defensive behaviors (Figure 5D).

Male Behaviors. Number of mounts did not differ significantly as a function of either prenatal treatment or training day (Figure 6A). However, prenatally-oil treated animals received more intromissions and ejaculations on the final training day compared to the first, as shown in Figure 6B and C respectively but this effect was not seen in androgen treated females, reflected in significant interactions: intromissions, $F(1,23)=4.44$, $p=0.046$, $\eta^2=0.16$; ejaculations, $F(1,23)=4.25$, $p=0.006$, $\eta^2=0.28$. Controls also received more intromissions ($p=0.002$, $d=1.38$) and ejaculations ($p=0.002$, $d=1.40$) on the final training day compared to prenatally TP-treated females. The effects are probably due to a lack of vaginal opening in prenatally TP-treated females.

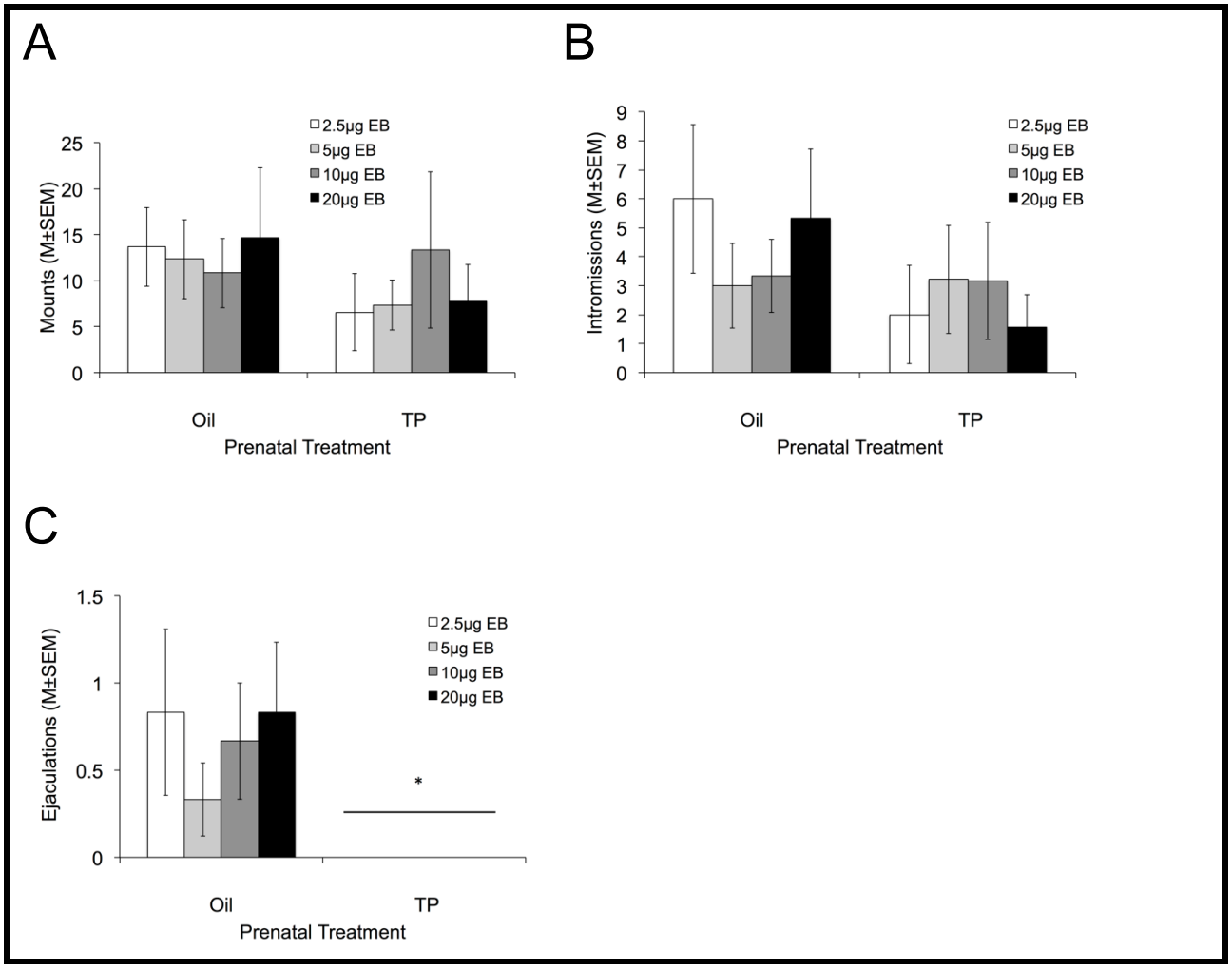

Figure 4. Varying doses of EB given in combination with 500µg P in prenatally Oil-treated or TP-treated females. The number of mounts (A) and intromissions (B) received were equal between prenatal treatment groups, but prenatally TP-treated females did not receive any ejaculations (C). *Significantly different from controls.

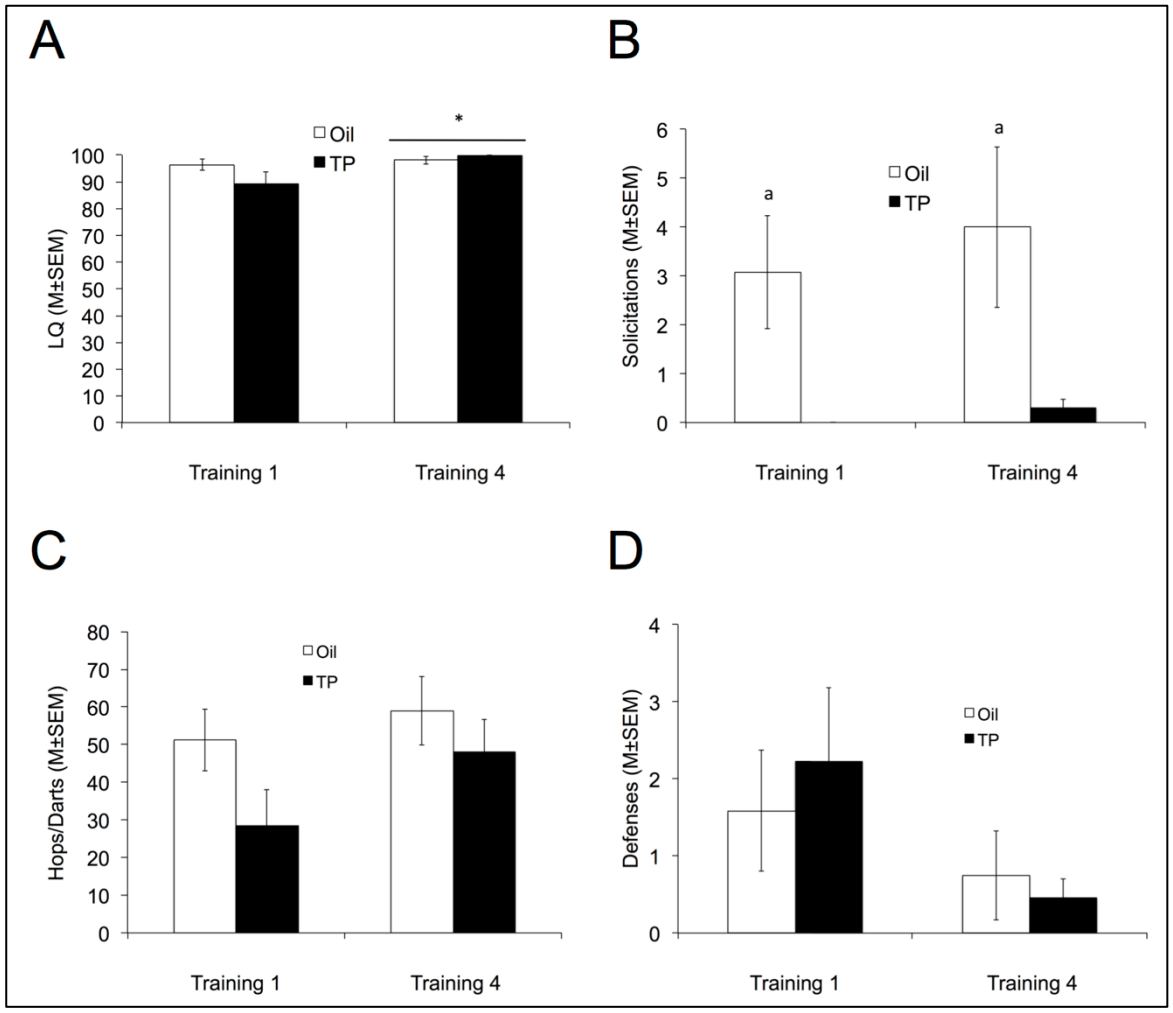

Figure 5. A subset of females from Phase 1 were given a 2-week hormone washout, followed by 4 sexual training sessions following EB (10µg) + P (500µg) priming. No prenatal treatment differences were found on LQ (A), hops/darts (C) or defenses (D), but fewer solicitations were observed in prenatally TP treated females overall (B). [a]Main effect of prenatal treatment.

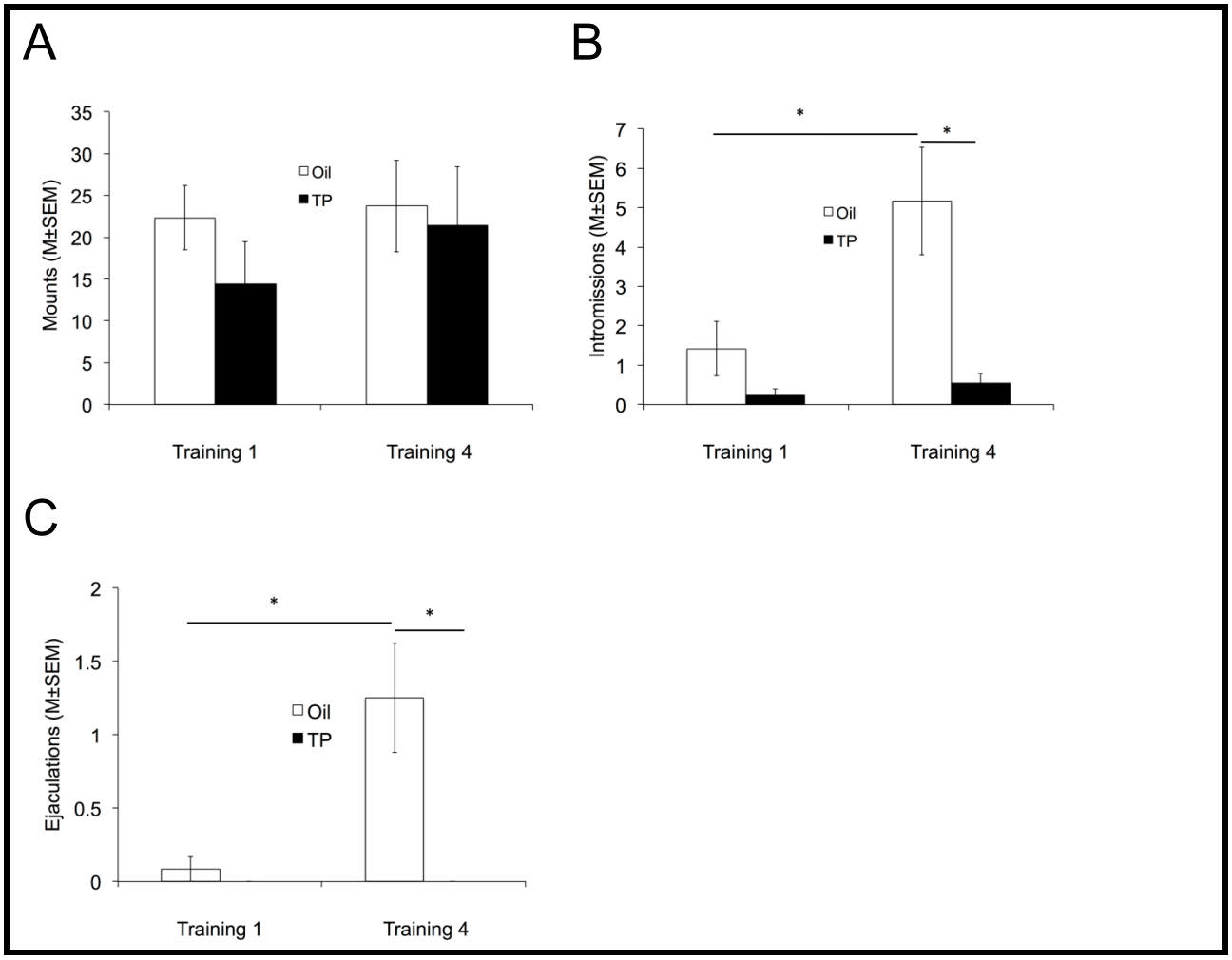

Figure 6. A subset of females from Phase 1 were given a 2-week hormone washout, followed by 4 sexual training sessions following EB (10µg) + P (500µg) priming. No prenatal treatment differences were found on the number of mounts received (A), but control females received significantly more intromissions (B) and ejaculations (C) on training day 4 compared to day 1, and on training day 4 they received significantly more intromissions and ejaculations than prenatally TP-treated females. *p<.05.

