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Possible Role of the Ventromedial Hypothalamus in Estrus Termination Following Vaginocervical Stimulation of the Rat.

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Abstract

Possible Role of the Ventromedial Hypothalamus in Estrus Termination Following Vaginocervical Stimulation of the Rat.

Myriam Lavoie

Vaginocervical stimulation (VCS) of the female rat during copulation is known to be the primary stimulus responsible for a decreased duration of the receptivity period called behavioral estrus. Central mechanisms by which VCS accelerates the onset of estrus termination in rats are not completely understood yet. Hence, the present study investigated the role of the ventromedial hypothalamus (VMH) in estrus termination using sexually experienced ovariectomized female rats. First, females were administered subcutaneous injections of estradiol benzoate and progesterone before being assigned to 4 different treatment groups. The first group received a microinfusion of a Na^+ channel blocker, tetrodotoxin (TTX), into the VMH an hour before receiving 50 manual VCSs, with a lubricated glass rod distributed over the course of 1h, while the second group received a microinfusion of TTX and sham VCSs. The remaining groups received a microinfusion of saline and their respective VCSs treatment. Each female was then tested for sexual behavior with sexually vigorous males in bilevel testing chambers 12, 16 and 20h after the beginning of the VCS treatment. Compared to females in the other groups, the infusion of TTX to the VMH delayed the decrease of lordosis responsiveness typical of estrus termination but no interaction between drug and stimulation treatments was established.

To my dear husband,

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1 Introduction

1.1 Female rat sexual behavior

Female rats typically display a complex interaction of appetitive, proceptive and consummatory behaviors during the receptivity period of behavioral estrus. In general, appetitive behaviors bring the subject into closer proximity to the incentive while consummatory behaviors occur when the animal has come into contact with the incentive and proceptive behaviors refine the contact (Pfaus, 1996; Pfaus, 1999; Pfaus, Smith & Coopersmith, 1999). In the female rat, appetitive behaviors such as anogenital investigation, ear wiggling and motor activities (e.g. hopping, darting and solicitation) serve to attract copulatory contact from the male, which normally result in the consummatory behavior, lordosis, necessary for intromission. Proceptive behaviors displayed by the female, such as pacing and defensive behaviors, impose the rate and intensity of intromissions and ejaculations from the male. At the beginning of a mating session, a female typically initiates copulation with an active solicitation after which she displays a prelordosis crouch and receives a mount from the sexually active male. Immediately after a successful mount with intromission, the female disengages abruptly from lordosis and runs away from the male while he grooms himself. Subsequent bouts, as described above, occur until ejaculation is achieved. After a post-ejaculatory period of 1 to 2 min., the female usually makes brief visits to the male and solicitates him more as he becomes more active and the pattern continues. As mating progresses, the pattern of behaviors displayed by the female changes to behaviors representative of estrus termination, a period of decreased willingness of the female to engage in sexual activity (Pfaus *et al.*, 1999; Pfaus, Smith, Byrne & Stephens, 2000).

Estrogen (E) and progesterone (P) gonadal hormones orchestrate many cellular responses in the brain to regulate the female willingness to copulate. First, E stimulates sexual receptivity, such as the lordosis reflex, via the activation of estrogenic-

concentrated cells in the ventromedial hypothalamus (VMH) (Carrer, Asch & Aron, 1974; Malsbury, Kow & Pfaff, 1977; Mathews & Edwards, 1977; Pfaff & Sakuma, 1979) and P facilitates sexual behavior when combined with E due to production of P receptors following estrogen-induced transcription (Blaustein & Feder, 1979). However, E and P are not the only stimuli to promote behavioral and neuroendocrine changes in female sexual behaviors.

Vaginocervical stimulation (VCS) provided by the male during copulation, or by the experimenter manually, to a female rat induces short- and long-term behavioral and neuroendocrine changes. For example, VCS given to ovariectomized (OVX) rats, not receiving E and P treatment, potentiates the lordosis reflex, making it the only known stimulus, other than E, to promote lordosis in the short-term (Rodriguez-Sierra, Crowley & Komisaruk, 1975). On the other hand, VCS can activate long-term events such as pregnancy and pseudopregnancy (Frye & Erskine 1990). Interestingly, different amounts of VCS induce different changes in female rats. Small amounts of VCS produce a short-term facilitation of both lordosis and pacing (Coopersmith, Candura & Erskine, 1996; Diakow, 1975; Erskine, 1989; Komisaruk & Diakow, 1973; Rajendren, Dubley & Moss, 1991; Rodriguez-Sierra *et al.*, 1975; Yang & Clemens, 1997) and has the ability to induce analgesia (Crowley, Jacobs, Volpe, Rodriguez & Komisaruk, 1976; Crowley, Rodriguez & Komisaruk, 1977; Crowley, Rodriguez & Komisaruk, 1977; Komisaruk & Larsson, 1971; Komisaruk & Steinman, 1986; Komisaruk & Wallman, 1977). As for small to moderate amount of VCS, it has the capacity of increasing sperm transport and fertility (Adler, 1969; Adler & Toner, 1986; Edmonds, Zoloth & Adler, 1972; Matthews & Adler, 1977), and the release of pituitary hormones LH, oxytocin and prolactin (Blake & Sawyer, 1972; Spies & Niswender, 1971; Terkel & Sawyer, 1978). As well, small to moderate amounts of VCSs have the capability of inducing pseudopregnancy (Adler, Resko & Goy, 1970; Coopersmith, Gans, Rowe & Erskine, 1996; Frye & Erskine, 1990; Komisaruk & Steinman 1986). Finally, large amounts of VCS can reduce the duration of

behavioral estrus (Coopersmith *et al.*, 1996; Erskine, 1985; Erskine & Baum, 1982; Erskine, Kornberg & Cherry, 1989; Hardy & Debold, 1971; Lodder & Zeilmaker, 1976; Pfaus *et al.*, 2000). The process by which behavioral estrus period can be shortened is largely unknown.

1.2 Manual vaginocervical stimulation

Manual VCS was designed to control the strength and the amount of stimulation females receive during an experiment. A cluster of VCS consists of inserting a lubricated glass rod of 4-5 mm o.d. with rounded and polished ends into the vagina four times at 1 sec intervals. The first 3 times, the rod is inserted in the anterior vagina for about 0.5 sec without touching the cervix and the fourth time the tip of the glass rod is pressed firmly against the cervix for about 2 sec (Pfaus, Marcangione, Smith, Manitt & Abillamaa, 1996). To mimic the typical stimulation a female receives from penile intromissions during an hour of copulation with a male rat, VCS is given using the glass rod and is distributed quickly in clusters of 5 every 6 min over the course of an hour, producing a total of 50 VCSs (Pfaus *et al.*, 1996; 2000).

Brain regions activated by VCS have been identified by numerous studies using the advantage of immediate-early genes produced by cells when activated. Namely, the medial preoptic area (MPOA), lateral septum (LS), bed nucleus of the stria terminalis (BNST), paraventricular hypothalamic nucleus, ventromedial hypothalamus (VMH), medial amygdala (MEA), arcuate, lateral habenula, ventral premammillary nuclei, mesencephalic central gray (MCG) and periduncular nuclei (Allen, Adler, Greenberg & Reivich, 1981; Blake & Sawyer, 1972; Dudley, Rajendren & Moss, 1992; Erskine, 1993; Pfaus, Kleopoulos, Mobbs, Gibbs & Pfaff, 1993; Pfaus, Jacob, Kleopoulos, Gibbs & Pfaff, 1994; Pfaus *et al.*, 1996; Polston & Erskine, 1995; Rowe & Erskine, 1993; Tetel, Getzinger & Blaustein, 1993; Tetel, Getzinger & Blaustein, 1994; Tetel, Celentano & Blaustein, 1994; Wersinger, Baum & Erskine, 1993) have been identified. VCS induced

experimentally is indistinguishable, in terms of the overall amount and pattern of Fos-immunoreactive cells (Fos-IR) induced in different brain regions, to an equivalent time of paced copulation in bilevel chambers (Pfaus *et al.*, 1993; 1996). In addition, the induction of Fos-IR can be reduced significantly (Rowe & Erskine, 1993) and the decrease in lordosis response typically observed as mating progresses can be abolished following transection of the pelvic nerve (Lodder & Zeilmaker, 1976). As a consequence of these studies, VCS is the primary stimulus during copulation responsible for the onset of estrus termination.

