The Role of the Clitoris in Sexual Behaviour and Reward In the Female Rat

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ABSTRACT

The Role of the Clitoris in Female Sexual Reward and Behaviour In the Rat

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Sexual behaviour encompasses a variety of sensory stimuli that makes sex rewarding in both humans and rats alike. Despite what we know about the clitoris' contribution to sexual pleasure in human females there is still a lack of research focusing on the function of the clitoris and its role in learning, sexual development, and sexual expression. The rat is a good animal model for the study of sexual function and dysfunction and can be used to add to our understanding of clitoral stimulation and function. Accordingly, the five chapters encompassing this thesis were designed to expand our comprehension of the role that the clitoris plays in female sexual behaviour and reward in the rat. Chapter 1 showed that the clitoris is important for interpreting genital signals that differentiate stimulation received from males during copulation. It also showed that sexual experience buffers female rat pacing behaviour from the disruptive effects of lidocaine administration to the clitoris. Chapter 2 showed that manual external clitoral stimulation (CLS) is rewarding as assessed by the development of robust conditioned place preference, and activates immediate early gene c-fos expression selectively in the medial preoptic area (mPOA) and medial amygdala (MeA). Chapter 3 showed that manual external CLS could be paired with a neutral olfactory stimulus in sexually naïve rats and induced a copulatory partner preference for a male bearing the odour cue during the female's first sexual experience with males. Moreover, if CLS was applied to sexually naïve rats in the presence of a scented but inaccessible male, those females solicited more an unscented male during their first sexual experience with males. Thus, the ability

of CLS to be a positive reinforcer is context-dependent. Chapter 4 examined the sexual reward state induced by paced mating or CLS and its dependence on ovarian hormones. The conditioned place preference (CPP) induced by CLS developed regardless of the hormonal state of the female, whereas paced-mating induced CPP required hormonal priming. This shows that the reward state induced by CPP is independent of ovarian hormones, whereas the sexual behaviors that entice males to mount and intromit (thus bringing the clitoris into contact with the male's penis and perineum at the female's preferred rate) are not. Finally, Chapter 5 showed that prior copulatory experience with males eliminated the ability of CLS alone to induce CPP, suggesting either that experience with full internal and external CLS provided by a male conspecific has a greater reward magnitude and/or that copulatory experience renders subsequent external CLS alone ineffective as an unconditioned stimulus due to sensory preconditioning. Taken together, these data show that external CLS is a powerful reinforcer to a sexually naïve female rat. Although its hedonic value is not dependent on ovarian hormones, the sexual behaviors required to receive it are, and copulatory experience appears to reduce or eliminate its hedonic value.

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LIST OF ABBREVIATIONS

mPOA: Medial preoptic area

CPP: Conditioned place preference

VCS: Vaginocervical stimulation

CLS: Clitoral stimulation

CLS5: Distributed clitoral stimulation at 5sec intervals

CLS1: Continuous clitoral stimulation at 1sec intervals

E: Estrogen

EB: Estradiol benzoate

P: Progesterone

OVX: Ovariectomized

LID: Lidocaine

VEH: Vehicle

CNTL: Control

CRL: Contact return latency

BOP: Behavioural observation program

M: Mount

I: Intromission

E: Ejaculation

VMH: Ventromedial hypothalamus

VMHvl: Ventromedial hypothalamus ventrolateral portion

BNST: Basolateral nucleus of the stria terminalis

MEA: Medial amygdala

MEApd: Posteriodorsal MEA

PVN: Paraventricular nucleus

Nacc: Nucleus accumbens

LS: Lateral septum

PirCtx: Piriform cortex

US: Unscented

SC: Scented

T2: Extinction trial

UCS: Unconditioned stimulus

LQ: Lordosis quotient

Me: Amygdala

CS: Conditioned stimulus

Fos-IR: Fos immunoreactivity

General Introduction

Sexual behaviour encompasses a wide variety of sensory experiences for all animals, including humans. These sensory experiences as a whole make sex rewarding and contribute to learning both during sexual development and the development of sexual behaviour and sexual preferences. In spite of the slow but steady increase in the understanding of female genital anatomy, physiology, and function (Figure 1), the relative contribution of individual components that make up those sensory experiences has yet to be fully understood. One area where there has been considerable interest and controversy is the role of the clitoris in sexual arousal and orgasm.

The growing body of literature that focuses on female sexual behaviour in the rat has provided many insights into the role played by genitosensory stimulation in reproduction and reward. Surprisingly, however, rat studies looking at the role of the clitoris in sexual reward and behaviour were never before conducted, despite the fact that rats have a pronounced clitoris (Figure 2) and have been used extensively as models for human sexual function and dysfunction (for review see: Giuliano et al., 2010; Kwangsung, 2006; Pfaus, Giuliano & Gelez, 2007). The goal of this thesis is to investigate the contribution of the clitoris in the experience of female sexual reward and partner preferences through the use of a rat model of clitoral stimulation and sexual behaviour.

The Clitoris in History

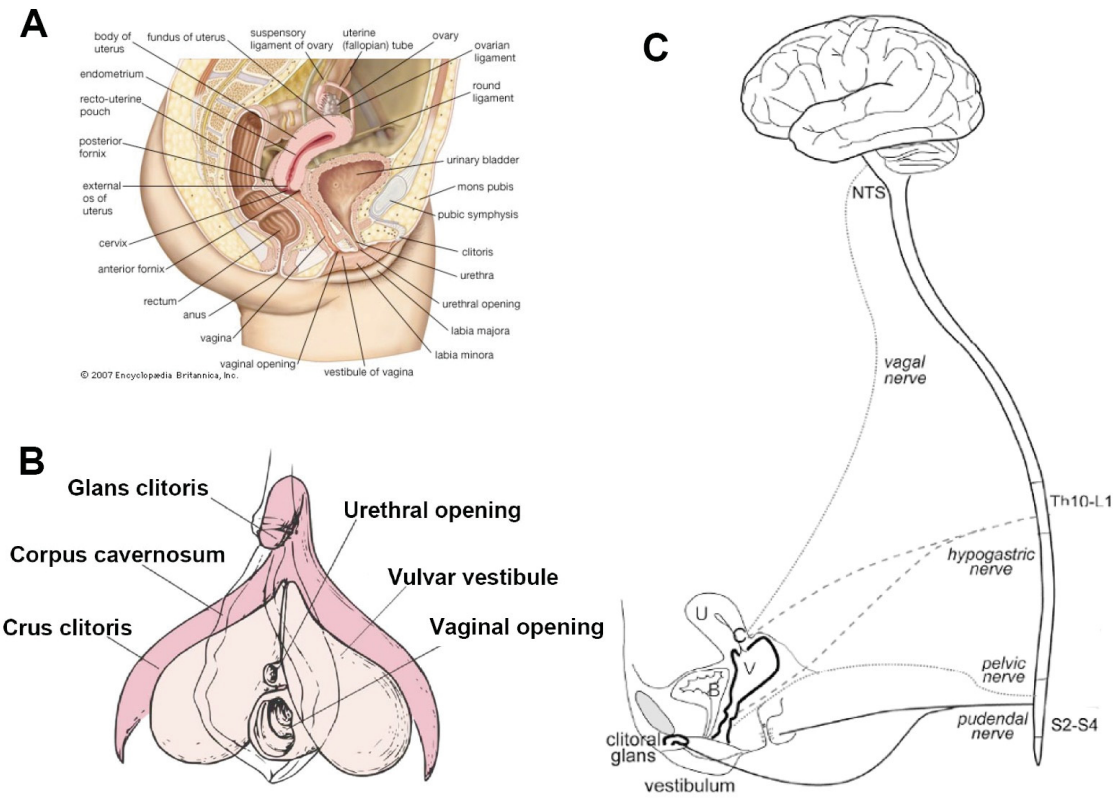
For over 5000 years the clitoris has been described as an organ of sexual pleasure. In spite of this there is still controversy about its existence, its anatomy, and above all its function (Moore & Clarke, 1995; Stringer & Becker, 2010). In the 17th century Regnier De Graaf gave one of the first comprehensive descriptions of

female genital anatomy and emphasized the use of the term “clitoris” in anatomical texts (De Graaf, 1668; Jocelyn & Setchell, 1972). Yet little or no mention is made of the clitoris in more recent anatomical texts, which is in stark comparison to the multiple pages devoted to penile anatomy (Gray, 1918; Sinnatamby, 1999; Standring, 2009). In fact, the exact anatomic description of the human clitoris was first produced in 1998 (O’Connell, Hutson, Anderson, & Plenter, 1998).

Freud (1905) generated a controversy concerning the function of the clitoris when he described clitoral orgasms as “infantile”, differentiating them from vaginal orgasms, which he considered “mature”. Freud’s analysis led to the idea that penile-vaginal intercourse was the most important component of female sexual satisfaction (Freud, 1962), and ignored the anatomical reality that the majority of the human clitoris is internal, making it likely that penile-vaginal intercourse stimulates both internal and external clitoris. Thus, vaginal orgasms are also clitoral orgasms. Kinsey et al. (1953) argued that Freud provided no empirical evidence for his theories and, in fact, embedded in them his own moral values (Kinsey et al., 1953; Pomeroy, 1972). In contrast, Kinsey et al. took the sexual histories of thousands of women and reported that women were not having orgasms from vaginal penetration to the degree that Freud would have interpreted as being “normal” in regards to psychosexual development. The majority of orgasms experienced by women in the Kinsey et al. sample were derived from stimulation of the external glans of the clitoris.

Subsequent laboratory observations made by Masters and Johnson (1966) found that the majority of women could achieve orgasm from clitoral stimulation alone, whereas only a minority achieved orgasm from vaginal stimulation. From these data, they concluded that all orgasms are physiologically the same and that the source originates with stimulation of the clitoris.

Figure 1: Human female genital anatomy and neurophysiology



A: Sagittal section of the pelvic and hypogastric plexus showing sexual and reproductive organs, including clitoris, vagina, cervix, and uterus. B: Anatomy of the clitoris positioned relative to vaginal structures and the urethra. C. Neurophysiology of the glans clitoris, bladder (B), vagina (V), cervix (C), and uterus (U) and their respective innervation by pudendal, pelvic, hypogastric, and vagus nerves.

The findings from Kinsey et al. and Masters and Johnson collided with the prevailing Freudian psychiatric view, but gained momentum socially and politically with the so-called “sexual revolution” of the 1960s. The Feminist movement of the 1960s and 70s viewed the apparent Freudian emphasis on vaginal orgasm as a negative source of influence on women’s feelings about their own bodies and their sex lives as a whole. Hite (1976) gathered thousands of responses from anonymous women regarding gender and sexuality, and dedicated a chapter on clitoral stimulation that focused on clitoral orgasms, rates of clinical orgasms from self-stimulation, and personal feelings about clitoral stimulation. The report revealed that women had a high degree of misunderstanding about their own bodies, held stigmas toward clitoral self-stimulation relative to heterosexual partner stimulation (and vaginal intercourse in particular), and expressed a high degree of dissatisfaction with their sexual experiences. At approximately the same time, the Boston Women’s Collective issued their famous sexual self-help book “Our Bodies Ourselves” (1970) that promoted women’s sexual health and education. The first edition dedicated a section to orgasms and distinctly described Freud’s interpretation of clitoral versus vaginal orgasms as false. It praised Masters and Johnson for disproving Freudian theory, describing yet again that all female orgasms happen in the same way, via the clitoris. Although the importance of the clitoris in orgasm was redeemed, no studies had expressly examined its role in sexual function or orgasm, nor had they quantified the contribution of external manual clitoral stimulation (CLS) relative to vaginocervical (VCS), or mixed clitoral-vaginal stimulation, in the generation of sexual pleasure and/or orgasm.

The most current research posits clitoral stimulation as a main source of sensory input for eliciting sexual pleasure during masturbation and partnered sexual

intercourse and orgasm in women (Mah & Binik, 2001; Goldstein, Graziottin, Heiman, 2004; Giuliano, Julia-Guilloteau 2006). However, studies have not examined the clitoris' contribution to sexual behaviour beyond its role in inducing orgasm. The female rat is a good model for the study of female sexual function because, along with it having a regular estrous cycle, the human and rat vaginas have a similar histologic structure.

Anatomy and Physiology of the Rodent Clitoris

The rat clitoris is virtually identical to that of the human. It is composed of paired corpus cavernosa and a large glans clitoris, which is visible externally as shown in Figure 2. It consists of a cylindrical, erectile organ, internally located between the preputial glans. The body of the clitoris is formed by sponge-like cavernosal tissue, similar to that of the penis, and composed of cavernosal spaces surrounded by a fibrous tunica albuginea, similar to human clitoral structure (Martin-Alguacil, Pfaff, Shelley, & Schober, 2008). The cavernosal space is lined by vascular endothelium and contains bundles of smooth muscle, with the urethra lined by erectile tissue (Martin-Alguacil et al., 2008). Both the clitoris and penis in rats and humans develop from the genital tubercle and are dependent on androgen exposure in utero (Marson, 1995).

From the clitoris to the CNS (afferents)

Sensory neurons arising from the glans of the clitoris form the dorsal nerve whereas a second, deeper nerve arises from the corpus cavernosum clitoridis. These afferents join, and eventually merge, with the pudendal nerve (Martin-Alguacil et al., 2008) that is the primary route through which sensory input from the clitoris in both the rodent and human is transmitted to the central nervous system.

Figure 2: External appearance of the rat clitoris, vagina, and anus.

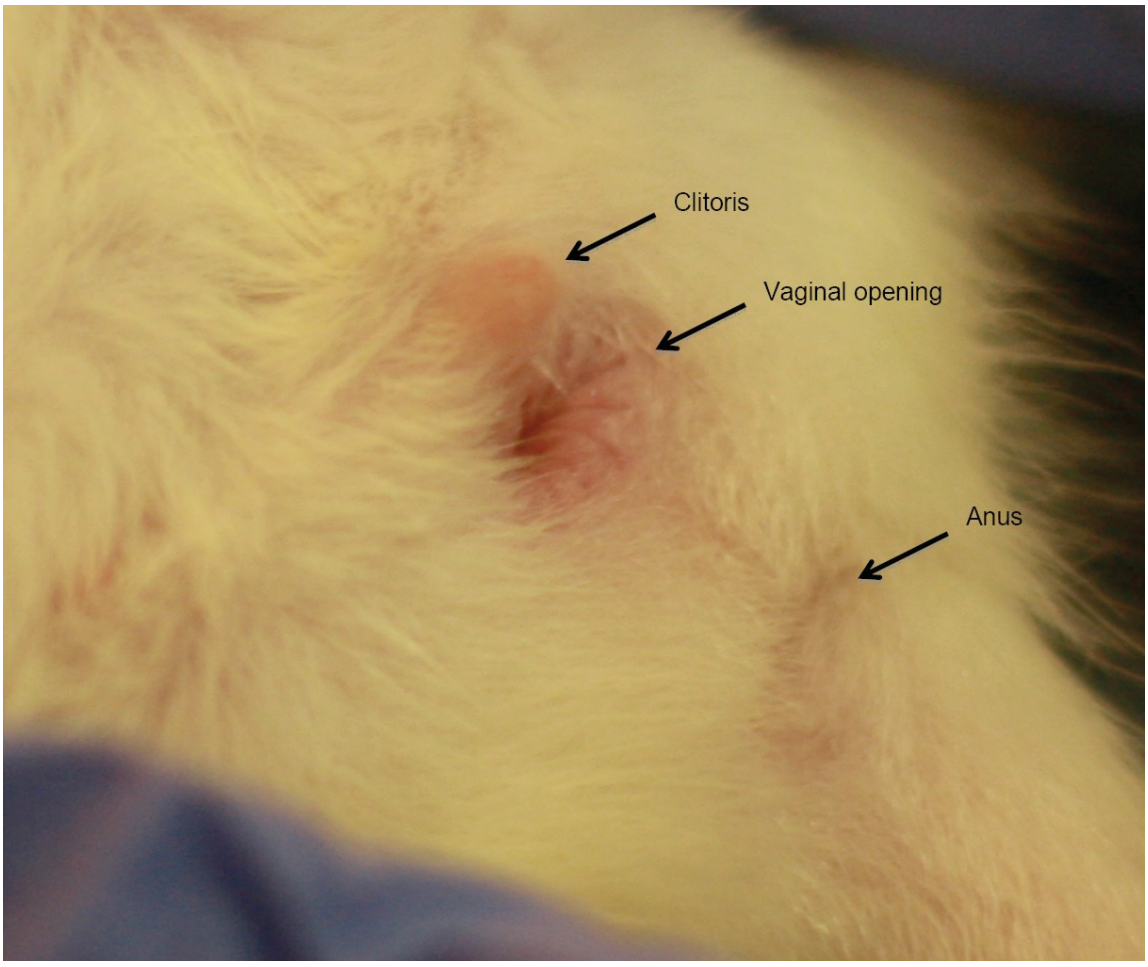


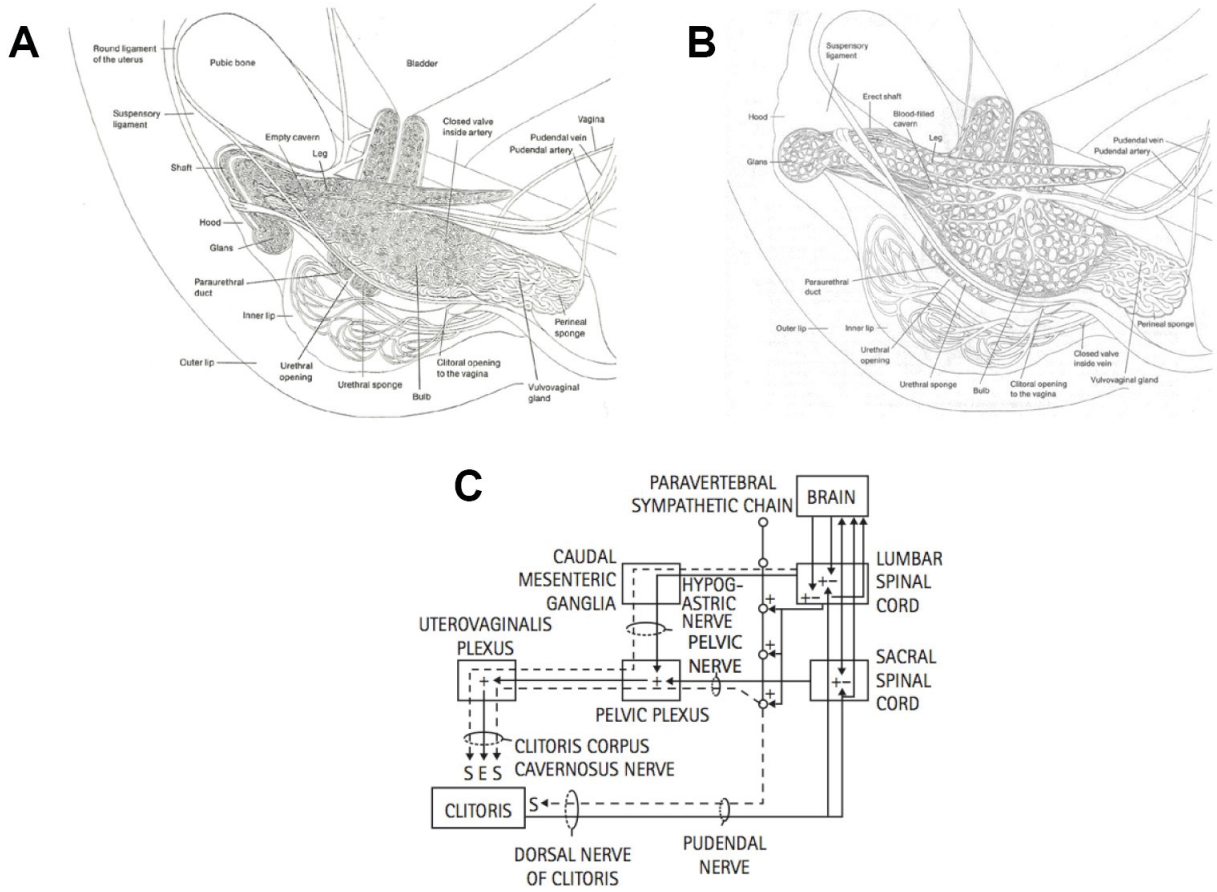
Photo by: M. Parada

In the rodent the pudendal nerve also has branches that are activated by cutaneous receptors in the flanks, posterior rump, perineum, and tailbase, which are analogous to genital regions in humans including the clitoris, perineum, and anus. These sensory fields in the rat are primarily activated by genital investigation and mounting by the male (Kow & Pfaff, 1973, McKenna & Nadelhaft, 1986, Pfaus, Manitt, & Coopersmith, 2006, Cruz et al., 2004). In the rodent these sensory fields overlap considerably and an examination of the contact that male rats make with these fields during mounting and intromissions show that the area surrounding the perineum and including the clitoris appear to be the most highly contacted (Adler, Davis, & Komisaruk, 1977; Pfaff, Montgomery, & Lewis, 1977). Pressure receptors in the vaginal canal, cervix, and uterus activate the hypogastric, pelvic, and vagus nerves when females receive vaginal intromissions. These also connect with sacral and lumbar spinal afferents, which lead directly to the brain (Berkley, Robbins, & Sato, 1993, Carlson & De Feo, 1965, Chinapen, Swann, Steinman, & Komisaruk, 1992, Komisaruk et al., 1996, Pfaus et al., 2006).

Brain areas that receive polysynaptic clitoral innervation have been mapped by injecting trans-synaptic retrograde tracers into the clitoris. These studies have shown staining in the nucleus paragigantocellularis, raphe pallidus, Barrinton's nucleus, ventrolateral central gray, regions of the hypothalamus, and the medial preoptic area (mPOA) (Marson, 1995). These are similar to brain regions activated by penile injections of pseudorabies virus (Marson, Platt, & McKenna, 1993). Whether these areas brain are, in fact, activated by clitoral stimulation remains to be determined. The findings are interesting however, because evidence suggests that these regions are involved in sensory processing for sexual responding. In particular,

mechanical stimulation of the cervix excites neurons in the mPOA (Haskins & Moss, 1983).

Figure 3: Vascular and neurophysiological mechanisms of clitoral arousal



A: non-erect clitoris; B: erect clitoris; C: neurophysiological pathways that underlie rat clitoral erection and sensory innervation. Adapted with permission from Martin-Alguacil, Pfaff, Shelley, & Schober (2007)

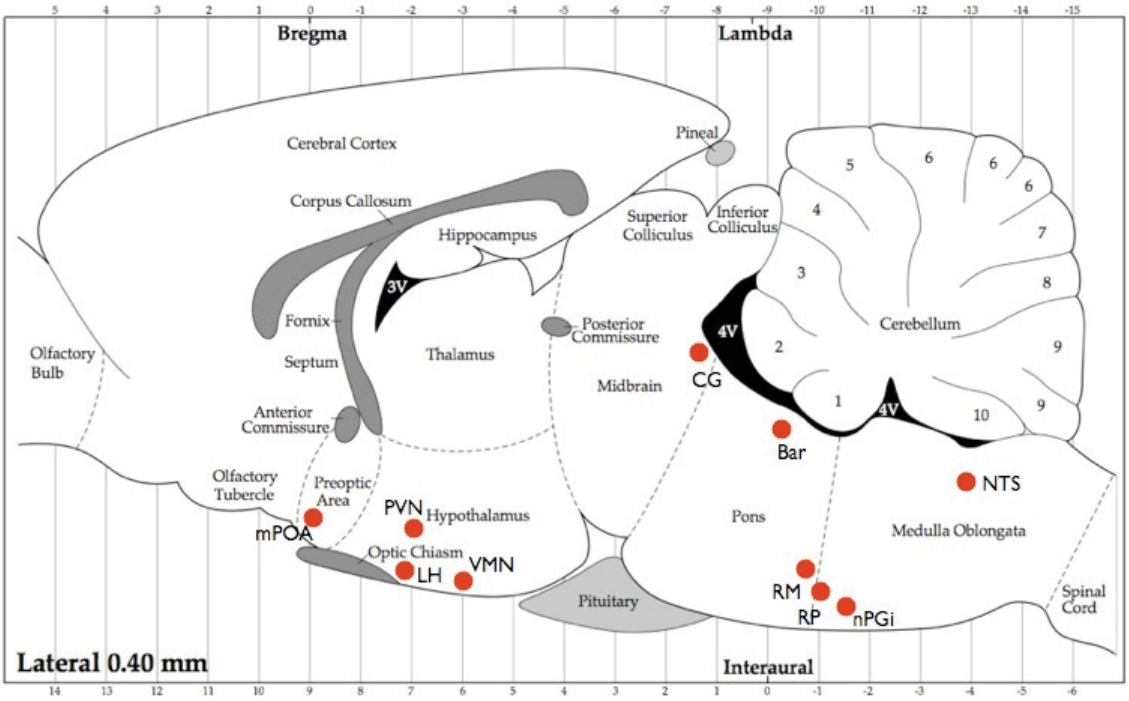
Activation of this region has also been demonstrated in response to VCS from either a male partner during copulation or from artificial manual stimulation (Pfaus, Kleopoulos, Mobbs, Gibbs, & Pfaff, 1993; Pfaus, Marcangione, Smith, Manitt, & Abillamaa, 1996; Tetel, Getzinger, & Blaustein, 1993; 1994; Wersinger, Baum, & Erskine, 1993). Further, bilateral ibotenic acid lesions of the mPOA have been shown to disrupt the development of a conditioned place preference (CPP) for VCS (Meerts & Clark, 2009), which raises the possibility that this is an important region in the circuitry underlying the reinforcing effects of female sexual behaviour. These data emphasize the importance of investigating the response of the mPOA to clitoral stimulation.

From the CNS to the clitoris (efferents)

Although the clitoris is considered to play primarily a sensory role in female sexual behaviour (Marson, 1995), afferent signals from the clitoris are relayed through neurons that activate a feedback loop that causes an erectile response of the clitoris (Figure 3). Clitoral erection, especially of the glans, increases the amount of sensory area that can be stimulated and thus adds to the pleasurable sensations derived from the clitoris during sexual interaction, much like erection of the glans penis increases its sensory field. Study of the mouse clitoris shows that the neural pathway from the brain and the afferent pathways that originate with the pudendal nerve both lead to specific regions in the sacral and lumbar spinal cord that connect with the pelvic nerve and hypogastric nerve (Martin-Alguacil et al., 2008). Both of these nerves feed back to the clitoris corpus cavernosus nerve (see figure 2). Stimulation of this network of nerves results in increased internal pudendal artery blood flow, leading to tumescence and extrusion of the glans; a response similar to

that of males during penile erection (Diederichs, Lue, Tanagho, 1991; (Pescatori et al., 1993).

Figure 4: Brain regions in the rat that receive polysynaptic inputs from the clitoris



Adapted from Marson (1995; 2006). Pseudorabies virus was injected into the clitoris and regions were assessed for transneuronal staining after 4 days.

Morphology in Response to Hormones

Clitoral physiology is altered by different hormonal states in both women and rats (Hall, 1938; Park et al., 2001; Yoon et al., 2001; For review see Giraldi et al., 2004). Early work on female genital tissue examined the effects of different hormones on the morphology of the female rat clitoris (Hall, 1938). Both intact and ovariectomized rats given injections of androstenedione, androstenediol, testosterone, and testosterone proprionate either alone or in combination with oestrone and progesterone show varying increases in clitoral size when examined following 3 weeks of daily treatment (the weakest effect from androstanediol and the strongest by testosterone proprionate) (Hall, 1938). The preputial sac also shows an increase in size and a significant change in the form of the epithelial layer that results in an appearance close to that of the penis; in some cases the epithelium appears even more fully developed than that of the normal penis (Hall, 1938). In vivo animal model studies show that estrogens modulate blood flow in the genitals and are critical for the maintenance of tissue structure and integrity (Park et al., 2001). Mimicking the response that would occur with genital stimulation during copulation by stimulation of the pelvic nerve increases, vaginal lubrication in hormonally intact rats but this response is reduced following ovariectomy and the full response is restored with estrogen replacement (Min et al., 2001). In contrast, treatment of ovariectomized animals with androgen alone or in combination with estradiol does not restore vaginal lubrication to control levels (Min et al., 1990). Removal of the ovaries also causes atrophy of the vagina and reduces epithelial cell maturation and this too is normalized by estrogen but not androgen treatment. As for the clitoris, removal of the ovaries results in clitoral structure alterations examined two weeks following ovary removal. Histological analysis of the tissue shows a decrease in

vascularity (visibility of vasculature) along with atrophy of the tissue. Estrogen replacement after ovary removal restores these changes (Yoon et al., 2001).

Hormonal Control of Female Sexual Behaviour

Notwithstanding the many similarities between the sexual physiology of humans and rodents there is a marked difference in their dependence on hormonal state for sexual behaviour and pleasure. Female rats have an estrus cycle that is approximately 4 days in length. On the Proestrus day of the cycle a peak of estrogen (E) followed by a peak of progesterone (P) levels occur within a 12 hr window of time. Ovulation occurs following the increase in P levels and is followed by an increase in the display of estrous behaviour 4-6 hrs later. During this time females will copulate with available males until they receive a sufficient amount of stimulation that induces pregnancy and sexual reward. Copulation can be observed in the laboratory setting when rats are gonadally intact and the phase of the ovulatory cycle is determined by the morphology of the vaginal epithelial cells (Marcondes, Bianchi, & Tanno, 2002). They can also be observed under hormonally induced conditions in which females are ovariectomized (OVX) and estradiol and progesterone are administered in such a way that the estrus cycle is mimicked (Moreines & Powers, 1977; Powers, 1970; Schwartz & Talley, 1965; Södersten & Hansen, 1977). Ovariectomy prevents impregnation and pseudopregnancy and allows for the experimental control of the periods of estrous behaviour. Estradiol benzoate (EB) will induce a low level of receptivity (they will show the lordosis reflex under EB-alone) without the full range of sexual behaviours. Administration of EB followed by P, 36-48 hours later will induce full lordosis reflexes along with the full display of appetitive behaviours like solicitations, hops and darts, and ear-wiggling (Baum, Södersten, & Vreeburg, 1974). Females will approach and solicit males, and control or “pace” the

rate of sexual contact they receive from males to a preferred rate (Beach, 1976; Erskine, 1989; McClintock, 1984; Pfaus, Smith, & Coopersmith, 1999). Pacing is therefore under the control of ovarian hormones and suggests that sexual reward may be as well. Corona et al. (2011) recently reported that a threshold amount of estrogen appears to be required for pacing to be able to induce a reward state that is strong enough to support a conditioned place preference in the rat (Corona et al., 2011). Since the full range of contact from the male is only possible under a sexually receptive state, penile intromissions and genital stimulation in general can only be received when females are sexually receptive (Erskine, 1989; Pfaff et al., 1977; Pfaus et al., 1999). Because low levels of estrogen produce low levels of receptivity (i.e. little to no lordosis reflex and reduced pacing) this reduces the amount of genital stimulation females can receive and thereby interferes with the development of a conditioned place preference. This suggests that it is not the hormones that are needed for sexual reward but the postures (lordosis) that are elicited by them.

It is currently unknown whether hormones are necessary for the experience of sexual reward through pacing of genital stimulation, including clitoral stimulation, or whether they are simply necessary for the development of a reward state.

In human females the expression of sexual behaviour per se is not dependent on hormonal states, therefore females have the ability to engage in sexual behaviour across the menstrual cycle. It is interesting however, the rates of copulation increase around the time of ovulation when women are most fertile (Puts, Dawood, Welling, 2012). Similarly, self-reported sexual desire in women increase a few days prior to ovulation and peak during ovulation (Stanislaw & Rice, 1988; Zuspan & Zuspan, 1979). Significant increases in orgasm frequency near the ovulatory phase have also

been reported (Puts et al., 2012). This would suggest that hormones have a permissive effect on sexual desire and copulatory behaviour in human females.

