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Neural Regulation of Sexual Solicitation in Female Syrian Hamsters: Role of Oxytocin

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NEURAL REGULATION OF SEXUAL SOLICITATION IN FEMALE SYRIAN HAMSTERS: ROLE OF OXYTOCIN

by

LUIS A. MARTINEZ

Under the Direction of Aras Petrulis

ABSTRACT

In most animal species, reproductive success depends critically on precopulatory or solicitational behaviors that occur prior to mating. The specific sensory systems and behavioral strategies employed in precopulatory behaviors vary across species; in all cases, however, animals must be able to identify potential mating partners and solicit sexual interest. Female Syrian hamsters (*Mesocricetus auratus*) engage in multiple forms of precopulatory behaviors that are preferentially expressed to males or their odors, including vaginal scent marking and sexual odor preference. Conspecific odors relevant for precopulatory behaviors are processed by a network of forebrain areas that includes the bed nucleus of the stria terminalis (BNST) and the medial preoptic area (MPOA). The precise functional and neurochemical mechanisms whereby these areas regulate the expression of precopulatory behaviors, however, are unknown. Therefore, the aim of this dissertation is to address the following research questions: (1) Is the

neuropeptide oxytocin (OT), acting within BNST or MPOA, necessary for the normal expression of odor-guided precopulatory behaviors? (2) Is BNST or (3) MPOA required for the preferential expression of vaginal marking or investigation towards male odors?, and (4) Does OT interact with social odor processing to regulate vaginal marking? We found that blockade of OT receptors (OTRs) in MPOA and BNST decreased vaginal marking to male odors. There was no effect of OTR blockade on sexual odor preference. Selective lesions of BNST also disrupted preferential vaginal marking responses to male odors, without affecting sexual odor preference. In contrast, lesions of MPOA disrupted odor preference without affecting vaginal marking responses. Finally, central blockade of OTRs eliminated the normal pattern of increased activation of neurons to male vs. female odors in BNST, but not MPOA. Considered together, these results suggest that OT normally acts within BNST to drive preferential vaginal marking responses to male odors via selective facilitation of neural responses to these odors, and further, that there are separate and distinct neural circuits that regulate different forms of odor-guided precopulatory behaviors in females.

INDEX WORDS: Olfaction, Reproduction, Sexual behavior, Sexual solicitation, Precopulatory, Appetitive, Oxytocin, Vasopressin, Medial preoptic area, Bed nucleus of the stria terminalis

NEURAL REGULATION OF SEXUAL SOLICITATION IN FEMALE SYRIAN HAMSTERS: ROLE OF OXYTOCIN

by

LUIS A. MARTINEZ

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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in the College of Arts and Sciences

Georgia State University

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DEDICATION

This dissertation is dedicated to my wife, *Jennifer*. I cannot begin to express how much you mean to me. You have always supported me and made me believe that this was possible. For that, and so much more, I love you with all my heart.

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LIST OF ABBREVIATIONS

ac, anterior commissure

ACo, anterior cortical amygdaloid nucleus

AH, anterior hypothalamus

AOBs, accessory olfactory bulbs

AOS, accessory olfactory system

ASD, autism spectrum disorders

AVP, arginine vasopressin

BLA, anterior division of the basolateral amygdaloid nucleus

BLP, posterior division of the basolateral amygdaloid nucleus

BMA, anterior division of the basomedial amygdaloid nucleus

BMP, posterior division of the basomedial amygdaloid nucleus

BNST, posterior division of the bed nucleus of the stria terminalis

BNSTpi, posterointermediate subdivision of the bed nucleus of the stria terminalis

