HORMONAL, EXPERIENTIAL, AND NEURAL REGULATION OF VENTROMEDIAL HYPOTHALAMIC GLUTAMATE IN THE FEMALE RAT

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ABSTRACT

Hormonal, Experiential, and Neural Regulation of Ventromedial Hypothalamic Glutamate in the Female Rat

Michaela Georgescu, PhD
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Previous experiments have shown that enhanced glutamate neurotransmission in the ventromedial hypothalamus by administration of glutamate or its selective receptor agonists kainate, N-methyl-D-aspartic acid or α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), results in a pronounced inhibition of proceptive and consummatory sexual behaviors. The five chapters encompassing this thesis were designed to expand our comprehension of the role that ventromedial hypothalamic glutamate plays in the control of sexual behavior in the female rat. The experiment presented in Chapter 1 shows that a substantial proportion of the neurons activated in the ventromedial hypothalamus by vaginocervical stimulation are glutamatergic, and that priming with estradiol benzoate and progesterone inhibits this activation. Chapter 2 investigated the effect of 6,7-Dinitroquinoxaline-2,3-dione (DNQX), a glutamate antagonist, on estrus termination, when infused in the ventromedial hypothalamus of female rats. DNQX effectively reversed the effect of vaginocervical stimulation on appetitive behaviors, lordosis quotient and lordosis magnitude, but had no effect on pacing, defensive behaviors, or male ejaculations. These results suggest that blocking glutamate transmission at the AMPA/kainate receptor delays onset of

estrus termination.The experiment presented in Chapter 3 analyzed the pattern of glutamate release in the ventromedial hypothalamus during a 2-hour copulation session with a sexually vigorous male following priming with oil, estradiol benzoate or extradiol benzoate and progesterone. The results suggest that glutamate release patterns vary as a function of hormonal priming as well as stimulation received from being in proximity of a sexually mature male. Chapter 4 tested the hypothesis that glutamate release may be subject to regulation by γ-aminobutryic acid (GABA), a neurotransmitter with cellular inhibitory properties and facilitative effects on lordosis upon infusion in the ventromedial hypothalamus. The results indicate that $GABA_A$ Rα1 are upregulated by estradiol benzoate because the percentage of neurons that contained glutamate and expressed those receptors doubled when animals were primed with estradiol benzoate or estradiol benzoate and progesterone relative to oil. The experiment presented in Chapter 5 identified the medial preoptic area, anterior hypothalamic area, paraventricular nucleus, anterior hippocampus, and medial amygdala as the nuclei that send GABAergic projections to the ventromedial hypothalamus. These nuclei have previously been found to play important roles in the control of female sexual behavior. The function of ventromedial hypothalamic glutamate is explored in the general discussion.

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DEDICATION

I'd like to dedicate this thesis to my grandparents, Ecaterina Georgescu, Dr. Gheorghe Georgescu, Constantin Alexandrescu, and Viorica Alexandrescu, whose value of knowledge and education instilled a thirst for it in me. Thank you for raising me, and I'm sorry for not being there when you needed me most. I will forever try to live up to the noble values that you have tried to pass on to me and to the rest of us.

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List of Abbreviations

A-P	anterior-posterior
AHA	anterior hypothalamic area
AHipp	anterior hippocampus
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AP-5	(2R)-amino-5-phosphonopentanoate
ARH	arcuate nucleus of the hypothalamus
BNST	bed nucleus of the stria terminalis
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CSF	cerebrospinal fluid
D-V	dorsal-ventral
DA	dopamine
DB	diagonal bands
DAB	3,3'-diaminobenzidine
DHP	5 alpha-pregnan-3,20-dione
DHEAS	5-androsten-3 beta-ol-17-one sulfate
DMH	dorsomedial hypothalamus
DNQX	6,7-Dinitroquinoxaline-2,3-dione
DPDPE	[D-Pen^2,D-Pen^5]-enkephalin
Fx	Fornix
E	estrogen
EB	estradiol benzoate
ELISA	Enzyme-linked immunosorbent assay
P	progesterone
PS	5-pregnen-3 beta-ol-20-one sulfate
FG	FluoroGold
GABA	γ-aminobutryic acid
GAD	glutamate decarboxylase
GnRH	gonadotrophin-releasing hormone
HPLC	high performance liquid chromatography
HPLC-UV	high performance liquid chromatography-ultraviolet
IR	immunorective
LH	lateral hypothalamus
LHA	lateral hypothalamic area
LM	lordosis magnitude
LQ	lordosis quotient

M-L	medial-lateral
MCG	midcentral gray
MDMA	methylenedioxy methamphetamine
MeA	medial amygdala
mPOA	medial preoptic area
mRNA	messenger ribonucleic acid
N	sample size
NE	norepinephrine
NGS	normal goat serum
NMDA	N-methyl-D-aspartic acid
O	oil
OVX	ovariectomy/ovariectomized
OXY	oxytocin
PRV	pseudorabies virus
PVN	paraventricular nucleus
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SF1	steroidogenic factor-1
SPN	spinal neurons?
TBS	tris buffer saline
THP	5 alpha-pregnan-3 alpha-ol-20-one
TTX	sodium channel blocker tetrodotoxin
VCS	vaginocervical stimulation
VGLUT	vesicular glutamate transporters
vl	ventrolateral
VMH	ventromedial hypothalamus
VMHvl	ventrolateral ventromedial hypothalamus
VS	ventral subiculum

CONTRIBUTION OF AUTHORS

CHAPTER 1: Vaginocervical stimulation induces Fos in glutamate neurons in the ventromedial hypothalamus: Attenuation by estrogen and progesterone

Authors:

Michaela Georgescu: Performed surgeries and perfusions, sliced the brains, ran the ICC, performed the microscopy, analyzed the data, wrote the manuscript.

Camille Sabongui: Performed surgeries and perfusions, sliced brains, assisted with the ICC, assisted with the microscopy.

Adina Del Corpo: Assisted with the surgeries and ICC.

Lina Marsan: Participated in the collection of the data.

James G. Pfaus: Conceptualized and designed the experiment, administered the VCS, conceptualized the way in which the data was analyzed, assisted with the writing of the manuscript.

CHAPTER 2. Effects of AMPA/Kainate receptor antagonism in the ventromedial hypothalamus on estrus termination in the rat

Authors:

Michaela Georgescu: Designed the experiment, performed surgeries, administered the VCS, conducted the experiment, collected, videoscored, and analyzed the data, wrote the manuscript.

Dave Cyr: Assisted with the surgeries, assisted with the administration of VCS, assisted in the conductance of the experiment.

James G. Pfaus: Conceptualized the experiment and assisted with the writing of the manuscript.

CHAPTER 3. Glutamate release in the ventromedial hypothalamus of the female rat: Modulation by ovarian hormones

Authors:

Michaela Georgescu: Conceptualized and designed the experiment, made probes, swivels, and designed the microdialysis chamber, performed all surgeries, conducted the microdialysis, conceptualized the data analysis, wrote the manuscript.

Veronica M. Afonso: Performed the HPLC analysis for glutamate, assisted with the conceptualization of the data analysis, assisted with the statistical analyses.

M. Dean Graham: Performed the statistical analyses.

James G. Pfaus: Assisted with the conceptualization of the experiment and the writing of the manuscript.

CHAPTER 4. Ovarian steroids increase $GABA_A$ receptor subunits on glutamate neurons in the ventromedial hypothalamus

Authors:

Michaela Georgescu: Designed the experiment, performed ovariectomies and perfusions, sliced the brains, performed the ICC, performed the microscopy, conducted the statistical analyses and wrote the manuscript.

Adina Del Corpo: Assisted with the microscopy.

James G. Pfaus: Conceptualized the experiment and assisted with the writing of the manuscript.

CHAPTER 5. GABAergic projections to the ventromedial hypothalamus of the female rat

Authors:

Michaela Georgescu: Designed the experiment, conducted the experiment, performed the microscopy, conceptualized and performed the data analysis, wrote the manuscript.

James G. Pfaus: Conceptualized the experiment and assisted with the writing of the manuscript.

General Introduction

With few exceptions, all behaviors have a beginning, middle, and end. For behaviors to be successful, arousal and attention must be directed at goals or incentives, and proper behavioral responses executed with a minimum of distraction. This requires coordinating internal processes, such as drive states or hormonal priming (that propel animals toward goals), with the internal representation of external incentives that "pull" animals toward them. For such behaviors to be competent, attention and movements must be focused on the goals themselves, to the exclusion of other potential goals or incentives that may be in the animal's environment at the time. Behaviors must also occur in the proper sequence, and once consummation of the goal has been achieved, advanced to the next phase (Pfaus, 1999; Toates, 1986). This requires the activation of inhibitory processes that can reduce the efficacy of competing drives or other potential incentives (Konorski, 1967). This type of organization was hypothesized by Pavlov (1929) and subsequently found in a variety of neural circuits, most notably in the visual system (Hubel and Wiesel, 1959) where excitatory centers and inhibitory surrounds (or vice versa) allow for focused visual attention. Thus, excitation and disinhibition become mechanisms by which behavior can be focused on the right goal at the right time.

The research in the present thesis examined such a disinhibitory mechanism in the control of sexual behavior in the female rat. For female rats to

go into sexual "heat", a sequential action of estrogen and progesterone is required. These actions stimulate steroid hormone receptors to act as transcription factors and create a constellation of protein synthetic effects that fundamentally alter the way in which females interact with males (Pfaff, 1980; 1999). Females essentially move from a behavioral repertoire of avoidance and fighting to one of solicitation, pacing, and lordosis. When sufficient stimulation is achieved to render them pregnant (or pseudopregnant), females will drop out of heat and reestablish a pattern of avoidance and fighting. The mechanisms by which this change occurs involve the coordinated action of the hypothalamus, limbic system, and motor output regions of the brain, along with cortical activity that provides the proper timing and context-specificity (Pfaus, 2009). In the ventromedial hypothalamus (VMH), implants of estradiol to ovariectomized (OVX) rats facilitates lordosis, whereas lesions of this region disrupt lordosis and the timing of other sexual responses. Although estradiol generates a number of proteins that are excitatory for sexual behavior (Kow and Pfaff, 2004), a conundrum exists in which infusions of the neural excitatory agent glutamate to the VMH *inhibit* female sexual behaviors, whereas infusions of the neural inhibitory agent γ-amino butyric acid (GABA) *facilitate* female sexual behaviors. This conundrum sets up the main question of this thesis: does estradiol and/or progesterone excite female sexual behavior by excitatory actions in the VMH, or do they influence female sexual behavior, in part, by inhibiting an inhibitory

system in the VMH that is involved in competing responses or avoidance behaviors?

Structure of sexual behavior in the female rat

Female rat sexual behavior occurs when females are primed sufficiently with estradiol and progesterone and have access to sexually mature males. Females approach males, and both engage in anogenital investigation. The female then initiates sexual interaction with the male through solicitations and hops and darts that focus his attention on her and compel him to chase her. A solicitation is characterized by a head-wise orientation towards the male followed by a quick runaway (McClintock, 1985). The male responds to solicitations with pursuit or chasing behavior. After a brief chase, the female adopts a pre-lordosis crouch, the male palpates the flanks and mounts the female, prompting her to adopt a full lordosis stance. Thus, the female responds to male mounts by engaging in lordosis, the sexually receptive posture of female rats, characterized by a dorsiflexion of the spinal cord. The lordosis posture involves raising the rump, permitting penetration of the penis in the vagina, which is called an intromission (Adler, 1969). Intromissions consist of several thrusts of the penis into the vagina, providing the female with vaginocervical stimulation (VCS), along with stimulation of the clitoris from the male's pelvic thrusting. Receipt of VCS prompts the female to terminate lordosis, disengage from the male and run away. The male then engages in genital grooming and, after a few seconds, resumes

the chase. This interaction occurs several times before the male ejaculates, at which point he rests for several minutes, and copulation begins again. Each copulatory bout that ends with an ejaculation is called an ejaculatory series. Copulation typically consists of several ejaculatory series before the female goes into a state of estrus termination, and takes herself out of the copulatory situation (McClintock, 1985).

The female controls the rate of intromissions by imposing physical distance between the male and herself, an important aspect of female rat sexual behavior called 'pacing'. The ability to pace allows females to enforce a preferred rate of copulation or terminate the copulation session altogether (Beach, 1976; Erskine, 1989; Pfaus, Smith, and Coopersmith, 1999). Females also engage in defensive behaviors such as prone positions and boxing to slow down the rate of copulation or to terminate copulation. Typically, as the number of ejaculatory series increases, solicitation rates decrease, lordosis loses intensity and pacing rates and defensive behaviors increase (Pfaus et al., 1999). Approach, solicitation and hopping and darting are often referred to as appetitive (Pfaus, 1996) or proceptive (Beach, 1976) behaviors, whereas lordosis is an unambiguous consummatory behavior (Pfaus, 1996). Female pacing is critical for sexual reward and reproduction. Females allowed to pace the rate of intromissions find copulation rewarding, as indicated by the acquisition of sexually conditioned partner and place preferences (Coria-Avila, Ouimet, Pacheco, Manzo, and Pfaus, 2005; Coria-Avila, Jones, Solomon, Gavrila,

Jordan, and Pfaus, 2006; Martinez and Paredes, 2001; Paredes and Alonso, 1997; Paredes and Vazquez, 1999). Pacing can occur in any context that allows the female room to solicit and pace, for example in bilevel chambers (Pfaus et al., 1999) where she can pace by running from level to level, or in unilevel pacing chambers bisected by a divider with holes that only the female can pass through. Pacing can also occur in open fields that allow the female room to engage in runaways and/or defensive responses (McClintock, 1985). Thus, even "nonpaced" copulation can, in circumstances where the female can enforce a rate of male stimulation, induce significant sexual reward in females (Meerts and Clark, 2007).

Females allowed to pace copulatory stimulation also have enhanced fertility and higher pregnancy rates (Adler and Toner, 1986; Edmonds, Zoloth, and Adler, 1972; Erskine, 1985; Frye and Erskine, 1990). In bilevel chambers, pacing can be assessed by the number of level changes per mount (Pfaus et al., 1999). In unilevel chambers, pacing can be assessed by contact return latencies (the amount of time it takes females to return through the hole(s) in the divider following receipt of a mount, intromission, or ejaculation), or, in the case of chambers with no divider, the interintromission interval of the male and/or the number of defensive behaviors per mount. Contact return latencies are typically shortest following mounts without intromission, slightly higher following mounts with intromission, and highest after ejaculation (Erskine, 1985), suggesting that the greater the intensity of the VCS, the higher the return latency. Pregnancy or

pseudopregnancy requires neuroendocrine changes induced by threshold amounts of VCS (Kornberg and Erskine, 1994; Lehmann and Erskine, 2004). Thus, pacing helps to ensure the receipt of sufficient amounts (and strength) of VCS to induce the neuroendocrine changes necessary for sperm transport and prolactin surges required for pregnancy (Adler, 1969), in addition to sexual reward (Pfaus, Kippin, and Coria-Avila, 2003).

Hormones and female sexual behavior

Female rats have estrus cycles of approximately 4-5 days.On a 12:12hr light/dark cycle, at 11AM on the day of Proestrus there is a peak in circulating estrogen (E) levels, followed by a peak in progesterone (P) levels between then and midnight of the same day. Ovulation occurs in the late afternoon of Proestrus, after which P levels increase. Females display estrous behavior (meaning full sexual receptivity) approximately 4-6 hr after ovulation, and will copulate until they receive a sufficient amount of stimulation to ensure pregnancy and/or sexual reward and satiety. In the laboratory setting, copulation can be observed in gonadally-intact females where the phase of the ovulatory cycle has been determined by the morphology of vaginal epithelial cells (Marcondes, Bianchi, and Tanno, 2002). Females can also be ovariectomized (OVX) to prevent impregnation and pseudopregnancy, and to allow experimental control over the periods of estrous behavior using exogenous hormone administration. The full array of appetitive and consummatory sexual behaviors is naturally

induced by E and P secreted by the ovaries. In the laboratory setting, administration of synthetic estrogens, such as estradiol benzoate (EB), induces a low level of receptivity without the complete range of appetitive (proceptive) behaviors, whereas administration of EB followed by P 36-48 hours later induces full lordosis, along with a full display of appetitive behaviors like solicitation, hops and darts, and ear-wiggling (Whalen, 1974).

Neurocircuitry of lordosis

Although female rats display a complex array of behaviors during copulation, lordosis is the most investigated aspect of copulatory behavior in studies pertaining to the neuroendocrinology of female sexual behavior (e.g., Frye, Murphy and Plateck, 2000; Luine, Wu, Hoffman, and Renner, 1999; Mathews and Edwards, 1977; Pfaff, Schwartz-Giblin, McCarthy, and Kow, 1994; Sakuma and Pfaff, 1983). Pfaff and colleagues described the neural mechanisms that mediate lordosis by postulating a four-module neural circuit that explains the behavioral correlates of hormonal effects in hypothalamic and limbic areas (Pfaff, 1980; Pfaff et al., 1994). The **spinal cord module** mediates sensorimotor reflexes, with pressure receptors on the flanks and hindquarters of the female evoking action potentials entering the spinal cord over dorsal roots L1, L2, L5, L6 and S1. The **hindbrain module** mediates activities across spinal levels, ascending fibers in the supraspinal loop travelling to the brainstem, terminating in the medullary reticular formation, in the dorsocaudal lateral vestibular nucleus

and in the midbrain (the peripeduncular region and the midbrain central gray). In turn, the **midbrain module** integrates input from the hypothalamus and motor control of the hindbrain, resulting in descending signals from the midbrain central gray and the mesencephalic reticular formation to the medullary reticulospinal neurons, which activate lumbar motor neurons that control the deep back muscles involved in lordosis. Finally, the **hypothalamic module** adds hormone dependence: input from the hypothalamus is sent to the midbrain central gray (MCG) via lordosis-related axons from E-sensitive neurons. Ovarian estrogens from the bloodstream cross the blood-brain barrier and, via actions at the alpha and beta receptors, turn on genes in the hypothalamus for the P receptor, adrenergic α_1 receptor, muscarinic receptors, enkephalin and opioid receptors, and oxytocin (OXY) and OXY receptors, and gonadotropin-releasing hormone (GnRH) and GnRH receptors (Kow and Pfaff, 2004; Parsons, Rainbow, Pfaff, and McEwen, 1982; Pfaff, 1980; Pfaff, 1999; Pfaff, Frohlich, and Morgan, 2002). In the preoptic area, estrogens turn on the gene for GnRH, GnRH receptor and prostaglandin-D synthase (Pfaff, 1999). Activation of these genes facilitates female sexual behavior and primes the brain to be selectively responsive to sexual incentives by increasing CNS arousal, remodeling the lordosis circuit, inducing efficient social recognition and partial analgesia, reducing anxiety, and synchronizing ovulation with lordosis (Mong and Pfaff, 2004; see review in Pfaff, Kow, Loose and Flanagan-Cato, 2008).

Hypothalamic regions that regulate female sexual behavior

Rich in steroid hormone receptors and an area of convergence of the neurotransmitter systems important in the facilitation of female sexual behavior, the hypothalamus contains nuclei that interact with each other to control various aspects of female sexual behavior.

Medial Preoptic Area (mPOA). The mPOA is generally associated with inhibition of lordosis and facilitation of proceptive behaviors (Hoshina, Takeo, Nakano, Sato, and Sakuma, 1994; Pfaff and Sakuma, 1979; Whitney, 1986). Lesions of the mPOA that spare fibers of passage facilitate lordosis and rejection behaviors and decrease solicitation and proceptive behaviors (Hoshina et al., 1994). Furthermore, electrical stimulation of the mPOA results in inhibition of lordosis (Hoshina et al., 1994; Moss, Paloutzian, and Law, 1974, Napoli, Powers, and Valenstein, 1972; Takeo, Chiba, and Sakuma, 1993). However, other experiments have shown that mPOA lesions can potentiate lordosis when females are tested in chambers that do not permit pacing, whereas when pacing is possible, lordosis is not potentiated (Whitney, 1986). Recent work from our laboratory suggests that the frequency of proceptive behaviors increases following (a) infusion of DA D1 agonists to the mPOA and priming with EB+P or (b) infusion of DA D2 agonists and priming with EB alone (Graham and Pfaus, a and b, submitted). These results suggest that DA D1/D2 activity in the mPOA may play an important role in the display of proceptive behaviors and, because the mPOA communicates with the VMH and vice-versa via efferent projections

(Conrad and Pfaff, 1975; Wagner, Eaton, Moore, and Lookingland, 1995), DA receptor activation may be involved in the mediation of the interplay between lordosis and proceptive behaviors.

Paraventricular Nucleus of the Hypothalamus (PVN). The PVN produces and releases OXY into the posterior pituitary, the spinal cord, and possibly other brain sites (Carmichael, Humbert, Dixen, Palmisano, Greenleaf, and Davidson, 1987; Swanson and Kuypers, 1980; Swanson, Sawchenko, Wiegand, and Price, 1980). In the peripheral circulation, OXY stimulates smooth muscle contraction of the uterus and myoepithelial cells of the mammary glands, expelling the fetus from the uterus and milk from the mammary glands (see Komisaruk and Steinman, 1986, for a review). OXY released within the brain acts as a neurotransmitter, and facilitates social recognition (Bielsky and Young, 2004), social motivation, approach behavior, maternal nurturing behaviors (Burbach, Young, and Russell, 2006), and pair bonding (Young and Wang, 2004). VCS activates PVN neurons that produce OXY (Komisaruk and Whipple, 2000). These neurons also project to the thoracic spinal cord where they release OXY. About 35% of oxytocinergic neurons in the PVN have E beta receptors, indicating that these PVN OXY neurons may be partially regulated by E (Simonian and Herbison, 1997).

Ventromedial Hypothalamus (VMH). The VMH plays a critical role in female sexual behavior. A large body of literature supports the idea that the VMH exerts facilitative influences on female sexual behavior. The ventrolateral (vl)

region of the VMH is particularly rich in E and P receptors (Stumpf, 1970; Pfaff and Keiner, 1973). Excitotoxic or excitolytic lesions of the VMH almost completely abolish females' ability to lordose in response to male mounts in rats (Malsbury, Kow and Pfaff, 1977; Matthews and Edwards, 1977; Pfaff, 1980; Pfaff and Sakuma, 1979b), hamsters (Malsbury, Kow, and Pfaff, 1977), and guinea pigs (Goy and Phoenix, 1963). E implants to the VMH of OVX females restore their ability to lordose (Dorner, Docke, and Moustafa, 1968; Rubin and Barfield, 1983). Binding of E to its receptors in the VMH induces protein synthetic changes (e.g., Etgen, 1987; Meisel and Pfaff, 1985) required to activate neuroendocrine systems conducive to sexual behavior. Together with somatosensory and olfactory cues from accessible sexual partner(s), VMH E-induced changes activate projections to the midbrain and to the brainstem that control the deep back muscles involved in lordosis (Pfaff, 1980; Rajendren, Dudley, and Moss, 1991). It was suggested that lesions of the VMH result in impairment of the ability to display lordosis because E-sensitive neurons are destroyed in the process (Pfaff and Sakuma, 1979b). Other evidence supports the idea that VMH lesions result in inhibition of lordosis because cell bodies critical in the hormone-sensitive neurocircuitry regulating lordosis are destroyed, or because axons of hormone-sensitive neurons passing through the VMH en route to other sites of the brain are damaged (Malsbury et al., 1977). P implants to the VMH of OVX females treated with E further facilitate sexual behavior by restoring proceptive behaviors (Barfield and Rubin, 1983). Also, electrical stimulation of the VMH results in

facilitation of lordosis in OVX rats primed with E (Pfaff and Sakuma, 1979a).

Etgen and Karkanias (1994) proposed a model of facilitation of lordosis by interactions between steroid hormones, OXY and norepinephrine (NE) in the VMH, in which OXY and NE act in concert to increase overall neuronal excitability. E and P upregulate OXY receptors and modify their distribution throughout the VMH. In response to VCS, OXY may be released from the posterior pituitary and increases NE release in the VMH (Vincent and Etgen, 1993). NE has a facilitative effect on sexual behavior in female rats (Etgen, 1990; Etgen, Ungar, and Petitti, 1992), and activation of $_1$-adrenoreceptors in the hypothalamus increases neuronal excitability (Kim, Dudley, and Moss, 1988; Kow and Pfaff, 1987). E potentiates these responses (Condon, Ronnekleiv, and Kelly, 1989). E, administered in doses that prime sexual behavior, facilitates release of NE in the VMH, which is inhibited in OVX animals, by decreasing NE's ability to provide negative feedback of its own release though the $_{2D}$-adrenoreceptor (Etgen and Karkanias, 1993). As such, OXY and NE act in synergy to increase excitability of VMH neurons, thereby promoting lordosis in response to male mounts in females primed with E. Conversely, dopamine applied iontophoretically decreases the electrical activity of VMH neurons (Chan, Dudley, and Moss, 1983) and activation of the D1 receptor in the VMH facilitates lordosis by activating progestin receptors in a ligand-independent manner (Mani, Allen, Clark, Blaustein, and O'Mailey, 1994; Mani, Mitchell, and O'Mailey, 2001).

"Paradoxical" effects of glutamate and GABA in the VMH

It follows from the lesion data that general neuronal excitation in the VMH should facilitate lordosis. Two important sets of data contradict this notion. The first involves the inhibitory effects of infusions of the excitatory neurotransmitter glutamate to the VMH on female sexual behavior (Kow, Harlan, Shivers, and Pfaff, 1985; McCarthy, Curran, and Feder, 1991; Georgescu and Pfaus, 2006a, b). Kow et al. (1985) first showed that microinfusions of glutamate, or its ionotrophic agonist kainate, to the VMH of OVX rats implanted subcutaneously with EB silastic capsules result in inhibition of lordosis induced by manual flank stimulation. A subsequent study showed that microinfusions of the glutamate agonist NMDA to the VMH of OVX rats primed with EB+P result in a rapid inhibition of lordosis induced by copulation with a sexually vigorous male (McCarthy et al., 1991).

Georgescu and Pfaus (2006a, b) conducted a neuropharmacological analysis of the effect of glutamate, its selective receptor agonists, and three of its selective receptor antagonists, on a full battery of appetitive and consummatory sexual behaviors in female rats. Agonists were tested in OVX rats primed with EB+P, whereas the antagonists were tested in OVX rats primed with EB+P or EB alone. Glutamate decreased hopping and darting; NMDA decreased lordosis quotients and lordosis magnitudes, and increased pacing and defensive behaviors; AMPA decreased solicitations, hopping and darting, lordosis quotients and magnitudes and increased defensive behaviors; and kainate decreased

solicitations, hops and darts, lordosis quotients and increased pacing and defensive behaviors. The glutamate antagonists tested were AP-5, antagonist of the NMDA receptor, CNQX and DNQX, both of which are antagonists of the AMPA/kainate receptor. The antagonists were tested on EB and EB+P-primed females. AP-5 infusions increased lordosis quotients in females primed with EB. CNQX increased solicitations, lordosis quotients and magnitudes, and the number of ejaculations by males in EB-primed females. In females primed with EB+P, CNQX increased solicitations. DNQX increased solicitations, hopping and darting, lordosis magnitudes and the number of ejaculations by males and decreased pacing in EB-primed females. In females primed with EB+P, DNQX increased solicitations and decreased pacing. Priming with EB and P may have induced sexual behavior that is already at optimum levels, causing a ceiling effect that masked the facilitative effect of the antagonists on lordosis and other behaviors. Overall, the behavioral pattern induced by increased glutamate transmission in the VMH was characterized by inhibition of sexual behaviors and activation of pacing and defensive behaviors, reminiscent of the state of estrus termination. Increased glutamate transmission in this region could also mediate states of non-receptivity, such as that following ovariectomy, or during pregnancy and lactation.

The second important set of data concerns the excitatory effects of infusions of the inhibitory neurotransmitter GABA to the VMH on female sexual behavior (Donoso and Zarate, 1981; McCarthy et al., 1990; McCarthy et

al.,1991). Bilateral infusions of GABA A agonists and antagonists indicate that GABA facilitates lordosis in the the medial hypothalamus and midbrain centray gray (MCG) and inhibits it in the mPOA and the anterior hypothalamic continuum (McCarthy et al., 1990; McCarthy, Pfaff, and Schwartz-Giblin, 1990). In the CNS, GABA is synthesized by two forms of glutamic acid decarboxylase (GAD) from glutamate. GAD-65 is activated by increased demand and synthesizes GABA for vesicular release; GAD-67 is active tonically and synthesizes GABA for metabolic purposes and non-vesicular release (Erlander and Tobin, 1991; see McCarthy et al., 1995). Antisense oligodeoxynucleotide against the GAD-67 reduces lordosis when administered into the medial hypothalamus or MCG, but not when administered in the mPOA (McCarthy et al., 1994). Infusion of antisense oligodeoxynucleotide to the mRNA for either form of GAD in the MCG reduced lordosis behavior (McCarthy et al., 1994). Interestingly, steroid hormones seem to modulate GABAergic activity in these areas by regulating its release, modulating the GABA A receptor synthesis and binding capacity, and by regulating GAD mRNA (for a review, see McCarthy, 1995). Briefly, E treatment was found to increase GAD-65 in the mPOA and decrease it in the dorsomedial hypothalamus (DMH; McCarthy, Kaufman, Brooks, Pfaff, Schwartz-Giblin, 1995). On the other hand, P increases binding affinity to GABA receptors (DeLorey and Olsen, 1994; Majewska, Harrison, Schwartz, Barker, and Paul, 1986). Furthermore, P metabolites (from most to least efficacious: THP [5 alpha-pregnan-3 alpha-ol-20-one], THDOC [5 alpha-pregnan-3 alpha,21-diol-20-one], P

[4-pregnen-3,20-dione], DHP [5 alpha-pregnan-3,20-dione],17-OH-P [17-hydroxyprogesterone], DHEAS [5-androsten-3 beta-ol-17-one sulfate] and PS [5-pregnen-3 beta-ol-20-one sulfate]) bind to the GABA A receptor complex (Frye and Duncan, 1994).

There is some controversy in the literature as to whether GABA is synthesized in the VMH or released from axon terminals of neurons originating elsewhere. Although many studies failed to detect mRNA for GAD-65 or GAD-67 within the VMH (Feldblum, Erlander, and Tobin, 1993; Fernandez-Guasti, Larsson, and Beyer, 1986; McCarthy et al., 1995; Okamura, Abitbol, Julien, Dumas, et al., 1990; Ovesjo, Gamstedt, Collin, and Meister, 2001; Ziegler, Cullinan, and Herman, 2002), GABA was detected through immunocytochemistry (Commons, Kow, Milner, and Pfaff, 1999). Because GAD enzymatic activity in the VMH is very high (Tappaz, Brownstein, and Kopin, 1977), Commons et al. postulate that GABA might be manufactured in the VMH by other forms of GAD (e.g., Coscina and Nobreaga, 1984; Edelhoff, Grubin, Karlsen, Adlern, Foster, Disteche, and Lernmark, 1993; Morley, Levine, Gosnell, and Billington, 1984; Nathan, Bao, Hsu, Aguilar, Wu, Kuo, and Wu, 1994), by a novel mechanism that remains unknown, or GABA is brought to the VMH through efferent projections from other areas in the brain. Another possibility is that neurons that do not synthesize GABA scavenge endogenous GABA released from afferents to the area (Commons et al., 1999; Im, Blakeman, and Davis, 1990).

VCS, Estrus termination, and the VMH

VCS inhibits sexual behavior by abbreviating the period of behavioral estrus (Lodder & Zeilmaker, 1976). VCS is received either from male intromissions and ejaculations, or from manual administration with a lubricated glass rod in a manner that mimics male intromissions during copulation (Pfaus, Kleopoulos, Mobbs, Gibbs, and Pfaff, 1993). Although VCS potentiates lordosis in the short-term, large amounts of VCS result in decreases in solicitations, hops and darts, and lordosis, and increases in pacing and defensive behaviors, a behavioral pattern reminiscent of estrus termination (Pfaus, Smith, Byrne, and Stephens, 2000) and of the effect of glutamate agonist infusions to the VMH (Georgescu and Pfaus, 2006a). Interestingly, bilateral infusions of the sodium channel blocker tetrodotoxin (TTX) to the VMH limited the ability of VCS to induce estrus termination in rats although TTX infusions to females that received sham VCS induced estrus termination on its own (Dobbek and Pfaus, 2001). VCS activates the immediate-early gene product Fos in the VMHvl (Pfaus et al., 1993; Tetel, Getzinger, and Blaustein, 1993; Tetel et al., 1994), and treatment with EB and P blunts this activation (Pfaus et al., 1996). Fos was not induced in that study in the absence of VCS, although a very small amount can be induced in the VMH within 2 hrs of P treatment to OVX rats maintained on EB (Auger and Blaustein, 1995). The Fos induction in the VMH by VCS is blunted in animals that receive EB+P relative to oil (Pfaus, Marcangione, Smith, Manitt, and Abillamaa, 1996; Tetel et al., 1993), although with sufficient amounts of VCS (30-50 distributed

stimulations) this blunting effect of the hormone priming could be overcome. The fact that steroid hormones reduced the cellular response to VCS in the VMH suggests that E and P inhibit cells activated by VCS. Could those cells be part of a neurochemical mechanism in the VMH that normally inhibits sexual behavior in the female?

Goals of the present thesis

The aim of this thesis was to examine more closely the putative inhibitory system in the VMH that is activated by VCS, that abbreviates the period of behavioral estrus, and that appears to be inhibited by E and P. It was hypothesized that this system is mediated by the activation of glutamate neurons in the area, and that steroid hormones inhibit this activation. Another hypothesis was that glutamate neurotransmission in the VMH may mediate the onset of estrus termination, and that glutamate release is induced by reception of VCS. Finally, we were interested in finding out whether E-related increases in GABA transmission in the VMH serve to deactivate glutamate neurons, and, if so, which brain regions may provide the VMH with GABA, since previous research has suggested that GABA is not synthesized by GAD in this area.

The first set of experiments investigated whether neurons that are activated in the VMH following VCS are glutamatergic and whether EB and P priming would suppress this response. Immunocytochemistry was used to determine whether Fos is induced in glutamate neurons following negligible (1) or

large (50) amounts of manual distributed VCS, and whether administration of steroid hormones EB and P, alone or in combination, would blunt this effect.

The second set of experiments explored the role of glutamate in the VMH in estrus termination. The effect of DNQX, antagonist of the AMPA/kainate receptor, was assessed on estrus termination induced by manually distributed VCS.

The third set of experiments used in vivo microdialysis to assess the pattern of glutamate release during a 2-hour copulatory session in different groups of OVX females primed with Oil, EB, or EB+P. It was hypothesized that glutamate release would increase as the copulatory session extended, and that steroid hormones EB and EB+P would partially inhibit the release. It was also hypothesized that extracellular concentrations of glutamate would be higher in oil-treated females relative to females treated with EB or EB+P.

The fourth and fifth sets of experiments investigated whether GABA is involved in the regulation of glutamate transmission in the VMH. The fourth set examined whether GABA A receptors exist on glutamate neurons using double immunocytochemistry to detect GABA A receptor subunits on the membrane of glutamate-stained neurons.

The fifth set of experiments sought to determine where GABA afferents to the VMH arise. The retrograde tracer FluoroGold (FG) was used to label neurons that project to the VMH, and GABA cell bodies were labeled using a specific antibody. Cell bodies stained for GABA that also expressed FG were counted and mapped.