Experiment 2b. Estradiol sensitization: Repeated administration of 2.5µg or 10µg EB alone.

Lordosis. Number of rats mounted on a given test day is shown in Table 2. As shown in Figure 7A, LQ was significantly higher in females treated with 10µg EB compared to those treated with 2.5µg EB, with significant main effects of EB at test 2 ($F(1,12)=21.20$, $p=0.001$, $\eta^2=0.64$), test 3, ($F(1,13)=4.59$, $p=0.05$, $\eta^2=0.26$), test 4, ($F(1,8)=7.98$, $p=0.022$, $\eta^2=0.50$), and test 6, ($F(1,10)=9.78$, $p=0.011$, $\eta^2=0.49$). On Test 5 LQ was significantly higher in oil than in TP treated females ($p=0.039$, $d=5.60$) but only at the 10µg dose of EB (significant dose x prenatal treatment interaction ($F(1,9)=5.14$, $p=0.050$, $\eta^2=0.36$).

Prenatally TP treated females displayed more LM1 (1.33±0.314) compared to oil treated females (0.367±0.302) (main effect of prenatal treatment, $F(1,21)=4.92$, $p=0.038$, $\eta^2=0.005$), yet the small effect size suggests that this difference is not an important one. Although a main effect of test was detected, $F(5,105)=2.40$, $p=0.042$, $\eta^2= 0.08$, the effect was small and Bonferroni post-hoc failed to detect any specific test differences. The interaction was not significant.

The frequency of LM2 generally increased across tests (main effect of test, $F(5,105)=6.44$, $p<0.001$, $\eta^2=0.19$). Specifically, more LM2 occurred on Tests 2 and 3 compared to Test 1 ($p=0.022$, $p=0.003$, respectively), and more LM2 were observed overall in females treated with 10µg EB (4.47±0.52) compared to 2.5µg EB (0.53±0.50) (main effect of EB treatment, $F(1,21)=29.70$, $p<0.001$, $\eta^2=0.56$). As shown in Figure 7B, females treated with 10µg EB displayed fewer LM2 on test 1 compared to all subsequent tests, and displayed more LM2 compared to females treated with 2.5µg EB on all but the first test (significant EB treatment by test interaction, $F(5,105)=4.53$, $p=0.001$, $\eta^2=0.14$). No effects of prenatal treatment were detected.

Significantly more LM3 were displayed by females treated with 10µg EB (1.93±0.34) compared to 2.5µg EB (0.21±0.32) (main effect of EB treatment, $F(1,21)=13.59$, $p=0.001$, $\eta^2=0.35$). The main effect of prenatal treatment, test, and the interaction effects were not statistically significant.

Appetitive and Defensive Behaviors. As shown in Figure 7C, prenatally oil-treated animals displayed significantly more solicitations compared to TP-treated females (main effect of prenatal treatment, $F(1,21)=4.55$, $p=0.045$, $\eta^2=0.12$), and more solicitations occurred with

Number of prenatally androgenized (TP) or control female rats (Oil) that were mounted on a given test day during the test phase of the estradiol sensitization paradigm, and included in the 2 way ANOVA analyses on LQ.

	Oil		TP	
	2.5µg EB (n=6)*	10µg EB (n=6)*	2.5µg EB (n=7)*	10µg EB (n=6)*
LQ_1	3	5	3	2
LQ_2	3	5	2	6
LQ_3	3	6	2	6
LQ_4	3	2	3	4
LQ_5	3	4	4	2
LQ_6	3	4	1	6

Note. EB: estradiol benzoate. TP: testosterone propionate. Numbers represent respective test day. *Original group sizes.

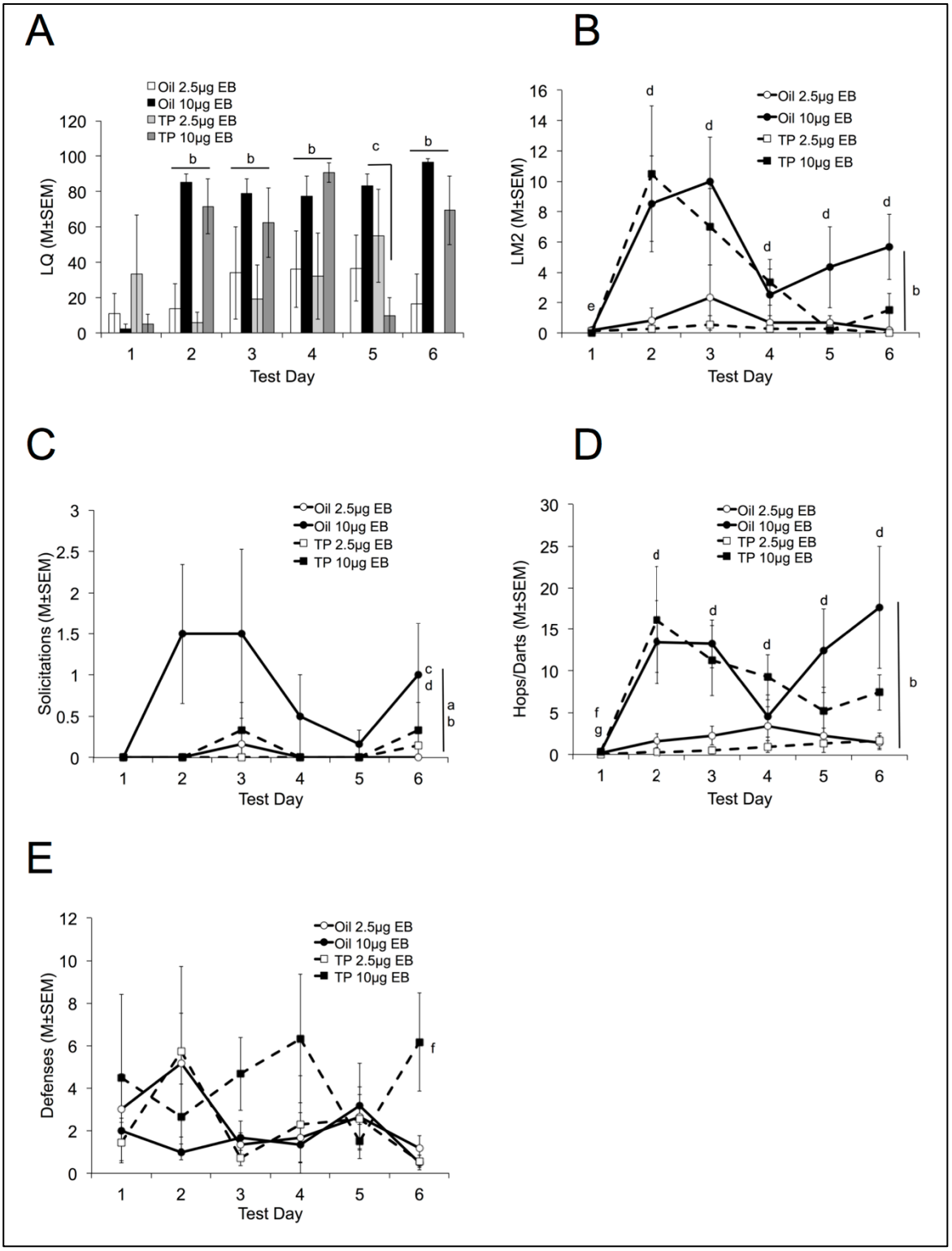

Figure 7. Sexually experienced OVX females treated prenatally with oil or TP were repeatedly treated with 2.5µg or 10µg EB every 4 days, 48hrs prior to each of six sexual behavior tests.

[a]Main effect of prenatal treatment, greater in oil treated than TP treated. [b]Main effect of EB treatment, greater in 10µg EB than 2.5µg EB. [c]Greater in prenatally oil treated compared to TP when treated with 10µg EB. [d]Significantly greater in 10µg EB compared to 2.5µg EB. [e]Less than tests 2-6 in females prenatally treated with TP and repeatedly tested with 10µg EB. [f]Overall, fewer hops/darts occurred on Test 1 compared to all subsequent tests. [g]Females treated with 10µg EB displayed fewer hops/darts on Test 1 compared to all subsequent tests. [f]More defensive behaviors in females treated with 10µg EB if prenatally exposed to TP.

10µg EB compared to 2.5µg EB (main effect of EB treatment, $F(1,21)=7.09$, $p=0.015$, $\eta^2=0.19$). For females treated with 10µg EB, fewer solicitations were observed in those prenatally treated with TP ($p=0.008$, $d=1.15$), and in those females prenatally treated with oil, more solicitations were elicited by 10µg EB than by 2.5µg EB ($p=0.003$, $d=1.36$) (significant EB treatment x prenatal treatment interaction, $F(1,21)=4.44$, $p=0.047$, $\eta^2=0.12$).

Significantly fewer hops and darts were observed on test 1 compared to all subsequent tests as shown in Figure 7D (main effect of test, $F(5,105)=4.42$, $p=0.001$, $\eta^2=0.14$), and more hops and darts were induced by 10µg EB (9.33±1.04) than 2.5µg EB (1.37±1.00) (main effect of EB treatment, $F(1,21)=30.13$, $p<0.001$, $\eta^2=0.58$). For females treated with 10µg EB, fewer hops/darts occurred on Test 1 compared to all subsequent tests, and significantly more hops and darts were induced by 10µg EB on tests 2-6 compared to 2.5µg EB (significant interaction between test and EB treatment, $F(5,105)=3.49$, $p=0.006$, $\eta^2=0.11$). There was no significant effect of prenatal treatment, and no other interaction effects were observed.

When hops/darts and solicitations were combined into a general measure of sexually appetitive behaviors, fewer appetitive behaviors were observed on test 1 compared to all subsequent tests (significant main effect of test, $F(5,105)=4.34$, $p=0.001$, $\eta^2=0.14$). Significantly more appetitive behaviors were seen in females treated with 10µg EB compared to 2.5µg EB (main effect of EB treatment, $F(1,21)=32.32$, $p<0.001$, $\eta^2=0.59$). Regardless of prenatal treatment, females treated with 10µg EB displayed significantly more appetitive behaviors on tests 2-6 compared to females treated with 2.5µg EB, and in females treated with 10µg EB, significantly more appetitive behaviors were displayed on Tests 2-6 compared to test 1 (significant interaction between Test and EB-treatment, $F(5,105)=3.45$, $p=0.006$, $\eta^2=0.11$). There was no significant effect of prenatal treatment, and no other interaction effects were observed. (Data not shown, but closely resembles that of hops/darts).