BNSTpl, posterolateral subdivision of the bed nucleus of the stria terminalis

BNSTpm, posteromedial subdivision of the bed nucleus of the stria terminalis

BNST-X, lesion of the posterior bed nucleus of the stria terminalis

Ce, central amygdaloid nucleus

E, estrogen

ER, estrogen receptor

f, fornix

FHVS, female hamster vaginal secretion

Fos, protein product of the immediate early gene *c-fos*

GDX, gonadectomized or castrated

GDX+T, GDX plus testosterone replacement

GP, globus pallidus

I, intercalated nuclei of the amygdala

ic, internal capsule

IEG, immediate early gene

IHC, immunohistochemistry

IP, intraperitoneal

LH, lateral hypothalamus

LPO, lateral preoptic area

LS, lateral septum

LV, lateral ventricle

MA or ME, medial nucleus of the amygdala

MeA, anterior division of the medial amygdala

MeP, posterior division of the medial amygdala

MLR, midbrain locomotor region

MOBs, main olfactory bulbs

MOE, main olfactory epithelium

MOS, main olfactory system

MPOA, medial preoptic area

MPOA-AH, medial preoptic area-anterior hypothalamus continuum

MPOA-X, lesions of the medial preoptic area

NAC, nucleus accumbens

NeuN, a neuron specific nuclear protein

ot, optic tract

OT, oxytocin

OTA, oxytocin receptor antagonist

OTR, oxytocin receptor

OVX, ovariectomized

ox, optic chiasm

P, progesterone

PAG, midbrain periaqueductal gray

PBS, phosphate buffered saline

PE, proestrus

Pir, piriform cortex

PLCo, posterolateral cortical amygdaloid nucleus

PMCo, posteromedial cortical amygdaloid nucleus

PR, progesterone receptor

PVH, paraventricular nucleus of the hypothalamus

SCN, superchiasmatic nucleus

SHAM, sham lesion of a specific brain area

SI, substantia innominata

sm, stria medullaris

SON, supraoptic nucleus

sox, supraoptic decussation

st, stria terminalis

V1aA, vasopressin 1a receptor antagonist

V1aR, vasopressin 1a receptor

VEn, ventral endopiriform nucleus

VMH, ventromedial nucleus of the hypothalamus

VTA, ventral tegmental area

VNO, vomeronasal organ

CHAPTER 1: INTRODUCTION

1.1 Overview

For animal species that reproduce sexually, precopulatory behaviors that aid in the coordination of mating with potential partners are critical for reproductive success (Beach, 1976). This is particularly true for animals that do not normally live in close proximity with members of the opposite sex, and come together only for mating (Petrulis, 2009; Thiessen and Rice, 1976). As the majority of animals, including humans, are not strictly sexually monogamous (Buss and Schmitt, 1993; Kleiman, 1977), the necessity of identifying and attracting a mate is a problem that animals must readdress throughout their reproductive lives. Although substantial research has gone into describing the specific types of signals and behavioral responses important for precopulatory behaviors, the basic neural mechanisms underlying these behaviors are not well understood. This is particularly the case for females, given that the preponderance of research has focused on copulatory, rather than precopulatory, behaviors. There is growing evidence to suggest that an interconnected set of limbic and hypothalamic nuclei may regulate female precopulatory behaviors elicited by male stimuli (Petrulis, 2009; Sakuma, 2008). It is not clear, however, how these areas process conspecific signals relevant for mating and generate appropriate behavioral responses dependent on the sexual identity of the signaler. This dissertation will focus on the underlying functional and neurochemical mechanisms whereby social signals are processed by two brain areas, the medial preoptic area (MPOA) and the bed nucleus of the stria terminalis (BNST), in order to generate preferential attraction and solicitational responses to opposite-sex stimuli in female Syrian hamsters.

1.2 Syrian Hamsters as a Model Species for Examining Female Precopulatory Behaviors

Female Syrian hamsters are an excellent model for studying the neural regulation of precopulatory behaviors for several reasons. First, Syrian hamsters display a suite of precopulatory responses that are highly stereotyped and readily observed in a laboratory setting, including preferential investigation of opposite-sex odors and vaginal scent marking. Vaginal marking involves the female moving forward while maintaining contact between the perineum and the underlying substrate, thereby depositing vaginal secretion (Johnston, 1977). This behavior appears to be important for mate solicitation, as females will vaginal mark to form a 'trail' linking her nesting area with that of a male (Lisk et al., 1983). Vaginal secretion found within vaginal marks is highly attractive to males (Johnston, 1974), and males will vigorously investigate and attempt to locate the source of the secretion (Johnston and Kwan, 1984; Johnston and Schmidt, 1979). In contrast to other animal models of female precopulatory behaviors, olfactory stimuli alone are sufficient to induce the expression of these behaviors in female hamsters. Females of this species preferentially investigate, and vaginal mark in response to, male vs. female odors, and this overall pattern of preferential responses to odors is maintained across multiple days of the reproductive cycle (Johnston, 1977; Petrulis and Johnston, 1999; Petrulis et al., 1999). Precopulatory behaviors in hamsters can therefore be assessed in the absence of males, thus allowing these behaviors to be assessed independently of other types of behaviors (i.e., copulation and aggression) that would otherwise be displayed by females when interacting with males.

As described above, the preferential expression of vaginal marking and investigatory responses towards males depends critically on the processing of chemosensory signals from conspecifics. This processing occurs within a ventral forebrain circuit that has been carefully described in hamsters and implicated in the regulation of sociosexual behaviors in this as well as other species (Newman, 1999). Therefore, vaginal marking and preferential investigation of male odors in female Syrian hamsters can serve as

a behavioral model system wherein to examine the specific neural mechanisms that underlie context-appropriate precopulatory behaviors.

1.3 Neural Substrates of Female Precopulatory Behaviors

Olfactory systems

In hamsters as well as other vertebrates, social odors are initially detected and processed by the main (MOS) and accessory (AOS) olfactory systems (Keller et al., 2009). These two systems are specialized for detecting either volatile or non-volatile chemosignals (MOS and AOS, respectively). Low molecular weight, volatile chemosignals are dissolved in the olfactory mucosa and activate receptors on sensory cells in the main olfactory epithelium (MOE) (Keller et al., 2009). These sensory cells project to mitral cells within the main olfactory bulbs (MOBs), that then relay chemosensory information to downstream targets, including the medial amygdala (MA), the anterior cortical amygdala (ACo), the posterolateral cortical amygdala (PLCo), and olfactory cortex (e.g., piriform and entorhinal cortex) (Davis et al., 1978; Kang et al., 2009). Higher molecular weight, non-volatile chemosignals are detected by the vomeronasal organ (VNO), a blind-ending structure found at the base of the nasal septum (Meredith, 1991; Zufall et al., 2002). Chemosignals are actively pumped into the VNO, and thereby gain access to receptors present on sensory neurons in the VNO epithelium. These neurons project to mitral cells in the accessory olfactory bulbs (AOBs), which relay chemosensory information to MA, the posteromedial cortical amygdala (PMCo) and BNST (Davis et al., 1978; Kang et al., 2009).