Chapter 1

Vaginocervical stimulation induces Fos in glutamate neurons in the ventromedial hypothalamus: Attenuation by estrogen and progesterone

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ABSTRACT

VCS induces the immediate early gene product Fos in the VMH of female rats. However, this induction is lower in ovariectomized rats that receive EB and P relative to an oil vehicle. We have observed that a substantial proportion of cells activated in the VMH by VCS stain for glutamate, and infusions of glutamate or its selective receptor agonists to the VMH inhibit both appetitive and consummatory sexual behaviors in females. This raises the possibility that VCS activates an inhibitory glutamate-driven system in the VMH, and that ovarian steroids blunt the activation, although it is not known whether EB or P, alone or in combination, lead to this effect. The present experiment examined the ability of VCS to induce Fos in glutamate neurons in the VMH of ovariectomized rats under 4 hormonal regimens: Oil, EB-alone, P-alone, or EB+P, following 1 or 50 distributed VCSs administered with a lubricated glass rod over the course of 1hr. Treatment with EB or P alone significantly reduced the number of glutamate neurons activated by 1 VCS, with P being more effective than EB. Treatment with EB+P also produced a significant reduction, but not to the extent of EB or P alone. Although EB and P work in synergy to activate sexual behavior in female rats, actions of EB or P alone are sufficient to blunt the ability of VCS to activate glutamate neurons in the VMH. It appears that ovarian steroids may "disinhibit" sexual responding in part by dampening the ability of VCS to activate glutamate neurons in the VMH. This may allow females to receive a sufficient number of intromissions for the activation of sexual reward and facilitation of pregnancy.

Introduction

The VMH plays a critical role in the control of lordosis and other female reproductive behaviors (Emery and Moss, 1984; Pfaff, 1980; 1999; Pfaff et al., 1994). It is rich in E and P receptors (Pfaff and Keiner, 1973), and lesions reduce lordosis and proceptive behaviors dramatically (Malsbury et al., 1977; Mathews and Edwards, 1977; Pfaff, 1980; Sakuma and Pfaff, 1979). Binding of estradiol in the VMH induces protein synthetic changes (e.g., Etgen, 1987; Meisel and Pfaff, 1985) that lead to the activation of neurochemical systems conducive to sexual behavior. This process enhances the ability of male-related somatosensory and olfactory cues to activate the VMH (Pfaff, 1980; Rajendren et al., 1991), which in turn controls outflow to midbrain structures, such as the central grey, and brainstem structures in the medullary reticular formation that control the contraction of axial muscles involved in spinal dorsiflexion. However, the VMH plays important roles in other behaviors, including feeding (Bray and York, 1998; Gold, Quackenbush, and Kapatos, 1972; Marshall, Barnett, and Mayer, 1955; Storlein and Albert, 1972), aggression (Adamec, 1991; Brayley and Albert, 1977; Sterner, Meisel, and Diekman, 1992), and pain (Borszcz, 2006). Engaging in sexual behavior thus requires not only the stimulation of neurochemical systems conducive to sexual activity, but also the active inhibition of neurochemical systems that give rise to competing responses (e.g., Konorski, 1967). It is possible therefore that part of the stimulation of lordosis or other sexual behaviors by estradiol involves an active disinhibition of neural systems

that normally inhibit sexual responding, as suggested originally by Steinach (1910), for the disinhibitory actions of testicular secretions on the clinging reflex in male frogs during the rut season.

Previous studies have shown that VCS increases neuronal excitability and glucose metabolism within E-concentrating regions of the forebrain, including the VMH (Allen, Adler, Greenberg, and Reivich, 1981; Blake and Sawyer, 1972; Darney and Terkel, 1990). Studies using Fos immunocytochemistry have shown significant activation of the ventrolateral VMH by VCS, and that this activation is inhibited by previous administration of EB and P (Blanger and Auger, 1995; Pfaus et al., 1993; Pfaus et al., 1996; Tetel, Getzinger, and Blaustein, 1993; Tetel, Getzinger and Blaustein, 1994). That steroid hormones reduce the cellular response to VCS in this area points to the presence of an inhibitory mechanism in this region that is directly or indirectly inhibited by ovarian hormones (Pfaus et al., 1996). Indeed, treatment of OVX rats with EB and P relative to no hormones differentially affects the pattern of cellular activation after different amounts of VCS in the VMH and other brain regions, including the mPOA, lateral septum, bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA; Pfaus et al., 1996). In the VMH, small amounts of VCS (1 to 20 distributed stimulations) result in moderate numbers of Fos-immunoreactive (IR) cells, whereas larger amounts of VCS (30 to 50 distributed stimulations) result in larger numbers of Fos-IR cells in EB- and P-primed females. This dynamic increase in Fos-IR cells with larger amounts of VCS suggests that large amounts of VCS diminish the

ability of ovarian hormones to inhibit the inhibitory VMH mechanism.

Harlan et al. reported that the ability of EB to maintain lordosis responding required hypothalamic action potentials (Harlan, Shivers, and Pfaff, 1983). In that study, blocking sodium conductance in the VMH and contiguous structures with tetrodotoxin resulted in a dose-dependent, reversible decline in lordosis. However, a subsequent study by Kow et al. (1985) showed that microinfusions of the excitatory neurotransmitter glutamate, or its ionotrophic agonist kainate, to OVX rats implanted subcutaneously with silastic capsules containing EB, dose-dependently inhibited lordosis induced by manual flank stimulation. McCarthy et al. (1991) infused the ionotrophic glutamate agonist NMDA to the VMH of OVX rats primed with EB and P and observed a rapid inhibition of lordosis induced by copulation with a sexually vigorous male. We recently examined the role of glutamate receptors in the VMH on the regulation of both appetitive and consummatory sexual behaviors in female rats (Georgescu and Pfaus, 2006a, b). Glutamate or its selective ionotrophic agonists or antagonists were infused bilaterally to the VMH of OVX rats primed with EB+P (agonists) or EB alone (antagonists). Glutamate and AMPA infusions decreased solicitations and lordosis, NMDA infusions decreased lordosis and increased defensive responses and level changes indicative of pacing. Kainate infusions decreased solicitations and lordosis, and increased defensive and pacing responses. Conversely, infusions of the AMPA receptor antagonists CNQX or DNQX increased solicitations and lordosis, and DNQX also increased lordosis and decreased

pacing and defensive responses. The NMDA receptor antagonist AP-5 increased lordosis.

Taken together, the overall effect of increased glutamate transmission in the VMH produces a behavioral state of sexual nonreceptivity, that resembles periods of estrus termination found after estrus, following ovariectomy, or during the juvenile period, pregnancy, or lactation, in which females do not display sexual responding. However, these states are hormone-dependent primarily, with input from multiple factors, external and/or endogenous, among which could be the glutamatergic system within the VMH. Increased glutamate transmission within the VMH is also reminiscent of the state of estrus termination, in which females display few if any solicitations, increased rejection responses, longer pacing intervals, and eventually a decrease in lordosis (Erskine and Baum, 1982; Lodder and Zeilmaker, 1976; Pfaus et al., 2000). Glutamate activation within the VMH is more likely involved in estrus termination than in the states of sexual non-receptivity mentioned above because estrus termination is only partially hormone-sensitive and largely dependent on coital stimulation, and in particular, intromissions from the male. The effect is more pronounced if the female is able to pace the rate of intromissions (Coopersmith et al., 1996; Erskine, 1985), and is eliminated by transaction of the pelvic nerve (Lodder and Zeilmaker, 1977), suggesting that VCS induced by paced intromissions activates an inhibitory system that abbreviates the duration of estrus. Paced copulation or manually-applied, distributed VCS (at intervals that approximate those of paced

intromissions), both facilitate estrus termination (Pfaus et al. 2000) and induce the immediate-early gene product Fos in the ventrolateral region of the VMH (Pfaus et al., 1993;1996; Tetel et al., 1993; 1994; Wersinger et al., 1993). Similar to the findings of Lodder and Zeilmaker, the Fos induction in the VMH by VCS is blocked by transaction of the pelvic, but not hypogastric or pudental nerves (Pfaus et al., 2006). Interestingly, treatment with EB and P blunts the ability of VCS to induce Fos in the VMH relative to OVX rats that receive VCS (Pfaus et al., 1996; Tetel et al., 1994).

Given the behavioral similarity between increased glutamate transmission in the VMH and estrus termination, and given that VCS potentiates estrus termination and induces Fos in the VMH, we asked whether Fos might be induced within glutamate neurons by VCS, and whether treatment with EB and P would blunt that effect. Accordingly, we examined the percetage of glutamate neurons co-labeled for Fos and the percentage of Fos neurons co-labeled for glutamate in the VMH of OVX rats primed with EB alone, P alone, EB+P and P, or the oil vehicle, 1 hr after the application of 1 or 50 manual VCSs. We hypothesized that 50 VCSs would result in more glutamate neurons co-labeled for Fos as well as more Fos neurons co-labeled for glutamate than 1 VCS. Also, we anticipated that the percentage of glutamate neurons co-labeled for Fos and the percentage of Fos neurons co-labeled for glutamate would be blunted by treatment with steroid hormones. Finally, we expected treatment with EB+P would be more effective than treatment with Oil, EB alone, or P alone, in reducing

the percentage of glutamate neurons co-labeled for Fos and the percentage of Fos neurons co-labeled for glutamate in both VCS conditions.

Methods

Animals and surgery

Forty sexually naïve female Long-Evans rats, weighing between 200-250g, were obtained from Charles-River Canada, Inc., St-Constant, QC. Females were housed in groups of five in large Plexiglas shoe-box cages with wood-chip bedding. The colony room was maintained on a reversed 12:12hr light/dark cycle (lights off at 08:00) at approximately 21°C with food and water continuously available. Females were OVX bilaterally through lumbar incisions following anesthesia induced by ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4mg/ml), mixed in a ratio of 4:3, respectively, and injected intraperitoneally. All OVX females were given a week of post-surgical recovery prior to sexual training. Forty Long-Evans males from the same breeder were used as conspecific stimuli. The males received a minimum of 10 prior sexual training sessions with receptive females before use and all were sexually vigorous copulators. The males were housed in groups of four in handing wire-mesh cages in the same colony room.

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

Hormone treatment and sexual stimulation

Full sexual receptivity was induced by subcutaneous injections of EB (10μg/ 0.1ml reagent grade sesame oil) 48 hours and P (500μg/ 0.1ml reagent grade sesame oil) 4 hours prior to sexual training sessions. All steroids were obtained from Steraloids (Hanover, NH). Females received four 30-min sexual training sessions at 4-day intervals prior to the study. Training sessions were conducted in bilevel chambers in the middle-third of the rat's dark cycle (13:00-15:00h). Females were then randomly distributed to one of 4 hormonal regimens: oil (O), EB+O, O+P, or EB+P (n=10/group) for an additional 4 cycles. Behavioral testing did not occur during this period. Following the last injection of O, EB, or P, females in all hormonal regimen conditions were randomly distributed to one of two VCS conditions: 1 or 50 VCSs (N=5/hormonal condition). Four hours after this last injection, VCS was administered as described by Pfaus et al., 1996; Pfaus et al., 2000). Briefly, VCS consisted of 1 or 50 manual VCSs with a lubricated glass rod. For animals receiving 50 VCSs, the stimulation was made in clusters of 5 insertions, each spaced 5 to 10 seconds apart. Clusters were distributed at 6-min intervals over the course of 1 hr. Rats receiving 1 VCS remained in the chamber for 1 hr following the stimulation.

All rats were sacrificed an hour following the first VCS by an overdose of sodium pentobarbital (120mg/kg, i.p.) and perfused intracardially with ice-cold phosphate-buffered saline (300mls) followed by ice-cold 4% paraformaldehyde in 0.1M phosphate buffer (300mls). Brains were removed, postfixed in 4% paraformaldehyde for 4h, and stored overnight in 30% sucrose at 4°C.

Immunocytochemistry

Brains were blocked around the area of the anterior hypothalamus, mounted on a chuck, and frozen 30µm coronal sections were cut from the VMH (corresponding to plates 27-34 in Paxinos and Watson, 1998) on a cryostat. Sections were washed in ice-cold 0.9% TBS, incubated for 30 minutes in a mixture of TBS and 30% hydrogen peroxide (H_2O_2) at 4°C, rinsed, and transferred for 90 minutes to a preblocking serum consisting of 0.05% triton TBS (50µl/100ml) and Normal Goat Serum (NGS; Vector Laboratories Canada, Burlington, ON, diluted 1:200) at 4°C. The sections were rinsed and incubated in a mixture of NGS, 0.05% triton TBS, and a rabbit polyclonal primary anti-glutamate antibody raised against L-glutamate conjugated to glutaraldehyde as the immunogen (Abcam, ab 8889; diluted 1:10 000) for 72 hr at 4°C. The manufacturer's information shows no measurable cross-reactivity with glutamate in peptides or proteins, no measurable cross-reactivity with other amino acids including GABA, and only low-affinity reactivity with D-glutamate, using ELISA. Specificity was confirmed previously in rat retinal tissue (Sun, Vingrys, and Kalloniatis, 2007). The sections were then rinsed and transferred to a secondary antibody solution consisting of 0.05% triton TBS, NGS, and a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Canada, Burlington, ON, diluted 1:200) for 1 hr at 4°C. Sections were rinsed and transferred to a solution of 0.2% triton TBS, and Vectastain *Elite* ABC solutions (diluted 1:55) for 2 hr at 4°C. Finally, the sections were rinsed in TBS, rinsed in 50mM Tris buffer (pH 7.6)

for 10 minutes, and incubated on an orbit shaker for 10 minutes in 0.05% 3,3'-diaminobenzidine (DAB) in 50mM Tris buffer with 3% H_2O_2 (solution buffered to pH 7.8 with drops of 6M NaOH), to catalyze the DAB. The reaction was stopped by rinsing in cold TBS. Sections were then incubated in a rabbit polyclonal primary antibody raised against residues 4-17 of the Fos protein (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72-h at 4°C. We have used this antibody in previous studies (e.g., Pfaus et al., 2006), and its specificity in vivo has been confirmed in rat brain tissue (Dai et al., 2004). Following secondary antibody and tertiary ABC reactions, sections were incubated in the DAB/Tris buffer/H_2O_2 solution with the addition of $NiCl_2$ to color the DAB chromagen product blue-black. Sections were rinsed in TBS to stop the reaction, mounted onto 75x25mm gel coated slides (Serum International), dehydrated in H_2O and ethanols (70%, 95%, and 100%, 10 min per solution) followed by immersion in Xylenes (Fisher Scientific) overnight to clear them. Slides were coverslipped with Permount, allowed to dry, cleaned, and subsequently examined under a Leitz Laborlux microscope.

Fos-positive cells, glutamate-positive cells, and double-labeled Fos/glutamate cells were counted bilaterally per slice in the VMHvl. Counting was performed by researchers blind to the animal's experimental group. Although the VMH is approximately 1.68mm in length along the sagittal plane, and is thus comprised of fifty-six 30μm-slices, counting was limited to 5 sections in which the Fos induction in the VMHvl was largest. We have shown previously that VCS

induces the most Fos within the anterior portion of the VMHvl (Pfaus et al., 1993; 1996), and the present study replicated this distribution. The VMHvl was easily identified because of its distinct shape, but landmarks such as the 3rd ventricle and hippocampus were used to place the VMH into anterior and posterior divisions (as done previously by Pfaus et al., 1993; 1996; 2006). Single-labeled Fos cells were observed as a black nucleus surrounded by a colorless cytoplasm, whereas glutamate cells were observed as a light brown cytoplasmic stain and clear nucleus. Double-labeled Fos/glutamate cells had a black nucleus surrounded by a light brown cytoplasm (Fig. 1) in one plane of view under the microscope. For all double-labelled cells counted, care was taken to focus the plane of view in and out to make sure the cells were not single-labelled and simply adjacent to one another (as can occur in a 30 μm section that is approximately 3 cell bodies' deep). All counts were made at 400 x (Fig. 1, bottom right). Mean numbers of single and double-labeled cells per section for each rat were obtained by dividing by 2 the sum of the activated cells counted on both sides for all sections counted. Thus the counts reported here are by hemisection. To equate animals in each group, the counts were converted into the proportion of glutamate neurons that contained Fos, and the proportion of Fos-positive cells that co-localized glutamate.

Data analysis

Separate 4 (Hormone Treatment) x 2 (VCS) analyses of variance (ANOVAs) were conducted on the percentage of glutamate cells that were co-

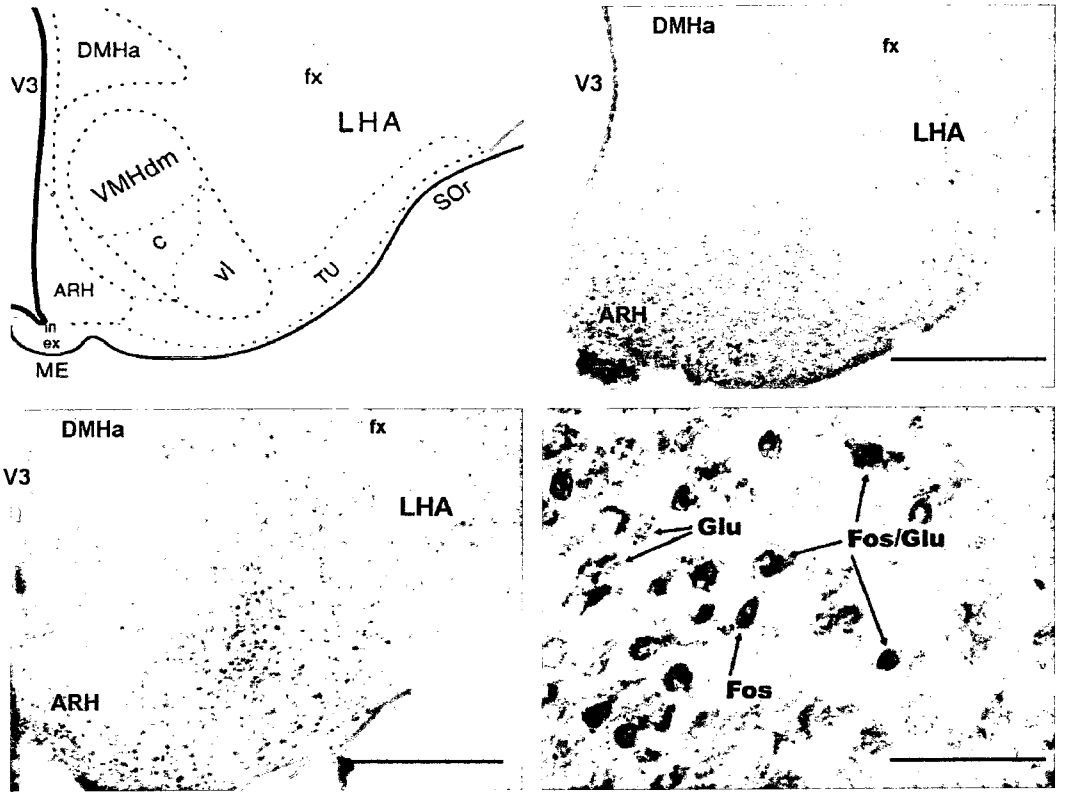

Figure 1. Top Left: Graphic representation of the VMH and its subcomponents (from the atlas of Swanson, 1992). Top Right: Glutamate staining alone in the VMH (20x). Scale bar = 500 µm. Bottom Left: Double-labelled Fos and glutamate neurons in the VMH (20x). Scale bar = 500 µm. Bottom Right: Higher magnification (40x) of the slice in the bottom left showing single-labelled Fos and glutamate cells, and cells labeled for both. Scale bar = 125 µm. All cells were counted in the VMHvl at this power. ARH: Arcuate nucleus of the hypothalamus. DMHa: Dorsomedial nucleus of the hypothalamus, anterior portion. LHA: Lateral hypothalamic area. Fx: Fornix.

labelled for Fos and the percentage of Fos cells that co-labelled glutamate. For each significant ANOVA, post-hoc comparisons of group means were conducted using the Tukey method for unequal sample sizes, $p<0.05$.

Results

As in our previous studies (Pfaus et al., 1993; 1996; 2006), Fos was induced by VCS in a cluster within the VMHvl (Fig. 1), and not in other VMH subregions. As depicted in Fig. 1, sections with the largest amount of Fos induction were found in the anterior region of the VMHvl, corresponding to Plates 27 to 29 in Paxinos and Watson (1998) and Swanson (1992). Accordingly, cell counts were thus constrained to this region of the VMH. Consistent with our previous findings, the total number of Fos-positive cells/hemisection in animals given 50 VCSs ranged from 11 to 52, depending on the hormone treatment. The total number of glutamate neurons/hemisection ranged from 15 to 64 in the regions that contained the highest Fos induction.

Table 1 shows the mean number of glutamate, Fos, and double-labelled cells found in the sections counted (+SEMs). As is readily apparent, the number of glutamate, Fos, and double-labelled neurons varied both by hormone treatment and VCS condition.

Number of glutamate-positive neurons

The ANOVA detected a significant main effect of Hormone, $F(3,30) = 19.11$, $p<0.0001$, but not VCS condition, $F(1,30) = 0.14$, ns. The ANOVA also detected a significant interaction of Hormone and VCS condition, $F(3,30) = 6.21$, $p<0.003$. Posthoc Tukey tests of the individual means revealed that the number of glutamate neurons was significantly lower overall in the groups that received EB or P, relative to the groups that received oil or EB+P. However, the number

Table 1. Cell counts by type as a function of hormone and VCS treatment

		VCS Condition	
Hormone	**Cell Type**	**1**	**50**
Oil	**Glu**	34.54±5.21	60.72±2.36
	Fos	20.00±1.84	30.20±0.96
	Fos+Glu	3.25±0.46	12.34±1.24
EB	**Glu**	37.50±2.13	25.32±5.89
	Fos	14.11±0.64	21.77±2.54
	Fos+Glu	2.16±0.18	4.67±0.81
P	**Glu**	20.58±3.32	21.62±2.30
	Fos	2.73±0.64	17.90±2.84
	Fos+Glu	1.33±0.28	4.06±0.32
EB+P	**Glu**	61.47±11.39	51.94±4.21
	Fos	8.75±0.89	25.60±1.60
	Fos+Glu	0.00±0.00	5.61±1.09

Table 1. Data are means ± SEM for all rats in each group. Means per hemisection were derived from 5 sections per rat that contained the most Fos, and the number of glutamate neurons and double-labeled cells were then counted in those sections. Results of the statistical analyses are presented in the text.

of glutamate-positive neurons was significantly higher in the oil-treated animals that received 50 VCSs relative to those that received 1 VCS.

Number of Fos-positive neurons

The ANOVA detected a significant main effect of Hormone, $F(3,30) = 25.50$, $p<0.0001$, and VCS condition, $F(1,30) = 108.19$, $p<0.000001$. The ANOVA also detected a significant interaction of Hormone and VCS condition, $F(3,30) = 3,18$, $p<0.04$. Posthoc Tukey tests of the individual means revealed that Fos induction was significantly higher overall in the oil-treated animals compared to the other groups, and significantly lower in the EB+P-treated animals compared to the other groups. In animals that received 1 VCS, both the P- and EB+P-treated groups had significantly lower amounts of Fos induced compared to the EB- or oil-treated animals. The groups did not differ significantly in Fos induction following 50 VCSs.

Number of double-labelled cells

The ANOVA detected a significant main effect of Hormone, $F(3,30) = 22.79$, $p<0.0001$, and VCS condition, $F(1,30) = 92.44$, $p<0.0001$. The ANOVA also detected a significant interaction of Hormone and VCS condition, $F(3,30) = 9.16$, $p<0.001$. Posthoc Tukey tests of the individual means revealed that overall the oil-treated rats had the highest number of double-labelled neurons compared to the other groups, which did not differ significantly from one another. However, in rats that received 1 VCS, there was an inverse, stimulation-dependent increase in double-labeled neurons between the EB+P-, P-, EB-, and oil-treated

groups, with the EB+P group showing significantly fewer double-labelled cells compared to the EB- and oil-treated groups, but not from the P-treated group. In rats that received 50 VCSs, the oil-treated group had significantly more double-labeled neurons compared to the other groups, which did not differ significantly from one another.

Percentage of glutamate cells co-labeled for Fos

The effect of VCS on the percentage of glutamate cells that co-labelled Fos in rats treated with the different hormonal regimens is shown in Fig. 2. The ANOVA detected a significant main effect of Hormone, $F(3,30) = 9.43$, $p<0.001$. Posthoc Tukey tests of the individual means revealed that the EB+P groups were significantly lower overall than the Oil, EB, or P groups, which did not differ from one another. There was also a significant main effect of VCS condition, $F(1,30) = 81.03$, $p<0.001$, but no significant interaction between Hormone and VCS condition.

Percentage of Fos cells co-labeled for glutamate

The effect of VCS on the percentage of Fos-IR cells co-labelled with glutamate is shown in Fig. 3. The ANOVA detected a significant main effect of Hormone, $F(3,30) = 5.82$, $p<0.01$. Posthoc Tukey tests of the individual means revealed that the EB+P groups were significantly lower overall than the Oil, EB, or P groups, which did not differ from one another. There was also a significant main effect of VCS condition, $F(1,30) = 6.97$, $p<0.01$, but no significant

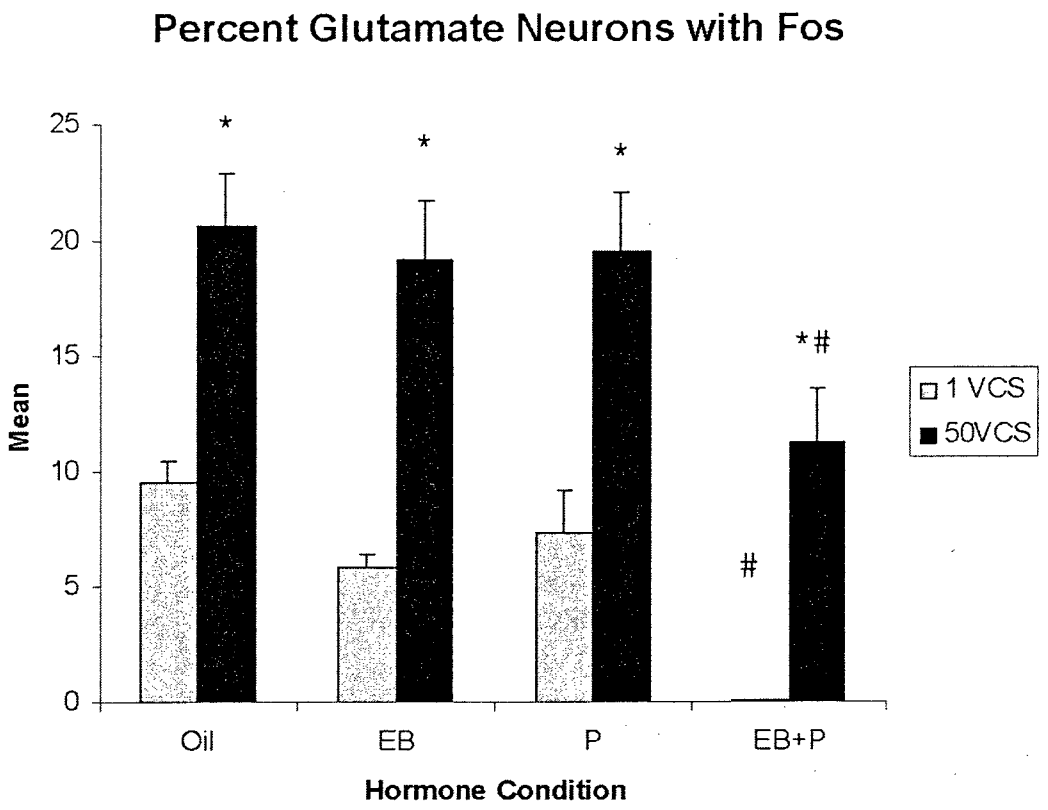

Figure 2. Percentage of glutamate cells that were co-labeled for Fos as a function of hormone priming (Oil, EB – Estradiol Benzoate, P - Progesterone, and EB+P) and vaginocervical stimulation (VCS). Data are means + SEMs.

interaction between Hormone and VCS condition.

Discussion

The present study shows that treatment of OVX rats with EB+P blunts the ability of VCS to activate Fos within glutamate neurons of the VMHvl. This effect was evident in both VCS treatment groups, suggesting that EB and P act

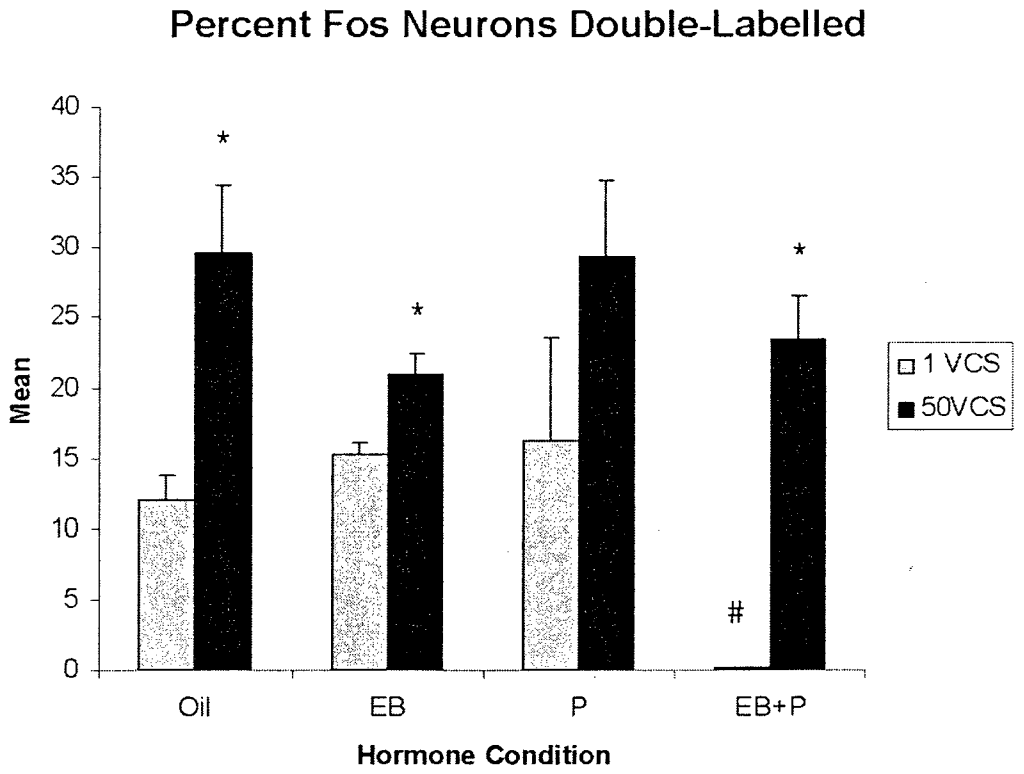

Figure 3. Percentage of Fos cells that were co-labeled for glutamate as a function of hormone priming (Oil, EB – Estradiol Benzoate, P - Progesterone, and EB+P) and vaginocervical stimulation (VCS). Data are means + SEMs.

synergistically to reduce the activation of glutamate neurons by VCS. In animals given 1 VCS, approximately 5 to 10% of glutamate cells were activated in the Oil, EB, and P groups (relative to none in the EB+P group), whereas in animals given 50 VCSs, 19 to 20% glutamate cells were activated in those three hormone groups (relative to 11% in the EB+P group). Percentages of glutamate neurons that also stained for Fos were significantly higher following all hormone treatments, indicating an overall inhibition by EB+P on the ability of VCS to activate Fos in glutamate neurons. Treatment with EB+P also resulted in

decreased amounts of Fos cells that co-labelled glutamate in the 1VCS group, however administration of 50 VCSs counteracted this effect. We have shown previously that sham VCS (holding the tailbase and raising the rump for 2 sec without applying VCS) does not induce Fos in the VMH (Pfaus et al. 1993; 1996). For this reason, we did not include a sham control in the present experiment.

There were significant differences in the total number of glutamate-positive neurons detected as a function of hormone treatment, along with the total number of Fos-positive and double-labeled neurons detected. In the case of glutamate neurons, there were significantly fewer detected overall in the EB- and P-treated rats, compared to the oil- and EB+P-treated rats. Although speculative, this could indicate a higher turnover of glutamate in the EB- or P-treated conditions, and we note that OVX rats receiving acute EB- or P-alone display more fighting with males during attempted copulation in bilevel chambers compared to either oil-treated or EB+P-treated rats (Pfaus et al., 1999). Oil-treated rats in that study were more likely to remain immobile and squeal during attempted mounts, whereas EB+P-treated rats engaged in appetitive and consummatory sexual behaviors. It may well be the case that glutamate release in the VMH increases active rejection responses, consistent with our previous findings for the effect of glutamate agonist and antagonist infusions to the VMH (Georgescu and Pfaus, 2006a, b). However, Fos induction overall, along with the number of double-labeled cells, was greatest in oil-treated rats compared to rats in the hormone-treatment groups. This suggests that EB and P may synergize to

inhibit the sensory coding of VCS in the VMH, the ability of glutamate to be released in response to different sensory cues, and to delay the initiation of rejection responses that characterize estrus termination. In a previous study (Pfaus et al., 2000), we found that treating females with EB+P on a 4- or 7-day hormone cycle abolished the VCS induction of estrus termination, whereas elements of estrus termination (e.g., reduced solicitations and increased defensive responses) were found in a time-dependent manner in animals treated with hormones at 14- or 28-day intervals. Those data, taken together with the current finding that VCS induces Fos in glutamate neurons, and with our previous finding that glutamate agonists infused to the VMH induce a pattern of behavior reminiscent of estrus termination in OVX, hormone-primed females (Georgescu and Pfaus, 2006a), strongly suggest that the activation of glutamate neurons in the VMH by VCS is an important inhibitory signal. This signal appears to gather strength progressively during copulatory contact (i.e., with increasing amounts of intromission-induced VCS) to decrease solicitations, increase pacing and defensive responses, and ultimately inhibit lordosis and other sexual behaviors altogether (e.g., Pfaus et al., 1999).

VCS produced by multiple mounts with intromissions has important effects on reward and reproduction. Female rats allowed to pace their copulatory contact with a sexually vigorous male find the stimulation rewarding, as indicated by the induction of significant sexually conditioned place and partner preferences following copulation in a unilevel pacing chamber (Coria-Avila et al., 2005; 2006;

Martinez and Paredes, 2001; Paredes and Alonso, 1997; Paredes and Vazquez, 1999). Interestingly, the ability to pace the intromissions at an optimal rate enhances fertility and increases the number of gonadally intact female rats that get pregnant (Adler and Toner, 1986; Edmonds et al., 1972; Erskine, 1985; Frye and Erskine, 1990). Moreover, the neuroendocrine changes induced by VCS that support pregnancy or pseudopregnancy (e.g., increased nightly prolactin surges), require a threshold amount of VCS (Kornberg and Erskine, 1994; Lehmann and Erskine, 2004). Pacing chambers are typically bisected by a divider with one or several small holes big enough to allow the female to cross but too small for the male. That effectively restricts the male to one side and allows the female to pace the copulatory contact by running between two sides. Erskine (1985) found that the "contact return latency" was lowest following mounts without intromission, longer following mounts with intromission, and longest after ejaculation. This suggests that the greater the intensity of the VCS, whether by duration of a single stimulus (e.g., ejaculation) or number over time (e.g., multiple intromissions), the greater the return latency. Female rats receive more intense intromissions from paced compared to nonpaced copulation, and 15 paced intromissions induce significantly more Fos in the VMH compared to 15 nonpaced intromissions (Erskine and Hanrahan, 1997). Thus, VCS from multiple paced intromissions in the rat serves to induce reward, enhance reproduction, and activate inhibitory neurochemical systems. The effect of EB and P to blunt the activation of the inhibitory glutamate neurons in the VMH by VCS may

therefore be a mechanism by which females can experience a requisite amount of VCS before estrus termination is engaged.

It appears that administration of P, whether alone or in conjunction with EB, results in stronger inhibition of VCS-induced Fos within the VMH. Previous research has established that prior to the administration of P, a rise in E levels is necessary for induction of receptivity to occur (Powers, 1970), because rises in E levels increase the expression of additional P receptors that regulate female sexual behavior (MacLusky and McEwen, 1978; 1980). Consequently, we expected that in both low and high VCS conditions, the pattern of cellular activation in animals primed with O and P alone would be similar. However, Fos induction in animals primed with P alone was significantly lower than both O-primed and EB+P-primed females, in both VCS conditions. Administration of P without prior EB does not induce sexual behavior, but still had the capacity to successfully blunt activation of the VMH by VCS.