1.3 Hypothalamic structures and estrus termination

The finding that the activation of Fos-IR cells is different in different brain regions (Pfaus *et al.*, 1996) has important implications regarding the function of those regions in the regulation of female sexual behavior. The study demonstrated that brain structures activated later in the test, when females received large amounts of VCS, were hypothalamic structures such as the ventrolateral part of the ventromedial hypothalamus (VMHvl) and the medial preoptic area (MPOA). The literature offers interesting results about these regions regarding their effects on female sexual behaviors. First, electrolytic and excitotoxic, axon sparing, lesions of the MPOA has been shown to facilitate lordosis whereas electrical stimulation has been shown to inhibit lordosis (Hoshina, Takeo, Nakano, Sato & Sakuma, 1994; Pfaff & Sakuma, 1979; Powers & Valenstein, 1972; Takeo, Chiba & Sakuma, 1993). More importantly, gonadal hormones and sexual stimulation from VCS may act in conjunction to control the inhibition of lordosis and the facilitation of certain appetitive sexual behaviors. In support, the application of estradiol to the MPOA inhibits neuronal activity (Hasegawa & Sakuma, 1990; 1993), VCS induces a rapid excitation of neurons in the MPOA (Blake & Sawyer, 1972; Dafny & Terkel, 1990) and Fos-IR within the MPOA following the application of VCS is enhanced by hormonal treatment (Pfaus *et al.*, 1996). Thus, the MPOA may contain circuits that control inhibi-

tory and facilitatory pathways for lordosis and certain appetitive sexual behaviors. In further support, a recent study by Ramos & Debold (1999) determined that protein synthesis in the MPOA is important for a VCS-induced decrease in estrus duration in hamsters. They demonstrated that the infusion of anisomycin, a protein synthesis inhibitor, delayed considerably the inhibition of lordosis following VCS from male hamsters, relative to females that received either intromissions following cholesterol infusions, or females that wore a vaginal mask during copulation to prevent penile intromission. Of particular interest, the latter finding suggests that VCS may induce estrus termination by initiating synthesis of proteins which function is to reinstate inhibition of receptivity. On that account, the activation of the MPOA during copulation may control sexual receptivity on one hand by inhibiting lordosis and on the other hand by stimulating pacing or proceptive behaviors.

Well-established data suggest that the VMH, in addition to the MPOA, contains neurochemical subsystems that can either inhibit or facilitate sexual receptivity. On one hand, intrahypothalamic infusions of neural excitatory agents, such as glutamate, KCl and Kainic acid, of E treated OVX female rats induced an inhibition of lordosis (Kow, Harlan, Shivers & Pfaff, 1985). On the other hand, infusions of acetylcholine, oxytocin, gonadotropin-releasing hormone and noradrenaline increase neuronal excitation in VMH slice preparations and also facilitate lordosis (Kow & Pfaff, 1988; Pfaff, Schwartz-Giblin, McCarthy and Kow, 1994). As well, microinfusion of N-methyl-S-aspartate (NMDA), an excitatory amino acid, into the VMH of OVX hormone treated rats, reduced significantly the measure of lordosis suggesting that increased excitatory amino acid activity inside the VMH inhibits lordosis (McCarthy, Curran & Feder, 1991). Following the same logic, electrophysiological experiments demonstrated that neural excitatory agents excite multiunit activity of the VMH hence suggesting the presence of a lordosis-inhibiting neural mechanism inside the VMH (Kow *et al.*, 1985). On the other hand, microinfusions of GABAA antagonist into the VMH of OVX hormone-treated rats resulted in an inhibition

of ongoing lordosis while infusion of GABA receptor agonist into the VMH resulted in a significant enhancement of lordosis (McCarthy, Malik & Feder, 1990). The latter finding indicates that increased GABAergic neurotransmission in the VMH facilitates lordosis. According to the above studies, an increased neuronal excitation of the VMH inhibits lordosis while an increased neuronal inhibition triggers lordosis. Interestingly, Pfaus & Sabongui (1996) found that VCS induces Fos-IR within glutamate neurons of the VMH, which suggests that VCS activates a lordosis-inhibiting system within the VMH. However, it is still not known whether the likely activation of the lordosis-inhibiting system signals the rapid termination of lordosis that allows the initiation of pacing (Pfaus *et al.*, 1999), or whether it signals a much later event such as estrus termination (Pfaus & Heeb, 1997; Pfaus *et al.*, 2000). It is interesting to consider that VCS might be activating an inhibiting system located in the VMHvl to stimulate the end of behavioral estrus.

1.4 Behavioral measures of estrus termination

Previous experiments have used lordosis as the ultimate measure of estrus in intact, naturally-cycling female rats mainly because a large amount of neuroendocrine, neurochemical and neuroanatomical information has been established on this reflexive behavior (Coopersmith *et al.*, 1996; Erskine *et al.*, 1989; Erskine, 1985). These studies usually expose the females to a period of copulatory interaction with the males during which they receive paced or nonpaced intromissions. Lordosis is then scored, 12, 16 and 20 h after the copulatory stimulation with new sexually vigorous males or using manual flank stimulation. Using this approach, these studies have established that paced intromissions lead to fewer stimulated lordosis postures at 20h compared to the same number of nonpaced intromissions. Paced mating gives the opportunity for the female to impose inter-intromission latency by running away from the male which then results in receiving stronger intromissive stimulation and consequently, maximizes the effectiveness of each intromission (Edmonds *et al.*, 1972; Erskine *et al.*, 1989; McClintock 1984). This type of

mating reflects better the type of situation females encounter in the wild. However, it is difficult to observe the complex integration of appetitive and consummatory behaviors displayed by female rats when they are tested in small chambers. Too often, the female is not allowed to solicit and pace the male for intromission efficiently and is left with defensive behaviors toward the male to impose the rate of intromissions and ejaculations. As a consequence, the display of these defensive behaviors makes it difficult to interpret the willingness of the female to copulate and decreases the incentive sexual stimuli value of these females to males (Everitt, 1990). In addition, males are quick at learning not to attempt to copulate with sexually nonreceptive females (Kippin, Talianakis, Schattmann, Bartholonew & Pfaus, 1998; Pfaus & Pinel, 1989). To counter these problems, new chambers were designed to allow the female to pace copulatory contacts (e.g. Erskine, 1989; Mendelson & Gorzalka, 1987; Yang & Clemens, 1997). One of these alternatives is the use of bilevel chambers (Mendelson & Gorzalka, 1987; Mendelson & Pfaus, 1989; Pfaus *et al.*, 1990). These are narrow in width and made up of two connected levels by a set of ramps on either side (Fig. 1). The use of bilevel chambers not only allows the female to pace and solicit copulatory contact from the male, it forces the animal to maintain an optimal sideways orientation which help the observer score behaviors with better accuracy.

By using bilevel chambers, previous studies established a complex patterns of appetitive and consummatory sexual behaviors performed by female rats which facilitate the identification of neurochemical or endocrine mechanisms associated with different aspects of female sexual motivation and performance (Pfaus *et al.*, 2000; Pfaus *et al.*, 1999; Pfaus *et al.*, 1996; Pfaus *et al.*, 1993). Pfaus *et al.* (2000), established that the display of appetitive and consummatory sexual behaviors by female rats changes as copulation progresses and these changes precede the passive avoidance of the male and the termination of lordosis responsiveness. VCS induced a dynamic pattern of estrus termination in OVX females primed with E and P starting with reduced solicitations,

increased interval between intromissions and increased number of active rejections. As well, they argued that females are still capable of showing lordosis when receiving flank stimulation by mounts but rarely do because they are aggressive towards males who attempt to mount. In support, 12h after receiving the VCS treatment, they observed the females while copulating with sexually active males and found that females displayed a decrease in appetitive responses and an increased in rejection responses prior to a decline in their ability to show lordosis reflex. The latter supports the idea that behaviors other than lordosis could be used as an indication of estrus termination in the female rat. In addition, by varying hormone treatments such that different groups of female were primed every 4, 7, 14 or 28 days using E and P hormones, their work demonstrated that steroid hormones have the capacity to alter the ability of VCS to facilitate estrus termination. The behavioral estrus termination pattern was enhanced in females receiving the VCS treatment and was at its most complete form in females receiving hormone priming every 28-day. On that account, estrus termination follows a progression of behavioral responses enhanced by a large amount of VCS and delayed by increased hormone sensitivity.

1.5 Purpose

The present study examined whether microinfusing tetrodotoxin (TTX), a sodium channel blocker, into the VMHvl of ovariectomized females primed with E and P every 28 days would alter the behavioral pattern during behavioral estrus termination in bilevel chambers 12, 16, and 20h after the administration of the first stimulation treatment. To control for individual differences in the number and in the strength of intromissions, manual VCS approximating the number of intromissions and ejaculation that a sexually receptive female would receive during an hour worth of copulation with a sexually active male rat in bilevel chambers (Pfaus *et al.*, 1993; 1996; 2000) was used. Females receiving the control infusion, buffer, and the stimulation treatment, VCS, were expected to

display lower measures of lordosis responsiveness and appetitive behaviors, and higher measures of rejection behaviors and pacing when compared to females receiving the drug infusion and the VCS treatment. Females receiving the infusion of buffer or TTX and the control stimulation, shamVCS, were expected to behave in a similar fashion as females receiving the drug infusion and the stimulation treatment, VCS.