There are several lines of evidence that implicate the principal components of MOS and AOS in the regulation of precopulatory behaviors in female hamsters. Lesions of the olfactory bulbs strongly decrease overall levels of vaginal marking (Kairys et al., 1980), an effect that can be partially replicated by zinc sulfate lesions of MOE (Johnston, 1992). Removal of VNO has no effect on overall levels of vaginal marking, and does not affect opposite-sex odor preference (Johnston, 1992; Petrulis et al., 1999).

Rather, VNO removal eliminates preferential vaginal marking to male vs. female odors (Petrulis et al., 1999). Together, these results demonstrate that the neural circuits regulating different forms of odor-guided precopulatory behaviors in females are already distinct at the level of primary olfactory structures. This distinction may continue to be maintained within downstream targets of these systems, since lesions of MA eliminate opposite-sex odor preference and decrease overall levels of vaginal marking, without disrupting preferential vaginal marking to male odors (Petrulis and Johnston, 1999).

BNST

In contrast to other areas that process olfactory information, little is known about the role of BNST in regulating odor guided precopulatory behaviors. BNST receives direct input from AOBs, as well as indirect olfactory input via MA and other amygdaloid nuclei (Wood and Swann, 2005). In male hamsters, neurons in BNST preferentially express Fos (an indirect marker of recent neuronal activation (Pfaus and Heeb, 1997)) in response to female vs. male odors (Maras and Petrulis, 2010a), and lesions of BNST decrease interest in female-derived chemosignals and completely eliminate opposite-sex odor preference (Been and Petrulis, 2010a; Powers et al., 1987). Furthermore, the connections between MA and BNST are critical for normal odor preference in males, as asymmetrical lesions of MA and BNST that functionally disconnect these two brain areas also eliminate sexual odor preference (Been and Petrulis, 2012). Although comparable data for female hamster sexual odor preference is lacking, there is some evidence that BNST is involved in sexual solicitation. Lesions of BNST decrease ultrasonic vocalizations in response to a male (Kirn and Floody, 1985), and large lesions of MPOA that damage BNST decrease vaginal marking in response to males (Malsbury et al., 1977). The precise role of BNST in preferential investigatory and solicitational responses to male odors, however, is not known.

MPOA

Another brain area that functions downstream of MA to regulate precopulatory behaviors is MPOA. In contrast to MA and BNST, MPOA does not receive chemosensory information from the olfactory systems directly; rather, this information reaches MPOA indirectly via BNST, MA, and other amygdaloid areas (Simerly and Swanson, 1986; Wang and Swann, 2006). Similar to BNST, MPOA is preferentially activated by opposite-sex odors in male hamsters (Maras and Petrulis, 2010a), and lesions of MPOA eliminate opposite-sex odor preference in males (Been and Petrulis, 2010b). There is also substantial evidence implicating MPOA in the regulation of odor-guided precopulatory behaviors in females. In rats, lesions of MPOA decrease solicitational behaviors towards, and time spent with, a sexually-experienced male, and disrupt the preference for intact male rat odors (Guarraci and Clark, 2006; Xiao et al., 2005). Although comparable data for the role of MPOA in sexual odor preference in female hamsters is not available, this area is involved in other precopulatory behaviors that can be induced by opposite-sex odors. Indeed, large, non-specific lesions of MPOA decrease vaginal marking during interactions with males (Malsbury et al., 1977), and ultrasonic vocalizations by females following exposure to male hamsters (Floody, 1989). It is not known, however, if MPOA plays a unique and dissociable role from that of BNST in the regulation of precopulatory behaviors in females.

1.4 Oxytocin and Female Sexual Behaviors

Although the specific neurochemical systems regulating female precopulatory behaviors have not been extensively examined, there is some evidence suggesting that the neuropeptide oxytocin may be involved. Oxytocin is produced by neurosecretory cells in the paraventricular and supraoptic nuclei of the hypothalamus (Lee et al., 2009). In addition to release into the circulatory system via projections to the posterior pituitary, oxytocinergic neurons in the hypothalamus also release oxytocin into the central nervous system. Central release of oxytocin is the consequence of either axonal or dendritic release, similar to other peptide systems (Bergquist and Ludwig, 2008; Ludwig and Leng, 2006). Only a single

form of oxytocin receptor (OTR) has been identified, a seven-transmembrane domain G-protein coupled receptor (Kimura et al., 1992). Binding of oxytocin to OTRs leads to increased release of calcium from intracellular stores, thereby increasing excitability of cells (Gimpl and Fahrenholz, 2001). The distribution of OTRs in the brain varies substantially across species (Barberis and Tribollet, 1996), and this variability has been proposed to underlie species-typical variations in sociality (Young et al., 1996).