The mechanism(s) by which P attenuates the induction of Fos in the VMH by VCS is not known, but it could involve the binding of P metabolites (e.g., 5 alpha-pregnan-3 alpha-ol-20-one) to GABA A receptors (Frye and Duncan, 1994). GABA in the VMH potently stimulates sexual receptivity (McCarthy et al., 1990; 1991), and VMH infusions of an antisense oligodeoxynucleotide against one of the GABA synthesizing enzymes, GAD-67, abolishes sexual behavior in female rats (McCarthy et al., 1994). That effect was not observed following infusion of the other GABA synthesizing enzyme, GAD-65. Acute EB significantly

decreases GAD-65 mRNA in the DMH, another region that shows a significant induction of Fos following VCS (Pfaus et al., 1993) or the presentation of olfactory stimuli associated with paced copulation (Coria-Avila and Pfaus, 2007), but increases it in the mPOA (McCarthy et al., 1995). Interestingly, the mPOA sends a large efferent projection to the VMH (Conrad and Pfaff, 1976), although the neurochemical identity of that projection is not known. P, in turn, augments the binding affinity of GABA A receptors (DeLorey and Olsen, 1994; Majewska et al., 1986). We note that VCS induces Fos in neurons of the VMH that contain intracellular P receptors (Auger et al., 1996). It may be the case that EB and P augment GABA transmission in the VMH, which inhibits glutamate neurons in such a way that an increased amount or intensity of VCS is required to overcome the inhibition.

There are other inhibitory transmitters in the VMH that could play a role in the disinhibition of female sexual behavior. Infusions of low doses of the selective δ opioid receptor agonist [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) to the VMH facilitates lordosis, whereas infusions of the selective δ receptor antagonist naltrindole inhibits lordosis and blocks the excitatory effect of DPDPE (Acosta-Martinez and Etgen, 2002a). In contrast, activation of μ opioid receptors in the VMH inhibits lordosis (Acosta-Martinez and Etgen, 2002b). All opioid receptors can induce neuronal hyperpolarization, and shorten stimulated action potentials. δ receptors accomplish this through an interaction with membrane Gi or Go proteins (Connor and Christie, 1999). As with GABA synthesis, EB increases the

synthesis of proenkephalin in the VMH (Lauber et al., 1990; Romano et al., 1990), suggesting that enkephalin actions at δ receptors could also be an intermediary of estrogen-induced disinhibition. It remains to be determined whether glutamate neurons possess GABA A and/or δ opioid receptors.

Kow et al. (1985) speculated that the VMH contains both excitatory and inhibitory neural systems for lordosis and other female sexual behaviors. Previous electrophysiological studies have confirmed that VCS can increase (fast) or decrease (slow) neuronal excitation in the VMH, but with different temporal profiles for each type of cellular response (Dafny and Terkel, 1990). In the present study, between 15 and 30% of the Fos induced by VCS in the different hormone-treatment groups was within glutamate neurons. In the animals that received 50 VCSs, that left an average of 15 to 20 Fos-positive neurons per section in each treatment group unaccounted for. It is not known what other types of neurons might be activated by VCS in the VMH. The VMH has connections with several opioid reward centers in the brain, including the mPOA, lateral hypothalamus, and MCG (Ban, 1975; Conrad and Pfaff, 1976; Fahrbach, Morrell, and Pfaff, 1989; Hennessey et al., 1990; Wise and Hoffman, 1992), and we note that infusions of the opioid receptor antagonist naloxone to the VMH or mPOA block the ability of paced copulation to induce a conditioned place preference (García Horsman, Agmo, and Paredes, 2008). Thus, activation of glutamate neurons in the VMH may direct the inhibition of sexual activity and activation of defensive responses that characterize estrus termination, whereas

the activation of other systems in the VMH may be involved in facilitating lordosis (e.g., Rodriguez-Sierra, Crowley, and Komisaruk., 1975), processing the rewarding effects of VCS, or in helping translate VCS into neuroendocrine reflexes that support pregnancy.

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Summary of Chapter 1

The experiment presented in Chapter 1 shows that a substantial proportion of the neurons activated in the VMH by VCS are glutamatergic. Priming with EB+P inhibited this activation. These data show that amounts of VCS previously shown to induce estrus termination trigger activation of twice as many glutamate cells than negligible amounts do. The amount of glutamate cells activated is halved by priming with EB+P regardless of the amount of VCS received. These results, taken together with previous research showing that activation of glutamate receptors within the VMH results in inhibition of appetitive and consummatory sexual behaviors and facilitation of pacing and defensive behaviors, suggest that increased glutamate neurotransmission in the VMH might be an important inhibitory signal to female sexual behavior, subject to inhibition by steroid hormones. The goal of the next chapter is to determine whether glutamate is involved in the mediation of estrus termination.

Chapter 2

Effects of AMPA/Kainate receptor antagonism in the ventromedial hypothalamus on estrus termination in the rat

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ABSTRACT

Infusions of glutamate or its selective receptor agonists to the VMH of female rats primed with EB and P inhibit both appetitive and consummatory aspects of sexual behavior. Conversely, infusions of selective glutamate receptor antagonists facilitate these measures in females primed with EB alone. Because VCS activates glutamate neurons in the VMH, and induces a faster termination of estrous behavior, the present study examined the effects of the AMPA/Kainate receptor antagonist DNQX on the induction of estrus termination by manual VCS. Ovariectomized, sexually-experienced rats were primed with EB and P and subsequently received either 1 or 50 distributed VCSs, over the course of an hour, 12 hrs before a test with sexually vigorous males. Half of the females in each stimulus group received bilateral infusions of 1 μl/side of either DNQX (19.8mMol/μl) or saline aimed at the VMH immediately prior to VCS or sham stimulation. Tests of sexual behavior began at 09:00 the next day at the beginning of the dark phase of their circadian cycle. Saline-infused females given VCS had lower lordosis quotients compared to females given sham stimulation. In contrast, females infused with DNQX prior to VCS displayed more appetitive behaviors and higher lordosis quotients and magnitudes compared to females infused with saline. The inhibition of estrus termination by DNQX in the VMH indicates that AMPA/Kainate receptors there mediate the effects of glutamate transmission by VCS. We suggest that hormonal inhibition of glutamate neurons in the VMH (Chapter 1) acts to disinhibit sexual behavior and insures that female

rats obtain a necessary number of intromissions for pregnancy before estrus termination begins.

1. Introduction

Microinfusions of the excitatory neurotransmitter glutamate, or its selective receptor agonists, to the VMH result in a rapid dose-related inhibition of lordosis and other sexual behaviors (Kow et al., 1985; Georgescu and Pfaus, 2006a; McCarthy et al., 1991). Kow et al. (1985) infused glutamate or its agonist kainate to the VMH of OVX females implanted subcutaneously with EB silastic capsules and observed a dose-related inhibition of lordosis induced by manual flank stimulation. McCarthy et al. (1991) infused the glutamate agonist NMDA to the VMH of females primed with E and P and observed a rapid inhibition of lordosis induced by copulation with a sexually vigorous male. More recently, the effects of glutamate, its selective receptor agonists AMPA, NMDA and kainate, and three of its selective receptor antagonists, DNQX, CNQX and AP-5, were tested on a full battery of appetitive and consummatory sexual behaviors (Georgescu and Pfaus, 2006a, b). We observed that in females primed with EB+P, glutamate decreased hopping and darting, kainate decreased lordosis quotients and solicitations and increased pacing, AMPA decreased solicitations, hoping and darting, lordosis quotients and lordosis magnitudes and increased defensive behaviors, whereas NMDA increased pacing and defensive behaviors and decreases lordosis quotients and lordosis magnitudes. Conversely, AP-5, an antagonist of the NMDA receptor, increased lordosis quotients in females primed with EB. CNQX, an antagonist of the AMPA/kainate receptor, increased the number of solicitations, lordosis quotients, lordosis magnitudes and number of

ejaculations by the males in EB-primed females and increased the number of solicitations in females primed with EB+P. DNQX, also antagonist of the AMPA/kainate receptor, increased the number of solicitations, hopping and darting, lordosis magnitudes and number of ejaculations by the males and decreased pacing in females primed with EB. In females primed with EB+P, DNQX increased solicitations and decreased pacing. Overall, those observations indicate that activation of glutamate receptors in the VMH results in inhibition of sexual behaviors and activation of pacing and defensive behaviors, a pattern reminiscent of estrus termination.

Estrus termination, induced naturally by coital stimulation and in particular by intromissions by the male, is characterized by an increased number of rejection responses, longer pacing intervals, decreased or absent solicitations, and decreases in lordosis quotients and magnitude (Erskine and Baum, 1982; Lodder and Zeilmaker, 1976; Pfaus et al., 2000). Manually-applied VCS using a lubricated glass rod at intervals similar to those of paced intromissions also facilitates estrus termination (Pfaus et al., 2000). VCS, whether manually-applied or received from male intromissions, induces the immediate-early gene product Fos in the ventrolateral region of the VMH (Blanger and Auger, 1995; Georgescu et al., 2009; Pfaus et al., 1993; 1996; Tetel et al., 1993; 1994; Wersinger, Baum, and Erskine, 1993). Transection of the pelvic nerve eliminates the effect of VCS on both estrus termination (Lodder and Zeilmaker, 1976) and Fos induction in the ventrolateral VMH (Pfaus, Manitt and Coopersmith, 2006), suggesting that VCS

activates an inhibitory system that abbreviates the duration of estrus. Because 15 to 30% of the Fos induced by VCS in the ventrolateral VMH is within glutamate neurons (Georgescu et al., 2009), it is likely that this inhibitory system is glutamate-driven and controlled by inputs to the VMH.

Treatment of OVX rats with the steroid hormones EB and P reduces the cellular response to VCS in the VMH (Blanger and Auger, 1995; Pfaus et al., 1993; Pfaus et al., 1996; Tetel et al., 1993; 1994) and blunts the ability of VCS to activate Fos within glutamate neurons in this area (Georgescu et al., 2009). Thus, we postulate that EB and P in the VMH inhibit the ability of VCS to activate the glutamate-driven system that brings about estrus termination, thereby allowing females to receive the requisite amount of sexual stimulation so neuroendocrine changes necessary for sexual reward and pregnancy can occur

The present experiment tested whether blockade of the AMPA/kainate receptor by the glutamate antagonist DNQX can affect the induction of estrus termination by manual VCS. DNQX was chosen on the basis of our previous work showing it to be the most effective glutamate antagonist in the facilitation of female sexual behavior (Georgescu and Pfaus, 2006b). DNQX or saline was administered prior to VCS, which was applied 12 hours before behavioral testing with a sexually vigorous male, as in Pfaus et al. (2000). The ability of VCS to induce a pattern of estrus termination was examined.

2. Methods

2.1. Subjects and procedure

Thirty-two sexually naïve Long-Evans females weighing between 200–250g were obtained from Charles River Canada, Inc., St. Constant, QC. Females were housed in groups of five in large Plexiglas cages with wood-chip bedding. Following cannulation, females were housed individually in plastic shoe-box cages (36 x 26 x 19 cm) with wood-chip bedding. The colony room was maintained on a reversed 12:12hr light/dark cycle (lights off at 08:00) at approximately 21°C with food and water continuously available.

Females were OVX bilaterally through lumbar incisions following aneasthesia induced by a 4:3 mixture of ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4mg/ml) injected intraperitoneally in a volume of 1ml/kg. One week of post-surgical recovery was given prior to sexual training. Thirty-two Long-Evans males from the same breeder were used as conspecific stimuli. The males received a minimum of 10 sexual training sessions with receptive females before use and all were sexually vigorous copulators. The males were housed in groups of four in hanging wire-mesh cages in the same colony room.

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

2.2. Hormonal injections and acquisition of sexual experience

Sexual receptivity was induced in females by subcutaneous injections of EB (10μg/0.1ml of reagent grade sesame oil) 48 hours and P (500μg/0.1ml of reagent sesame oil) four hours prior to sexual training sessions. Steroids were obtained from Steraloids (Hanover, NH). Females received ten 30-min copulatory training sessions at 4-day intervals in bilevel chambers in the middle-third of their dark-cycle (13:00-15:00).

2.3. Cannulations, infusions and VCS administration

After the 10th trial of sexual behavior, females were taken off hormones for 26 days. They were cannulated bilaterally to the VMH between day 10 and 19 of this 26-day period. Cannulations were performed under anesthesia with sodium pentobarbital (65mg/kg) injected intraperitoneally. Cannulation coordinates were 1mm lateral to the midline on either side of Bregma, 0mm anterior to Bregma, and 8.5mm ventral to Dura, at a 5-degree elevation angle of the skull. The tip of the cannula guide ended 1mm above the desired target area. Cannula blockers were cut so that they would protrude 0.5mm from the guide cannula. The 22-gauge infusion cannulae were cut to 1mm longer than the guide cannula. Guide cannulae, cannulae blockers, injection cannulae and dust caps were obtained from Plastic One. Females were given seven days of *post*-surgical recovery before testing began. On day 26, EB was injected at 13:00 and on day 28, P was injected at 17:00. Four hours later, half the females were infused

bilaterally with 1μl of 19.8mM DNQX (Georgescu and Pfaus, 2006b), the other half with 1μl saline. Females were randomly assigned to the groups. The drug and saline were infused at a rate of 1μl/minute for one minute using an infusion pump (Harvard Apparatus, Pump 22). The infusor was left in for another full minute and a half to ensure full absorption. The concentration of DNQX was obtained by diluting the DNQX into warm phosphate buffer (pH = 7) and vortexed until the DNQX was completely dissolved. In the case of saline infusions, a 0.1M phosphate buffer with a pH of 7 was infused in a volume of 1μl/side. Immediately following the infusions, females in the DNQX and saline groups were randomly assigned to receive 1 or 50 VCSs. VCS was administered as described previously by Pfaus et al. (1996; 2000), with a lubricated glass rod in clusters of 5, each spaced 2 seconds apart. Clusters of 5 VCSs were applied at 6-minute intervals over the course of one hour for a total of 50 VCSs.

2.4. Behavioral testing, perfusions and histology

The following day, at 09:00, females were tested in bilevel chambers for 30 min with sexually vigorous males. Females were then sacrificed in order to verify proper cannulae placement. They were injected intraperitoneally with 1ml of sodium pentobarbital and perfused intracardially using a 50ml syringe filled with ice-cold phosphate buffer saline followed by 50ml of 4% paraformaldehyde in 0.1M phosphate buffer. The brains were placed in a 4% paraformaldehyde solution for 4 hours, then transferred into a 30% sucrose solution overnight. They

were frozen in dry ice and sliced into coronal sections using a cryostat. The exclusion criteria was set such that animals with one or both guide cannulae ending outside of the boundaries of the VMH were excluded from the study (N = 9), leaving data solely from subjects that had correct bilateral (N = 23) cannulations to the VMH included in the analyses. Cannulation placement data from subjects included in the statistical analyses are shown in Fig. 4.

2.5. Behavioral analyses

Testing sessions were videotaped and scored using a computerized event recorder customized for female sexual behavior (Cabilio, 1996). The frequency of appetitive behaviors (solicitations and hops and darts), pacing (number of level changes in the bilevel chamber), defensive behaviors (including boxing, fighting, kicking and prone defensiveness, characterized by the female lying on her back), and male ejaculations, were included in the analyses. Lordosis was analyzed from two perspectives: lordosis reflex magnitude (LM, on a 1 to 3 scale), and lordosis quotient (LQ, lordosis:mount ratio). LM and LQ were calculated as in Hardy and DeBold (1971).

2.6. Statistical analyses

A 2 (stimulation: 1VCS vs. 50VCSs) x 2 (treatment: DNQX vs. saline) between subjects ANOVA was conducted to assess the effects of VCS and DNQX treatment, and their interaction on each of the behaviors. Tukey *post hoc* analyses were conducted when statistical significance was detected to specify

where the significant differences lay. Only significant main effects and interactions between these variables are reported.

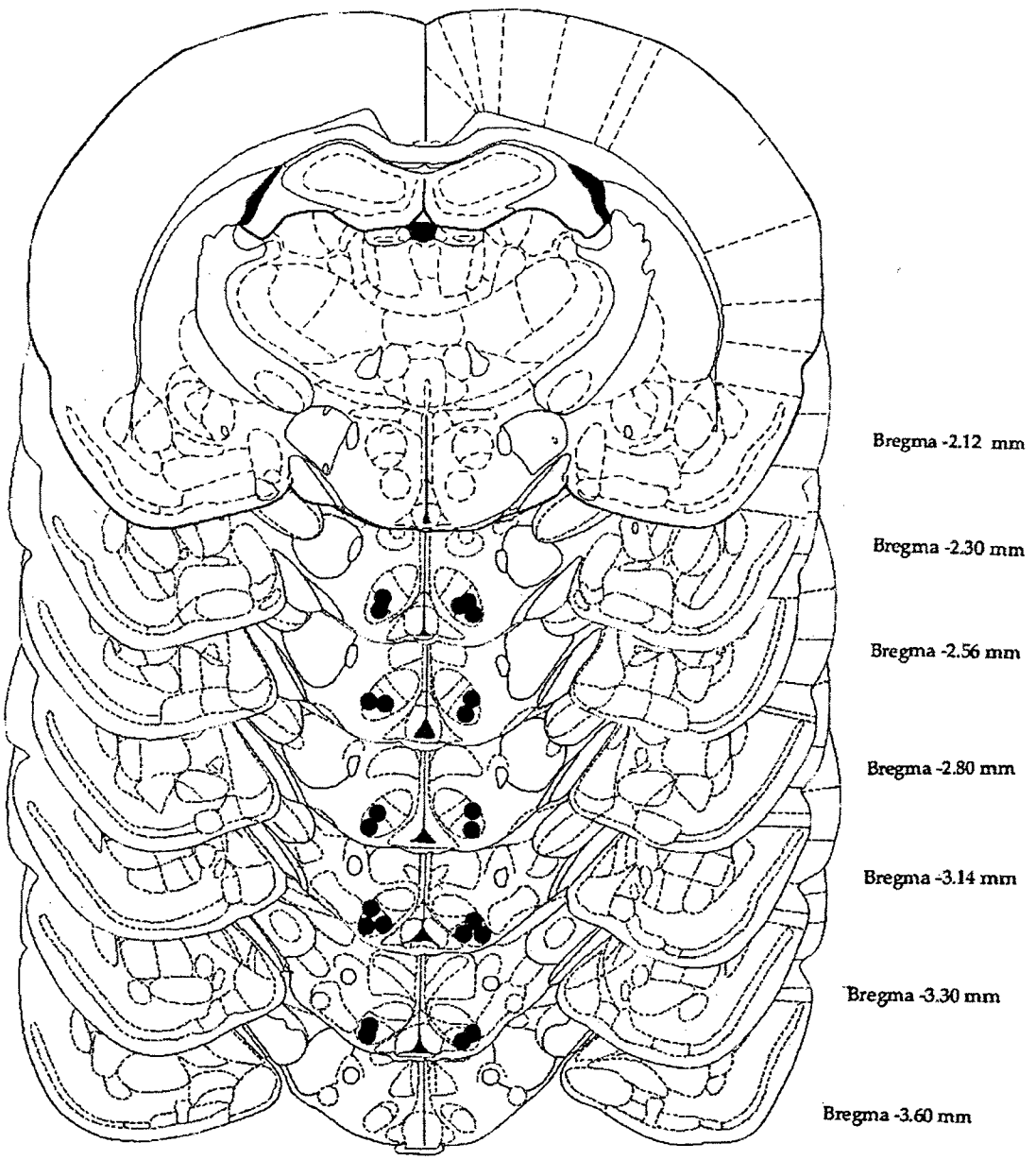

Figure 4. Placement data for females with correct bilateral cannulations.

3. Results

The effect of 1VCS vs. 50VCSs, and DNQX vs. saline infusions on the number of appetitive behaviors (solicitations and hops and darts), pacing, LQ, LM, defensive behaviors and male ejaculations are shown in Fig. 5.

3.1. Appetitive behaviors

A significant interaction was detected between drug and VCS treatment, $F(3, 19) = 6.285$, $p < 0.01$. A Tukey post hoc analysis revealed that females in the 50VCS/DNQX group engaged in significantly more appetitive behaviors than females in the 50VCS/saline group, whereas in the 1VCS condition, animals that received DNQX did not engage in more appetitive behaviors than those that received saline.

3.2. Level changes

A significant interaction between drug and VCS treatment was detected, $F(3,19) = 4.154$, $p < 0.05$, post hoc analyses revealing that animals in the 1VCS/saline group changed levels significantly more than animals in the 50 VCS/saline group, whereas animals that received DNQX engaged in similar numbers of level changes regardless of the amount of VCS received.

3.3. Lordosis quotients

The ANOVA detected a significant interaction between drug and VCS treatment, $F(3,17) = 8.141$, $p<0.01$. Post hoc analyses revealed that animals in the saline group that received 1VCS had significantly higher LQs than animals

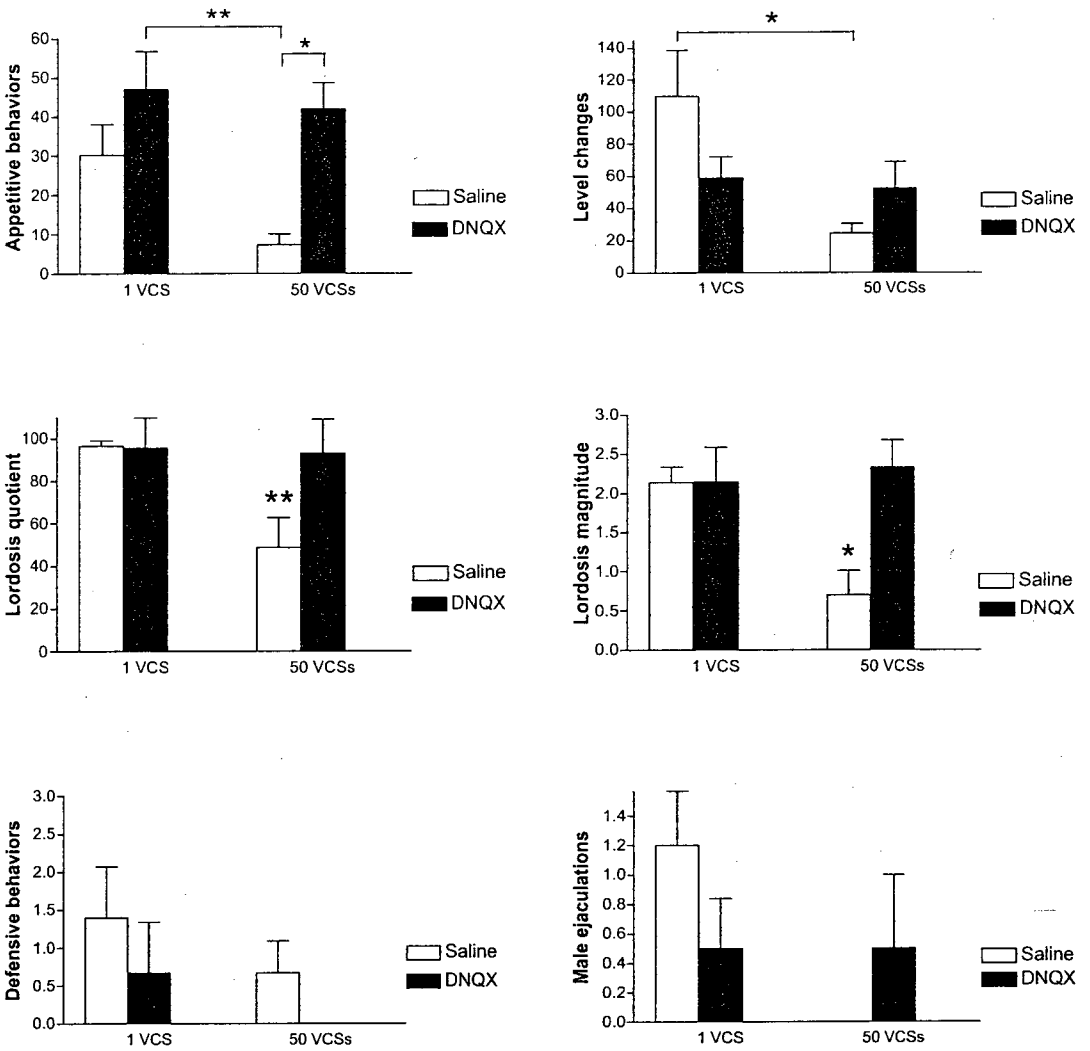

Figure 5. The effect of Saline or DNQX infusions on the mean number of appetitive behaviors, level changes, lordosis magnitude, lordosis quotient, defensive behaviors and male ejaculations in females that received 1 or 50 VCSs. Data are means ± SEMs. (* : $p<0.05$; **: $p<0.01$)

that received 50VCSs, whereas animals that received DNQX displayed similar LQs.

3.4. Lordosis magnitudes

The ANOVA detected a significant interaction between drug and VCS

treatment, $F(3,19) = 4.874$, $p<0.05$. Post hoc analyses revealed that animals in the saline group that received 1VCS had significantly higher LMs than animals that received 50VCSs, whereas animals that received DNQX had similar LMs.

3.5. Defensive behaviors and ejaculations

The ANOVAs did not detect significant interactions between drug and VCS treatment on the number of defensive behaviors displayed by the females or on the number of male ejaculations.

4. Discussion

The results of the present experiment indicate that blockade of the AMPA/kainate receptor by its selective receptor antagonist DNQX fully reverses the inhibitory effects of VCS administered 12 hours prior to behavioral testing with a sexually vigorous male. Despite having been subjected to amounts of VCS that were previously shown to induce estrus termination (Pfaus et al., 2000), females that received DNQX were still displaying solicitations and lordosis in response to male mounts, whereas females that received saline were not. More specifically, treatment with DNQX eliminated the differences observed in the saline group between animals that received 1 VCS and those that received 50 VCSs in terms of number of appetitive behaviors, including solicitations and hops and darts, pacing, lordosis quotient and lordosis magnitude. Altogether, females that received DNQX behaved similarly regardless of the amount of VCS received. Those females also behaved similarly to those that received saline and 1VCS.

This strongly suggests that blockade of the AMPA/kainate receptor by DNQX effectively eliminated the induction of estrous termination by VCS and prolonged the period of behavioral estrus. Thus, glutamate appears to play a role in the onset of estrus termination. It may drive an inhibitory system in the VMH that is sensitive to the endocrine status of the female, because EB and P blunt the activation of VMH glutamate neurons by VCS (Georgescu et al., 2009).

Increased glutamate neurotransmission within the VMH may result in inhibition of female sexual behavior in several circumstances. First, glutamate may mediate inhibition of sexual behavior observed during periods of sexual non-receptivity, such as during the juvenile period, following ovariectomy, when adult females are not in estrus, or during pregnancy and lactation. Glutamate levels have been analyzed in OVX females primed with oil, EB, or EB+P at baseline and during copulation (Chapter 3). In animals that received oil, glutamate levels were low at baseline and peaked following introduction of the male into the chamber. This suggests that in absence of hormones, sensory information obtained by being in proximity of a sexually mature male is enough to activate release of glutamate in the VMH. In animals primed with EB, glutamate levels at baseline were higher than those collected from animals in the oil group, and decreased following introduction of the male into the chamber. In animals primed with EB+P, glutamate release was low throughout the entire copulation session, confirming previous results in which EB and P inhibited activation of glutamate

neurons in the VMH. These results suggest that glutamate release is sensitive to different aspects of the sexual interaction in each hormonal regimen.

Another possibility is that glutamate facilitates mechanisms that mediate behaviors incompatible with sexual activity. This idea was first introduced by Steinach (1910), in regards to the disinhibitory actions of testicular secretions on the clinging reflex during the rut season in male frogs. Ovarian hormones might induce sexual behavior in part by inhibiting glutamate release in the VMH, which may be activating behaviors incompatible with sexual behavior. In fact, there are important neural mechanisms within the VMH that participate in the regulation of feeding (Bray and York, 1998; Gold et al, 1972; Marshall et al, 1955; Storlein and Albert, 1972), aggression (Adamec, 1991; Brayley and Albert, 1977; Sterner et al., 1992), and pain (Borszcz, 2006). As such, studies on hypoglycemia have found that glutamate release from steroidogenic factor-1 neurons within the VMH is an important mechanism within the neurocircuitry that underlies prevention of hypoglycemia (Tong, McCrimmon, Dhillon, Choi, Kramer, et al., 2007). It is therefore possible that glutamate release in the VMH is inhibitory to sexual behavior but facilitative to other behaviors such as feeding.

A third possibility is that glutamate neurotransmission is involved in the onset of estrus termination. This hypothesis is supported by results of the present experiment, which clearly show that blocking glutamate neurotransmission at the AMPA/kainate receptor reverses the effects of VCS on estrus termination. Estrus termination is characterized by decreases in appetitive behaviors and lordosis,

and increases in pacing and defensive behaviors (Coopersmith, Candurra, and Erskine, 1996; Erskine and Baum, 1982; Lodder and Zeilmaker, 1976; Pfaus et al., 2000). Glutamate release may be prompted by reception of VCS in amounts sufficient to abbreviate the period of estrus. As the number of ejaculatory series increases, inhibiting sexual behavior would allow the female to engage in pacing and rejection/defensive behaviors to keep the male away. Indeed, increased glutamate transmission in the VMH results in inhibition of appetitive behaviors and lordosis and facilitation of pacing and defensive behaviors. If glutamate is involved in mediation of pacing, then it may also play an important role in pregnancy/pseudopregnancy and fertility. Thus, an in-depth analysis of glutamate's role in pacing and defensive behaviors is warranted.

Bilateral infusions of glutamate agonists kainate and NMDA increased pacing rats in OVX females primed with EB+P (Georgescu and Pfaus, 2006a). AMPA and NMDA infusions also increased the number of defensive behaviors in which females engaged during copulation. Overall, infusions of glutamate agonists to the VMH resulted in marked increases in fighting and locomotor activity, including level changing, which was used as a measure of pacing (Georgescu and Pfaus, 2006a). Conversely, blockage of glutamate's AMPA/kainate receptor by DNQX resulted in significant reductions in pacing rates relative to saline infusions (Georgescu and Pfaus, 2006b). Glutamate antagonists did not have an effect on defensive behaviors. Thus, we have argued previously (see Georgescu et al., 2009), that glutamate may exert its inhibitory

role on female sexual behavior by activating pacing and defensive behaviors. In the present experiment, infusion of DNQX did not result in significant decreases in pacing or defensive behaviors.

However, animals that received 50VCSs and that were treated with saline had very low pacing and defensive behavior rates, but had the option to sit on one of the two ledges of the bilevel chambers where males typically do not attempt to mount them. As mentioned above, several experiments have consistently shown that increases in pacing rates and defensive behaviors accompany decreases in appetitive responses and lordosis (Coopersmith, Candurra, and Erskine, 1996; Erskine and Baum, 1982; Lodder and Zeilmaker, 1976; Pfaus et al., 2000). What may explain the lack of increases in pacing or defensive behaviors in our saline/50VCSs group is that males used in the present experiment received a lot of sexual experience with females under various hormonal regimens. Overtime, these males become particularly attuned to the hormonal state of their female counterparts: they quickly learn not to mount non-receptive females and to adjust the rate of copulation according to the females' level of receptivity. Therefore, females in the 50VCS/Saline group did not have to increase pacing rates or engage in defensive behaviors to control the rate of copulation because the males quickly reduced their attempts to mount them since they were no longer in behavioral estrus, as reflected by decreases in appetitive behaviors, LQs and LMs. Had these males been less sexually experienced, the rates of pacing and frequency of defensive behaviors in the

50VCS/Saline group would almost certainly be higher, rendering the effect of DNQX inhibitory on these behaviors.

If glutamate is involved in mediation of pacing and defensive behaviors, then it plays an important role in ensuring that the number and timing of intromissions received by the female are optimal for the neurochemical changes necessary for successful impregnation to occur. Adler (1969) reported that if too few intromissions are received, the likelihood of pregnancy is reduced. When intromissions are too closely spaced, the same effect occurs (Edmonds, Zoloth, and Adler, 1972). Others have observed that pacing intervals depend on the strength of stimulation: during copulation in pacing chambers, females exit 41% of the time following a mount without intromission, 71% of the time following an intromission and 100% of the time following an ejaculation (Erskine, 1985). The length of time spent away from the male follows the same rule: inter-mounts intervals are shortest, followed by inter-intromissions intervals, followed by inter-ejaculations intervals (Berman, 1961; Gilman, Mercer and Hitt, 1979; Krieger, Orr, and Perper, 1976; Pierce and Nuttall, 1961). If glutamate participates in the regulation of pacing and defensive behaviors, it is probably also involved in mediation of the interplay between pacing, proceptive behaviors and lordosis. If so, glutamate neurons within the VMH must communicate directly with neurons that mediate proceptive behaviors in the mPOA, the activation of which is generally associated with inhibition of lordosis and facilitation of pacing and proceptive behaviors (Hoshina et al., 1994; Modianos, Delia, and Pfaff, 1976;

Whitney, 1986). Interestingly, the VMH has direct projections to the mPOA (Fahrbach et al., 1989), and, in turn, the mPOA has projections to the ventrolateral VMH (Conrad and Pfaff, 1976; Anderson and Shen, 1980), although the neurochemical nature of these projections is unknown. Thus, the VMH sends projections to the mPOA, perhaps activating appetitive and pacing behavior, and the mPOA to the VMH, activating lordosis by inhibiting glutamate via release of neurochemicals with facilitative effects on lordosis in the VMH but inhibitory cell properties (see Georgescu et al., 2009, for a review of neurochemicals with these properties).

In summary, the glutamate signal within the VMH is inhibitory to sexual behavior. Glutamate receptor activation results in strong inhibitory effects on lordosis and appetitive sexual behaviors and activation of pacing and defensive behaviors. Conversely, blockade of the AMPA/kainate receptor by selective receptor antagonist DNQX results in facilitation of sexual behaviors and inhibition of pacing and defensive behaviors. The present experiment demonstrated that microinfusions of DNQX effectively reverse estrus termination, suggesting that activation of glutamate receptors within the VMH is involved in the onset of estrus termination.

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Summary of Chapter 2

The results presented in Chapter 2 show that blocking glutamate neurotransmission at the AMPA/kainate receptor reverses the effects that VCS has on estrus termination. DNQX or saline were infused prior to administration of 1 or 50 VCS. Following administration of 50VCs, females infused with DNQX were still in heat, whereas animals infused with saline were not. More specifically, DNQX effectively reversed the effect of VCS on appetitive behaviors, lordosis quotient and lordosis magnitude, but had no effect on pacing, defensive behaviors, or male ejaculations. Overall, the results presented in Chapter 2 suggest that blocking glutamate transmission at the AMPA/kainate receptor delays onset of estrus termination. The goal of the experiment presented in the next chapter was to measure glutamate release in the VMH of females primed with Oil, EB, and EB+P while they had access to sexually vigorous males.

Chapter 3

Glutamate release in the ventromedial hypothalamus of the female rat during copulation: Modulation by ovarian hormones

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ABSTRACT

Bilateral infusions of glutamate or its selective receptor agonists within the VMH of EB and P primed female rats inhibit both appetitive and consumatory aspects of sexual behavior. Conversely, infusions of selective glutamate receptor antagonists facilitate these measures in females primed with E-alone. Because VCS activates glutamate neurons in the VMH and induces a faster termination of estrus behavior, the present study examined the effects of hormonal priming with vehicle (oil) (O), EB, and EB+P on glutamate release within the VMH. All animals were sexually experienced. Females in the O and E groups received either O or E, respectively, 4 hours prior to testing. Animals in the EB+P group were primed with EB 48 hours prior to priming with P, and tested 4 hours later. Testing was conducted over 4h10mins, including a 2 hour-period of habituation to the testing chamber, a 30 minute-period of baseline sample collection, and a 100-minute period during which a sexually vigorous male was introduced into the testing chamber. Glutamate was collected through a microdialysis probe inserted to the vlVMH and subsequent dialysates were analyzed using HPLC. Dialysates were collected every 20 minutes following introduction of the male in the testing chamber. Statistical analysis of percent changes of glutamate from baseline levels revealed a significant interaction between hormonal regimen and time elapsed since introduction of the male into the chamber. More specifically, priming with O resulted in significant increases in glutamate release from baseline once the male was introduced to the chamber. Priming with EB+P increased the % change of glutamate release from baseline levels, whereas

priming with EB alone resulted in decreases of the % change of glutamate release from baseline levels. Within 60 minutes following introduction of the male to the chamber, glutamate levels returned to baseline in all three groups. These results, along with other data from our laboratory lend support to the existence of a complex interaction between glutamate and GABA within the VMH under different hormonal regimens. It is not known, however, whether other neurotransmitter systems may mediate this interaction.