Female Pacing, Genital Stimulation, Reward, and Reproduction

The ability of female rats to control or “pace” the rate of copulatory stimulation they receive from males is a critical component of sexual reward and reproduction. Pacing induces a positive hedonic state that supports the development of CPP (Oldenburger, Everitt, & de Jonge, 1992; Paredes & Alonso, 1997; Paredes & Vazquez, 1999). Given the choice of a scented male that was previously paired with paced mating or a non-scented male that was paired with non-paced mating female rats will selectively approach, solicit, and selectively receive intromissions and ejaculations from the males bearing the scent cue; known as conditioned partner preference (Coria-Avila et al., 2006; Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005; Erskine, 2005). Pacing can occur in different contexts that will allow females the ability to solicit and runaway, as in those that provide obstacles for females to be able to run away or engage in defensive behaviours (i.e. bilevel or unilevel paced mating chambers; McClintock, 1984). However, even in environments that do not easily allow pacing, females can enforce paced genital stimulation through defensive responses alone and still induce a rate of stimulation that is rewarding (Meerts & Clark, 2007). This suggests that pacing or the control of copulatory stimulation itself is not necessarily rewarding, but instead it is the distribution of the stimulation at an interval that is preferred that is rewarding (Jenkins & Becker, 2003). It has been demonstrated that 15 distributed manual VCSs given by the experimenter will also induce a reward state in female rats (Meerts & Clark, 2009). This indicates that the presence of the male and the odour cues provided by him are not necessary for sexual reward.

Females that pace the rate of copulatory stimulation have enhanced fertility and higher rates of pregnancy (Adler & Toner, 1986; Edmonds, Zoloth, & Adler, 1972; Erskine, 1985; Frye & Erskine, 1990). Pregnancy or pseudopregnancy (mentioned previously) is induced by threshold amounts of VCS and requires important neuroendocrine changes (Freeman, 1979; 1988; Kornberg & Erskine, 1994; Lehmann & Erskine, 2004; Smith, Freeman, & Neill, 1975). Pacing therefore helps to ensure that females receive the necessary amounts of VCS to induce those neuroendocrine changes required for pregnancy (Adler, 1969). Evidence from other animals indicates that clitoral stimulation may play a role in the rate of pregnancy. Experiments on cows indicate that administration of manual clitoral stimulation following artificial insemination hastened ovulation and resulted in higher rates of pregnancy compared to controls (Randel, Short, Christensen, & Bellows, 1973). This finding however, has not been consistent when compared to studies using other forms of clitoral stimulation, demonstrating that a particular type or particular timing of clitoral stimulation is important for these effects on pregnancy (Cooper, Newman, Schermerhorn, & Foote, 1985). Currently it is not fully known whether other types of genital stimulation received by females during copulation, like clitoral stimulation, could induce a hedonic state strong enough to support the development of a conditioned place or a conditioned partner preference in the rat.

Male rats are attracted to the clitoris, and during sexual interaction will lick around the female's entire anogenital region. Analysis of the chemistry of the clitoral gland secretions in the rat indicates that the clitoris contains several chemical compounds, one of which serves as a sex-attracting chemical (Kannan & Archunan, 2001). Pelvic thrusting from male mounts and intromissions stimulate the female's flanks, rump, tailbase, perineum, and perivaginal surfaces, which include the clitoris

itself (Pfaff et al., 1977). In addition, intromissions consist of several thrusts of the penis into the vagina, which provide VCS. Since the vagina, cervix, and clitoris overlap in the neural circuitry it is possible that this stimulation provided externally through pelvic thrusting during mounts, and mounts with intromissions, generates reward-related sensory inputs through the clitoris itself. We are still learning what clitoral stimulation contributes to the neural circuitry that controls pacing behaviour.

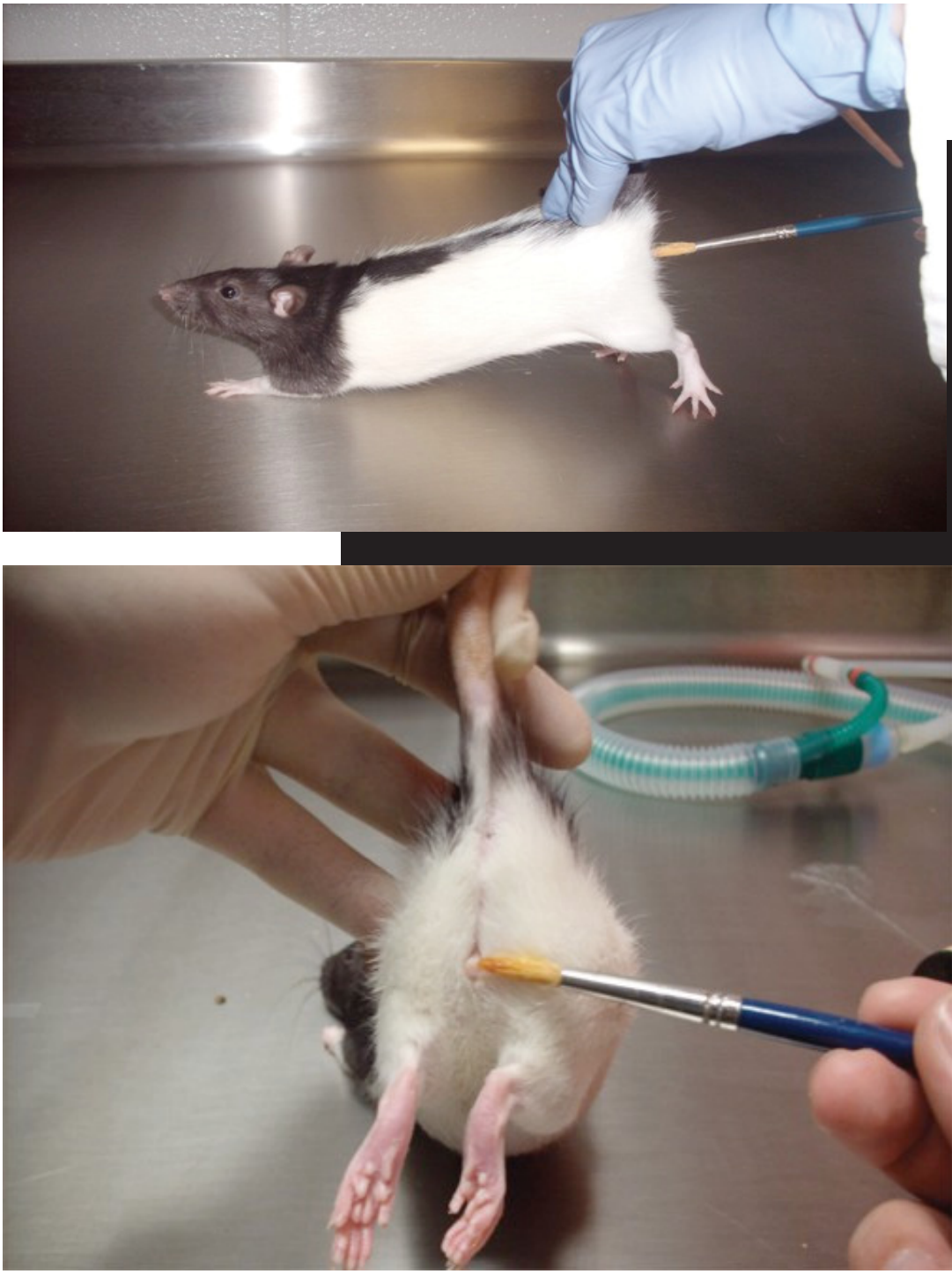
Clitoral stimulation

To study the role of the clitoris in sexual behavior, sexual reward, and reproduction, it was necessary to design a procedure that would provide clitoral stimulation to awake rats under controlled experimental conditions. A procedure was designed based on the artificial vaginocervical stimulation (VCS) procedure used in Pfaus et al. (1993); based on Fos activation in response to differing amounts of (VCS) following an hour of copulation. Clitoral stimulations were made by lifting the base of female's tail and lightly brushing the clitoris with a stroke of a small, soft-bristle number 4 paintbrush dipped in K-Y® Jelly (Figure 5). Two types of stimulations were used to examine the differences between distributed vs. continuous stimulation of the clitoris. The first type of stimulation was one stroke every 5 seconds (CLS5); the second type was one stroke per second (CLS1).

Goals of the present thesis

The aim of this thesis was to examine the role that clitoral stimulation plays in female sexual behaviour and female sexual reward in the rat. The main hypothesis was that the clitoris is an organ of pleasurable sensory input and that it plays an important role in the development and expression of female sexual reward and behaviour. First, it was predicted that clitoral stimulation would contribute to the acquisition and maintenance of typical patterns of pacing behaviour; second, that

Figure 5: Method of experimental stimulation of the rat clitoris



Photos courtesy of: M. Parada

clitoral stimulation would result in the development of reward states powerful enough to establish associations between genitosensory reward and both contextual and partner cues; third, that stimulation of the clitoris would activate neural circuits known to be involved in sexual behaviour; fourth, that hormones mediate the reward experienced from clitoral stimulation; and finally that sexual experience devalues the reinforcing effect of clitoral stimulation.

The first set of experiments investigated whether clitoral anesthesia affected the display of paced mating in female rats and whether any effect of this might be permanent. Lidocaine hydrochloride injections were administered to the clitoral gland prior to several copulatory sessions with a male to prevent any sensory information from being transmitted through the clitoris during sexual behaviour. The behaviour of lidocaine treated females was then compared to females that received saline injections or no injections.

The second set of experiments explored whether clitoral stimulation (CLS) induces a hedonic state strong enough to develop CPP. The second part of this chapter examined the neural sites activated by different types of clitoral stimulation using the expression of Fos protein as a marker of neuronal activation.

The third set of experiments investigated whether the same form of CLS used to develop a significant CPP is rewarding enough to develop a conditioned partner preference. These experiments use a neutral almond odour on an inaccessible male partner paired manual distributed CLS.

The fourth set of experiments tested the hypothesis that estrogen and progesterone are necessary for the development and expression of sexual reward induced by paced mating and CLS.

The fifth and final set of experiments examined whether prior sexual experience devalues the rewarding effect of clitoral stimulation and blocks the induction of CPP to distributed CLS that is normally induced in sexually naive animals. Females in three groups are allowed varying levels of sexual experience with a copulatory partner in a female paced bilevel chamber and subsequently conditioned to distributed CLS in a place preference chamber.

Chapter 1

Clitoral anesthesia disrupts paced copulation in the female rat

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ABSTRACT

In humans clitoral stimulation during sexual contact is associated with pleasure. The present experiment investigated the effect of blocking genitosensory stimulation of the clitoris with lidocaine during copulation in female rats on a measure of female sexual motivation: pacing behaviour. Sexually naïve, ovariectomized female rats were treated with 10ug estradiol benzoate 48h and 500ug progesterone 4h prior to mating sessions with a sexually vigorous stimulus male. Females were given two sets of 5 trials. In the first set, females received an injection (0.05cc) of either 2% lidocaine (LID), saline (VEH), or no injection (CNTL) to the clitoral sheath under isoflurane anesthesia. In the second set, females previously injected with lidocaine were switched to saline and vice versa for another 5 trials. Rats were then placed on one side of a paced mating chamber and allowed to copulate for 30min. Overall time spent with the males, number of solicitations, contact return latencies (CRLs) following male mounts, intromissions, and ejaculations; the frequency of entrances and exits from the male chamber, and frequency of mounts, intromissions, ejaculations, were recorded. At the end of the first trial set, females in the LID group had a greater number of entrances/exits but spent significantly less time in the presence of the male during the copulatory bout than CNTL animals. These females also showed significantly longer CRLs after ejaculations than VEH and CNTL groups. On the 10th trial, LID females switched to vehicle treatment no longer differed from controls in entrance/exit numbers, time spent with males or ejaculation CRLs. We suggest that that clitoral stimulation in the rat serves as both a reward signal and a detector of differences in copulatory stimuli that are critical to pacing and potentially the initiation of pregnancy.

INTRODUCTION

In natural and laboratory environments female rats control the rate of copulatory stimulation they receive from males by means of approach and avoidance behaviours known collectively as “pacing” (Bermant, 1961; Erskine, 1985; McClintock & Adler, 1978). Pacing behaviour increases the likelihood that mating stimulation will initiate luteal function thus enhancing the probability of pregnancy when the female is paired with a fertile male or pseudopregnancy if the male is infertile (Adler, 1969; Adler, Resko, & Goy, 1970; Baumgardner & Dewsbury, 1980; Chester & Zucker, 1970; Terkel & Sawyer, 1978). The ability of females to pace or control the initiation and rate of sexual stimulation also leads to a positive reward state that induces both conditioned place and partner preferences (Oldenburger, Everitt, & de Jonge, 1992; Paredes & Alonso, 1997; Paredes & Vazquez, 1999; Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005; Coria-Avila et al., 2006) and it has been suggested that the rewarding aspects of pacing is associated with its functional consequences (Diamond, 1970).

In general, pacing rate, i.e. the time between solicitations of the male by the female, increases as a function of the specific type of sexual stimulation that she receives. Contact return latencies (CRLs) are highest following ejaculations, which provide the greatest amount of stimulation from deep thrusting, and shortest following mounts, which provide the least amount of stimulation (McClintock, Adler, 1978; McClintock, Anisko, 1982; Bermant, 1961; Gilman, Mercer & Hitt, 1979; Krieger, Orr, Perper, 1976; Pierce, Nuttall, 1961). These data suggest that females differentiate mounts, intromissions, and ejaculations. Intromissions and ejaculations both provide vaginocervical stimulation (VCS), an important component of the genitosensory stimulation that females receive during copulation. Whether supplied

during sexual interactions or manually VCS facilitates lordosis and pacing behaviours, induces analgesia, and facilitates estrus termination (Komisaruk, Diakow, 1973; Diakow, 1975; Erskine, 1989; Rajendren, Dudley, Moss, 1991; Coopersmith, Candurra, Erskine, 1996; Pfaus et al., 2000' Yang, Clemens, 1997). Vaginocervical stimulation also induces the release of luteinizing hormone, oxytocin, and prolactin, which is associated with ovulation and pregnancy (Blake, Sawyer, 1972; Spies, Niswender, 1971; Terkel, Sawyer, 1978). Stimulation from intromissions and manually applied VCS activates pressure receptors in the vaginal canal, cervix, and uterus, which activate the hypogastric, pelvic, and vagus nerves (Berkley, Robins, Sato, 1993; Carlson, DeFeo, 1965; Chinapen, Swann, Steinman, Komisaruk, 1992; Kondo, Shinoda, Yamanouchi, Arai, 1990). Manual VCS alone has been shown to induce a reward state strong enough for the development of conditioned place preference when it is administered at intervals that females prefer (Meerts, Clark, 2009) which highlights its importance in the reward value of sexual stimulation received by females.

VCS is not the only form of tactile stimulation that female rats receive during sexual interaction, however. Stimulation of the flanks, rump, and tail base during male mounts aid in the induction of the lordosis reflex enabling males to gain vaginal intromission and ejaculation. The lordosis posture also allows contact between the lower perineal area of the female and the genitals of the male during male thrusting (Pfaff et al., 1977) and this lower perineal contact stimulates the clitoris and, in turn, the pudendal nerve (Pacheco et al., 1989).

Studies of the effects of clitoral stimulation in humans have largely focused on its function as a relay of pleasurable sensory information (Masters et al., 1986, Giuliano et al., 2002, van Netten et al., 2008) and, more recently, on its influence on vaginal

muscle function (Shafik et al., 2008). In the rat, clitoral innervation and vasculature during sexual arousal has been studied (Peters et al., 1987, Pacheco et al., 1989, Giuliano et al., 2001, Cruz et al., 2004, Hannan et al., 2011) along with its morphology following hormone administration (Hall, 1938; Welsh et al., 2010; Munarriz, 2003; Pessina et al., 2006). Little is known, however, about the contribution of clitoral stimulation to sexual behaviour in female rats.

The present study examined the role of clitoral stimulation in female sexual behaviour in rats through the use of clitoral anesthesia using the local anesthetic agent lidocaine hydrochloride. Females were injected with lidocaine in the clitoral gland immediately prior to copulation with a sexually vigorous male. Female solicitations, pacing, and lordosis behaviours were examined after 5 sessions of Lidocaine treatment and compared to both a vehicle injection group and a no injection control group. A second set of 5 reversal sessions followed during which females in the lidocaine group were given vehicle injections and those in the vehicle group were given lidocaine injections. Sexual behaviours were observed at the end of the 10 sessions to investigate any permanent effects of the anesthetic on sexual behaviour. We hypothesized that blocking sensory information from the clitoris would result in an increase in solicitations and a possible increase in the time spent with the male during the copulatory tests. We also hypothesized that any effect of the anesthetic on sexual behaviour would dissipate on further testing. Finally, we hypothesized that females initially treated with vehicle and given lidocaine during the reversal trials would show an increase in solicitations at the end of the reversal sessions.

MATERIALS AND METHOD

Animals and surgery

Sexually naive female Long-Evans rats, weighing 200 to 250 g, were obtained from Charles River Canada, Inc., St. Constant, QC. Animals were housed in groups of two in shoebox cages in a colony room maintained on a reversed 12:12 h light/dark cycle (lights off at 08:00 h) at approximately 21C. Food and water were continuously available. Females were ovariectomized bilaterally through lumbar incisions under intraperitoneal (i.p.) injections (1 ml/kg of body weight) of ketamine hydrochloride (50 mg/ml) and Xylazine hydrochloride (4 mg/ml) anesthetic mixed in a ratio of 4:3 respectively. All females were given 1 week of post surgical recovery and maintained for the duration of the experiment on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 ml of sesame oil) 48 h and progesterone (P; 500 µg in 0.1 ml of sesame oil) 4 h prior to testing.

Male sex training

Long-Evans males from the same breeder were given 10, 30-min sessions of sexual training with different sexually receptive females prior to testing in order to generate stable baseline rates of sexual responding (n=20 per group). Males were considered good copulators if they mounted females within 15 sec of the presentation of the female.

All animal procedures conformed to the guidelines of the Canadian Council on Animal Care, and were approved by the Concordia University Animal Research Ethics Committee.

Injections and Testing Procedure

Thirty-six females (n= 12 per group) were assigned to one of three groups: LID-VEH; VEH-LID, and CONTROL. Females in the LID-VEH and VEH-LID groups received one injection immediately prior to each testing session based on their group assignment such that females in the LID-VEH group received lidocaine injections

before the first 5 of the 10 trials and vehicle injections for the last 5 trials. Order of injections was reversed for rats in the VEH-LID group and those in the Control group remained uninjected throughout. Females were either given an injection (0.05cc) of 2% lidocaine hydrochloride (Sigma Aldrich Canada) in 0.9% physiological saline (LID), 0.9% physiological saline (VEH), or no injection (CNTL) to the clitoral sheath under Isoflurane anesthesia approximately 5-10 min prior to the test. This time was taken to ensure that females were fully alert at the beginning of the test. All injections were conducted using a standard U-100 (1ml) insulin syringe to reduce pain. A single sexually vigorous stimulus male was placed into one side of the mating chamber approximately 5-10 min before the introduction of the female. Following the injection, females were placed on the opposite side of a paced mating chamber (38 cm H x 60 cm W x 38 cm deep) bisected by a wall containing 4 holes, large enough for the females to pass through but not the males. Under these conditions, females will spontaneously control the contact with the male through the ability to enter or leave the side of the test chamber to which the male is confined (Erskine, 1985). All tests lasted 30 minutes and were recorded for later scoring using the Behavioural Observation Program (BOP) (Cabilio, 1996).

Observational coding

The behavioural measures of interest were the latency and frequency of solicitations (headwise orientation toward the male followed by a runaway, as in McClintock, 1984) including hops & darts, lordosis frequency, male mounts (M), intromissions (I), and ejaculations (E), the frequency of entrances/exits following male behaviour, and the total time spent with each male. Defensive behaviours toward the males were also recorded when females displayed a rearing posture with or without also striking the male with the forepaws to prevent him from mounting

(Barnett, 1963). To examine pacing behaviour the frequency with which each female left the compartment containing the male after receiving a mount, intromission, or ejaculation was expressed as a percentage of the total numbers of each type of stimulus received (percent exits) (Coopersmith, Candura, Erskine, 1996). Secondly, the contact-return latency (CRL) was calculated as the average time taken to return to the male side of the chamber following male contact (mount, intromission, or ejaculation).

Statistical Analysis

Only data from females that satisfied the criteria for sexual receptivity and those that satisfied with the pacing criteria were included in the respective statistical analysis, i.e., females that did not approach or enter the male's side of the pacing chamber and/or females that did not show a lordosis posture in response to male mounts were not included in the analysis. The effects of eliminating clitoral stimulation on sexual behaviour were examined at 3 time points. A one-way between groups ANOVA was used on test 1 to investigate any immediate effects of this manipulation on sexual behaviour. A second ANOVA was run at test 5 to assess the effects of repeated sexual experience in the absence of clitoral stimulation on sexual behaviour. A final ANOVA was run at test 10 following the reversal sessions to examine whether local anaesthesia of the clitoris had permanent effects on sexual behaviour and whether sexual experience blocked the effects of lidocaine. Any statistically significant effects were further examined using a Tukey test for pairwise comparisons. Effect sizes are reported using eta squared.

RESULTS

Phase 1

All the females satisfied the criteria for sexual receptivity and thus were included in the analysis for receptive behaviour however any females that did not cross into the male's side of the chamber were not included in the analyses for pacing behaviour.

Acute effects of lidocaine on female sexual behaviour

There were no significant differences on either proceptive or receptive behaviours among the groups on the first test suggesting that lidocaine injections to the clitoris had no immediate effect sexual behaviour (Data not shown).

Chronic effects of lidocaine on female sexual behaviour

At test 5, rats in the LID-VEH group had a higher number of exits and entrances to and from the male's side of the pacing chamber than females in the other groups (Fig 1). Analysis of these data showed a significant main effect of group ($F(2, 33) = 4.746$, $p = 0.01$, $\eta^2 = 0.47$) and further investigation showed a significant difference between LID-VEH and CNTL groups.

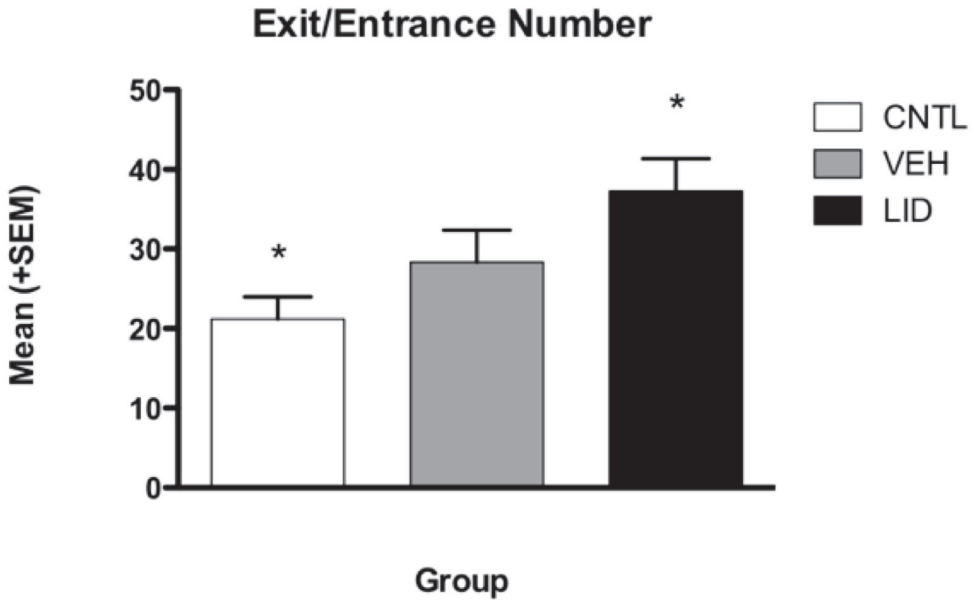

Figure 1.

Mean number (+SEM) of exits/entrances from the male side of the pacing chamber at session 5. Females in the lidocaine (LID) group have a significantly higher number of exits/entrances than females in the CNTL group (N=36)

(*indicates significance $p<0.05$).

Lidocaine also altered the time spent in the male's side of the pacing chamber $F(2, 33) = 3.643$, $p = 0.03$, $\eta^2 = 0.42$. Females in the LID-VEH group spent significantly less time in close proximity of the male compared to the VEH group. A trend towards significance was seen between the LID and CNTL animals (Fig 2).

Females in the LID group had a higher contact return latency following ejaculations compared to the CNTL group. Analysis of these data showed a trend towards significance between the groups at test 5, $F(2, 30) = 3.048$, $p = 0.06$, $\eta^2 = 0.41$ (Fig 3).

Finally, there were no significant differences between the groups in the number of hops/darts, number of solicitations, or latency to solicit at test 5.

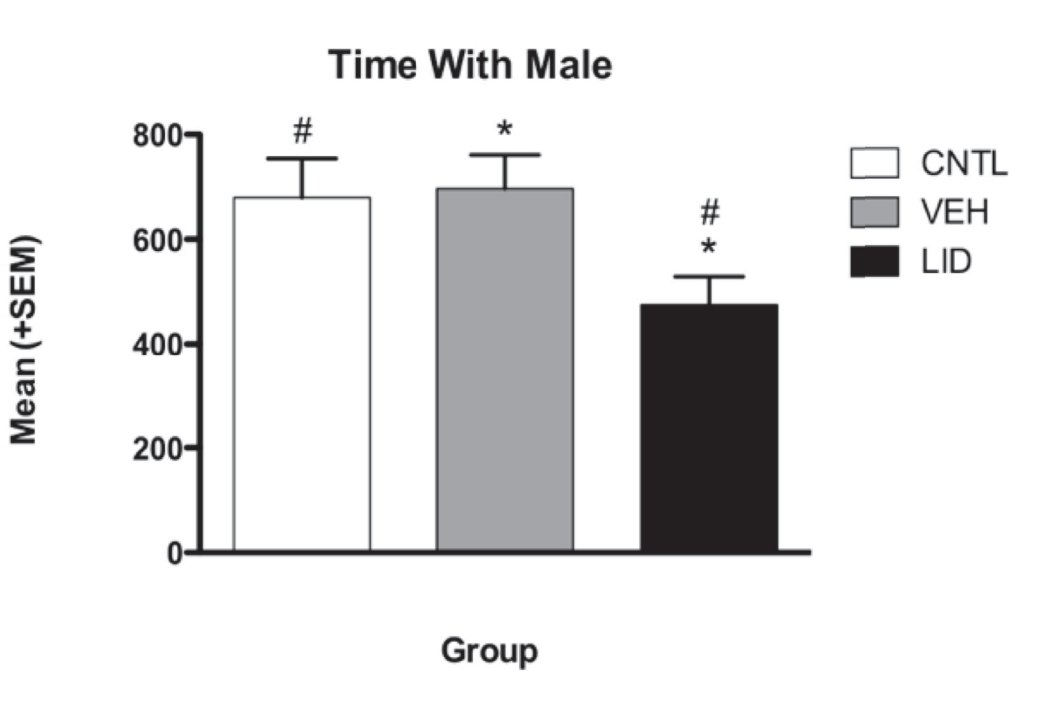

Figure 2.

Mean time (sec) +SEM spent in the male's side of the pacing chamber at test 5. Females in the Lidocaine group (LID) spent less time in close proximity of the male compared to females in the vehicle (VEH) and no injection control (CNTL) group (N= 36)(* indicates significance $p<0.05$, # indicates a trend at $p<0.06$).

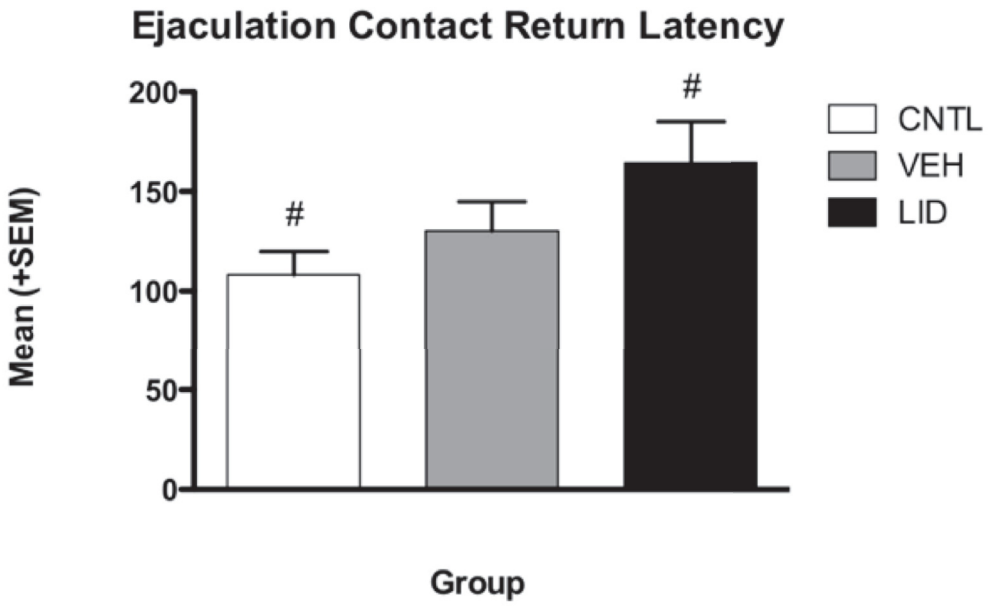

Figure 3.

Mean latency (sec) +SEM for females to return to the male's side of the pacing chamber (ECRL) following an ejaculation at test 5. Females in the lidocaine (LID) group took significantly longer to return to the male's side of the pacing chamber compared to no injection control (CNTL) females (N=33)(*indicates significance $p<0.05$).

Phase 2

Long-term effects of Lidocaine and the effects of sexual experience

Females that had received lidocaine in the first set of sessions and were subsequently reversed to vehicle (lid-VEH) had a higher number of intromissions than the CNTL group. Analysis of these data show a significant effect of group at test 10, $F(2, 29) = 3.704$, $p = 0.04$, $\eta^2 = 0.45$ (Fig 4).

Secondly, females in the lid-VEH group had shorter inter intromission intervals than females in the CNTL group. Analysis of these data also showed a main effect of group $F(2, 28) = 3.392$, $p = 0.05$, $\eta^2 = 0.44$ (Fig 5).

Finally, there were no significant differences in the number of entrances/exits, the total time spent with the male, or contact return latencies at this time point.

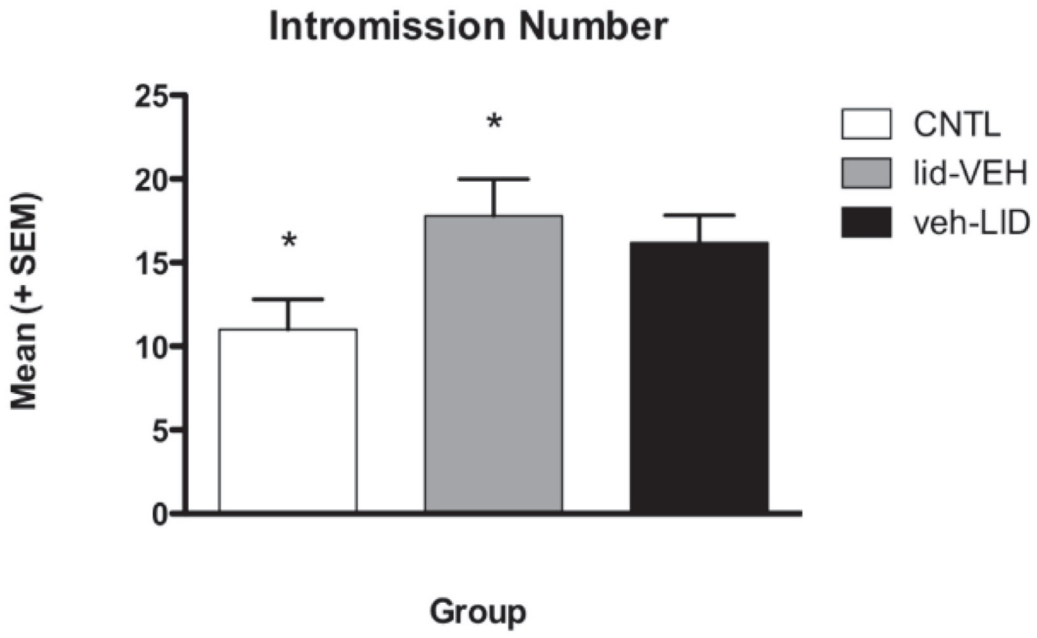

Figure 4.