Females treated with 10µg EB were significantly more defensive if they were prenatally treated with TP (4.31±0.97) compared to Oil (1.61±0.40) ($p=0.016$, $d=1.51$), and prenatally TP treated females were more defensive when treated with 10µg EB compared to 2.5µg EB ($p=0.048$, $d=2.10$) (significant interaction between EB treatment and prenatal treatment, $F(1,21)=4.32$, $p=0.050$, $\eta^2=0.15$).

Male Behaviors. More mounts were observed on test 3 compared to test 1 (main effect of test, $F(5,105)=3.47$, $p=0.006$, $\eta^2=0.13$) as shown in Figure 8A, and more mounts and

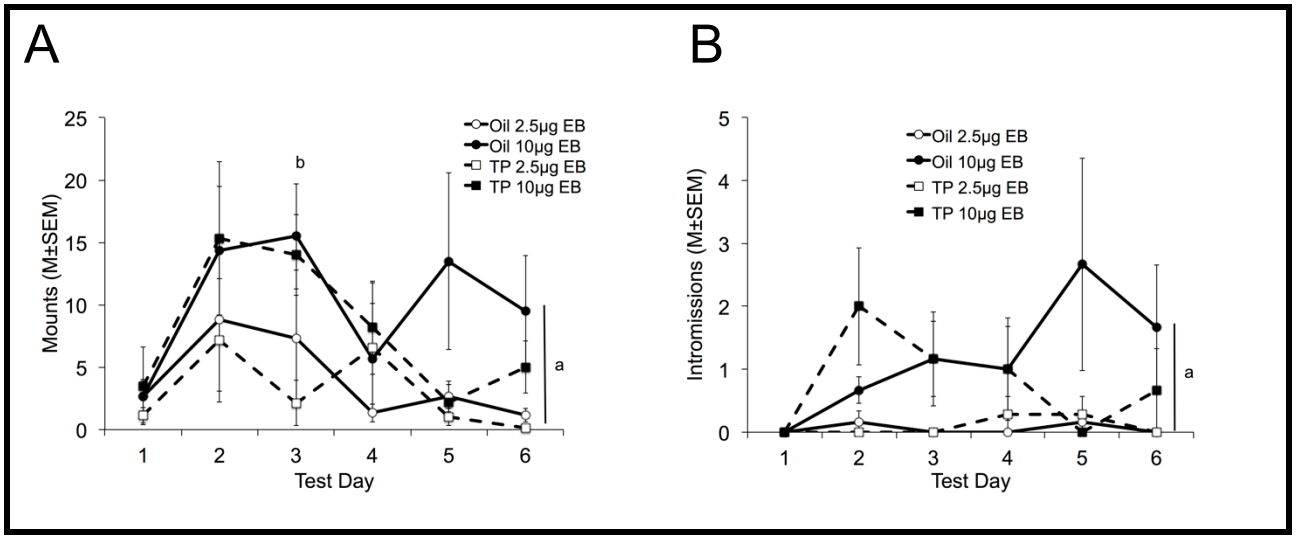

Figure 8. Average number of mounts (A) and intromissions (B) received from males in sexually experienced OVX females treated prenatally with oil or TP and repeatedly treated with 2.5µg or 10µg EB every 4 days, 48hrs prior to each of six sexual behavior tests. [a]Main effect of EB treatment, greater in 10µg EB versus 2µg EB. [b]More mounts overall compared to Test 1.

intromissions (Figure 8B) were received by females treated with 10µg EB compared to 2.5µg EB (main effect of EB, mounts: $F(1,21)=15.64$, $p=0.001$, $\eta^2=0.41$; intromissions: $F(1,21)=12.46$, $p=0.002$, $\eta^2=0.36$). More ejaculations were received by prenatally oil-treated females (0.097±0.027) compared to prenatally TP-treated females (0.00±0.026) (main effect of prenatal treatment, $F(1,21)=6.51$, $p=0.019$, $\eta^2=0.16$), and those treated with 10µg EB (0.097±0.027) received significantly more ejaculations than those treated with 2.5µg EB (0.00±0.026) (main effect of EB treatment, $F(1,21)=6.51$, $p=0.019$, $\eta^2=0.16$). No other effects were detected

Discussion

The goal of this study was to test whether PNAF rats were desensitized to the activational effects of EB on lordosis and appetitive sexual behaviors in adulthood. This was tested in two ways, first using varying doses of EB administered in combination with P, and second by subjecting the animals to an established estradiol-sensitization paradigm in which females are first given sexual training followed by a hormone washout period before being repeatedly treated with EB alone (Jones et al., 2013; Jones & Pfaus, 2014). The greater anogenital distance and size of the SDN-POA suggest that the prenatal TP manipulation was effective. However, the results of the behavioral analyses indicated that prenatal androgen treatment did not permanently desensitize females to the activational effects of EB. Although sexually naïve females were initially desensitized to the acute effects of EB+P within the varying EB doses administered (Experiment 1), this difference was largely overcome experientially by repeated testing with EB+P (training phase of Experiment 2). Moreover, sexually experienced females treated repeatedly with 10µg EB every four days experienced sensitization to the effects of EB on sexual behavior, whereas females treated with the 2.5µg dose of EB did not, regardless of prenatal treatment. Interestingly, PNAF displayed a consistent deficit in the display of full sexual solicitations. Together these data illustrate that prenatal androgen treatment does not permanently desensitize females to the activational effects of EB, and suggest that the decreased sensitivity can in part be overcome by repeated hormone exposure and sexual experience.

The current data are in accord with prior reports that the behavioral deficits induced by prenatal androgen treatment can be overcome by modifying the estradiol priming conditions. Prior studies have shown that both PNAF and males gonadectomized in adulthood display normal levels of lordosis if estradiol is administered in a pulsed manner (Södersten et al., 1983;

Olster & Blaustein, 1988). The present studies demonstrate that the repeated administration of EB (alone or in combination with P) at four-day intervals (as occurs during the rat's natural ovarian cycle) is capable of overcoming the initial deficit on lordosis measures as well as some appetitive measures observed in PNAFs. The recovery of hops/darts seen in our study parrallel's the report by Södersten (1976) on ear wiggling in PNAF and males, yet is in contrast to the lack of hops/darts seen in males reported by Olster and Blaustein (1988). The magnitude of the androgenization, hormone priming regimen, strain differences, or the testing apparatus may all contribute to that discrepancy. We have extend the effect of prenatal androgen treatments on the recovery of appetitive measures of sexual behavior by showing that PNAF appear to have a chronic deficit in the ability to display full solicitations. In the current study animals were tested in the uni-level four-hole pacing chamber, and although the female has the ability to display full solicitations in this type of chamber (e.g., Jones & Pfaus, 2014), they are more readily observed in the bi-level pacing chamber (e.g., Pfaus et al., 1999). Very little is known about the underlying neural mechanisms that control varying forms of solicitations; therefore, it would be interesting to test whether this behavioral deficit is also observed in the bi-level pacing chamber, and if so, this may provide a useful paradigm to study those underlying mechanisms. We recently found that AMPA receptor activation within the vlVMH attenuates the sensitization of appetitive sexual behaviors by repeated EB treatments, mimicking the effect of repeated vaginocervical stimulation during copulation (Jones, S.L., Farisello, L., Mayer-Heft, N., Pfaus, J.G., *submitted*). As such, it is possible that prenatal TP administration results in higher glutamatergic signaling in the vlVMH, which is inhibitory to sexual behavior (Georgescu et al., 2009; 2012; 2014; Georgescu & Pfaus, 2006a; 2006b; Kow et al., 1985). However, we cannot rule out the possibility that full solicitations would be restored to normal levels had the training phase been extended, or with the administration of EB alone (i.e., without access to a male) which potentiates the sensitization of hops/darts and solicitations (Jones & Pfaus, 2014). Nonetheless, here we have shown that a moderate disruption to normal female sexual differentiation (by a single TP injection on PN18, as opposed to for example, longer androgen treatment or the use of males), led to a chronic disruption in the female to display full solicitations.

The retained sensitivity of PNAFs to EB and P on most measures of sexual behavior following repeated testing may be due to the presence of the ovaries throughout the early post-natal period, as well as puberty, which is another well-recognized critical period. Gerall et al.,

(1973) showed that females that were OVX early in life (within 10 days of birth) were behaviorally less sensitive in adulthood to the activational effects of EB+P compared to those OVX later (at 35 or 60 days after birth), suggesting that retention of the ovaries throughout development enhances the behavioral sensitivity to EB in rats. Although this suggests that had we OVX our females prior to puberty the behavioral deficits may not have been overcome with repeated testing under EB+P priming, the data by Gerall et al. (1973) also show that the differences in sensitivity were only apparent on the first three tests, and were eliminated as of the fourth test, up until the final test day (day 7, with tests occurring at 8-10 day intervals). Our EB+P training data are consistent with Gerall et al.'s data, and further suggest that the behavioral deficits can be overcome by repeated testing with EB+P.

Sexually appetitive behaviors are activated, at least in part, by activation of P receptors in hypothalamic regions. Estradiol induces P receptors in sex-relevant brain areas such as the mPOA and VMH (MacLusky & McEwen, 1978; Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994), such that P can subsequently bind its receptors in these regions to potentiate lordosis and activate sexually appetitive behaviors (Etgen & Barfield, 1986; Mani, Blaustein, Allen, Law, O'Malley, & Clark, 1994; Rubin & Barfield, 1983). Females fully primed with EB+P display high levels of both hops/darts and solicitations, whereas very few of those behaviors are induced by acute administration of EB alone. Hops and darts occur in close proximity to the male, enticing him to mount, and solicitations occur more distally (as they include a runaway) and entice the male to chase her, and as such the size of the testing environment influences the expression of these behaviors (Erskine, 1989). When tested in a context that allows the expression of both solicitations and hops/darts, for example the unilevel 4-hole pacing chamber or the bilevel chamber, hops/darts are often referred to as partial solicitations and full solicitations are interpreted as indicative of greater sexual motivation (McClintock, 1984; Pfaus et al., 1999). However the neural mechanisms underlying the different components of appetitive sexual behaviors are not well-understood, although it is clear they can be selectively activated or inhibited by different pharmacological or behavioral manipulations (e.g., Gelez et al., 2013; Jones & Pfaus, 2014). The present data also suggest differential mechanisms underlying subcomponents of sexually appetitive behaviors, and one likely candidate could include differential activation of P receptors.

Estradiol not only induces the synthesis of P receptors, but also the production of neuroprogesterone within hypothalamic astrocytes (Sinchak et al., 2003), and the administration of a P receptor blocker RU486, or neuroprogesterone synthesis inhibitors on the fourth test attenuates sexually appetitive behaviors (hops/darts and ear wiggles) in adrenalectomized-OVX females when using a similar sensitization paradigm (Micevych et al., 2008). We have also found that chronic administration of RU486 disrupts the maintenance of sensitized sexual behaviors by repeated EB in OVX females (Jones, S.L., Gardner-Gregory, J., Pfaus, J.G., *submitted*). Estradiol-induced P receptors are attenuated in PNAFs (Foecking et al., 2005), which together with the present findings suggest that estrogen-induced P receptors are involved in the induction of appetitive behaviors by repeated EB. Estrogen-positive feedback, which is sexually dimorphic, appears to involve the production of neuroprogesterone synthesis in hypothalamic astrocytes by the activation of a membrane-initiated signaling mechanism by estradiol (Kuo & Micevych, 2012). Kuo and Micevych (2012), using the four-core genotype model in mice (see Arnold & Chen, 2009), found that regardless of chromosomal sex, estrogen positive feedback does not occur in phenotypic males (those that develop testes), and proposed that estrogen-induced neuroprogesterone synthesis is sexually-dimorphic and dependent on the perinatal steroid hormone environment. If this mechanism occurs on a continuum, then variations in the ability of EB to induce neuroprogesterone, perhaps in combination with reduced P receptor synthesis (Foecking et al., 2005), could explain why overall, fewer solicitational behaviors were induced in PNAFs treated with EB-alone in the current study.