Introduction

The VMH is generally recognized as a neural substrate that is central in the control of female sexual behavior. It is rich in E and P receptors (Pfaff and Keiner, 1973) that induce protein synthetic changes conducive to sexual behavior (e.g., Etgen, 1987; Meisel and Pfaff, 1985). Lesions of the VMH result in dramatic decreases in lordosis and proceptive behaviors, such as solicitation and hopping and darting (Malsbury, Kow, and Pfaff, 1977; Matthews and Edwards, 1977; Pfaff, 1980; Pfaff and Sakuma, 1979). Implants of EB to the VMHvl of ovariectomized rats reestablish lordosis in response to flank stimulation (Rubin and Barfield, 1983a), whereas implants of P to the VMH of E-primed rats reestablish solicitation to levels observed in E and P-primed animals (Rubin and Barfield, 1983b). A robust induction of Fos protein is observed in the VMH following VCS, suggesting that this nucleus is activated by VCS (Blanger and Auger, 1995; Pfaus et al., 1993; Pfaus et al., 1996; Tetel et al., 1993; Tetel et al., 1994). Steroid hormones blunt this activation following administration of small to moderate amounts of VCS, but lose their ability to do so following larger amounts (Pfaus et al., 1996). Thus, EB and P facilitate sexual behavior, yet inhibit cellular activation following VCS in the VMH. This suggests that the cells that are inhibited by EB and P do not participate in facilitation of sexual behavior and may actually have an inhibitory function. As females receive amounts of VCS that have previously been shown to induce estrus termination, EB and P lose their ability to inhibit those cells, suggesting they participate in the inhibition of sexual behavior.

We observed that 15-30% of cells activated in the VMHvl by VCS also stain for glutamate (Georgescu et al., 2009). Infusions of glutamate agonists to the VMH result in an overall inhibition of female sexual behavior. Kow et al. (1985) first noted a dose-dependent decline in lordosis induced by manual flank stimulation in OVX rats implanted subcutaneously with estrogen capsules following infusions of glutamate or its ionotrophic agonist kainate to the VMH. McCarthy et al. (1991) also reported rapid inhibition of lordosis induced by copulation with a sexually vigorous male following infusions of ionotrophic glutamate agonist NMDA to the VMH of OVX rats primed with EB and P. We conducted a systematic analysis of the role of glutamate receptors on both appetitive and consumatory sexual behaviors, and noted that activation of glutamate receptors has an inhibitory effect on female sexual behavior (Georgescu and Pfaus, 2006a). More specifically, infusions of glutamate and its selective receptor agonists AMPA, NMDA and kainate to the VMH of OVX females primed with EB+P decreased lordosis and solicitation and increased defensive and pacing behaviors during copulation with sexually vigorous males. Infusions of glutamate receptor antagonists DNQX and CNQX increased lordosis and solicitations and decreased defensive and pacing behaviors in OVX females primed with EB (Georgescu and Pfaus, 2006b). Overall, activation of glutamate receptors resulted in decreases in lordosis and solicitation, and increases in pacing and defensive behaviors. These results suggest that increased glutamate transmission in the VMH may result in an overall inhibition of sexual behavior.

The aim of the present experiment was to examine the pattern of glutamate release during copulation. We hypothesized that glutamate release would increase as the copulatory session extends, and that steroid hormones EB and EB+P would partially inhibit this release.

Methods

Subjects

Twenty naïve female Long-Evans rats weighing between 200-250g were obtained from Charles-River Canada, Inc., St-Constant, QC, and housed in groups of five in large Plexiglas shoebox cages with wood-chip bedding. The colony room was maintained on a reversed 12:12hr light/dark cycle (lights off at 08:00) at approximately 21 ℃ with food and water ad libitum. Prior to ovariectomy, females were anesthetized with ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4mg/ml), mixed in a ratio of 4:3 and injected intraperitoneally, in a volume of 1ml/kg. All OVX females were given a week of post-surgical recovery prior to sexual training. Ten Long-Evans males from the same breeder were used as sexual stimuli. The males were housed in groups of five in hanging wire-mesh cages in the same colony room. All animals received 10 sexual training sessions in microdialysis chambers.

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

Hormone treatment and experimental design

Full sexual receptivity was induced by subcutaneous injections of EB (10µg/ 0.1ml reagent grade sesame oil) 48 hours and P (500µg/ 0.1ml reagent grade sesame oil) 4 hours prior to sexual training sessions. All steroids were obtained from Steraloids (Hanover, NH). Females and males received five 30-min sexual training sessions in microdialysis chambers at 4-day intervals prior to the study. Training sessions were conducted in the middle-third of the rat's dark cycle (14h-16h). Females were then randomly distributed to one of 3 hormonal regimens: Oil (Oil+Oil), EB (EB+Oil), or EB+P (n=6/group) for an additional 4 cycles. Following the last injection and a week following cannulation to the VMH, individual testing of each female with a male began. Females were placed in microdialysis chambers for 6 hours, during which 3 baseline glutamate levels were collected in the hour just prior to testing. Each baseline sample was collected over 20 minute-bouts. A male was then introduced into the chamber. The copulatory session was videotaped and dialysates were collected every 20 minutes for 2 hours, resulting in 6 dialysate samples, along with 3 baseline samples for analysis.

Cannula implantation

Females were anesthetized with sodium pentobarbital (65mg/kg) injected ip. Unilateral 22-gauge guide cannulae cut to 7.2mm in length were implanted using a Kopf stereotaxic instrument. Stereotaxic coordinates were relative to Bregma and implants were made with the head tilted upward at a 5 degree

angle: A-P: 0mm; M-L: ±1mm; and D-V: -7.2mm ventral to dura. Cannula blockers were cut so that they would protrude 0.5mm from the guide cannula. Guide cannulae, cannulae blockers, and dust caps were obtained from Plastics One (Roanoke, VA). Females were given seven days of post-surgical recovery before testing.

Microdialysis probe

Dialysis probes were custom made in the laboratory, as described by Sorge, Rajabi, and Stewart (2005). The semipermeable dialysis membrane was 1mm long, perfusing artificial CSF at a rate of 0.03l/min. The probes were inserted 5 hours prior to collection of the baseline samples, and 6 hours prior to collection of samples during testing with males.

Microdialysis chamber

Microdialysis chambers were also custom made at Concordia University. Each box was 38 cm × 60 cm × 38 cm in size with clear Plexiglas walls and bedded floors covered by evenly spaced stainless steel tubing.

Glutamate detection

Glutamate concentrations were determined by HPLC-UV. The HPLC-UV system consisted of a dual piston pump (SSI, from Lab Alliance), a 4 mm reverse-phase column (15 cm × 4 mm, Haisil 5µm, Lab Alliance), and a refrigerated autosampler injector injector valve (Model AS3000, Lab Alliance)

with a 100µl sample loop. An LC305 UV detector (Lab Alliance) was used for the analysis of glutamate.

The precolumn derivatization of glutamate was performed with an *o*-phthalaldehyde/mercaptoethanol reagent (0.4 M borate, 0.04 M phthalaldehyde, and 0.4 M 2-mercaptoethanol, pH 9.3). Briefly, 10 µl of the reagent was added to and mixed with the samples by the microsampler. After a 60sec reaction period at 6°C in the microsampler, 20µl of the mixture was injected onto the column. The elution of glutamate was achieved with a mobile phase consisting of 0.2M sodium phosphate and 13% acetonitrile, at a flow rate of 0.9ml/min. After the appearance of the glutamate peak on the chromatogram, a 40% acetonitrile was used to accelerate the elution of other peaks on the column. The detection limit was 15 pmol/injection. Sample levels are reported uncorrected for in vitro recovery.

Perfusion and histology

Once the testing was concluded and all dialysates were collected, females were sacrificed by overdose of sodium pentobarbital (120 mg/kg) in order to verify proper cannulae placement. Prior to the perfusion, microdialysis probes were inserted into the guide cannulae. Females were then perfused intracardially using a 50ml syringe of ice-cold phosphate-buffered saline followed by 50ml of 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and placed in a 4% paraformaldehyde solution for 4 hours, and into a 30% sucrose solution overnight. Brains were then blocked around the area of the anterior

hypothalamus, mounted on a chuck, and sliced into coronal sections using a cryostat. All animals that had microdialysis probes ending 0.5mm below the VMH were included in the study (Oil: N=4; EB: N=5; EB+P: N=5; see Fig. 6).

Behavioral data collection

All sessions were recorded and videoscored using the Behavioral Observation Program (Cabilio, 1996). Behavioral data was recorded during the 2-hour copulation session and videoscored in bouts of 20 minutes, resulting in six 20-min sets of behavioral data corresponding to each collected dialysate sample.

Results

Statistical analyses of basal glutamate release

Prior to the male-stimuli exposure, basal glutamate concentrations were obtained from means of three consecutive samples that differed from one another by no more than 10%. A 3 (Hormone Treatment: Oil, EB, EB+P) x 1 (basal glutamate concentration measured in pg/10µl) ANOVA performed on glutamate concentrations (pg/10µl) revealed a marginal effect of Hormone Treatment on basal glutamate concentration ($P = 0.092$). Posthoc Tukey's HSD analysis ($P < 0.05$, two-tailed) on individual means revealed that the EB-primed females had significantly higher basal glutamate release than the O-primed females (Fig. 7).

Statistical analyses of glutamate release during copulation

A 3 (between subjects, Hormone Treatment: Oil, EB and EB+P;) x 7

(within subjects, Sampling Time: basal average sample prior to introduction of the male into the chamber; six consecutive 20-mins bout samples in presence of sexually vigorous male stimulus: at minute 20, 40, 60, 80, 100, 120 minutes following introduction of the male into the chamber) mixed factorial ANOVA was

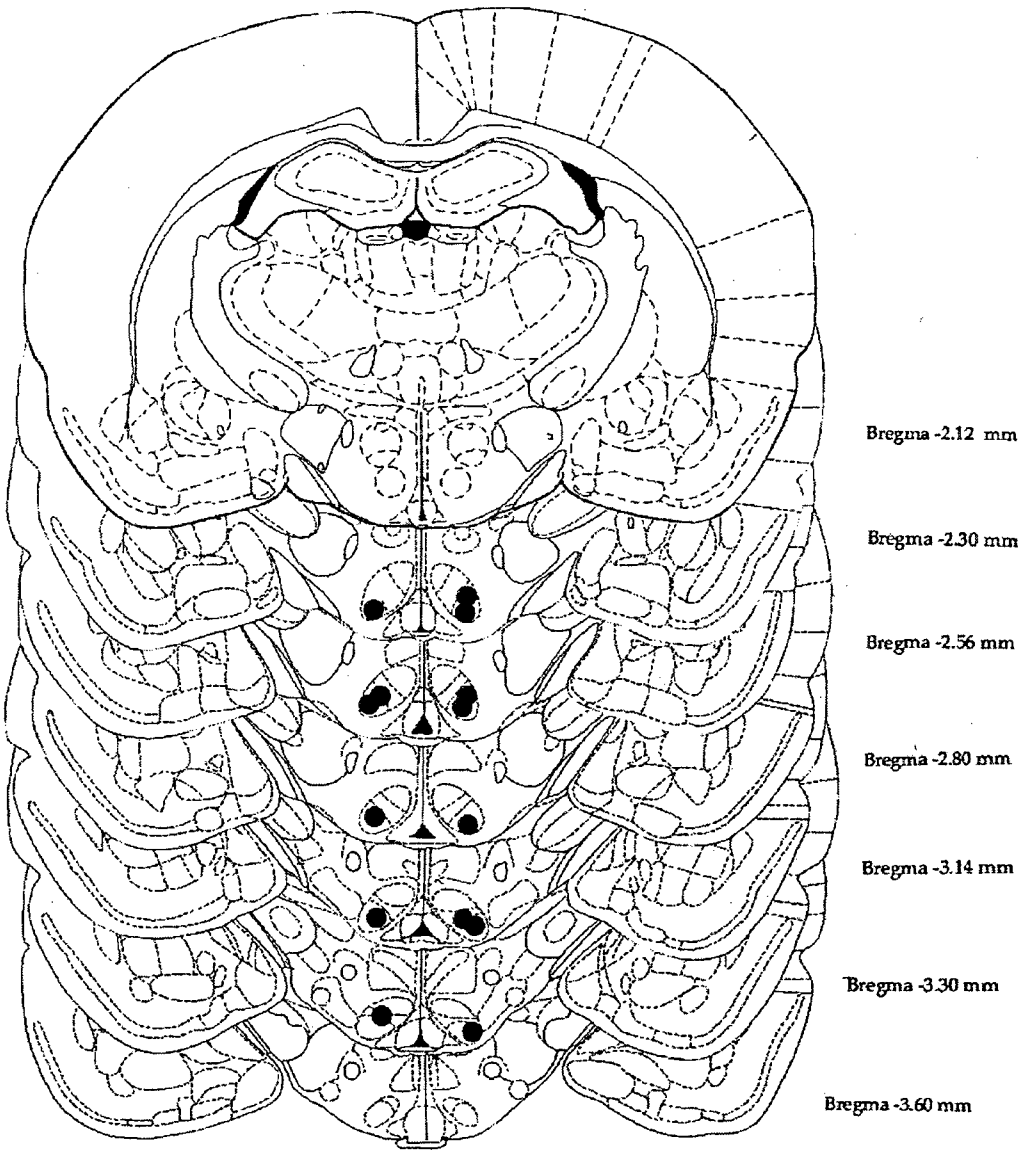

Figure 6. Placement data for females in the O, EB, and EB+P group included in the analyses.

performed on percent changes from basal concentrations (see Fig. 7). The ANOVA showed a significant interaction between Hormone Treatment and Sampling Time [F(12, 66) = 2.43, P < 0.01] and a main effect of Sampling Time

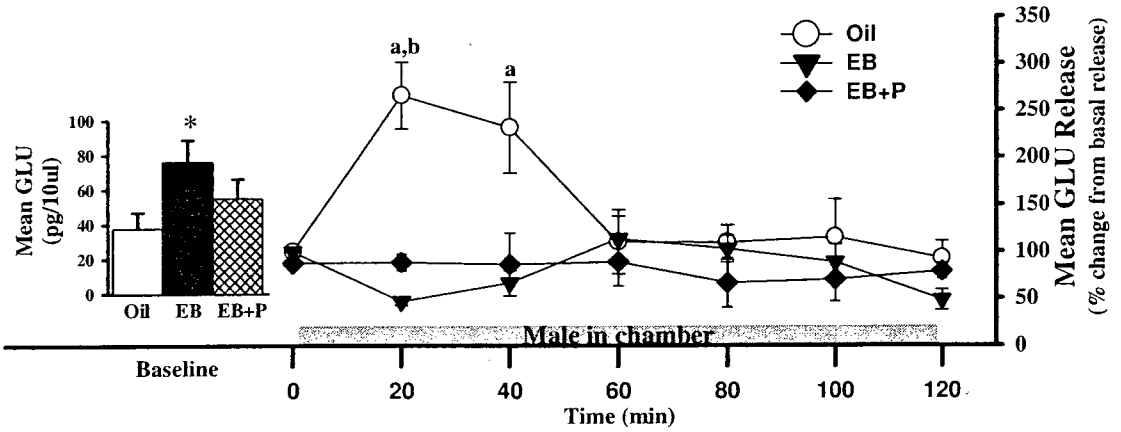

Figure 7. Left: Baseline glutamate release in the VMH as a function of hormonal priming. Right: Percent change from baseline glutamate levels in the VMH as a function of hormonal priming and time spent in presence of a male. (*: $p<0.05$; a: Oil significantly different than EB; at min. 20, $p<0.001$; at min. 40, $p<0.05$; b: Oil significantly different than EB+P, $p<0.001$). Data are means ± SEMs.

[F(6, 66) = 3.24, P < 0.001]. A two-tailed post hoc Tukey HSD analysis ($p<0.05$) on individual means revealed that percent changes from baseline glutamate release were significantly different between the Oil group and the EB ($p<0.001$) and EB+P ($p<0.001$) groups at minute 20. At minute 40, percent changes from baseline glutamate release was significantly larger in the Oil group than in the EB group ($p< 0.05$). There was a trend towards statistical significance between the Oil group and EB+P group ($p=0.053$). No other differences were statistically significant at any time point between the 3 hormone groups.

In the Oil group, pairwise comparisons revealed that glutamate levels significantly increased from baseline at 20 minutes ($p=0.019$). In this same group,

percent change from baseline glutamate at 20 minutes was significantly higher than at 60 minutes ($p=0.009$), 80 minutes ($p=0.019$), 100 minutes ($p=0.003$), and 120 minutes ($p=0.002$). Percent change from baseline glutamate levels was also significantly higher at 40 minutes than at 60 minutes ($p=0.042$), 100 minutes ($p=0.026$), and 120 minutes ($p=0.019$), and there was a trend towards significance at baseline (0.073) and at 80 minutes (0.085). In the EB group, glutamate levels significantly decreased from baseline at minute 20. There were no other statistically significant differences in this hormone group. In the EB+P group, there were no significant differences at all in percent change from baseline glutamate levels at any time point.

A 3 (between subjects, Hormone Treatment: Oil, EB and EB+P;) x 3 (within subjects, Sampling Time: basal average sample prior to introduction of the male into the chamber; two consecutive 20-mins bout samples in presence of sexually vigorous male stimulus: at minute 20 and 40 following introduction of the male into the chamber) analysis of covariance (ANCOVA) revealed a significant interaction [$F(2, 10) = 9.644$, $p = 0.005$], and a significant effect of Hormone Treatment [$F(4, 20) = 4.942$, $p = 0.006$]. Factoring out baseline glutamate release levels on percent change from baseline glutamate levels did not eliminate the significant differences described above between the Oil group and EB group ($p<0.05$) at minute 20; Oil group and EB+P group ($p<0.05$) at minute 20; Oil group and E group ($p<0.05$) at minute 40; and EB group at baseline and EB group at minute 20 ($p<0.05$).

Statistical analyses of raw glutamate release during copulation

A 3 (between subjects, Hormone Treatment: Oil, EB and EB+P;) x 7 (within subjects, Sampling Time: basal average sample prior to introduction of the male into the chamber; six consecutive 20-mins bout samples in presence of sexually vigorous male stimulus: at minute 20, 40, 60, 80, 100, 120 minutes following introduction of the male into the chamber) mixed factorial ANOVA was performed on raw glutamate release concentrations (see Fig. 8). The ANOVA showed a significant interaction between Hormone Treatment and Sampling Time [$F(4, 22) = 4.35$, $P < 0.01$]. A Bonferroni post hoc analysis ($p<0.05$) on individual means revealed that glutamate release in the Oil group at minute 20 was significantly higher than at baseline ($p<0.05$). Glutamate release in the Oil group at minute 20 was also significantly higher than in the EB group, at minute 20 ($p<0.05$). No other statistically significant differences were observed.

Statistical analyses of female behavior

Females engaged in sexual behavior characteristic of the hormonal regimen to which they were assigned: females in the Oil group were not sexually receptive, therefore they did not engage in sexual behavior at all; females in the EB group had low proceptivity and receptivity rates, whereas females in the EB+P group had high rates of proceptivity and receptivity. Reported here are within-groups differences during the 2-hour copulation session in each hormone treatment group for the following behaviors: appetitive behaviors, which include solicitations and hops and darts, lordosis magnitude, lordosis quotient, number of male mounts, intromissions, defensive behaviors, and ejaculations by the males (Fig.

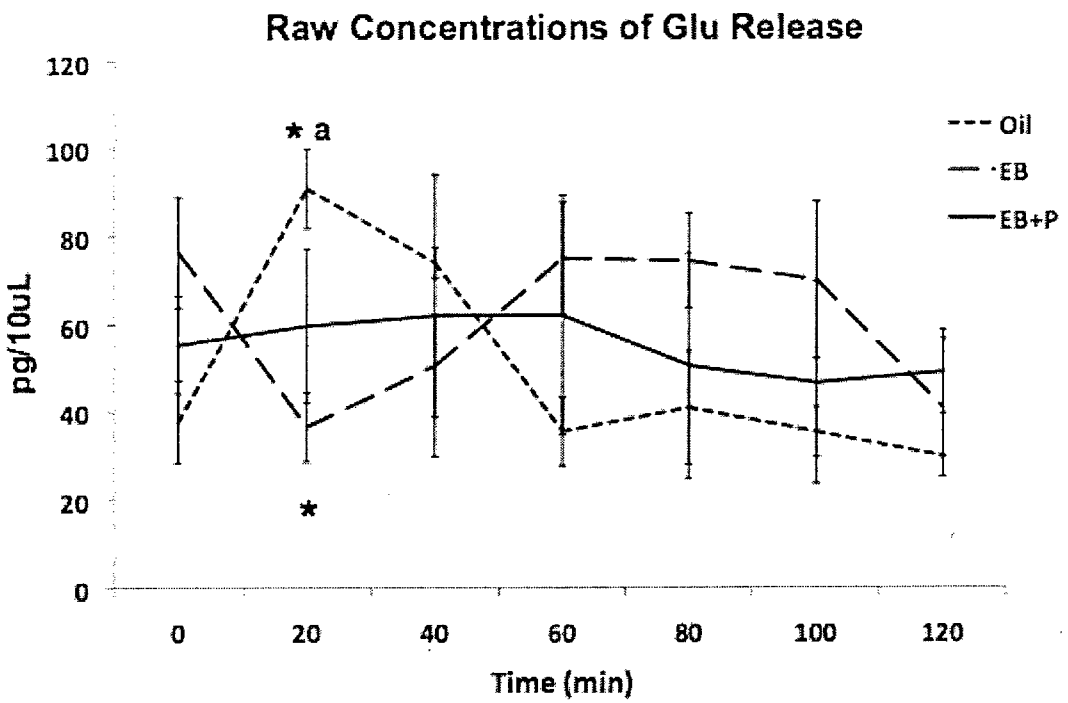

Figure 8. Raw glutamate release in the VMH as a function of hormonal priming. and time spent in presence of a male. (*: basal release significantly different than release at minute 20, $p<0.05$; a: Oil significantly different than EB; at min. 20, $p<0.05$). Data are means ± SEMs.

In Oil and EB-primed females, appetitive behaviors, LMs, LQs, male mounts, intromissions, defensive behaviors and number of ejaculations by the males were all zero or close to zero, and not significantly different from one another throughout the 120-min copulatory session.

In EB+P-primed females, appetitive behaviors gradually decreased from the first 20 min until the end of the session. The decrease became significant as of min 60 ($p=0.002$). LMs and LQ were high and not significantly different from each other throughout the entire copulatory session. The number of male mounts decreased significantly from min 60 to min 120 ($p=0.038$). The number of

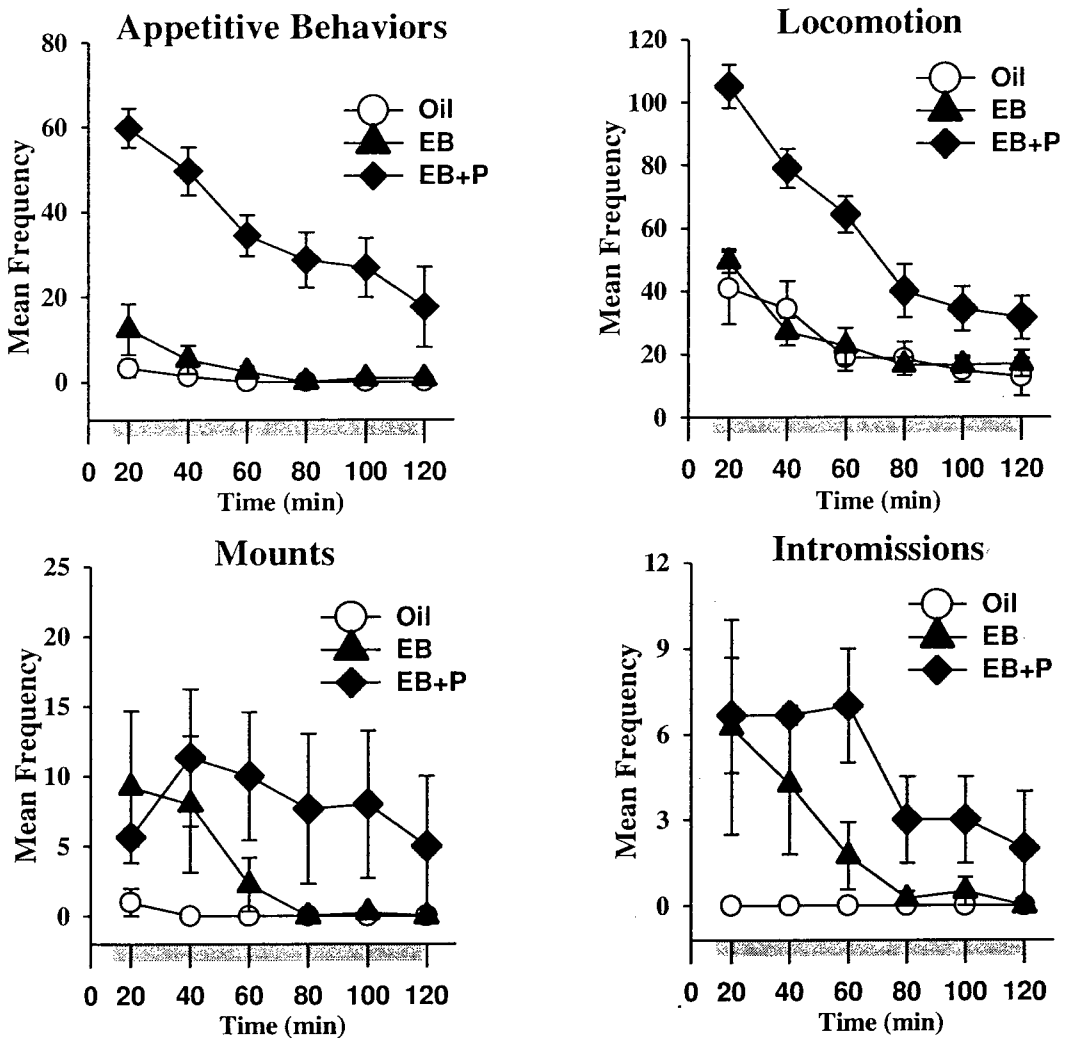

Figure 9a. Effect of hormonal priming on mean frequency of appetitive behaviors, locomotion, mounts and intromissions. Data are means ± SEMs.

intromissions. There was no statistically significant difference in the number of intromissions or defensive behaviors throughout the 120 minute-session. The number of male ejaculations gradually decreased throughout the session, becoming significant as of minute 40 ($p=0.016$).

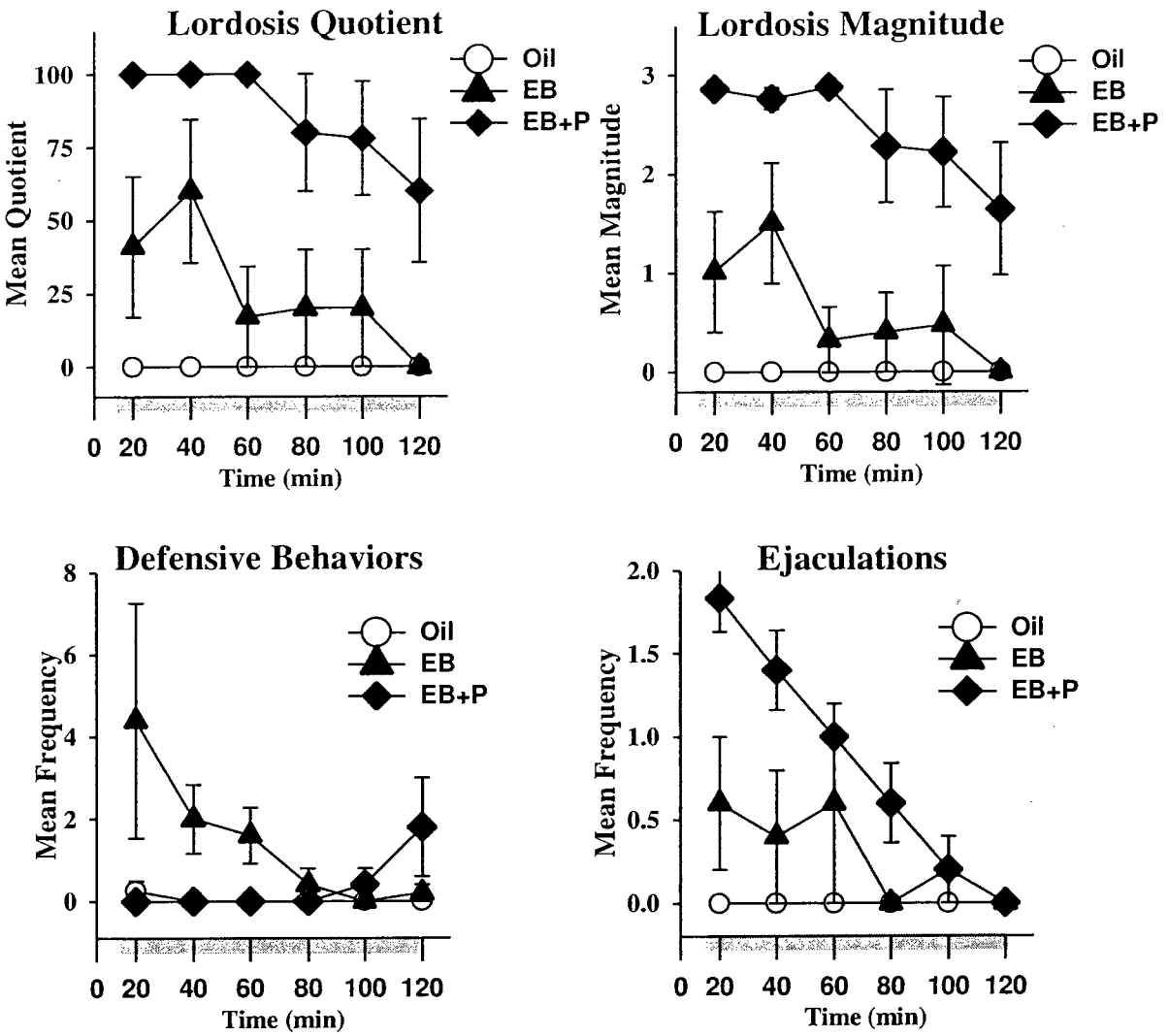

Figure 9b. Effect of hormonal priming on mean lordosis quotients and magnitudes, and mean frequency of defensive behaviors and male ejaculations. Data are means ± SEMs.

Discussion

These findings suggest that glutamate release patterns in the VMH vary as a function of hormonal priming and reactivity to male stimuli. In absence of hormones, glutamate levels were low at baseline and dramatically increase once a male is introduced into the chamber. Locomotion was highest during the 20 min immediately following introduction of the male into the chamber, when females

ran away from males that attempted to mount them. Those males learned rapidly not to mount sexually non-responsive females. Within an hour, glutamate returned to baseline levels, as did locomotion. No appetitive behaviors, intromissions, lordosis, ejaculations or defensive behaviors were observed in the Oil group during the entire 2 hour-session. Instead, these females explored the chamber, groomed themselves, observed the behavior of the male, and slept. It is thus possible that, in absence of hormones, the observed increases in glutamate release result in inhibition of sexual behavior to allow exploration, grooming, observation and sleeping to occur. In females primed with EB alone, baseline levels were significantly higher than in the Oil group. Glutamate release decreased following introduction of the male into the chamber, when frequency of appetitive behaviors, mounts, intromissions, ejaculations, defensive behaviors, lordosis magnitude and lordosis quotients were at their highest. Glutamate increased back to baseline levels within the following 40 minutes, as the frequency of solicitations, locomotion, mounts, intromissions, defensive behaviors, lordosis magnitude and lordosis quotients gradually decreased. From minute 60 on, glutamate decreased to levels observed during the first 20 minutes following introduction of the male into the chamber. As of minute 80 there was virtually no sexual activity. In the EB+P group, glutamate levels did not change at all from baseline levels throughout the copulatory session, despite significant changes in sexual behavior.

Although females in the Oil group did not receive any intromissions, upon introduction of the male into the chamber, glutamate levels peaked to reach the

highest rate of release observed at any time point among the three hormone groups. We have previously observed that 1 manually-administered VCS with a lubricated glass rod is not sufficient to trigger activation of glutamate neurons in the VMH. This suggests that in absence of hormones, somatosensory information obtained by being in proximity to a sexually mature male is enough to activate glutamate release. Meanwhile, when females were primed with EB+P, glutamate did not differ from baseline levels despite receipt of about 30-40 VCSs. These results are consistent with data showing that steroid hormones inhibit the ability of VCS to activate glutamate neurons but inconsistent with data showing that large amounts of VCS override E and P's ability to inhibit activation of glutamate neurons (Georgescu et al., 2009).

VCS induced activation of glutamate neurons when it was administered manually in amounts previously shown to induce estrus termination (Georgescu et al., 2009). In the present experiment, VCS was received from male intromissions in amounts insufficient to induce estrus termination. At the end of the 2-hour copulation session females were still in heat, even though appetitive behaviors, lordosis magnitude and lordosis quotients decreased. However, the number of male mounts, intromissions and ejaculations also decreased throughout the session, such that females did not have to resort to defensive behaviors to impose a slower rate of copulation. We did not measure pacing because the behavioral testing was not conducted in chambers that permit its quantification, like bilevel or pacing chambers do (Mendelson and Gorzalka, 1987). It would be interesting to measure glutamate release in EB+P-primed

females as they reach estrus termination, either from manually administered VCS, or from copulation with sexually vigorous males over a longer period. The males used as sexual stimuli in this experiment were sensitive to females' cues in regards to sexual receptivity. They learned particularly fast not to mount sexually non-receptive females or to mount/intromit/ejaculate when solicited. This could be due to the fact that these males were part of a pool of males used exclusively as sexual stimuli in many experiments in the laboratory. Thus, these males received extensive sexual experience with females primed with oil, EB or EB+P, and may have learned to adjust their behavior accordingly.

In females primed with EB alone, we expected glutamate release to be partially inhibited by EB, therefore somewhere between the release observed in the Oil group and that in the EB+P group. The pattern of release in this group, however, was different than what we predicted. First, at baseline, glutamate levels were significantly higher than in the Oil group. Second, glutamate release decreased once males were introduced into the chambers. Finally, at the end of the copulatory session, glutamate levels did not return to baseline but to levels observed at minute 20. This was the lowest level reached by glutamate at any time among the 3 hormone groups. The behavioral state induced by priming with EB alone does not exist in reproductively mature females in the wild. In the laboratory setting, priming with EB alone is often employed to induce a low level of receptivity (e.g. Georgescu and Pfaus, 2006b; Mills, Sohn, and Micevych, 2004). It is not unprecedented that the addition of P to hormonal priming with EB alone reverses the effects of EB. For instance, treatment with EB alone induces

low sexual receptivity and correlates with activation and internalization of mu-opioid receptors in the mPOA (Mills et al., 2004). Subsequent administration of P reverses the EB-induced activation and internalization of the mu-opioid receptor, and induces sexual receptivity. Griffin and Flanagan-Cato (2008) also report that E and P exert opposing actions on VMH neuronal morphology: E treatment alone reduced the length of long primary dendrites in the VMHvl, whereas subsequent treatment with P induced long primary dendrite elongation within 4 hours. The authors suggest that treatment with E alone may attenuate oxytocinergic and noradrenergic release to inhibit female mating behavior. Although E is generally recognized to promote sexual behavior, Griffin and Flanagan-Cato propose that E may also generate mechanisms to limit this behavior until P levels rise. The authors explain that this paradox may reflect the parallel effects that E receptor activation has on other cellular processes (Pfaff and Schwartz-Giblin, 1988), such as increased spine density on short primary dendrites (Calizo and Flanagan-Cato, 2000). Therefore, priming with E alone and E+P may exert divergent actions on inter-cellular and neuronal morphology processes, however, in synergy, E+P might induce full sexual receptivity by providing both neuronal excitability and interneuronal connectivity (Griffin and Flanagan-Cato, 2008).