OTRs are expressed in BNST and MPOA in a number of mammalian species (Campbell et al., 2009; Lee et al., 2008; Veinante and Freund-Mercier, 1997). In hamsters, however, OTRs are present in BNST but not in MPOA or other hypothalamic areas (Dubois-Dauphin et al., 1992). Despite this evidence, numerous studies have examined the effects of oxytocin acting within MPOA on sociosexual behaviors in this species. Injections of oxytocin into MPOA decrease aggressive responses towards a non-aggressive intruder in female hamsters, whereas injections of an oxytocin receptor antagonist (OTA) increase aggressive responses (Harmon et al., 2002a). Conversely, copulatory behavior in female hamsters is facilitated by oxytocin injections into MPOA and inhibited following injections of OTA (Whitman and Albers, 1995), similar to reports in female rats (Caldwell et al., 1989, 1994). It is interesting to note that OTA injections into the lateral ventricles inhibit precopulatory as well as copulatory responses in female rats (Witt and Insel, 1991), but that effects on precopulatory responses are not seen following injections directly into MPOA, the ventromedial hypothalamus or the ventral tegmental area (Caldwell et al., 1994). This suggests that other brain areas (e.g., MA or BNST) may mediate the effects of OTA on precopulatory behaviors in rats. In female hamsters, the effect of oxytocin on precopulatory responses has only been examined for a single behavior, ultrasonic vocalizations. This behavior is facilitated by injections of oxytocin into MPOA (Floody et al., 1998). It is not known, however, if endogenous oxytocin acts within either MPOA or BNST to regulate vaginal marking and opposite-sex odor preference in female hamsters.

1.5 Goal of Dissertation

The overarching goal of this dissertation is to examine the functional and neurochemical mechanisms that underlie the regulation of odor-guided precopulatory behaviors in female hamsters. In order to address this goal, I will be examining the following research questions: (1) Is oxytocin acting within MPOA or BNST necessary for vaginal marking and sexual odor preference in female Syrian hamsters? (2) Is BNST or (3) MPOA functionally required for vaginal marking and investigatory responses to conspecific odors? and (4) Does oxytocin interact with social odor processing within BNST to regulate vaginal marking? Taken together, these experiments will provide critical insight into the neural processes whereby females direct solicitational responses to appropriate social targets.

CHAPTER 2: BLOCKING OXYTOCIN RECEPTORS INHIBITS VAGINAL MARKING TO MALE ODORS IN FEMALE SYRIAN HAMSTERS

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2.1 Abstract

In Syrian hamsters (*Mesocricetus auratus*), precopulatory behaviors such as vaginal scent marking are essential for attracting a suitable mate. Vaginal marking is dependent on forebrain areas implicated in the neural regulation of reproductive behaviors in rodents, including the medial preoptic/anterior hypothalamus (MPOA-AH). Within MPOA-AH, the neuropeptide oxytocin (OT) acts to facilitate copulation (lordosis), as well as ultrasonic vocalizations towards males. It is not known, however, if OT in this area also facilitates vaginal marking. In the present study, a specific oxytocin receptor antagonist (OTA) was injected into MPOA-AH of intact female Syrian hamsters to determine if oxytocin receptor-dependent signaling is critical for the normal expression of vaginal marking elicited by male, female, and clean odors. OTA injections significantly inhibited vaginal marking in response to male odors compared to vehicle injections. There was no effect of OTA on marking in response to either female or clean odors. When injected into the bed nucleus of the stria terminalis (BNST), a nearby region to MPOA-AH, OTA was equally effective in decreasing marking. Finally, the effects of OTA appear to be specific to vaginal marking, as OTA injections in MPOA-AH or BNST did not alter general locomotor activity, flank mark-

ing, or social odor investigation. Considered together, these results suggest that OT in MPOA-AH and/or BNST normally facilitates male odor-induced vaginal marking, providing further evidence that OT generally supports prosocial interactions among conspecifics.

2.2 Introduction

In female mammals, successful reproduction depends upon a suite of precopulatory behaviors that enhance the probability of locating and attracting a suitable mate (Petrulis, 2009; Takahashi, 1990). Precopulatory behaviors may be particularly important for reproductive success in species where adult members of the opposite sex live in isolation from each other, such as the Syrian hamster (Gattermann et al., 2001; Johnston, 1977). Vaginal marking is one form of precopulatory behavior in this species that serves to solicit potential mates (Huck et al., 1985; Lisk et al., 1983). This highly stereotyped behavior involves the female moving forward while maintaining contact between the perineum and the underlying substrate, thereby depositing vaginal secretion (Johnston, 1977). In agreement with its role as a precopulatory behavior, vaginal marks are highly attractive to male hamsters (Johnston and Schmidt, 1979; Johnston, 1974; Kwan and Johnston, 1980), and males may use vaginal marks to locate females for mating (Lisk et al., 1983). Furthermore, the expression of vaginal marking is critically dependent on internal hormonal state and external signals important for synchronizing mating activity (Johnston, 1977). Vaginal marking levels are highest on the reproductive cycle day of proestrus, and are completely absent on the following cycle day (estrus) when females are sexually receptive and will engage in mating (Johnston, 1977; Petrulis and Johnston, 1997). Vaginal marking is also preferentially directed towards males, as females will vaginal mark at higher levels in response to male odors than in response to female odors or clean bedding (Johnston, 1977; Petrulis and Johnston, 1999; Petrulis et al., 1998, 1999, 2000).