Mean number (+SEM) of intromissions that females received at test 10 of the reversal sessions. Females previously treated with lidocaine and now treated with vehicle (lid-VEH) had a significantly higher number of intromissions compared to females in the no injection control (CNTL) group (N=32) (*indicates significance $p<0.05$).

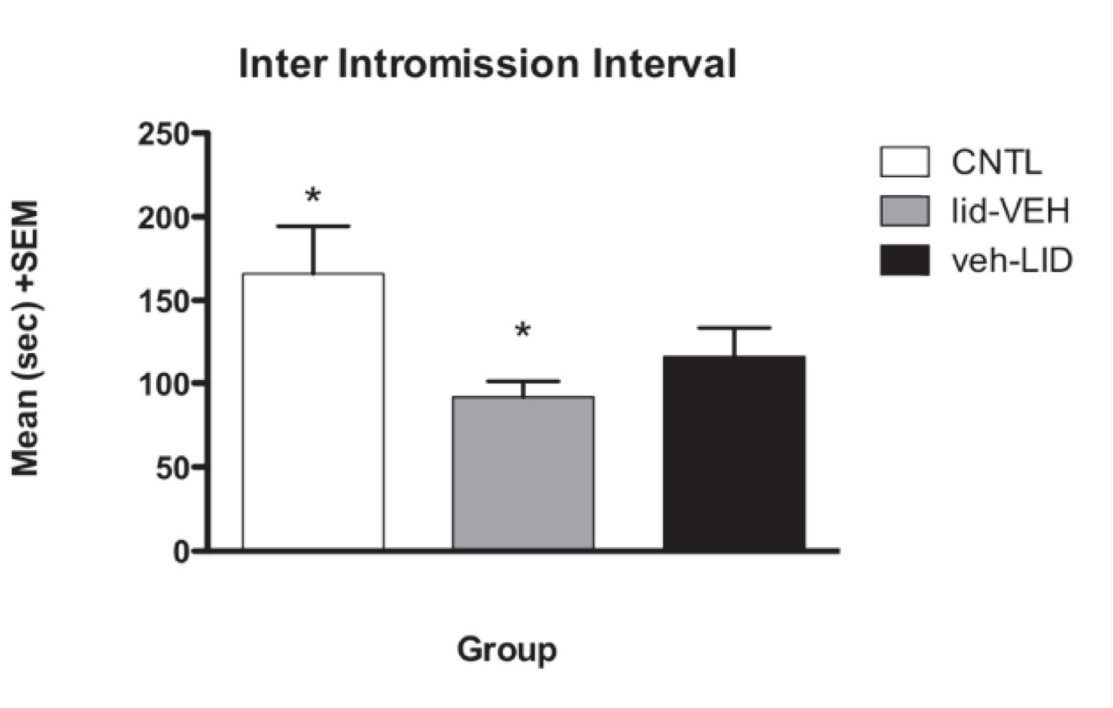

Figure 5.

Mean (sec) +SEM interval between intromissions (III) received by females at test 10 of the reversal sessions. Females previously treated with lidocaine and now treated with vehicle (lid-VEH) had a significantly shorter inter intromission interval compared to the no injection control (CNTL) group (N= 31) (*indicates significance $p<0.05$).

DISCUSSION

The present study examined the effects of clitoral anesthesia on sexual behaviour in the female rat. It was hypothesized that sensory information received from clitoral stimulation by male thrusting and intromissions during copulation is rewarding, and that blocking the reward would result in a disruption of solicitations and pacing behaviours. Although lidocaine injections to the clitoris did not significantly alter solicitations or hops & darts, it induced a higher number of entrances to the male's chamber and reduced the total time spent with the male compared to VEH and CNTL females. These data suggest that reducing clitoral stimulation during sexual interaction changes pacing behaviour, which itself serves as a method of controlling genitosensory stimulation (McClintock, Adler, 1978; McClintock, Anisko, 1982; Bermant, 1961; Peirce, Nuttall, 1961; Erskine 1989). It is also indicative of an overall lessening of reward since females are spending less time in the male's compartment and more time away from the male.

An examination of the individual components of pacing, mainly contact return latencies to mounts, intromissions, and ejaculations, an effect of clitoral anesthesia on the ejaculation CRLs was found. Females in the LID group displayed longer contact return latencies compared to females in the CNTL group, however this difference was no longer present at test 10 following the reversal trials. Given that sensory input from the external portion of the clitoris is blocked during lidocaine treatment, theoretically, internal cervical input would remain intact. Activation of internal cervical input through ejaculations could result in a dampening of the reward input during sexual behaviour, especially through ejaculations, which provide the greatest intensity of sensory stimulation and activate the deeper areas of the cervix.

The effect was temporary since it was subsequently reversed once lidocaine was no longer administered and clitoral sensory input was restored.

Our findings suggest a possible inhibitory effect from cervical stimulation that may be overriding influential input from clitoral stimulation. Injections of lidocaine block neuronal signaling in the sensory neurons of the clitoris and would prevent the transmission of sensory information through the pudendal nerve to the spinal cord. Females in the LID group therefore received a combination of flank, rump, and cervical stimulation without the participation of clitoral stimulation. It has been demonstrated previously that repeated cervical stimulation induces estrus termination in female rats (Pfaus, Smith, Byrne, Stephens, 2000). Artificial VCS is known to increase neuronal excitability within estrogen-concentrating regions of the forebrain, including the ventromedial hypothalamus (VMH), which is inhibited by previous administration of ovarian hormones (Auger and Blaustein, 1995; Pfaus et al., 1993, 1996; Tetel et al., 1993, 1994). This inhibition occurs within glutamate neurons of the VMHvl (Georgescu, Sabongui, Del Corpo, Pfaus, 2009) and coincides with behaviours indicative of estrus termination, analogous to increased pacing responses in the LID females (Pfaus et al., 1999). Contact return latencies increase with increasing stimulation intensity, hence the pattern of CRLs in response to mounts is shorter compared to that of ejaculations (Erskine, 1989; Erskine and Hanrahan, 1997). If sensory stimulation from the clitoris serves as a reward signal for paced genital stimulation a higher CRL to ejaculations in the LID group could be a result of more concentrated inhibitory stimulation to the cervix as a result of blunted reward signal from the clitoris.

The medial preoptic area (mPOA) controls solicitations and pacing, along with other appetitive sexual responses in female rats. Electrolytic lesions of this area that

extend to portions of the basolateral nucleus of the stria terminalis (BNST) result in less social contact with males during a paced mating task and a delay in the time to return to the male's side of a paced mating chamber (Yang, Clemens, 2000). Targeted neurotoxic lesions of the mPOA induce similar effects to those found by Yang and Clemens (2000) on pacing behaviour as well as decreased entrances and exits to the male's side and decreased solicitations, but do not affect lordosis (Hoshina, Takeo, Nakano, Sato, Sakuma, 1994; Guarraci, Megroz, Clark, 2004). Our findings are partly analogous with these data. Females with clitoral anesthesia had longer contact return latencies after ejaculations compared to control females and spent more time away from the males, despite having a higher number of entrances/exits compared to controls.

Further, lesions of the mPOA block the ability of VCS to induce a significant conditioned place preference, suggesting that the altered patterns of pacing behaviour observed following these lesions is attributed to a decrease in the reinforcing effects of VCS (Meerts, Clark, 2009). However, VCS includes the stimulation of a combination of nerves that show a considerable amount of overlap with nerves that serve the clitoris. They are the proximal perineal branch of the sacral plexus innervating the skin between the clitoris and vagina, the viscerocutaneous branch of the pelvic nerve, and the distal perineal branch of the pudendal nerve that innervates the skin lateral to the vaginal opening (Cruz et al., 2004; Kow, Pfaff, 1973; Pfaus, Manitt, Coopersmith, 2006). Overlap of these nerves has been suggested as a mechanism to ensure that somatosensory information necessary to trigger reproduction actually reaches the spinal cord and brain. Stimulation during copulation would activate reproductive and reward-related circuits in the central nervous system simultaneously. Vaginocervical stimulation is known to

activate lumbar and sacral regions of the spinal cord and regions in the brain such as the mPOA, and ventromedial hypothalamus (VMH) (Pfaus, Manitt, Coopersmith, 2006). It is possible that VCS may include some aspects of internal clitoral stimulation, which could stimulate the mPOA and the VMH simultaneously. During copulation, clitoral and cervical stimulation may lead to two different events. First, CLS from male thrusts, mounts, and intromissions activates reward and second, cervical stimulation from intromissions and ejaculations activates estrus termination. This is analogous to male sexual stimulation in that ejaculations lead to both reward and sexual exhaustion.

Our primary findings were observed at trial 5. The number of entrances, time spent in the male's compartment, and ejaculation CRLs for females in the LID group were no longer significantly different from VEH or CNTLs at trial 10 indicating a reversal of clitoral anesthesia in phase 2. Thus, sensory input from the clitoris during the development of sexual experience is important for the crystallization of pacing given that the return of clitoral sensory input reversed the effect on pacing behaviour. Females initially treated with vehicle and reversed to lidocaine were not significantly different from controls at test 10. This indicates that sexual experience de-values the reinforcing effect of clitoral stimulation and blocks the ability of lidocaine to induce its effect on pacing in females. Similar effects have been observed in male rats given penile anesthesia with lidocaine (Pfaus et al., 2012). Males received either 1,2,3,4,5, or 10 prior sexual experiences to one ejaculation before the application of topical lidocaine or saline to the penis. Those with 10 prior sexual experiences showed no significant effect of penile anesthesia on the proportion of mounting, intromitting, or ejaculating compared to controls. Sexual experience has also been shown to protect against the disruptive effects of castration (Lisk & Heiman, 1980), anosmia (Thor &

Flennelly, 1977), and penile deafferentiation (Lodder, 1975). Thus, sexual experience in female rats results in patterns of pacing behaviour that become relatively fixed and independent of genital stimulation.

In phase 2, females that were reversed from lidocaine to vehicle displayed significantly shorter inter intromission intervals and received a significantly greater number of intromissions compared to controls. However, these measures were not significantly different from females that were reversed from vehicle to lidocaine, although the pattern of behaviours was in the same direction. Inter-intromission interval patterns are typically longer during paced compared to non-paced tests and the number of paced intromissions that are necessary for estrus abbreviation is typically around 10 (Erskine, Kornberg, Cherry, 1989) which is the number of intromissions received by the CNTL females. The decreased inter intromission intervals and increased number of intromissions in the lid-VEH animals once again shows a significant difference in pacing strategy. However, in this phase, the difference may be a result of clitoral over sensitivity or simply a compensation for the lack of CLS caused by phase 1 treatment. The dampening effect on reward that occurred at test 5 in the LID females was demonstrated in part by decreased overall time with males. At test 10, following reversal to vehicle, females increased their contact with the males allowing a greater number of intromissions and a shorter period between those intromissions. This is likely indicative of their restored clitoral input, which has to be adapted into their pacing strategy. This provides further support that clitoral sensory input is reward-related and important to the development of pacing behaviour in females.

In summary, our data demonstrate that the clitoris transmits important genitosensory information from male copulatory contact. First, the enhancement of

ejaculation contact return latencies after clitoral anesthesia suggests that normal clitoral stimulation may oppose the inhibitory actions of extended cervical stimulation after ejaculation, possibly by providing a reward signal that maintains the activation of neural systems involved in sexual excitation. Second, we demonstrate that clitoral sensory input is important for pacing behaviour and may affect the likelihood of pregnancy given that females spend more time away from males and show longer contact return latencies to ejaculations. Finally, that sexual experience in female rats serves as a protective factor against elements that disrupt sexual behaviour and that input from the clitoris is important for the crystallization of pacing behaviour, given that sexually experienced females in the VEH group were not affected by lidocaine treatment in phase 2. The mPOA and VMH may form an important convergence system for the interpretation of genitosensory information coming from the clitoris and vagina and cervix for both reward and estrus termination.

SUMMARY OF CHAPTER 1

In chapter one we examined the effect of blocking genitosensory input from the clitoris on the sexual behaviour of sexually receptive female rats. Clitoral anesthesia resulted in impairments on both pacing behaviours such as exit/entrance number and ejaculation CRL but also on the overall time spent with males in the paced mating chamber compared to controls. These impairments were present only for females that were initially treated with lidocaine during the first phase of testing and reversed to vehicle during the second phase. Second, females that showed these impairments from lidocaine treatment were no longer different from controls at the end of the second phase following reversal to vehicle. Third, females initially treated with vehicle during the first phase and reversed to lidocaine during the second phase showed no impairments in sexual behaviours compared to controls at the end of the second phase. Based on these findings we can conclude that the clitoris is involved in detecting sensory information during copulatory behaviour that is utilized to regulate sexual contact with males and that clitoral sensory input is especially necessary during the development of pacing behaviour when females are essentially sexually inexperienced. It can also be concluded that clitoral sensory input while gaining sexual experience may act as a reward signal given that without it, females will still show a motivation to copulate but will spend less overall time in close proximity to their male counterparts indicating a dampening of the reward value of sexual contact. To examine this further it is necessary to assess the reward value of clitoral stimulation on its own and whether areas of the brain typically involved in regulating sexual behaviour, genitosensory information, incentive salience and reward are activated differentially to clitoral stimulation.

Chapter 2

Clitoral Stimulation Induces Conditioned Place Preference and Fos Activation in the Rat

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ABSTRACT

The present study examined the ability of clitoral stimulation (CLS) to induce conditioned place preference (CPP) and Fos protein in the brain. Ovariectomized, hormone-primed Long-Evans rats were randomly assigned to receive either distributed CLS (1 stimulation every 5 seconds for 1 minute prior to being placed in one distinctive side of a nonbiased CPP box for 2 min, after which the cycle of stimulation and CPP exposure were repeated for 4 more cycles, totaling 60 stimulations) or continuous CLS (1 stimulation per second for 1 min with 2 min in one side of the CPP box, repeated for 4 more cycles, totaling 300 stimulations). Two days later, females were placed into the other side of the CPP box without prior stimulation. CPP was tested after 5 sequential exposures each of CLS and no stimulation. Females given distributed stimulation developed a significant CPP whereas females given continuous stimulation did not. CLS induced Fos in hypothalamic and limbic structures, including the nucleus accumbens, piriform cortex, arcuate nucleus, and dorsomedial portion of the ventromedial hypothalamus, compared to no stimulation. However, distributed CLS induced more Fos in the medial preoptic area than continuous CLS or no stimulation. In contrast, continuous CLS induced more Fos in the posteroventral medial amygdala compared to no stimulation. These data indicate that CLS induces a reward state in the rat and a pattern of Fos activation in regions of the brain that process genitosensory input, incentive salience, and reward.

INTRODUCTION

The ability of female rats to control or “pace” the initiation and rate of copulation supports the development of a positive hedonic state that results in a conditioned place preference (CPP; Oldenburger et al., 1992; Paredes and Alonso, 1997; Paredes and Vazquez, 1999). Female rats will also selectively approach, solicit, and receive intromissions and ejaculations from males bearing an odour (e.g., almond) or strain cue (pigmented or albino) associated with paced copulation (Coria-Avila et al., 2005; 2006). Both conditioned place and partner preferences are blocked by treatment with the opioid receptor antagonist naloxone during conditioning (Coria-Avila et al., 2008; Paredes and Martinez, 2001) whereas the dopamine antagonist flupenthixol blocks the development of conditioned partner preference for an odor, but not for strain cues (Coria-Avila et al., 2008) or conditioned place preference in female rats (García Horsman & Paredes, 2004). Naloxone infusions to regions of the hypothalamus medial preoptic area (mPOA) or ventromedial hypothalamus (VMH) or limbic system medial amygdala (MEA) but not nucleus accumbens, block the development of pacing-induced CPP (García Horsman et al., 2008), indicating that opioid actions within those structures play an important role in pacing-induced sexual reward.

Despite knowledge of the putative neurochemical mechanisms involved in linking place or partner cues to sexual reward in female rats, very little is known about the role of sensory stimulation received during copulation in this regard. Such stimulation could be olfactory or genitosensory in nature, and could arise from simple exposure to males or from the distributed stimulation received during each paced (or otherwise controlled) mount with pelvic thrusting and intromission from the male. Indeed, the development of significant CPP for copulation can occur in female rats in

conditions where pacing is not optimized (Meerts & Clark, 2007), suggesting that pacing or control per se is not necessarily the rewarding element in copulation, but rather the distribution of stimulation at some preferred interval that is rewarding (Jenkins & Becker, 2003). Moreover, one recent study showed that 15 distributed artificial vaginocervical stimulations (VCSs) induced a reward state in female rats (Meerts & Clark, 2009), indicating that olfactory or visual cues provided by the presence of a male are not necessary for sexual reward. It is not known whether other types of genitosensory stimulation received by female rats during copulation could support the development of a sexual CPP, or whether electrical stimulation of the nerves that innervate the genitosensory tract (pelvic, pudendal, hypogastric, vagus), might stimulate a reward state.

The clitoris is an important organ for genitosensory stimulation. In women, the clitoris is regarded as a gland that relays localized pleasurable sensory input (e.g., Giuliano et al., 2002; Masters et al., 1986; van Netten et al., 2008) and influences vaginal muscle function (Shafik et al., 2008). It has been studied in rats with regard to its innervation and vasculature during sexual arousal (Peters et al., 1987; Pacheco et al.,1989; Giuliano et al., 2001; Cruz, et al., 2004) and its morphology in response to hormones (Hall, 1938; Munarriz et al., 2003; Pessina et al., 2006). However, its role in carrying sensory information during sexual activity in the female rat is currently unknown. However, the lordosis posture of the female facilitates the male's ability to mount with pelvic thrusting and intromission. This interaction stimulates the female's flanks, rump, tailbase, perineum, and perivaginal surfaces, which include the clitoris (Pfaff, Montgomery, Lewis, 1977), in addition to allowing the male to make penile intromission, which provide VCS. It is thus possible that clitoral stimulation in

the rat could carry reward-related sensory inputs that are generated externally from the pelvic thrusting of the male during a mount and mounts with intromission.

In addition to CPP, paced copulation with intromission, or artificial distributed VCS, induces a faster termination of estrus (Coopersmith et al., 1996; Erskine and Baum, 1982; Lodder and Zeilmaker, 1976; Pfaus et al., 2000) and pseudopregnancy (Coopersmith and Erskine, 1994; Erskine et al., 2004; Lehmann and Erskine, 2004; Terkel, 1986). VCS activates the immediate-early gene product Fos in lumbar and sacral regions of the spinal cord, and in hypothalamic and limbic regions of the brain such as mPOA, posteriodorsal MEA (MEApd), bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN), and VMH (Dudley et al., 1992; Erskine 1993; Pfaus et al., 1993; 1996; Pfaus and Heeb, 1997; Tetel et al., 1993; Wersinger et al.,1993). These regions also show differential activation in response to distributed VCS under different hormone priming regimens (Pfaus et al., 1996). Olfactory stimuli associated with paced copulation also induce Fos in those brain regions (Coria-Avila and Pfaus, 2007). One study using transneuronal tracing following pseudorabies virus injections to the clitoris has shown afferent input to lumbar and sacral spinal cord, and regions of the hypothalamus such as mPOA, PVN, and VMH (Marson, 1995), suggesting that clitoral stimulation (CLS) and VCS could carry a complex array of information relevant for reward and reproduction.

Given the lack of information on the functional role of the rat clitoris in sexual behaviour, the present study asked whether different types of CLS (continuous vs. distributed) could induce a reward state of sufficient intensity to induce CPP. We also examined where those two types of CLS induced Fos within hypothalamic and limbic structures previously shown to be activated by VCS or paced copulation.

MATERIALS AND METHODS

Animals and Surgery

Sexually naive female Long Evans rats, weighing 200 to 250 g, were obtained from Charles River Canada, Inc., St. Constant, QC. Animals were housed in groups of two in shoebox cages in a colony room maintained on a reversed 12:12 h light/dark cycle (lights off at 08:00 h) at approximately 21^{o} C. Food and water were continuously available. Females were ovariectomized bilaterally through lumbar incisions under intraperitoneal (ip) injections (1ml/kg of body weight) of ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4 mg/ml) anesthetic mixed in a ratio of 4:3 respectively. All females were given one week of post surgical recovery and maintained for the duration of the experiment on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 ml of sesame oil) 48 hours and progesterone (P; 500 µg in 0.1 ml of sesame oil) 4 hours prior to testing.

Long Evans males from the same breeder were given 10 prior sexual experiences with sexually receptive females to generate maximal baseline rates of sexual responding (n=20 per group).

All animal procedures conformed to the guidelines of the Canadian Council on Animal Care, and were approved by the Concordia University Animal Research Ethics Committee.

CPP apparatus

Conditioning was conducted in 8 identical tri-coloured PVC plastic rectangular boxes (21 x 21 x 68 cm), each containing three chambers separated by guillotine doors (Med-Associates Inc; St. Albans, VT). The two large end chambers were separated by a smaller center choice chamber, which was used on the pre-exposure

and test days. One of the main chambers had black walls and a wire mesh floor; the other had a white wall located across from the guillotine door and a stainless steel rod floor with larger spaces. All floors were raised 5 cm to reduce the accumulation of urine and feces. Through the use of a computer interface, time spent in each chamber was recorded by means of infrared beam crossings. In each of the two end chambers, there were two beams separated by 8 cm. A rat was considered to be in an end chamber if the beam furthest from the door was broken. If only the beam closest to the door was broken, the rat was said to be in the center choice chamber. During conditioning and testing, the room was kept in semidarkness with only a single lamp reflecting light off one wall of the room.

Conditioning procedure

All conditioning and testing was conducted at 4-day intervals, 4 hr after P injections, during the middle third of the rats' dark circadian cycle (as in Pfaus, Smith, Coopersmith, 1999). The place conditioning procedure consisted of three phases: pre-exposure, conditioning, and a final CPP test. During the pre-exposure phase, each female was placed in the center choice chamber of the CPP box with the guillotine doors removed to allow access to the entire apparatus for 15 min. The amount of time spent in each chamber was monitored and used to assess unconditioned preferences.

During the conditioning phase, females were randomly assigned to one of three stimulation conditions. The first group (n = 20) received continuous clitoral stimulation (CLS 1). CLS was applied once per second over the course of 1 min, after which females were placed into the previously non-preferred side of the CPP box for 2 minutes, after which they were removed and given CLS again. The cycle of CLS for 1 min and placement into the CPP apparatus for 2 min was repeated for a

total of 5 cycles, which totaled 300 stimulations. A second group (n = 20) received distributed CLS (CLS 5). This consisted of 1 CLS every 5 seconds for 1 min, after which females were placed in the previously non-preferred side of the apparatus for 2 minutes. The cycle of CLS and placement into the CPP apparatus was repeated for a total of 5 cycles, which totaled 60 stimulations. A third group (n = 19) received sham (CNTL) clitoral stimulation prior to placement into the CPP apparatus. Each of the conditioning sessions lasted a total of 15 min per animal. On alternate days females received general handling stimulation, but no clitoral stimulation, and were placed into the previously preferred side of the CPP apparatus.

Clitoral stimulations were performed by the experimenter; the stimulations were made by lifting the base of the female's tail and lightly brushing the clitoris with one stroke of a small, soft-bristle number 4 paintbrush dabbed with K-Y® jelly. As with manually applied stimuli, the intensity was likely not exactly the same every time; however care was taken to avoid brushing too strongly and to avoid stimulating the vagina. Sham stimulations were done by lifting the base of the tail but not touching the clitoris. Stimulation parameters were chosen specifically to examine distributed vs. continuous sensory stimulation of the clitoris. The 5-sec interval was chosen on the basis of unpublished observations that fully primed female rats in bilevel chambers receive mounts with pelvic thrusting in bouts with approximately 5 seconds between successive mounts before an intromission is achieved. The 1-sec interval was therefore chosen to provide continuous stimulation relative to the more distributed stimulation that occurs under normal circumstances.

After 10 conditioning sessions (5 reinforced and 5 non-reinforced) a final 15-minute preference test was conducted 24-hours after the last conditioning trial in the same manner as the initial preference test.

Statistical analysis for CPP

Pre-exposure and CPP test outcomes were determined by the total time spent in each chamber. To ensure that there was no initial bias of the chambers, a within-subjects repeated measures ANOVA was used to assess the effect of Chamber. Based on Paredes and Alonso (1997) there were two criteria used to determine whether place preference conditioning was successful. A preference score (time in the reinforced compartment/ (time in the reinforced compartment + time in the non-reinforced compartment) was expected to increase significantly between pre-test and post-test. To account for the possibility that the rats are spending most of their time in the grey (neutral) chamber, a difference score was also calculated (difference between the time spent in the non-reinforced cage and time spent in the reinforced cage) and was expected to decrease significantly after conditioning.

Preference scores and difference scores were analyzed for each stimulation type across pre-test and post-test using a paired-samples t-test to examine the change in scores prior to, and following, clitoral stimulation (Dominguez-Salazar et al., 2005; Meerts & Clark, 2007; Meerts & Clark, 2009). As per Paredes and Alonso (1997) a significant increase in the preference score and a significant decrease in the difference score is the main criteria when a conditioned place preference is developed.

Fos activation by CLS

One week following the CPP experiment the same females were primed with EB and P as above, placed in a Plexiglas cage identical to their home cages (36cm x 26cm x 19cm), and allowed to acclimate for 20 minutes, after which females were randomly re-assigned to one of the three CLS groups described above (to eliminate any previous effects of conditioning on the activation of Fos by CLS). Females

remained in the cage and were sacrificed 1-hour following the initiation of CLS and perfused to prepare tissue for immunocytochemistry.

Histology and immunocytochemistry

All rats were sacrificed by overdose of sodium pentobarbital (120mg/kg, i.p.) and perfused intracardially with phosphate buffered saline (250ml) and followed by 4% paraformaldehyde in 0.1M phosphate buffer (250ml). Brains were extracted and placed in fresh 4% paraformaldehyde for 4-hours and then overnight in 30% sucrose at 4^{o} C.

Frozen coronal brain sections (40 µm) were cut from each brain through the medial prefrontal cortex and to the level of the ventral tegmental area on a cryostat. The sections were washed in cold Tris-buffered saline (TBS) and incubated sequentially with 30% hydrogen peroxide (H_2O_2) in TBS for 30-min at room temperature with 3% Normal goat serum (NGS) in 0.05% Triton-TBS for 90-min at 4-degrees C, with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72-h at 4-degrees C, with biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200) in 0.05% Trition-TBS with 3% NGS for 1-hour at 4-degrees C, and avidin-biotinylated-peroxidase complex (Vectastain ELITE ABC KIT, Vector Laboratories Canada; diluted 1:55) for 2-hours at 4-degrees C. Sections were washed in TBS (3x5 min) between each incubation. Immunoreactions were stained by sequential treatments at room temperature with 50mM Tris for 10-min, 3,3'-diaminobenzidine (DAB) in 50-mM Tris (0.1ml of DAB/Tris buffer, pH 7.8) for 10-min, and 8% nickel chloride (400 µl per 100ml of DAB/Tris buffer + H_2O_2). Sections were then mounted on gel-coated slides and allowed to dry, then dehydrated in alcohols (70%, 90% and

100%, 10 min each, respectively), cleared in Xyline (2 hrs), coverslipped, and examined under a Leitz light microscope.

Histological and statistical analyses

Tissue sections were examined at 40X, and the number of Fos-positive cells was counted bilaterally for each region from 3 sections per area per rat using Scion Image 1.63. The regions were defined by using the atlas of Paxinos and Watson (1986): mPOA (medial preoptic area), VMH (ventromedial hypothalamus), MeA (medial amygdala), BLA (basolateral amygdala), NAcc (nucleus accumbens), VTA (ventral tegmental area), PirCtx (piriform cortex), LH (lateral habenula), BNST (basal nucleus of the stria terminalis), ArcN (Arcuate Nucleus). A mean was calculated for each area in each rat from the 3 bilateral sections per area, and statistical analyses was conducted for 5 rats in each stimulation group (15 sections per group for each brain area, as we have done previously for Fos induction by VCS; Pfaus et al., 1993; 1996). A between-subjects analysis of variance (ANOVA) was performed to assess differences in Fos induction between females that received CLS 1, CLS 5, and sham stimulation. For each significant ANOVA, post hoc analysis of mean differences was made using the least significant difference (LSD) method, $p<0.05$.

RESULTS

CPP associated with manual clitoral stimulation

Rats that received CLS 5 (distributed CLS) showed both a significant increase in preference scores at the post-test following the conditioning trails ($t(19) = -3.394$, $p<0.05$) and a significant decrease in difference scores ($t(19) = 3.213$, $p<0.05$) (Fig. 1). Rats that received CLS 1 (continuous CLS) or sham stimulation did not show any significant change in preference or difference scores.

Fos induction by CLS

Examination of brain sections from the medial prefrontal cortex back to the level of the ventral tegmental area of the midbrain revealed clusters of Fos in areas associated with tactile genitosensory stimulation. Specifically Fos was found in and around the mPOA, VMH, MEA, BLA, NAcc, VTA, PirCtx, LH, BNST, and ArcN (see Table 1). Although clusters of Fos were present in these areas only the mPOA and the MEApv had significantly different levels of Fos in response to the type of CLS received.

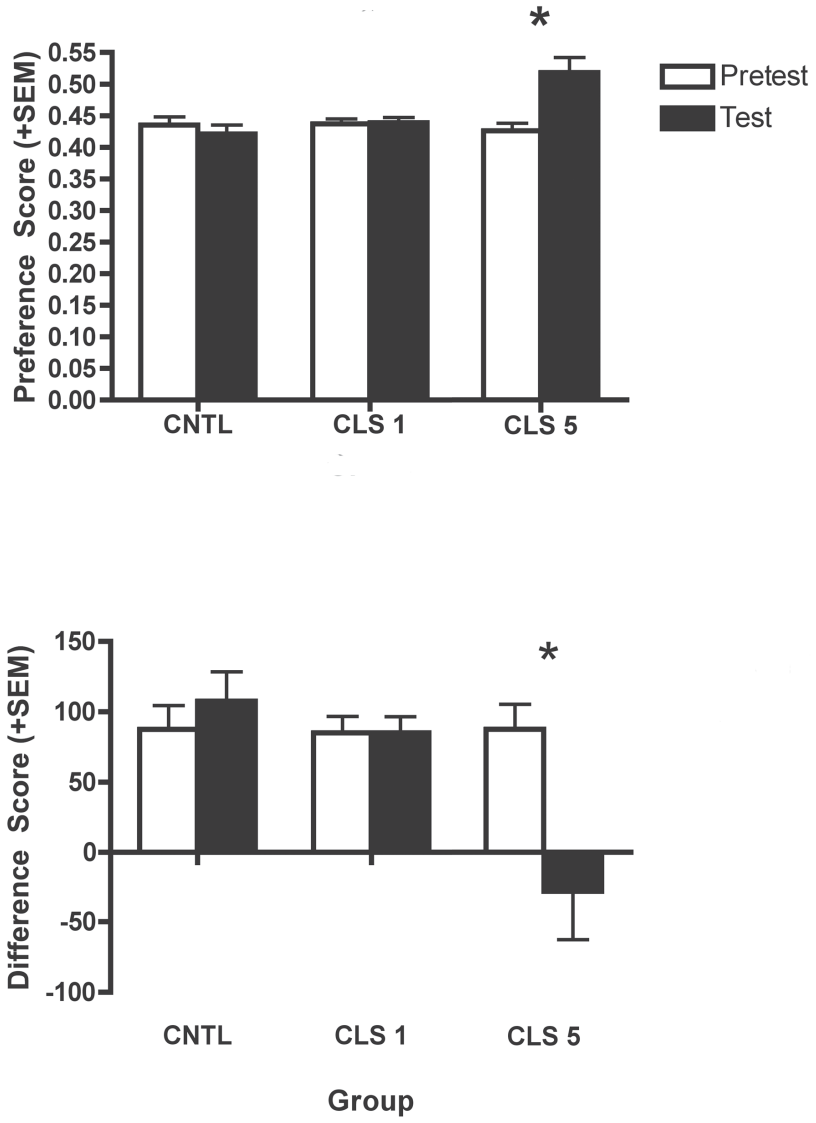

Figure 1.