It has also been proposed that sexual dimorphisms can be the result of chromatin remodeling occurring during the critical perinatal period (Gagnidze et al., 2010). The aromatization of testicular testosterone to estradiol in the brain, and its subsequent signaling through ER is critical for sexual differentiation of neural structures (Gagnidze et al., 2010; McCarthy, 2008; McCarthy et al., 2008; Patchev, Götz, & Rohde, 2004). ERs recruit coregulators, before binding estrogen-response elements on DNA, which alter the rate of transcription. Nuclear receptor coregulators can induce chromatin remodeling by acetylating, deacetylating, or methylating histone proteins (Gagnidze et al., 2010). Histone acetylation and deacetylation are typically associated with gene transcription and repression, respectively. Histone methylation can either activate or silence gene transcription, and long-term gene silencing can be induced by lysine methylation of histones (Gagnidze et al., 2010). Many nuclear

ER coregulators have been identified, and are expressed in a sexually dimorphic manner (Auger et al., 2002; Jessen, Kolodkin, Bychowski, Auger, & Auger, 2010; Misiti, Koibuchi, Bei, Farsetti, & Chin, 1999). As such, it was proposed that testicular steroid hormone secretion leading to ER activation can affect chromatin remodeling, leading to the activation of genes relevant to male sexual behavior, and the silencing of those involved in female sexual behavior (Gagnidze et al., 2010). This proposed mechanism could potentially explain the long-lasting effect of inhibited sexual solicitations in PNAFs. It will be interesting to test whether ER mediated chromatin remodeling is sensitive to sexual experience in adulthood, which would not be surprising given the epigenetic literature, and could explain the restoration of sexual behaviors by EB+P in PNAFs. In fact, perinatal exposure to estradiol as well as maternal grooming during the early post-natal period methylates the ERα promoter region, associated with a down-regulation of ERα mRNA (Champagne et al., 2006; Kurian et al., 2010), which shows that somatosensory experiences can epigenetically modify genes coding ER. It has also recently been shown that estradiol administration induces chromatin remodeling in the mPOA and VMH (Gagnidze, Weil, Faustino, Schaafsma, & Pfaff, 2013). Additionally, some sex differences are inherent in the sex chromosomes (Arnold, 2009), as they appear prior to the differentiation of the gonads, or the secretion of testicular hormones (Burgoyne, 1993; Burgoyne et al., 1995; Dewing et al., 2006; Thornhill & Burgoyne, 1993). As such, it will be important for future studies to assess sexual dimorphisms induced by differential gene expression between chromosomal sexes, and/or the interaction between sex steroid hormones and the sex chromosomes which can be examined using the four core genotype model (see Arnold & Chen, 2009).

Conclusions

Although PNAFs that are OVX in adulthood are initially less sensitive to the activational effects of EB+P on female sexual behavior, this can be overcome experientially by repeated mating following priming with EB+P. Moreover, the PNAF brain remains relatively sensitive to EB in adulthood, since behavioral differences on LQ and hops/darts dissipated following repeated hormone priming in combination with mating. Nevertheless, prenatal androgen treatment appears to interfere with the normal expression of full sexual solicitations. Although the underlying mechanisms of the sensitization of sexual behaviors by repeated administration of EB are unknown at this time, EB induction of neuroprogesterone and/or P receptors, sexual

dimorphisms in chromatin remodeling, and the interaction between steroid hormones and gene function due to chromosomal sex differences are interesting possibilities.

CHAPTER 5: GENERAL DISCUSSION

This thesis established that the administration of EB to the OVX rat every four days, in a pattern that mimics the natural ovarian cycle of the rat, generates a behavioral sensitization such that sexual behavior is potentiated with its repeated administration. The first set of experiments (Chapter 2) characterized the dosing regimens required to induce and counteract sensitization of sexual behaviors by EB, and assessed the role of two primary candidates: P (and its receptor) and copulatory stimulation. It was established that a dosing regimen of 5μg EB every eight days counteracts the sensitization while inducing a relatively stable baseline that can be used to assess facilitative effects of compounds on female sexual behavior (Chapter 2.1, Experiment 1). To date, the methodology has been applied in at least one preclinical model which showed the facilitative role of the pharmacological agent flibanserin on female sexual desire (Gelez et al., 2010). It was also established that estradiol sensitization was robustly induced by 10μg EB administered at 4-day intervals, and that it occurs in OVX rats in the absence of the adrenals, suggesting that neither ovarian nor adrenal P play a role in the sensitization (Chapter 2.1, Experiment 2). However, when PR were repeatedly blocked on the day of testing, using RU486 from the onset of the sensitization paradigm, lordosis and appetitive behaviors were facilitated on earlier tests, but were later inhibited, suggesting that PR activation may be involved in the maintenance of the sensitized response (Chapter 2.2). It was also determined that if the female is given the opportunity to copulate on every test session, the sensitization of sexually appetitive behaviors is attenuated compared to females that are not given that opportunity (Chapter 2.3).

The primary aim of Chapter 3 was to more thoroughly assess the behavioral and neural mechanisms associated with copulation that contribute to the attenuation of estradiol sensitization of appetitive sexual behaviors. First it was found that repeated administration of 10μg EB in the absence of other manipulations (ACF group) had the strongest effect on behavioral sensitization, and that effect was attenuated by VCS received either from the male or artificially from the experimenter on intermediate tests, suggesting that VCS activates mechanisms that are inhibitory to those that induce estradiol sensitization (Chapter 3.1). Given that VCS is also inhibitory to the duration of estrus, and that part of that inhibitory mechanism occurs via glutamate transmission in the VMH, the next set of experiments assessed whether mechanisms of estrous termination are also involved in the attenuation of estradiol sensitization

(Chapter 3.2). First it was found that repeated AMPA receptor activation within the VMH (in place of VCS) attenuates the sensitization of appetitive sexual behaviors by repeated administration of EB (Chapter 3.2, Experiment 1). Next it was discovered that females treated with EB alone (i.e., those that display a stronger sensitization), also have a quicker onset of estrous termination, particularly following VCS (Chapter 3.2 Experiment 2). This finding is particularly interesting as it suggests that their brains are not only more sensitive to the activational effects of EB on sexual behaviors, but are also more sensitive to the termination of those behaviors. As such, it appears that repeated administration of EB in the absence of copulation sensitizes circuits required not only for the induction of sexual behavior, but also the termination of that behavior, and the effect is potentiated in response to reproductively relevant stimuli.

Sensitization is most robust if EB is repeatedly administered in the absence of the opportunity to copulate. Surprisingly, a low dose of EB (2μg EB) - that had repeatedly been shown to be suboptimal for inducing female sexual behavior in Long-Evans rats in the absence of subsequent P administration - resulted in the sensitization of sexually appetitive behaviors if the female was not given access to a sexually vigorous male on each episode of heat (Chapter 2.3). That finding was replicated and extended when a similar effect was observed in females repeatedly treated with 10μg EB but left undisturbed in their home cage (ACF) on intermediate tests (Chapter 3.1). Those females displayed the greatest increase in the number of sexually appetitive behaviors on the final test day. Together those findings illustrate that the repeated administration of EB in the absence of sexual somatosensory stimulation from the male is sufficient to sensitize female sexual behaviors once the female is given access to a male. These findings are particularly relevant to studies examining the neurophysiological effects of EB, especially those using doses that were previously believed to be behaviorally "subthreshold". Moreover, given that repeated administration of EB alters an overt behavioral response, any neurophysiological responses to acute EB treatment are surely different from those following repeated treatments.

In Chapter 4, the estradiol sensitization paradigm was used as a diagnostic tool to test EB sensitivity in two paradigms of female sexual inhibition. In the first study, whether the sexual behavior deficits reported by the use of corncob bedding in the animal's housing chambers could be overcome by repeated administration of EB was assessed (Chapter 4.1). However, corncob

bedding did not disrupt the display of sexual behaviors when females were treated with EB+P, much to the delight of the researchers who use our animal care facility. In contrast, although housing animals on corncob bedding did not prevent estradiol sensitization, if those females were housed in a room separate from males (CC/F) their sexual behavior was inhibited. This finding is important as it may help explain why only some research groups report sexual inhibition by corncob bedding, and moreover it suggests that continuous exposure to male cues can overcome inhibitory effects of corncob bedding. In the next study, whether prenatally androgenized females would display estradiol sensitization was assessed (Chapter 4.2). Despite a behavioral deficit following an acute treatment of EB+P, when tested in the estradiol sensitization paradigm their sexual behavior was nearly identical to prenatally oil-treated controls. This finding is important as it challenges the notion that early androgen exposure in females permanently impairs all female sexual behavior, by illustrating that the deficits observed following acute EB can be overcome with repeated administration. Thus, prenatally androgenized females retain their sensitivity to EB.

Overall, the primary findings of this thesis illustrate that sexual behaviors in the female rat sensitize to the repeated administration of EB, that the effect is robust as it occurs with varying doses at varying dosing intervals, and that the magnitude of the sensitization interacts with sexual experience and exposure to male cues. Sexual behavior in the female rat is dependent on E2 signaling in sex-relevant brain regions, and in particular the vlVMH. The data in this thesis show that estradiol sensitization is attenuated both by VCS (which alters neural activity in those same brain regions) as well as by pharmacological manipulations (i.e., AMPA receptor activation) within the VMH. Taken together, these findings illustrate that the female brain becomes increasingly sensitive to the activational effects of EB on sexual behavior with its repeated administration, and extend the hypothesis that glutamate signaling within the VMH is inhibitory to sexual behavior. Moreover, the data illustrate the flexibility in female rat sexual strategies as a function of prior sexual experience.

Role of the VMH in competing motivated behaviors

One of the primary findings in this thesis is that the receipt of VCS on prior successive episodes of heat attenuates the sensitization of appetitive sexual behaviors induced by the repeated administration EB, and that the effect is mimicked by repeated AMPA receptor

activation within the vlVMH. There are clearly other factors involved in the attenuation, since females repeatedly administered 2μg EB and placed with a sexually vigorous male fail to display behavioral sensitization, an effect that cannot be dependent on VCS since they do not receive intromissions (see Chapter 2.1, Figure 3e). Nonetheless, the finding that AMPA receptor activation attenuated the sensitization extends our hypothesis that glutamate signaling in the VMH is inhibitory to sexual behaviors. Moreover, when considered in the context of feeding behaviors, the data suggest that glutamate signaling within the VMH can act as a switch between these competing motivated behaviors.

It is becoming increasingly clear that the VMH has both excitatory and inhibitory pathways with respect to sexual behavior, as first proposed by Kow et al., (1985). It also plays a very important role in feeding and energy homeostasis by integrating peripheral signals of nutrient status and adiposity (Routh, 2010). Moreover E2 modulates the activity of glucose responsive neurons within this region (Kow & Pfaff, 1985) The VMH can therefore act as a switch between the execution of sexual behavior and feeding depending on the hormonal state of the animal. It is well recognized that OVX, which abolishes the expression of sexual behavior, results in a dramatic increase in food intake and weight gain compared to ovary-intact control animals, whereas administration of E2 reverses those effects (Asarian & Geary, 2002; 2006; Butera, Wojcik, & Clough, 2010; Geary & Asarian, 1999; Wade, 1972). Some of those effects are driven by E2 actions within the VMH and the nearby ARC (Frank, Brown, & Clegg, 2014). EB infusions to the VMH inhibit feeding (Nunez, Gray, & Wade, 1980), and disrupting ERα expression in the vlVMH disrupts the ability of E2 to reduce body weight, leads to a decrease in energy expenditure and an increase in visceral adiposity, and abolishes sexual behavior (Musatov, Chen, Pfaff, Kaplitt, & Ogawa, 2006; Musatov et al., 2007). Moreover, leptin, an anorexigenic hormone released from fat cells that leads to the inhibition of feeding, stimulates lordosis (Garcia-Juárez, Beyer, & Gómora-Arrati, 2013; Wade, Lempicki, Panicker, Frisbee, & Blaustein, 1997), but only if the animals are fed ad-lib, since in food deprived animals not only is the facilitation by leptin abolished, it actually amplifies sexual inhibition (Wade et al., 1997).