Although the sensory stimuli and hormonal signals important for vaginal marking have been examined to a considerable extent (Been and Petrulis, 2007), less is known about the brain areas and specific neurochemicals that may be important for regulating this behavior. One likely candidate is the neu-

ropeptide oxytocin (OT) acting within the medial preoptic/anterior hypothalamus (MPOA-AH). OT is expressed in cell bodies and fibers within MPOA-AH (Whitman and Albers, 1998), and large, bilateral electrolytic lesions of MPOA-AH inhibit vaginal marking (Malsbury et al., 1977). Injection of OT into MPOA-AH of female hamsters (Whitman and Albers, 1995) or rats (Caldwell et al., 1989) enhances the expression of lordosis, a reflexive copulatory posture assumed by females (Beach, 1967). In contrast, blockade of oxytocin receptors (OTRs) in MPOA-AH via injection of a specific OTR antagonist (OTA) inhibits lordosis in both these species (Caldwell et al., 1990; Pedersen and Boccia, 2002; Whitman and Albers, 1995). In addition to effects on lordosis, OT in MPOA-AH also increases the production of ultrasonic vocalizations (Floody et al., 1998), a pericopulatory behavior that induces approach in males (Floody, 1981).

Taken together, the existing data suggest that OT in MPOA-AH may normally facilitate vaginal marking. To test this hypothesis, we injected proestrous female hamsters with OTA or vehicle into MPOA-AH and measured vaginal marking in response to stimuli present in male, female, and clean cages (Experiment 1). Given that vaginal marking is preferentially directed towards opposite-sex, rather than same-sex, stimuli (Petrulis, 2009), we predicted that OTA injections into MPOA-AH would decrease vaginal marking to male, but not to female or clean, cage odors. Following the results of Experiment 1, two subsequent experiments were conducted to assess the anatomical and functional specificity of OTA on vaginal marking. In Experiment 2, injections were made into either MPOA-AH or the bed nucleus of the stria terminalis (BNST), a brain area located immediately dorsal to MPOA-AH that contains both OT immunoreactivity and OTR binding in hamsters (Dubois-Dauphin et al., 1992; Whitman and Albers, 1998), and vaginal marking was measured in response to male odors only. Finally, we measured the effects of OTA on social odor investigation to determine if alterations in odor investigation might underlie the effects of OTA on male odor-induced vaginal marking (Experiment 3).

2.3 Methods

Subjects

Female Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratories (Wilmington, MA, USA) at approximately 22 days of age. Subjects were individually housed upon arrival within the animal facility in an all-female room. In addition to experimental subjects, a separate group of male and female Syrian hamsters served as odor donors. Odor donors were also purchased from Charles River Laboratories as sexually mature adults (approximately 60 days of age). Odor donors were either individually housed or group housed (3-4 same sex animals per cage); in all cases, odor donors were unrelated to, and had no previous contact with, subjects prior to use in experiments. All animals were housed in solid-bottom Plexiglas cages (43 cm x 22 cm x 20 cm) containing corncob bedding and cotton nesting material (Nestlets; Ancare, Bellmore, NY) and maintained on a reversed light cycle (14:10 light:dark; lights out at 9 am). Behavior testing occurred during the first four hours of the dark portion of the light cycle. Food and water were available *ad libitum*. Animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and approved by the Georgia State University Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Estrous cycle monitoring

Adult subjects were examined daily for eight consecutive days to determine stage of the estrous cycle. Subjects were gently restrained while vaginal secretion was manually extruded and the consistency of the secretion was examined (Orsini, 1961). Estrous cycles were also monitored for eight days following surgery to ensure that the procedure had not disrupted cyclicity. For all experiments, the cycle day immediately prior to behavioral estrus was defined as proestrus, whereas the two cycle days imme-

diately following behavioral estrus were defined as diestrous day 1, and diestrous day 2, respectively. In all cases, day refers to the dark phase of the light cycle.

Surgery

At two to three months of age, subjects were unilaterally implanted with guide cannulae. Subjects were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p.; Nembutal, Ovation Pharmaceuticals, Deerfield, IL) and placed within a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with ear- and incisor-bars positioned such that the top of the skull was level. Each subject was then fitted with a single 4-mm 26-gauge guide cannula (Plastics One, Roanoke, VA), implanted at a 10° angle and directed at the following coordinates: 1.1 mm anterior to bregma, 1.7 mm lateral to the midline suture, and 4.0 mm ventral to dura. Injection cannulae were cut to extend the additional distance necessary to reach from the tip of the guide cannula to the target brain region. For Experiments 1 and 3, injection cannulae extended a total distance of 7.3 mm ventral to dura, effectively targeting the juncture between MPOA-AH and BNST. For experiment 2, injection cannulae extended either 6.8 mm or 7.8 mm ventral to dura, in order to more specifically target BNST or MPOA-AH, respectively. In all cases, stereotaxic coordinates were derived from published neuroanatomical plates for the Syrian hamster brain (Morin and Wood, 2001). Guide cannulae were secured to the skull with dental cement and skull screws, and immediately prior to the completion of the surgical procedure, subjects were injected subcutaneously with an analgesic agent (Ketofen; Fort Dodge Animal Health, Fort Dodge, IA). All subjects were allowed to recover for at least 10 days prior to behavioral testing.