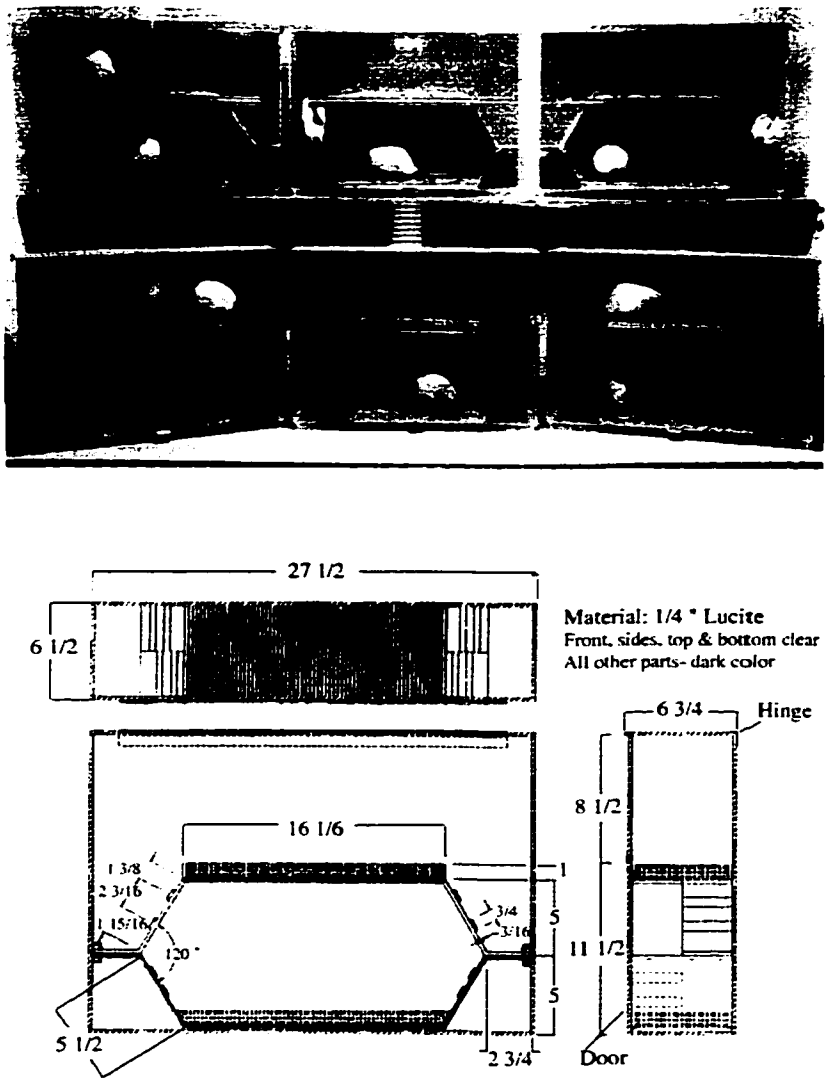

Figure 1. Photograph of a typical copulatory test using bilevel chambers (upper) and schematic representation of a bilevel chamber (lower) from (Pfaus *et al.*, 1999).

2 *Methods*

2.1 Animals

Thirty-two Long-Evans female rats (150 - 200 g) were obtained from Charles River Canada, St-Constant, Québec). They were housed singly in stainless steel cages (18 x 24 x 18 cm) in a colony room maintained on a reversed 12:12 h light/dark cycle at a temperature of approximately 21 °C, for a period of two months. They were then transferred to plastic cages (32 x 26 x 19 cm) in the same conditions as above. Food and water were continuously available. Intact Long-Evans male rats that served as stimulus males had at least 10 tests of sexual behaviors in the testing bilevel chambers prior to the start of the experiment. These males were sexually active and initiated copulatory activity with the females within 15 sec of being placed into the testing chambers.

2.2 Surgery (ovariectomy)

Anesthetized females were ovariectomized bilaterally via lumbar incisions. A mixture of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml), mixed at a ratio of 4:3, respectively, was administered as the anesthetic using intraperitoneal injections (i.p.). A volume of 0.125 ml per 1 kg of body weight was injected in each female a few minutes before the beginning of the surgery. All females were given a week of postsurgical recovery prior to the tests of sexual behavior.

2.3 Hormones and tests of sexual behavior

Females were habituated to the testing bilevel chambers (fig. 1) 2 days prior to the first sexual behavior test. They were given 10 standard 35 min tests of sexual behavior in bilevel chambers with sexually experienced males prior to cannulation surgery. For each test, the female was placed into a bilevel chamber for at least 5 min prior to the introduction of the male for a period of 30 min test of sexual behavior. These tests were conducted at 4 day intervals during the middle third of the rats' dark circadian cycle. Each

female received subcutaneous injections of estradiol benzoate (EB; 10 μg in 0.1 ml sesame oil) 48 h, and progesterone (P; 500 μg in 0.1 ml of sesame oil) 4 h, before each test to induce full sexual receptivity.

2.4 Preliminary VCS test for Fos-IR

To ensure that VCS was applied and activated the VMH in a similar fashion as in previous studies, a preliminary test was performed. A female was given E and P treatment to induce full sexual receptivity, as described above, prior to receiving 50 VCSs distributed manually in cluster of 5 at 6 min intervals over the course of 1 h . The female was sacrificed, 75 min after the application of the first VCS, by an overdose of sodium pentobarbital (120 mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, On) to be perfused intracardially with ice-cold phosphate-buffered saline (PBS: 300 ml) followed by ice-cold 4 % paraformaldehyde in 0.1 M phosphate buffer (PB: 300 ml). Immediately after, the brain was removed from the skull, postfixed in fresh 4 % paraformaldehyde for a duration of 4 h, then manually blocked, stored in 30 % sucrose at 4 °C for a minimum of 12 h and subsequently frozen using dry ice to prepare for slicing.

Frozen coronal brain sections (30 μm) of the VMH area were cut (corresponding to plates 27-36 of Paxinos and Watson 1998) using a sliding microtome. These sections were then washed in ice-cold Tris Buffered Saline (TBS) and incubated with an affinity-purified monoclonal antibody raised against residues 4-17 of the N-terminal sequence of human Fos protein (NCI-BCB Repository, Quality Biotech, Camden, NJ; diluted 1:8000) at 4 °C for 48 h in 0.05 % Triton X-100 with 1 % normal horse serum. Then, brain sections were rinsed in ice-cold TBS and incubated at 4 °C for 1 h with a rat-adsorbed biotinylated antimouse IgG made in horse (Vector Laboratories, Burlingame, Ca; diluted 1:33.33) in 0.05 % Triton X-100 with 1 % normal horse serum. Afterward, sections were rinsed in ice-cold TBS and incubated at 4 °C for 2 h in an avidin-horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; diluted 1:55:55). Once more,

sections were rinsed with TBS, then rinsed in 50 mM Tris buffer (pH 7.6), and rinsed for 10 min in 0.05 % 3,3'-diaminobenzidine (DAB) in 50 mM Tris buffer at room temperature. The sections were then incubated on an orbit shaker for 10 min in DAB/Tris buffer with the addition of 3 % H_2O_2 (100 µl per 100 ml of DAB/Tris buffer, pH 7.8) to catalyze the DAB and 8 % $NiCl_2$ (400 µl per 100 ml of DAB/Tris buffer + H_2O_2) to color the DAB chromagen product blue-black. Finally, brain sections were rinsed in TBS to stop the reaction, mounted onto gel-coated slides, dehydrated with ethanols, cleared in xylene, protected using a coverslip, and examined under a microscope.

2.5 Cannulation surgery

7 to 10 days prior to the experimental test, females were anesthetized using a mixture of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml), mixed at a ratio of 4:3, respectively, and injected in a volume of 0.125 ml per 1 kg of body weight. Double guide cannulae (26 gauge, 2 mm center to center; from Plastics One Inc., Roanoke, VA) were stereotaxically implanted in all females. The head was secured in the stereotaxic frame with the tooth bar set at 5. The double cannula along with the injector were aimed to stop 1 mm dorsal to the ventrolateral (vl) VMH using fixed coordinates. At bregma, 1.0 mm lateral to the midline and 7.5 ventral to the dura (AP: 0; ML: 1.0; DV: 7.5) cannulae were inserted. They were secured in place by using 4 skull screws and dental cement. To prevent entry of foreign particles dummy internal cannulae were used which fitted inside guide cannulae and protruded about 0.5 mm. An injector cannula fitted inside the guide cannula to infuse the drug and protruded 1 mm.