Part of the mechanism through which E2 regulates food intake and energy balance is by altering the brain's sensitivity to orexigenic and anorexigenic hormones, which stimulate and inhibit feeding respectively. For example, E2 administration attenuates the orexigenic effects of melanin concentrating hormone (Messina, Boersma, Overton, & Eckel, 2006) and neuropeptide

Y (Jessica Santollo, 2008). Estradiol also enhances the anorexigenic effects of centrally administered leptin and insulin in gonadectomized animals (Clegg, Brown, Woods, & Benoit, 2006; Clegg, Riedy, Smith, Benoit, & Woods, 2003), at least in part via classic ERα as well as mER signaling in a subset of neurons located within the ARC, which project to the VMH (Frank et al., 2014; Sinchak & Wagner, 2012).

Glutamate transmission within the VMH appears to be a key switch for the onset of feeding and the control of energy homeostasis, and the inhibition of sexual behavior. For example, blocking the ability of VMH cells to release glutamate by selectively knocking out VGLUT2 (vesicular glutamate transporter type 2, necessary for the packaging and consequently the release of glutamate into the synapse) prevents the activation of systems that counter hypoglycemia (e.g., the release of glucagon), suggesting that glutamate transmission in the VMH is involved the activation of systems that stimulate food intake (Tong, Ye, McCrimmon, Dhillon, & Choi, 2007). Similarly, glutamate release is increased in the nearby mediobasal hypothalamus (particularly the ARC, though a weaker response was also seen in the VMH of animals with incorrect placements) within 1-2 seconds of oral contact with a food pellet, and the increase is further potentiated in response to a palatable high-fat food pellet, whereas this increase was not observed in response to water ingestion (Guyenet, Matsen, Morton, Kaiyala, & Schwartz, 2013). Glutamate release within the VMH appears to be under inhibitory control of GABA, since bicuculline, a GABA-A receptor antagonist, increases glutamate transmission, indicating that glutamate is released by a mechanism of disinhibition (Booth et al., 2010).

In summary, the VMH is a hypothalamic integrator of peripheral and central signaling, that orchestrates behavioral outputs associated with at least two competing motivated behaviors: sex and feeding. As just reviewed in this section, one important and likely mechanism that could regulate these behavioral systems is glutamate signaling, which may act on discrete neuronal populations of estrogen sensitive neurons within the VMH (Akesson et al., 1994).

Other potential mechanisms of behavioral sensitization to estradiol

Across the lifespan of the female rat, sexual behavior is predominantly in a state of sexual inhibition. Sexual behavior only appears once the ovarian cycle has been initiated in puberty, and then only occurs for a small window of time (approximately 12-20 hours) throughout her 4-5 day ovarian cycle. If the female has the opportunity to copulate, sexual behavior will be inhibited

during a two to six week period of pseudopregnancy or pregnancy, respectively. Sexual behavior again declines with age, as ovarian cyclicity becomes disrupted (beginning around 10 months of age). As such, a mechanism whereby sexual behavior becomes sensitized must involve a process of disinhibition particularly within the VMH, and potentially through increased GABA-ergic signaling to reduce glutamate transmission in this region. This is a possibility since it has been shown that glutamate release occurs through a process of disinhibition by GABA acting at GABA-A receptors in the VMH (Booth et al., 2010).

This thesis has provided indirect evidence of this by demonstrating that behavioral sensitization to EB is attenuated by repeated infusions of the ionotropic glutamate receptor agonist, AMPA, infused into the VMH; this suggests that glutamate transmission is attenuated by repeated EB treatments, potentially by sensitizing GABAergic transmission on glutamate neurons, as previously proposed (Georgescu et al., 2014). GABA-A receptors exist on VMH glutamate neurons, and the percentage of glutamate neurons expressing GABA-A receptors is approximately doubled by EB or EB+P treatments (Georgescu, Del Corpo, & Pfaus, in preparation). As such, repeated treatment with E2 may sensitize GABAergic transmission onto those glutamate neurons within the VMH, thereby acting to disinhibit sexual behavior. In addition, the administration of E2 also triggers the upregulation of a number of systems within the hypothalamus and limbic regions (Micevych & Sinchak, 2007; Pfaff, 1999), including PR, nP, dopamine, enkephalins, and opioid receptors; thus, a number of converging systems may be involved in the behavioral sensitization. Some of the primary candidates include mER, opioids, nP, dopamine, as well as estrogen-induced epigenetic changes.

Membrane estrogen receptor signaling. Although E2 administration is necessary for the display of female sexual behavior, lordosis only begins to occur approximately 24 hours later, which led early investigators to believe that E2's actions on sexual behavior occur through genomic (i.e., long, taking hours or days) mechanisms. However it is now clear that the induction of sexual behavior following E2 treatment also involves rapid actions through mER signaling.

Estradiol has rapid membrane actions within the VMH to promote sexual behavior (Kow & Pfaff, 2004). Priming with a membrane impermeable form of E2 (E2 bound to bovine serum albumin, BSA), followed by a behaviorally ineffective dose of EB was as effective at inducing lordosis as two injections of free E2, and this membrane potentiation of lordosis likely occurs through activation of protein kinases A and C (PKA, PKC) (Kow & Pfaff, 2004). Similarly,

GPR-30 (G-protein coupled receptor 30, also known as GPER1, or G-protein coupled ER1) which has a high binding affinity for E2, has been shown to be sufficient in the activation of lordosis behavior in EB+P treated OVX mice (Anchan et al., 2014). Thus, repeated EB treatment might also sensitize mER signaling to potentiate sexual behaviors.

One interesting possibility of how E2 can induce long-term changes in cell signaling through membrane receptors and ultimately lead to a potentiation of sexual behaviors was proposed by Caldwell (Caldwell, 2002). In this model membrane steroid receptor complexes interact with G-protein coupled receptors (such as oxytocin receptors) to alter the affinity of the steroid hormone ligand-binding sites. More specifically, Caldwell proposed that the membrane receptor complex has multiple binding sites for different steroid hormones, in particular E2 and P. Based on the initial observations by Frye, Mermelstein, DeBold (1992) that sexual behavior in the hamster could be altered by infusing membrane impermeable P-BSA in the ventral tegmental area, Caldwell and Moe (1999) later reported that E-BSA given in combination with oxytocin enhanced LQ and the quality of the lordosis posture. Together those findings suggest that steroid hormones have the ability to activate membrane receptors, and that membrane receptors can interact with oxytocin receptors to facilitate sexual behavior. Moreover, E-BSA and P-BSA can act on the same receptors at times, something that is unique to membrane bound receptors, (since E2 and P intracellular receptors are separate proteins) (Caldwell, Walker, Rivkina, Pedersen, & Mason, 1999), providing a unique way to alter subsequent binding ability. For example, the priming dose of E2 may bind to an allosteric site, opening new sites for subsequent E2 or P binding (Caldwell, 2002). Uncoupling the binding sites from the G-protein using G-protein antagonists increased high-affinity P-BSA binding and decreased high-affinity E-BSA binding (Caldwell, 2002; Caldwell & Moe, 1999). Thus, membrane bound receptor complexes with multiple steroid hormone binding sites and their ability to interact with G-proteins to open new binding sites might explain the priming effect of E2, whereby subsequent injections of E2 induce higher levels of receptivity.

Membrane estrogen-receptor signaling also occurs in the ARC by forming a complex with mGluR, and activation and deactivation of this complex is involved in the inhibition and facilitation of sexual behavior (lordosis) through opioid receptor signaling (recently reviewed in Micevych & Sinchak, 2013), and is important in the timing of sexual behavior. In fact, opioid receptors are also primary candidates in the underlying mechanism.

Opioids. E2-induced opioid receptor signaling in the mPOA and VMH is one likely potential candidate in the sensitization of sexual behaviors. There are four known opioid receptors: delta (δ), kappa (κ), mu (μ), and opioid receptor-like receptor 1 (ORL-1). In general, activation of δ receptors in the VMH (Micevych & Sinchak, 2007; Pfaus & Pfaff, 1992) and ORL-1 (Sanathara, Moraes, Kanjiya, & Sinchak, 2011) facilitate sexual behavior, whereas μ receptor activation inhibits sexual behavior (Pfaus & Pfaff, 1992), particularly within the mPOA (Sinchak, Shahedi, Dewing, & Micevych, 2005). Ovarian hormones increase ORL-1 expression in the mPOA and increases mRNA expression of its endogenous agonist OFQ/N (orphanin-FQ/nociceptin) in the VMH (Sinchak, Romeo, & Micevych, 2006). Infusions of OFQ/N to the mPOA (Dewing et al., 2007; Micevych & Sinchak, 2007; Sinchak & Micevych, 2003; Sinchak, Dewing, Cook, & Micevych, 2007) or VMH (Micevych et al., 1996; Micevych & Sinchak, 2007; Sinchak et al., 2007; Sinchak, Hendricks, Baroudi, & Micevych, 1997) facilitate lordosis in females treated with physiological doses of E2.

Initially, E2 actually inhibits female sexual behavior during the first 20-24 hours of administration, through actions in the ARC which result in μ-opioid receptor activation within the mPOA. The ARC, in addition to its role in feeding, plays an important role in the inhibition and disinhibition of female sexual behavior by E2. Estradiol induces rapid membrane-initiated signaling within the ARC, which sends projections to the mPOA, and a subset of μ-opioid receptor containing neurons in the mPOA project to the VMH (Sinchak & Wagner, 2012) (see Figure 1.1). Within the ARC, mER form a complex with mGluR, such that E2 binding signals the release of beta-endorphins within the mPOA, causing μ-opioid receptor activation and internalization, to inhibit lordosis (Micevych, Kuo, & Christensen, 2009). This μ-opioid receptor activation is maintained for at least 48 hours in OVX females treated with EB alone (Sanathara et al., 2011; Sinchak & Micevych, 2001; Sinchak & Wagner, 2012). The circuit is deactivated by subsequent administration of P, a high dose of E2, or the administration of the opioid receptor antagonist naloxone (Acosta-Martinez & Etgen, 2002; Eckersell, Popper, & Micevych, 1998; Sinchak & Micevych, 2001). Thus, repeated EB treatments may sensitize the deactivation of this inhibitory system.

μ-opioid receptor activation also fluctuates with the natural ovarian cycle in the rat, such that it is active during periods of sexual inhibition (diestrus and estrus) and deactivated on the evening of proestrus when sexual behavior emerges (Sinchak & Micevych, 2003). Sinchak and

Wagner (2012) proposed that a bolus dose of E2 administered 24-48 hours following the priming dose down-regulates the mER-mGluR1 complex within the ARC, through a down-regulation of ERα. This hypothesis is supported by a preliminary report that ERα antagonists administered 44 hours after a priming dose of E2 reduces mER-mGluR1 activation, coincident with an increase in lordosis (as cited in Sinchak & Wagner, 2012). Since E2 downregulates its own receptor (Lauber et al., 1991a; Lauber, Romano, Mobbs, Howells, & Pfaff, 1990), this hypothesis may also hold true for EB induced sensitization. Frequent EB priming may down-regulate mERα within the ARC, thus reducing mERα-mGluR1 signaling, thereby reducing μ-opioid receptor signaling in the mPOA, and reducing this inhibitory mechanism (thereby facilitating lordosis). In females maximally primed with EB+P, μ-opioid receptor agonists infused within the mPOA or VMH inhibits lordosis (Acosta-Martinez & Etgen, 2002; Pfaus & Pfaff, 1992; Sinchak & Micevych, 2001) consistent with the idea that opioid release induces a state of satiety, and the inhibition of sexual behavior.