Drug injections

OTA ($[(\text{CH}_2)_5^1, \text{Tyr}(\text{Me})^2, \text{Thr}^4, \text{Orn}^8, \text{des-Gly-NH}_2^9]$ -vasotocin; Bachem, Torrance, CA) was diluted in sterile saline to a final concentration of 900 μM and stored in aliquots of 10 μl at -20°C until use. OTA is a highly selective OTR antagonist that demonstrates a 17-fold greater potency for antagonizing OTRs

compared to vasopressin 1a receptors (V1aRs) (Manning et al., 1989). Thirty minutes prior to behavioral testing, subjects were injected via guide cannulae with either 200 nl of OTA (900 μ M) or vehicle (saline). This concentration and volume of OTA is effective in eliciting behavioral effects in female hamsters when administered 30 minutes prior to testing (Harmon et al., 2002a). OTA or vehicle was delivered to the target brain area through an injection cannula connected to a 1 μ l Hamilton syringe. Subjects were lightly restrained for the 20 seconds while the drug was injected, and for an additional 20 seconds post-injection to allow the drug to diffuse away from the tip of the injection cannula.

Scent-marking tests

Odor stimuli and apparatus

Stimuli for scent-marking tests (Experiments 1 and 2) consisted of vacated male cage odor stimuli, vacated female cage stimuli, or stimuli present in an empty clean cage. Male and female cage stimuli came from odor donors housed individually for six to ten days. Estrous cycles of stimulus females were not monitored; however, this time frame comprised at least one complete four-day estrous cycle, and therefore it is likely that cage stimuli were representative of multiple cycle days. Approximately one to four hours prior to use, odor donors were removed from their cages along with any food pellets and caked urine present in the corncob bedding, and the soiled cotton nesting material was distributed evenly across the bottom of the cage. Clean cage stimuli consisted of clean cages containing unsoiled corncob bedding and nesting material. Immediately prior to use, a 40 cm x 19 cm x 0.4 cm perforated Plexiglas plate was placed over the bedding in the donor cages. The surface of the plate was painted with black non-toxic chalkboard paint (Rust-oleum, Vernon Hills, IL) such that an unpainted cross divided the plate into four equally sized quadrants. This plate facilitated the observation and quantification of vaginal marking behavior by elevating the subject out of the bedding material and increasing contrast; in

addition, the divisions on the plate provided a means for quantifying locomotor activity throughout the course of the behavioral tests.

Testing protocol

For each 15-minute scent-marking test, females were placed within a soiled stimulus cage and the number of vaginal and flank marks were scored live using a hand counter. Vaginal and flank marking are discrete, stereotyped events that are easily recognized (Johnston, 1977). A vaginal mark was scored each time the female moved forward with tail deflected upwards while maintaining contact between the perineum and the underlying substrate. A flank mark was scored each time the female moved forward while maintaining contact between the flank region and the side of the stimulus cage. Tests were recorded using an overhead camera connected to a VHS video recorder. Videos were scored for the number of quadrant entries by researchers blind to the experimental conditions of the subjects, with inter-rater reliability of 90% or greater. Entry into a quadrant was scored whenever greater than 50% of the body mass of the subject crossed from one quadrant into another.

Prior to cannula surgery, all subjects were tested on proestrus in a prepared clean cage to screen for adequate vaginal marking behavior. Subjects that failed to mark at least five times during this initial screening test were removed from the study. This criterion resulted in the removal of 25 subjects that were initially included in scent-marking tests (29% of initial subjects). Following screening, the remaining subjects were given an additional pre-test on proestrus in their assigned odor condition. Subjects in Experiment 1 were tested in male cages, female cages, or clean cages, whereas subjects in Experiment 2 were only tested in male cages. This pre-test served to familiarize subjects with the future stimulus environment/condition. After recovering from surgery, each subject was tested on two consecutive proestrous days in its assigned odor condition. One test followed injection of OTA, whereas the other test followed injection of vehicle. The order of drug presentation was counterbalanced across subjects.

Odor investigation tests

Apparatus

For odor investigation tests (Experiment 3), the apparatus consisted of a modified 51 cm x 25.5 cm x 30.5 cm glass aquarium with opaque paper lining the exterior of the four vertical glass walls and the glass floor. A black line drawn parallel to the short axis of the apparatus bisected the available floor space, allowing activity levels to be quantified. Three 8 cm square acrylic odor containers were attached along one short wall of the apparatus. Each odor container had a perforated front door to allow subjects to investigate the volatile components of the stimuli without allowing direct access to the contents of the container. The top of the apparatus was secured with a clear Plexiglas lid, allowing for overhead video recording of the subject throughout the behavioral test.