Mean + SEM preference scores (top) and difference scores (bottom) on the pre-test (pre-conditioning, white bars) and test (post-conditioning, black bars) for female rats that received sham stimulation (CNTL), continuous stimulation (CLS 1), or distributed stimulation (CLS 5) (n=20 per group). Rats receiving distributed stimulation displayed CPP for the compartment associated with that treatment. This coincides with a significant decrease in difference score, *$p<0.05$.

Fos in the mPOA

In the mPOA (Fig. 2), distributed CLS produced a clustered pattern of activation located around the medial, central, and most ventral aspect of the medial preoptic nucleus, with a scattering of induction in the medial, dorsal aspect of this nucleus. This pattern of activation was not induced by continuous or sham CLS. For the induction in the mPOA, the ANOVA detected a significant main effect of CLS, $F(2,15) = 19.388$, $p<0.0001$. Post-hoc comparisons revealed that only distributed CLS induced Fos over that observed in the continuous CLS and control groups (Fig. 3).

Fos in the MEA

In the MEA (Fig. 2), both distributed and continuous CLS induced a cluster of Fos activation located within the ventromedial portion of the MEApv only, with scattered Fos cells radiating laterally away from this cluster. Little to no Fos cells were observed in other regions of the MEA including the MEApd. For the induction in the MEApv, the ANOVA detected a significant main effect of CLS, $F(2, 15) = 5.104$, $p<0.05$. Post-hoc comparisons revealed that continuous CLS produced a significantly greater amount of Fos activation in the MEApv compared to the control group (Fig. 3).

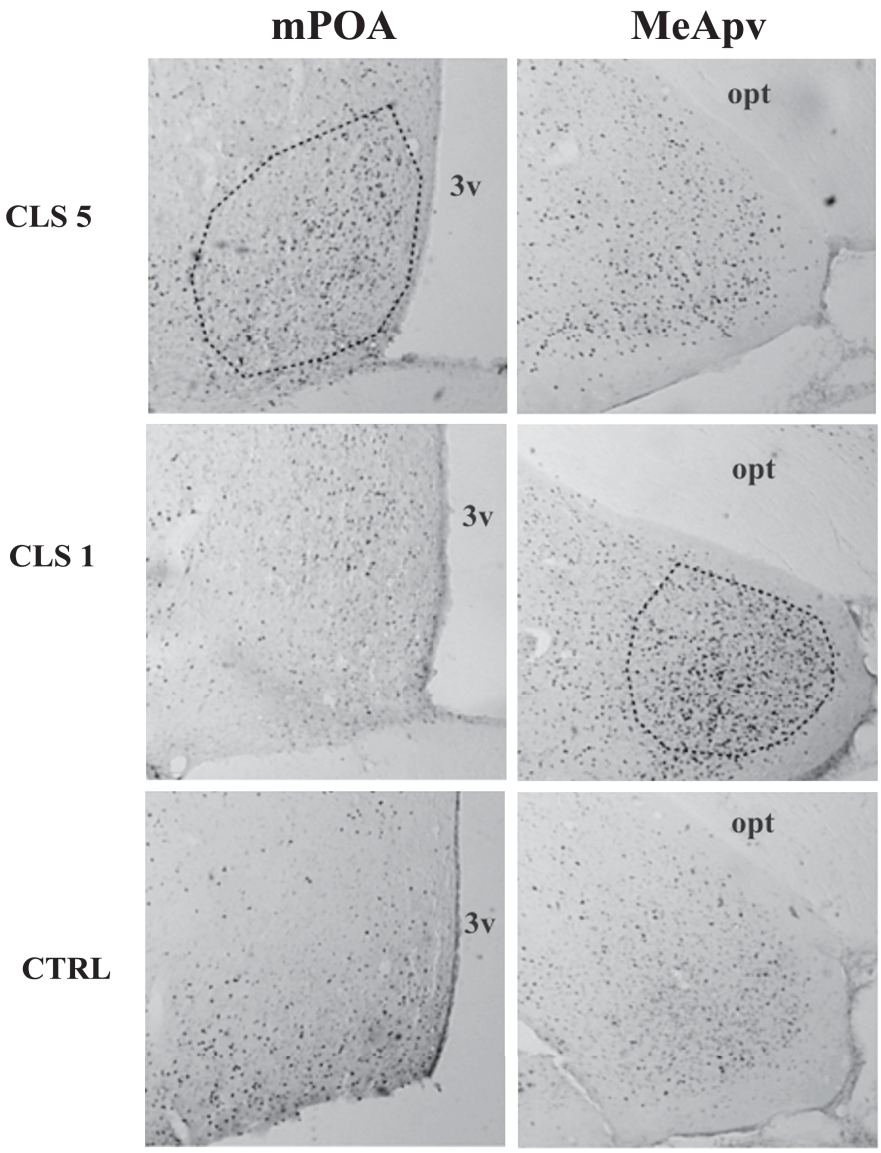

Figure 2.

Representative digitized images of the mPOA (left side) and MEApv (right side) taken at 40x. Areas that were analyzed for Fos-IR are outlined in black. Fos activation for the mPOA as a function of stimulation type (CLS 5: distributed, CLS 1: continuous, CTRL: sham stimulation) is displayed along the left side and Fos activation for the MEApv is displayed along the right side. 3V, third ventricle; opt, optic tract.

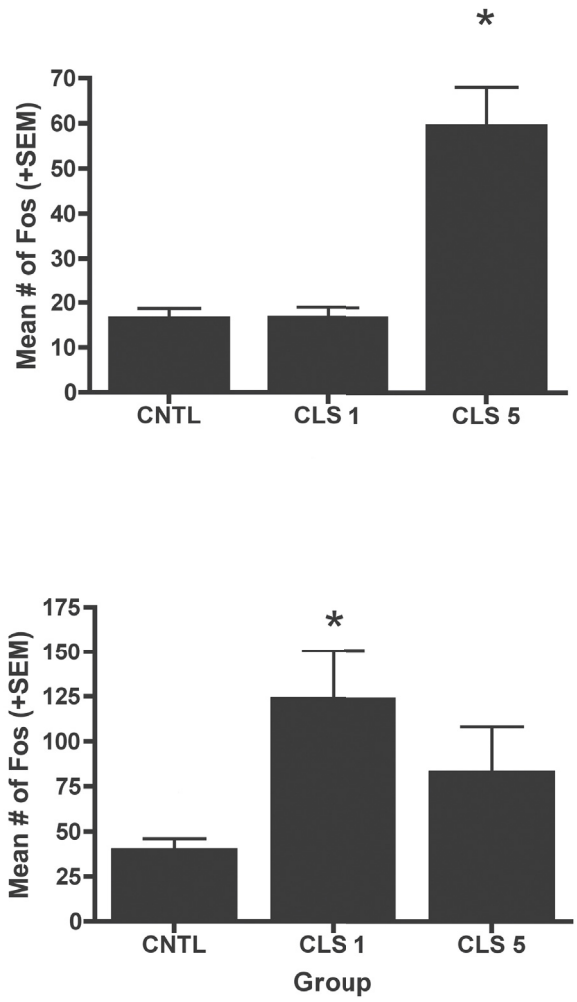

Figure 3.

Mean number of Fos cells/side (+SEM) as a function of different types of CLS are shown for the mPOA (top) and MEApv (bottom) for females receiving no stimulation (CNTL), continuous stimulation (CLS 1), and distributed stimulation (CLS 5) (n=5/stimulation condition). Rats receiving distributed stimulation showed a significantly greater number of Fos cells in the mPOA than rats receiving continuous stimulation and no stimulation. Rats receiving continuous stimulation showed a significantly greater number of Fos cells in the MEApv compared to no stimulation, *$p<0.05$.

Area	Stimulation condition		
	CNTL	CLS 1	CLS 5
Nacc			
shell	45.9 ± 9.7	38.1 ± 8.9	64.9 ± 17.5
core	33.2 ± 9.5	56.6 ± 18.9	53.4 ± 14.6
mPOA	16.8 ± 1.8	17.05 ± 1.9	59.7 ± 8.2*
LS	17.7 ± 5.9	28.7 ± 12.3	37.9 ± 7.3
BNST	6.7 ± 4.5	12.8 ± 5.6	19.7 ± 4.3
VMH	32.3 ± 6.3	42.7 ± 14.6	29.1 ± 2.6
Arc	37.0 ± 6.6	40.4 ± 7.5	50.7 ± 4.5
MEApv	39.4 ± 6.5	123.2 ± 27.2*	82.6 ± 15.6
LH	20.8 ± 5.4	28.5 ± 7.7	27.7 ± 5.8
PirCtx	91.0 ± 10.8	140.6 ± 26.5	83.0 ± 13.1
VTA	57.0 ± 11.0	47.0 ± 9.2	56.4 ± 20.2

Average numbers of Fos-positive cells in different hypothalamic and limbic structures as a function of sexual stimulation (mean +/-SEM).

Table 1.

Average numbers of Fos-positive cells in different hypothalamic and limbic structures as a function of sexual stimulation (mean +/- SEM).

DISCUSSION

The present study demonstrates both a behavioural and neural response to clitoral stimulation in the rat. This study identified manual distributed CLS as a strong inducer of sexual CPP and also that CLS induces Fos protein within regions of the brain generally associated with tactile genitosensory stimulation when females are in a receptive state. Further, the ability of CLS to induce Fos-immunoreactivity in the mPOA and the MEApv is dependent on the type of CLS that is received by the female, with distributed CLS resulting in a significantly larger number of cells with Fos in the mPOA, and continuous CLS resulting in a significantly larger number of cells with Fos in the MEApv.

We suggest that the rat clitoris, like that of the human, carries reward-related tactile stimulation during sexual interaction to the brain. In the rat, however, a distributed form of stimulation is necessary to induce reward of sufficient intensity to support CPP, similar to recent studies using VCS as the tactile cue (Meerts & Clark, 2009). We speculate that the clitoris may well be stimulated by pelvic thrusting during mounts and intromissions, and ejaculations during copulation. Thus, together with VCS, CLS may form an important component of sexual reward.

Distributed CLS induced a significantly greater number of Fos cells in the mPOA compared to continuous CLS and controls. It has been shown previously that mechanical stimulation of the cervix excites neurons in the mPOA (Haskins and Moss, 1983). Further, expression of Fos-immunoreactivity has also been shown in this region in response to vaginocervical stimulation both from a male partner during copulation and from artificial manual stimulation (Pfaus et al., 1993, 1996; Tetel et al., 1993; 1994a; Wersinger et al., 1993). It is interesting that different subregions of the mPOA appear to be activated by CLS and VCS. Whereas VCS activates a

central and medial region (Pfaus et al., 1996), CLS activates a more ventral region of the mPOA. This suggests a specific area of activation for clitoral stimulation. This also suggests that clitoral stimulation during copulation may be independent of other forms of tactile stimulation (e.g., flank stimulation), and may contribute information to the mPOA about the timing of pleasurable anogenital sensations. A similar effect was observed within the MEA. Previous work has shown that VCS activates Fos in cells of the MEApd in a linear fashion, with more activation in response to increasing amounts of stimulation (Erskine, 1993; Pfaus et al., 1993; 1996; Polston and Erskine, 1995; Tetel et al., 1993). However the present study did not find Fos activation in the MEApd, but rather in the posteroventral MEA (MEApv) in response to continuous CLS. This suggests that specific regions of the MEA respond to different forms of genitosensory stimulation with VCS activating the MEApd and CLS activating the MEApv. The idea that separate regions of the amygdala contribute differentially to female sexual behaviour is not new. Lesion studies of the corticomedial amygdala have reported a reduction of the lordosis response whereas lesions of the basolateral and lateral nuclei of the amygdala facilitate lordosis (Masco & Carrer, 1980; McGinnis et al., 1978; Nance et al., 1974).

Neural implants of estrogen to the MEApd have been shown to facilitate lordosis (Lisk & Barfield, 1975) and VCS in females primed with estrogen results in an increase in Fos-immunoreactivity in this same region but only to threshold levels in the MEApv (Pfaus et al.,1996). Lidocaine infusions to the medial amygdala, which include regions around the MEApv and MEApd, block the induction of pseudopregnancy when administered both before and after receiving intromissions, which has been suggested to provide evidence that the MEApd may modulate neuroendocrine and behavioural functions that are activated by VCS (Coopersmith

et al., 1996; Pfaus et al., 1996). It is possible that activation of the MEApv by a large amount of CLS may also contribute to the modulation of neuroendocrine and behavioural functions.

Like distributed CLS, distributed VCS induces significant CPP (Meerts & Clark, 2009). There is considerable overlap in the sensory innervation of both clitoris and vagina, including the proximal perineal branch of the sacral plexus that innervates the skin between the clitoris and vagina, the viscerocutaneous branch of the pelvic nerve, and the distal perineal branch of the pudendal nerve that innervates the skin between the vagina and anus, and the distal perineal branch of the pudendal nerve and the proximal perineal branch of the sacral plexus that innervates the skin lateral to the vaginal opening (Cruz et al., 2004). Cruz et al. (2004) suggested that such overlap might help to ensure that somatosensory information necessary to trigger reproductive and non-reproductive reflexes actually reaches the spinal cord and brain. It is therefore possible that a mount with intromission and pelvic thrusting from a male rat activates the clitoris, vagina, and cervix of the female, thus activating reward- and reproductive-related circuits of the spinal cord and brain simultaneously, including the mPOA and VMH. It is important to note that although CLS and VCS are strong inducers of CPP and thus sexual reward during copulation, it is possible that pacing copulation or distributing stimulation to induce pseudopregnancy drives reward. Females may pace the behaviour to ensure that enough intromissions occur to induce ejaculation and pregnancy, and that this may be “more” rewarding than the behaviour itself. It is also possible that the reward value of the somatosensory stimulation drives the reproductive consequences, or that the two occur by simultaneous activation of reward- and reproductive-related brain systems. It would be interesting to examine pseudorabies virus traces at

different times after injection, given that the virus travels approximately 1 synapse per day. It would also be instructive to perform selective unilateral lesions of reward- (e.g., VTA, NAcc, mPOA, VMH) and reproductive- (VMH, mPOA, MEApd) related brain regions prior to the pseudorabies virus injection, to examine the actual synaptic flow of afferent information.

It is also important to note that the stimulations given in this experiment differed not only in terms of distribution but the total number received during a given conditioning session. Females in the distributed group received a total of 60 clitoral stimulations during the 15-minute period in contrast to the 300 stimulations received by females in the continuous group. It is therefore possible that CPP developed as a result of the total number of stimulations rather than the pattern of distribution. However, in this case, the results of the current study would suggest that more is less in terms of the activation of CPP and Fos in the mPOA, a site that plays a critical role in linking sexual reward to appetitive sexual responses, such as solicitations in females (Hoshina et al., 1994; Paredes, 2009; Pfaus, 2009). This is in contrast to studies that have revealed a positive relationship between the amount or intensity of VCS and the induction of Fos in various brain regions (Erskine 1993; Pfaus et al., 1993; 1996; Tetel et al., 1993; Wersinger et al.,1993), and strongly suggests that an optimal timing exists for the conduction of reward-related CLS.

Finally, the parameters for the stimulation conditions used in the present study were chosen specifically to examine continuous vs. distributed CLS. They were based on unpublished observations from our laboratory that mounting stimulation often occurs in bouts that consist of several trains of pelvic thrusting prior to intromission and dismount. We do not yet know whether the stimulation parameters used here were in any way optimal for particular behavioural end-points (e.g., CPP,

partner preference, estrus termination, etc.). It is important to acknowledge that there is inconsistency throughout the literature regarding artificial VCS and its method of application, the intensity of the stimulation, and the timing, which makes it difficult to approximate what a female rat would experience in the real world. Attempts have been made to mimic the timing of intromissions to what is observed on average during a copulatory bout (Meerts & Clark, 2009) or to compare Fos activation from differing amounts of artificial VCS to what is seen following an hour of copulation (Pfaus et al., 1993). However, it is not known whether the same temporal parameters used for artificial VCS would induce a similar pattern of Fos induction with CLS. The present study found statistical trends toward activation of other regions (e.g., BNST, LS, PirCtx), and it may be the case that other patterns of CLS will result in significant increases (or decreases) in Fos induction in those regions. Further work will need to address the optimization of CLS, although it may be the case that individual differences will exist for female rats in the optimization of CLS for reward, as is acknowledged in women (Masters et al., 1986).

In summary, the present study confirms that clitoral stimulation is a form of tactile genitosensory stimulation that has a reinforcing value strong enough to induce CPP. This type of conditioned preference occurs when the stimulation is distributed rather than continuous, and induces Fos-immunoreactivity in areas previously associated with the processing of genitosensory stimulation for reward, estrus termination, and pseudopregnancy. Throughout a copulatory bout females typically receive both CLS and VCS from mounts. CLS likely occurs when the male's testicles or pelvis come into contact with the clitoris during pelvic thrusting, whereas VCS occurs during intromissions and ejaculations. This would stimulate both the vagina and cervix and also the clitoris through internal contact with the nerves that

feed into it. Because the innervation of the clitoris and vagina share common sensory pathways in humans and other animals (e.g., Foldes & Buisson, 2009), receiving both distributed CLS combined with VCS may be an important sensory experience that links reward with reproduction.

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SUMMARY OF CHAPTER 2

What is the reward value of clitoral stimulation and what brain areas are activated in response to this form of rewarding genitosensory stimulation? In chapter two we examined these questions using a Pavlovian paradigm to assess incentive salience and subsequently, brain activation in response to either distributed clitoral stimulation or continuous clitoral stimulation or no stimulation. It was observed that clitoral stimulation does have incentive salience strong enough to induce a conditioned place preference, however, only stimulation that was distributed induced this effect. Further, an examination of brain sites associated with incentive salience, genitosensory stimulation and reward revealed that the mPOA is an important site for the interpretation of clitoral stimulation since Fos activation was significantly higher in response to distributed clitoral stimulation compared to continuous clitoral stimulation and no stimulation. The medial amygdala was a second site to respond differentially to the type of clitoral stimulation. More Fos was activated in response to continuous stimulation compared to distributed stimulation and no stimulation. These findings in indicate several important points. First, that clitoral stimulation is rewarding enough to induce an association between the reward state produced by the stimulation itself and stimuli in the immediate environment; so much so that rats will spend an increased amount of time in a chamber that was previously not preferred. Second, that the brain responds specifically to clitoral stimulation and that these sites can differentiate between the specific patterns of stimulation to produce both a reward state and may contribute to the modulation of neuroendocrine responses.

What is the nature of the reward from clitoral stimulation during copulation? It has been shown that female rats can form preferences for particular copulatory

partners based on cues from partners that are associated with rewarding stimuli. Since clitoral stimulation is received along with several other forms of tactile stimulation during copulation, it would be necessary to examine whether distributed clitoral stimulation on its own could contribute to the formation of a partner preference when paired with an unconditioned odour cue on a copulatory partner. This is the basis for chapter 3.

Chapter 3

Context alters the ability of clitoral stimulation to induce a sexually-conditioned partner preference in the rat

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ABSTRACT

We have shown previously that clitoral stimulation (CLS) of female rats induces significant conditioned place preference (CPP), indicating that it is rewarding. The present study asked whether CLS could induce a conditioned partner preference. In the first experiment, sexually naïve females received 10 alternating trials of CLS and No-CLS in the presence of a male rat behind a wire-mesh screen. For one group, CLS was made in the presence of the male scented with almond extract. On alternating trials, those females received sham CLS in the presence of an unscented male behind the screen. The order was reversed for the other group. After 5 trials in each condition, females were placed into an open field with two sexually vigorous males, one scented and the other unscented. Contrary to expectation, females displayed a preference for the male associated with sham CLS. Upon re-examination of the videos during conditioning, it was observed that females were attempting to solicit the male behind the screen following CLS. The second experiment examined whether a partner preference could be conditioned by associating CLS with the almond odor alone. A new group of sexually naive females received the same CLS-odor, No-CLS-No Odor pairings as above, but with the odor presented on cotton gauze in the chamber. During the final open field test, those females selectively solicited the scented male. We conclude that CLS that induces CPP also induces conditioned partner preference. However, we propose that CLS in the presence of an inaccessible male created a sexual inhibitory state for female rats.

INTRODUCTION

The clitoris is an important organ for genitosensory stimulation in women. It relays localized pleasurable sensory input (Masters & Johnson 1988; Giuliano, Rampin, Allard, 2002; van Netten, Giorgiadis, Niewenbug, Kortekaas, 2008) and influences vaginal muscle function (Shafik, El Sibai, Shafik, 2008). Both human and animal studies have focused on its innervation and vasculature during sexual arousal, and its morphology in response to hormones (Hall, 1938; Peters, et al. 1987; Pacheco, et al., 1989; Giuliano, et al., 2001; Cruz, et al., 2004; Munarriz, et al., 2003; Pessina, et al., 2006). Recent studies in rats have also begun to elucidate the chemistry of the clitoral gland, blood flow in response to clitoral nerve stimulation, and the neural circuits that innervate the clitoris using anterograde tracers (Vachon et al., 2000; Kannan & Archunan, 2001; Marson & Murphy, 2006). Despite this knowledge, the role of the clitoris in carrying sensory information during sexual activity in the female rat is not well understood.

The lordosis posture assumed by the female during sexual activity facilitates the male's ability to mount and intromit, and mounting with pelvic thrusting by the male stimulates the female's flanks, rump, tailbase, perineum, and perivaginal surfaces, which include the clitoris (Pfaff, Montgomery, Lewis, 1977). Lordosis also allows the male to achieve penile intromission, which provides vaginocervical stimulation (VCS) to the female. Female rats find the ability to control or "pace" the initiation and rate of copulatory contact with males rewarding, as indexed by conditioned place preference (CPP; Jenkins & Becker, 2003; Oldenburger et al., 1992; Paredes & Alonso, 1997). Paredes and Alonso (1997) and Paredes and Vazquez (1999) showed that female rats would develop CPP for distinctive sides of a place preference box that was associated with paced copulation, relative to

nonpaced copulation. In those studies, pacing occurred in a unilevel chamber bisected by a divider with holes cut out of the bottom that allowed the female, but not male, to cross from one side to the other and thus regulate her contact with the male. The nonpaced condition consisted of the same chamber without the divider. Thus, female rats find optimal rates of copulatory contact rewarding, and such rates are essentially distributed in time in the female's control. Interestingly, Adler (1978) showed that gonadally-intact female rats were more likely to get pregnant after 5 intromissions if the interintromission intervals provided by the male were distributed in time. Moreover, when copulating in groups in large, semi natural open fields, female rats often impose delays between successive intromissions (McClintock, 1984), suggesting that the timing of intromissive stimulation serves both reward and reproductive functions.

What is the nature of the rewarding stimulus? Recently, we have shown that clitoral stimulation (CLS) administered with a lubricated paintbrush in a distributed fashion to females in a sexually receptive state induced strong CPP (Parada et al., 2010). Fos protein was activated by distributed CLS within regions of the brain generally associated with tactile genitosensory stimulation, including the ventral portion of the medial preoptic area (mPOA). This suggests that CLS may contribute information to the mPOA about the timing of pleasurable genital sensations. Thus, we proposed that the rat clitoris, like that of the human, carries reward-related tactile stimulation during sexual interaction to the brain, and that a distributed form of stimulation is necessary to induce reward of sufficient intensity to support CPP. This was reminiscent of studies using VCS as the tactile cue (Meerts and Clark, 2009). Moreover, Cibrian-Llanderal and colleagues (2010) found that in Wistar rats, CLS administered prior to copulation increased proceptive behaviours in ovariectomized,

hormone-primed females and enhanced reproduction in gonadally intact females that received greater than 9 intromissions. Those data strengthen the conclusion that the timing of CLS may form an important component of both sexual reward and fertility.

Olfactory stimuli play an important role in the expression of sexual behaviour in the female rat. The odors of male rats are attractive to females (Bermant, 1961; Bermant & Westbrook, 1966; Eliasson & Meyerson, 1975; Pfaus, Smith, & Coopersmith, 1999) and such odors increase Fos activation in a number of hypothalamic and limbic structures (Pfaus et al., 1993; Tetel et al., 1993; Wersinger et al., 1993). Although conspecific odors regulate sexual behaviour through activation of accessory olfactory pathways (Pfaus & Heeb, 1997), neutral odors that activate main olfactory pathways can be paired with sexual reward states in male and female rats, and come to regulate neuroendocrine and behavioural functions (Coria-Avila & Pfaus, 2007; Graham & Desjardins, 1980; Kippin, Cain, & Pfaus, 2003; Pfaus, Kippin, Coria-Avila, 2003). For example, Coria-Avila and colleagues (2005) found that associating almond-scented males with paced copulation induced a conditioned partner preference for the almond scented males. Females in the "paired" group received paced copulations with a scented male and unpaced copulations with an unscented male versus females in the "unpaired" group, which received the opposite association. During a final preference test with access to both a scented and unscented male in an open field, paired females showed a greater number of solicitations towards the scented males and the majority chose the scented male for their first ejaculation. This demonstrated that females can learn to associate a neutral odour with sexual reward and that sexual behaviour and partner preferences can be modified on the basis of experience with sexual reward.

The present study asked whether a neutral odor paired with the same distributed CLS used previously to induce CPP (Parada et al., 2010) could induce a conditioned partner preference. Two experiments were conducted. In the first, a neutral almond odor was applied to the neck and head area of a male rat placed behind a wire-mesh screen during presentations of CLS. In the second, the neutral almond odor was presented on a cotton ball during presentations of CLS.

MATERIALS AND METHODS

EXPERIMENT 1

Animals and Surgery

Sexually naïve female Long-Evans rats, weighing 200 to 250g, were obtained from Charles River Canada, Inc., St-Constant, QC. Animals were housed in groups of two in shoebox cages in a colony room maintained on a reversed 12:12 h light/dark cycle (lights off at 08:00h) at approximately 21^{o}C. Food and water were continuously available. Females were ovariectomized (OVX) bilaterally through lumbar incisions under intraperitoneal (ip) injections (1ml/kg of body weight) of ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4mg/ml) anesthetic, mixed in a ratio of 4:3 respectively. All females were given one week of post surgical recovery and maintained for the duration of the experiment on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10ug in 0.1 ml of sesame oil) 48 hrs, and progesterone (P; 500ug in 0.1 ml of sesame oil) 4 hrs, prior to testing. Sexually experienced male Long-Evans rats from the same breeder were used as stimuli. If scented, males were smeared with almond extract (Blue Ribbon, Etobicoke, ON, Canada) on the back of their necks and around the ears so that females could detect the odor easily.

All animal procedures conformed to the guidelines of the Canadian Council on Animal Care, and were approved by the Concordia University Animal Research Ethics Committee.

Male Sex Training

Male rats received a minimum of 10, 30-min sessions of sexual behaviour training with females other than the ones used in this study to ensure that they would copulate during the final open field partner preference test. Training was done in unilevel pacing chambers (60 L x 40 W x 40 H cm). Males were considered good copulators if they mounted females within 15 seconds of starting the training session.

Partner Preference Chambers

Conditioning was conducted in 10 Plexiglas chambers (38 cm H x 60 cm W x 38 cm deep) equipped with a wire mesh screen, which acted as a divider to keep the males inaccessible to the females. Five chambers were located in a particular room of the laboratory and were assigned as scented chambers and the other 5 assigned as unscented chambers. This was done to avoid any cross contamination of odours within the chambers and across conditioning sessions.

Conditioning Procedure

All conditioning sessions were conducted at 4-day intervals, 4 hrs after P injections, during the middle third of the rats' dark circadian cycle (as in Pfaus, Smith, Coopersmith, 1999). Females were assigned randomly to one of three stimulation conditions. The CLS-SC (clitoral stimulation-scented) group received CLS 5 (CLS every 5 sec for 1 min, applied as in Parada et al., 2010) followed by a 2-min period in the chamber with the scented male behind the screen for 5 cycles over a 15-min period. CLS was applied by the experimenter who lifted the base of the female's tail and lightly brushed the clitoris with one stroke of a small, soft-bristle number 4

paintbrush dabbed with K-Y® jelly. As with manually applied stimuli, the intensity was likely not exactly the same each time; however care was taken to avoid brushing too strongly and to avoid stimulating the vagina. Sham stimulations were done by lifting the base of the tail but not touching the clitoris.

On alternating trials, the females received sham CLS 5 prior to exposure to an unscented male behind the screen. The CLS-US (clitoral stimulation-unscented) group received the reverse order of presentation (CLS 5 prior to exposure to an unscented male, and sham CLS 5 prior to exposure to the scented male). The control group (CNTL) received sham CLS 5 prior to exposure to both scented and unscented males.

Males were placed in the chambers 5 min prior to the beginning of the conditioning session on one side of the bisecting wire mesh screen. This allowed females to see, hear, and smell the males, but not have any other physical interaction.

Open-Field Test

Following the 10 conditioning sessions, 5 with CLS and 5 with sham CLS for the paired and unpaired groups, and all without CLS in the control group, a final 30-min preference test was conducted 4 days after the last conditioning trial. This is the first full encounter with a stimulus male where females are allowed to copulate freely with both a scented and unscented male. The partner preference test was conducted in an open field (60H x 120 W x 120 L cm) that was divided into three compartments by a four-hole Plexiglas divider. The holes were made large enough for only the females to be able to move across the different compartments. At the beginning of the test the female was placed in the middle while a scented male and an unscented male was placed in either of the two remaining compartments. All rats were able to roam freely within their compartments. Females were free to choose which male they

interacted and copulated with. Each test was recorded so that scoring could be done at a later time using Behavioural Observation Program (Cabilio, 1996). The behavioural measures of interest were the latency and frequency of solicitations (headwise orientation toward the male followed by a runaway, as in McClintock, 1984), hops and darts, mounts, intromissions, and ejaculations, lordosis frequency, frequency of entrances/exits, and the total time spent with each male. Also examined were the proportions of females that chose either scented or unscented males for their first solicitation, first entrance, mounts, and ejaculations during this single preference test.

Finally, all defensive behaviours toward the males were recorded. This typically involved a female displaying a rearing posture and striking the male with the forepaws to prevent him from mounting, or rolling on her back to kick the male to prevent him from mounting. Both were defined as a “defensive hit” (Barnett, 1963).

Statistical Analysis

Due to the absence of homogeneity of variance in the data, the latencies and frequencies of each behavior were compared for the scented versus unscented male using a nonparametric Wilcoxon Signed Ranks test. This was done separately for CLS-SC, CLS-US, and CNTL animals. Chi-square analysis were also performed to examine the proportion of females that received their first mount, first intromission, and first ejaculation from either scented or unscented males, and to examine the choice of male for first solicitation, first hop/dart, and first entrance. A conservative Bonferroni correction was applied to the Wilcoxoin tests to correct the potential elevation of experiment-wise error per dependent measure made because 3 contrasts were made per measure. The Bonferroni correction was 0.05/3 = 0.0166, constituting the new p value used to denote statistical significance between

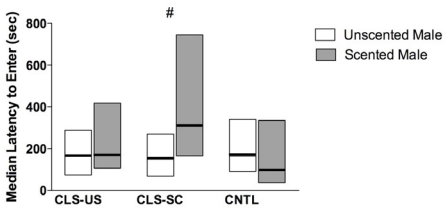
behaviors toward the scented or unscented males. We regarded p-values between 0.0166 and 0.05 as “trends” toward significance. Females that did not copulate with either male were removed from analysis.