2.6 Tetrodotoxin solutions

Tetrodotoxin stock solutions were made by dissolving 1 mg of tetrodotoxin (TTX, Sigma Chemicals, St-Louis, Mo) into 10 ml of 20 mM citrate buffer (Na citrate:citrate ratio, 55:45, *p*H 4.45) as used previously (Kohane, Yield, Lu, Langer, Strichartz & Berde 1998). The TTX stock solution was diluted with buffer to a ratio of 1:100, prior to infu-

sion. The concentration of TTX used was 5 ng (Harlan, Shivers, Kow & Pfaff, 1983) in a volume of 500 nl saline. The infusion lasted 1 min. The injector was left inside the guide cannula for 2 additional min prior to its withdrawal and was replaced by the dummy cannula. The infused fluid was never observed to seep out of the guide.

2.7 Hormone, Infusion and VCS treatments (experimental conditions)

After the 10th copulatory test, females were maintained on at least another 4 day cycle of EB until they received the habituation treatment to VCS. On the day of habituation, each female received the P treatment at 17:00 h and was administed 50 VCSs distributed manually using a lubrificated glass rod in clusters of 5 at six-min intervals over the course of 1 h, as described previously (Pfaus *et al.*, 1996). Females were then assigned randomly to one of the four experimental conditions. All females were maintained on a 28 day cycle of EB and P treatment (EB was administed 26 days and P was administered 28 days, after VCS) prior to the drug infusion and VCS treatment application. The infusion was performed at 20:00 h, the VCS treatment at 21:00 h and the behavioral estrus termination was tested the next day at 09:00, 13:00 and 17:00 h.

Each experimental condition consisted of 2 treatments. First, an infusion into the VMHvl of either 0.5 µl TTX (5 ng in 0.5 µl citrate buffer) or 0.5 µl citrate buffer an hour prior to the second treatment. The latter consisted of 50 VCSs distributed manually or sham VCS (in which the female was held by the base of the tail, her tail and rump raised and anogenital region exposed, for an amount of time equal to that for the application of 5 consecutive VCSs) at six-min intervals over the course of 1 h, as described previously (Pfaus et al., 1996). The VCS treatment was administered at 21:00 h and estrus termination was tested the following day at 09:00, 13:00 and 17:00 h. Females were assigned randomly and evenly to one of the 4 possible combinations of treatments.

2.8 *Behavioral estrus termination tests*

Behavioral estrus termination tests began 12, 16, and 20 h following the first VCS treatment and lasted approximately 30 min. Each female was placed into a bilevel chamber for 5 min prior to the introduction of the first sexually receptive stimulus male. The male was allowed to copulate with the female to one ejaculation or 10 min of copulation (in the event that ejaculation did not take place), after which it was removed from the chamber to be replaced by a second sexually active male, 5 min later, as described previously (Pfaus *et al.*, 2000). The test would end following ejaculation of the second male or 10 min of copulation. In the event, males did not exhibit typical sexual arousal behaviors such as investigation of the female anogenital area and pursuing her, within 1 min of the copulatory test, males were immediately changed by another sexually active male rat. Males not getting intromissions were sexually primed at the end of the experimental day with sexually receptive stimulus females to keep them sexually vigorous for subsequent tests with other groups of females.

2.9 *Behavioral scoring*

Each estrus termination test was videotaped and scored subsequently using a computerized event recorder program (Cabilio, 1998). This study focused on the behaviors of the females during the tests without taking into account the males' responses to females' solicitations and rejections. Incidents of level changing (as a measure of locomotion), proceptive hops and darts, active solicitation (defined as the number of times each female moved from one level which contained the male and made a headwise orientation followed by a runaway back to the original level (McClintock, 1984)), pacing (number of level changes per mount), lordosis (calculated as quotient and reflex intensity (Fig. 2) (Hardy, 1971)), and passive (ledge sitting) and active rejection (boxing postures, fighting, defeat) were recorded as in previous studies (Pfaus *et al.*, 1999: 2000). Female sexual behaviors quantified from both ejaculatory series were used in the present study.

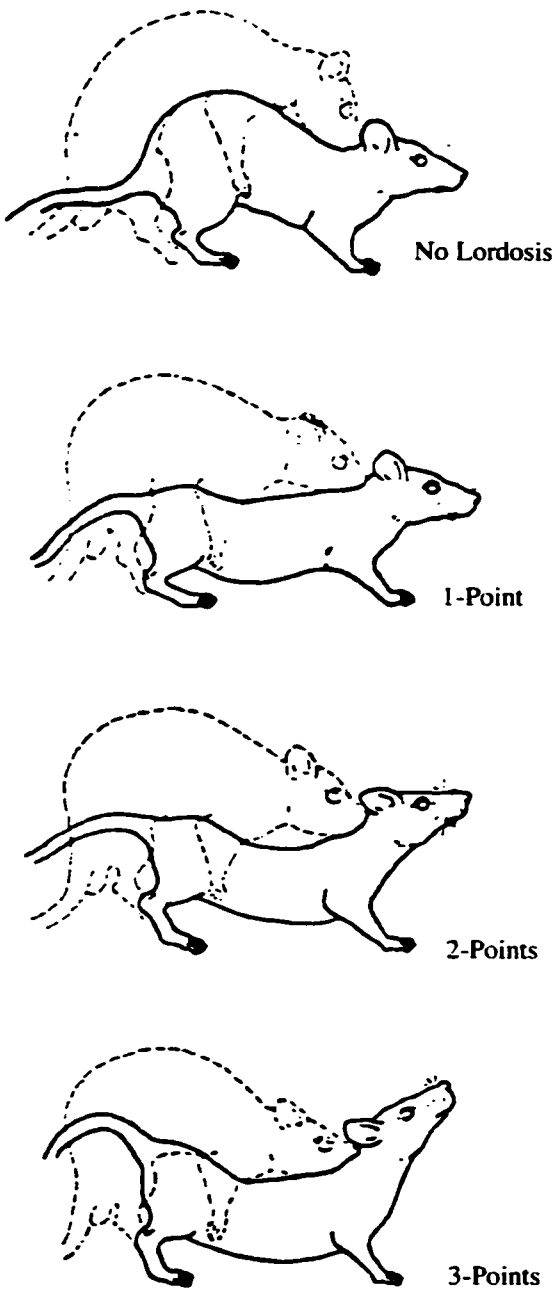

Figure 2. Lordosis magnitude representation of the 3-point scale scoring used in the present experiment. (Hardy & Debold, 1971)

2.10 Staining and cannulae placements

1 to 2 weeks following the end of the test, females were sacrificed using an overdose of sodium pentobarbital (120 mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, On) and then perfused intracardially as described in the preliminary test section (2.4). Following the slicing of frozen coronal brain sections (30 μm) through the end of cannulae tracks, sections were mounted onto gel-coated slides, dipped into cresyl violet for 6 min, differentiated using 95% ethanol, dehydrated with ethanol, cleared in hemo-D, protected using a coverslip, and examined under a microscope for cannulae placements.

2.11 Statistical analyses

To investigate the role of the VMHvl on estrus termination behaviors, only females with infusion sites within 2 mm, dorso-lateral, to the VMHvl were included. Each behavioral measure was analysed using a 2 (drug treatment) x 2 (stimulation condition) x 3 (test time) analysis of variance (ANOVA), with drug infusion and stimulation as between-subjects factors and test time as the repeated measure. Values were considered significant at $P < .05$.

3 *Results*

3.1 Preliminary Fos-IR test

Microscopic examination of brain sections from the area showed that VCS induced Fos-IR in a similar pattern, inside the VMHvl, to that of Pfaus *et al.*, 1993; 1996. Representative digitized images of the VMH illustrating VCS induced Fos-IR from this study (Fig. 3). The pattern of Fos-IR VCS induced in this preliminary test is similar to the pattern of Fos-IR VCS induced in the study by Pfaus *et al.*, 1993; 1996.

3.2 Experiment

All females displayed a pattern of estrus termination in which lordosis intensity and quotient were low while rejection behaviors occurred at a high frequency and pacing ratio was high. Incidents of hops and darts were only observed during the first sex test (12h after beginning of VCS treatment) in only one female and at a very low frequency. Active solicitations were not observed at any testing time for any female in any group. Consequently, hops, darts and active solicitation measures were ignored in the current analysis.

3.2.1 Infusion sites

Histological analysis, using cresyl violet stain, revealed that cannulae placements were above the site of interest, VMHvl. In the event that some of the drug might make it inside the VMHvl, placements within an area of 2mm, dorso-lateral, to the VMHvl were used for the statistical analysis. These placements are represented on a histological reconstruction of infusion sites (Fig. 4). A total of 27 females were used in the present analysis.