The mechanism by which EB and P work to inhibit glutamate activation (Georgescu et al., 2009) and glutamate release in the VMH is not known, but could involve increased GABAergic neurotransmission in the area. GABA infusions to the VMH facilitate sexual receptivity (McCarthy et al., 1990; 1991), whereas antisense oligodeoxynucleotide against one of the enzymes that

synthesizes glutamate into GABA, GAD-67, abolishes sexual behavior altogether (McCarthy et al., 1994). EB increases GAD-67 mRNA in the dorsomedial hypothalamus and decreases it in the MPOA (McCarthy et al., 1990), regions that are both involved in the mediation of female sexual behavior (Pfaus et al., 1993; Coria-Avila and Pfaus, 2007) and that have efferent projections to the VMH (Dai et al., 1998; Fahrbach et al., 1989). In turn, P increases binding affinity of GABA A receptors (DeLorey and Olsen, 1994; Majewska et al., 1986), and P metabolites (e.g., 5 alpha-pregnan-3 alpha-ol-20-one) bind to GABA A receptors (Frye and Duncan, 1994). Thus, EB and P may inhibit glutamate release or increase the amount and/or intensity of VCS required to activate glutamate neurons within the VMH by augmenting GABA neurotransmission in the VMH. E also increases synthesis of proenkephalin in the VMH (Lauber et al., 1990; Romano et al., 1990), rendering actions at δ receptors another possible intermediary of E-induced disinhibition. Infusions of δ receptor agonist [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) to the VMH facilitate lordosis, whereas infusions of its selective receptor agonist naltrindole inhibit lordosis (Acosta-Martinez and Etgen, 2002). Whether glutamate neurons possess GABA A and/or δ opioid receptors remains to be determined.

We have previously hypothesized that glutamate results in inhibition of female sexual behavior in the VMH either by (1) inhibiting sexual behavior during periods of sexual nonreceptivity; (2) facilitating behaviors incompatible with sexual behavior; and/or (3) by facilitating estrus termination by activating pacing and inhibiting lordosis (Georgescu et al., 2009, Chapter 2). The results of the

present experiment support the idea that glutamate may inhibit sexual behavior during periods of sexual nonreceptivity when females receive somatosensory information by being in proximity to sexually vigorous males and when they are engaged in other behaviors, such as grooming and exploring their surroundings. Because estrus termination did not occur, no statement can be made regarding glutamate's involvement in estrus termination. However, no changes in glutamate levels were observed in any hormone group once locomotion became minimal and stable, namely during the last hour of testing. Therefore, the results could be interpreted as providing partial support to our hypothesis that glutamate mediates pacing, since pacing involves locomotion, and glutamate levels were low when locomotion was low.

Glutamate release might be controlled by efferent projections from areas that mediate the intromission mnemonic. The intromission mnemonic refers to the encoding of the amount and pattern of VCS necessary for initiation of the neuroendocrine changes required for successful impregnation (Oberlander and Erskine, 2008). The MeApd and the CA 1 region of the hippocampus, both of which have efferent projections to the VMH (Fahrbach et al., 1989), are the neural substrates involved in the encoding of this mnemonic. Oberlander and Erskine (2008) suggested that this mnemonic encoding mechanism might result from altered synapse density in VCS-activated regions (Oberlander and Erskine, 2008). Fos induction is robustly induced in the MeA following VCS (Erskine, 1993; Pfaus et al., 1993; Polston and Erskine, 1995; Tetel et al., 1993; Pfaus et al., 1996). In the CA 1 region of the hippocampus, a small amount of Fos

(between 5 and 20 cells) is induced following flank stimulation or copulation in EB+P primed females (Pfaus et al., 1993). In animals primed with Oil, Fos induction is moderate following copulation and flank stimulation. Since these areas send GABAergic projections to the VMH (Georgescu et al., 2009), it is possible that glutamate release within the VMH is controlled by these projections, rendering appetitive and consumatory behaviors contingent upon the intromission mnemonic. This could work to ensure that the amount and pattern of VCS is synchronized with the pattern necessary for initiation of neuroendocrine changes required for fertility and successful impregnation to occur. We hypothesize that EB might upregulate GAD within these areas, resulting in increases in production of GABA, which exerts its inhibition of glutamate release within the VMH according to the intromission mnemonic.

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Summary of Chapter 3

The experiment presented in Chapter 3 showed that glutamate release patterns vary as a function of hormonal priming during a 2-hour copulation session with a sexually vigorous male. In females primed with Oil, glutamate release increased upon introduction of a male into the chamber, despite absence of intromissions. In females primed with EB, glutamate release decreased upon introduction of a male into the chamber. Glutamate release returned to baseline levels within an hour both in the Oil and EB groups. In females primed with EB+P, glutamate release was low throughout the 2-hour copulation session, despite receipt of moderate amounts of VCS though intromissions and ejaculations by the male. Those data suggest that glutamate release patterns vary as a function of hormonal priming as well as stimulation received from being in proximity of a sexually mature male. The experiment presented in the next chapter aimed to determine whether glutamate neurotransmission in the VMH is subject to regulation by GABA.

Chapter 4

Short Communication: Ovarian steroids increase $GABA_A$ receptor subunits on glutamate neurons in the ventromedial hypothalamus

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ABSTRACT

Infusions of glutamate or its selective receptor agonists to the VMH of OVX female rats primed with EB and P inhibit both appetitive and consummatory sexual behaviors, whereas glutamate receptor-selective antagonists facilitate these measures in females primed with EB-alone or EB+P. Conversely, bilateral infusions of GABA to the VMH facilitate lordosis, and may be acting through a mechanism of disinhibition, in which GABA hyperpolarizes glutamate neurons. For this to occur, GABA receptors would have to exist on glutamate neurons. The present study examined this possibility using immunocytochemical detection of GABA A subunits in the cell membranes of glutamate neurons in the VMHvl of female rats primed with O, EB, or EB+P (N = 5/group). Statistical analyses of the percentage of double-labeled cells revealed that females receiving O displayed significantly fewer double-labeled neurons than females receiving EB alone or EB+P, which did not differ from one another. These results suggest that estrogen may prime sexual behavior in the female rat, in part, by activating inhibitory GABAergic inputs to glutamate neurons in the VMHvl.

1. Introduction

Previous research has shown that glutamate and its selective receptor agonists AMPA, kainate and NMDA, have inhibitory effects on sexual behavior in the VMH of the female rat (Georgescu and Pfaus, 2006a; Kow et al., 1985; McCarthy et al., 1991). This inhibition is characterized by decreases in appetitive behaviors, lordosis magnitudes and quotients, and increases in pacing and defensive behaviors. Conversely, infusions of glutamate antagonists DNQX and CNQX to the VMH of OVX females primed with EB facilitate sexual behavior, as indicated by increases in solicitation, hops and darts, lordosis magnitudes, lordosis quotients and male ejaculations, and decreases in pacing (Georgescu and Pfaus, 2006b).

The behavioral pattern induced by activation of glutamate receptors in the VMH is reminiscent of that observed during estrus termination. Indeed, VMH infusions of glutamate antagonist DNQX completely reversed the inhibitory effects of VCS on the length of the period of estrus (Chapter 2). This suggests that glutamate may be involved in the onset of estrus termination by inducing sexual inhibition as the VCS received reaches amounts required for pregnancy/pseudopregnancy. Results from another study (Chapter 1) in which a larger percentage of glutamate neurons became activated (measured by co-localization of c-fos) following large amounts of VCS (50) relative to negligible

amounts corroborate that indeed, VMH glutamate activation may be involved in the onset of estrus termination. Furthermore, results from a microdialysis experiment revealed that VMH glutamate release during copulation depends on female's hormonal regimen (Chapter 3). In absence of hormones, when females are not sexually receptive, glutamate levels increased relative to baseline once sexually vigorous males were introduced in the chamber, even though these females did not receive any intromissions. When females were primed with EB, glutamate levels were high at baseline, decreased upon introduction of the male into the chamber, and returned to baseline when sexual activity ceased, 60 minutes into the session. In EB+P-primed females, glutamate levels did not change from baseline throughout the 2-hour session. These data suggest that VMH glutamate release patterns vary as a function of the hormonal priming regimen and the sensory input received from being in proximity of a sexually vigorous male.

If increased glutamate neurotransmission within the VMH is inhibitory to sexual behavior, then a neurochemical agent with inhibitory effects on cellular actions yet facilitative effects on sexual behavior likely inhibits its release during periods of sexual receptivity. The inhibitory neurotransmitter GABA has both of these properties. Infusions of muscimol, a GABA A receptor agonist, to the VMH of OVX females primed with EB+P facilitate lordosis, whereas infusions of bicuculline, a GABA A receptor antagonist, inhibit lordosis (McCarthy et al., 1990). Also, infusions of antisense oligodeoxynucleotide against GAD-67, one of

the enzymes that synthesizes glutamate into GABA, reduce lordosis for 1-2 days (McCarthy et al., 1994). Interestingly, EB increases GAD-65 mRNA in the mPOA, and decreases it in the DMH, regions that are both implicated in the mediation of various aspects of sexual behavior, and that have projections to the VMH (McCarthy et al., 1995). Progesterone augments the binding affinity to GABA receptors (DeLorey and Olsen, 1994; Majewska et al., 1986), and P metabolites, such as 5 alpha-pregnan-3 alpha-ol-20-one, bind to $GABA_A$ receptors (Frye and Duncan, 1994). Thus far, however, whether $GABA_A$ receptors are localized on glutamate neurons in the VMHvl is not known. The purpose of this study was to determine whether glutamate neurons within the VMH possess $GABA_A$ receptors, and if so, whether these receptors are subject to upregulation by steroid hormones EB and P.

2. Methods

2.1. Subjects

Fifteen Long Evans females, weighting between 200-250g were obtained from Charles- River, Inc., St-Constant, QC. Females were housed in groups of five in large Plexiglas shoe-box cages with wood-chip bedding. The colony room was maintained on a reversed 12:12hr light/dark cycle (lights off at 08:00) at approximately 21°C with food and water continuously available. Females were OVX bilaterally through lumbar incisions following anesthesia induced by ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4mg/ml), mixed in

a ratio of 4:3, respectively, and injected intraperitoneally. All OVX females were given a week of post-surgical recovery.

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

2.2. Hormone Treatment

Females were randomly distributed to one of 3 hormone groups: Oil+Oil, EB+Oil, EB+P. The Oil+Oil group received an injection of Oil (0.1ml reagent grade sesame oil) 48 hours prior to another injection of Oil. The EB+Oil group received an injection of EB (10µg/ 0.1ml reagent grade sesame oil) 48 hours prior to an injection of Oil. The EB+P group received an injection of EB 48 hours prior to an injection of P (500µg/ 0.1ml reagent grade sesame oil). Hormones were administered for 4 cycles prior to perfusion. All steroids were obtained from Steraloids (Hanover, NH).

2.3. Immunocytochemistry for glutamate neurons and $GABA_A$ receptor staining

Females were sacrificed 4 hours following the last hormonal injection by an overdose of sodium pentobarbital (120mg/kg, i.p.) and perfused intracardially with ice-cold phosphate-buffered saline (300mls) followed by ice-cold 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer (300mls).

Brains were removed, postfixed in 4% paraformaldehyde and 1% glutaraldehyde solution for 4h, and stored overnight in 30% sucrose at 4°C. Brains were blocked around the area of the anterior hypothalamus, mounted on a chuck, and frozen. Thirty µm coronal sections were cut from the VMH (corresponding to plates 28-34 in Paxinos and Watson, 1998) on a cryostat. Sections were washed in ice-cold 0.9% TBS, incubated for 30 minutes in a mixture of TBS and 30% hydrogen peroxide (H_2O_2) at 4°C, rinsed, and transferred for 90 minutes to a preblocking serum consisting of 0.05% triton TBS (50µl/100ml) and Normal Goat Serum (NGS; Vector Laboratories Canada, Burlington, ON, diluted 1:200) at 4°C. The sections were rinsed and incubated in a mixture of NGS, 0.05% triton TBS, and a rabbit polyclonal primary anti-l-glutamate antibody conjugated to glutaraldehyde as the immunogen (Abcam, ab 8889; diluted 1:10 000) for 72 hr at 4°C. The manufacturer's product information sheet states that the antibody has no cross-reactivity with glutamate in peptides or proteins, no measurable cross-reactivity with other amino acids including GABA, and only low-affinity reactivity with d-glutamate, using ELISA. Sun et al. previously established antibody specificity previously in rat retinal tissue (Sun et al., 2007).

After incubation with the primary antibody, sections were rinsed and transferred to a secondary antibody solution consisting of 0.05% triton TBS, NGS, and a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Canada, Burlington, ON, diluted 1:200) for 1 hr at 4°C. Sections were rinsed and transferred to a solution of 0.2% triton TBS, and Vectastain *Elite* ABC solutions

(diluted 1:55) for 2 hr at 4°C. Finally, the sections were rinsed in TBS, rinsed in 50mM Tris buffer (pH 7.6) for 10 minutes, and incubated on an orbit shaker for 10 minutes in 0.05% 3,3'-diaminobenzidine (DAB) in 50mM Tris buffer with 3% H_2O_2 (solution buffered to pH 7.8 with drops of 6M NaOH), to catalyze the DAB. The reaction was stopped by rinsing in cold TBS.

Sections were then incubated in a rabbit polyclonal primary antibody raised against the N-terminus of $GABA_A$ Rα1 (Santa Cruz, Product Cat. # SC-7348, diluted 1:100) in 0.05% Triton-TBS with 3% NGS for 72-h at 4°C. The $GABA_A$ Rα1 can cross-react with $GABA_A$ Rα2, $GABA_A$ Rα3, and $GABA_A$ Rα5 of mouse, rat, and human origin; it is not cross-reactive with other $GABA_A$ Rα subunits (information from the product description provided by Santa Cruz Biotechnology, Inc.). Antibody specificity was established previously by Western blot in other laboratories (Delgado and Schmachtenberg, 2008; Delgado, Vielma, Kahne, Palacios, and Schmachtenberg, 2009; Foley, Stanton, Price, Cunningham, Hasser, and Heesh, 2003). Following secondary antibody and tertiary ABC reactions, sections were incubated in the DAB/Tris buffer/H_2O_2 solution with the addition of $NiCl_2$ to color the DAB chromagen product blue-black. Sections were rinsed in TBS to stop the reaction, mounted onto 75x25mm gel coated slides (Serum International), dehydrated in H_2O and ethanols (70%, 95%, and 100%, 10 min per solution) followed by immersion in Xylenes (Fisher Scientific) overnight to clear them. Slides were coverslipped with Permount, dried

overnight, cleaned, and subsequently examined under a Leitz Laborlux microscope.

3. Results

$GABA_A$ Rα1-positive cells, glutamate-positive cells, and double-labeled $GABA_A$ Rα1/glutamate cells were counted bilaterally per slice in the VMHvl. Counting was performed by researchers blind to the animal's experimental group. Although the VMH is approximately 1.68mm in length along the sagittal plane, and is thus comprised of fifty-six 30μm-slices, counting was limited to the 10 sections in which the VMHvl was at its largest diameter. The VMHvl is easily identified because of its distinct shape, but landmarks such as the 3rd ventricle and hippocampus were used to place the VMH into anterior and posterior divisions (as done previously by Pfaus et al., 1993; 1996; 2006). Glutamate-positive cells and double-labeled $GABA_A$ Rα1/glutamate cells were counted bilaterally per slice. Glutamate cells were manifested as a light brown cytoplasmic stain, whereas $GABA_A$ Rα1-positive cells were manifested by a dark brown stain in the cytoplasmic membrane. Double-labeled $GABA_A$ Rα1/glutamate cells had a light brown cytoplasm with a darker brown stain in the cytoplasmic membrane (Fig. 9) in one plane of view under the microscope. For all double-labeled cells counted, care was taken to focus the plane of view in and out to make sure the cells were not single-labeled and simply adjacent to one another (as can occur in a 30 μm section that is approximately 3 cell bodies' deep). All

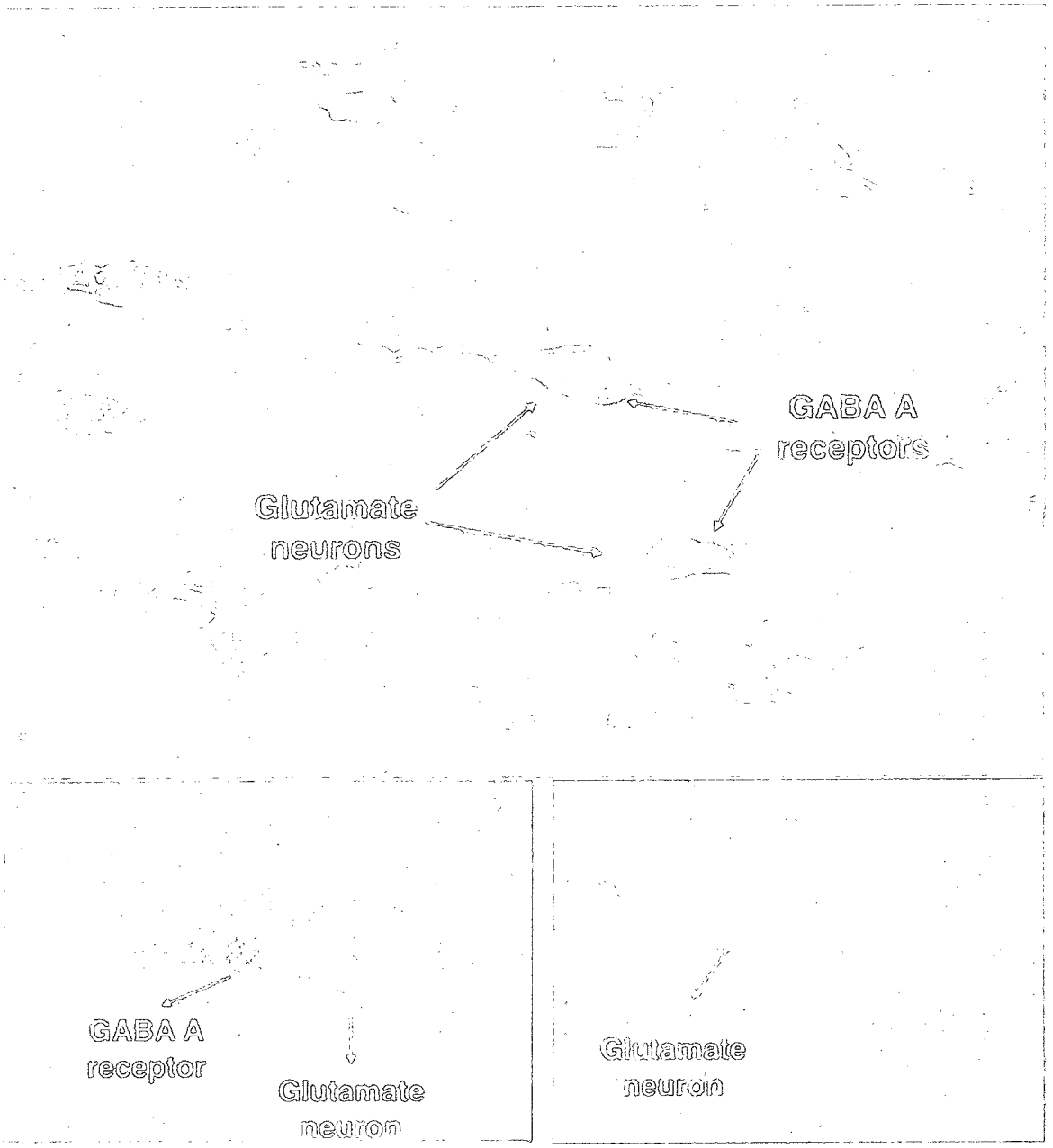

Figure 10. Example of brain tissue obtained from an animal primed with estrogen and progesterone. Double-labeled neurons are brown with darker brown coloration around the edges of the cytoplasm, whereas glutamate neurons without GABA receptors do not have the darker coloration around the cytoplasm. Top picture was taken at magnification 60x, bottom pictures at 100x.

counts were made at 40x. Mean numbers of single and double-labeled cells per

section for each rat were obtained by dividing by 2 the sum of the activated cells counted on both sides for all sections counted. Thus the counts reported here are by hemisection.

A one-way between-subjects analysis of variance (ANOVA) revealed a significant interaction between hormonal priming and the % of double-labeled neurons, $F(2, 14) = 41.932$, $p<0.001$ (Fig. 10). Tukey HSD post hoc comparisons revealed that the percentage of double-labeled neurons was significantly lower in the Oil group than in the EB ($p<0.001$) or EB+P ($p<0.001$) groups, which did not differ from one another.

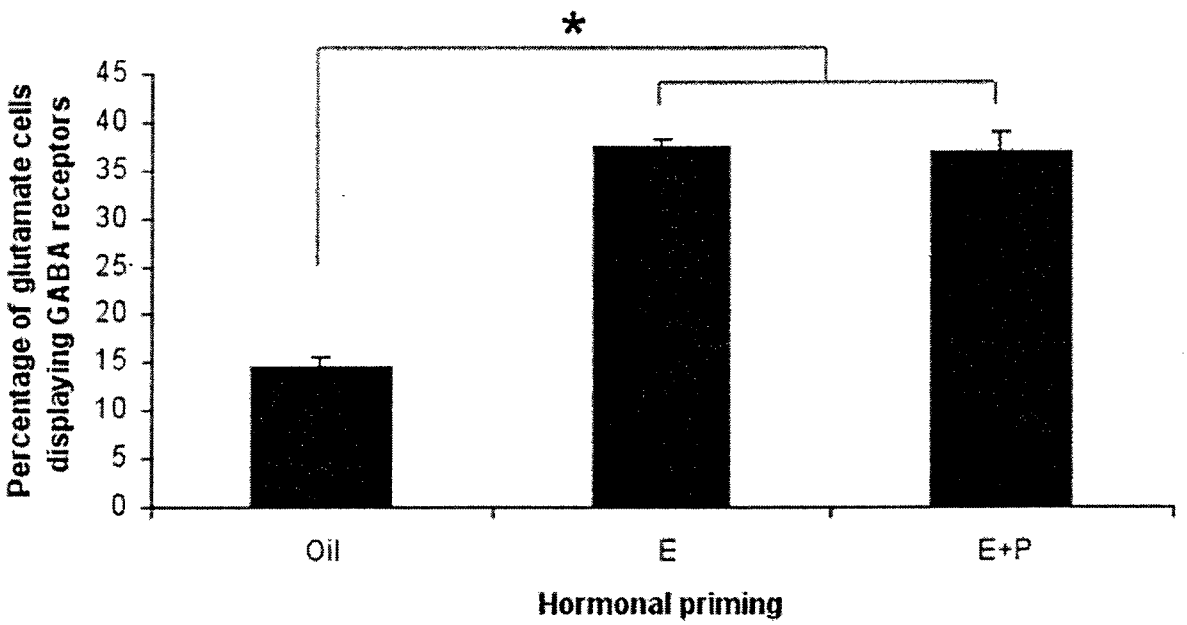

Figure 11. Percentage of glutamate cells displaying GABA receptors in the VMH of Oil, Estrogen, and Estrogen + Progesterone primed female rats. Data are means ± SEMs.

4. Discussion

The results from this experiment indicate that $GABA_A$ receptors exist on glutamate-containing neurons in the VMH and are upregulated by EB, as the percentage of neurons that express $GABA_A$ receptors more than doubled when animals were primed with EB or EB+P, relative to the oil control. The addition of P to the hormonal regimen did not result in increases in the expression of $GABA_A$ receptors on these neurons. E has previously been shown to upregulate the production of GAD-65, one of the enzymes that synthesizes GABA from glutamate, in the mPOA (McCarthy et al., 1990), whereas P increases the binding affinity of the GABA A receptor (DeLorey and Olsen, 1994; Majewska et al., 1986). Previous research showed that E decreases ß2 and increases γ2 mRNA subunits of the GABA A receptor in the POA, but increases ß3 mRNA in the VMH (Petersen, Reeves, Keller, Gardner, Mahan, and McCrone, 1993b). Together with the present results, these findings suggest that subunits of the GABA A receptor are subject to regulation by E. Thus, GABA may participate in the inhibition of neurons that contain glutamate in the VMH when circulating E levels are high, thereby inhibiting the effects that glutamate transmission has in this region on sexual behavior. This may be a mechanism that ensures that females can receive the requisite amount of VCS during copulation to produce the neuroendocrine changes necessary for pregnancy to occur.

The present experiment employed an antibody raised against glutamate to mark glutamate cells within the VMH. Analysis of glutamate-IR cells maybe be

compromised by the ubiquitous presence of glutamate in all cells, because glutamate antibodies not only identify glutamate that is manufactured for vesicular release but also cytoplasmic pools of metabolic glutamate for enzymatic biosynthesis of GABA (Fonnum and Hassel, 1995; Storm-Mathisen, Danbolt, and Ottersen, 1995; Ziegler, Cullinan, and Herman, 2002). Since the discovery of the vesicular glutamate transporters VGLUT-1, -2, and -3, glutamate cells have been labeled in immunocytochemical studies using antibodies raised against its vesicular transporters, the second of which (VGLUT-2) is the predominant one in the hypothalamus, including the VMH (Ziegler et al., 2002). Since then, a number of studies verified previous reports of distributions of glutamatergic neuronal populations throughout the CNS using mRNA expression of VGLUT-1 and VGLUT-2 (Aihara, Mashima, Onda, Hisano, Kasuya, Hori, Yamada, et al., 2000; Bellocchio, Reimer, Fremeau, and Edwards, 2000; Bellocchio, Hu, Pohorille, Chan, Pickel, and Edwards, 1998; Fremeau, Troyer, Pahner, Nygaard, Tran, Reimer, Bellocchio, Fortin, Storm-Mathisen, and Edwards, 2001; Fujiyama, Furuta, and Kaneko, 2001; Hayashi, Otsuka, Morimoto, Hirota, Yatsushiro, Takeda, Yamamoto, and Moriyama, 2001; Hisano, Hoshi, Ikeda, Maruyama, Kanemoto, Ichijo, Kojima, Takeda, and Nogami, 2000; Ni, Rosteck, Nadi and Paul, 1994; Ni, Wu, Yan, Wang, and Paul, 1995; Takamori, Rhee, Rosenmund, and Jahn, 2000). The combined distributions of VGLUT-1 and VGLUT-2 mRNA expression were found in regions previously shown to display glutamate neurons identified by other immunocytochemical techniques.

Also, in situ hybridization studies have failed to detect GAD-65 or GAD-67 mRNA within the VMH (Feldblum et al., 1993, Fernandez-Guasti et al., 1986; McCarthy et al., 1995; Ovesjö et al., 2001), suggesting that (1) GABA in the VMH is released from axon terminals of neurons originating elsewhere, (2) GABA within this region is manufactured by a novel mechanism not yet identified, or (3) neurons within the VMH scavenge GABA released from afferents to the VMH (Commons et al., 1999). In any case, these reports suggest that without the presence of GAD-65 or GAD-67 in the VMH, the glutamate-IR cells detected in the present study cannot represent pools of glutamate destined for biosynthesis to GABA. We recently found that GABAergic input to the VMH is provided by efferent projections from the mPOA, the AHA, the PVN, the AHipp and the MeA, regions that play important roles in the mediation of female sexual behavior (Chapter 5). Nonetheless, the presence of $GABA_A$ receptors on glutamate neurons within the VMH needs further investigation using experimental techniques that permit quantitative analyses, such as in situ hybridization, perhaps using VGLUT-2 as a marker for glutamatergic cells.

The present data provide a mechanism by which GABA projected to the VMH could inhibit glutamate neurotransmission. Thus far, we have shown that increased glutamate neurotransmission in the VMH has an inhibitory effect on female sexual behavior (Georgescu and Pfaus, 2006a), and that diminishing its actions in this area facilitates sexual behavior (Georgescu and Pfaus, 2006b) and delays VCS-induced estrus termination (Chapter 2). However, the circumstances

in which endogenous glutamate action comes into play in the VMH remain unclear. We have postulated a few hypotheses. First, VMH glutamate may be involved in onset of estrus termination because blockade of the AMPA/kainate receptor by glutamate antagonist DNQX effectively reversed VCS-induced estrus termination (Chapter 2). Second, increased VMH glutamate neurotransmission may work to inhibit sexual behavior during periods of sexual non-receptivity, such as following ovariectomy, puberty and lactation, or during phases of the estrous cycle during which females are not sexually receptive. In a previous experiment, glutamate release was larger in animals primed with Oil than in animals primed with EB or EB+P, and that, upon introduction of the male into the chamber, suggesting that during states of non-receptivity, sensory information received from being in proximity of a sexual mature male leads to increases in VMH glutamate release (Chapter 3). During states of sexual receptivity however, circulating estrogens appear to upregulate synthesis of GAD-65 in brain regions that have GABAergic projections to the VMH, resulting in increases in GABA release within the VMH, which in turn may inhibit glutamate neurons within that area. Because the VMH also mediates feeding (Bray and York, 1998; Gold et al., 1972; Marshall et al., 1955; Storlein and Albert, 1972), aggression (Adamec, 1991; Brayley and Albert, 1977; Sterner et al., 1992) and pain (Borszcz, 2006), increased glutamate neurotransmission within the area may be involved in activation of these behaviors and inhibition of lordosis and other sexual behaviors.

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Summary of Chapter 4

The experiment presented in Chapter 4 shows that GABA A receptors exist on glutamate containing neurons within the VMH. Those receptors may be upregulated by EB since the percentage of neurons that contain glutamate and express those receptors doubled when animals were primed with EB or EB+P relative to Oil. Thus, GABA may participate in the inhibition of glutamate release in the VMH when estrogen levels are high, thereby disinhibiting sexual behavior perhaps to allow females to receive requisite amounts of VCS to induce the neuroendocrine changes necessary for pregnancy to occur. These results await replication with in situ hybridization. The goal of the next chapter was to identify the areas that send GABAergic projections to the VMH, since previous research has shown that the VMH is devoid of GAD, the enzyme that synthesizes glutamate into GABA.

Chapter 5

GABAergic projections to the ventromedial hypothalamus of the female rat

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ABSTRACT

GABA transmission in the VMH potentiates female sexual behavior in part by inhibiting glutamate interneurons in this region that activate defensive responses. Previous studies have failed to detect mRNA of the GABA synthesizing enzyme GAD within the VMH, suggesting that the GABA transmission comes from efferents projected to the VMH. The goal of this experiment was to identify brain areas that have GABAergic projections to the VMH. Female Long Evans rats received bilateral infusions to the VMH of 0.15 μl of a 2% solution of FluoroGold, a fluorescent retrograde tracer. Animals were sacrificed 10 days following infusions, brains were removed, and immunocytochemistry for detection of GABA was performed on 30 μm coronal brain slices. FluoroGold labeling was found in the lateral septum, the diagonal band of Broca, the BNST, the mPOA, the AHA, the lateral hypothalamus, and the MeA. However, GABA was co-localized only in neurons projecting from the BNST, the mPOA, the AHA and the MeA, steroid hormone concentrating regions that are also involved in the control of sexual behavior in female rats. We postulate that ovarian steroid actions in one or all of those regions activate GAD, thereby increasing the availability of GABA for release in the VMH, which subsequently inhibits glutamate neurons to promote sexual behavior in female rats.

1. Introduction

Increased GABAergic neurotransmission to the VMH facilitates sexual and reproductive behaviors in female rats (Donoso and Zarate, 1981; McCarthy et al., 1990). Infusions of muscimol, a GABA A receptor agonist, to the VMH of OVX females primed with EB had a facilitative effect on lordosis, whereas VMH infusions of bicuculline, a GABA A receptor antagonist, have an inhibitory effect on lordosis (McCarthy et al., 1990). On the other hand, glutamate or its selective receptor agonists NMDA, kainate or AMPA, have inhibitory effects on sexual behavior when infused to the VMH of OVX females primed with EB and P (Kow et al., 1985; McCarthy et al., 1991; Georgescu and Pfaus, 2006a), and infusions of glutamate antagonists CNQX and DNQX to this area have facilitative effects on a battery of sexual behaviors in OVX females primed with EB (Georgescu and Pfaus, 2006b). Furthermore, cells that contain glutamate are activated in the VMH following large amounts of VCS (Chapter 1), and blocking glutamate neurotransmission by infusions of DNQX to the area reverses the effects that VCS has on estrus termination (Chapter 2). We postulate that GABA may inhibit glutamate release in the VMH to permit lordosis in response to male mounts.

GABA is synthesized in neurons from its precursor glutamate by one of 2 forms of glutamic acid decarboxylase (GAD), GAD-65 and GAD-67 (Erlander and Tobin, 1991; Kaufman et al., 1991). Numerous studies have detected very low levels of GAD mRNA or failed altogether to detect it in the VMH (Feldblum et al.,

1993; Fernandez-Guasti et al., 1986; McCarthy et al., 1995; Okamura et al., 1990; Ovesjo et al., 2001; Ziegler et al., 2002).

Besides a 2kDA difference in molecular weights (Erlander et al., 1991; Kaufman et al., 1991), it is now generally recognized that GAD-65 is activated by increased demand and synthesizes pools of GABA for vesicular release, whereas GAD-67 is active tonically and synthesizes GABA for metabolic purposes and non-vesicular release (Erlander and Tobin, 1991; see McCarthy et al., 1995). The presence of GABA and high affinity GABA A receptors within the VMH is well-documented in the literature (McCarthy, Coirini, Schumacher, Johnson, Pfaff, Schwartz-Giblin, and McEwen, 1992; O'Connor, Nock, and McEwen, 1988; Ovesjo et al., 2001; Schumacher, Coirini, and McEwen, 1989; Chapter 4), suggesting that the GABA active in this nucleus is released from axon terminals of neurons with nuclei originating elsewhere. The large majority of GABAergic cells are intrinsic interneurons with short axons, exerting local control over cellular excitability (Brown et al., 1990; Herbison et al., 1991; Leranth et al., 1985; Reichling and Basbaum, 1990), suggesting that the areas that send GABAergic projections to the VMH must be in close vicinity. Studies on the distribution of GAD-65 and GAD-67 in the hypothalamus and surrounding areas have found that the mPOA, the AHA, and the DMN are the richest in GAD mRNA signals, followed by the zona incerta, the arcuate nucleus, the suprachiasmatic nucleus, and the LHA (Okamura et al., 1990). Treatment with E was found to

increase GAD-65 mRNA in the mPOA and decrease it in the DMN (McCarthy et al., 1995).

The purpose of this experiment was to identify the brain areas from which GABAergic projections to the VMH originate. We postulate that ovarian steroid actions in one or all these regions activate GAD, thereby increasing the availability of GABA for release in the VMH, which subsequently inhibits glutamate neurons there to promote sexual behavior in female rats.

2. Methods

2.1. Subjects

Seven intact Long Evans females, weighing between 200-250g were obtained from Charles-River, Inc., St. Constant, QC. Females were housed in groups of three and four in large Plexiglas shoe-box cages with wood-chip bedding. The colony room was maintained on a reversed 12:12hr light/dark cycle (lights off at 08:00) at approximately 21°C with food and water continuously available.

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

2.2. FluoroGold injections

All animals were anesthesized with sodium pentobarbital (65mg/kg)

injected intraperitoneally prior to unilateral cannulation to the VMH. Cannulation coordinates were 1mm lateral to the midline on either side of Bregma, 0mm anterior to Bregma, and 8.5mm ventral to Dura, at a 5-degree elevation angle. The tip of the cannula guide ended 1mm above the desired target area. Cannula blockers were cut so that they would protrude 0.5mm from the guide cannula. The 22-gauge infusion cannulae were cut to 1mm longer than the guide cannula. Guide cannulae, cannulae blockers, injection cannulae and dust caps were obtained from Plastics One. Four out of the 7 animals infused had correct placements and successful FG infusion (see Fig. 11 for placements).