EXPERIMENT 1 RESULTS

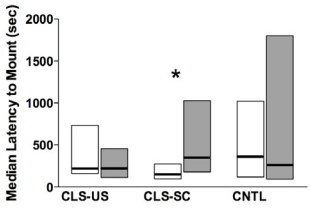
CLS-Scented

Females that received CLS in the presence of the scented male (CLS-SC) showed a trend towards a shorter latency to enter the unscented (CLS-US) male’s chamber (Mdn = 149.4 sec) compared to the scented male’s chamber (Mdn = 312.15 sec), z= -2.30, p= 0.020, r = 0.55 (figure 1a). These females also had a significantly shorter latency to receive their first mount from the unscented males (Mdn = 157.2 sec) compared to the scented males (Mdn = 355.60 sec), z= -2.417, p= 0.016, r = 0.57 (figure 1b). Finally, these females also showed a significantly shorter latency to receive their first ejaculation from the unscented males (Mdn = 457.6 sec) compared to the scented males (Mdn = 1800.0 sec), z= -2.54, p=0.01, r= 0.60 (figure 1c). The percentage of females in the CLS-SC group that first entered the scented male chamber compared to the unscented male chamber differed significantly. A greater proportion of these females entered the unscented male’s chamber (78%) compared to the scented male’s chamber (22%), $X^2(1)=5.56$, p=0.02. A greater proportion of females in the CLS-SC group received their first mount from the unscented males (78%) than from the scented males (22%), $X^2(1)=5.56$, p=0.02. Finally, a greater proportion of the females in the CLS-SC group also received their first ejaculation from the unscented males (79%) compared to the scented males (21%), $X^2(1)=4.57$, p=0.03. (Figure 2a, b, c). Those effects were not observed in either the CLS-US or CNTL groups.

a.



b.



c.

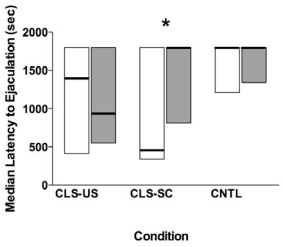


Figure 1.

Median latency (sec) to enter the unscented and scented male's chamber (a.) to receive the first mount (b.). And to receive the first ejaculation (c.) for females in the CLS-Unscented (CLS-US) group (n=19), CLS-Scented (CLS-SC) group (n=18), and control (CNTL) group (n=10). Females in the CLS-SC group had a tendency to show a shorter latency to enter the unscented male's chamber, $z=2.30$, $p=0.02$, $r=0.55$ (a.), they received their first mount from the unscented males compared to the scented males, $z= -2.417$, $p=0.016$, $r=0.57$ (b.), and received their first ejaculation from the unscented male earlier than from the scented male, $z=-2.54$, $p=0.01$, $r=0.60$ (c.). The box represents the inter quartile range. The dark lines across represent the median (* represents $p<0.05$) compared with the corresponding groups.

CLS-Unscented and Control groups

No significant differences were found for the frequency of hops/darts, solicitations, number of entrances, mounts, intromissions, ejaculations, and total time spent with each male for the CNTL group. The latencies to display first hop/darts, solicitations and first entrance into the male chambers and the latencies to receive the first mount, intromission, and ejaculation were also not significantly different for the scented or unscented males in the CNTL group. This confirms previous findings that rats do not display an innate preference for the almond odour (Kippin et al., 1998; Coria-Avila et al., 2005).

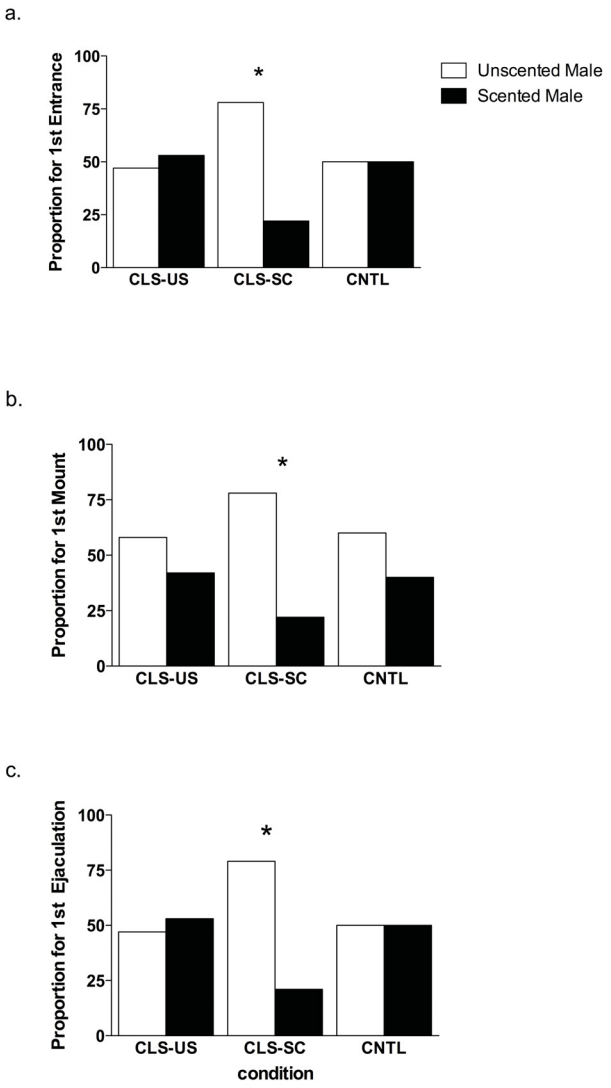


Figure 2.

Proportion of females to show first entrance (a.) to receive their first mount (b.) and to receive their first ejaculation (c.) from either male in the CLS-Unscented (CLS-US) group (n=19), CLS-Scented (CLS-SC) group (n=18), and control (CNTL) group (n=10). A significantly greater proportion of females in the CLS-SC group entered the unscented male's chamber, received their first mount, and received their first intromission from the unscented males, *$p<0.05$, chi-square test, comparing scented versus unscented groups.

DISCUSSION

We have shown previously that female rats develop CPP for a distinctive environment paired with distributed manual CLS with a fine paintbrush (Parada et al., 2010). The present experiment examined whether this effect would extend to conditioned partner preference, given previous findings that paced copulation which results in significant CPP also results in a significant partner preference for males bearing a neutral almond odour paired with paced copulation (Coria-Avila et al., 2005, 2006). However, in the present experiment, females given distributed CLS in the presence of an inaccessible male bearing an almond odour entered primarily the unscented male's chamber first, and received their first mounts and first ejaculations from the unscented male. Conversely, females in the CLS-US group received more mounts from the scented male during the test, although this effect was not statistically significant. Thus, females in the scented group displayed a significant preference for the male not associated with CLS. Although this could be taken as evidence that CLS does not induce a partner preference, and in fact may induce partner avoidance, we were skeptical given the fact that females during the conditioning trials were observed to actively solicit the males (headwise orientation toward the male followed by a runaway, as in McClintock, 1984) following CLS. An alternative explanation for those data would be that females associated a state of lowered sexual attractiveness with the male behind the screen, given that it was impossible to solicit sexual interaction successfully with the inaccessible male. The magnitude of this effect may have been higher in the condition in which CLS was received relative to the sham CLS conditions.

A similar phenomenon has been observed in male rats. Males ejaculate preferentially with females scented with almond if the males are in the presence of

the odour following ejaculation. However, if the males are allowed only 5 intromissions (without ejaculation) in the presence of a scented female, their preference switches to the unscented female (Kippin & Pfaus, 2001). In light of those findings we asked in the second experiment whether CLS in the presence of a neutral almond odour alone could induce a subsequent preference for a male bearing that odour.

MATERIALS AND METHODS

EXPERIMENT 2

A second set of OVX, sexually naïve Long-Evans rats were used in Experiment 2. All surgical and recovery procedures were the same as in Experiment 1, as were the partner preference chambers used for the final test.

Conditioning Procedure

As in Experiment 1, conditioning sessions were conducted at 4-day intervals, 4 hrs after P injections, during the middle third of the rats' dark circadian cycle. Females were assigned to the same three groups as in Experiment 1. However, in this experiment, females in the CLS-SC group were given CLS-5 in the presence of a cotton swab that had been saturated with almond extract, with the almond odour smeared throughout the conditioning chamber and the swab left on the opposite side of the wire-mesh divider. The females received sham CLS-5 in the presence of a cotton swab saturated with distilled water and smeared throughout the conditioning chamber, with the swab placed on the opposite side of the wire-mesh divider. The CNTL group was given sham CLS-5 in the presence of the cotton swab saturated with distilled water or almond extract. Females received 10 alternating conditioning sessions, 5 reinforced and 5 non-reinforced, as in Experiment 1.

Open-Field Test

The final partner preference test was conducted using the same open field as in Experiment 1. The female was placed in the middle compartment whereas a scented male and an unscented male were placed on either of the two lateral compartments. Once again, this is the first full encounter with male counterparts. All rats were able to roam freely within their compartments. Females were free to choose which male she interacted and copulated with. Each test was recorded so that scoring could be done at a later time using the Behavioural Observation Program (Cabilio, 1996). The same behavioural measures of interest were analyzed as in Experiment 1.

Statistical Analysis

The same analyses were conducted as in Experiment 1. Females that did not copulate were removed from analysis.

EXPERIMENT 2 RESULTS

CLS-Scented

Females that received CLS in the presence of the cotton swab saturated with almond odour had a significantly higher frequency of solicitations towards the scented males (Mdn=21) compared to the unscented males (Mdn=6), $z= -3.18$, $p=0.001$, $r=-0.96$ (see figure 3). Females in this group also displayed a trend toward spending significantly more time with the scented male (Mdn = 360.8 sec) compared to the unscented male during the open field test (Mdn = 237.2 sec), $z= -1.92$, $p= 0.055$, $r = -0.53$, and a significantly higher frequency of hops/darts towards the scented males (Mdn=42) compared to the unscented males (Mdn=29), $z= -2.44$, $p=0.01$, $r=-0.68$ (see figures 4, 5). Finally, females in the CLS-SC group displayed a trend towards a greater number of defensive hits towards the unscented

males (Mdn=2.0) compared to the scented males (Mdn=1.0), z= -2.08, p=0.037, r=-0.58 (see figure 6).

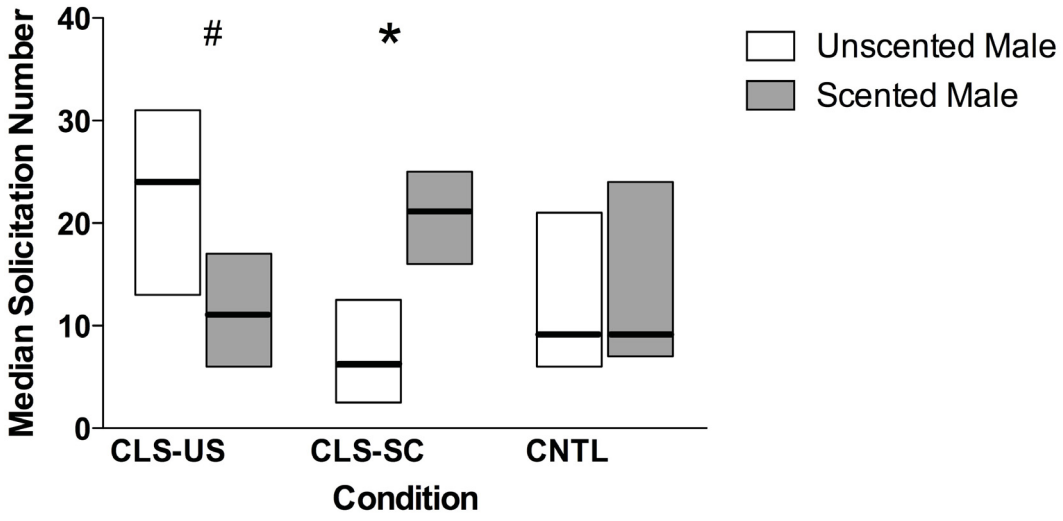


Figure 3.

Median number of solicitations displayed by females in the CLS-Unscented (CLS-US) group (n=11), CLS-Scented (CLS-SC) group (n=13), and control (CNTL) group (n=7). Females in the CLS-SC group displayed a significantly higher number of solicitations toward the scented males than the unscented males, $z=-3.18$, $p=0.001$, $r=-0.24$. Females in the CLS-US group showed a trend toward a higher number of solicitations toward the unscented males $z=-1.89$, $p=0.05$, $r=-0.57$. The box represents the inter quartile range. The dark lines across represent the median (*represents $p<0.05$) compared with the corresponding groups.

CLS-Unscented

Females that received CLS in the presence of the cotton swab saturated with distilled water displayed a trend toward a higher frequency of solicitations toward the unscented males (Mdn=24) compared to the scented males (Mdn=11), z=-1.89, p=0.05, r=-0.57 (see figure 3), and higher frequency of hops/darts towards the unscented males (Mdn=33) compared to the scented males (Mdn=19), although this effect was not statistically significant, z= -1.78, p=0.075, r=-0.49 (see figure 5). No other effects approached significance.

Control

No significant differences were found for the frequency of hops/darts, solicitations, mounts, entrances, intromissions, ejaculations, and total time spent with each male for the CNTL groups. The latencies to display first hop/darts, solicitations and first entrance into the male chambers and the latencies to receive the first mount, intromission, and ejaculation were also not significantly different for the scented or unscented males in the CNTL group.

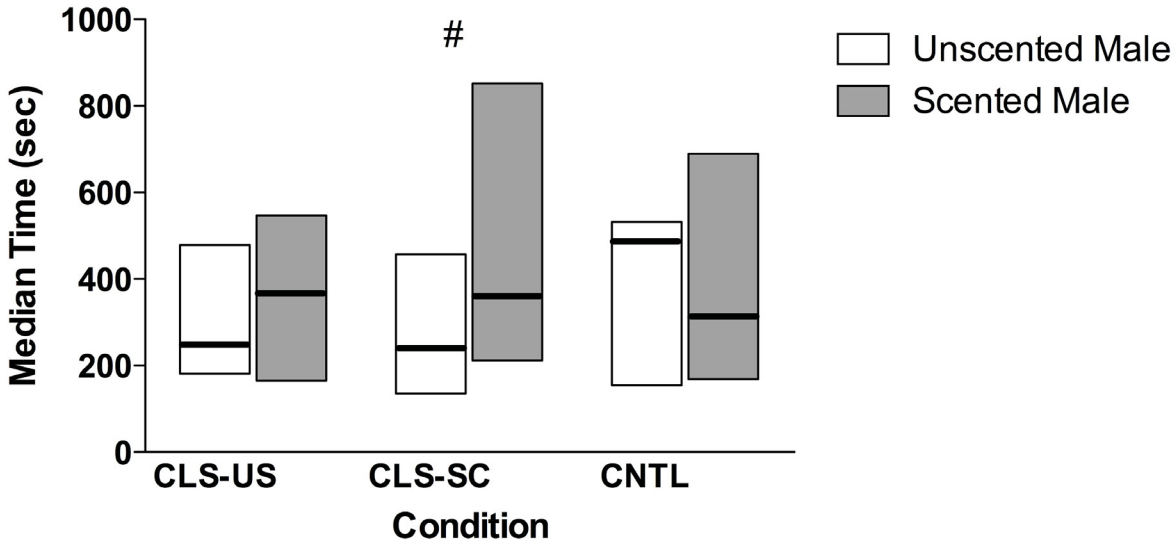


Figure 4.

Median time (sec) spent with the scented and unscented males for females in the CLS-Unscented (CLS-US) group (n=11), CLS-Scented (CLS-SC) group (n=13), and control (CNTL) group (n=7). Females in the CLS-SC group showed a tendency to spend more time with the scented males than with the unscented males $z=-1.92$, $p=0.05$, $r=-0.53$ compared with the corresponding groups. The box represents the inter quartile range. The dark lines across represent the median (# represents $p<0.05$) compared with the corresponding groups.

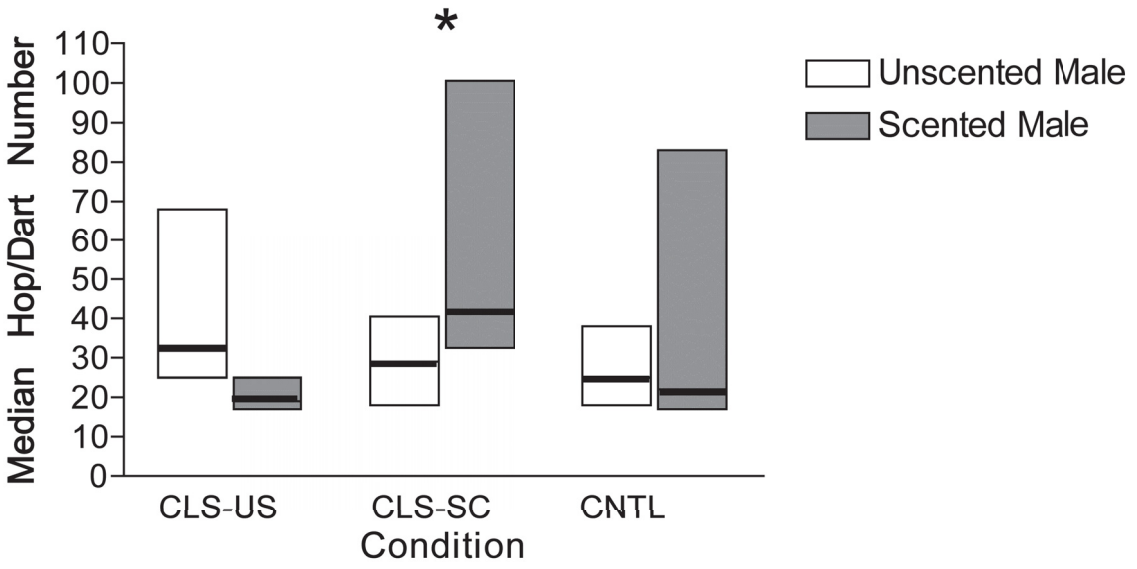


Figure 5.

Median number of hops/darts displayed by females in the CLS-Unscented (CLS-US) group (n=11), CLS-Scented (CLS-SC) group (n=13), and control (CNTL) group (n=7). Females in the CLS-SC group displayed a significantly greater number of hops/darts toward the scented males than the unscented males $z=-2.44$, $p=0.014$, $r=-0.18$. The box represents the inter quartile range. The dark lines across represent the median (* represents $p<0.0166$) compared with the corresponding groups.

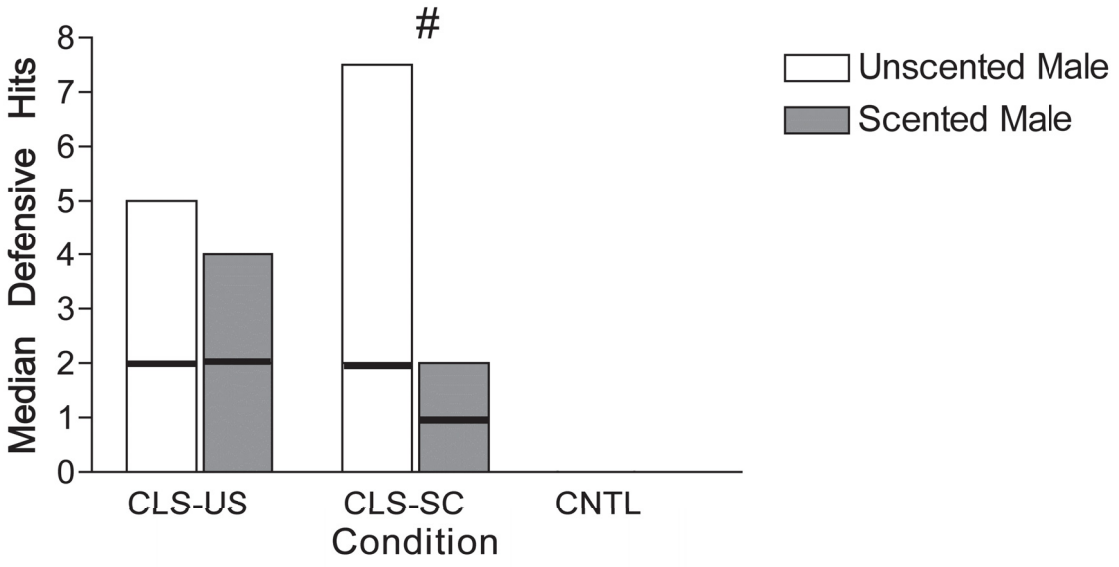


Figure 6.

Median number of defensive hits displayed by females in the CLS-Unscented (CLS-US) group (n=11), CLS-Scented (CLS-SC) group (n=13) toward the scented and unscented males. Females in the CLS-SC group displayed a trend toward a greater number of defensive hits toward unscented males $z=-2.08$, $p=0.037$, $r=0.16$. The box represents the inter quartile range. The dark lines across represent the median (# represents $p<0.05$) compared with the corresponding groups.

GENERAL DISCUSSION

The present study shows that the ability of female rats to display a conditioned preference for an olfactory cue associated with the reward state induced by clitoral stimulation depends on the context. Experiment 1 revealed a negative association between distributed CLS and an olfactory cue (scented or unscented) presented to sexually naïve females on an inaccessible male behind a wire-mesh screen. In a post-conditioning choice test, females copulated with males associated with the No-CLS state. Preference was demonstrated through earlier entrance latencies to the unscented male chamber, first mounts received by the unscented males, first shorter latency to ejaculations received by the unscented males and greater proportion of first frequency of ejaculations received from the unscented males. At first glance, those data seemed to indicate that the CLS state might have been aversive. We hypothesized however, that the inaccessibility of the male partner during the conditioning sessions may have induced an aversive or negative state between the CLS (which stimulated sexual desire) and the olfactory cue on the male. The second experiment asked the same question, but with the olfactory cue presented on cotton gauze rather than on an inaccessible male. Accordingly, females showed a significantly higher frequency of hops/darts, solicitations, and a lower frequency of defensive behaviours towards the males bearing the odour previously paired with CLS compared to unscented males. Females in the control group did not display a significant preference for either male, indicating that the almond scent alone did not carry unconditional appetitive value. Thus, females in the second experiment demonstrated a learned association between the CLS reward state and the odour during conditioning trials that was transferred to the male during their first sexual experience.

Although females in the CLS-SC group of the second experiment solicited the scented males more than the unscented males, and received more mounts from the scented males, they did not display a preference to receive the scented male's ejaculations. Females allowed to copulate with scented males during their initial sexual experiences solicit, copulate with, and receive ejaculations from, scented males versus unscented males when given a final test with both males in an open field (Coria-Avila et al., 2005; 2006; 2008a,b). Taken together with the present data, CLS appears to enhance elements of sexual desire (e.g., increases in solicitation, decreases in rejection responses) toward males bearing the odour, but is not sufficient to induce a mate preference involving the choice of male to receive ejaculations from.

We suggest that CLS induced sexual desire in both experiments, but that the inability of the male to pursue and mount the female in the first experiment created a state akin to "teasing", in which the desire induced by CLS could not be fulfilled by solicitations that led to successful copulation with the male. Females in the second experiment did not display solicitations during conditioning, indicating that the presence of the male in the first experiment was a salient cue for subsequent sexual activity. Although the precise nature of the male cue is unknown, it is likely something distal and could be related to pheromones, major histocompatibility complexes, and/or vocalizations. Female rats can differentiate males from females at a distance (Ismail, Gelez, Lachapelle, Pfaus, 2009), and also between gonadally intact and castrated males (Afonso & Pfaus, 2006), as well as males of pigmented and albino strains (Coria-Avila et al., 2006).

In males, thwarted copulation (usually mounts without the possibility of intromission) leads to displacement behaviours and aggression (Calhoun, 1972;

Hansen & af Hagelsrum, 1984). Although the opioid reward state induced by ejaculation is necessary for the development of both CPP (Ågmo & Berenfeld, 1990; Miller & Baum, 1987; Tenk, Wilson, Zhang, Pitchers, Coolen, 2009) and conditioned ejaculatory preference (Kippin & Pfaus, 2001), conditioning is stronger when it is preceded by a larger number of intromissions and/or longer ejaculation latencies (Ismail et al., 2008). However, experience with intromissions alone is sufficient to induce significant CPP in sexually naïve, but not experienced, male rats (Tenk et al., 2009), and to crystallize normative patterns of copulatory behaviour, including ejaculation latencies, in male rats (Whalen, 1961). Indeed, thwarting ejaculation by removing the female following 7 intromissions conditions male rats to ejaculate on or before the 7th intromission (Silberberg & Adler, 1974). Sexually experienced male rats learn not to attempt copulation with sexually non-receptive females (Pfaus & Pinel, 1989), and pairing an odour with that experience leads males to reject sexually receptive females bearing the odour on subsequent tests (Kippin et al., 1998). Although terms such as “teasing” and “frustration” used to describe the state in females of experiment 1 are colloquial analogies used to aid in the understanding of the phenomenon, further work would be necessary to determine the implicit state of the females during this situation. At this stage, it is more precise to describe the results of the first experiment as indicative of decreased attraction to inaccessible males paired with clitoral stimulation.

Female rats develop strong CPP following experience with paced copulation, in which the female controls the initiation and rate of copulatory stimulation she receives from a male (Martinez & Paredes, 2001; Paredes & Alonso, 1997; Paredes & Martinez, 2001; Paredes & Vazquez, 1999). Paced copulation with a scented male partner also results in a significant conditioned partner and mate preference

(Coria-Avila et al., 2005, 2006; 2008a,b). Such preference is inferred by significantly more solicitations, hops and darts, and a decrease in rejection responses, toward scented versus unscented males in a final open-field test, and the choice of receiving that male's first and often subsequent ejaculations. In Experiment 2, distributed CLS induced a significant preference for females to solicit, hop and dart, spend more time with, and not reject, the male associated with it. However, those females did not show a significant ejaculatory preference. This raises the intriguing possibility that the reward state induced by distributed CLS in female rats is of a smaller magnitude than that induced by paced copulation, which provides the full compliment of sexual stimuli to the female including vaginocervical stimulation (VCS) from intromissions and ejaculations, CLS from mounts with pelvic thrusting, flank stimulation from male forepaw palpations, proprioceptive feedback from being chased by the male, and the "cognitive" awareness of being able to control the rate of copulation by approach and withdrawal from the male. Previous studies have shown that paced VCS enhances both sperm transport and the induction of nightly prolactin surges that support the maintenance of progesterone release by the corpora lutea (Erskine, 1989; Matthews & Adler, 1978). Paced copulation is highly effective in inducing luteal function, faster estrus termination, and pregnancy (Bermant & Westbrook, 1966; Erskine, 1989; Lodder & Zeilmaker, 1976). It is not yet known whether CLS alone produces similar hormonal or reproductive effects.

Vaginocervical stimulation includes both stimulation of the cervix and the internal clitoris. VCS induces Fos in the medial preoptic area (mPOA), bed nucleus of the stria terminalis (BNST), ventrolateral ventromedial hypothalamus (VMHvl), and posterior dorsal medial amygdala (MEApd) (Pfaus et al., 1993; 1996; Tetel et al., 1993; 1994; Wersinger et al., 1993). VCS induces an inhibitory state that includes a

faster termination of estrus (Georgescu et al., 2009; Lodder & Zeilmaker, 1976; Pfaus et al., 2000), and results in pseudopregnancy (Coopersmith et al., 1996; Lehmann & Erskine, 2004; Polston et al., 2001). Those inhibitory effects are blocked by pelvic nerve cuts (Lodder & Zeilmaker, 1976), which results in decreased activation of hypothalamic and amygdaloid regions noted above (Pfaus et al., 2006). Distributed CLS activates the mPOA, but not the other regions (Parada et al., 2010), suggesting that VCS activates two signals, one that stimulates proceptive behaviours and reward in the short term, and another that produces a longer term activation of inhibition involved in estrus termination and pseudopregnancy.

The mPOA may be an important integrator of the reward and reproductive signals induced by CLS and VCS (Pfaus et al., 2010). Lesions of the mPOA virtually eliminate all appetitive sexual behaviours including solicitations and hop/darts and disrupt the display of pacing in female rats (Hoshina, Takeo, Nakano, Sato, Sakuma, 1994). The VMH and mPOA are interconnected structures that together control the temporal patterning of solicitations, pacing, and lordosis. The overlap of activation of those brain regions mirrors the overlap in the sensory innervation of the clitoris and vagina (Cruz et al., 2004). This overlap has been suggested to help ensure that somatosensory information necessary to trigger reproductive and non-reproductive reflexes actually reaches the brain (Cruz et al., 2004). Therefore, a mount with intromission would activate the clitoris, vagina, and cervix of the female, which includes both reproductive and reward-related circuits including the mPOA and VMH, and elsewhere. Stimulation of the external clitoris alone is strong enough to induce a preference in females to solicit particular males, but is not strong enough to induce a full mate preference. This could be addressed by combining manual CLS and VCS, or by examining the effects of clitoral versus cervical anaesthesia.

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SUMMARY OF CHAPTER 3

The experiments presented in chapter 3 demonstrate that distributed clitoral stimulation has the ability to induce a reward state strong enough for the development of a conditioned partner preference. A female's first experience with sexual reward occurred in a context that was not associated with a copulatory partner. However, the reward state was strong enough that the cues associated with that state during conditioning were transferred onto a partner bearing those cues in a preference test. When the female's first experience with sexual reward was paired with an inaccessible partner, the cues associated with that partner were also conditioned to the sexual experience and were expressed as a preference for an alternative partner not bearing those cues. Most importantly, the preferences expressed in each of the experiments were copulatory preferences but not mate preferences. This indicates that clitoral stimulation has a reward value that is high enough to induce sexual desire in females but is not sufficient enough to induce a preference for a mate to receive ejaculations from. It is likely natural pacing, that includes all forms of tactile stimulation induces a much higher reward state and thusly results in the development of mate preferences. Still, these experiments reveal the importance of this singular form of stimulation and its contribution to paced mating induced partner preferences. The goal of the next chapter is to determine whether ovarian hormones mediate the development of conditioned associations between the reward state induced by clitoral stimulation and the cues paired with that reward state.

Chapter 4

The role of ovarian hormones in sexual reward states of the female rat

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ABSTRACT

To what extent does the reward value of sexual stimulation in females depend on ovarian hormones? The effect of estradiol benzoate (EB) and progesterone (P) were examined on the acquisition and expression of sexual reward induced by paced copulation and clitoral stimulation (CLS) in ovariectomized (OVX) rats. In Experiment 1 we examined the expression of a pacing induced conditioned place preference (CPP). Ovariectomized, hormone-primed rats were given experience with paced copulation associated with one side of a CPP apparatus. Changing hormonal status prior to the final CPP test did not alter pacing-induced CPP. However, subsequent partial extinction of CPP was observed only in rats primed with EB+P, a treatment previously shown to induce sexual desire and receptivity. In Experiment 2, significant CLS-induced CPP developed in ovariectomized rats regardless of hormone priming. Our results show that the expression of the sexual reward state induced by paced copulation, and CLS in particular, is independent of hormone priming. We propose that ovarian hormones sensitize sensory and motor pathways necessary for sexual behaviour and stimulation to induce reward.

INTRODUCTION

A major question in behavioural endocrinology and clinical therapy alike concerns the actual role played by sex hormones in priming sexual responses and sexual reward. Many women experience a decline in sexual arousal and desire after natural or surgical menopause, and hormone replacement therapy often reinstates these components of sexual response (Bitzer & Brandenburg, 2009; Sherwin et al., 1985; Sherwin, 1988). Validated rat models of human sexual response exist (Pfaus et al., 2003: Pfaus et al., 2012) that show the same relationship. During copulation in rats, male stimulation of the flanks and rump of the female induces a lordosis posture, the dorsoflexion of the back that facilitates the male's mounts, pelvic thrusts and intromissions. Mounting and thrusting by the male further stimulates the female's rump, flanks, tailbase, and perivaginal surfaces including the external clitoris (Pfaff et al., 1977), whereas penile intromission stimulates the internal clitoris and cervix (Marson & Murphy, 2006; Pfaus et al., 2006; Pfaus et al., 1996). This contact is only possible when the female is in a sexually receptive state, which is under the control of cyclic estradiol and progesterone in gonadally intact, naturally cycling females, or when EB followed by P is administered exogenously to OVX rats (Erskine, 1989; Pfaff et al., 1977; Pfaus et al., 1999). Females control the initiation and rate of this stimulation by means of solicitation and pacing behaviour, in which the female entices the male to chase and copulate by means of headwise orientations, runaways, hops, and darts, that end in a lordosis crouch (McClintock, 1983). Lordosis, in turn, allows the male to mount and gain vaginal penetration, which stimulates both the clitoris and cervix in a distributed manner.