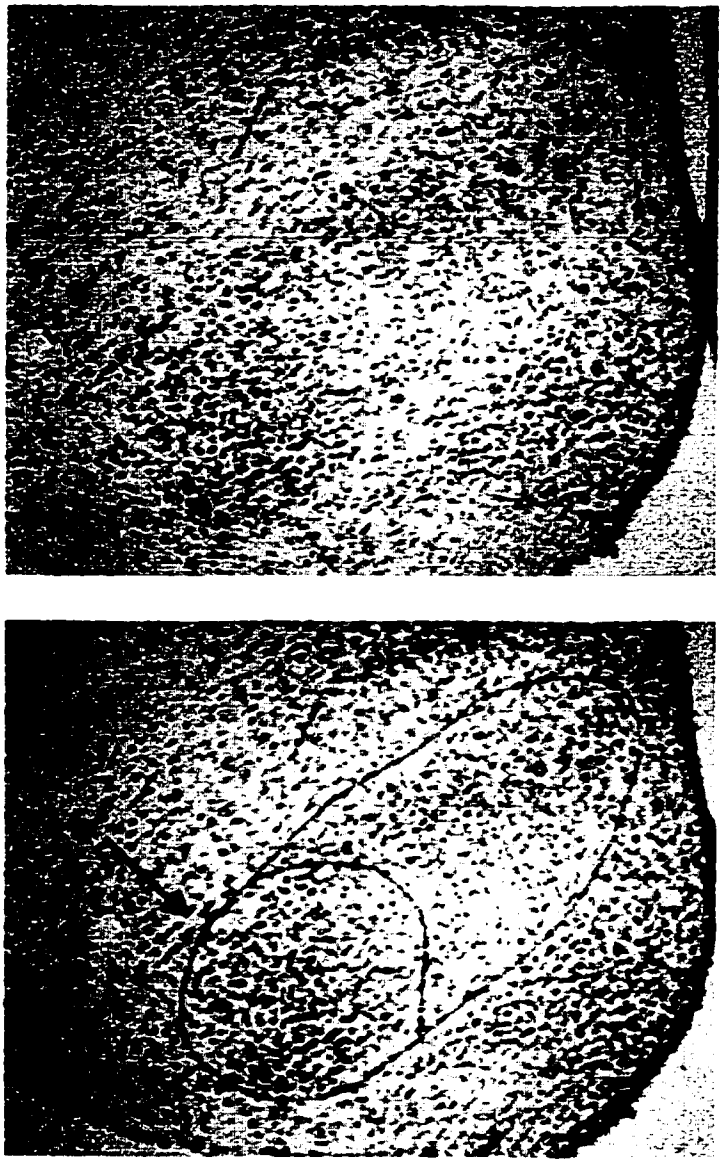

Figure 3. Digitized images of a brain section using cresyl violet to illustrate the VMH. The image below outlines the VMH structure and the arrow points at the VMHvl area.

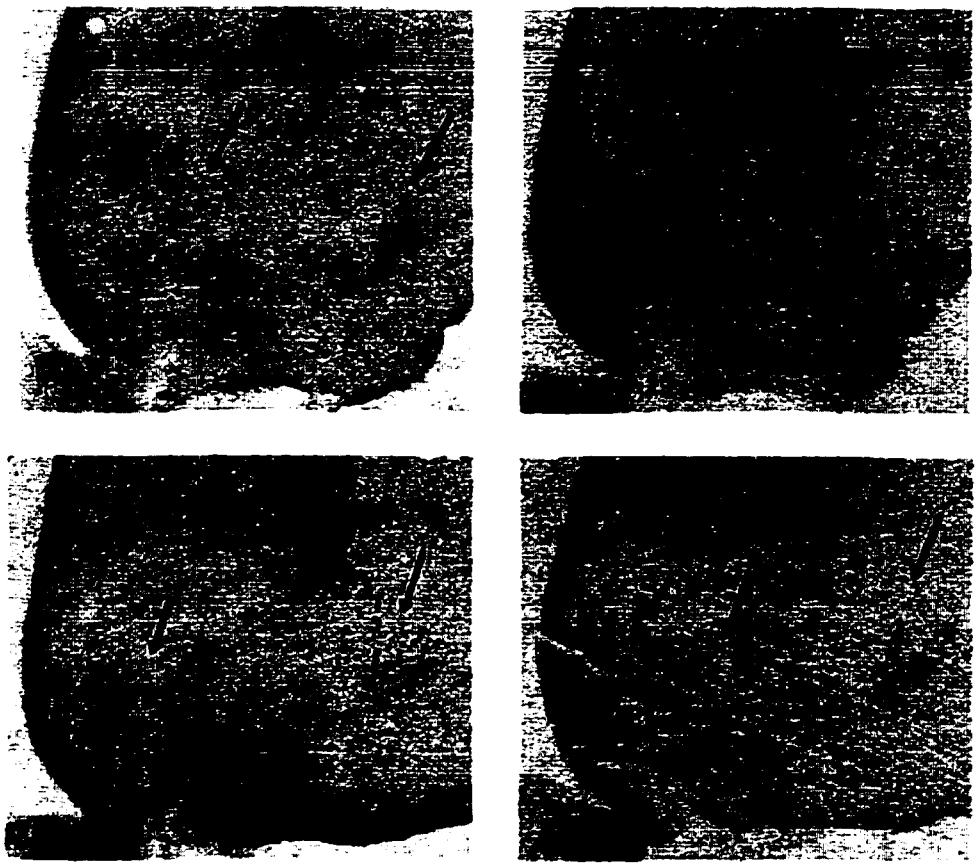

Figure 4. Representative digitized images of VCS induced Fos-IR induction in the VMHvl area taken at 40x. Arrows on right point at VMHvl and arrows on left point at the arcuate nucleus.

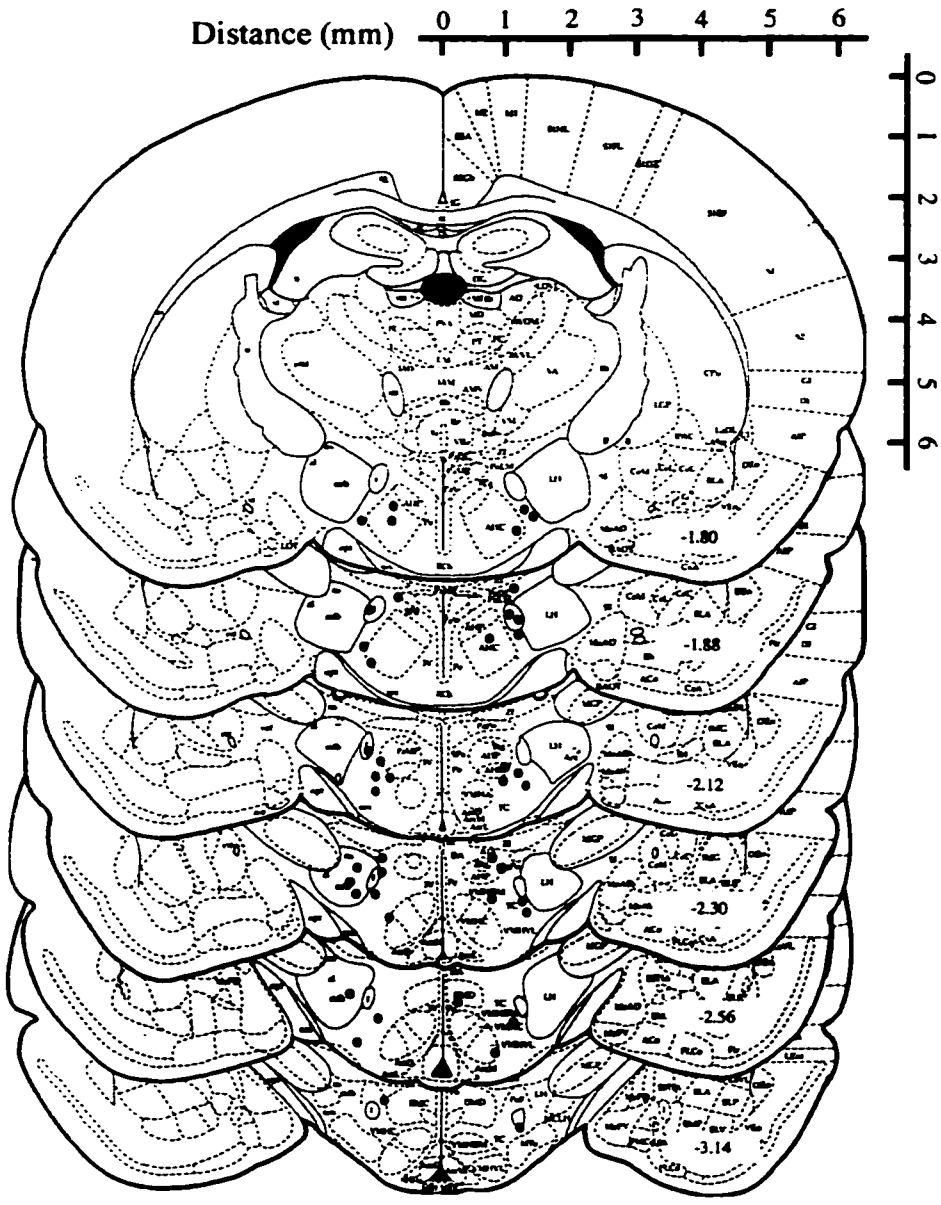

Figure 5. Histological reconstruction of infusion sites of subjects used in the present experiment.