Stimuli

Odor stimuli were collected from group-housed odor donor hamsters (3-4 same-sex animals per cage, bi-weekly cage changes). During collection, approximately 50 ml of soiled corncob bedding and 12 g of soiled cotton nesting material were placed in a one-quart re-sealable plastic collection bag. In addition, separate damp gauze pads were used to wipe down the walls of the cage, the anogenital region, and the bilateral flank glands of odor donors, and these pads were included in the collection bag. Vaginal secretion from odor donor females was collected onto an additional gauze pad by gently palpating the vaginal area with a disposable probe, and included in female odor stimuli collection bags. Hamsters investigate female odors collected on different days of the estrous cycle at relatively equivalent levels (Johnston, 1980); therefore, female stimuli (vaginal secretion, cage stimuli, and body odors) were collected irrespective of cycle day of odor donors and were likely representative of multiple cycle days. Clean odor stimuli consisted of 10 ml of clean corncob bedding, four grams of clean cotton nesting material, and one clean cotton gauze pad. Once collected, all odor stimuli were stored at 4°C until 30

minutes prior to use. Male and female odor stimuli were stored for up to one month and discarded if not used within that time.

Testing protocol

Subjects were tested in the apparatus on two consecutive proestrous days. Following recovery from cannula implant surgery, subjects were first tested with clean odor stimuli in each of the three odor containers (clean odor test). This test served to habituate the subjects to the testing protocol. Subjects were then divided into two groups based on drug condition (OTA or vehicle). On the subsequent proestrous day, subjects were injected according to assigned drug condition and 30 minutes later placed into the choice apparatus (odor preference test). For this test, one of the two outer odor containers contained male odor stimuli, the other outer container contained female odor stimuli, and the center container contained clean odor stimuli. This pattern of odor box placement was designed to maximize the discriminability of male and female odors.

Subjects were allowed to freely explore the apparatus for 10 minutes and upon completion of the test, subjects were removed and the apparatus was cleaned thoroughly with 70% ethanol. Odor containers were emptied and cleaned with 70% ethanol. Prior to testing with another subject, fresh odor stimuli were added to the odor containers and the containers were replaced within the apparatus. The left/right positioning of the male and female odor stimuli containers was counterbalanced across subjects.

All behavior tests were recorded using an overhead video camera connected to a VHS video recorder. Videos from each subject were then digitized and scored using the Observer for windows, version 5.0 (Noldus Information Technology B.V., Wageningen, the Netherlands). Researchers blind to the experimental conditions of the subjects scored the videos, with an inter-rater reliability of 90% or greater. Within each test, the number of entries into each of the two compartments and the duration of investigation of each of the three odor containers were scored. Investigation was scored whenever the

snout of a subject came within one cm of the perforated front panel of an odor box. Entry into a compartment was scored whenever greater than 50% of the body mass of the subject crossed from one compartment into another.

Histology and brain injection site verification

Upon completion of behavioral testing, subjects were administered a lethal dose of sodium pentobarbital (0.2 ml, i.p.; SleepAway, Fort Dodge Animal Health, Fort Dodge, IA), and 200 nl india ink was infused via cannulae. Brains were removed, post-fixed in 10% neutral buffered formalin and 50 μ m sections were taken using a vibrating microtome (Vibratome, Richmond, IL). Sections were examined under light microscopy for ink penetration and compared against published neuroanatomical plates for the Syrian hamster brain (Morin and Wood, 2001). The microinjection site was considered to be within the section of tissue containing the most ventral location of deposited ink.

Data analysis

All data were analyzed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL). Data were first examined to determine if the assumptions of parametric statistical tests were met. When assumptions were violated, an applicable non-parametric alternative was employed. For all statistical tests, results were considered to be statistically significant if $p < .05$.

All measures for Experiment 1 (number of vaginal/flank marks and number of quadrant entries) were first examined using mixed-design 2 X 3 ANOVAs with drug (OTA or vehicle) as a within-subjects factor and odor stimulus (male, female or clean) as a between-subjects factor. Statistically significant interactions were further examined using paired-samples t-tests (effects of drug within each odor condition), and through separate one-way ANOVAs followed by pairwise comparisons using Tukey's HSD (effects of odor within each drug condition). For Experiment 2, mixed-design 2 X 2 ANOVAs were run with drug (OTA or vehicle) as a within-subjects factor and brain area (MPOA-AH or BNST) as a between-

subjects factor for all behavioral measures (number of vaginal/flank marks, number of quadrant entries and grooming duration).

For Experiment 3, odor investigation durations (clean odor tests) were examined using mixed-design 2 X 3 ANOVAs with drug (OTA or vehicle) as a between-subjects factor and odor stimulus box location (left, center, or right) as a within-subjects factor. Friedman tests followed by Wilcoxon sign-ranks tests with Bonferroni correction were used to examine the main effect of odor stimulus and the simple main effect of odor stimulus within each drug condition, respectively. For odor preference tests, the duration of odor box investigation was adjusted, such that the time each subject spent investigating the left, center, and right odor boxes during clean odor tests was subtracted from the time spent investigating odor boxes in corresponding positions during odor preference tests. This adjustment corrected for any innate biases in investigation due to odor box positioning. Data were then analyzed as described for clean odor tests. For both clean odor tests and odor preference tests, the number of compartment entries was analyzed via Mann-Whitney U tests.