Female rats find the ability to control or pace the initiation and rate of copulatory contact rewarding, as indicated by the development of a conditioned

place preference (CPP) to paced, but not nonpaced copulation (Martinez & Paredes, 2001; Paredes & Alonso, 1997; Paredes & Vazquez, 1999). This copulatory CPP was abolished if females were given systemic injections of the opioid receptor antagonist naloxone prior to each conditioning trial (Paredes & Martinez, 2001), but not the mixed D1/D2 dopamine receptor antagonist flupenthixol (Garcia Horsman & Paredes, 2004). Indeed, artificial clitoral stimulation (CLS) or vaginocervical stimulation (VCS) applied in a distributed manner over time to OVX rats primed with EB and P induced significant CPP (Meerts & Clark, 2009; Parada et al., 2010). Moreover, paced copulation or distributed CLS induce a conditioned partner preference in female rats for males bearing an odor (e.g., almond) associated with the sexual reward state (Coria-Avila et al., 2005; Parada et al., 2011), an effect that was abolished by systemic administration of the opioid antagonist naloxone for paced copulation (Coria-Avila et al., 2008) but not flupenthixol (Coria-Avila et a., 2008), showing the dependence of sexual reward on the activation of opioid systems.

Corona, Camacho, Garcia-Horsman, Guerrero, Oganda, and Paredes (2011) reported recently that a threshold amount of estradiol is required for paced copulation to induce a significant CPP. Significant CPP developed in all but the group receiving the lowest dose of EB, suggesting that for paced copulation to induce a reward state sufficient to support CPP a threshold amount of estradiol is necessary.

The present study addressed the dependence of sexual reward on ovarian hormones in two experiments. The first asked whether OVX females primed with EB + P during CPP paired with paced copulation would express a significant CPP under EB and P, E alone, or oil (unprimed controls) hormone priming. Subsequently, we

asked whether these females would display significant extinction of paced-copulation induced CPP when primed with EB and P, EB alone, or oil if placement of the females into the CPP box was not preceded by paced copulation. It was hypothesized that paced mating would induce a significant CPP for all animals during the final preference test. Second, based on findings by Corona et al., (2011) we hypothesized that the EB alone and EB and P groups would show an extinction of CPP developed by paced copulation.

The second experiment asked whether CPP would be acquired under the same hormone-priming groups following artificial CLS. Because artificial CLS was applied by the experimenter, the lordosis behaviour of the female along with paced copulatory interaction with a male were not confounding factors as they would be in experiment 1, therefore hormone priming could be manipulated during the conditioning phase of the place preference paradigm. We hypothesized that only the EB alone and EB and P groups would develop a significant CPP to manual CLS.

MATERIALS AND METHOD

Animals and surgery

Sexually naïve, female, Long-Evans rats were purchased from Charles River Canada Inc. (St-Constant, QC). All animals were housed in groups of two in shoebox cages in a colony room maintained on a reversed 12:12 h light/dark cycle (lights off at 08:00 h) at 21^{o}C. Food and water were continuously available. One week after arriving at the colony females were bilaterally ovariectomized (OVX) through lumbar incisions under intraperitoneal (ip.) injections (1 ml/kg of body weight) of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml) anesthetic mixed at a ratio of 4:3 respectively. All animals were given an additional 2 weeks of post-surgical recovery time before testing. All animal procedures conformed to the

guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Steroid hormones

EB (10 µg) and P (500 µg) were purchased from Steraloids, Inc. (Newport, RI USA), dissolved in reagent-grade sesame oil (Sigma), and injected subcutaneously in a constant volume of 0.1 ml. An equal volume of sesame oil was used as the control solution. Depending on the condition, EB or oil was injected 48 h, and P or oil 4 h before each conditioning trial and/or CPP test. Hormone priming doses chosen for these experiments were based on previous studies done in our lab that consistently induce sexual receptivity in females (Parada et al., 2010; Parada et al., 2011; Pfaus et al., 1999).

CPP apparatus

CPP conditioning was conducted in 4 tri-coloured PVC plastic rectangular chambers (21 x 21 x 68 cm) each containing three compartments separated by guillotine doors (as in Parada et al., 2009). The two large end compartments were separated by a smaller center choice compartment, which was used on the pre-exposure and final test days. One of the end compartments was “dark” and had black walls and wire mesh floors. The other end compartment was “light” and white walls and a stainless steel rod floors with large spaces between the rods. All floors were raised 5 cm so that urine and feces could fall under the rats. Through the use of a computer interface, time spent in each compartment was recorded by means of infrared laser beam crossings. In each of the two end compartments there were two beams separated by 8 cm. A rat was considered to be inside a compartment once both beams in that respective chamber were broken. If only one beam was broken

the rat was considered to still be in the center compartment. Lights were on during conditioning sessions.

Expression and extinction of paced copulation-CPP

All conditioning and testing was conducted at 4-day intervals during the middle third of the rats' dark circadian cycle. There were four phases: an initial preference test, conditioning, a final preference test after conditioning, and a partial extinction test. During the initial preference test OVX females (N=36) were placed into the CPP chamber with the guillotine doors raised to allow access to the entire apparatus for 30 min. The amount of time in each compartment of the chamber was monitored and used to assess unconditioned preference (typically for the darker side). During conditioning, females were primed fully with 10 μg of EB 48 h and 500 μg of P 4 h before being tested with sexually vigorous male rats in a pacing chamber that contained a central Plexiglas divider (Kippin et al., 2003, Kippin et al., 2004, Pfaus et al., 2009). With the divider, females could pace copulation by running from side to side, forcing the male to chase them. Without the divider, females could not pace the copulatory contact efficiently. Females were given 6 alternating copulatory conditioning trials that lasted 30 min each, 3 with the divider, and 3 without the divider, counterbalanced in terms of first presentation. Immediately after paced trials, females were placed into their initially non-preferred side of the CPP box for an additional 30 min. After nonpaced trials, females were placed into their initially preferred side of the CPP box for an additional 30 min. The time chosen for exposure to the CPP apparatus was based on the length of the paced and nonpaced trials. CPP was tested 4 days after the final conditioning trial to be able to manipulate the female's hormonal state prior to the final preference test. Prior to the CPP test, females were randomly assigned to one of three hormone groups and received

EB+P, E-alone, or the oil vehicle (n=12/group). Females were then placed into the start compartment of the CPP chamber, and allowed free access to both distinctive compartments. After the test, females were maintained on their respective hormone treatments and put on a partial extinction schedule, in which the maintenance of CPP was tested again 4 days after the first CPP test but without prior copulation.

CLS-CPP procedure

As in the first experiment, conditioning and testing were conducted at 4-day intervals during the middle third of the rat's dark circadian cycle. Females (N=36) were given their initial unconditioned preference test followed by CLS conditioning and a final preference test. During the conditioning phase females were assigned to one of three hormone-priming regimens. The first group (n=12) received 0.1ml of sesame oil at 48 h and 4 hrs prior to the conditioning sessions (Oil group) and was considered the unprimed control group. The second group (n=12) received 10 µg of EB 48 h and sesame oil 4 h prior to conditioning sessions (EB group). The final group (n=12) received 10 µg of EB 48 h and 500 µg of P 4 h before conditioning sessions (EB+P group). During the conditioning sessions females received distributed CLS (CLS 5). Rats were given one stimulation every 5 seconds for a period of 1 min and placement into the initially non-preferred side of the CPP apparatus for 2 min. This was repeated for a total of 5 cycles. Each conditioning session lasted a total of 15 min. On alternate days females received general handling stimulation, but no clitoral stimulation, and were placed in the previously preferred side of the CPP apparatus. Clitoral stimulation was administered by the experimenter and was accomplished by lifting the base of the tail and lightly brushing the clitoris with one stroke of a small bristle #4 paintbrush dabbed in K-Y Jelly®. Because the strokes were given manually the intensity was likely not the same every

time; however, care was taken to avoid brushing too strongly and to avoid stimulating the vagina. Sham stimulation was done by lifting the base of the tail but not touching the clitoris.

After 10 conditioning sessions (5 reinforced and 5 non-reinforced) a final 15-minute preference test was conducted 24-hours after the last conditioning trial in the same manner as the initial preference test. The time of the conditioning sessions, the number of trials, and the timing of the pre-test and final test were based on previous experiments conducted in our lab that reliably induced a significant CLS-CPP (Parada et al., 2010).

Statistical analysis

The development of CPP was assessed using three measures. The total time spent in the reinforced chamber, a preference score (time in the reinforced compartment/[time in the reinforced compartment + time in the non-reinforced compartment]) and a difference score (time in the non-reinforced chamber – time in the reinforced chamber). All three measures are compared between the initial preference test and the final preference test (Paredes, 2010). Time spent in the reinforced chamber and the preference scores are expected to significantly increase from pre-test to post-test, the difference score is expected to significantly decrease.

The data collected for pacing-induced CPP and CLS induced CPP were subjected to one-tailed t-tests comparing scores in the pre-test to scores at test within the groups. One-tailed t-tests were chosen since the data for raw time and preference scores were expected to increase, the difference scores were expected to decrease. The data collected to examine extinction of pacing induced CPP were subjected to two-tailed t-tests since our interest was to examine weather the mean of

the initial preference test was either higher or lower at the extinction trial. Cohen's d was used as a measure of effect size.

RESULTS

Effect of hormone priming on the retention of pacing-induced CPP

Time in the Reinforced Chamber

One-tailed t-test revealed that rats receiving paced mating paired with the initially non-preferred compartment of the CPP chamber significantly increased the time spent in the reinforced chamber from pre-test to test regardless of hormonal state during the test. Females in the oil vehicle group showed a significant increase from pre-test to test, $t(11) = -3.038$, $p=0.006$, $d=1.20$. Rats in the EB-alone group also showed a significant increase from pre-test to test, $t(11) = -3.129$, $p=0.005$,$d=1.16$. Finally, rats in the EB+P group showed a significant increase in the time in the reinforced chamber from pre-test to test $t(11) = -2.12$, $p=0.028$, $d=0.96$ (Fig. 1A).

Preference Score

The t-test revealed a significant increase in the preference score from pre-test to test, $t(11) = -3.24$, $p=0.004$ for the Oil group. Rats in the EB-alone group also showed an increase in the preference score from pre-test to test, $t(11) = -4.13$, $p=0.001$. Finally, in the EB+P group, preference scores also increased from pre-test, to test, $t(11) = -2.14$, $p=0.017$, $d=0.99$ (Fig. 1B).

Difference Score

The t-test revealed a significant decrease in the difference scores of the Oil group from pre-test to test, $t(11) = 3.30$, $p=0.035$, $d=1.23$. Rats in the EB-alone group also showed a significant decrease in difference scores from pre-test to test,

t(11)= 3.974, p=0.001, d=1.39. Finally, in the EB+P group, difference groups also significantly decreased from pre-test to test, t(11)= 2.65, p=0.011, d=1.08 (Fig. 1C).

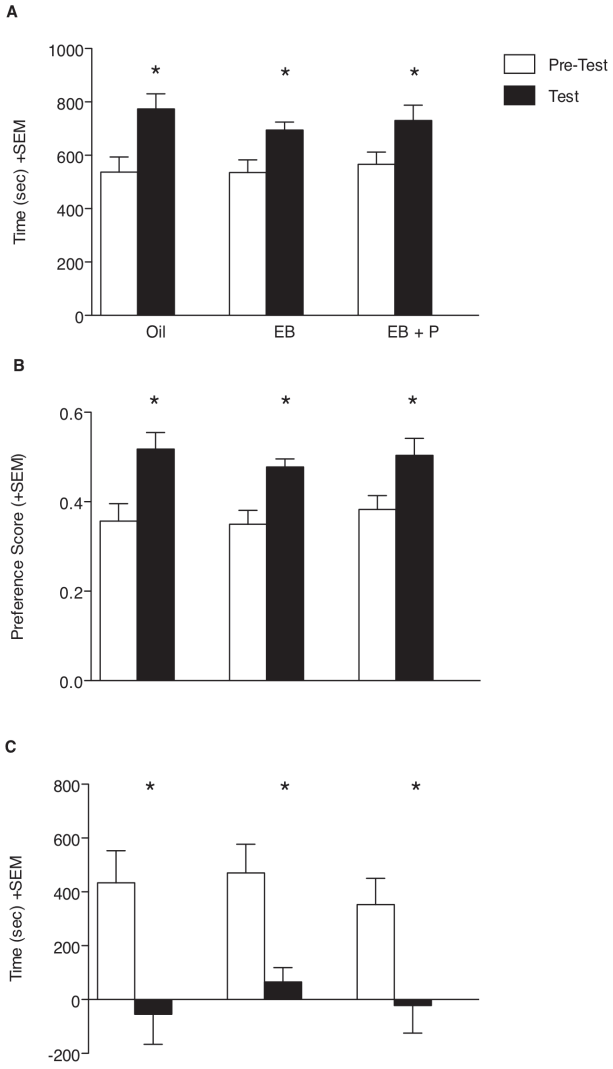

Figure 1.

Mean + SEM Raw scores (A), preference scores (B), and difference scores (C) on the pre test (pre-conditioning, white bars) and post-test (post-conditioning, black bars) for female rats that received paced mating and were tested under 3 hormone priming conditions; no hormones (Oil), estrogen estradiol benzoate (EB alone), and estradiol benzoate combined with progesterone (EB+P) (n=12 per group). Rats tested under all three hormone conditions displayed CPP for the compartment associated with paced mating. This coincides with a significant increase in preference score and a significant decrease in difference score, *$p<0.05$.

Effect of hormone priming on partial extinction of pacing-induced CPP

Time in the Reinforced Compartment

A two-tailed t-test between T2 (extinction trial 2) and the initial preference test revealed a significant difference from pre-test to T2 for the Oil group, t(11)= -2.732, p=0.020, d=1.14. Rats in the EB-alone group also showed a significant difference between pre-test and T2, t(11)= -3.108, p=0.010, d=1.45. In contrast, rats in the EB+P group did not show a significant difference between pre-test and T2, t(11)= -1.385, p=0.194, d= -0.64 (Fig. 2A).

Preference Score

The t-test revealed a significant difference between the preference score at pre-test and T2, t(11)=-3.52, p=0.005, d=1.41 for the Oil group. The EB-alone group also showed a significant difference between preference score at pre-test and T2, t(11)=-4.64, p=0.001, d=1.90. Finally, the EB+P group did not show a significant difference between pre-test preference scores and the T2 preference scores, t(11)=-1.385, p=0.194, d=0.51 (Fig. 2B).

Difference Score

The t-test revealed a significant difference in the difference score between the pre-test and T2 for the Oil group, t(11)=3.42, p=0.006, d=1.32. Rats in the EB-alone group also showed a significant difference between difference scores in the pre-test and T2, t(11)=4.661, p=0.001, d=1.822. Rats in the EB+P group did not show a significant difference in the difference score between pre-test and T2, t(11)=1.701, p=0.117, d=0.64 (Fig. 2C).

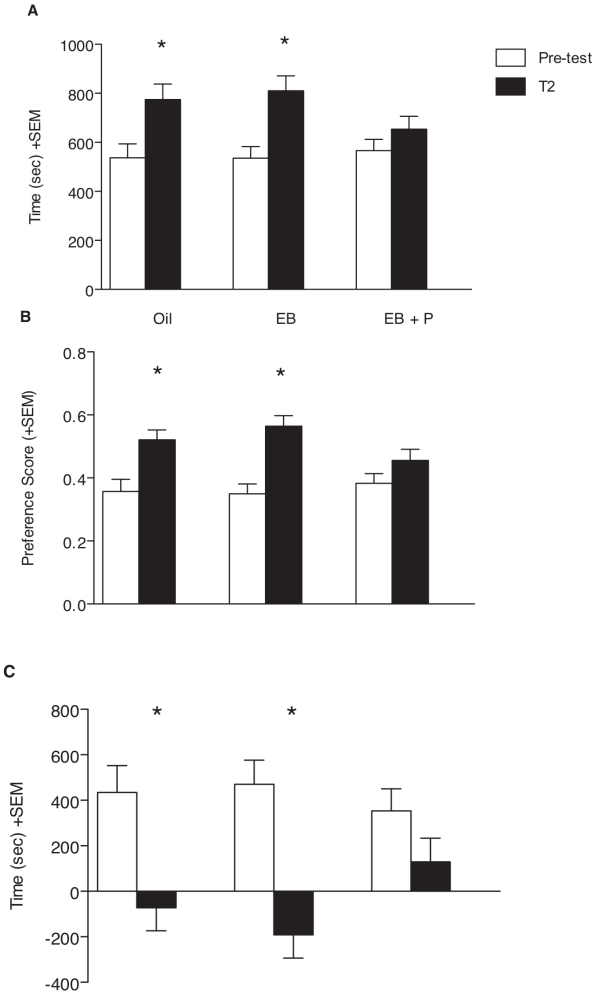

Figure 2.

Mean + SEM Raw scores (A), preference scores (B), and difference scores (C) on the pre test (pre-conditioning, white bars) and extinction trial (T2, black bars) for female rats that received paced mating and were tested under 3 hormone priming conditions; no hormones (Oil), estrogen estradiol benzoate (EB alone), and estradiol benzoate combined with progesterone (EB+P) (n=12 per group). Rats tested under Oil and EB conditions displayed CPP for the compartment associated with paced mating at T2. This coincides with a significant increase in preference score and a significant decrease in difference score. Rats in the EB+P no longer displayed a significant CPP at T2, *$p<0.05$.

Effects of hormone priming on the development of CLS-induced CPP

Time in the Reinforced Chamber

Females primed with oil vehicle alone (Oil group) showed a significant increase in time spent in the reinforced chamber from the pre-test to the test following conditioning trials, t (11) = -3.068, p=0.005, d=1.27. Rats in the EB-alone group showed an increase in reinforced time from pre-test to test, t(13)= -4.044, p=0.0005, d=1.49. Rats in the EB+P group also showed a significant increase from pre-test to test, t(12)=-2.653, p=0.01, d=0.902 (Fig. 3A).

Preference Score

Rats in the Oil group showed a significant increase in the preference score from pre-test to test, t(11)=-3.73, p=0.015, d=1.37. Rats in the EB-alone group showed a significant increase in the preference score from pre-test to test, t(13)=-4.72, p=0.000, d=1.81. Rats in the EB+P groups also showed a significant increase in preference scores from pre-test to test, t(12)=-2.37, p=0.017, d=1.05 (Fig. 3B).

Difference Score

Rats in the Oil group showed a significant decrease in the preference score from pre-test to test, t(11)=3.486, p=0.002, d=1.23. Rats in the EB-alone group showed a significant decrease in the difference score from pre-test to test, t(13)=4.69, p=0.000, d=1.59. Finally, rats in the EB+P group also showed a significant decrease in the difference score from pre-test to test, t(12)=2.28, p=0.041, d=0.90 (Fig. 3C).

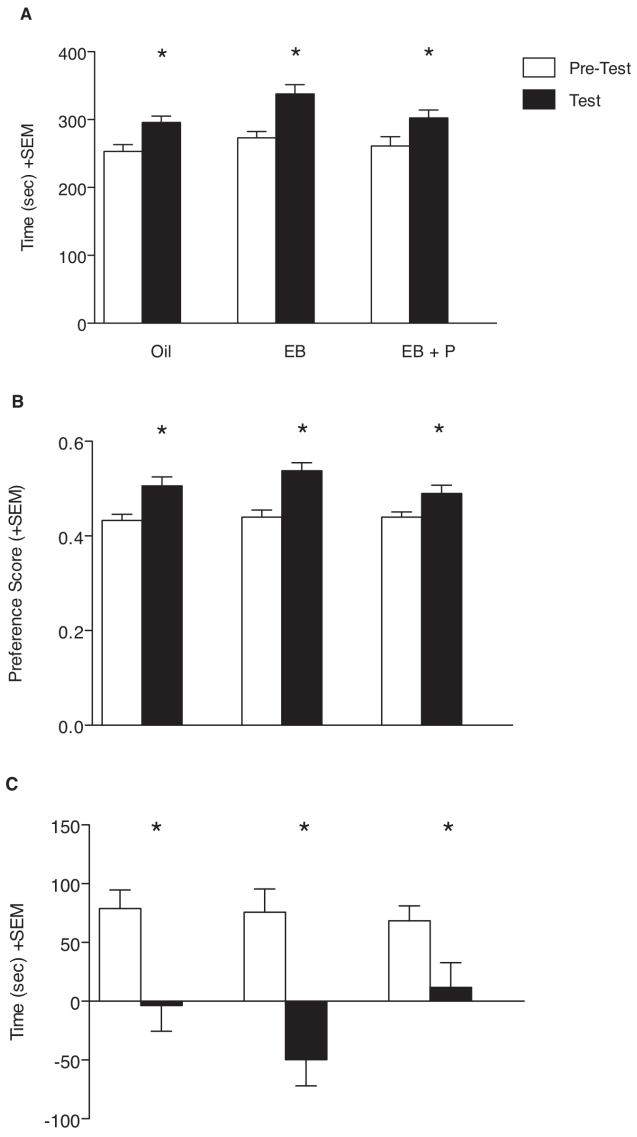

Figure 3.

Mean + SEM Raw scores (A), preference scores (B), and difference scores (C) on the pre test (pre-conditioning, white bars) and post-test (post-conditioning, black bars) for female rats that received manual clitoral stimulation (CLS 5) under 3 hormone priming conditions; no hormones (Oil), estrogen estradiol benzoate (EB alone), and estradiol benzoate combined with progesterone (EB+P) (n=12 per group). Rats receiving CLS displayed CPP for the compartment associated with CLS at post-test, *$p<0.05$.

DISCUSSION

The current studies examined the expression and acquisition of sexual reward states in the female rat. In experiment 1 the expression of place preference for paced copulation was assessed under three hormonal states; EB+P, EB-alone, or Oil control. Regardless of hormonal state at the time of expression for the reward, paced copulation was a strong inducer of sexual CPP. Subsequently, during the extinction trial when females in all three hormone priming groups were re-tested for CPP only females in the EB+P group did not show significant CPP. In experiment 2 the acquisition of CPP with CLS was tested in OVX rats that received EB+P, EB-alone, or Oil. Significant CPP developed regardless of hormonal state. Taken together, these data suggest that ovarian hormones control the behavioural and postural adjustments necessary for rewarding CLS to be experienced, but that the reward value of CLS is independent of the hormonal state.

Clearly, the acquisition of CPP induced by paced copulation requires ovarian hormones to drive sexual motivation and behaviour. This is necessary for female rats to display lordosis, which in turn allows them to receive CLS from male pelvic thrusts and intromissions (Pfaff, Montgomery, & Lewis, 1977). However, once the sexual reward state induced by paced copulation (and presumably CLS received at the female's preferred rate) has been associated with the environmental cues of the CPP chamber, hormones are not required to drive the expression of CPP. Thus, the memory of sexual reward that drives CPP was not dependent on the hormonal state of the animal.

In contrast to the expression of CPP, its extinction was dependent on hormonal state. Females tested during the extinction trial (T2) under Oil or EB-alone treatment continued to display a significant preference for the chamber associated

with paced mating. Females tested under EB+P no longer expressed a significant preference, indicative of a partial extinction effect. Thus, at test, females in a hormonal state that motivated them to seek out sexual interaction were sensitive to the extinction contingency, whereas those not in a sufficient hormonally driven motivational state did not experience the contingency. Because females primed with EB+P are in a state of behavioural estrus, they are more sensitive to tactile, olfactory, auditory and visual cues that would typically initiate approaches and withdrawals from a male partner in a pattern that is beneficial for reproduction (pacing) and that facilitates their receipt of CLS and the induction of a sexual reward state. We hypothesize that the desire for sexual reward, driven by hormonal priming, stimulates an excitatory state that, without the reward, results in faster extinction of the previously developed place preference. In contrast, when there is little or no desire for sexual reward (Oil group and EB-alone group) and no reward is present, either extinction does not occur, or it requires more time for non-pairings with the UCS to produce it. Thus, we argue that hormones play an important role in priming excitatory systems for sexual desire and behaviour (Pfaus, 2009). Engaging in solicitations, pacing, and lordosis at the preferred rate produces a reward state (Martinez & Paredes, 2001) and also enhances pregnancy (Coopersmith & Erskine, 1994). However, the present data show that the reward state itself at the time of expression is hormonally independent even if the behaviour that brings it about during the development of the reward state is not.

Recently, Corona et al., (2011) reported that a threshold amount of estradiol is required for paced copulation to induce significant CPP. In that study, separate groups of sexually naïve female albino (Wistar) rats were OVX and treated with 25, 5, 2.5, 1.25, or 0.625 μg of EB 48 h and 0.5 mg of P 4 h before each of six

alternating test sessions of paced copulation (ended after 10-15 intromissions with the male) vs. no sex and paired with one side of a conditioned place preference chamber. Significant CPP developed in all but the group receiving the lowest dose of EB, suggesting a threshold of estradiol is necessary for paced copulation to induce a reward state sufficient to support CPP. However, females in the lowest EB group displayed significantly lower lordosis-mount ratios (lordosis quotients, LQs), and thus received significantly fewer intromissions from the males relative to the other groups. They also showed higher mount return latencies (latency to reenter the male's compartment after receiving a mount) relative to the other groups, although that effect did not reach statistical significance. Given the present data, it is likely that the lowest EB dose was not sufficient to induce the sexual desire, arousal, and lordosis necessary for CLS and/or VCS to be experienced fully. Interestingly, CPP was tested between 24 and 72 h after the final conditioning trial, and without prior hormone administration, suggesting that the memory of sexual reward in the EB dose groups that developed it was not affected by the relative lack of plasma estradiol when tested.

The data of experiment 2 demonstrate that a threshold amount of EB is not necessary for the sexual reward state induced by CLS to be experienced. Because CLS is administered manually, a lordosis posture is not required for females to experience it. This is in contrast to genital stimulation received from mounts, intromissions, and ejaculations during natural paced copulation paradigms. Because the lordosis posture is mediated by the presence of EB, we conclude that a threshold amount of estradiol is necessary for the activation of sexual desire and lordosis. Hormones are therefore necessary to condition a copulatory CPP given that females need hormones to copulate. However, in light of experiment 2, when we lift the tail

and provide external CLS artificially, the requirement for hormones is no longer present since lordosis is not necessary for us to be able to administer CLS, one form of sexual stimulation.

According to Pfaff (1999), drive states like female sexual motivation or desire are hormone-sensitive, which when activated, direct behaviour by lowering the threshold to respond to sexual stimuli (estrogen mediated). In rats, this triggers both solicitations and the lordosis reflex, which is entirely dependent on estrogen, and allows for the further processing of sexual stimulation of the genitals, i.e. the clitoris. Hormones are activating the behaviour, which allows CLS (sexual stimulation) to be experienced from male stimulation. These experiments confirm Pfaff's hypothesis that hormones set up drive states that activate desire as with paced mating, but further indicate that the reward value of sexual stimulation from CLS is independent of ovarian hormones. This may help explain the apparent inconsistency that postmenopausal women without hormone replacement continue to achieve orgasm through masturbation while rates of desire for partnered sexual activity diminish (Cain et al., 2003). Although many cultural and psychosocial factors exist for masturbation in postmenopausal women, the most obvious reason is to obtain sexual gratification (e.g., Hite, 1976). Based on our current findings, therefore, the experience of sexual gratification does not require the presence of circulating ovarian hormones.

Acknowledgements

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SUMMARY OF CHAPTER 4

The experiments in chapter 4 demonstrate that hormones control the behavioural and postural adjustments that are necessary for receiving sexual reward. Specifically, they are necessary for the postural adjustment needed for the receipt of clitoral stimulation during copulation to induce a reward state. Further, we now know that the reward value of clitoral stimulation is not dependent on the receptive state of the female. This finding has important implications for the understanding of female sexual desire and sexual function. In humans, post-menopausal women typically report a decline in sexual desire however, this is inconsistent with their reported levels of sexual self stimulation (masturbation). This indicates that circulating hormones are more directly tied to levels of sexual desire but, as with our findings, sexual gratification (reward) is independent of this. The consistency between the current animal and human data is promising.

The goal of the next chapter is to examine whether the contribution of clitoral stimulation as a signal of reward is influenced by prior sexual experience. Is the impact of clitoral stimulation in sexual reward of equal importance throughout the development of sexual experience or does repeated sexual experience diminish the value of clitoral stimulation so that it no longer supports the development of a conditioned place preference?

Chapter 5

Sexual experience blocks the ability of clitoral stimulation to induce a conditioned place preference in the rat

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ABSTRACT

We have previously established the rewarding value of clitoral stimulation (CLS) through the demonstration that manual, distributed, CLS induces a significant conditioned place preference (CPP) and conditioned partner preference in naïve, hormonally primed and non-hormonally primed rats. The present experiment asks whether previous sexual experience might inhibit the ability of clitoral stimulation to induce a conditioned place preference.

Female Long-Evans rats were ovariectomized and treated with 10 µg of estradiol benzoate (EB) 48 h and 500 µg of progesterone (P) 4 h prior to receiving either 0, 1, or 5 consecutive copulatory sessions with a sexually vigorous male. Each copulatory session ended when females received at least 1 ejaculation. Females then experienced 10 alternating trials of distributed CLS or no CLS paired with one side of a non-biased CPP box under the same hormone-priming regimen. Females that experienced 5 consecutive copulatory sessions did not develop a significant place preference indicated by both a preference score (the proportion of time spent in the reinforced chamber) and a difference score (time in the non-reinforced chamber minus the time in the reinforced chamber) compared prior to and following the 10 conditioning trials. This suggests that repeated copulatory experience might induce a desensitization of the genitosensory circuit since copulation includes both clitoral, and vaginocervical stimulation from mounts plus intromissions. Alternatively, repeated sexual experience prior to conditioning may generate a UCS pre-exposure effect that cannot be altered when manual clitoral stimulation is paired with a new environment.

INTRODUCTION

A cascade of reinforcing events is necessary for the development of baseline rates of sexual responses in rats. The perception of sex odours, anticipating and chasing receptive partners, and in males, receiving penile stimulation during mounts, intromissions, and ejaculations, are all rewarding events necessary for the development and expression of copulatory behaviour (Whalen, 1961; see Pfaus et al., 2012).

Early studies reported disruptions of copulatory behaviour in male rats following disruptions of olfactory processing and disruptions of penile sensory stimulation. For example, the induction of anosmia following olfactory bulbectomy or zinc sulfate treatment, castration, penile deafferentiation and penile anesthesia all impair sexual behaviour in male rats (Heimer, Larsson, 1967; Beach 1948; Beach, Holtz, 1946; Lodder, 1976). However, the impairments caused by those disruptions were typically observed in males with relative sexual inexperience (e.g., few or no prior sexual contacts with females). In contrast, other work has shown that sexual experience ameliorates the effects of such disruptors (Thor & Flanelly, 1977; Lisk & Heiman, 1980; Lodder, 1975; Pfaus et al., 2012). For example, males that have been limited to mounting females are less likely to copulate compared to males that have received intromissions or intromissions with ejaculations (Whalen, 1961). Experience with intromissions has even been shown to help males learn to run through a maze more accurately to access a female partner. Males that have experience with ejaculations display faster run times in a straight-arm runway over males experienced with intromissions only and males that are sexually naive (Lopez, Olster, Ettenberg, 1999). We have shown that penile anesthesia with lidocaine in males with 0 or 1 prior sexual experience to ejaculation results in significantly fewer

males that mount, intromit, or ejaculate relative to controls. However, males with 10 prior sexual experiences with ejaculation are completely unaffected by penile anesthesia and display latencies to mount, intromit, and ejaculate that are no different from saline controls (Pfaus et al, 2012). Thus, experience with penile stimulation automates behavioural responses and reduces the need for complex triggering stimuli for males to copulate effectively. Sexual experience seems to reduce the saliency of individual stimulatory components that were previously necessary for successful copulation in the less experienced animal. By reducing the need for complex triggering stimuli, sexual experience helps to sensitize animals to stimuli that are most important and desensitize animals to those that are less important for effective copulation. The result in highly experienced animals is an increase in the speed of the initiation of copulation and the development of baseline rates of sexual responding (Larsson, 1956; Pfaus, Wilkins, 1995). Thus, with repeated experience, sexual behaviours become “habitual”. This process most likely involves the automation of motor patterns via the striatum and cerebellum to ultimately crystallize behaviour and make it less dependent on the individual sensory components that were previously necessary.