Following stereotaxic surgery, 0.15uls of a 2% FG solution diluted in phosphate buffer saline was infused to the VMH through an infusion cannula linked by plastic tubing to a 1ul Hamilton syringe in an infusion pump (Harvard Apparatus, Pump 22). The infusion took place over 1 minute. Ten minutes were allowed for full absorption to take place.

2.3. Immunocytochemistry for GABA staining

Animals were perfused 10-14 days following infusion of FG, on the afternoon of proestrus. Females were sacrificed by an overdose of sodium pentobarbital (120mg/kg, i.p.) and perfused intracardially with ice-cold phosphate-buffered saline (300mls) followed by ice-cold 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer (300mls). Brains were removed, postfixed in 4% paraformaldehyde and 1% glutaraldehyde solution for 4h, and

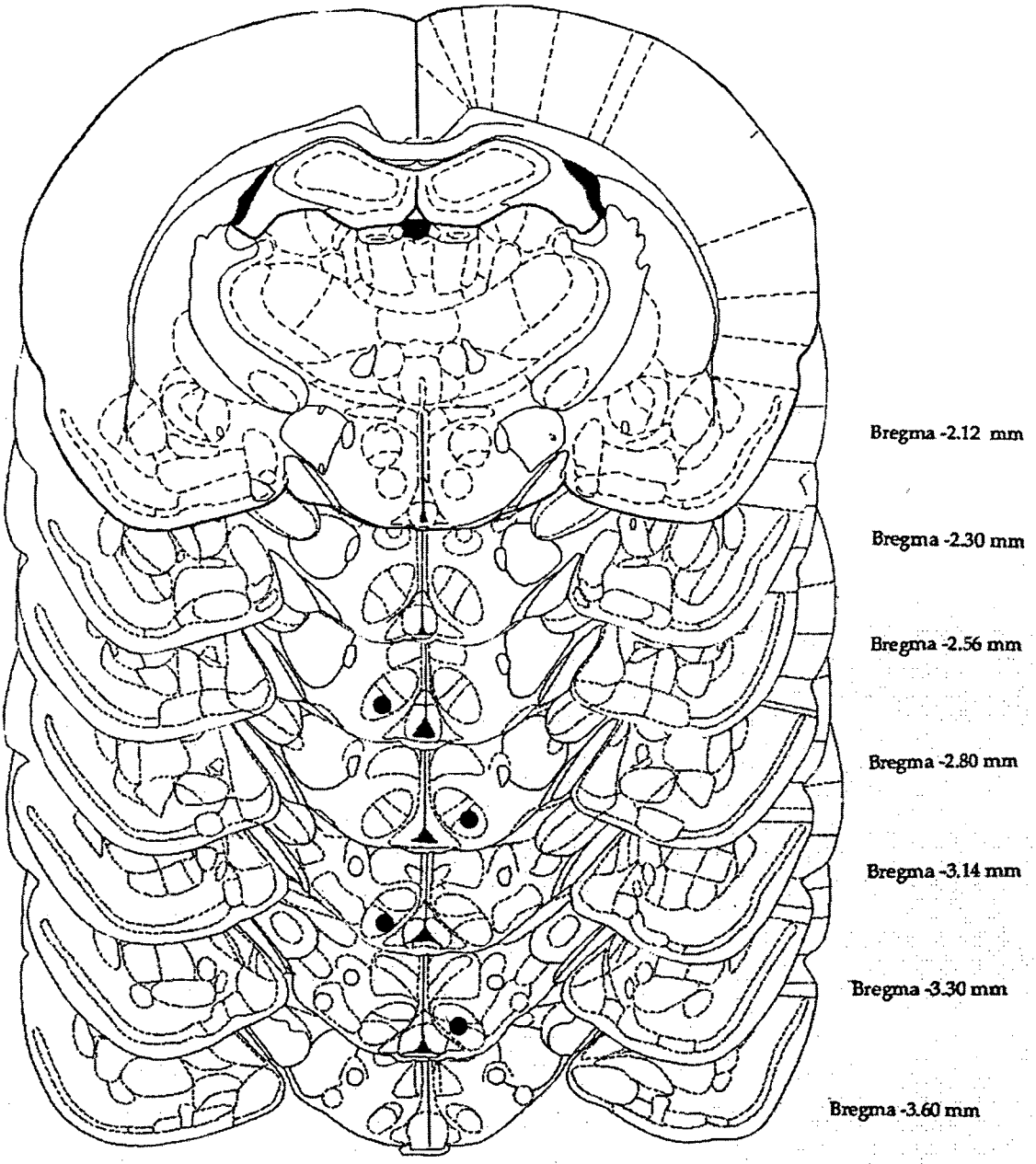

Figure 12. Infusion placement data.

stored overnight in 30% sucrose at 4°C. Brains were then mounted on a chuck and cut on a cryostat into 30µm coronal sections. One out of every two sections

was collected. Sections were washed in ice-cold 0.9% TBS, incubated for 30 minutes in a mixture of TBS and 30% hydrogen peroxide (H_2O_2) at 4°C, rinsed, and transferred for 90 minutes to a preblocking serum consisting of 0.05% triton TBS (50µl/100ml) and Normal Goat Serum (NGS; Vector Laboratories Canada, Burlington, ON, diluted 1:200) at 4°C. The sections were rinsed and incubated in a mixture of NGS, 0.05% triton TBS, and a primary rabbit anti-GABA antibody (Sigma, product number A-2052; dilution 1:10 000) for 72 hr at 4°C. The antibody was isolated by affinity chromatography on GABA bound to epoxy activated Sepharose 6B, which removes essentially all rabbit serum protein and immunoglobulins which do not bind specifically to GABA. An immuno-binding technique was used to test specificity of this antibody and showed positive binding with GABA, and negative binding with bovine serum albumin, L-glutamic acid, L--alanine, DL--amino-butyric acid, L--amino-butyric acid, L--amino-n-butyric acid and glycine at a dilution of 1:10 000 (Zhang, Li, Kelly and Wu, 1998). The sections were then rinsed and transferred to a secondary antibody solution consisting of 0.05% triton TBS, NGS, and a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Canada, Burlington, ON, diluted 1:200) for 1 hr at 4°C. Sections were rinsed and transferred to a solution of 0.2% triton TBS, and Vectastain *Elite* ABC solutions (diluted 1:55) for 2 hr at 4°C. Finally, the sections were rinsed in TBS, rinsed in 50mM Tris buffer (pH 7.6) for 10 minutes, and incubated on an orbit shaker for 10 minutes in 0.05% DAB in 50mM Tris buffer with 3% H_2O_2 (solution buffered to pH 7.8 with drops of 6M NaOH), to

catalyze the DAB. The reaction was stopped by rinsing in cold TBS.

Brain slices were then mounted on slides and coverslipped following application of VectaShield (Vector Laboratories Canada, Burlington, ON). The slides were kept at 4° Celcius, protected from light. Slides were examined under a Leica microscope with ultraviolet epifluorescence and transmitted light illumination, as in Zhang et al. (1998).

3. Results

In the 4 animals with correct placements and successful FG infusion, FG-positive cells, GABA-positive cells, and double-labeled FG/GABA cells were counted unilaterally in the slices obtained from each of the areas previously shown to contain projections to the VMH. These areas include the lateral septum (LS), medial septum (MS), diagonal band (DB), BNST, mPOA, AHA, suprachiasmatic nucleus (SCN), PVN, lateral hypothalamus (LH), MeA, anterior hippocampus (AHipp), and ventral subiculum (VS). Counting was limited to every second brain section from each area. Brain regions were identified using the Rat Brain Atlas (Paxinos and Watson, 1998). Under ultraviolet epifluorescence, single-labeled GABA cells manifested as a dark cytoplasm and FG cells manifested as a fluorescent yellow cytoplasmic stain. Double-labeled FG/GABA cells were observed as illuminated dark cytoplasms (Fig. 12) in one plane of view under the microscope. For all double-labeled cells counted, care was taken to focus the plane of view in and out to ensure that cells were not single-labeled and

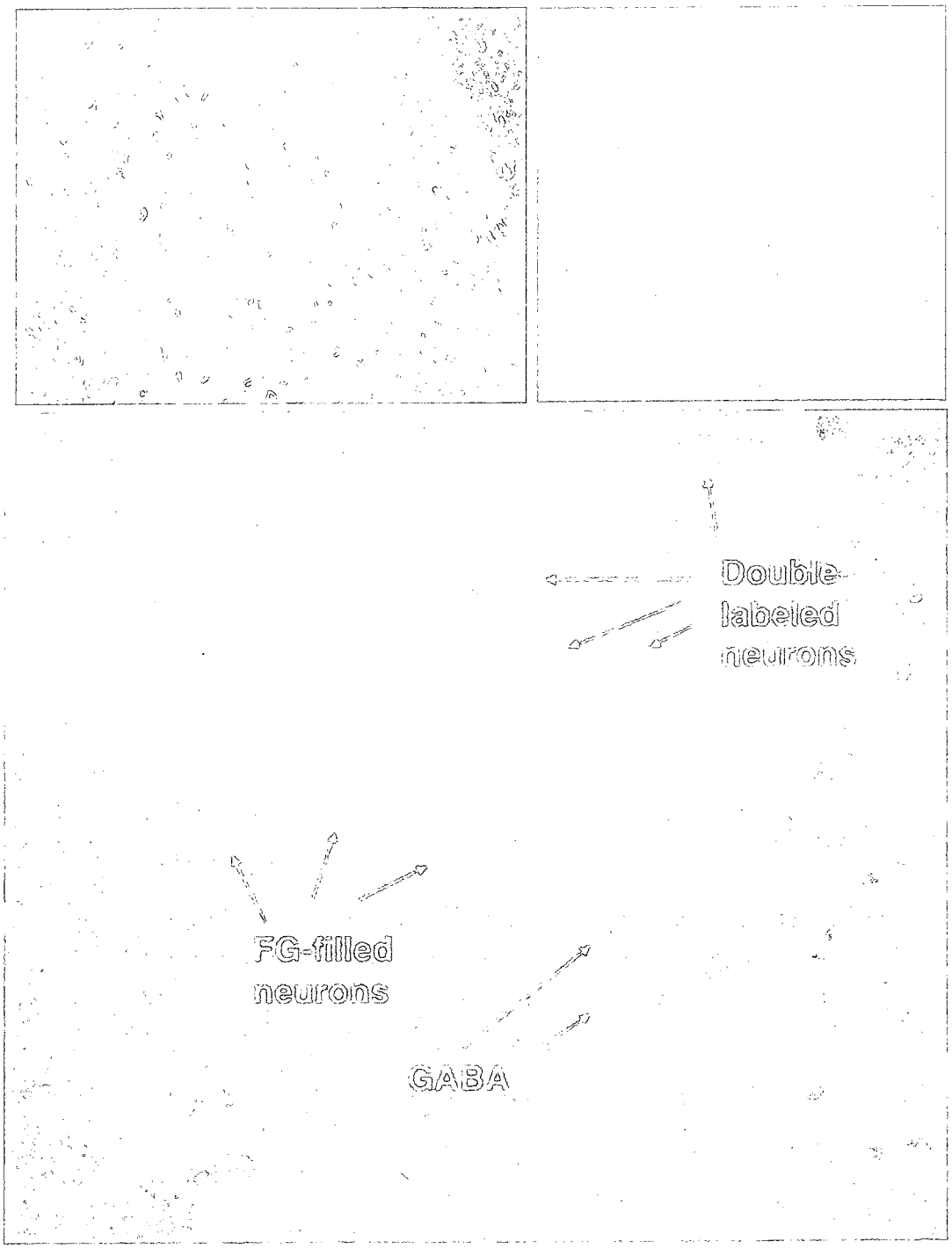

Figure 13. Photographs of brain tissue obtained from an area that contains GABA (top left, magnification X20); an area that has projections to the VMH (top right, magnification X60); and from the mPOA, one of the areas that has important GABAergic projections to the VMH (bottom, magnification X40).

simply adjacent to one another (as can occur in a 30μm section that is approximately 3 cell bodies' deep). All counts were made at 40x magnification.

Mean numbers of single FG-labeled cells, GABA-labeled cells, and double-labeled cells per section were thoroughly counted in the sections collected from each of the brain areas named above for one rat (except for the mPOA, in which FG-labeled, GABA-labeled and double-labeled cells were counted in all 4 animals, see Tables 2 and 3). Then, qualitative categories were created for the number of FG-positive cells, GABA-positive cells, and FG/GABA-positive cells. In the remaining 3 brains, counting of the number of FG-positive cells, GABA-positive cells and FG/GABA positive cells was restricted to analysis and subsequent assignment into one of the categories created for each type of labeled cell. Each brain area was categorized as having either 'under 15', 'between 15 and 24', or 'over 24 FG-positive cells'. The same areas were categorized as having either 'under 15', 'between 15 and 35', or 'over 35' GABA-positive cells. Finally, each area was categorized either as 'rarely presenting with double-labeled cells', 'always presenting with double-labeled cells', or 'presenting with numerous double-labeled cells'.

To report the contribution of each nucleus to the total number of GABAergic projections to VMH, the number of double-labeled cells in each nucleus was divided by the total number of double-labeled cells in all nuclei and multiplied by 100. This way, a percentage of the total number of GABAergic projections to the VMH was obtained for each brain area (Table 2). This

Brain area	FG	GABA	FG and GABA	% FG/GABA from each area
LS	++		‡	
MS	+		‡	
DB	+		‡	
BNST	++	†	‡‡	♦
mPOA	+++	†††	‡‡‡	♦♦♦
AHA	+++	†††	‡‡‡	♦♦
SCN	+	†††	‡	
PVN	++	††	‡‡‡	♦♦
LH	++	†	‡‡	♦
MeA	+++	†††	‡‡‡	♦♦
AHipp	+++	††	‡‡	♦♦♦
VS	+	†	‡	

+ Under 15 FG-filled neurons present in 30μm section through this nucleus
++ Between 15 and 24 FG-filled
+++ Over 24 FG-filled neurons

† Under 15 GABA-labeled neurons present in 30μm section through this nucleus
†† Between 15 and 35 GABA-labeled neurons
††† Over 35 GABA-labeled neurons

‡ Double-labeled neurons rarely present in a 30μm section through this nucleus
‡‡ Double-labeled neurons always present
‡‡‡ Numerous double-labeled neurons present

Under 5% of total GABAergic projections to the VMH are from this nucleus
Between 5% and 15% of total GABAergic projections to the VMH are from this nucleus
Over 15% of total GABAergic projections to the VMH are from this nucleus

Abbreviations: FG, Fluorogold; LS, lateral septum; MS, medial septum; DB, diagonal band; BNST, bed nucleus of the stria terminalis; mPOA, medial preoptic area; AHA, anterior hypothalamic area; SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; LH, the lateral hypothalamus; MeA, medial amygdala; AHipp, the anterior hippocampus; VS, the ventral subiculum.

Table 2. Distribution of FG-filled neurons, GABA-labeled neurons, and FG/GABA-labeled neurons following FG infusion to the VMH, along with rounded percentage of GABA neurons projecting from each area to the VMH.

Brain	Number of FG-labeled neurons	Number of GABA-labeled neurons	Number of double-labeled neurons
1	>200	>400	98
2	>220	>300	110
3	>180	>340	87
4	>200	>350	105

Table 3. Number of FG-labeled, GABA-labeled and double-labeled cells in the mPOA of each of the 4 animals with correct FG infusion placements.

calculation was performed using the cell counts of the brain from one animal, namely the only brain in which all type of cells and double-labeled cells were counted.

4. Discussion

The goal of this experiment was to identify brain areas with GABAergic projections to the VMH, as GAD isoforms are not found within this brain region (Feldblum et al., 1993; Fernandez-Guasti et al., 1986; McCarthy et al., 1995; Okamura et al., 1990; Ovesjo et al., 2001; Ziegler et al., 2002). FG infusions identified a number of areas previously shown to have projections to the VMH, including the LS, the DB, the BNST, the mPOA, the AHA, the SCN, the PVN, the LH, the MeA, the AHipp and the VS (Fahrback et al., 1989). Of these areas, the BNST, mPOA, AHA, SCN, PVN, LH, MeA, and AHipp also displayed a substantial number of GABA-labeled neurons. However, the mPOA, AHA, PVN,

AHipp, and the MeA, accounted for approximately 94% of GABAergic projections to the VMH, with the BNST and LH sharing the rest of the efferents. It is important to note that because the FG signal was very strong at the site of infusion, it was impossible to assess whether neuronal populations within the DMN, located above the VMH, might send GABAergic projections to the VMH. Other reports have noted prominent GAD mRNA containing cell populations in the DMN (McCarthy et al., 1995), making it possible that this potentially important source of efferent GABA projection to the VMH was missed in the present study.

4.1. Role of the mPOA

The mPOA, which contained by far the largest amount of GABAergic neurons projecting to the VMH (~45%), plays a major role in the control of female sexual behavior. It is one of the steroid concentrating brain regions that has also been associated with inhibition of lordosis and facilitation of proceptive behaviors (Hoshina et al., 1994; Pfaff and Sakuma, 1979; Whitney, 1986). Excitotoxic lesions that spare fibers of passage throughout the mPOA facilitate lordosis and rejection behaviors, and decrease solicitation and other proceptive behaviors (Hoshina et al., 1994). Electrophysiological studies have shown inhibition of lordosis following electrical stimulation of the area (Hoshina et al., 1994; Pfaff and Sakuma, 1979; Takeo et al., 1993). Immunocytochemical studies have shown that the larger the amount of VCS received, the larger is the induction of Fos within the mPOA, as is the case within the VMH (Pfaus et al., 1996). We have

previously shown that a substantial amount of VMH cells that stained for Fos following 50 VCSs also stains for glutamate (Georgescu et al., 2009), and that increased glutamate transmission within the VMH has an inhibitory effect on lordosis and other aspects of sexual behavior. These studies, taken together with our data, suggest that there is an interplay between neuronal activity within the mPOA and the VMH, perhaps to ensure that behaviors that are mutually exclusive, such as lordosis and proceptive behaviors, do not occur at the same time. We speculate that increased glutamate neurotransmission within the VMH may inhibit lordosis, thus allowing neuronal activity within the mPOA to activate proceptive behaviors. This interplay between the VMH and mPOA neurons is possible via reciprocal projections between the VMH and the mPOA.

Interestingly, increased GABAergic neurotransmission in the VMH facilitates lordosis whereas in the mPOA, it has the opposite effect (McCarthy et al., 1990). Furthermore, GAD 65 mRNA levels in the magnocellular POA are increased, whereas GAD 67 mRNA levels are decreased following estrogen treatment (McCarthy et al., 1995). GAD 65 synthesizes GABA for vesicular release whereas GAD 67 manufactures GABA for non-vesicular release and metabolic purposes (Erlander and Tobin, 1991). Estrogen receptors and GAD are colocalized in a large percentage of cells within the POA (Herbison et al., 1992; Sagrillo and Selmanoff, 1994; Flugge et al., 1986). Thus, estrogen upregulates synthesis of GAD 65 within the mPOA, which in turn synthesizes glutamate into GABA, providing the mPOA with substantial GABA pools ready for vesicular

release. It is highly possible that the important GABAergic projection from the mPOA to the VMH increases GABAergic neurotransmission in the VMH and consequently inhibits release of glutamate. In the absence of GABAergic release from the axon terminals of neurons originating in the mPOA, glutamate is released in the VMH and exerts inhibitory effects on lordosis. The interplay between neuronal activity between the mPOA and the VMH and its function in female sexual behavior remains an emerging story.

4.2. Role of the PVN

The PVN is another steroid-concentrating brain region that contained about 15% of the GABAergic projections to the VMH. Induction of Fos following copulation with intromission or manual VCS has been consistently reported in this nucleus (Pfaus et al., 1993; Flanagan-Cato and McEwen, 1995). The PVN is a major source of OXY to the posterior pituitary and the rest of the brain and spinal cord (Carmichael, Humbert, Dixen, Palmisano, Greenleaf, and Davidson, 1987; Swanson and Kuypers, 1980; Swanson, Sawchenko, Wiegand, and Price, 1980). Blood-borne OXY from posterior pituitary release stimulates smooth muscle contraction of the uterus and myoepithelial cells of the mammary glands, expelling the fetus from the uterus and milk from the mammary glands (see Komisaruk and Steinman, 2001, for a review). Sansone and Komisaruk (2001) report that VCS activates oxytocinergic neurons within PVN, neurons that project to the thoracic spinal cord where they release OXY, which has a stimulatory

effect (direct and/or indirect) on the sympathetic division of the autonomic nervous system, accounting for the dilation of the pupil, and perhaps the increase in heart rate and blood pressure observed during VCS (Komisaruk and Whipple, 2000). PVN neurons are labeled after PRV injection into the clitoris (Marson, 1995). Interestingly, the VMH also has efferent projections to the PVN (Lin and York, 2004). VMH and PVN neurons might communicate during copulation to regulate lordosis quotients and magnitudes according to rates of smooth muscle contractions in the uterus and vagina that facilitate sperm transport (Pacheco, Martinez-Gomez, Whipple, Beyer, Komisaruk, 1989; Shafik, El-Sibai Mostafa, Shafik, 2005; Toner and Adler, 1986) to optimize successful impregnation. More specifically, PVN neurons that receive input from the clitoris and the vagina might release GABA into the VMH in such a way as to control the rate of intromission. Thus, GABA might be released to permit females to lordose, and not released when females engage in pacing. It is also possible that this interaction between VMH and PVN neurons might contribute to OXY release, thus controlling smooth muscle contractions and activation of the sympathetic nervous system. These potential mechanisms of action are interesting routes for future investigation.

4.3. Role of the MeA and Hippocampus

GABAergic projections from the MeA and hippocampus accounted for a fifth of the total number of GABAergic projections to the VMH. The functional role of the MeA in female sexual behavior has been studied extensively. Strong

induction of Fos is observed in the MeA following VCS (Erskine, 1993; Pfaus et al., 1993; Polston and Erskine, 1995; Tetel et al., 1993; Pfaus et al., 1996) induction that is reduced substantially following transection of the pelvic nerve (Rowe and Erskine, 1993), indicating that the MeA receives direct input from VCS. The MeA is generally considered as a neural substrate that integrates olfactory, pheromonal and genital stimulation received from projections from the accessory olfactory bulbs (Alheid, de Olmos, and Beltramino, 1995) and the peripeduncular nuclei (Jones, Burton, Saper, and Swanson, 1976).

Although it is widely recognized that the hippocampus is involved in learning and memory, the functional role that it plays in female sexual behavior has not been elucidated. A moderate amount of Fos is induced in the dentate gyrus following copulation and VCS (Pfaus et al., 1993). In the CA 1 region of the hippocampus, Fos induction is lower following both copulation and VCS (Pfaus et al., 1993). However, work on pseudopregnancy mnemonics attributes important roles both to the hippocampus and the MeApd in the encoding of the amount and pattern of VCS necessary for initiation of the neuroendocrine changes required for successful impregnation (Oberlander and Erskine, 2008). Increased ARC expression was detected in the hippocampus following pseudopregancy-inducing VCS (Yang, Oberlander and Erskine, 2007). The authors suggest that although this mnemonic encoding mechanism is not understood, it might result from altered synapse density in VCS-activated regions. Interestingly, both the MeApd and the hippocampus show indirect evidence of synaptic pruning or modification

of neuronal phenotypes following VCS (Dafny and Terkel, 1990; Yang et al., 2007). The density of synapses in the MeApd and hippocampus being highest on proestrus (Woolley and McEwen, 1994; Rasia-Filho et al., 2004) might enable less active synapses to prune after VCS (Oberlander and Erskine, 2008). We speculate that glutamate release may be controlled by GABAergic afferents from these regions such that the rate and strength of intromissions are synchronized with the intromission mnemonic.

4.4. Role of the AHA, the BNST and the LH

The AHA, the LH and the BNST account for about 20% of all GABAergic neurons projecting to the VMH. Although the AHA alone accounted for about 75% of this percentage, its role in female sexual behavior remains relatively unexplored, as is the case with the LH. Pfaus et al. (1993) reported moderate amounts of Fos induction in both areas following copulation or 50 VCSs in presence of EB+P, and negligible amounts in absence of EB+P. E receptors are abundant both in the AHA and the LH (Pfaff and Keiner, 1973) further implicating these structures in the control of female sexual behavior. The AHA also has projections to the MCG (Pfaff, 1999), which is rich in E receptors (Pfaff and Keiner, 1973), and lesions of the tracts that connect the VMH to the MCG severely disrupt lordosis (Hennessey, Camak, Gordon, and Edwards, 1990).

The BNST, like the MeA, seems to be an area of convergence for olfactory, hormonal and somatosensory stimulation, as it receives input from the

pudendal and pelvic nerve following flank stimulation and VCS, respectively (Bueno and Pfaff, 2976; Berkley, Hotta, Robbins, and Sato, 1990; Rowe and Erskine, 1993). A robust induction of Fos is observed in the BNST following copulation or VCS, both under priming with EB+P and Oil, and both regions display a substantial induction of Fos following copulation with sexually vigorous males and following 50VCSs (Pfaus et al, 1993).

4.5. Steroid modulation of GABA

Numerous studies suggest that GABA release in the hypothalamus changes as a function of the estrus cycle because steroid hormones alter its neurotransmission, modulate GAD, modulate the GABA A receptor binding capacity, and modulate GABA A receptor synthesis (see McCarthy, 1995, for a review). Initially, it was reported that GABA turnover rates in the hypothalamus are lower in OVX rats, and increase with E treatment (Frankfurt, Fuchs, and Wuttke, 1984; Mansky, Mestres-Ventura, and Wuttke, 1982). Subsequently, McCarthy et al. showed that GABA release in the hypothalamus of receptive females is 25% greater than in postreceptive females and 20% greater than in OVX females, whereas in the mPOA, GABA release in receptive females was 18% less than in postreceptive females and 32% less than in OVX females (McCarthy et al., 1991). In receptive females, GABA release was 25% greater than in the mPOA. A study using E autoradiography and immunocytochemistry for GAD showed that about 20% of E-binding cells were GAD-IR, and 35% of E-

binding cells were GAD-IR in the mPOA and AHA (Flugge, Oertel, and Wuttke, 1986), although other researchers suggest those numbers to be as high as 100% (Sagrillo and Selmanoff, 1995). Similar observations were made in the septum and the BNST (Flugge et al., 1986). These reports suggest that GABA release in the hypothalamus is directly regulated by E. Studies on the effect of steroid hormones on GAD synthesis show that E upregulates GAD-65 in the mPOA and the MCG, and downregulates it in the DMN and zona incerta, but E also downregulates GAD-67 in the mPOA and upregulates it in the DMN and in the MCG (McCarthy et al., 1995). Experiments on steroid modulation of GABA A receptor binding show that E alters agonist binding at the GABA A receptor via classic genomic steroid actions (see McCarthy, 1995, for a review), and that P enhances agonist binding (Lopez-Colome, McCarthy, and Beyer, 1990). Finally, E and P regulate the number of GABA receptors (Maggi and Perez, 1984). Petersen and colleagues reported that E decreases ß2 and increases γ2 mRNA subunits of the GABA A receptor in the POA, and increases ß3 mRNA in the VMH, although the function of this effect is not known (Petersen et al., 1993). Thus, via genomic steroid actions in discrete brain nuclei, E increases or decreases mRNA levels for specific subunits of the GABA A receptor and synthesis of GABD, and upregulates GABA A rα receptors, providing inhibitory input with activated or depressed behavioral correlates in specific brain regions (McCarthy, 1995, Petersen et al., 1993; Chapter 4). Our results show that GABAergic projections to the VMH originate from neural substrates important in

the regulation of female sexual behavior. It is likely that E actions in these areas control GABA release in the VMH.

4.6. Methodological considerations

Replication of results from this experiment using confocal microscopy is imperative to provide quantitative analyses and interpretation of these data. Also, because GABA can be utilized by neurons for non-vesicular release or metabolic purposes, it is impossible to ascertain that the projections identified in this experiment actually release GABA in the VMH. However, because the VMH is devoid of GAD (Okamura et al., 1990, McCarthy et al., 1995, Ziegler, Cullinan, and Herman, 2002) yet GABA-IR neurons and axon terminals are abundant in the area (Commons et al., 1999), we favor the interpretation that part of the projections identified in this experiment release GABA into the VMH. Electron microscopic analysis of these circuits is therefore necessary.

5. Acknowledgments

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Summary of Chapter 5

The experiment presented in Chapter 5 identified the nuclei that send GABAergic projections to the VMH. Interestingly, the nuclei that contain large amounts of GABAergic projections to the VMH have all been previously found to play important roles in the control of female sexual behavior. These areas include the mPOA, with the largest number of GABAergic projections, followed by the AHA, the PVN, the AHipp, and the MeA. Discussed next are the data presented in the previous chapters and their implication in terms of glutamate function in the VMH.

General Discussion

The experiments presented in this thesis were designed to expand our understanding of the role that ventromedial hypothalamic glutamate plays in the control of sexual behavior in the female rat, and to determine whether its transmission could be controlled by GABA.

The results of the experiments in Chapter 1 showed that a substantial proportion of the neurons activated in the VMH by VCS are glutamatergic, and that priming with EB+P inhibits this activation. Amounts of VCS previously shown to induce estrus termination (50) triggered activation of twice as many glutamate cells than a single VCS. The number of glutamate cells activated was decreased by half in females primed with EB+P, regardless of the amount of VCS received. Those results, taken together with previous data showing that activation of glutamate receptors within the VMH results in inhibition of sexual behavior (Kow et al., 1985; McCarthy et al., 1991; Georgescu and Pfaus, 2006a), strongly suggest that increased glutamate transmission in this area is an important inhibitory signal for female sexual behavior, and that its activation is inhibited by priming with EB+P.

Chapter 2 investigated whether blocking glutamate neurotransmission at the AMPA/kainate receptor would have an effect on estrus termination induced by VCS. The results showed that animals infused with DNQX prior to administration of 50VCS at 9 pm were still in heat the next morning, whereas

animals infused with saline were not. DNQX reversed the effect of VCS on appetitive behaviors, lordosis quotient and lordosis magnitude, but had no effect on pacing, defensive behaviors, or the number of ejaculations the males were able to achieve. These results suggest that blocking glutamate transmission at the AMPA/kainate receptor delays onset of estrus termination.

The experiments presented in Chapter 3 analyzed the pattern of glutamate release in the VMH of females during a 2-hour copulation session with a sexually-vigorous male following priming with Oil, EB or EB+P. In females primed with Oil, glutamate release increased upon introduction of the male into the chamber despite an absence of sexual behavior by the male. In females primed with EB alone, glutamate release decreased when a male was introduced into the chamber, although in both Oil and EB groups, glutamate release returned to baseline levels within 1 hr. In females primed with EB+P, glutamate release was low throughout the 2-hour copulation session, despite receipt of VCS through intromissions and ejaculations by the male. Those results suggest that glutamate release patterns vary as a function of hormonal priming, but are also affected by the stimulation (e.g., olfactory) received from being in the proximity of a sexually mature male.

Chapter 4 examined whether glutamate release could be subject to regulation by GABA. For this to be possible, GABA receptors should be located on the membrane of glutamate neurons. The results showed that $GABA_A$ R1 subunits are upregulated by EB. The percentage of neurons that contained

glutamate and expressed those receptor subunits doubled when animals were primed with EB or EB+P, relative to Oil.

The experiment presented in Chapter 5 used infusions of the retrograde marker FluoroGold to the VMHvl combined with GABA immunocytochemistry to identify brain regions that send GABAergic projections to the VMH. Regions that had a large overlap included the mPOA, AHA, PVN, AHipp, and MeA. The mPOA had the largest amount of overlap, indicating that it is one of the primary sources of GABA to the VMH. Given the role of the mPOA in the control of appetitive aspects of female sexual behavior and the excitatory effects of GABA in the VMH, this finding suggests an interaction between the mPOA and VMH that controls the timing and patterning of female sexual behavior, especially perhaps the toggling between approach, solicitation, and runaway (mPOA), followed by lordosis (VMH). This interaction may also control the induction of estrus termination. In both cases, the drive for this interaction comes from estradiol, which appears to set up the ability of GABA to inhibit glutamate and thus disinhibit female sexual behavior.

Disinhibition as a mechanism of sexual excitation

Steinach (1910) proposed that the activation of a particular behavior involves mechanisms that facilitate the display of that behavior specifically and mechanisms involved in the active inhibition of competing behaviors. This was proposed with reference to hormone action, and in particular to the "disinhibitory"

effects of testicular secretions on the clinging reflex during the rut season in male frogs. Thus, the neuroendocrine mechanisms that facilitate sexual behavior might involve both excitatory actions that facilitate sexual responses directly, and also inhibitory actions on neural systems that normally inhibit sexual responses (either as a normal function of sexual satiety, as with estrus termination, or as a function of the induction of behaviors that are incompatible with sexual activity, such as feeding). This requires a nervous system designed not only to translate neuronal excitatory states into behavior, but that also sends out inhibitory influences to regions that control incompatible states (Konorski, 1967). This was proposed with reference to conditioned excitation and inhibition by Pavlov (1927), and found electrophysiologically in the visual system by Hubel and Wiesel (1959).

Estrogens activate both cell surface and cytosolic receptors that change electrophysiological and molecular properties of neurons (Mermelstein, Becker, and Surmeier, 1996; Pfaff, 1999). Those changes alter cellular excitation and up- and down-regulate protein products that act as neurotransmitters, enzymes, and receptors. This creates a neurochemical state that primes an animal's attention toward sex-related incentive stimuli and facilitates appropriate motor responses to bring animals from distal to proximal so that sexual interaction can occur. Following receipt of sufficient stimulation (which can be genitosensory and involve the activation of systems for reward, satiety, and sedation, Pfaus, 2009), animals become refractory. The activation of those inhibitory systems feeds back to inhibit excitatory systems for sexual behavior, allowing animals to move to the

"next phase", which could involve sleeping, feeding, nest building, etc., behaviors that are incompatible with sexual responding. In female rats, this refractoriness is referred to as "estrus termination" which marks a progressive transition from high appetitive behaviors such as solicitations coordinated with pacing and lordosis at the beginning of sexual interaction with males, to a decline in those behaviors, with appetitive behaviors decreasing before consummatory behaviors along with an increase in defensive and rejection responses (Pfaus et al., 2000).

For example, copulation with sexually vigorous males for 1 hr provides a large array of sensory stimulation, including olfactory and accessory olfactory stimulation from male odors, clitoral stimulation from mounts with pelvic thrusting, and a large amount of VCS induced by intromissions and ejaculations. Some of those stimuli facilitate, whereas others inhibit sexual responding. For instance, olfactory stimuli provided by the male activate appetitive approach and solicitations in OVX females primed with EB+P (Afonso, Woehrling, and Pfaus, 2006), but activates rejection responses in OVX, oil-treated females (Pfaus et al., 2000). Vocalizations made by males during the early phase of copulation facilitate the maintenance of lordosis (White, Cagiano, and Barfield, 1990). Mounts with pelvic thrusting but without intromission can reduce rejection responses in OVX rats without hormone replacement (Hardy and Debold, 1971), and it has recently been shown that manual distributed clitoral stimulation alone (using a paint brush) induces sexual reward in hormonally-primed females (Parada, Chamas, Censi, Coria-Avila, and Pfaus, 2010). Although as little as two

manually applied VCSs can potentiate lordosis (Rodriguez-Sierra, Crowley, and Komisaruk, 1975), a larger number of intromissions or manually applied VCSs results in a potent abbreviation of estrus, including the suppression of appetitive approach and solicitations, decrease in lordosis magnitude, and the facilitation of rejection responses (Bermant and Westbrook, 1966; Blandau, Boling, and Young 1941; Erskine and Baum, 1982; Hardy and Debold, 1972; Pfaus et al., 2000). The inhibitory effect of VCS provided by multiple intromissions and ejaculations is prevented following transaction of the pelvic nerve (Lodder and Zeilmaker, 1976), which also prevents the induction of Fos protein in the VMHvl and elsewhere by manual distributed VCS (Pfaus, Manitt, and Coopersmith, 2006). Pelvic nerve transaction also alters the display of pacing in female rats (Erskine, 1992).