In addition, it has been proposed that the μ-opioid receptor signaling in the mPOA circuit is important in the timing of sexual behavior, involved in both its onset and offset (Micevych & Sinchak, 2007; Sinchak & Wagner, 2012). Such a mechanism may help explain the quicker onset of estrous termination in EB/Alone females that received VCS. Estrous termination was more readily apparent in EB/Alone females following VCS compared to those that copulated (Chapter 3.2), suggesting that the offset of sexual behavior was accelerated in those females, further indicating a potential role for this circuit in the underlying mechanism. It would be interesting to test μ-opioid receptor activation in the mPOA is modulated by afferent signals from the pdMeA for example, following VCS, which could be one other way in which VCS might modulate the expression of female sexual behavior. Thus, opioid signaling within the mPOA and VMH, initiated at least in part via E2's actions within the ARC may be involved in both the induction and inhibition of the behavioral sensitization to EB.

Mechanisms of estradiol sensitization may also occur via changes in δ opioid receptor signaling. Delta opioid agonists infused in the lateral ventricles or the VMH facilitate lordosis (Acosta-Martinez, González-Flores, & Etgen, 2006; Pfaus & Pfaff, 1992) and sexually appetitive behaviors such as hops, darts and present postures (Pfaus & Pfaff, 1992). Since E2 upregulates pro-enkephalin in the VMH (Lauber et al., 1990; Romano, Mobbs, Lauber, Howells, & Pfaff, 1990), an endogenous opioid which binds δ-opioid receptors, repeated treatment with E2 may

sensitize the release of pro-enkephalin signaling through δ-opioid receptors in the VMH, contributing to the behavioral sensitization of sexual behaviors. Similarly, repeated EB administration may sensitize ORL-1 signaling within the VMH, since ORL-1 activation by OFQ/N in the VMH is necessary for the facilitation of lordosis in females treated with E2-alone (in an E2 facilitation paradigm) (Sanathara et al., 2011).

Neuroprogesterone. Neuroprogesterone may be particularly relevant in the sensitization of appetitive sexual behaviors. Micevych et al. (2003) and Sinchak (2003) reported that nP is induced by E2 and is involved in the initiation of the LH surge, which occurs around the time that sexual behavior is displayed. Given that sexual behaviors are reliably reinstated by the co-administration of EB+P, tested whether E2-induced nP is also involved in the increased behavioral sensitivity experienced in female rats treated repeatedly with E2 (in an E2-facilitated paradigm) was examined (Micevych et al., 2008). OVX/ADX rats were administered 10μg EB every 4 days and tested for sexual behavior 52 hours later. On the fourth test they received an injection of unconjugated E2 (50μg), which led to maximal LQ and a significant increase in appetitive behaviors (hops, darts, ear wiggles). Pre-treating the animals with RU486 (a PR antagonist), AGT (aminoglutethimide, a P450 side chain cleavage inhibitor), or trilostane (3-β-hydroxysteroid dehydrogenase inhibitor, which blocks the synthesis of P from pregnenolone) failed to prevent the facilitation of LQ by repeated exposure to EB on the 4^{th} test. However, appetitive behaviors were significantly attenuated, illustrating that the facilitation of proceptive behaviors are PR dependent, and furthermore that this facilitation is dependent on E2-induced synthesis of nP (Micevych et al., 2008). Although that study used an E2-facilitated paradigm, nP may also play a role in our paradigm when EB is administered every four days. Repeated E2 treatment may lead to a progressive increase in nP signaling, (e.g., by increasing the efficacy of nP synthesis, or increasing PR density), leading to a potentiation of sexual behavior with repeated EB administration. If this does occur, it would explain why the competitive PR antagonist RU486 only inhibited female sexual behavior on later tests (Chapter 2.2), once there was enough nP to compete with.

Dopamine. Dopamine (DA) is also a primary candidate in the sensitization of sexual behaviors, particularly within the mPOA, striatum, nucleus accumbens (NAcc) and pdMeA. Dopaminergic systems are key players in sexual excitation once steroid hormones have set the stage by controlling attention and behavioral responses towards incentive stimuli and outflow of

the autonomic nervous system that controls sympathetic nervous system activation, for example by increasing heart rate and parasympathetic activation to increase blood flow to the genitals (Hull et al., 1999; Pfaus, 2009).

Dopamine within the mPOA plays an important role in female sexual behavior, particularly the induction of appetitive sexual behaviors (Graham & Pfaus, 2010; 2012; 2013), and its effects are sensitive to hormonal status of the female. Within the mPOA, extracellular DOPAC, a DA metabolite, fluctuates across the estrous cycle, rising in late proestrus, coinciding with the expression of female sexual behavior (Luine, 1993). Similarly, extracellular DA levels increase in the mPOA in OVX rats primed with EB+P, although not with acute EB administered alone (Matuszewich et al., 2000), and that DA increase occurs approximately 3.5 hrs after the P injection (the time when P activated sexual behavior is induced), and a further increase occurs following presentation of male (Matuszewich et al., 2000). The ability of DA receptor agonists infused in the mPOA to facilitate female sexual behavior is also dependent on the hormonal status of the female through varying effects on its receptor subtypes. D2 receptor activation facilitates sexual behavior in OVX rats primed with EB-alone (Graham & Pfaus, 2010) whereas blocking D2 receptors or activating D1 receptors is facilitative in females treated with EB+P (Graham & Pfaus, 2012). As such, it has been proposed that differences in the activation of DA receptor subtypes are subject to the hormonal status of the female, such that when the female's hormonal state is one of sexual excitation (e.g., fully primed with EB+P) D1 receptor activation is favored (Graham & Pfaus, 2012). Thus, it would be interesting to test whether chronic E2 treatment shifts the DA receptor ratio activity within the mPOA in favor of the state induced by fully priming with EB+P (for example from D2 to D1). Dopamine also has the ability to activate PR in a ligand-independent manner and to facilitate P-dependent (i.e., appetitive) sexual behaviors (Mani et al., 1996; Mani, Allen, Clark, Blaustein, & O'Malley, 1994a; Mani, Reyna, Chen, Mulac-Jericevic, & Conneely, 2006; Mani & Portillo, 2010; Power, Mani, Codina, Conneely, & O'Malley, 1991), and may play a role in the enhancement of lordosis behavior in a mating-induced enhancement paradigm (Auger et al., 1997).

Dopamine is also well-recognized as one of the mechanisms involved in the locomotor sensitization to psychomotor stimulants and stress (and the cross-sensitization between the two) (Kalivas & Stewart, 1991). Females, through estradiol-dependent mechanisms, are more sensitive to the sensitization effects of psychomotor stimulants (e.g., Hu & Becker, 2003; Zhao

& Becker, 2010; Zhen, Goswami, Abdali, Frankfurt, & Friedman, 2006) and vulnerability to that behavioral sensitization may be partly programmed by the early perinatal environment by altering DAergic systems (Meaney, Brake, & Gratton, 2002). Estradiol enhances behavioral sensitization to psychomotor stimulants in adult female rats (Hu & Becker, 2003), and similarly, an increase in locomotor activity is required in sexual behavior, for instance to engage the animal in motivational approach behaviors, as well as with respect to active solicitations which require an orientation towards the sexual incentive (the male) followed by a runaway. Dopamine release within the nucleus accumbens (NAc) helps orient the female towards a sexually active male in anticipation of sexual reward (Jenkins & Becker, 2003a), and increases with the presentation of a sexually vigorous male, and a further increase occurs during copulation (Pfaus, Damsma, Wenkstern, & Fibiger, 1995). Estradiol also stimulates the release of DA in the striatum and NAc during mating (Becker, Rudick, & Jenkins, 2001; Jenkins & Becker, 2003a; Mermelstein & Becker, 1995; Pfaus et al., 1995), and this increase is more pronounced if the copulatory stimulation is received at her preferred interval (such as in a paced mating chamber) (Jenkins & Becker, 2003a; Mermelstein & Becker, 1995). Moreover, paced mating behavior is disrupted by lesions to the NAc (Guarraci, Megroz, & Clark, 2002; Jenkins & Becker, 2001) and striatum (Jenkins & Becker, 2001), at least in part through direct actions of E2 on these regions (L. Xiao & Becker, 1997). Thus, repeated E2 may sensitize the mesolimbic DAergic system to facilitate sexual behavior, or alter the female's reproductive strategies (since paced mating behavior was disrupted in EB sensitized animals, Chapter 2.3).

Dopamine transmission within the pdMeA is also implicated in the facilitation of sexual behavior. Ovarian hormones increase tyrosine-hydroxylase (the rate-limiting enzyme in DA synthesis) in the pdMeA (Holder et al., 2010), and it has recently been reported that DA within the pdMeA enhances PR signaling (Holder, Veichweg, & Mong, 2015), which is associated with the increase in sexually appetitive behaviors following the administration of methamphetamine (Holder et al., 2010; 2015; Holder & Mong, 2010). Infusions of D1 receptor agonists to the pdMeA facilitate sexually appetitive behaviors (hops, darts, ear wiggles) (Holder et al., 2015; Mascó & Carrer, 1984), whereas D1 receptor blockade prevents the facilitation by methamphetamine (Holder et al., 2015). The facilitation may occur through MeA efferents terminating in the VMH, since destruction of the connections from the MeA to the VMH impairs lordosis (Mascó & Carrer, 1984), yet lesioning the MeA does not impair sexual behavior (Mascó

& Carrer, 1984). In addition, OVX rats that had previously been treated with amphetamine (at doses that induce locomotor sensitization) display greater levels of appetitive sexual behaviors such as hops and darts, solicitations, and female-male mounting, the latter of which is considered a super-solicitational behavior (Afonso, Mueller, Stewart, & Pfaus, 2009b). Moreover, this super-solicitational behavior was associated with greater Fos-IR in the MeA, as well as the mPOA and VMH (Afonso, Lehmann, Tse, Woehrling, & Pfaus, 2009a). Taken together, these findings suggest that increased DA signaling within the MeA may be associated with the sensitization of appetitive sexual behaviors following repeated administration of EB.

Epigenetic modifications. Acting as a transcription factor through classic genomic mechanisms, E2 binds its receptors, dimerizes, and activates estrogen-response elements on DNA to initiate transcription, upregulating a number of systems some of which are themselves transcription factors (e.g., PR). An important regulatory step in transcription by hormone receptors involves the interaction with cofactors, including histone acetyltransferase (HATs), histone methyltransferase (HMT) and histone deacetylases (Gagnidze et al., 2013), which are involved in chromatin remodeling, and can activate (e.g., HAT and HMT) or suppress (e.g., histone deacetylation) gene transcription. It has recently been shown that E2 administration upregulates cofactors associated with the synthesis of PR in the mPOA and VMH in adult female mice (Gagnidze et al., 2013). As such, repeated administration of EB may, through its effects on epigenetic mechanisms, amplify transcription of systems that are facilitative to both appetitive and consummatory aspects of sexual behavior.

It has also been proposed that neurons retain their perinatal ability to switch on or off gene silencing mechanisms (Wilson & Westberry, 2009). ERα and ERβ are highly expressed in the rodent cerebral cortex early in postnatal life but they decline as the animal approaches puberty, but middle cerebral artery occlusion increases ERα mRNA. As such, it has been proposed that E2 can be neuroprotective by returning to an earlier stage of developmental programming ERα gene expression, potentially through changes in DNA methylation, which can reversibly silence genes (Wilson & Westberry, 2009; Wilson, Westberry, & Prewitt, 2008). If this mechanism is in fact conserved, then repeated administration of E2 may facilitate such a process within the hypothalamus, to silence genes involved with sexual inhibition, while facilitating those associated with sexual excitation (particularly ERα).

Is behavioral sensitization adaptive or maladaptive?

Behavioral sensitization broadly refers to an enhanced level of behavioral responding (e.g., locomotor activity) after repeated exposure to a stimulus (e.g., psychomotor stimulant, hormones, shock) (Kalivas & Stewart, 1991; Steketee & Kalivas, 2011), and can be adaptive or maladaptive. For example, pain sensitization can be adaptive to prevent further injury by generating a withdrawal reflex and a more complex behavioral response to escape future encounters with the stimuli (Latremoliere & Woolf, 2009). Moreover, repeated exposure to the painful stimuli lowers the pain threshold and amplifies the response, which increases the protective function of this system on the organism (Latremoliere & Woolf, 2009). However, pain sensitization can become maladaptive in some clinical syndromes (Latremoliere & Woolf, 2009).