In contrast to males, little is known about the influence of sexual experience in the acquisition of baseline rates of sexual responding in female rats. Although appetitive and consummatory female sexual behaviours, such as solicitations, pacing, and lordosis, are stimulated by the ovarian hormones estradiol and progesterone (P) (Powers, 1970; Pfaff, 1970; Pfaff, 1980; Pfaff, Schwartz-Giblin, McCarthy, Kow, 1994; Erskine, 1985; Pfaus, Smith, Coopersmith, 1999; Brandling-Bennett, Blasberg, Clark, 1999; Fernandez-Guasti, Vega-Matuszczyk, Larsson, 1991), the role of sexual experience is not well understood. Females do however

modify their behaviour as a function of their environment to maximize their ability to pace the stimulation they receive from males (Pfaus et al., 1999), and such experience reliably induces a sexual conditioned place preference (CPP) in female rats that are allowed to pace copulatory contact with males under their own control (Paredes, Vazquez, 1999; Paredes, Alonso, 1997; Martinez, Paredes, 2001). Indeed, early sexual experience that has its opioid reward value blunted by treatment with the opioid receptor antagonist naloxone results in female rats that avoid males, despite full hormonal priming with estradiol benzoate (EB) and progesterone (Pfaus et al., 2012).

We have shown previously that distributed external clitoral stimulation (CLS) in naive rats is rewarding and acts as a positive reinforcer in studies of conditioned place and partner preference (Parada, Chamas, Censi, Coria-Avila, Pfaus, 2010; Parada, Abdul-Ahad, Censi, Sparks, Pfaus, 2011). Further, when CLS is given in the presence of an inaccessible male bearing a neutral odour, females will solicit those males during training trials, however they seem to develop a form of avoidance and will solicit males not associated with the odour. This response pattern does not occur however, when females are conditioned with CLS to the odour alone. Interestingly, females that receive CLS in the presence of the odour cue will solicit and prefer males bearing that odour at a later time during a partner preference test (Parada, Abdul-Ahad, Censi, Sparks, Pfaus, 2011). Thus, with no male cues present during conditioning of CLS to the odour cue, CLS acts as a reward on its own. In the presence of an inaccessible male, however, it acts to excite the female to solicit the male. Would previous copulatory experience with full CLS through intromissions make females less responsive to external CLS alone, just as copulatory experience makes male rats less dependent on penile stimulation?

We currently, know little about what effect previous sexual experience would have on the reward value of clitoral stimulation. Sexual experience with a copulatory partner would provide external clitoral stimulation from the thrusts of male mounts (Pfaff, Montgomery, Lewis, 1977) and internally via intromissions. We hypothesize that this experience would de-value manual external clitoral stimulation when administered on its own. Evidence from our previous work leads us to this hypothesis. Females given 5 sexual experiences prior to treatment with a 2% lidocaine solution, injected into the clitoral sheath, showed no differences in sexual behaviour compared to controls. In contrast, sexually naive females treated with lidocaine showed differences in the total time spent with the males, a greater number of entrances and exits from the male side of the testing chamber, and significantly longer contact return latencies to ejaculations compared to controls (Parada, Sparks, Censi, Pfaus, in prep). This demonstrates that sexual behaviour in females, once crystallized, is less susceptible to the effects of lidocaine treatment and that input from the clitoris is less necessary for proper copulatory behaviour once females are experienced.

The aim of the present study is to examine whether sexual experience can de-value the reinforcing effect of distributed CLS and block the development of conditioned place preference in ovariectomized (OVX), hormone-primed females. Rats were randomly assigned to one of three experience groups; naïve, low experience (1 copulatory sessions) and high experience (5 copulatory sessions) with a sexually vigorous male. Following the experience sessions, females were tested using a conditioned place preference paradigm (as in Parada et al., 2010) and paired with manual distributed clitoral stimulation.

Based on our previous findings we hypothesize that females that have a high degree of sexual experience will not show a conditioned place preference to distributed CLS compared to females that have little or no sexual experience.

MATERIALS AND METHODS

Animals and surgery

Sexually naive female Long-Evans rats were purchased from Charles River Canada, Inc., St. Constant, QC. Animals weighing 200 to 250g, were housed in groups of two in shoebox cages in a colony room maintained on a reversed 12:12 h light/dark cycle (lights off at 08:00 h) at approximately 21^{o}. Food and water were continuously available. Females were ovariectomized bilaterally through lumbar incisions under intraperitoneal (ip.) injections (1ml/kg of body weight) of ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4mg/ml) anesthetic mixed in a ratio of 4:3 respectively. All females were given one week of post surgical recovery and maintained for the duration of the experiment on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 ml of sesame oil) 48 hours and progesterone (P; 500 µg in 0.1 ml of sesame oil) 4 hours prior to testing.

Long-Evans males from the same breeder were given 10 prior sexual experiences with sexually receptive females to generate maximal baseline rates of sexual responding (n=20 per group).

All animal procedures conformed to the guidelines of the Canadian Council on Animal Care, and were approved by the Concordia University Animal Research Ethics Committee.

Sexual experience and CPP apparatus

Females were randomly assigned to one of 3 sexual experience groups. Females remained naive (CNTL), or received 1, or 5 (N=36) copulatory sessions

with a sexually vigorous male prior to conditioning. Each copulatory session ended when females received at least 1 ejaculation. Copulatory experience sessions took place in a bilevel chamber where females could pace the copulatory stimulation they received from their male counterparts by changing levels. Females in the multiple experience session groups were given copulatory sessions every 4 days. The first conditioning session took place 4 days following the last experience session.

Conditioning was conducted in 4 identical tri-coloured PVC plastic rectangular boxes (21 x 21 x 68 cm), each containing three chambers separated by guillotine doors (manufactured at Concordia University, QC). The two large end chambers were separated by a smaller center choice chamber, which was used on the pre-exposure and test days. One of the main chambers had black walls and a wire mesh floor; the other had white walls located across from the guillotine door and a stainless steel rod floor with larger spaces. The floors were raised 5cm from the ground for proper collection of urine and feces and for proper cleaning between each animal tested. Using a computer interface the time spent in each of the chambers was recorded by means of infrared laser beam crossings. Infrared beams located inside each of the end chambers were located 8 cm apart. A rat was considered to be inside one of the end chambers if both beams on one side were broken. If only the beam closest to the guillotine door was broken the rats was considered to still be in the center chamber. Lights were left on during conditioning.

Conditioning procedure

Conditioning and test days were conducted at 4-day intervals, 4h following P injections, during the middle third of the rats dark circadian cycle (as in Pfaus et al., 1999). The place preference conditioning procedure consisted of three phases: an initial preference test, conditioning, and final preference test. During the initial

preference test females were placed in the middle chamber with the guillotine doors raised to allow access to all three chambers for 15 min. The amount of time in each of the chambers was monitored and used to assess unconditioned preferences.

During the conditioning phase, females from the three experience groups were given distributed CLS (CLS 5), which has previously been shown to induce a significant CPP (see Parada et al., 2010). Clitoral stimulation consisted of 1 CLS every 5 sec for 1 min, after which females were placed into the previously non-preferred side of the CPP chamber for 2 min. The cycle of CLS and placement into the chamber was repeated for a total of 5 cycles, which totaled 60 stimulations. Each conditioning session lasted a total of 15 min per animal. On alternating days females were given sham stimulation by lifting the base of the tail, but no CLS was given, and placed into the previously preferred side of the CPP chamber.

Clitoral stimulations were administered manually by the experimenter; the stimulations were made by lifting the base of the tail and lightly brushing the clitoris with a small bristle number 4 paintbrush dabbed with K-Y® Jelly. After 10 conditioning sessions (5 reinforced, 5 non-reinforced) a final 15-min preference test was conducted 24-hours following the final conditioning trial in the same manner as the initial preference test.

Statistical analysis for CPP

The outcome of the pre-exposure and CPP tests were determined by the total raw time spent in each of the three chambers. A within-subjects repeated measures ANOVA was used to examine any initial bias of the chambers. If any initial preference was detected females were reinforced to the initially non-preferred side of the chamber. Based on Paredes (2010) three main measures were used to assess the development of CPP. The raw time spent in the reinforced chamber during the

pre-test and during the post-test was expected to significantly increase. The preference score (time in the reinforced chamber/(time in the reinforced compartment + time in the non-reinforced compartment)) was also expected to significantly increase from pre-test to post-test. Finally, the difference score (difference between the time spent in the non-reinforced chamber - time in the reinforced chamber) was expected to significantly decrease from pre- to post-test. This was calculated to account for the possibility that the rats are spending most of their time in the neutral chamber.

Raw time, preference scores, and difference scores were analyzed for each experience group across pre-test and post-test using a paired-samples t-test to examine the change in scores prior to, and following, clitoral stimulation (Dominquez-Salazar, Camacho, Paredes, 2005; Meerts and Clark, 2007; Meerts and Clark, 2009). As per Paredes and Alonso (1997) a significant increase in the raw score, preference score and a significant decrease in difference score are the main criteria when a conditioned place preference is developed. Cohen's d was used as a measure of effect size.

RESULTS

CPP associated with previous sexual experience

Rats that had no previous sexual experience prior to conditioning (Naive) showed a significant increase in raw time spent in the reinforced chamber from pre-test (M= 259.3 sec, SD= 51.17) to post-test (M= 304.3 sec, SD=42.89) ($t(11) = -2.746$, $p= 0.009$, $d= -0.953$), an increase in preference score from pre-test (M=0.43, SD=0.04) to post-test (M= 0.50, SD=0.06) ($t(11) = -2.573$, $p=0.012$, $d= -1.07$) and a significant decrease in difference score from pre-test (M=69.17, SD=47.87) to post-test (M=4.83, SD=75.49) ($t(11) = -2.507$, $p= 0.014$, $d= 1.01$).

Rats that had 1 sexual experience prior to conditioning also showed a significant increase in raw time in the reinforced chamber from pre-test (M=265.6 sec, SD=49.45) to post-test (M=370.3, SD=86.19) (t (11) = −4.183, p=0.000, d= −1.49), an increase in the preference score from pre-test (M=0.407, SD=0.06) to post-test (M=0.518, SD=0.114) (t (11) = −3.157, p=0.004, d= −1.19) and a significant decrease in difference score from pre-test (M= 120.0, SD=86.35) to post-test (M=-27.79, SD=163.8) (t(11) = -2.928, p=0.006, d= 1.12).

Rats that had 5 sexual experiences prior to conditioning did not show a significant increase in both the raw or preference scores and no significant decrease in the difference scores (FIG 1-3).

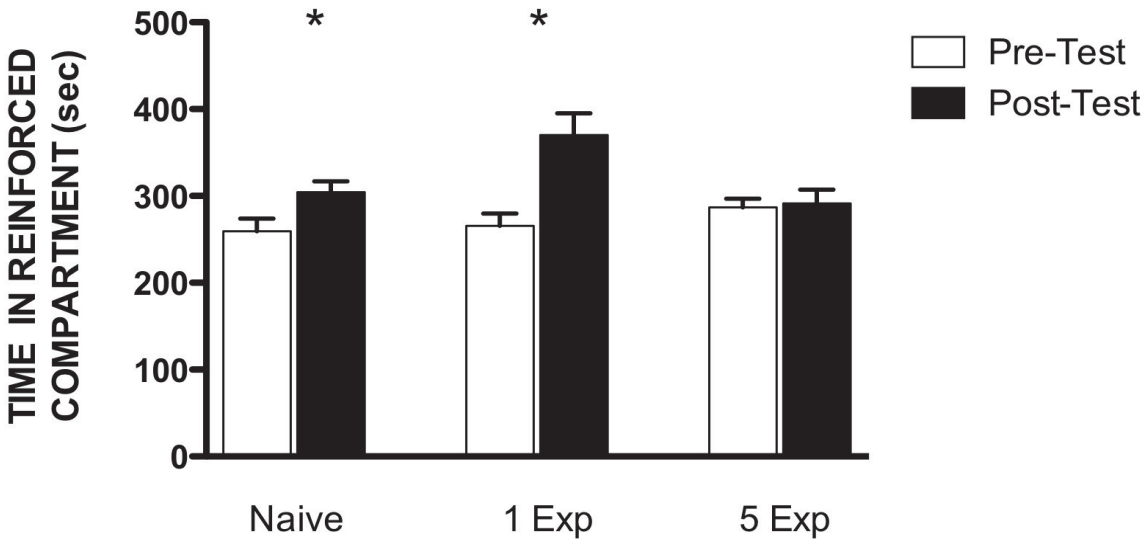

Figure 1.

Time spent in the reinforced compartment between pre-test (white bars) and post-test (black bars) in the conditioned place preference paradigm after zero (Naive), 1, or 5 previous sexual experiences. Pre-test and post-test were compared in all three sexual experience groups. Data are expressed as mean +SEM (N=12 for each group). *Different from the pre-test, $p<0.05$.

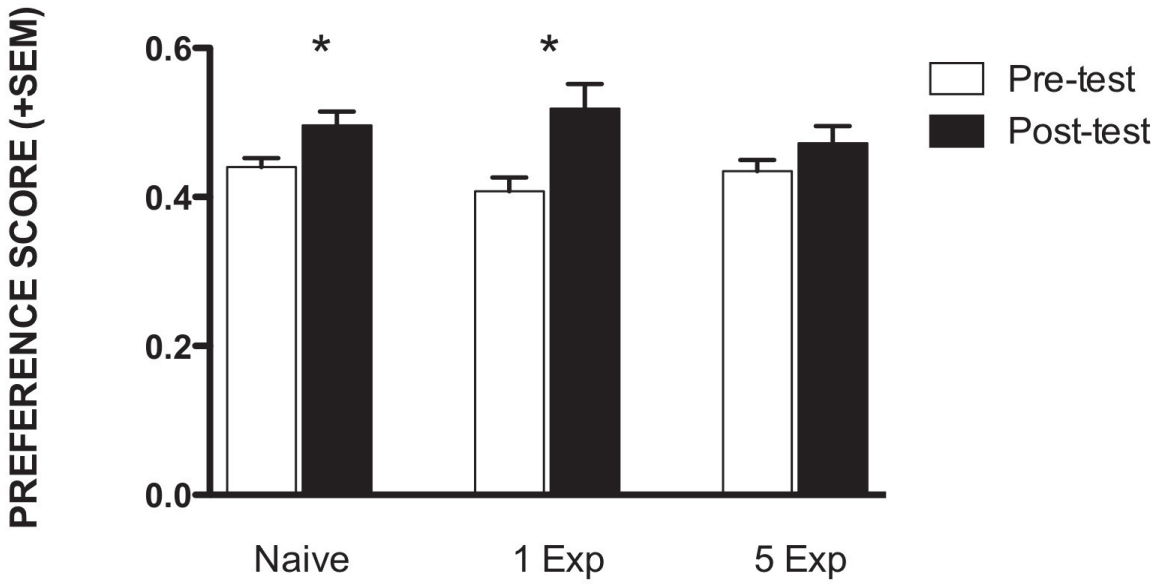

Figure 2.

Preference scores between pre-test (white bars) and post-test (black bars) in the conditioned place preference paradigm after zero (Naive), 1, or 5 previous sexual experiences. Pre-test and post-test were compared in all three sexual experience groups. Data are expressed as mean +SEM (N=12 for each group). *Different from the pre-test, $p<0.05$.

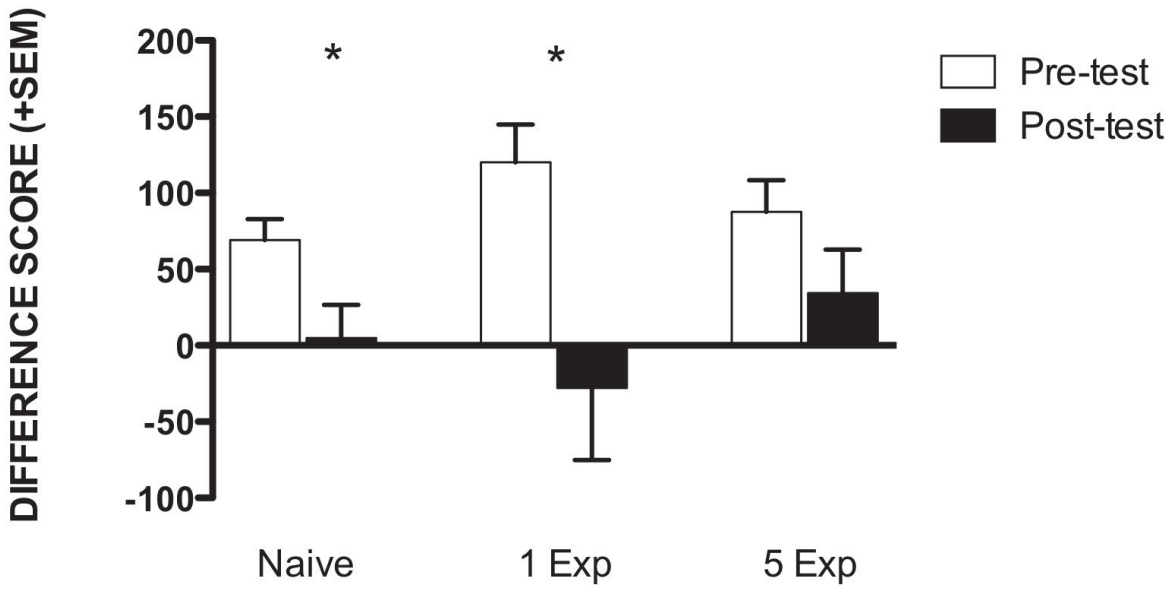

Figure 3.

Difference scores between pre-test (white bars) and post-test (black bars) in the conditioned place preference paradigm after zero (Naive), 1, or 5 previous sexual experiences. Pre-test and post-test were compared in all three sexual experience groups. Data are expressed as mean +SEM (N=12 for each group). *Different from the pre-test, $p<0.05$.

DISCUSSION

We have shown previously that external CLS in sexually naïve female rats induces significant CPP (Parada et al., 2010), whereas external CLS in the presence of an inaccessible male results in conditioned partner avoidance (Parada et al., 2011). CLS in the latter study induced females to solicit the inaccessible male, indicating that its signaling value changed from a reinforcer in its own right, to a promoter of appetitive responding in the presence of male cues. The present study asked whether copulatory experience could also change the value of CLS. Females that received 5 prior sexual multi-ejaculatory copulatory experiences in a bilevel chamber did not develop a significant CPP to external CLS, whereas females with either no prior copulatory experience, or those with 1 multi-ejaculatory experience, developed significant CPP to external CLS. Thus, copulatory experience in which females receive auditory and olfactory stimulation from males, flank stimulation from male mounts, external CLS from male pelvic thrusts, and internal clitoral and cervical stimulation (VCS) from male intromissions and ejaculations, appears to devalue external CLS as a reward. One experience, however, is not sufficient to devalue the rewarding effect of external CLS, suggesting that a threshold amount of this compound stimulation must occur.

Based on these and previous data we speculate that clitoral stimulation is an important reinforcer for the development and expression of sexual behaviour in female rats, similar to the way that penile stimulation is important for the development and expression of sexual behaviour in male rats (Beach, 1946; Manzo et al., 2008; Whalen, 1961). Impairments in male copulatory behaviour occur as a result of disruptions in the processing of penile stimulation such as anosmia, castration, and penile deafferentiation or anesthesia. Male rats under penile

anesthesia with lidocaine show significant impairments in their ability to mount and intromit, however, once they have gained a sufficient degree of sexual experience with penile stimulation lidocaine treated rats are no different from controls (Pfaus et al., 2012).

Our current data are similar to earlier work using clitoral anesthesia. Naive ovariectomized female rats received 5 consecutive injections of 2% lidocaine to the clitoris immediately prior to being placed in a paced mating chamber with a sexually vigorous male partner. Females in this group displayed significantly less time with males during copulatory sessions, increased number of entrances/exits, and a significantly increased contact return latency to ejaculations compared to females that received an injection of saline and no injection controls. In contrast, females that received injections of saline, which were subsequently reversed to lidocaine for a second phase of copulatory sessions, showed no effect of lidocaine on sexual behaviour and pacing compared to the former lidocaine treated animals (Parada, Sparks, Censi, Pfaus, in prep). Previous sexual experience blocked clitoral anesthesia from interfering with the normal pacing repertoire of the rat. This demonstrates that the sensory stimulation received from the clitoris during copulation has important contributions to the development of sexual behaviour in females however, once this behaviour is crystallized, its importance as a singular entity is reduced.

Previous experiments using CLS have typically involved sexually naive animals and have demonstrated robust effects of clitoral stimulation on the development of both place preference and partner preference paradigms (Parada, et al., 2010; Parada et al., 2011). Clitoral stimulation for the naive animal represents a reward signal strong enough to induce a positive hedonic state that can be

associated with environmental and partner stimuli. Once experienced, CLS transitions from a reward signal to a signal of arousal, suggesting that there is a developmental trajectory for CLS in the female rat. Clitoral stimulation during sexual experience, which includes the full compliment of male stimuli such as flank, rump, and tail stimulation, becomes a much richer source of sexual stimulation in the female. With experience, the clitoris loses its place as only a reward related signal.

In males, sexual experience also acts as a protective factor against treatments that disrupt sexual behaviour. For example, sexually naive male rats placed in a novel environment typically show reductions in mounts, intromissions, and ejaculations. A large proportion of them do not copulate. Having extensive sexual experience prior to exposure to a new environment drastically diminishes this effect (Pfaus, Wilkins, 1995). Sexual experience also seems to be protective against the stressful effects of acute predator odour exposure and other stressors (Spritzer, Weinburg, Viau, Galea, 2009; Edinger, Frye, 2007; Westenbroek, Snijders, den Boer, Gerrits, Fokkema, Ter Horst, 2005). In their study, Pfaus and Wilkins (1995) showed that administration of opioid receptor antagonist naloxone to sexually naive male rats facilitated sexual behaviour similar to having several pre-exposures to the novel environment. Naloxone had no effect on sexually experienced males. They suggest that exposure to novelty induces the release of endogenous opioid activity that leads to the disruption of sexual behaviour.

Endogenous opioids released during sexual behaviour are critical for conditioning of both place and partner preferences in male rats (Parra-Gamez, Garcia-Hidalgo, Salazar-Juarez, Anton, Paredes, 2009; Agmo, Gomez, Irazabal, 1994, Szechtman, Hershkowitz, Simantov, 1981; Ismail et al., 2011; Agmo, Berenfeld, 1990). Exogenous administration of naloxone has been shown to prevent

the acquisition of conditioned level changing in bilevel chambers and conditioned ejaculatory preferences in male rats (Van Furth, Wolterink-Donselaar, van Ree, 1994, Ismail et al., 2009). Naloxone also reduces instrumental responses for a female presented under a second-order schedule of reinforcement (Everitt, 1990) and abolishes previously acquired conditioned place preference for a sexually receptive female (Hughes, Everitt, Herbert, 1990). In females, naloxone injections block the expression of conditioned place preference induced by paced mating (Paredes, Martinez, 2001). We have also shown that naloxone administration prior to conditioning trials that pair paced mating with a male bearing a neutral odour prevents the development of a conditioned partner preference and reduces the number of solicitations overall, despite the females being fully primed with steroid hormones (Coria-Avila, Solomon, Vargas, Lemme, Ryan, Menard, Gavrila, Pfaus, 2008). These studies demonstrate that opioids play a strong role in the development of conditioned associations that are relatively resistant to extinction. It has further been shown that the sites of action where this effect takes place include the medial preoptic area (mPOA), the ventromedial hypothalamus (VMH), and the amygdala (Me) (Garcia-Horsman, Agmo, Paredes, 2008).

The mPOA is a critical site for the control of solicitations in female rats (Graham, Pfaus, 2010; Hoshina et al., 1994), and appears to integrate both genitosensory and chemosensory cues in females to generate sexual desire (Pfaus, 2009). We have shown that the mPOA is activated in response to distributed clitoral stimulation that can induce a significant CPP (Parada et al., 2010). This site is also important for the induction of CPP in response to artificial vaginocervical stimulation (VCS) (Meerts & Clark, 2009). Bilateral ibotenic acid lesions of the mPOA result in a failure to display significant CPP for VCS (Meerts & Clark, 2009). It is possible that

with enough pre-exposure to copulation, permanent changes occur in the mPOA through opioid release that crystallize the ability of contextual or partner cues to prime the reward state. This explanation is reminiscent of the phenomenon of sensory preconditioning (Seidel, 1959), in which experience with an unconditioned stimulus in the absence of a particular neutral cue makes it difficult to condition a subsequent association between the two. Females in the current study were exposed to full (internal and external) CLS and VCS through mounts with intromission and multiple ejaculations with males during copulation in bilevel chambers. Assuming that the bilevel chambers had become the conditioned contextual stimulus (CS) associated with copulatory stimulation of the clitoris and cervix, it would be difficult to then associate a new context with external CLS alone and have it be of sufficient intensity to acquire significant predictive value.

Similarly, once the reward from full CLS and VCS is experienced during copulation with a sexually vigorous male (who also provides potentially attractive olfactory and auditory stimulation to the female), manual stimulation of the external clitoris is no longer of sufficient intensity to induce a reward state. Indeed, CLS applied in the presence of a sexually vigorous male (that provides both unconditioned olfactory and auditory stimuli to the female) may well change it's meaning entirely. In our previous study (Parada et al., 2010), we noticed that CLS in the presence of an inaccessible almond-scented male behind a wire-mesh screen elicited full solicitations directed toward the male (headwise orientation to the male followed by hops or runaways; McClintock, 1984; Pfaus et al., 1999). However, in a subsequent partner preference test, the females chose to solicit and receive ejaculations from the unscented male over the scented male, suggesting that solicitations that are not followed by chasing and copulation on the part of the male is

aversive. Importantly for the present analysis, CLS in that study may have induced a state of sexual arousal and/or desire, but not reward per se. In contrast, CLS applied to sexually naïve females in the presence of a neutral almond odour on gauze generated a subsequent partner preference for the male bearing the odour during their first copulatory experience. Taken together with the present results, we suggest that copulatory experience de-values the salience of external CLS as a reward, and that male cues appear to redefine it as a stimulus of sexual arousal or desire.

General Discussion

Despite a considerable amount of anecdotal evidence, it is only recently that scientific studies have begun to address the role of the clitoris in the sexual function of humans and animals. The overarching goal of this thesis was to use an experimental approach to study the role of the clitoris and clitoral stimulation in female sexual behaviour in the rat, and most particularly its contribution to sexual reward.

The experiments described in Chapter 1 examined the effect of eliminating sensory stimulation arising from the clitoris on the sexual behaviour of female rats by administering lidocaine, a local anaesthetic. Clitoral stimulation is one form of tactile stimulation that female rats receive from mounts and intromissions with pelvic thrusting. The ability of female rats to control or pace this stimulation leads to a positive reward state that induces both CPP and sexually-conditioned partner preferences (Coria-Avila et al., 2006; Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005; Oldenburger, Everitt, & de Jonge, 1992; Paredes & Alonso, 1997; Paredes & Vazquez, 1999). We predicted that blocking clitoral sensation during copulation in naive female rats would impair proceptive behaviour. Contrary to our prediction we found no effect of our manipulation on proceptive behaviour although it did produce changes in pacing behaviour and reduced the total time that lidocaine treated females spent with males relative to VEH and CNTL groups. The differences in behaviour induced by local anaesthesia of the clitoris did not persist in the absence of lidocaine. By theoretically “turning off” clitoral sensory input during copulation two things might be occurring: First, the remaining cervical stimulation overrides what was previously a combination of clitoral and cervical stimulation and induces an inhibitory effect, since repeated cervical stimulation has been shown to result in an

inhibition of sexual behaviour in females (Pfaus, Smith, Byrne, & Stephens, 2000). Second, there is an overall lessening of reward during copulation in females treated with lidocaine given that those females are spending less time in close proximity to their male counterparts. Although clitoral stimulation is a source of rewarding sensory information, it is not the only source since we did not see an overall reduction in solicitation frequency, hops/darts, or latency to solicit the males. We conclude that clitoral stimulation is an important component to the reward value of paced copulation.

Both sexually experienced and sexually naive females were included in the studies described in Chapter 1. Local anesthesia of the clitoris had different effects on pacing behaviour in these groups. Females that were sexually naïve during phase 1 displayed a higher number of exits and entrances to and from the male's side of the pacing chamber and significantly less time in close proximity of the male compared to control females. Sexually experienced females in phase 2 did not display any effects of lidocaine on sexual behaviour. This suggests a stronger contribution of clitoral sensation to the sexual behaviour of naïve females. This supposition was confirmed in studies described in Chapter 5, the results of which showed that 5 consecutive sexual experiences prior to CLS paired with CPP blocks the development of CPP. The question brought forward from these studies is, what is clitoral stimulation to the naive female? Based on the findings presented in chapters 1 and 5, once sexually experienced, we hypothesize that clitoral stimulation transforms from a reward signal to a signal of arousal. Females in the sexually experienced group displayed small changes in preference scores and difference scores in the same direction as those in the sexually naïve group even though those changes were not significant. Copulation with a male partner that includes a variety

of tactile and olfactory stimuli, including clitoral stimulation, becomes a much richer source of sexual stimulation for the female. It was shown that females with a small amount of previous sexual experience still develop a significant place preference, suggesting that clitoral stimulation received on its own still has a reward value. With greater previous sexual experience CPP is no longer displayed. Vaginocervical stimulation is also rewarding enough to induce CPP in sexually naïve females (Meerts & Clark, 2009). This suggests that with increasing copulatory experience the clitoris loses its place as a reward-related signal only and becomes integrated with other sources of genitosensory information (i.e. VCS, flank, tailbase). In males, penile sensory information is critical to the development of typical patterns of copulatory behaviour. This has been demonstrated through the use of topical lidocaine application to the penis in sexually inexperienced males. Rats treated with lidocaine show impairments in the frequency of mounts intromissions and ejaculations, however those that are sexually experienced prior to lidocaine treatment show no impairments in sexual behaviour (Pfaus et al., 2012). The current findings suggest that clitoral and penile sensory stimulation follow similar developmental trajectories.

Although difficult to examine in humans, the development of sexual experience that begins with external clitoral stimulation during early masturbation to external and internal clitoral stimulation during sexual intercourse may follow similar trajectories in the rat. In humans, reports of sexual satisfaction based on rates of masturbation versus penile-vaginal intercourse in women reveal that the frequency of masturbation is inversely associated with sexual satisfaction, whereas the frequency of penile-vaginal intercourse is directly and strongly associated with sexual satisfaction (Brody & Costa, 2009; Philippsohn & Hartmann, 2009). Recent

reports of solitary-masturbatory versus average sex-with-partner orgasms in women reveal that masturbatory orgasms are less flushing in sensation, and generate less general spasms, pleasurable satisfaction, emotional intimacy (in the presence of a partner), and ecstasy, compared to their descriptions of sex-with-partner orgasms (King, Belsky, Mah, & Binik, 2010). These data may be an indication that stimulation of the clitoris alone loses potency as a reward once women have had penile-vaginal intercourse; however there was no direct comparison in women of clitoral stimulation and quality of masturbation prior to and following experience with penile-vaginal intercourse, so caution must be taken when interpreting those findings. Further, there are many other socio-cultural factors that must be taken into account with reports of human sexual satisfaction; however these studies did attempt to separate reports of sexual satisfaction from reports of relationship satisfaction.

Animal studies allow the experience of reward to be assessed using well-validated paradigms such as CPP, in which the rewarding value of a stimulus is inferred from a change in the amount of time that rats will spend in an environment that has previously been associated with that stimulus. This approach was used in the experiments described in Chapter 2. The results demonstrated that rats increase the time that they spend in an area where they have received clitoral stimulation, and that a preference for this environment is dependent on the pattern of clitoral stimulation they receive. This clearly indicates that clitoral stimulation has reward value. Using the same conditioning paradigm we examined the effect of hormonal state of the female on the reward value of clitoral stimulation and found that in contrast to sexual behaviour itself, the reward value of clitoral stimulation did not depend on level of estrogen and progesterone. Because females of many species are sexually responsive only at particular points of the hormonal cycle, it has been

assumed that sexual reward can only be experienced during these periods of sexual receptivity. The current evidence suggests that estrogen and progesterone are important for setting up the behaviours and postural adjustments necessary to receive reward but that the reward from clitoral stimulation can be experienced regardless of hormonal state.