The present data indicate that ovarian steroids, especially estradiol, set the stage for appetitive and consummatory sexual responding in females not only by activating excitatory systems associated with olfactory and auditory attention and sensitivity to genital and flank stimulation, but by blunting the activation of inhibitory systems by VCS. It can be postulated that doing so allows females to receive enough VCS to stimulate visceral and neuroendocrine reflexes associated with pregnancy, thus preventing them from entering estrus termination too early. For example, an optimal amount of VCS promotes sperm transport (Adler, 1969; Adler and Toner, 1986; Matthews and Adler, 1977) presumably by induction of a cervicouterine reflex that sucks sperm from the cervix into the uterus and through the epindymal folds of the uterus toward the

fallopian tubes (Shafik, El-Sibai, Shafik, and Shafik, 2005). VCS also promotes the induction of nightly prolactin surges in rats that are necessary for the maintenance of progestin secretion by the corpora lutea prior to the full growth of the placenta. Nightly prolactin surges are critical for the maintenance of pregnancy through the first trimester (Adler and Toner, 1986). Consistent with this, females allowed to pace their copulatory contact with males in unilevel pacing chambers bisected by a 1-hole divider receive "stronger" intromissions from males and show a faster onset of estrus termination relative to females not allowed to pace (same chamber with the divider removed; Erskine and Baum, 1982). Pacing copulation at their preferred rate also induces an opioid reward state in females that promotes the development of sexually conditioned place preference (Paredes and Alonso, 1997; Paredes and Vazquez, 1999; Paredes and Martinez, 2001) and partner preference (Coria-Avila, Ouimet, Pacheco, Manzo, and Pfaus, 2005; Coria-Avila, Gavrila, Boulard, Charron, Stanley, and Pfaus, 2008; Coria-Avila, Solomon, Vargas, Lemme, Ryan, Mėnard, Gavrila, and Pfaus, 2008).

In the human clinical literature, disinhibition is a common "mechanism" associated with enhanced sexual activity in individuals prone to hypoactive sexual arousal or desire because of too much endogenous inhibition (Bancroft, 1999), or with engaging in risky sex (Beckman and Ackerman, 1995; Colfax and Guzman, 2006). Indeed, many drugs of abuse have been considered "prosexual" because they result in disinhibited sexual responding. Use of psychomotor

stimulants like amphetamine, caffeine, cocaine, methylenedioxy methamphetamine (MDMA or "Ecstasy), or depressants such as alcohol or heroin, are often used in sexual situations where they are believed to increase sexual arousal or desire, or to enhance the intensity of sexual stimulation during sexual intercourse (Abel, 1984; Buffum and Moser, 1986; Kall, 1992; Pfaus and Gorzalka, 1987; Semple, Zians, Strathdee, and Patterson, 2009). Some of those effects may be direct, such as the facilitation of erection or an enhanced sensory awareness that can amplify sexual stimulation and intensify orgasm. Other effects, however, may be indirect, and stem from a general cognitive disinhibition that prompts individuals to engage in highly arousing ("naughty"), promiscuous, unsafe, "marathon", or even violent sexual activity without regard to its consequences. In humans, it is also important to consider those effects in light of cultural belief in the power of drugs to disinhibit sexual activity and thus provide an "excuse" for otherwise unacceptable sexual behavior (Leigh, 1990).

Although rats are obviously not subject to the same cultural influences as humans, they can be trained to inhibit their sexual responses. For example, sexually experienced male rats learn not to copulate with sexually nonreceptive females (Pfaus and Pinel, 1989), and male and female rats can associate a neutral olfactory cue (almond) with frustrated sexual activity or sexual nonreward and learn to avoid even sexually receptive or active partners that bear the inhibitory cue (Kippin and Pfaus 2001; Parada et al., in preparation). High doses of alcohol or psychomotor stimulants inhibit sexual behavior in both male and

female rats (Pfaus, Wilkins, Dipietro, Benibgui, Toledano, Rowe, and Couch, 2010), but lower doses of alcohol or cocaine can disrupt the display of both types of inhibition (Pfaus and Pinel, 1989; Pfaus et al., 2010). Although it is not known how drugs of abuse disinhibit sexual behavior, the existence of a disinhibitory mechanism for sexual behavior, as has been elucidated in the research reported here, suggests that they could work in a similar fashion to disrupt the activation of neurochemical mechanisms that underlie the learned inhibition.

Role of VMH glutamate

The VMH is a critical part of the hypothalamic module that integrates the hormonal control of lordosis and other female sexual behaviors. Electrolytic or excitotoxic lesions of the VMH decrease lordosis and appetitive sexual behaviors (Pfaff, 1980), although females may recover from those effects (La Vaque and Rodgers, 1975; Pfaff and Sakuma, 1979b). Estradiol binds to cytosolic estrogen receptors in the VMH (Pfaff and Keiner, 1973) and application of crystalline estradiol to the VMHvl of OVX rats potentiates lordosis in response to manual flank stimulation (Pfaff, 1980). In addition to sexual behavior, the VMH is involved in the control of feeding (Bray and York, 1998; Gold et al., 1972; Marshall et al., 1955; Storlein and Albert, 1972), aggression (Adamec, 1991; Brayley and Albert, 1977; Sterner et al., 1992), and pain (Borszcz, 2006), activities that are incompatible with sexual responding. It is therefore likely that the VMH has both excitatory and inhibitory systems for sexual behavior, of which glutamate neurons

appear to exert important inhibitory control. What is the role of glutamate? There are at least three possibilities.

I. VMH glutamate may mediate states of sexual non-receptivity:

Glutamate release may work to inhibit female sexual behavior in several circumstances. First, increased glutamate neurotransmission may account for states of sexual non-receptivity, such as the juvenile period, pregnancy, lactation, following ovariectomy, or during phases of the estrus cycle during which females are not sexually receptive. The experiment presented in Chapter 3 analyzed glutamate release following ovariectomy as well as following priming with EB and EB+P. The results suggest that glutamate release patterns are contingent upon the hormonal priming regimen, and sensitive to the type of sensory stimulation received from being in proximity to a sexually mature male. As such, OVX females not receiving hormones had low baseline glutamate levels that rapidly increased upon introduction of a male into the chamber. OVX females received a small number of male mounts and no intromissions. These data refute the hypothesis that glutamate mediates states of sexual non-receptivity; rather, they suggest that in the absence of circulating E and P, being in proximity of a sexually mature male without reception of VCS is sufficient to trigger significant increases in glutamate release from baseline levels. Therefore, finding themselves in a sexual context, along with the visual, auditory, olfactory and tactile stimulation received from the sexually mature male, seem to be the cues that trigger glutamate release in OVX females. Results from Chapter 1 support

this explanation: the number of activated glutamate neurons was low in OVX females that received 1VCS, whereas when 50 VCSs were administered, this number more than doubled. These findings suggest that the VMH may act as a hormonal regulator of sensory sexual stimulation. It is possible that during states of sexual non-receptivity, the function of glutamate release in the VMH is to induce running as part of a suite of defensive responses, so females can get away from males that attempt to mount them or that engage in anogenital investigation. Very high levels of pacing were observed in bilevel chambers following administration of glutamate and its agonists (Georgescu and Pfaus, 2006a).

II. VMH glutamate may facilitate display of behaviors incompatible with sexual behavior: A second possibility is that glutamate facilitates behaviors that are incompatible with sexual activity. The VMH is involved in the mediation of feeding (Bray and York, 1998; Gold et al., 1972; Marshall et al., 1955; Storlein and Albert, 1972), aggression (Adamec, 1991; Brayley and Albert, 1977; Sterner et al., 1992), and pain (Borszcz, 2006). Studies on the role of VMH glutamate in feeding have revealed that glutamate release by steroidogenic factor-1 (SF1) neurons in the VMH plays an important role in the neurocircuitry involved in the prevention of hypoglycemia (Tong et al., 2007). Conversely, relative excesses of VMH GABA contribute to hypoglycemia-associated autonomic failures (Chan, Cheng, Herzog, Czyzyk, Zhu, Wang, McCrimmon, Seashore, and Sherwin, 2008), whereas infusions of the GABA agonist muscimol to the VMH increases

food intake in a dose-dependent manner (Kelly, Rothstein, and Grossman, 1979). Although it is well known that OVX females eventually become overweight (Wade, 1975), it is not known whether this is a result of decreased GABAergic and/or increased glutamatergic tone in the VMH. The decreased GABAergic tone may be secondary to low circulating estrogen levels, leading to low GAD-65 production in areas that send GABAergic projections to the VMH. Females with VMH lesions also become markedly obese (Tepperman, Brobeck, and Long, 1943; Han, 1968; Cox and Powley, 1981; Bernardis and Skelton, 1965; Han, Lin, Chu, Mu, and Liu, 1965).

Glutamate released in the VMH may inhibit female sexual behavior but facilitate feeding (Tong et al., 2007). However, it is likely that only a proportion of the glutamate released in this area is utilized by neurons involved in mediation of sexual behavior, another proportion being utilized by neurons involved in mediation of feeding, and so on for each of the functions controlled by the VMH. It is possible that the VMH has neuronal populations that are primarily involved in the control of feeding, and other neuronal populations that are primarily involved in the control of sexual behavior. Yet another possibility is that under certain conditions, such as when females are hungry, the glutamate and GABA released from VMH neurons is utilized for cellular mechanisms that underlie feeding behavior. Under other conditions, such as when E and P levels are high and females are in heat, glutamate and GABA released from the VMH may exert their effects mainly on sexual behavior.

A number of experiments are necessary to elucidate the interaction between GABA, glutamate, and steroid hormones, and their effects on feeding and sexual behavior. First, microdialysis should be employed to determine if/how GABAergic tone varies in presence of circulating ovarian hormones. Second, GABA and GABA agonist infusions should be administered to the VMH of OVX females primed with Oil, EB and EB+P to test whether these infusions lead to increased or decreased food intake following different hormonal priming regimens. Conversely, it would be important to determine whether increased glutamate neurotransmission within the VMH leads to increased food intake. Finally, glutamate and GABA release should be measured in intact females during the 4 phases of the estrous cycles while females have access both to food and to a sexually vigorous male. Correlations between behavior and neurotransmitter release could reveal the conditions under which glutamate and GABA are released as a function of the behavior in which the female is engaged.

III. VMH glutamate may mediate onset of estrus termination: A third possibility is that VMH glutamate release is involved in the onset of estrus termination. Results from Chapter 2 strongly support this hypothesis: blockade of the AMPA/kainate receptor by glutamate antagonist DNQX reversed the effects of VCS on estrus termination. As such, appetitive behaviors, including solicitations and hops and darts, lordosis magnitudes and lordosis quotients were maintained in females infused with DNQX despite reception of amounts of VCS sufficient to end the period of estrus in females that were infused with saline prior

to VCS. Estrus termination is characterized by decreases in solicitation, lordosis magnitudes and quotients, and increases in pacing and defensive behaviors (Coopersmith, Candurra, and Erskine, 1996; Erskine and Baum, 1982; Lodder and Zeilmaker, 1976; Pfaus, Smith, Byrne, and Stephens, 2000). The behavioral pattern induced by glutamate agonist infusions to the VMH is reminiscent of that observed during estrus termination (Georgescu and Pfaus, 2006a). It is possible that reception of amounts of VCS sufficient to induce pregnancy or pseudopregnancy trigger the release of glutamate from neurons within the VMH. Then, areas that receive input from the vagina and cervix and send GABAergic projections to the VMH might downregulate production of GAD-65, leading to decreased GABAergic output into the VMH. These areas include the mPOA, the PVN, the Hipp, the MeApd and the AHA (Pfaus et al., 1993). Estrogen was previously shown to upregulate production of GAD-65 in the mPOA, an area rich in GAD mRNA-containing neuronal populations (McCarthy, Kaufman, Brooks, Pfaff, and Schwartz-Giblin, 1995). Although speculative, it is also possible that, as in the mPOA, E upregulates production of GAD-65 in areas that receive sensory input from the vagina, cervix and clitoris and that contain GABAergic projections to the VMH. Again, a number of experiments are required to test this hypothesis. First, we need to establish if GAD-65 synthesis is modulated by different hormonal priming regimens in each of the areas that contain GABAergic projections to the VMH. Also, it would be interesting to know how different amounts of VCS impact GAD-65 synthesis in areas that contain GABAergic

projections to the VMH. Finally, VMH glutamate and GABA levels must be measured during tests of estrus termination.

Role of GABA projections to the VMH

Results from Chapter 4 show that GABA A receptors exist on glutamate neurons within the VMH, but in situ hybridization studies have shown that the VMH is devoid of GAD, the GABA synthesizing enzyme (Okamura et al., 1990, McCarthy et al., 1995, Ziegler, Cullinan, and Herman, 2002). The experiment presented in Chapter 5 identified the mPOA, the PVN, the MeA, the hippocampus, the AHA, the BNST and the LH as areas that send GABAergic projections to the VMH. About 45% of these originate in the mPOA, 15% in the PVN, 20% in the MeA and the hippocampus, and 20% from the AHA, the BNST, and the LH.

The mPOA is important in the control of proceptive behaviors, especially solicitations, and communicates with the VMH via afferent and efferent projections. Electrical stimulation of the mPOA inhibits lordosis (Hoshina et al., 1994; Pfaff and Sakuma, 1979; Takeo et al., 1993), whereas mPOA lesions that spare fibers of passage facilitate lordosis and rejection behaviors, and decrease solicitation and other proceptive behaviors (Hoshina et al., 1994). It is possible that an interplay between neuronal activity within the mPOA and VMH exists, such that when mPOA projection neurons release GABA in the VMH, glutamate release is inhibited and females display lordosis. Without GABAergic inhibitory

input to the area, glutamate is released, lordosis is inhibited, and females engage in running and defensive behaviors. This way, behaviors that are mutually exclusive do not occur at the same time.

The PVN was another area with a substantial number of GABAergic projections to the VMH. The PVN is important in the production and release of OXY into the posterior pituitary, brain, and spinal cord (Carmichael et al., 1980; Swanson et al., 1980). Blood-borne OXY stimulates smooth muscle contraction of the uterus and myoepithelial cells of the mammary glands (see Komisaruk and Steinman, for a review). PVN neurons are labeled following PRV injections into the clitoris (Marson, 1995), and oxytocin neurons within the PVN project onto SPN neurons labeled with PRV following injections in the vagina (Marson and Murphy, 2006). The VMH has efferent projections to the PVN (Lin and York, 2004), suggesting that VMH and PVN neurons communicate during copulation to perhaps syncronize lordosis with contractions of the uterus and vagina, this way facilitating sperm transport to optimize impregnation (Pacheco et al., 1989; Shafik et al., 2005; Toner and Adler, 1986). It would be interesting to know if GABA-releasing neurons from the PVN with axon terminals in the VMH communicate with oxytonergic neurons that mediate blood oxytocin levels.

The MeA and hippocampus also contained GABAergic projections to the VMH. These brain areas could be involved in the control of glutamate release by ensuring that female rats synchronize the strength and pattern of intromissions according to the intromission mneumonic that is controlled by mechanisms within

these two areas (Yang et al., 2007; Oberlander and Erskine, 2008). Further research is needed to explore these neural interactions, as well as to clarify the roles of the BNST, the AHA and the LH in the mediation of glutamate release within the VMH.

Steroid modulation of GABAergic inhibition of VMH glutamate

GABA agonists acting at GABA A in the VMH facilitate lordosis; however such agonists have the opposite actions in the mPOA and MCG (McCarthy et al., 1990; McCarthy et al., 1991). There is substantial evidence that GABA transmission in the VMH is regulated by estrogens. First, VMH GABA content is larger in sexually receptive than non receptive females (McCarthy et al., 1991). This is corroborated by other studies in which E treatment increased VMH GABA release (Frankfurt et al., 1984; Mansky et al., 1982). GAD, the GABA synthesizing enzyme, is also subject to regulation by E in brain areas in the vicinity of the VMH, including the mPOA, DMN, MCG, and the zona incerta (McCarthy et al., 1995). Also, E treatment regulates the number of GABA receptors whereas P enhances the binding affinity of these receptors (Chapter 4; DeLorey and Olsen, 1994; Majewska et al., 1986; Maggi and Perez, 1984; Petersen et al., 1993). A large number of E-receptive cells are also GAD-IR in the mPOA, AHA and BNST (Flugge et al., 1986; Herbison et al., 1991; Sagrillo and Selmanoff, 1997), indicating that steroid modulation of GABA turnover might be direct. Thus, E may well upregulate the synthesis of GAD within the mPOA,

which in turn synthesizes more GABA, providing mPOA projection neurons with substantial GABA pools ready for vesicular release. We postulate that GABA may participate in the inhibition of glutamate release in the VMH when E levels are high, thereby disinhibiting sexual behavior perhaps to allow females to receive requisite amounts of VCS to induce the neuroendocrine changes necessary for pregnancy to occur. Whether estrogens regulate production of GAD in the PVN, MeA, hippocampus and the other areas with GABAergic projections to the VMH remains to be determined.

Alternative inhibitory influences in the VMH

Glutamate transmission in the VMH could also be inhibited via activation of δ opioid receptors. Infusions of the selective δ receptor agonist [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) to either the lateral ventricles or VMH resulted in a facilitation of lordosis (Acosta-Martinez and Etgen, 2002a; Pfaus and Pfaff, 1992). Bilateral infusions of low doses of DPDPE to the VMH of OVX females primed with EB and low doses of P facilitated lordosis, whereas high doses inhibited sexual receptivity. Conversely, infusions of the δ opioid receptor antagonist naltrindole to the VMH blocked lordosis. δ receptors interact with membrane Gi or Go proteins to induce neuronal hyperpolarization and shorten stimulated action potentials (Connor and Christie, 1999). As with GABA, EB increases synthesis of the enkephalin precursor proenkephalin in the VMH (Lauber et al., 1990; Romano et al., 1990). Thus, lordosis might be facilitated by

EB-induced increases in synthesis of proenkephalin which binds to δ receptors, hyperpolarizing the host neuron. Whether those neurons are also glutamatergic remains to be determined.

Conclusion

The five experiments encompassing this thesis were designed to elucidate the mechanism(s) through which increased glutamate neurotransmission within the VMH results in inhibition of female sexual behavior. These experiments showed that (1) glutamate neurons are activated by large amounts of VCS and that steroid hormones inhibit this activation; (2) decreasing glutamate neurotransmission within the VMH by blockage of the AMPA/kainate receptors delays estrus termination; (3) glutamate release depends on the hormonal priming regimen and received sensory stimulation; (4) GABA is one of the chemical agents that may participate in the inhibition of glutamate release; and (5) areas important in female sexual behavior send GABAergic projections to the VMH. Based on these data, we have proposed 3 possible mechanisms of action by which VMH glutamate inhibits sexual behavior in the female rat. First, glutamate may mediate sexual inhibition during states of sexual non-receptivity. Second, glutamate may activate other behaviors mediated by the VMH that are incompatible with sexual behavior, such as feeding. Third, increased glutamate neurotransmission may bring about estrus termination.

References

Abel, E.L. (1984). Opiates and sex. *Journal of Psychoactive Drugs. 16*, 205-216.

Acosta-Martinez, M., & Etgen, A.M. (2002a). The role of delta-opioid receptors in estrogen facilitation of lordosis behavior. *Behavioral Brain Research, 136*, 93-102.

Acosta-Martinez, M., & Etgen, A.M. (2002b). Activation of mu-opioid receptors inhibits lordosis in estrogen and progesterone-primed female rats. *Hormones and Behavior, 41*, 88-100.

Adamec, R.E. (1991). Partial kindling of the ventral hippocampus: identification of changes in limbic physiology which accompany changes in feline aggression and defense. *Physiology and Behavior, 49*, 443-453.

Adler, N.T. (1969). Effects of the male's copulatory behavior on successful pregnancy of the female rat. *Journal of Comparative and Physiological Psychology, 69*, 613-622.

Adler, N.T. & Toner, J.P. (1986). The effects of copulatory behavior on sperm transport and fertility in rats. *Annals of the New York Academy of Science, 474*, 21-32.

Afonso, V.M., Woehrling, A., & Pfaus, J.G. (2006). Sensory mediation of female-male mounting in the rat: I. Role of olfactory cues. Physiology and Behavior, 87, 857-862.

Aihara, Y., Mashima, H., Onda, H., Hisano, S., Kasuya, H., Hori, T., Yamada, S.,

Tomura, H., Yamada, Y., Inoue, I., Kojima, I., & Takeda, J. (2000). Molecular cloning of a novel brain-type Na(+)-dependent inorganic phosphate cotransporter. *Journal of Neurochemistry, 74*, 2622-2625.

Alheid, G.F., de Olmos, J.S., & Beltramino, C.A. (1995). Amygdala and extended amygdala. In G. Paxinos (Ed.), *The Rat Nervous System, 2nd Edition*, Academic Press, Sand Diego, 495-578.

Andersen, C.H. & Shen, C.L., (1980). Efferents of the medial preoptic area in the guinea pig: An autoradiographic study. *Brain Research Bulletin, 5*, 257-265.

Auger, A.P. & Blaustein, J.D. (1995). Progesterone enhances an estradiol-induced increase in Fos immunoreactivity in localized regions of female rat forebrain. *Journal of Neuroscience, 15*, 2272-2279.

Auger, A.P., Moffatt, C.A., & Blaustein, J.D. (1996). Reproductively-relevant stimuli induce Fos-immunoreactivity within progestin-receptor containing neurons in localized regions of female rat forebrain. *Journal of Neuroendocrinology, 8*, 831-838.

Ban, T. (1975). Fiber connections in the hypothalamus and some autonomic functions. Pharmacology, *Biochemistry and Behavior, 3*, 3-13.

Bancroft, J. (1999). Cardiovascular and endocrine changes during sexual arousal and orgasm. *Psychosometric Medicine, 61*, 290-291.

Beach, F.A. (1976). Sexual attractivity, proceptivity and receptivity in female mammals. *Hormones and Behavior, 7*, 105-138.

Beckman, L.J. & Ackerman, K.T. (1995). Women, alcohol, and sexuality. *Recent*

Developments in Alcoholism, 12, 267-285.

Bellocchio, E.E., Hu, H., Pohorille, A., Chan, J., Pickel, V.M., & Edwards, R.H. (1998). The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission. *Journal of Neuroscience, 18*, 8648-8659.

Bellocchio, E.E., Reimer, R.J., Fremeau, R.T.Jr., & Edwards, R.H. (2000). Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science, 289*, 957-960.

Berkley, K.J., Hotta, H., Robbins, A., & Sato, Y. (1990). Functional properties of afferent fibers supplying reproductive and other pelvic organs in pelvic nerve of female rat. *Journal of Neurophysiology, 63*, 256-272.

Bermant, G. (1961). Response latencies of female rats during sexual intercourse. *Science, 133*, 1771-1773.

Bermant, G. & Westbrook, W.H. (1966). Peripheral factors in the regulation of sexual contact by female rats. *Journal of Comparative Physiology and Psychology, 61,* 244-250.

Bernardis, L.L. & Skelton, F.R. (1965). Blood pressure in female and male rats following ventromedial hypothalamic lesions placed at four different ages. *Proceedings of the Society of Experimental Biology and Medicine, 120*, 756-760.

Bielsky, I.F. & Young, L.J. (2004). Oxytocin, vasopressin, and social recognition in mammals. *Peptides, 25,* 1565-1574.

Blandau, R.J., Boling, J.L., & Young, W.C. (1941). The length of heat in the albino rat as determined by the copulatory response. *Anatomical Record, 79*, 453-463.

Borszcz, G.S. (2006). Contribution of the ventromedial hypothalamus to generation of the affective dimension of pain. *Pain, 123*, 155-168.

Bray, G.A., & York, D.A. (1998). The MONA LISA hypothesis in the time of leptin. *Recent Progress in Hormone Research, 53*, 95-117.

Brayley, K.N., & Albert, D.J. (1977). Suppression of VMH-lesion-induced reactivity and aggressiveness by electrical stimulation ventral to the anterior septum in the rat. *Physiology and Behavior, 18*, 567-571.

Brown, T.J., MacLusky, N.J., Leranth, C., Shanabrough, M., & Naftolin, F. (1990). Progestin receptor-containing cells in guinea pig hypothalamus: Afferent connections, morphological characteristics, and neurotransmitter content. *Molecular and Cellular Neuroscience, 1*, 58-77.

Bueno, J. & Pfaff, D.W. (1976). Single unit recording in hypothalamus and preoptic area of estrogen-treated and untreated ovariectomized female rats. *Brain Research, 101*, 67-78.

Buffum, J. & Moser, C. (1986). MDMA and human sexual function. *Journal of Psychoactive Drugs, 18*, 355-359.

Burbach, J.P., Young, L.J., & Russell, J. (2006). Oxytocin: Synthesis, secretion and reproductive functions. In: Neill, J.D. (Ed.), *Knobil and Neill's Physiology of Reproduction*. Elsevier, pp. 3055-3128.

Cabilio, S. (1996). Rodent Sex Behavior Scoring Program. Unpublished computer software program, Concordia University.

Calizo, L.H. & Flanagan-Cato, L.M. (2000). Estrogen selectively regulates spine density within the dendritic arbor of the rat ventromedial hypothalamic neurons. *Journal of Neuroscience, 20*, 1589-1596.

Carmichael, M.S., Humbert, R., Dixen, J., Palmisano, G., Greenleaf, W., & Davidson, J.M. (1987). Plasma oxytocin increases in the human sexual response. *Journal of Clinical Endocrinology and Metabolism, 64*, 27-31.

Chan, O., Cheng, H., Herzog, R., Czyzyk, D., Zhu, W., Wang, A., McCrimmon, R.J., Seashore, M.R., & Sherwin, R.S. (2008). Increased GABAergic tone in the ventromedial hypothalamus contributes to suppression of counterregulatory responses after antecedent hypoglycemia. *Diabetes, 57*, 1363-1370.

Chan, A., Dudley, C.A., & Moss, R.L. (1983). Action of prolactin, dopamine and LHRH on ventromedial hypothalamic neurons as a function of ovarian hormones. *Neuroendocrinology, 36*, 397-403.

Colfax, G. & Guzman, R. (2006). Club drugs and HIV infection: A review. *Clinical and Infectious Diseases, 42*, 1463-1469.

Commons, K.G., Kow, L.M., Milner, T.A., & Pfaff, D.W. (1999). In the ventromedial nucleus of the rat hypothalamus, GABA-immunolabeled neurons are abundant and are innervated by both enkephalin- and GABA-immunolabeled axon terminals. *Brain Research, 816*, 58-67.

Condon, T.P., Ronnekleiv, O.K., & Kelly, M.J. (1989). Estrogen modulation of the alpha-1-adrenergic response of hypothalamic neurons. *Neuroendocrinology, 50*, 51-58.

Connor, M. & Christie, M.D. (1999). Opioid receptor signaling mechanisms. *Clinical and Experimental Pharmacology and Physiology, 16*, 493-499.

Conrad, L.C. & Pfaff, D.W. (1975). Axonal projections of medial preoptic and anterior hypothalamic neurons. *Science, 190*, 1112-1114.

Coopersmith, C., Candurra, C., & Erskine, M.S. (1996). Effects of paced mating and intromissive stimulation on feminine sexual behavior and estrus termination in the cycling rat. *Journal of Comparative Psychology, 110*, 176-186.

Coria-Avila, G.A., Gavrila, A.M., Boulard, B., Charron, N., Stanley, G., & Pfaus, J.G. (2008). Neurochemical basis of conditioned partner preference in the female rat: II. Disruption by flupenthixol. *Behavioral Neuroscience, 122*, 396-406.

Coria-Avila, G.A., Jones, S.L., Solomon, C.E., Gavrila, A.M., Jordan, G.J., & Pfaus, J.G. (2006). Conditioned partner preference in female rats for strain of male. *Physiology and Behavior, 88*, 529-537.

Coria-Avila, G.A., Ouimet, A.J., Pacheco, P., Manzo, J., & Pfaus, J.G. (2005). Olfactory conditioned partner preference in the female rat. *Behavioral Neuroscience, 119*, 716-725.

Coria-Avila, G.A. & Pfaus, J.G. (2007). Neuronal activation by stimuli that predict

sexual reward in female rats. *Neuroscience, 148*, 623-632.

Coria-Avila, G.A., Solomon, C.E., Vargas, E.B., Lemme, I., Ryan, R., Ménard, S., Gavrila, A.M., & Pfaus, J.G. (2008). Neurochemical basis of conditioned partner preference in the female rat: I. Disruption by naloxone. *Behavioral neuroscience, 122*, 385-395.

Coscina, D.V. & Nobrega, J.N. (1984). Anorectic potency of inhibiting GABA transaminase in brain: Studies of hypothalamic, dietary and genetic obesities. *International Journal of Obesity, 8*, 191-200.

Cox, J.E. & Powley, T.L. (1981). Prior vagotomy blocks VMH obesity in pair-fed rats. *American Journal of Physiology, 240*, 573-583.

Dafny, N. & Terkel, J. (1990). Hypothalamic neuronal activity associated with onset of pseudopregnancy in the rat. *Neuroendocrinology, 51*, 459-467.

Delgado, L.M. & Schmachtenberg, O. (2008). Immunohistochemical localization of GABA, GAD65, and the receptor subunits $GABA_{A_{\alpha 1}}$ and $GABA_{B1}$ in the Zebrafish Cerebellum. *Cerebellum, 7*, 444-450.

Delgado, L.M., Vielma, A.H., Kahne, T., Palacios, A.G., & Schmachtenberg, O. (2009). The GABAergic system in the retina of neonate and adult Octodon degus, studied by immunohistochemistry and electroretinography. *The Journal of Comparative Neurology, 514*, 459-472.

Delorey, T.M. & Olsen, R.W. (1994). GABA and glycine. In G.J. Siegel, B.W. Agranoff, R.W. Albers, and P.B. Molinoff (Eds.), *Basic neurochemistry* (5th ed.). Raven, New York, 389-399.

Dobbek, N. & Pfaus, J.G. (2001). Unpublished manuscript, Concordia University.

Donoso, A.O. & Zárate, M.B. (1981). Changes in prolactin release caused by GABA and endogenous GABA increase in rats. *Brain Research Bulletin, 7*, 359-64.

Dorner, G., Docke, F., & Moustafa, S. (1968). Differential localization of a male and a female hypothalamic mating centre. *Journal of Reproduction and Fertility, 17*, 583-586.

Edelhoff, S., Grubin, C.E., Karlsen, A.E., Alder, D.A., Foster, D., Disteche, C.M., & Lernmark, A. (1993). Mapping of glutamic acid decarboxylase (GAD) genes. *Genomics, 17*, 93-97.

Edmonds, S., Zoloth, S.R., & Adler, N.T. (1972). Storage of copulatory stimulation in the female rat. *Physiology and Behavior, 8*, 161-164.

Emery D.E., & Moss, R.L. (1984). Lesions confined to the ventromedial hypothalamus decrease the frequency of coital contacts in female rats. *Hormones and Behavior, 18*, 313-329.

Erlander, M.G. & Tobin, A.J. (1991). The structural and functional heterogeneity of glutamic acid decarboxylase: A review. *Neurochemistry Research, 16*, 215-226.

Erskine, M.S. (1985). Effects of paced coital stimulation on estrus duration in intact cycling rats and in ovariectomized and ovariectomized-adrenalectomized hormone-primed rats. *Behavioral Neuroscience, 99*, 151-161.

Erskine, M.S. (1989). Solicitation behavior in the estrous female rat: A review. *Hormones and Behavior, 23*, 473-502.

Erskine, M.S. (1992). Pelvic and pudendal nerves influence the display of paced mating behavior in response to estrogen and progesterone in the female rat. *Behavioral Neuroscience, 106*, 690-697.

Erskine, M.S. (1993). Mating-induced increases in FOS protein in preoptic area and medial amygdala of cycling female rats. *Brain Research Bulletin, 32*, 447-451.

Erskine, M.S. & Baum, M.J. (1982). Effects of paced coital stimulation on termination of estrus and brain indoleamine levels in female rats. *Pharmacology, Biochemistry and Behavior, 17*, 857-861.

Erskine, M.S. & Hanrahan, S.B. (1997). Effects of paced mating on c-fos gene expression in the female rat brain. *Journal of Neuroendocrinology, 9*, 903-912.

Etgen, A.M. (1987). Inhibition of estrus behavior in rats by intrahypothalamic application of agents that disrupt nuclear binding of estrogen-receptor complexes. *Hormones and Behavior, 21*, 528-535.

Etgen, A.M. (1990). Intrahypothalamic implants of noradrenergic antagonists disrupt lordosis behavior in female rats. *Physiology and Behavior, 48*, 31-36.

Etgen, A.M. & Karkanias, G.B. (1994). Estrogen regulation of noradrenergic signaling in the hypothalamus. *Psychoneuroendocrinology, 19*, 603-610.

Etgen, A.M., Ungar, S., & Pettiti, N. (1992). Estradiol and progesterone modulation of norepinephrine neurotransmission: Implications for female reproductive behavior. *Journal of Neuroendocrinology, 4*, 255-271.

Fahrbach, S.E., Morrell, J.I., & Pfaff, D.W. (1989). Studies of ventromedial hypothalamic afferents in the rat using three methods of HRP application. *Experimental Brain Research, 77*, 221-233.

Feldblum, S., Erlander, M.G., & Tobin, A.J. (1993). Different distributions of GAD65 and GAD67 mRNAs suggest that the two glutamate decarboxylases play distinctive functional roles. *Journal of Neuroscience Research, 34*, 689-706.

Fernández-Guasti, A., Larsson, K., & Beyer, C. (1986). Lordosis behavior and GABAergic neurotransmission. *Pharmacology, Biochemistry and Behavior, 24*, 673-6.

Flanagan-Cato, L.M. & McEwen, B.S. (1995). Pattern of Fos and Jun expression in the female rat forebrain after sexual behavior. *Brain Research, 673*, 53-60.

Flügge, G., Oertel, W.H., & Wuttke, W. (1986). Evidence for estrogen-receptive GABAergic neurons in the preoptic/anterior hypothalamic area of the rat brain. *Neuroendocrinology, 43*, 1-5.

Foley, C.M., Stanton, J.J., Price, E.M., Cunnigham, J.T., Hasser, E.M., & Heesch, C.M. (2003). $GABA_A$ α_1 and α_2 receptor subunit expression in rostral ventrolateral medulla in nonpregnant and pregnant rats. *Brain*

Research, 975, 196-206.

Fonnum, F. & Hassel, B. (1995). Glutamate synthesis, metabolism, and uptake. In: Stone TS, editor. *CNS Neurotransmitters and Neuromodulators: Glutamate*. New York: CRC Press, 19-34.

Fremeau, R.T.Jr., Troyer, M.D., Pahner, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., Bellocchio, E.E., Fortin, D., Storm-Mathisen, J., & Edwards, R.H. (2001). The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron, 31*, 247-60.

Frye, C.A., & Duncan, J.E. (1994). Progesterone metabolites, effective at the GABA A receptor complex, attenuate pain sensitivity in rats. *Brain Research, 643*, 194-203.

Frye, C.A. & Erskine, M.S. (1990). Influence of time of mating and paced copulation on induction of pseudopregnancy in cyclic female rats. *Journal of Reproduction and Fertility, 90*, 375-385.

Frye, C.A., Murphy, R.E., & Platek, S.M. (2000). Anti-sense oligonucleotides, for progestin receptors in the VMH and glutamic acid decarboxylase in the VTA, attenuate progesterone-induced lordosis in hamsters and rats. *Behavior and Brain Research, 115*, 55-64.