The infusion of TTX induced mild seizures, within 5 min after infusion, in 2 out of 16 rats. These rats laid quietly with their eyes closed in their home cage for a maximum of one hour, after which they showed no sign of sedation. Histology revealed sites of infusion to be respectively within 1mm of the anterior hypothalamus (AH) and 1mm lateral to the AH . Yet, 3 other females had their cannula placed at proximity to those sites without showing any signs of seizure after TTX infusion.

3.2.2 Lordosis

Effects of drug treatments and stimulation conditions on the lordosis quotient (LQ) and lordosis reflex magnitude (LM) are shown in Fig 5. Using repeated dependent measures of LQ, the ANOVA detected a significant main effect of stimulation condition, $F(1,23) = 5.74$, $p < .0251$. Groups receiving 50 VCSs were significantly lower than sham VCSs groups and that effect was independent of the drug treatment females received and the time of testing after the VCS treatment. As well, the ANOVA detected a significant main effect of test time, $F(2,46) = 58.357$, $p < .0001$, where LQ values decreased with time. The ANOVA did not detect a main effect of drug treatment. Although the ANOVA did not detect an interaction effect between stimulation condition and test time, $F(2,46) = 2.818$, $p < .0702$, groups of females receiving VCS treatment tended to perform lower LQ faster in time than groups of females receiving sham treatment. As for lordosis reflex magnitude, the ANOVA detected a significant main effect of drug treatment, $F(1,23) = 5.182$, $p < .033$. Females receiving TTX infusions displayed higher lordosis magnitudes than females receiving buffer infusions, and this effect was independent of stimulation treatment and test time. In addition, the ANOVA detected a significant main effect of test time, $F(2,46) = 50.603$, $p < .0001$, where LM values decreased with time. An interaction between drug treatment and test time was detected by the ANOVA, $F(2,46) = 4.43$, $p < .018$. Groups of females receiving buffer infusions performed lower LM faster in time than groups of females receiving TTX infusions.

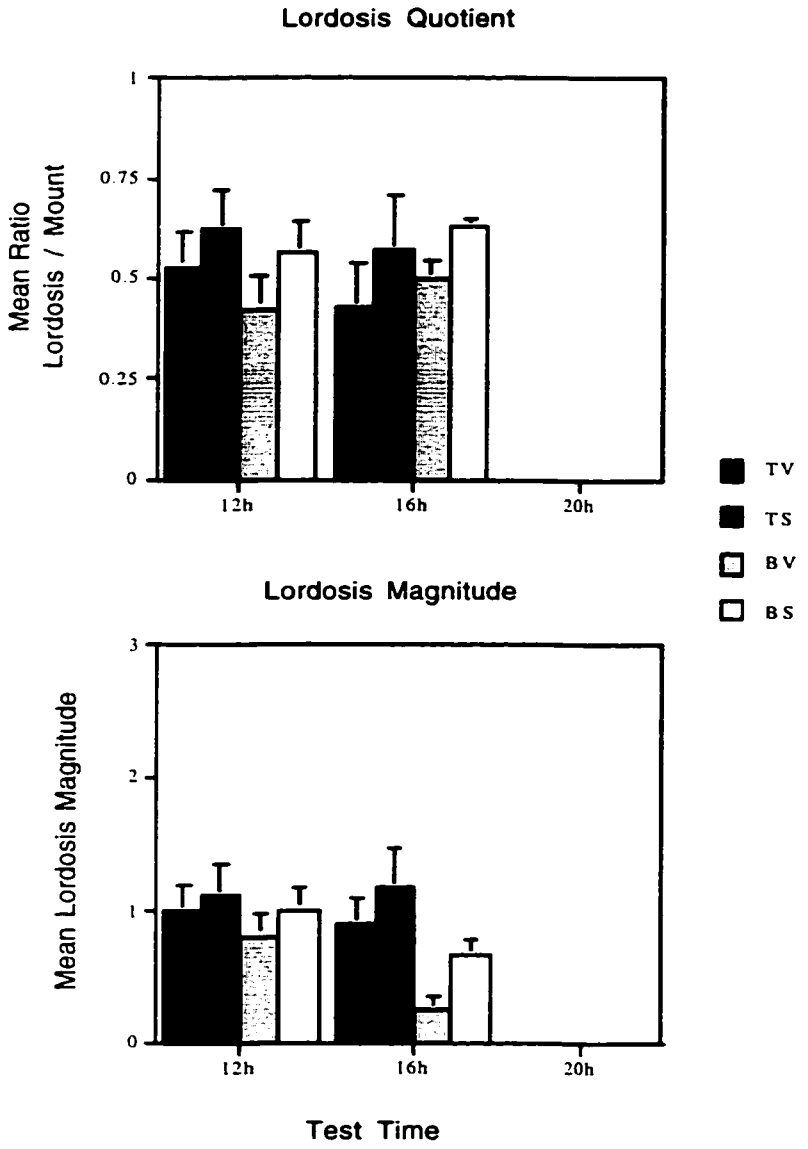

Figure 6. Mean measures of lordosis quotient (above) and lordosis magnitude (below) for each treatment group with positive standard error bars. TV females received TTX and VCS treatments (N=8; black bar) while TS females received TTX and sham VCSs treatments (N=6; dashed bars). BV females received buffer infusion and VCS treatment (N=8; grey bars) and BS females received buffer infusion and sham VCSs (N=5; clear bars).

3.2.3 Pacing

Effects of drug treatments and stimulation conditions on pacing are shown in fig. 7. The ANOVA detected no significant main effect of drug treatment or stimulation condition and no significant interaction effect. Although the ANOVA did not detect any significant effect of test time, $F(2,46) = 2.078$, $p < .137$, figure 7 suggests an increase in pacing as testing progressed. However, the large error bar, at 20h for TTX-VCS group, exists because 2 females displayed very large numbers (38 and 54) of pacing behaviors while the other 6 females of that group displayed low numbers (0 to 6) of pacing behaviors.

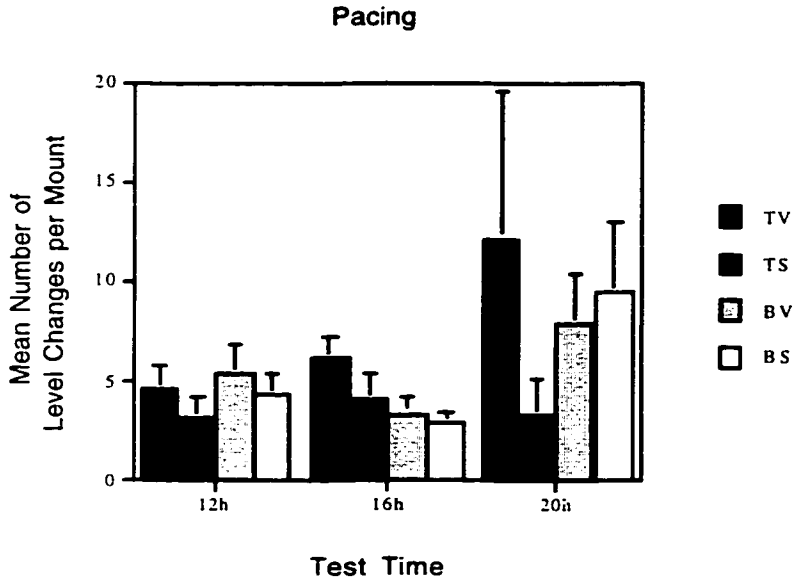

Figure 7. Mean frequency measures of pacing behaviors for each treatment groups with positive standard error bars. TV females received TTX and VCS treatments (N=8; black bar) while TS females received TTX and sham VCSs treatments (N=6; dashed bars). BV females received buffer infusion and VCS treatment (N=8; grey bars) and BS females received buffer infusion and sham VCSs (N=5; clear bars).

3.2.4 Rejection

Effects of drug treatments and stimulation conditions on active (AR) and passive rejection (PR) are shown in fig. 6. The ANOVA detected no significant main effects and no interaction effects for AR, but the data are variable. In the TTX-VCS group, one female showed a very high frequency of AR at 16h (i.e. 69 with the next highest number of value 26). Other females oscillated between 1 to 19 AR during the 16h test. As for the large error bar at 20h, a similar pattern occurred. One female of the TTX-VCS group, exhibited 48 AR behaviors while the next highest number of AR being 11. Again, other groups show similar outlier presence with one or two females displaying large numbers of AR compared to other females in the group.

The ANOVA detected no significant main effects and no interaction effects for PR. However, an overall tendency towards an increased in proportion of time spent PR with test time was observed, $F(2,46) = 2.935$, $p < .064$, for all groups of females. Data on PR were also quite variable.