There is evidence that in humans, levels of sexual desire and arousal fluctuate across the menstrual cycle (Bancroft, Sanders, Davidson, & Warner, 1983; Van Goozen, Wiegant, Endert, Helmond, & Van De Poll, 1997). Levels of sexual desire and the frequency of sexual behaviour and orgasm all increase during the ovulatory phase of the menstrual cycle that coincide with elevations in estrogen and progesterone (Stanislaw & Rice, 1988; Puts et al., 2012) however, it is well known that in humans, sexual behaviour and reward can still be experienced the menstrual cycle. Research on postmenopausal women show stable rates of masturbation and a full capacity to experience orgasm following menopause without hormone replacement, while rates for partnered sexual activity decrease (Avis et al., 2009; Cain et al., 2003; Penteado, Fonseca, Bagnoli, Assis, & Pinotti, 2004). Increased frequency of masturbation could be to compensate for decreased interest in partnered sexual activity. It is possible that masturbation in this population is solely for clitoral stimulation; however specific descriptions of the type of stimulation are not reported. Although other social or biological factors may play into the decrease in rates for partnered sexual activity (ex. reports of pain experienced through partnered sex), it is clear that sexual gratification can continue to be experienced independently of hormones in humans and in rats.

Chapter 3 asked whether CLS was a stimulus with strong enough hedonic value that it could be used to condition a positive association with a neutral odor and

induce a subsequent sexual partner preference for a random male bearing the odor. Females in the first experiment received 10 alternating trials of CLS and no-CLS in the presence of an inaccessible male. For one group, the inaccessible male paired with CLS was bearing a neutral almond odour whereas for the second group males were without odour. Following 10 trials (5 in each condition), females were allowed the choice of copulating with two sexually vigorous males, one scented and one unscented. Females that received CLS in the presence of an inaccessible scented male would show a preference for those males when given the choice to copulate between a scented and unscented male partner. It was unexpected that females in the CLS-scented group displayed a preference for males that were not associated with CLS since we had previously shown that female rats develop CPP for a distinctive environment paired with CLS (Parada, Chamas, Censi, Coria-Avila, & Pfaus, 2010). As per conditioning that developed in our CPP study in chapter 2, this effect would not extend to conditioned partner preference, however during observations of the conditioning sessions females did display solicitation behaviour following CLS demonstrating that females were, in fact, sexually receptive towards the inaccessible males. In experiment 2, a new group of females received the same conditioned pairings of CLS and odour, but here the odour was presented on a piece of cotton gauze in the chamber. This time, during the open field test, females in the CLS-scented group selectively solicited the scented male. Our conclusion was that CLS given in the presence of an inaccessible partner created a sexual inhibitory state for females, which prevented conditioning to the males associated with the almond odour. Given that paced copulation has been shown to induce a significant partner preference for males bearing a neutral almond odour (Coria-Avila et al., 2005, 2006) we hypothesize that blocking the ability to pace sexual interaction

following stimulation of the clitoris acted as a negative reinforcer of clitoral stimulation. Therefore, the use of clitoral stimulation as a positive reinforcer for conditioning is context-dependent. Clitoral stimulation induces sexual desire whether in the context of an inaccessible male or not. However, the inaccessibility of the male partner prevents CLS induced sexual desire to be fulfilled with successful copulation that is typical following solicitations. Therefore, CLS can be a signal of upcoming sexual activity or inactivity depending on the context. In addition, the process that takes place when female rats are learning about potential copulatory partners is not static. In our experiment, we successfully blocked the ability of the females from interacting with males during the conditioning phase. While the rats were still able to receive visual, auditory, olfactory, and extremely minimal tactile cues, the mesh wall did not allow for any flank, tail, rump, or genital stimulation from the male. Our findings suggest that active physical interaction with a male partner (chasing and being chased) is an important part of the sexual learning experience for female rats. This experiment demonstrates that manual CLS possesses a strong enough reward value that receipt of this form of stimulation by itself is enough to set off a cascade of events involving enough activation of sensory afferents leading to mPOA activation that is then blunted by preventing interaction with the male partner and is expressed at the behavioural level. We suggest that activation of the mPOA leads to an increase in sexual arousal that is then “frustrated” by the inability to interact with the male. The mPOA is an important integrator of reward and reproductive signals (Pfaus et al., 2010) and lesions of this region eliminate appetitive sexual behaviours including solicitations and hops/darts in addition to disruptions in the display of pacing (Hoshina et al., 1994). Other forms of sexual stimulation known to induce a significant CPP, like VCS (Meerts & Clark, 2009), have yet to be tested using the

paradigm adopted in experiment 1 of chapter 3. The use of a paradigm such as this would help us to better understand the contributory magnitude of each form of tactile stimulation involved in sexual behaviour of female rats.

Chapter 4 extended the focus on the reward value of sexual stimulation through an examination of the sexual reward state induced by paced mating and manual clitoral stimulation and its dependence on estrogen and progesterone. In the first experiment a significant CPP was expressed for paced copulation regardless of the hormonal state under which the females were tested. At the time of expression paced copulation was still a strong inducer of sexual CPP whether under the influence of EB+P, EB-alone, or oil control. However, following a four-day period, when females were tested a second time under the same hormonal conditions only females in the EB+P group failed to show significant CPP. The second experiment tested the dependence of sexual reward on estrogen and progesterone during the acquisition phase of conditioning. Females received CLS while under EB+P, EB-alone, or oil control paired with one side of a conditioned place preference chamber. A significant CPP developed under all three hormonal conditions. We speculate that the desire for sexual reward, driven by hormonal priming of EB+P, stimulates an excitatory state that results in faster extinction of the previously developed place preference if the reward is not presented. However, in hormonal states where there is little or no desire for sexual reward and reward is not presented extinction does not occur as quickly. Previous work in female rats has demonstrated that EB+P facilitates extinction of behaviours not associated with reward (Milad, Igoe, Lebron-Milad, & Novales, 2009) indicating that EB+P might alter extinction learning through a mechanism independent of the presence/absence of reward. Milad et al., (2009) describe an EB+P facilitation of extinction to fear conditioning. It is well established

that P possess anxiolytic effects when metabolized into alloprenanolone and that these effects are mediated by the GABA-A receptor (for review see Andréen et al., 2009). Further, progesterone receptor agonists, such as the one used in Milad et al., (2009) study mifepristone, blocks glucocorticoid receptors, which are hypothesized to be involved in fear extinction (Engin & Treit, 2007; Gourley, Kedves, Olausson, & Taylor, 2009; Yang, Chao, & Lu, 2006). Thus, extinction of fear conditioning by ovarian hormones may occur by an entirely different mechanism than the one observed in chapter 4, as there is no obvious fear state associated with the extinction of a paced copulation-induced CPP.

The findings of Chapter 4 lend support to Pfaff's (1999) theory that drive states like female sexual motivation or desire are hormone-sensitive and direct behaviour by lowering the threshold to respond to sexual stimuli. They further indicate that the reward value of sexual stimulation from CLS is independent of estrogen and progesterone. We argued that these findings might serve as an explanation for the inconsistency that postmenopausal women without hormone replacement continue to achieve orgasm through masturbation while rates of desire for partnered sexual activity diminish (Cain et al., 2003). Cain et al. explain that these ratings do not seem to be linked to satisfaction with one's sexual relationship therefore suggesting that the differences in behaviour are linked specifically to changes in aspects of sexual functioning. Unfortunately, studies that focus on the sexual practices of women before and after menopause, especially those that focus on

desire for and frequency of different types of sexual stimulation, are severely lacking. Although it would be possible to conduct a study of this kind, the difficulty in tracking sexual behaviour throughout the lifespan, and in particular during such a transitional period in women's lives, is a likely the reason why one has not yet be conducted.

Still, it has been demonstrated in one study that 68% of women continue to be sexually active during menopause and that enjoyment of sex is not dependent on whether participants are under hormonal therapy (Hess et al., 2009).

Chapter 5 demonstrated that clitoral stimulation induces reward strong enough for the development of a conditioned place preference but only when females are sexually naive. Female rats given 5 previous sexual interactions with a sexually experienced male did not develop a conditioned place preference when given manual CLS in a place preference chamber. However, females allowed a small degree of previous sexual experience were still able to develop a significant CPP to CLS.

When female rats engage in their first copulatory interactions, they are exposed to an array of visual, auditory, olfactory, and tactile cues provided by copulatory partners. These cues serve to reinforce proper sexual behaviour that will lead to successful outcome such as reward and pregnancy. This is based on the knowledge that pacing behaviour is also rewarding and that proper spacing of genital stimulation leads to hormonal responses that lead to pregnancy. These responses are less likely to occur when pacing is not allowed/blocked (Paredes & Vazquez, 1999; Coria-Avila & Pfaus, 2007; Coopersmith & Erskine, 1994). It is plausible that during the development of sexual behaviour the clitoris is more sensitive to tactile stimulation and that distributed CLS helps to hone the females' ability to pace the receipt of the stimulation that she receives from the male. As females gain more sexual experience the continued receipt of both external and internal clitoral and cervical stimulation from mounts and intromissions devalues the type of external manual CLS used in the present experiments as a reward in its own right. Since a minimal amount of sexual experience was not enough to block the development of

CPP, this suggests that a threshold amount of experience is necessary for clitoral stimulation to lose its reward value. This finding is not restricted to females, male penile stimulation seems to follow the same stimulation dependent change. Pfaus et al. (2012), showed that penile anesthesia using lidocaine impaired the ability of sexually naive males to mount, intromit and ejaculate. These impairments were partially blocked with intermediate levels of previous sexual experience and completely blocked with 10 sessions of previous sexual experience. With one previous sexual experience lidocaine still completely impaired their ability to copulate. Three sexual experiences increased mounting by 70% and intromissions by 20% however ejaculations were still completely impaired. By 5 experiences mounts had completely recovered and intromissions reached about 80%. Only approximately 40% could ejaculate however (Pfaus et al., 2012). The threshold for females seems to be lower than that of males if we compare these aspects of male sexual behaviours to our findings from chapter 5. The findings presented in Chapter 1 further support this comparison.

In Chapter 1, females with 5 sexual experiences prior to treatment with lidocaine showed no significant impairments in their ability to pace sexual stimulation from a sexually experienced male. These findings reinforce the profound effect that sexual experience has on physiology, making animals resistant to disruptors of sexual behaviour (Pfaus et al., 2012). Learning the appropriate behaviours to gain access to sex partners is so crucial to successful reproduction that it renders the high reward value of clitoral and penile stimulation in naive animals a necessary part of this learning process. The experiments conducted in Chapter 5 are the first to show the importance of sexual experience in female rats and the first demonstrate

that sexual experience in females has equal importance to sexual experience in males in the development and expression of sexual behaviour.

Overall, an underlying theme that emerges from this thesis is that the sensory information from the clitoris can take on several meanings depending on the context within which it is experienced and the previous sexual experience of the animal. In Chapter 2, we clearly demonstrated that pairing distributed stimulation with one side of a conditioned place preference chamber had enough incentive salience that rats showed the development of a preference for that side of the chamber. This finding established that the clitoris is an organ that transmits rewarding sensory stimulation to the brain. We have subsequently tested the minimum number of sessions that rats would require to develop a significant CPP to CLS 5 and we have learned that only 2 pairings of CLS 5 with one side of the chamber are needed to express conditioning (see Appendix 1). Therefore, CLS is a strong inducer of CPP and acts as a signal of reward.

The reward signal from CLS is likely expressed in several brain regions, although it's value in inducing solicitations likely depends on its activation of the mPOA (Graham & Pfaus, 2010, 2012; Hoshina et al., 1994). CLS-induced Fos in this region was distinct from that reported from other forms of genital stimulation (e.g., VCS) which activates a separate sub-region of this area (Pfaus et al., 1993). Greater amounts of CLS may act also as a trigger of hormonal responses necessary for the induction of pregnancy. CLS 1 was found to induce more Fos-IR in the MeApv compared to CLS 5 and CTRL. This region is known to be involved in the initiation of pseudropregnancy and has been suggested as a modulator of neuroendocrine and behavioural responses that are activated by VCS (Coopersmith et al., 1996a,b; Pfaus et al., 1996). Thus, high enough amounts of CLS may also

serve a similar purpose making it not only a signal of reward but also, a signal of endocrine changes that are necessary to induce pregnancy. Vaginocervical stimulation, either from a male during paced copulation, or received manually in a distributed manner, leads to a fast termination of estrus (Lodder & Zeilmaker, 1978; Pfaus et al., 2000). Although Chapter 1 provides evidence that CLS alters pacing behaviour in female rats, it is still unknown whether an optimal amount of CLS would also lead to estrus termination. If not, then CLS may not be activating the inhibitory part of the pseudopregnancy/pregnancy pathway. Activation of glutamate neurons in the ventrolateral VMH is a critical component of the termination of estrus induced by VCS (Georgescu & Pfaus, 2008a, b; Georgescu, Cyr & Pfaus, 2012; Georgescu et al., 2010). Although CLS activates Fos in the VMH, the pattern of activation is scattered and not restricted to the ventrolateral region where the critical set of glutamate neurons reside (Appendix 4). Thus, CLS may well activate only the excitatory aspects of copulatory responding.

The context in which CLS occurs can modify the “meaning” of the stimulation as either a reward in its own right or a signal for upcoming sexual activity. In Chapter 3, CLS 5 was given to females in the presence of either an unscented or scented inaccessible male versus no stimulation in an attempt to induce a conditioned partner preference. Following a final preference test females were given the opportunity to mate with a male of their choice, scented or unscented. Females in the CLS-SC group displayed an overall preference for the male not associated with CLS. In the second experiment, we demonstrated that removal of the male from the conditioning sessions and replacement with only the scent or no scent placed on gauze induced a conditioned partner preference when that scent was placed on a copulatory partner in the final preference test.

An analysis of Fos-IR within the mPOA compared activation in response to CLS 5-alone, CLS paired with the previously neutral odour, CLS paired with the previously neutral odour on an inaccessible partner and no stimulation CNTL. Clitoral stimulation paired with a previously neutral odour induced significantly more Fos-IR compared to CLS-alone, CLS paired with an odour on an inaccessible partner and no stimulation controls. Clitoral stimulation on its own (CLS-alone) induced more Fos compared to no stimulation controls (See appendix 2 & 3). These data show that clitoral stimulation, although rewarding, can serve as a signal beyond that of pleasure and will induce a state of excitation that can itself be associated with upcoming sexual activity or alternatively, sexual non-activity. This association is strong enough to drive females to solicit males not previously associated with clitoral stimulation. The state of excitation has been transformed into a state of sexual inhibition.

Sexual experience can also act as a contextual variable for the female herself. The reward value of clitoral stimulation is reduced to a level that is not as significant when received on its own and not with the full compliment of stimuli received during copulatory behaviour with a male partner. In this context, clitoral stimulation becomes a promoter of appetitive responding in the presence of male cues.

Conclusion and future directions

The present results show that the clitoris transmits important genitosensory information from male copulatory contact. Clitoral stimulation can act as a signal of reward and a signal of upcoming sexual activity or non-activity, which in turn leads to the development of place and partner preferences. Rewarding CLS activates a specific region of the mPOA that differentiates it from other forms of genital stimulation. The fact that this occurred from distributed and not massed CLS

suggests that there is an optimal timing for the conduction of reward-related CLS. Optimal CLS combined with the overlap in sensory pathways that are also stimulated via the vagina during copulation (e.g., VCS) may link reward with reproduction, and thus be critical in the optimal expression of pacing behavior in the female rat. Further, the reward experienced from CLS in the rat is independent of estrogen and progesterone, which are only important for setting up the behaviours and postural adjustments needed to experience reward. Finally, sexual experience that includes CLS is important for sexual development in the rat and acts as a buffer for disruptors of sexual behaviour in experienced animals.

These studies have laid the groundwork to study the role of the clitoris in sexual behavior, sexual reward, and the formation of sexual partner preferences, As with any new scientific direction, the experiments have stimulated more questions than they have answered. For example, although the reward value of CLS is not dependent on estrogen and progesterone, is it possible that CLS during behavioral estrus could modulate cervical signals that induce estrus termination. Understanding the connection between the VMH, activated by VCS, and the mPOA, activated by CLS needs to be explored in more detail. An examination of Fos induction in the VMH in response to VCS during anesthesia of the clitoris would remove the reward signal from CLS when given during different phases of the estrus cycle, specifically at estrus termination.

There is also much more to be studied in terms of the neurochemical signals transmitted as a result of CLS during copulation. The activation of different neurochemical systems in the hypothalamus or limbic system during CLS (e.g., dopamine, glutamate, and GABA) needs to be explored using in-vivo microdialysis. Such experiments could help explain how the reward signal from CLS changes in

both sexually inexperienced and experienced animals. Given the lack of pseudorabies virus transfection into mesolimbic structures, notably the NAc, it is likely that any activation of dopamine transmission in that region would originate from mPOA efferents to the VTA (Tobiansky et al., 2012) that are activated by CLS. This method could also be used further to examine the termination of a copulatory bout once females reach estrus termination and what neurochemicals may play a role in this process.

Chapter 3 explored the development of partner preferences in response to CLS. Although partner preference in terms of selective solicitations was induced by CLS, an ejaculatory preference (mate choice) was not. This contrasts with the ability of female rats to display both solicitational and ejaculatory preference for a male bearing and odor that was paired previously with paced copulation, in which the female has received both CLS and VCS. One question to examine could address whether an ejaculatory preference would be induced by cervical stimulation alone or would full CLS with VCS bring about a mate choice? Perhaps CLS acts as a signal of arousal in addition to that of reward that mixes with cervical stimulation and the release of oxytocin to induce a mate choice.

The data from Chapter 4 show that the reward value of CLS is independent of estrogen and progesterone. This may help to explain an inconsistency in the literature showing that postmenopausal women without hormonal replacement that lose interest in partnered sexual interaction continue to achieve orgasm through masturbation (Cain et al., 2003). Because the experience of sexual pleasure and reward from CLS does not require circulating estrogen and progesterone, but sexual behaviour in response to partnered stimulation does, this would serve as a plausible explanation. Indeed, the desire for sexual gratification may not diminish without

hormone replacement, whereas the ability to engage in sexual interaction with a partner may be more susceptible. It would be necessary to conduct longitudinal studies on rates of sexual behaviour, including masturbation, in human females across all stages of sexual development, from a period prior to experience with penile-vaginal sexual intercourse, to pre and post menopause, to glean a comprehensive answer.

In summary, the clitoris of the rat, like that of a human, contributes important genitosensory input for sexual reward. It is hoped that the methods developed in the present thesis and the data they generated can be utilized and improved upon to ask further “human” questions in ways that rats can answer them.

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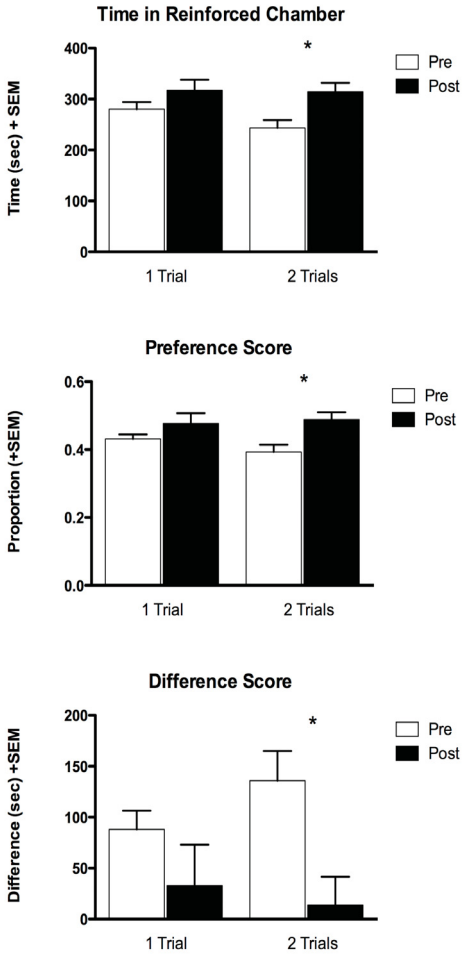
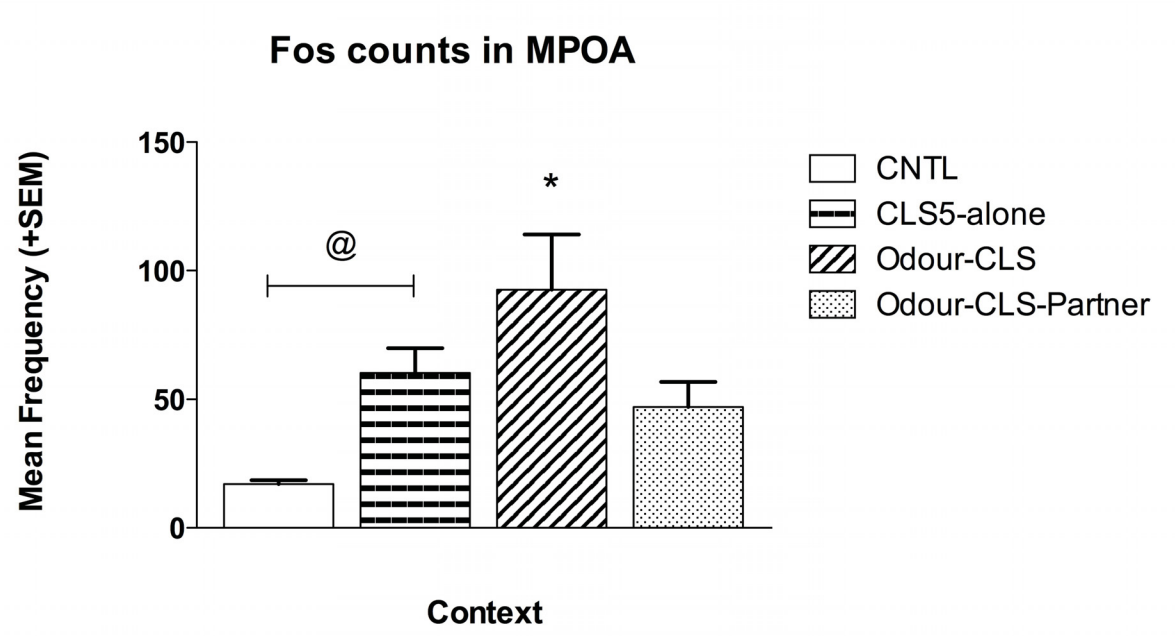
Appendix 1



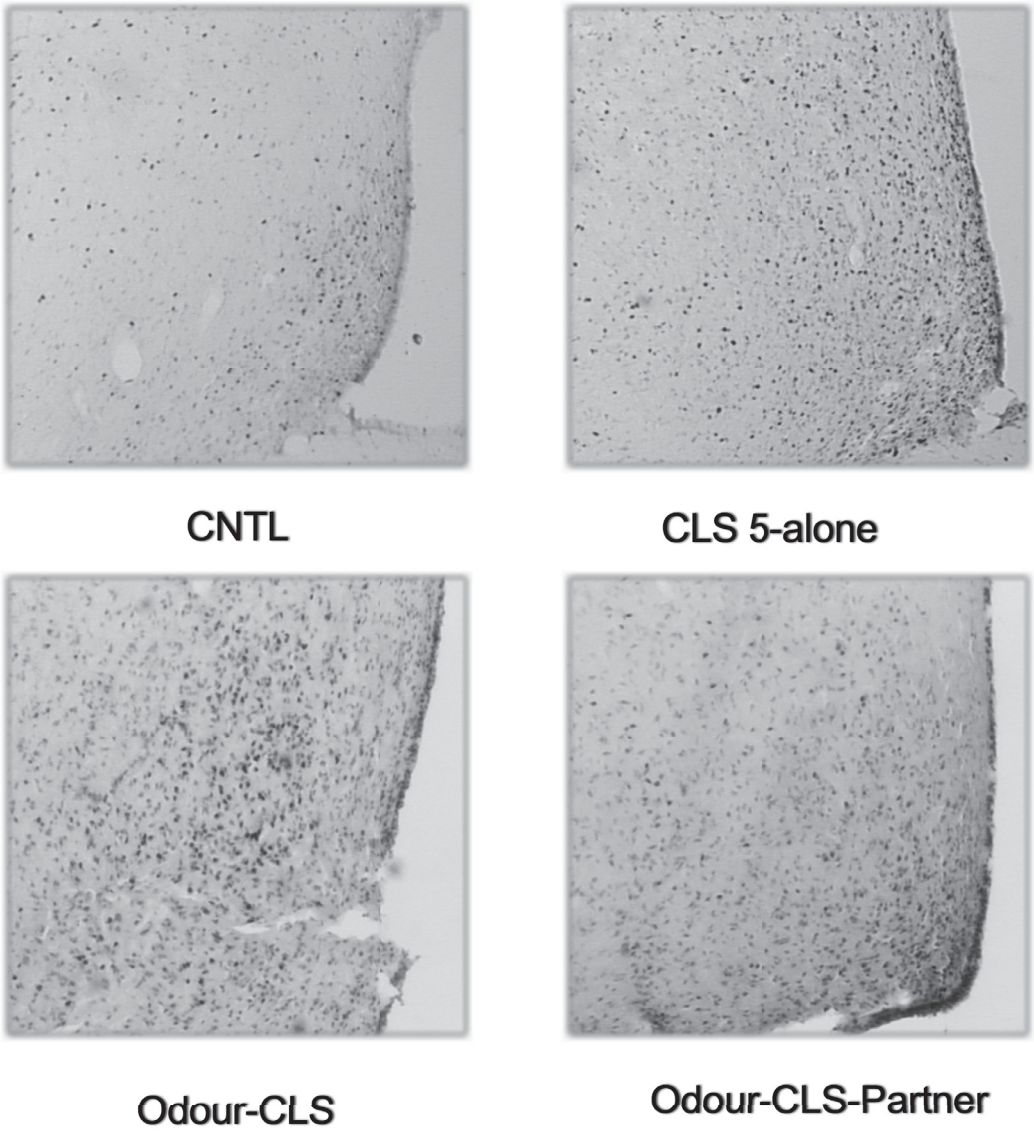
Mean (+ SEM) raw time in reinforced chamber (sec) (M=280.1, SD=48.38 1 Trial Pre; M=317.6, SD=70.91 Post) (M=243.6, SD=52.55 2 Trials Pre; M=314.5, SD=60.52 Post) (a.) preference score (M=0.43, SD=0.05 1 Trail Pre; M=0.47, SD=0.105 Post) (M=0.39, SD=0.07 2 Trials Pre; M=0.49, SD=0.07 Post) (b.) and difference score (M=88.00, SD=63.76 1 Trial Pre; M=32.92, SD=139.4 Post) (M=135.9, SD=101.1 2 Trials Pre; M=13.92, SD=95.87 Post) (c.) between pre-test (white bars) and post-test (black bars) for females following 1 trial of CLS 5 versus no stimulation and 2 trials of CLS 5 versus no stimulation (n=12 per group). Paired samples t-test was used to assess differences between pre and post-test. * Indicates significance at $p<0.05$.

Appendix 2



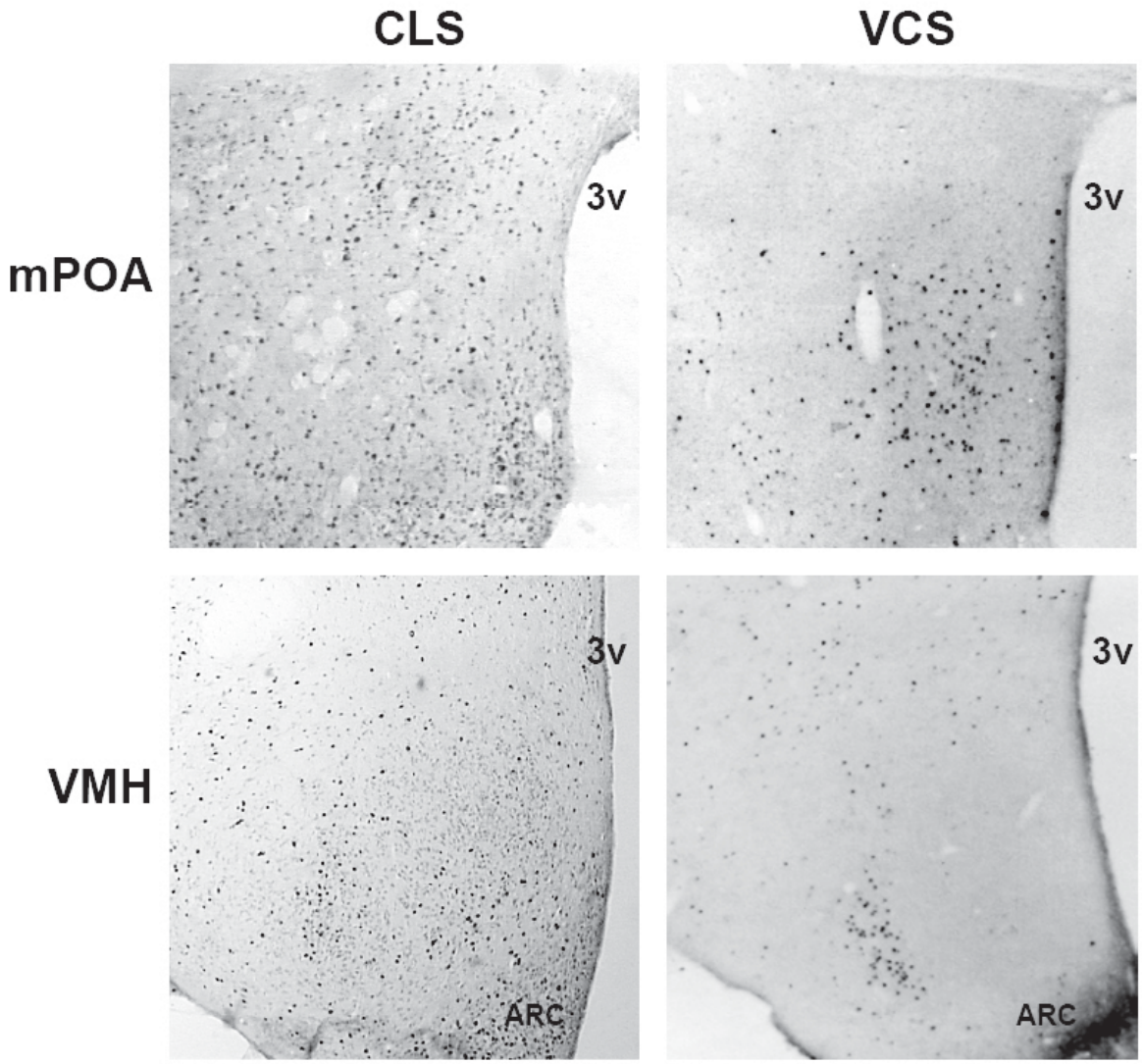
Mean number of Fos-IR cells/side (+SEM) in the medial preoptic area (mPOA) as a function of context within which CLS5 is received. 1-way ANOVA was used to examine differences in Fos counts between context conditions. The ANOVA detected a significant main effect of context $F(3,18)=6.87$, $p<0.05$. Post hoc comparison indicates a significant difference between Odour-CLS (n=5, M=92.57, SD=48.10), CNTL (n=6, M=17.05, SD=3.8), CLS5-alone (n=6, M=60.28, SD=23.75), and Odour-CLS-Partner (n=5, M=47.07, SD=21.72).

Appendix 3



Representative digitized images of the medial preoptic area (mPOA) stained for Fos-IR by immunocytochemistry under different CLS5 context conditions. Pictures were taken at 40X for CNTL (no stimulation), CLS5-alone (clitoral stimulation unpaired), Odour-CLS (clitoral stimulation paired with odour), and Odour-CLS-Partner (clitoral stimulation paired with odour on a partner) using a Leitz microscope.

Appendix 4



Representative digitized images of the medial preoptic area (mPOA) and ventromedial hypothalamus (VMH) stained for Fos-IR by immunocytochemistry in response to distributed clitoral stimulation (CLS) and vaginocervical stimulation (VCS). Pictures taken at 40X using a Leitz light microscope.