Estradiol's ability to sensitize central nervous system functioning with respect to psychomotor stimulants can also be maladaptive or adaptive. For example, in OVX rats, E2 administration (5μg/day for two days) lowered the threshold for intracranial self-stimulation, and the threshold was even lower if cocaine was co-administered (5mg/kg for 5 days), indicating that E2 sensitized the reward system (which can be adaptive in some instances) but also amplified the rewarding effects of cocaine (Galankin, Shekunova, & Zvartau, 2010). Females also display a stronger behavioral sensitization of psychomotor behaviors in response to psychomotor stimulants such as cocaine and amphetamine, which may be due in part to a facilitative role of E2 (Forgie & Stewart, 1993; 1994; Peris, Decambre, Coleman-Hardee, & Simpkins, 1991), which increases striatal DA release (Becker, 1999). In humans, chronic amphetamine use induces a sensitized covert behavioral response such that paranoid behaviors progressively increase to the point of psychosis (Robinson & Becker, 1986). Interestingly, E2 appears to protect against psychotic (positive) symptoms (Kulkarni et al., 2014). In a behavioral model of schizophrenia, where OVX rats are first sensitized to AMPH, the antipsychotic effects of haloperidol (represented by a decrease in locomotor sensitization) following the administration of E2 at a dose meant to mimic that of proestrus, enhanced the effects of haloperidol (i.e., resulted in a further reduction in locomotor activity following an AMPH challenge) (Madularu, Shams, & Brake, 2014). It was proposed that this ameliorating effect of E2 may be in part via a blunted release of NAc DA in response to haloperidol treatment, or changes in striatal DA receptor subtype binding and/or affinity states (Madularu et al., 2014).

It was proposed that heightened sensitivity to E2 or reduced sensitivity to the inhibitory effects of VCS may serve as an adaptive mechanism to keep ovary intact cycling females in heat for a longer period of time, if they have not mated for a long period of time or have recently been pseudopregnant (Pfaus et al., 2000). There is evidence that behavioral sensitization to EB occurs with aging, suggesting that females that have been through more ovarian cycles are more sensitive to E2. Cooper (1977) showed that aged (25 months) females (both sexually-naïve and retired breeders) are more sensitive to EB than their younger (3 months) counterparts following OVX. Females were OVX then administered repeated daily injections of E2 (0.5µg/kg or 1µg/kg). The older females displayed lordosis a day sooner (31 hrs after EB treatment) than the younger females, accompanied by a progressive increase in LQ, reaching max five days later, which was maintained until the end of the experiment (three additional days), and was significantly higher than the younger females during that period. Moreover, once EB treatment had ceased, the older females continued to display significantly higher LQ compared to the younger animals, and continued to display lordosis for at least one day longer than their younger counterparts. This important study suggested that the aged female brain is more sensitive to the activational effects of EB, translating into higher and more prolonged expression of sexual behavior.

Thus, a mechanism of estradiol sensitization of sexual behaviors may be adaptive for species survival. But if that is true, what then would be the purpose of pseudopregnancy in a naturally cycling female? Why would the brain be set up to cease sexual behavior for an extended period of time (equivalent to 3-4 ovulatory cycles) and prepare the body for pregnancy, in the absence of fertilization? One answer may be that the rat brain contains mechanisms to optimize its potential for reproduction. Pseudopregnancy is a common phenomenon in other animals such as mice and dogs (Gobello, La Sota, & Goya, 2001), and even occurs to some extent in humans (Tarín, Hermenegildo, García-Pérez, & Cano, 2013) (although the underlying mechanisms in humans are likely more complex), yet its evolutionary purpose is not known. However, pseudopregnancy appears to sensitize the latency to onset of maternal behavior, when examined after its natural termination (Steuer, Thompson, Doerr, Youakim, & Kristal, 1987), which may be further evidence of an adaptive behavioral response for species survival. Taken together, it appears more and more obvious that the female rat brain is set up to optimize reproductive success, potentially through a series of "failsafe" mechanisms, one of which is

estradiol sensitization of sexual behaviors by enhancing excitatory neural mechanisms to activate, and reducing inhibition of systems that inhibit sexual behavior, as initially proposed by Pfaus et al. (2000), while also sensitizing the onset of estrous termination and presumably pseudopregnancy, particularly upon receipt of reproductively relevant stimulation (i.e., VCS).

Estradiol sets the stage for transition to the next phase

Although E2 sets up a state of sexual excitation such that the animal can respond appropriately to olfactory, auditory and somatosensory sexual stimulation, it also acts by reducing inhibitory neural processes. Powers and Valenstein (1972) were the first to show that one of the actions of E2 is to reduce neural inhibition that prevents the display of lordosis. They found that OVX females with lesions to the mPOA were more sensitive to the activational effects of EB on lordosis, and that those females stayed receptive for a longer period of time, showing that for lordosis to occur, EB must be removing inhibition within the mPOA in the intact animal. Similar disinhibitory effects of E2 have also been reported more recently in the VMH, whereby E2 reduces neural processes that are inhibitory to sexual behavior, by reducing glutamate transmission (Georgescu et al., 2009; 2014).

The ability of E2 to reduce the activation of systems that are inhibitory to sexual behavior may allow females to receive sufficient amounts of VCS required for the activation of the neuroendocrine events necessary to promote fertilization and sustain pregnancy (Georgescu et al., 2009; 2012; 2014; Pfaus et al., 2000). Without such a mechanism she might enter estrous termination too soon, since a requisite amount of stimulation is necessary for sperm transport (Adler & Toner, 1986; Adler, 1969; Matthews & Adler, 1978; Toner, Attas, & Adler, 1987) and the twice daily prolactin surges required for maintaining pregnancy (Adler & Toner, 1986). This thesis extends that idea by illustrating that the ability of E2 to activate excitatory systems and inhibit inhibitory systems interacts with the receipt of VCS during previous episodes of heat.

It was proposed previously that heightened E2 sensitivity or a lower sensitivity to the inhibitory effects of VCS may enhance reproductive success in ovary intact cycling females that have not mated for long periods of time, perhaps by extending the time they are in behavioral estrus at least long enough to receive a requisite number of intromissions and VCSs (Pfaus et al., 2000). However the present data suggest an alternate, though compatible, mechanism. Increasing doses of EB extend the amount of time that females will display lordosis (Quadagno,

McCullough, & Langan, 1972), yet females that received repeated EB treatments but were not given the opportunity to mate for six consecutive tests were at a later stage of estrous termination and were more sensitive to the inhibitory effects of VCS (Chapter 3.2). Thus, although E2 may extend the period of behavioral estrus (Albert et al., 1991), its repeated administration sensitizes systems that lead to its quicker termination, particularly following receipt of VCS, thereby *shortening* the period of behavioral estrus. Thus, E2 not only primes the circuits necessary for the activation of sexual behavior given the appropriate sexual stimuli and inhibits systems that act in opposition to those of sexual behavior (e.g., feeding, or on a neurochemical level glutamate transmission in the vlVMH), it also sensitizes the activation of systems required to shift the animal into the next phase (i.e., the termination of estrus, and presumably a return to behaviors that are incompatible with sexual behavior, such as feeding, nest building, and sleeping). Moreover, its sensitizing effects interact with prior sexual experience, particularly VCS. In addition, it appears that in EB treated females, repeatedly stimulating a mechanism that has been shown to be inhibitory to sexual behavior (AMPA receptor activation in the vlVMH), alters the female's sexual behavior pattern, by reducing appetitive aspects of sexual behavior while facilitating the expression of LM3 (Chapter 3.2 Experiment 1), a behavior that potentiates the onset of pseudopregnancy. This finding highlights the plasticity and reproductive adaptability of the female brain to the interaction between E2 and sexual experience.

The present data in combination with evidence in the field allows the generation of a rate-limiting rule of E2's enhancement and termination of sexual behavior: repeated cyclic exposure to E2 sensitizes the activation sexual behaviors in the OVX rat, which is a function of the dose and administration interval, while the rate of sensitization and duration of its expression, is limited by the sexual stimulation received (Figure 5.1). What is the applicability of the rule, and how does it translate into different scenarios? The neuroendocrine and experiential effects of behavioral estrus set the stage for the subsequent phase, beginning with estrous termination. For example, with the receipt of VCS sexual behavior becomes inhibited, while promoting physiological actions (such as prolactin release) that lead to a change in behavior (such as nest building and feeding) to prepare the animal to enter the next phase: pregnancy. Metabolic changes associated with pregnancy are a necessary adaptation to prepare the body for massive protein synthesis (fetal growth, parturition, lactation), and include increased

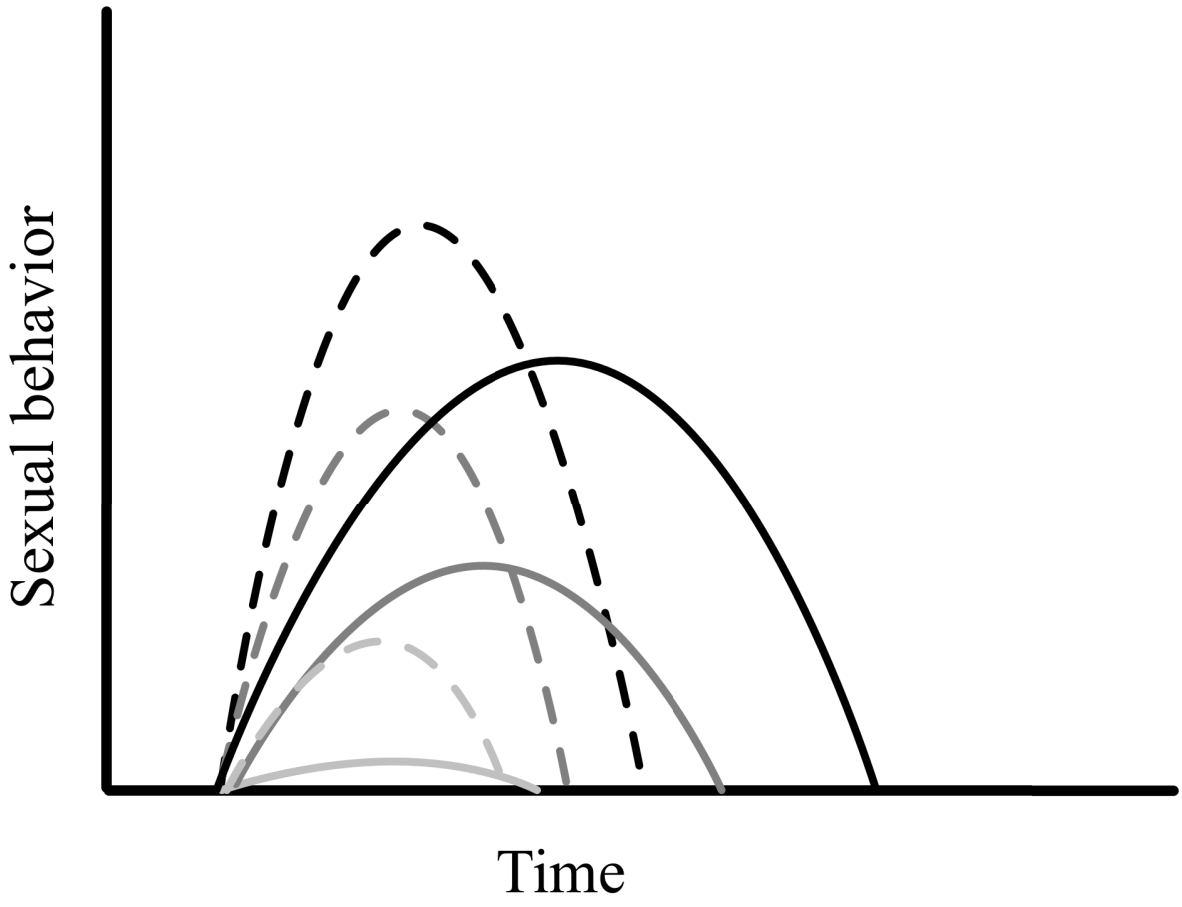

Figure 5.1 Rate-limiting rule of the ability of estradiol to sensitize mechanisms that facilitate as well as terminate sexual behavior. The rate of sensitization of female sexual behavior by estradiol is a function of dose (increasing color intensities represent increasing EB doses, e.g., 2, 5, 10μg), administration frequency (not shown), and sexual experience. Relative rates of sensitization are depicted as the upward arm of each line, whereas descending arms represent offset of the behavior in a given episode of heat in EB sensitized animals. As the dose increases the rate of sensitization increases, yet the rate is limited by the receipt of sexual stimulation. The repeated administration of EB without the opportunity to copulate (dashed lines) induces a robust sensitization, yet sexual behavior terminates quicker relative to females given the opportunity to copulate on each test (solid lines).