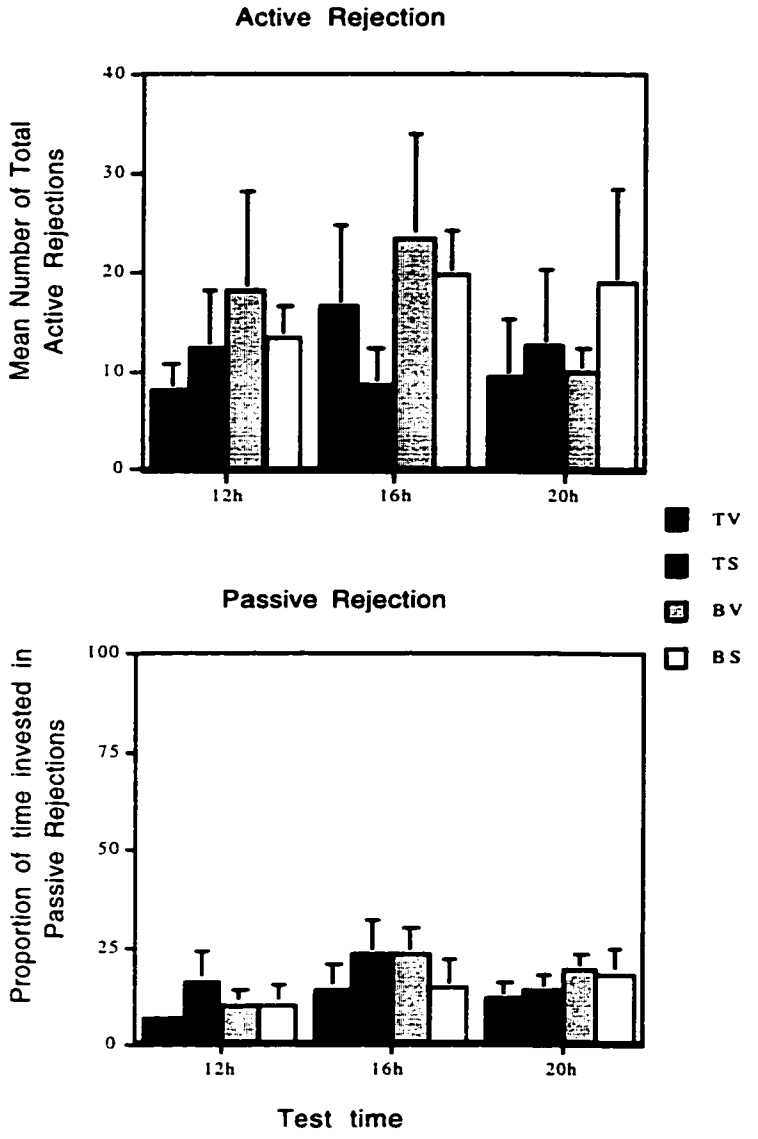

Figure 8. Mean frequency measures of active rejections (above) and proportion of time spent panel sitting, passive rejection (below), for each treatment groups with positive standard error bars. TV females received TTX and VCS treatments (N=8; black bar) while TS females received TTX and sham VCSs treatments (N=6; dashed bars). BV females received buffer infusion and VCS treatment (N=8; grey bars) and BS females received buffer infusion and sham VCSs (N=5; clear bars).

3.2.5 Level Changes

Effects of drug treatments and stimulation conditions on the number of level changes (LC) are shown in fig. 7. The ANOVA detected a significant main effect of drug treatment, $F(1,23) = 5.007$, $p < .036$, independent of stimulation treatment and time factor. Overall, females receiving TTX showed lower numbers of LC than females receiving buffer infusions. As well, the ANOVA detected a significant main effect of stimulation, $F(1,23) = 9.049$, $p < .0063$, independent of drug treatment and time factor. Females in VCS groups showed higher numbers of LC than females in sham groups. A significant main effect of time was also detected by the ANOVA, $F(2,46) = 7.122$, $p < .002$, where the total number of LC decreased over time. Although the ANOVA detected no significant interaction effect, the data suggest a tendency for a main drug and sex interaction effect, $F(2,46) = 2.764$, $p < .11$, and a time and drug interaction effect, $F(2,46) = 2.42$, $p < .101$. First, TTX-saline females tended to show lower LC than buffer-saline females while the buffer-saline group tended to show lower LC than buffer-VCS females. As well, TTX-VCS females tended to display higher LC that TTX-sham females. Second, females receiving buffer infusions tended to show lower LC, at 12h and 16h, than females receiving TTX infusions. A similar display of LC was observed at 20h.

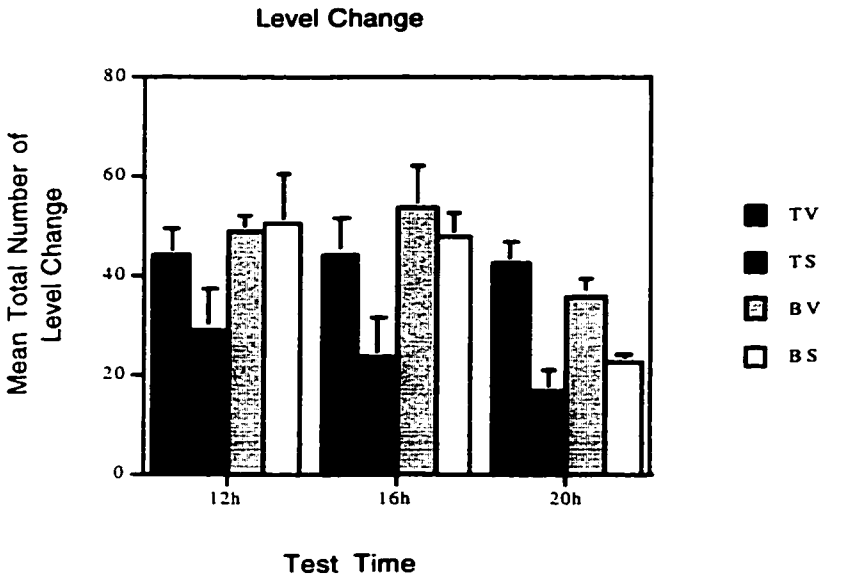

Figure 9. Mean frequency measures of level changes, a measure of locomotion, for each treatment groups with positive standard error bars. TV females received TTX and VCS treatments (N=8; black bar) while TS females received TTX and sham VCSs treatments (N=6; dashed bars). BV females received buffer infusion and VCS treatment (N=8; grey bars) and BS females received buffer infusion and sham VCSs (N=5; clear bars).

4 *Discussion*

Although brain mechanisms of estrus termination in female rats are not yet understood, studies point to the VMH and MPOA as regions likely to possess neurochemical subsystems that either facilitate or inhibit sexual receptivity (Kow *et al.*, 1985; McCarthy 1995; McCarthy *et al.*, 1990; McCarthy *et al.*, 1991; Pfaus & Sabongui 1996). It is not known yet whether the activation of cells in the VMH by VCS is linked to the rapid termination of lordosis and the initiation of pacing typically following an intromission (Pfaus *et al.*, 1999), or whether it is activating a long-term event such as estrus termination (Pfaus & Heeb, 1997). Estrogen-concentrating cells in and around the VMH are essential for estrogenic induction and maintenance of lordotic responsiveness (e.g. Carrer *et al.*, 1974; Malsbury *et al.*, 1977; Mathews & Edwards, 1977; Pfaff & Sakuma, 1979). In addition, it has been established that drugs interfering with synthesis of messenger RNA or protein can disrupt the estrogenic action on lordotic responsiveness when infused into the VMH (Quadagno, Shryne & Gorski, 1971; Quadagno & Ho, 1975; Rainbow, Davis & McEwen, 1980; Shivers, Harlan, Parker & Moss, 1980). Furthermore, in order to demonstrate that hypothalamic cells require action potentials to activate lordotic behaviors, Harlan *et al.* (1983), infused drugs that blocked voltage-dependent sodium channels (such as procaine, bupivacaine and tetrodotoxin) into the VMH of conscious estrogen-treated female rats and measured the lordotic responsiveness of the female to these drugs. They found that infusion of local anesthetics, procaine and bupivacaine, has no effect on lordotic responsiveness whereas infusion of tetrodotoxin, which blocks voltage-dependent sodium channels using a different mechanism of action from traditional local anesthetics, resulted in a reversible decline in lordotic responsiveness. The first significant drop was observed 40 min after the infusion while the minimum drop was reached 2 - 4 h after and complete recovery occurred 12 - 24 h after. Harlan *et al.* argued

that the lordotic responsiveness change induced by TTX into hypothalamic cells may be caused by an action on neurons intrinsic to hypothalamus or efferent to it. They argued further that estrogen requires action potentials from lordosis-relevant hypothalamic cells to potentiate the lordosis reflex. Linking their main finding to the functional significance of Fos-IR, VCS effects may result from changes in protein synthesis via Fos-induction into activated brain regions, such as the MPOA and the VMH to result in a decrease in sexual receptivity. In fact, a recent study by Ramos & Debold (1999), demonstrated that protein synthesis in the MPOA is important for a VCS-induced decreased in estrus duration. A protein synthesis inhibitor, anisomycin, was infused into the MPOA of steroid primed hamster females and a delayed inhibition of lordosis following VCS from male hamsters, relative to females that received either intromissions following cholesterol infusions, or females wearing a vaginal mask during copulation was observed. This study established that induction of estrus termination depends on the initiation of protein synthesis in the MPOA of hamster females.