Fujiyama, F. Furuta, T., & Kaneko, T. (2001). Immunocytochemical localization of candidates for vesicular glutamate transporters in the rat cerebral cortex. *Journal of Comparative Neurology, 435*, 379-87.

García Horsman, S.P., Ågmo, A., & Paredes, R.G., (2008). Infusions of naloxone

into the medial preoptic area, ventromedial hypothalamus, and amygdala block conditioned place preference induced by paced mating behavior. *Hormones and Behavior, 54*, 709-716.

Georgescu, M. & Pfaus, J.G. (2006a). Role of glutamate receptors in the ventromedial hypothalamus in the regulation of female rat sexual behaviors. I. Behavioral effects of glutamate and its selective receptor agonists AMPA, NMDA and Kainate. *Pharmacology, Biochemistry and Behavior, 83*, 322-332.

Georgescu, M. & Pfaus, J.G. (2006b). Role of glutamate receptors in the ventromedial hypothalamus in the regulation of female rat sexual behaviors. II. Behavioral effects of selective glutamate receptor antagonists AP-5, CNQX, and DNQX. *Pharmacology, Biochemistry, and Behavior, 83*, 333-341.

Georgescu, M., Sabongui, C., Del Corpo, A., Marsan, L., & Pfaus, J.G. (2009). Vaginocervical stimulation induces Fos in glutamate neurons in the ventromedial hypothalamus: Attenuation by estrogen and progesterone. *Hormones and Behavior, 56*, 450-456.

Gilman, D.P., Mercer, L.F., & Hitt, J.C. (1979). Influence of female copulatory behavior on the induction of pseudopregnancy in the female rat. *Physiology and Behavior, 22*, 675-678.

Gilman, D.P., Mercer, L.F., Hitt, J.C., Gold, R.M., Quackenbush, P.M., & Kapatos, G. (1972). Obesity following combination of rostrolateral VMH cut

and contralateral mammillary area lesion. *Journal of Comparative Physiology and Psychology, 79*, 210-218.

Gold, R.M., Quackenbush, P.M., & Kapatos, G. (1972). Obesity following combination of rostrolateral to VMH cut and contralateral mammillary area lesion. *Journal of Comparative Physiology and Psychology, 79*, 210-218.

Goy, R.W. & Phoenix, C.H. (1963). Hypothalamic regulation of female sexual behaviour: Establishment of behavioral oestrus in spayed guinea-pigs following hypothalamic lesions. *Journal of Reproduction and Fertility, 5*, 23-40.

Graham, M.D. & Pfaus, J.G. (2010a. submitted to *Pharmacology, Biochemistry & Behavior*). Role of dopamine in the medial preoptic area on female rat sexual behaviour: I. Effects of dopamine agonists.

Graham, M.D. & Pfaus, J.G. (2010b. submitted to *Pharmacology, Biochemistry & Behavior*). Role of dopamine in the medial preoptic area on female rat sexual behaviour: II. Effects of dopamine antagonists.

Griffin, G.D. & Flanagan-Cato, L.M. (2008). Estradiol and progesterone differentially regulate the dendritic arbor of neurons in the hypothalamic ventromedial nucleus of the female rat (Rattus norvegicus). *Journal of Comparative Neurology, 510*, 631-640.

Han, P.W. (1968). Energy metabolism of tube-fed hypophysectomized rats bearing hypothalamic lesions. *American Journal of Physiology, 215*, 1343-1350.

Han, P.W., Lin, C.H., Chu, K.C., Mu, H.Y., & Liu, A.C. (1965). Hypothalamic obesity in weanling rats. *American Journal of Physiology, 209*, 627-631.

Hardy, D.F. & DeBold, J.F. (1971). Effects of mounts without intromission upon the behavior of female rats during the onset of estrogen-induced heat. *Physiology and Behavior, 7*, 643-645.

Hardy, D.F. & DeBold, J.F. (1971). Effects of mounts without intromission upon the behavior of female rats during the onset of estrogen-induced heat. *Physiology and Behavior, 7*, 643-645.

Harlan, R.E., Shivers, B.D., & Pfaff, D.W. (1983). Estrogenic maintenance of lordotic responsiveness: Requirement for hypothalamic action potentials. *Brain Research, 268*, 67-78.

Hayashi, M., Otsuka, M., Morimoto, R., Hirota, S., Yatsushiro, S., Takeda, J., Yamamoto, A., & Moriyama, Y. (2001). Differentiation-associated Na+-dependent inorganic phosphate cotransporter (DNPI) is a vesicular glutamate transporter in endocrine glutamatergic systems. *Journal of Biological Chemistry, 276,* 43400-43406.

Hennessey, A.C., Camak, L., Gordon, F., & Edwards, D.A. (1990). Connections between the pontine central grey and ventromedial hypothalamus are essential for lordosis in female rats. *Behavioral Neuroscience, 104*, 477-488.

Herbison, A.E., Augood, S.J., & McGowan, E.M. (1992). Expression of glutamic acid decarboxylase messenger RNA in rat medial preoptic area neurones

during the oestrous cycle and after ovariectomy. *Brain Research and Molecular Brain Research, 14*, 310-316.

Herbison, A.E., Heavens, R.P., Dye, S., & Dyer, R.G. (1991). Acute action of oestrogen on medial preoptic gamma-aminobutyric Acid neurons: Correlation with oestrogen negative feedback on luteinizing hormone secretion. *Journal of Neuroendocrinology, 3*, 101-106.

Hisano, S., Hoshi, K., Ikeda, Y., Maruyama, D., Kanemoto, M., Ichijo, H., Kojima, I., Takeda, J., & Nogami, H. (2000). Regional expression of a gene encoding a neuron-specific Na(+)-dependent inorganic phosphate cotransporter (DNPI) in the rat forebrain. *Brain Research and Molecular Brain Research, 83*, 34-43.

Hoshina, Y., Takeo, T., Nakano, K., Sato, T., & Sakuma, Y. (1994). Axon-sparing lesion of the preoptic area enhances receptivity and diminishes proceptivity among components of female rat sexual behavior. *Behavioral Brain Research, 61*, 197-204.

Hubel, D.H. & Wiesel, T.N. (1959). Receptive fields of single neurones in the cat's striate cortex. *Journal of Physiology, 148*, 574-591.

Im, W.B., Blakeman, D.P., & Davis, J.P. (1999). Na^+ -dependent GABA transport system scavenges endogenous external GABA and prevents desensitization of GABA A receptors in rat cerebrocortical synaptoneurosomes. *Brain research, 521*, 143-147.

Jones, E.G., Burton, H., Saper, C.B., & Swanson, L.W. (1976). Midbrain,

diencephalic and cortical relationships of the basal nucleus of Mynert and associated structures in primates. *Journal of Comparative Neurology, 167*, 385-419.

Käll, K.I. (1992). Effects of amphetamine on sexual behavior of male i.v. drug users in Stockholm--a pilot study. *AIDS Education and Prevention, 4*, 6-17.

Kaufman, D.L., Houser, C.R., & Tobin, A.J. (1991). Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *Journal of Neurochemistry, 56*, 720-723.

Kelly, J., Rothstein, J., & Grossman, S.P. (1979). GABA and hypothalamic feeding systems. I. Topographic analysis of the effects of microinjections of muscimol. *Physiology and Behavior, 23*, 1123-1134.

Kendrick, K.M., Keverne, E.B., Hinton, M.R., & Goode, J.A. (1992). Oxytocin, amino acid and monoamine release in the region of the medial preoptic area and bed nucleus of the stria terminalis of the sheep during parturition and suckling. *Brain Research, 569*, 199-209.

Kim, Y.I., Dudley, C.A., & Moss, R.L. (1988). α2-adrenoceptro-mediated inhibition of hypothalamic norepinephrine release. *Journal of Neuroscience, 13*, 3448-3455.

Kippin, T.E. & Pfaus, J.G. (2001). The nature of the conditioned response mediating olfactory conditioned ejaculatory preference in the male rat. *Behavioral Brain Research, 122*, 11-24.

Komisaruk, B.R. & Steinman, J.L. (1986). Genital stimulation as a trigger for neuroendocrine and behavioral control of reproduction. *Annals of the New York Academy of Sciences, 474*, 64-75.

Komisaruk, B.R. & Whipple, B. (2000). How does vaginal stimulation produce pleasure, pain, and analgesia? *Progress in Pain Research and Management, 17*, 109-13.

Konorski, J. (1967). *Integrative activity of the brain.* Chicago: University of Chicago Press.

Kornberg, E. & Erskine, M.S. (1994). Effects of differential mating stimulation on the onset of prolactin surges in pseudopregnant rats. *Psychoneuroendocrinology 19*, 357-371.

Kow, L.M. & Pfaff, D.W. (1987). Responses of ventromedial hypothalamic neurons in vitro to norepinephrine: Dependence on dose and receptor type. *Brain Research, 413*, 220-228.

Kow, L.M. & Pfaff, D.W. (2004). The membrane actions of estrogens can potentiate their lordosis behavior-facilitating genomic actions. *Proceedings of the National Academy of Sciences U S A, 101*, 12354-12357.

Kow, L.M., Harlan, R.E, Shivers, B.D., & Pfaff, D.W. (1985). Inhibition of the lordosis reflex in rats by intrahypothalamic infusion of neural excitatory agents: Evidence that the hypothalamus contains separate inhibitory and facilitory elements. *Brain Research, 341*, 26-34.

Krieger, M.S., Orr, D., & Perper, T. (1976). Temporal patterning of sexual

behavior in the female rat. *Behavioral Biology, 18*, 379-86.

La Vaque, T.J. & Rodgers, C.H. (1975). Recovery of mating behavior in the female rat following VMH lesions. *Physiology and Behavior, 14*, 59-63.

Lauber, A., Romano, G.J., Mobbs, C.V., Howells, R.D., & Pfaff, D.W. (1990). Estradiol induction of proenkephalin messenger RNA in hypothalamus: Dose-response and relation to reproductive behavior in the female rat. *Brain Research and Molecular Brain Research, 8*, 47-54.

Lehmann, M.L. & Erskine, M.J. (2004). Induction of pseudopregnancy using artificial VCS: Importance of lordosis intensity and prestimulus estrous cycle length. *Hormones and Behavior, 45*, 75-83.

Leigh, B.C. (1990). "Venus gets in my thinking": Drinking and female sexuality in the age of AIDS. *Journal of Substance Abuse, 2*, 129-145.

Léránth, C., Sakamoto, H., MacLusky, N.J., Shanabrough, M., & Naftolin, F. (1985). Estrogen responsive cells in the arcuate nucleus of the rat contain glutamic acid decarboxylase (GAD): An electron microscopic immunocytochemical study. *Brain Research, 331*, 376-81.

Lin, L. & York, D.A. (2004). Amygdala enterostatin induces c-Fos expression in regions of hypothalamus that innervate the PVN. *Brain Research,1020*, 147-153.

Lodder, J. & Zeilmaker, G.H. (1976). Role of pelvic nerves in postcopulatory abbreviation of behavioral estrus in female rats. *Journal of Comparative Physiology and Psychology, 90*, 925-929.

Luine, V.N., Wu, V., Hoffman, C.S., & Renner, K.J., 1999. GABAergic regulation of lordosis: Influence of gonadal hormones on turnover of GABA and interaction of GABA with 5-HT. *Neuroendocrinology, 69,* 438-45.

MacLusky, N.J. & McEwen, B.S. (1978). OE modulates progestin receptor concentrations in some rat brain regions but not in others. *Nature, 274*, 276-278.

MacLusky, N.J. & McEwen, B.S. (1980). Progestin receptors in rat brain: Distribution and properties of cytoplasmic progestin-binding sites. *Endocrinology, 106*, 192-202.

Majewska, M.D., Harrison, N.L., Schwartz, R.D., Barker, J.L., & Paul, S.M. (1986). Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science, 232*, 1004–1007.

Malsbury, C.A., Kow, L.M., & Pfaff, D.W. (1977). Effects of medial hypothalamic lesions on the lordosis response and other behaviors in female golden hamsters. *Physiology and Behavior, 19*, 223-237.

Mani, S.K., Allen, J.M., Clark, J.H., Blaustein, J.D., & O'Malley, B.W. (1994). Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. *Science, 265*, 1246-1249.

Mani, S.K., Mitchell, A., & O'Malley, B.W. (2001). Progesterone receptor and dopamine receptors are required in Delta 9-tetrahydrocannabinol modulation of sexual receptivity in female rats. *Proceedings of the National Academy of Science, U S A., 98,* 1249-1254.

Marcondes, F.K., Bianchi, F.J., & Tanno, A.P. (2002). Determination of the estrous cycle phases of rats: Some helpful considerations. *Brazilian Journal of Biology, 62*, 609-614.

Marshall, N.B., Barrnett, R.J., & Mayer, J. (1955). Hypothalamic lesions in goldthioglucose injected mice. *Proceedings of the Society for Experimental Biology and Medicine, 90*, 240-4.

Marson, L. (1995). Central nervous system neurons identified after injection of pseudorabies virus into the rat clitoris. *Neuroscience Letters, 190*, 41-44.

Martinez, I. & Paredes, R.G. (2001). Only self-paced mating is rewarding in rats of both sexes. *Hormones and Behavior, 40*, 510-517.

Matthews, M. & Adler, N.T. (1977). Facilitative and inhibitory influences of reproductive behavior on sperm transport in rats. *Journal of Comparative Physiology and Psychology, 91*, 727-741.

Mathews, D. & Edwards, D.A., (1977). The ventromedial nucleus of the hypothalamus and the hormonal arousal of sexual behaviors in the female rat. *Hormones and Behavior, 8*, 40-51.

McCarthy, M.M., Coirini, H., Schumacher, M., Johnson, A.E., Pfaff, D.W., Schwartz-Giblin, S., & McEwen, B.S. (1992). Steroid regulation and sex differences in [^{3}H]muscimol binding in hippocampus, hypothalamus and midbrain in rats. *Journal of Neuroendocrinology, 53*, 472-479.

McCarthy, M.M., Curran, G.H., & Feder H.H. (1991). Excitatory amino acid modulation of lordosis in the rat. *Neuroscience Letters, 126*, 94-97.

McCarthy, M. M., Kaufman, L.C., Brooks, P.J., Pfaff, D.W., & Schwartz-Giblin, S. (1995). E modulation of mRNA levels for the two forms of glutamic acid decarboxylase (GAD) in female rat brain. *Journal of Comparative Neurology, 360*, 685-697.

McCarthy, M.M., Malik, K.F., & Feder, H.H., (1990). Increased GABAergic transmission in medial hypothalamus facilitates lordosis but has the opposite effect in preoptic area. *Brain Research, 507*, 40-44.

McCarthy, M.M., Masters, D.B., Fiber, J.M., López-Colomé, A.M., Beyer, C., Komisaruk, B.R., & Feder, H.H. (1991). GABAergic control of receptivity in the female rat. *Neuroendocrinology, 53*, 473-479.

McCarthy, M.M., Masters, D.B., Rimvall, K., Schwartz-Giblin, S. & Pfaff, D.W. (1994). Intracerebral administration of antisense oligodeoxynucleotides to GAD65 and GAD67 mRNAs modulate reproductive behavior in the female rat. *Brain Research, 636*, 209-20.

McCarthy, M.M., Pfaff, D.W., & Schwartz-Giblin, S. (1990). GABA in the midbrain central gray facilitates lordosis in the rat. *Society of Neuroscience Abstracts, 16*, 380-383.

McClintock, M.K. (1985). Group mating in the domestic rat as a context for sexual selection: Consequences for the analysis of sexual behaviour and neuroendocrine responses. *Advances in the Study of Behavior, 14*, 1-50.

Meerts, S.H. & Clark, A.S. (2007). Female rats exhibit a conditioned place preference for nonpaced mating. *Hormones and Behavior, 51*, 89-94.

Meisel, R.L. & Pfaff, D.W., (1985). Specificity and neural sites of action of anisomycin in the reduction of facilitation of female sexual behavior in rats. *Hormones and Behavior, 19*, 237-251.

Mendelson, S.D. & Gorzalka, B.B. (1987). An improved chamber for the observation and analysis of the sexual behavior of the female rat. *Physiology and Behavior, 39*, 67-71.

Mermelstein, P.G., Becker, J.B., & Surmeier, D.J. (1996). Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. *Journal of Neuroscience, 16*, 595-604.

Mills, R.H., Sohn, R.K., & Micevych, P.E. (2004). Estrogen-induced mu-opioid receptor internalization in the medial preoptic nucleus is mediated via neuropeptide Y-Y1 receptor activation in the arcuate nucleus of female rats. *Journal of Neuroscience, 24,* 947-955.

Modianos, D.T., Delia, H., & Pfaff, D.W. (1976). Lordosis in female rats following medial forebrain bundle lesions. *Brain Research, 636*, 209-220.

Mong, J.A. & Pfaff, D.W. (2004). Hormonal symphony: Steroid orchestration of gene modules for sociosexual behaviors. *Molecular Psychiatry, 9*, 550-556.

Moss, R.L., Paloutzian, R.F., & Law, T.O. (1974). Electrical stimulation of forebrain structures and its effects on copulatory as well as stimulus-bound behavior in ovariectomized hormone-primed rats. *Physiology and Behavior, 12*, 997-1004.

Morley, J.E., Levine, A.S., Gosnell, B.A., & Billington, C.J. (1984). Neuropeptides

and appetite: Contribution of neuropharmacological modeling. *Federation Proceedings, 43*, 2903-2907.

Napoli, A., Powers, J.B., & Valenstein, E.S. (1972). Hormonal induction of the behavioral estrus modified by electrical stimulation of the hypothalamus. *Physiology and Behavior, 9*, 115-117.

Nathan, B., Bao, J., Hsu, C.C., Aguilar, P., Wu, R., Yarom, M., Kuo, C.Y., & Wu, J.Y. (1994). A membrane form of brain L-glutamate decarboxylase: Identification, isolation, and its relation to insulin-dependent mellitus. *Proceedings of the National Academay of Sciences, U S A, 91*, 242-246.

Ni, B., Wu, X.,Yan, G.M., Wang, J., & Paul, S.M. (1994). Regional expression and cellular localization of the Na(+)-dependent inorganic phosphate cotransporter. *Proceedings of the National Academy of Science USA, 91*, 5607-5611.

Oberlander, J.G. & Erskine, M.S. (2008). Receipt of vaginal-cervical stimulation modifies synapsin content in limbic areas of the female rat. *Neuroscience, 153*, 581-593.

O'Connor, L.H., Nock, B., & McEwen, B.S. (1988). Regional specificity of gamma-aminobutyric acid receptor regulation by estradiol. *Neuroendocrinology, 47*, 473-81.

Okamura, H., Abitbol, M., Julien, J.F., Dumas, S., Bérod, A., Geffard, M., Kitahama, K., Bobillier, P., Mallet, J., & Wiklund, L. (1990). Neurons containing messenger RNA encoding glutamate decarboxylase in rat

hypothalamus demonstrated by in situ hybridization, with special emphasis on cell groups in medial preoptic area, anterior hypothalamic area and dorsomedial hypothalamic nucleus. *Neuroscience, 39*, 675-99.

Ovesjö, M.L., Gamstedt, M., Collin, M., & Meister, B. (2001). GABAergic nature of hypothalamic leptin target neurones in the ventromedial arcuate nucleus. *Journal of Neuroendocrinology, 13*, 505-16.

Pacheco, P., Martinez-Gomez, M., Whipple, B., Beyer, C., & Komisaruk, B.R. (1989). Somato-motor components of the pelvic and pudendal nerves of the female rat. *Brain Research, 490*, 85-94.

Parada, M., Chamas, L., Censi, S., Coria-Avila, G., & Pfaus, J.G. (2010). Clitoral stimulation induces conditioned place preference and Fos activation in the rat. *Hormones and Behavior, 57*, 112-118.

Parada, M., Censi, S., Abdul Ahad, F., & Pfaus, J.G. (in preparation). Clitoral stimulation induces conditioned partner preference in the rat.

Paredes, R.G. & Alonso, A. (1997). Sexual behavior regulated (paced) by the female induces conditioned place preference. *Behavioral Neuroscience, 111*, 123-128.

Paredes, R.G. & Martínez, I. (2001). Naloxone blocks place preference conditioning after paced mating in female rats. *Behavioral Neuroscience, 115*, 1363-1367.

Paredes, R.G. & Vazquez, B. (1999). What do female rats like about sex? Paced mating. *Behavioral Brain Research, 105*, 117-127.

Parsons, B., Rainbow, T.C., Pfaff, D.W., & McEwen, B.S. (1982). Hypothalamic protein synthesis essential for the activation of the lordosis reflex in the female rat. *Endocrinology, 110*, 620-624.

Pavlov, I. P. (1927). *Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex*. Translated and Edited by G. V. Anrep. London: Oxford University Press.

Paxinos, G. & Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates* (4th edition). Academic Press, San Diego.

Peterson, S.L., Reeves, A., Keller, M., Gardner, E., Mahan, L.C., & McCrone, S. (1993). Effects of estradiol (E2) and progesterone on expression of mRNAs encoding GABA A receptor subunits. *Society for Neuroscience Abstracts, 19*, 489.13.

Pfaff, D.W. (1980). *Estrogens and Brain Function*. New York: Springer-Verlag.

Pfaff, D.W. (1999). *Drive: Neurobiological and Molecular Mechanisms of Sexual Motivation*. Cambridge, MA: MIT Press.

Pfaff, D.W., Frohlich, J., & Morgan, M. (2002). Hormonal and genetic influences on arousal – sexual and otherwise. *Trends in Neuroscience, 25*, 45-50.

Pfaff, D.W. & Keiner, M. (1973). Atlas of estradiol-concentrating cells in the central nervous system of the female rat. *Journal of Comparative Neurology, 151*, 121–158.

Pfaff, D.W., Kow, L.M., Loose, M.D., & Flanagan-Cato, L.M. (2008). Reverse engineering the lordosis behavior circuit. *Hormones and Behavior, 54*, 347-

354.

Pfaff, D.W. & Sakuma, Y. (1979a). Facilitation of the lordosis reflex of female rats from the ventromedial nucleus of the hypothalamus. *Journal of Physiology, 288*, 189-202.

Pfaff, D.W. & Sakuma, Y. (1979b). Deficit in the lordosis reflex of female rats caused by lesions in the ventromedial nucleus of the hypothalamus. *Journal of Physiology, 288*, 203-210.

Pfaff, D.W. & Schwartz-Giblin, S. (1988). Cellular mechanisms of female reproductive behaviors. In E. Knobil and J. Neil (Eds.), *The Physiology of Reproduction.* New York: Raven, pp. 1487-1568.

Pfaff, D.W., Schwartz-Giblin, S., McCarthy, M.M., & Kow, L.M. (1994). Cellular and molecular mechanisms of female reproductive behaviors. In Knobil, E. et al. (Eds.). *The Physiology of Reproduction* (2nd Ed), Vol. 2. New York: Raven Press, 107-220.

Pfaus, J.G. (2009). Pathways of sexual desire. *Journal of Sexual Medicine, 6*, 1506-1533.

Pfaus, J.G., Dobbek, N., & Lavoie, M. (in preparation). Role of the ventromedial hypothalamus in estrus termination induced by vaginocervical stimulation.

Pfaus, J.G. & Gorzalka, B.B. (1987). Selective activation of opioid receptors differentially affects lordosis behavior in female rats. *Peptides, 8*, 309-317.

Pfaus, J.G., Kleopoulos, S.P., Mobbs, C.V., Gibbs, R.B., & Pfaff, D.W. (1993). Sexual stimulation activates c-fos within estrogen concentrating regions of

the female rat forebrain. *Brain Research, 624*, 253-267.

Pfaus, J.G., Manitt, C., & Coopersmith, C.B. (2006). Effects of pelvic, pudendal, or hypogastric nerve cuts on Fos induction in the rat brain following vaginocervical stimulation. *Physiology and Behavior, 89*, 627-636.

Pfaus, J.G., Marcangione, C., Smith, W.J., Manitt, C., & Abillamaa, H. (1996). Differential induction of Fos in the female rat brain following different amounts of vaginocervical stimulation: Modulation by steroids hormones. *Brain Research, 741*, 314-330.

Pfaus, J.G. & Pinel, J.P.J. (1989). Alcohol inhibits and disinhibits sexual behavior in the male rat. *Psychobiology, 17*, 195-201.

Pfaus, J.G., Smith, W.J., Byrne, N., & Stephens, G. (2000). Appetitive and consommatory behaviors of females rats in bilevel chambers: II. Patterns of estrus termination following vaginocervical stimulation. *Hormones and Behavior, 37*, 96-107.

Pfaus, J.G., Smith, W.J., & Coopersmith, C.B. (1999). Appetitive and consommatory behaviors of females rats in bilevel chambers: I. A correlational and factor analysis, and the effects of ovarian steroids. *Hormones and Behavior, 33*, 224-240.

Pfaus, J.G., Wilkins, M.F., Dipietro, N., Benibgui, M., Toledano, R., Rowe, A., & Couch, M.C. (2010). Inhibitory and disinhibitory effects of psychomotor stimulants and depressants on the sexual behavior of male and female rats. *Hormones and Behavior, in press.*

Peirce, J.T. & Nuttall, R.L. (1961a). Duration of sexual contacts in the rat. *Journal of Comparative Physiology and Psychology, 54*, 585-587.

Peirce, J.T. & Nuttall, R.L. (1961b). Self-paced sexual behavior in the female rat. *Journal of Comparative Physiology and Psychology, 54*, 310-313.

Pinel, J.P., Pfaus, J.G., & Christensen, B.K. (1992). Contingent tolerance to the disruptive effects of alcohol on the copulatory behavior of male rats. *Pharmacology, Biochemistry and Behavior, 41*, 133-137.

Polsten, E.K. & Erskine, M.S. (1995). Patterns of induction of the immediate-early genes c-fos and egr-1 in the female rat brain following differential amounts of mating stimulation. *Neuroendocrinology, 62*, 370-84.

Powers, J.B. (1970). Hormonal control of sexual receptivity during the estrous cycle of the rat. *Physiology and Behavior, 5*, 831-835.

Rajendren, G., Dudley, C.A., & Moss, R.L. (1991). Role of ventromedial nucleus of hypothalamus in male-induced enhancement of lordosis in female rats. *Physiology and Behavior, 50*, 705-710.

Rasia-Filho, A.A., Fabian, C., Rigoti, K.M., & Achaval, M. (2004). Influence of sex, estrous cycle and motherhood on dendritic spine density in the rat medial amygdala revealed by the Golgi method. *Neuroscience, 126*, 839-847.

Reichling, D.B. & Basbaum, A.L. (1990). Contribution of brainstem GABAergic circuitry to descending antinociceptive controls: II. Electron microscopic immunocytochemical evidence of GABAergic control over the projection

from the periaqueductal gray to the nucleus raphe magnus in the rat. *Journal of Comparative Neurology, 302*, 378-393.

Rodriguez-Sierra, J.F., Crowley, W.R., & Komisaruk, B.R. (1975). Vaginal stimulation in rats induces prolonged lordosis responsiveness and sexual receptivity. *Journal of Comparative Physiology and Psychology, 89*, 79-85.

Romano, G.J., Mobbs, C.V., Lauber, A., Howells, R.D., & Pfaff, D.W. (1990). Differential regulation of proenkephalin gene expression by estrogen in the ventromedial hypothalamus of male and female rats: Implications for the molecular basis of a sexually differentiated behavior. *Brain Research, 536*, 63-68.

Rowe, D.W. & Erskine, M.S. (1993). c-Fos proto-oncogene activity induced by mating in the preoptic area, hypothalamus and amygdala in the female rat: Role of afferent input via the pelvic nerve. *Brain Research, 621*, 25-34.

Rubin, B.S. & Barfield, R.J. (1983). Induction of estrous behavior in ovariectomized rats by sequential replacement of estrogen and progesterone to the ventromedial hypothalamus. *Neuroendocrinology, 37*, 218-224.

Rubin, B.S. & Barfield, R.J. (1983a). Induction of estrous behavior in ovariectomized rats by sequential replacement of estrogen and progesterone to the ventromeidal hypothalamus. *Neuroendocrinology, 37*, 218-224.

Rubin, B.S. & Barfield, R.J. (1983b). Progesterone in the ventromedial

hypothalamus facilitates estrous behavior in ovariectomized, estrogen-primed rats. *Endocrinology, 113*, 797-804.

Sagrillo, C.A. & Selmanoff, M. (1997). Castration decreases single cell levels of mRNA encoding glutamic acid decarboxylase in the diagonal band of broca and the sexually dimorphic nucleus of the preoptic area. *Journal of Neuroendocrinology, 9*, 699-706.

Sansone, G.R. & Komisaruk, B.R. (2001). Evidence that oxytocin is an endogenous stimulator of autonomic sympathetic preganglionics: The pupillary dilatation response to vaginocervical stimulation in the rat. *Brain Research, 898*, 265-271.

Schumacher, M., Coirini, H., & McEwen, B.S. (1989). Regulation of high-affinity GABAa receptors in specific brain regions by ovarian hormones. *Neuroendocrinology, 50*, 315-320.

Semple, S.J., Zians, J., Strathdee, S.A., & Patterson, T.L. (2009). Sexual marathons and methamphetamine use among HIV-positive men who have sex with men. *Archives of Sexual Behavior, 38*, 583-590.

Shafik, A., El-Sibai, O., Shafik, I., & Shafik, A.A. (2005). Uterine effect of cervical buffeting: Role of the cervicouterine reflex in coitus. *Journal of Reproductive Medicine, 50*, 837-843.

Simonian, S.X. & Herbison, A.E. (1997). Differential expression of estrogen receptor alpha and beta immunoreactivity by oxytocin neurons of rat paraventricular nucleus. *Journal of Neuroendocrinology, 9*, 803-806.

Sorge, R.E., Rajabi, H., & Stewart, J. (2005). Rats maintained chronically on buprenorphine show reduced heroin and cocaine seeking in tests of extinction and drug-induced reinstatement. *Neuropsychopharmacology, 30*, 1681-1692.

Steinach, E. (1910). Geschlechtstrieb und echt sekundäre Geschlechtsmerkmale als Folge der innersekretorischen Funktion der Keimdrüsen (Sex Instinct and Genuine Secondary Sex Characteristics as a Result of Internal Secretion of the Gonads). *Zentralblatt für Physiologie, 24*, 551-563.

Sterner, M.R., Meisel, R.L., & Diekman, M.A. (1992). Forebrain sites of estradiol-17 beta action on sexual behavior and aggression in female Syrian hamsters. *Behavioral Neuroscience, 106*, 162-171.

Storlien, L.H. & Albert, D.J. (1972). The effect of VMH lesion, lateral cuts and anterior cuts on food intake, activity level, food motivation, and reactivity to taste. *Physiology and Behavior, 9*, 191-197.

Storm-Mathisen, J., Danboldt, N.C., & Ottersen, O.P. (1995). Localization of glutamate and its membrane transport proteins. In: Stone TS, editor. *CNS neurotransmitters and modulators: Glutamate*. New York: CRC Press, pp. 1-16.

Stumpf, W.E. (1970). Estrogen-neurons and estrogen-neuron systems in the periventricular brain. *American Journal of Anatomy, 129*, 207-217.

Sun, D., Vingrys, A.J., & Kalloniatis, M. (2007). Metabolic and functional profiling of the normal rat retina. *Journal of Comparative Neurology, 505*, 93-114.

Swanson, L.W. & Kuypers, H.G. (1980). The paraventricular nucleus of the hypothalamus: Cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *Journal of Comparative Neurology, 194*, 555-570.

Swanson, L.W., Sawchenko, P.E., Wiegand, S.J., & Price, J.L. (1980). Separate neurons in the paraventricular nucleus project to the median eminence and to the medulla or spinal cord. *Brain Research, 198*, 190-195.

Takamori, S., Rhee, J.S., Rosenmund, C., & Jahn, R. (2000). Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature, 407*, 189-194.

Takeo, T., Chiba, Y., & Sakuma, Y. (1993). Suppression of the lordosis reflex of female rats by efferents of the medial preoptic area. *Physiology and Behavior, 53*, 831-838.

Tappaz, M.L., Brownstein, M.J., & Kopin, I.J. (1977). Glutamate decarboxylase (GAD) and gamma-aminobutyric acid (GABA) in discrete nuclei of hypothalamus and substantia nigra. *Brain Research, 125*, 109-121.

Tepperman, J., Brobeck, J.R., & Long, C.N.H. (1943). The effects of hypothalamic hyperphagia and of alterations in feeding habits on the metabolism of the albino rat. *Yale Journal of Biology and Medicine, 15*, 855-874.

Tetel, M.J., Getzinger, M.J., & Blaustein, J.D. (1993). Fos expression in the rat

brain following vaginal-cervical stimulation by mating and manual probing. *Journal of Neuroendocrinology, 5*, 397-404.

Tetel, M.J., Getzinger, M.J., & Blaustein, J.D., (1994). Estradiol and progesterone influence the response of ventromedial hypothalamic neurons to tactile stimuli associated with female reproduction. *Brain Research, 646*, 267-272.

Toates, F. (1986). *Motivational systems*. Cambridge University Press.

Toner, J.P. & Adler, N.T. (1986). Influence of mating and vaginocervical stimulation on rat uterine activity. *Journal of Reproduction and Fertility, 78*, 239-49.

Tong, Q., Ye, C., McCrimmon, R.J., Dhillon, H., Choi, B., Kramer, M.D., Yu, J., Yang, Z., Christiansen, L.M., Lee, C.L., Choi, C.S., Zigman, J.M., Shulman, G.I., Sherwin, R.S., Elmquist, J.K., & Lowell, B.B. (2007). Synaptic glutamate release by ventromedial hypothalamic (VMH) neurons is part of the neurocircuitry that prevents hypoglycemia. *Cell Metabolism, 5*, 383-393.

Vincent, P.A. & Etgen, A.M. (1993). Steroid priming promotes oxytocin-induced norepinephrine release in the ventromedial hypothalamus of female rats. *Brain Research, 620*, 189-194.

Wade, G.N. (1975). Some effects of ovarian hormones on food intake and body weight in female rats. *Journal of Comparative Physiology and Psychology, 88*, 183–193.

Wagner, C.K., Eaton, M.J., Moore, K.E., & Lookingland, K.J. (1995). Efferent projections from the region of the medial zona incerta containing A13

dopaminergic neurons: a PHA-L anterograde tract-tracing study in the rat. *Brain Research, 677*, 229-237.

Wersinger, S.A., Baum, M.J., & Erskine, M.S. (1993). Mating-induced FOS-like immunoreactivity in the rat forebrain: A sex comparison and a dimorphic effect of pelvic nerve transaction. *Journal of Neuroendocrinology, 5*, 557-568.

White, N.R., Caggiano, R., & Barfield, R.J. (1990). Receptivity of the female rat (Rattus norvegicus) after male devocalization: A ventral perspective. *Journal of Comparative Psychology, 104*, 147-151.

Whitney, J.F. (1986). Effect of medial preoptic lesions on sexual behavior of female rats is determined by test situation. *Behavioral Neuroscience, 100*, 230-235.

Wise, R.A. & Hoffman, D.C. (1992). Localization of drug reward mechanisms by intracranial injections. *Synapse, 10*, 247-263.

Wooley, C.S. & McEwen, B.S. (1994). Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. *Journal of Neuroscience, 14*, 7680-7687.

Yang, J.J., Oberlander, J.G., & Erskine, M.S. (2007). Expression of FOS, EGR-1, and ARC in the amygdala and hippocampus of female rats during formation of the intromission mnemonic of pseudopregnancy. *Devepmental Neurobiology, 67*, 895-908.

Young, L.J. & Wang, Z. (2004). The neurobiology of pair bonding. *Nature*

Neuroscience, 7, 1048-1054.

Zhang, D.X., Li, L., Kelly, J.B., & Wu, S.H. (1998). GABAergic projections from the lateral lemniscus to the inferior colliculus of the rat. *Hearing Research, 117*, 1-12.

Ziegler, D.R., Cullinan, W.E., & Herman, J.P. (2002). Distribution of vesicular glutamate transporter mRNA in rat hypothalamus. *Journal of Comparative Neurology, 448*, 217-229.