food intake, reduced energy expenditure, and more efficient nutrient absorption (Grattan & Le Tissier, 2014). Initially two daily surges of prolactin release from the pituitary maintains the P secreting corpora luteum, for approximately 12 days (Levine, 2014), after which the placenta in a fertilized female would take over. In the case of pseudopregnancy, the absence of the placenta results in luteolysis, a drop in P levels, and the resumption of the ovarian cycle. In a fertilized female, P production transitions to the placenta, and complex neuroendocrine events initially inhibit the expulsion of the fetus followed by a release of that inhibition to deliver the offspring (Kota et al., 2013). Just prior to parturition there is a dramatic reduction in P, and an increase in E2 and prolactin, and this state in turn alters the females' physiology and metabolism that must occur in the next state: lactation. The metabolic changes associated with lactation have recently been extensively reviewed (Woodside, Budin, Wellman, & Abizaid, 2012).

In summary, the neural consequence of repeated E2 administration interacts with sexual stimulation received to prepare the animal for the next phase. Since the phase that follows behavioral estrus requires the disinhibition of feeding, which presumably occurs at least in part through glutamatergic signaling in the VMH, it would be interesting to test differences in feeding behaviors in E2 sensitized animals following varying stimulations (e.g., EB/Alone vs EB/Male), or following the receipt of VCS in a pattern that is sufficient to induce pseudopregnancy. In the former case we might expect feeding behaviors to be greater (or a quicker onset) in those females that copulate with every episode of heat (compared to those that do not) since the receipt of VCS on each copulatory session activates mechanisms that are inhibitory to sexual behavior. In contrast, in the latter case it would be expected that feeding behaviors would be greater in females repeatedly treated with EB without an opportunity to copulate (e.g., after eight injections) particularly following VCS, since estrous termination was accelerated in that group. If these findings were supported, they would provide evidence that an increased behavioral sensitivity induced by E2 in one phase (i.e., sexual behavior) translates into increased behavioral responses in a subsequent, competing phase (i.e., feeding).

Additional applications of the phenomenon of estradiol sensitization

In Chapter 4 the estradiol sensitization paradigm was applied to two scenarios of sexual inhibition; yet another circumstance where sexual behavior is inhibited is with aging. As the female rat ages, ovarian steroid hormone secretion is disrupted (Borchardt, Lehman, &

Hendricks, 1980; Chambers & Phoenix, 1986; Cooper & Linnoila, 1977; Gerall, Dunlap, & Sonntag, 1980; Huang, Steger, & Bruni, 1978), resulting in abnormal patterns of sexual behavior. Although lordosis remains relatively intact in aged females that have transitioned into a period known as constant estrus, or in aged OVX rats treated with EB+P, the expression of appetitive sexual behaviors remains impaired compared to their younger counterparts (Borchardt et al., 1980; Chambers & Phoenix, 1986; Cooper & Linnoila, 1977; Gerall et al., 1980). We reported a similar impairment in female sexual behavior in aged ovary-intact Wistar rats tested in bi-level chambers, such that their behavior resembled that of younger OVX rats in estrous termination (Jones & Pfaus, 2007). Whether older female rats would sensitize to repeated treatment of EB is not known, although there is some evidence that chronically high levels of E2 is in fact inhibitory to female sexual behavior in aged females (Jones, Ismail, King, & Pfaus, 2012; Jones, 2009), suggesting that the ability of the brain to sensitize to E2 is disrupted with aging (perhaps due to an already heightened sensitivity to E2 (Cooper, 1977), as previously discussed, which may shunt them into an inhibitory state). Continuous treatment with testosterone propionate (an aromatizable androgen) via a subcutaneously implanted capsule can significantly increase the expression of lordosis and appetitive sexual behaviors, although the effect is short-lasting (Jones et al., 2012). Maintaining that facilitation may instead require periodic hormone treatments (e.g., every 4 days to mimic younger ovarian release), as previously proposed (Jones et al., 2012). Testing whether aged OVX females would sensitize to the repeated administration of EB, would allow a more thorough investigation of E2 sensitivity in the aged female brain. In fact, there may also be a sensitive window of opportunity to conserve the facilitative effects of E2 on rat sexual behavior with aging, as has been reported with E2's role in the conservation of cognitive function as women transition into menopause (e.g., Sherwin, 2005).

Further implications of the phenomenon of estradiol sensitization

Veterinary medicine. Our paradigm of estradiol sensitization may be particularly relevant to those in veterinary medicine, as it can be used as a diagnostic tool to test decreased sensitivity to E2, and potentially as a means to enhance reproductive behaviors and success. However, it would first be important to test whether in ovary-intact females repeated episodes of heat in the absence of mating would also enhance subsequent sexual behavior, and second whether that manipulation would improve fertility. One way this can be tested is using normally-

cycling ovary-intact females that are first mated (with vasectomized males, preventing impregnation), then tested for sexual behavior after varying numbers of complete cycles have passed. If repeated episodes of heat in the absence of mating potentiates sexual behaviors in naturally-cycling animals, then one would expect that the display of sexual behaviors would increase within group from the initial test to the final test, and that those females that complete more ovarian cycles prior to the second sexual behavior test would display higher levels of sexual behavior as the number of cycles increased (e.g., 1, 2, 4) compared to the other groups. If that were indeed the case, then it would be interesting to test whether those females that show higher levels of sexual behavior would be more likely to become impregnated once given access to a sexually vigorous (gonad-intact) male. If that were the case, and if it generalized to other species, it may be one way in which reproductive success could be enhanced in endangered species.

Sexual response cycles. Human data also reflect variability and flexibility in female sexual responses. This variability was first described in a linear sexual response model proposed by Masters and Johnson's (1966) and later revised by Kaplan (1974) which begins with sexual desire, followed by excitement, plateau, orgasm, and a resolution phase. In this model, sexual responses can differ between women or within the same woman at different times. The linear model has been generally replaced by circular models (Basson, 2005) which incorporate more cognitive factors, such as the desire for intimacy, pleasure and satisfaction, all of which must be considered in the context of the individual's relationship (Basson, 2005). Moreover, they emphasize the interaction between women's sexual desire and physiological arousal.

In women there exists discordance between subjective (self-report) and objective (e.g., vaginal blood flow) measures of arousal, in contrast to men who have a high concordance. A woman's physiological arousal occurs not only to images of their preferred sex, for example to heterosexual porn in heterosexual women, but also to homosexual porn (of men or women) as well as to nonhuman stimuli, despite discordance with their self-reports (Chivers & Bailey, 2005; Chivers, Rieger, Latty, & Bailey, 2004). Interestingly, the discordance between increased vaginal blood flow and measures of sexual desire in women has also been analogously shown in female rats. Whereas compounds that increase genital blood flow and sensitivity also increase sexual motivation in male rats (Chu et al., 2008), compounds that increase genital blood flow in female rats can in fact alter female sexual behavior (by increasing the contact-return latency following

ejaculation, suggesting heightened genital sensitivity to intense VCS) without affecting motivational components (such as hops/darts, or partner preference). This illustrates that increased physiological genital arousal does not necessarily translate into an increase in appetitive sexual motivation (Clark, Meerts, & Guarraci, 2009; Guarraci, 2010), analogous to the dissociation that is reported in women. Comparisons between human and rat sexual behavior (Pfaus, 1996) and the use of rodents in preclinical models of sexual function and dysfunction (Giuliano & Pfaus, 2006; Giuliano et al., 2010) have been thoroughly discussed.

The difference in appetitive behaviors found between females that copulate on every test (EB/Male) compared to those that are not given that opportunity (EB/Alone or ACF) may reflect differences in spontaneous and reactive sexual desire, as proposed in Basson's model (Basson, 2005). Basson's model stipulates that initially, sexual desire is typically absent and becomes active through a complex set of internal and external factors including a desire for emotional intimacy, and a need to increase a woman's sense of well-being and self-image (e.g., feeling attractive and loved) which can be partly dependent on the hormonal and physiological state of the woman (Basson, 2005). As such, in this model it is proposed that female sexual desire is responsive to the context, and only in some women is sexual desire spontaneous. It is spontaneous desire that may be related to hormonal status and pharmacological manipulations, and subject to increase with a new relationship (Basson, 2005; Klusmann, 2002). Is it possible then that the difference in appetitive measures of sexual behavior between animals that repeatedly copulate and those that do not reflect different aspects of sexual desire? Perhaps the greater display of appetitive behaviors displayed by animals not given the opportunity to copulate reflects an aspect of spontaneous desire.

Sand and Fisher (Sand & Fisher, 2007) found that Basson's model appears to best explain sexual responses of women with sexual dysfunction. Women were asked to identity themselves with one of three sexual response models (Masters and Johnson, Kaplan, or Basson's models); the distributions were equal among the three, and importantly 10% of women felt that none of the available categories best fit their sexual response. This report is particularly interesting as it demonstrates the variability in sexual responses between women; however it also reflects a major hindrance in our current understanding of female sexual responses given that we really have no good model in women. Nonetheless, an animal model of variations in different aspects of sexual desire, as we currently understand them, may help identify endocrine and physiological

underpinnings of differences in sexual responses between women, and how variability in those responses are altered by prior sexual experience. In closing, although patterns of sexual stimulation have been shown to improve fertility in rodent research as discussed in this thesis (e.g., distributed clitoral stimulation, paced VCS) limited evidence suggests that fertility is dependent on any aspect of human sexual response cycles or sexual stimulation (e.g., Levin, 2002; 2011). However, this may reflect our limited understanding of the parameters underlying the variability in women's sexual responses and sexual function, and only with the progression of sexual research can we truly address this question.

Conclusions

This thesis has established that repeated administration of E2 in combination with sexual experience converge within brain regions relevant to the expression of sexual behavior resulting in different sensitivities and behavioral baselines. The expression of those behaviors changes not only within an episode of heat (e.g., Bennett, Blasberg, & Blaustein, 2002; Blaustein et al., 2009), but the data in the present thesis show that sexual experience across multiple episodes of heat also changes the expression of sexual behaviors on subsequent sexual encounters. The differential sensitivities and behavioral outcomes that occur with repeated E2 and sexual experience may to be set up to increase the probability of reproductive success. In addition the data in the current thesis have shown that the repeated administration of EB, even at doses that have previously been shown to be subthreshold, sensitizes an estrogen-dependent behavior. This suggests that E2 has differential actions on neural physiology and behavior depending on the dose, and whether it is administered acutely or chronically, and that those effects interact with experience. Thus, these findings have important implications for all studies examining the role of E2 on neural physiology and behavior.

Finally, the findings of the current thesis have broad implications. On a proximal level the phenomenon of estradiol sensitization has implications for the design of preclinical models of female sexual function, and all studies examining the effects of E2 on neural function and resulting behavior in female rats. On a more distal level, the work has implications for understanding neural mechanisms of sensitization, and potentially as a way to enhance reproductive function (which may be pertinent to other species) and increase our understanding of the variations in female sexual response cycles.

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