The pattern of Fos-IR inside the VMH following the application of VCS in the preliminary test successfully replicated the pattern of Fos-IR inside the VMH following the application of VCS treatment in Pfaus *et al.* (1996; 2000). A pattern of Fos-IR similar to that of manual VCS occurs following copulatory stimulation from the male (Pfaus *et al.*, 1993). The fact that the application of VCS induced the same pattern of Fos-IR inside the VMH indicates that the technical procedure was well described in previous studies. Hence, this technique can be used to investigate further effects of VCS on different aspects of female reproductive behaviors and mechanisms.

Even if the infusion of the drug, in the present study, was not inside the VMHvl, but within an area of 2 mm dorso-lateral to it, the infusion might have resulted in a spread of the drug to some of the hypothalamus. A dye infusion test performed in Harlan, Shivers, Kow & Pfaff (1982) suggests that the drug infusion immediately dorsal to the VMH would cover the entire nucleus. An area of stain 1-2 mm from the cannula tip in each

direction and along the cannulae tracks could be seen in sections from brains removed 20 min after the dye infusion. As well, Harlan *et al.* suggested that the volume of visible dye might change as a function of time following infusion. Since effects of drug, in the present study, were observed at a time much later than 20 min after the infusion, it is possible that some of the drug might have diffused to the VMHvl. In the event diffusion reached part of VMHvl, the present study may indicate effects that would be substantially stronger if the total amount of drug was infused successfully inside the VMHvl. In fact, the present study may be used in conjunction with a study aiming successfully at the VMHvl to support the location specific effect of the treatment. In other words, the present infusion sites could be used to demonstrate that 1-2 mm away from the VMHvl, the same effects are not as powerful. Therefore, results from the present experiment may have important implications to better understand the role of the VMH in estrus termination following VCS.

The stimulation treatment had a significant effect on lordosis quotient. Females receiving 50 VCSs following the drug treatment decreased their lordosis quotient significantly more than females receiving sham VCSs following the drug treatment, and that effect is independent of the drug treatment and test time. The latter finding supports the idea that a large number of VCS reduces the duration of behavioral estrus, hereby decreasing lordosis measurements. As well, the drug treatment had a significant main effect on both lordosis magnitude and total level change numbers. Females receiving a TTX infusion prior to stimulation decreased their lordosis magnitude significantly more than females receiving the control treatment prior to stimulation, and that effect was independent of the stimulation condition females received and test time. The latter finding suggests that some of the drug might have reached the VMH to inhibit neural mechanisms involved in lordosis responsiveness control. Interestingly, females receiving TTX infusions displayed a significantly lower total number of level changes than females receiving buffer infusions which may indicate an effect of the drug on locomotion. How-

ever, no interaction effect between drug treatment and stimulation condition, can be drawn from the present study. Unless a future study includes a larger number of animals and successfully aims at the VMHvl, it will be hard to know if the VMHvl has any role in estrus termination.

The statistical analysis identified a significant time effect for both lordosis quotient and magnitude, an effect independent of drug and stimulation treatment. Females in all groups decreased their lordosis responsiveness as testing proceeded. A time effect on lordosis is indicative of estrus termination which replicated the well-established fact that lordosis measures decrease as mating progresses (e.g. Erskine 1985; Erskine & Baum 1982; Erskine *et al.*, 1989; Pfaus *et al.*, 2000). As well, a significant time effect was identified, by the present statistical analysis, on the total number of level changes female accomplished. Females typically decreased their number of level changing as a function of time which indicate a decrease in activity level typical of estrus termination. As for passive rejection, although the statistical analysis did not identify significant effects on passive rejection behaviors, the present data suggests that females increased their proportion of time devoted to passive rejection behaviors as a function of test time which would replicate the finding that females invest more time in passive rejection behaviors as estrus termination progresses (Pfaus *et al.*, 2000).

Although no significant effect was identified on pacing behaviors, data tend toward an increase in pacing as testing progressed. Such a tendency is in accordance with observations that females display higher pacing as mating progresses (Coopersmith *et al.*, 1996; Pfaus *et al.*, 2000). Pacing is an important behavioral measure for estrus termination since females tend to increase pacing to impose larger inter-intromission latency on the male (e.g. Pfaus *et al.*, 1999; Pfaus *et al.*, 2000). Considering that no drug treatment effect was identified by the statistical analysis for pacing behaviors, it is possible that the region around the VMHvl does not play a role in the control of this behavior. However,

more investigation and a larger N is needed to establish the role of the VMH on pacing behaviors in the female rat.

Results of this study confirm previous findings that VCS, successfully applied, has an effect on behavioral estrus. First, females receiving large amounts of stimulation, 50 VCSs, prior to copulation, displayed lower lordosis quotients that females not receiving VCS which consistent with the idea that females will decrease their responsiveness to lordosis as the number of intromission increases (Coopersmith *et al.*, 1996). In further support, the data showed a significant time effect for both lordosis quotient and magnitude. Overall, females decreased their lordosis responsiveness as a function of time which indicates the occurrence of estrus termination. As well, the data suggested that TTX diffused slightly to the VMH. By doing so, females receiving TTX infusions displayed higher lordosis magnitude scores than females infused with buffer, supporting the argument that the VMH is involved in the control of lordosis responsiveness (Pfaff *et al.*, 1994). More investigation need to be done in order to establish clearly the role of the VMHvl in the control of estrus termination behaviors.

Following the finding that many VCS-responsive neurons contain estrogen receptors (Tetel, Celentano & Blaustein, 1994), work by Pfaus *et al.* (1996) and Tetel *et al.* (1994) have demonstrated that Fos protein induction in certain brain regions (such as the VMH and caudal BNST) following VCS can be diminished by priming treatment with estrogen (48h) and progesterone (4h), before VCS. First, Tetel et al. (1994) demonstrated hormone effects on VCS induced Fos-IR using a VCS treatment consisting of inserting a plastic seringe plunger attached to a force gauge into the vagina and administering 2 sessions of 5 min separated by a 3 min interval of no stimulation. Each session consisted of 400 g of force applied on the vagina and cervix in alternating periods of 10 sec on and off. Prior to the administration of the VCS treatment, they injected priming doses of E and P to one group of female and oil to another group of females to then look for the expression of Fos-IR within brain regions typically expressing Fos-IR following VCS. They found that

hormone priming decreased VCS-induced Fos-IR and that this effect was localized within the VMH. Furthermore, Pfaus *et al.* (1996) established that cells within different estrogen-concentrating regions are differentially sensitive to VCS and that gonadal hormones alter the ability of VCS to induce Fos-IR. Following different amount of VCSs, applied, using the lubricated glass rod, in cluster of 5 every 6 min, the hormone treatment enhanced the number of Fos-IR found in the mPOA and MEApd. However, the opposite effect was observed in the LS, BNST and VMH following low amount of VCSs and no effect in the MCG whatsoever. These findings have important implications in the interpretation of Fos protein induction by VCS. The enhancement of cells showing Fos-IR following VCS indicates that the hormone treatment facilitates cellular responses to VCS in those regions. Yet, it does not necessarily indicate that an increase in Fos-IR has anything to do with the direct facilitation of a particular sexual behavior. Females perform an array of complex responses to VCS and any of those could be involved by the activation of Fos-IR. Even so, it is interesting to consider the possibility that VCS-induced Fos response into the VMH mediated by estrus termination occurs via protein synthesis as is the case for the MPOA, in hamsters (Ramos & Debold, 1999).

Finally, it is of crucial importance that a study establishes clearly the role of the VMHvl in the control of estrus termination behaviors. Such study would take the approach of the present study but would aim successfully at the VMHvl and would have a larger N. In the event a study establishes that the VMH is indeed involved in the control of estrus termination behaviors, another study could use a protein synthesis inhibitor, such as anisomycin (Ramos & Debold, 1999). That study would consist of an infusion treatment and a stimulation treatment. Two groups of females would receive infusions of a protein synthesis inhibitor inside the VMHvl prior to the administration of either 50 VCSs or sham VCS. Two other groups would receive cholesterol infusions into the VMHvl followed by their respective VCS treatment. Establishing whether VCS-induces

Fos-IR into the VMHvl mediated estrus termination via protein synthesis would contribute greatly to the understanding of estrus termination in the female rat.

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