2.4 Results

Histology

Subjects were included in the study only if the tip of the injection cannula was located within MPOA-AH (Experiment 1: $N = 30$; Experiment 3: $N = 27$) or within either MPOA-AH or BNST (Experiment 2: $N = 20$) (Figure 2.1). Across experiments, a total of eight subjects were removed due to injection cannula tips located in the bed nucleus of the anterior commissure ($n = 1$), the paraventricular nucleus ($n = 1$), the posterior paraventricular nucleus ($n = 1$), the dorsomedial anteroventral thalamic nucleus ($n = 1$), the lateral hypothalamus ($n = 1$), or immediately dorsal to the posteromedial BNST at the level of the fornix ($n = 3$).

Experiment 1: Effects of OTA in MPOA-AH on male, female, and clean cage odor-induced vaginal marking

Microinjections of OTA into MPOA-AH decreased vaginal marking in response to male, but not in response to female or clean, odors (Figure 2.2). The interaction of drug by odor stimulus on the number of vaginal marks was statistically significant, $F(2,27) = 5.485, p = .010$. In the male odor condition ($n = 11$), OTA injections into MPOA-AH significantly decreased the number of vaginal marks compared to vehicle injections, $t(10) = -3.420, p = .007$. In contrast, OTA did not impair vaginal marking in either the female odor condition ($n = 10$), $t(9) = .805, p = .442$, or the clean odor condition ($n = 9$), $t(8) = -1.348, p = .214$. Although there was a statistically significant effect of odor condition on the number of vaginal marks for both OTA- and vehicle-injected subjects (Vehicle: $F(2,27) = 15.334, p < .001$; OTA: $F(2,27) = 5.391, p = .011$), post-hoc comparisons revealed that marking was significantly higher in the male odor condition compared to the female odor condition for OTA-injected subjects, whereas for vehicle-injected subjects, marking was higher in the male odor condition compared to either the clean or female odor conditions. No significant effects of drug, odor condition, or drug by odor condition interaction, were observed for the number of flank marks or the number of quadrant entries (Table 2.1).

Experiment 2: Effects of OTA in MPOA-AH or BNST on male odor-induced vaginal marking

OTA injections into MPOA-AH ($n = 10$) or BNST ($n = 10$) significantly decreased the number of vaginal marks to male odors compared to vehicle injections, $F(1, 18) = 6.172, p = .023$ (Table 2.2). No significant effect of brain area or interaction of drug by brain area on the number of vaginal marks was observed, however. Similar to results from Experiment 1, there were no significant effects of brain area, drug, or brain area by drug interaction on the number of flank marks or quadrant entries (Table 2.2).

Experiment 3: Effects of OTA in MPOA-AH on social odor investigation

Clean odor tests

When tested prior to group assignment, subjects differentially investigated the three odor boxes when the same stimuli (clean cage materials) were presented in each box, $\chi^2(2) = 20.222$, $p < .001$. Specifically, subjects investigated the center box less than either the left box, $z = -4.060$, $p < .05$, or the right box, $z = -3.820$, $p < .05$. The investigation times for the two outer boxes, however, were not significantly different, $z = -.793$, $p > .05$. In addition, there were no pre-existing differences between the OTA ($n = 16$) and vehicle ($n = 11$) groups for odor box investigation or number of midline crosses (Table 2.3).

Odor preference tests

Irrespective of OTA ($n = 16$) or vehicle ($n = 11$) injection, subjects differentially investigated male, clean and female cage stimuli, $\chi^2(2) = 21.308$, $p < .001$ (Figure 2.3). Subjects investigated male odors more than either clean odors, $z = -4.178$, $p < .05$, or female odors, $z = -3.695$, $p < .05$. The main effect of drug and the drug by odor stimulus interaction on the duration of odor investigation were not significant. Furthermore, the effect of drug on the number of midline crosses was not significant (Table 2.3).

2.5 Discussion

We show here, for the first time, that disrupting OT signaling via injection of OTA decreases vaginal marking induced by male, but not female or clean, odors. This decrease in vaginal marking cannot be attributed as a general effect of OTA on scent marking behavior or as an indirect effect of OTA on general locomotor activity, as OTA injections did not significantly alter the number of quadrant entries or flank marks. Injections of OTA into MPOA-AH or BNST were equally effective in decreasing marking, suggesting that either brain area may mediate the effects of OTA. Finally, OTA does not alter the normal pattern of investigation of social odors by female hamsters, as OTA-injected subjects investigated male, female, and clean cage odors at levels equivalent to vehicle-injected subjects. Taken together, these re-

sults support our hypothesis that OT normally facilitates the elevated levels of vaginal marking induced by male odors, through its actions within MPOA-AH and/or BNST.

Role of OT within MPOA-AH or BNST in scent marking

Vaginal marking behavior

The results of the present study indicate that the decrease in vaginal marking observed following injections of OTA on male odor-induced vaginal marking may be mediated by either MPOA-AH or BNST. This is consistent with previous studies examining the effects of either OT or OTA in MPOA-AH on other reproductive behaviors, as these studies had injection sites nearby, and likely including, BNST (Caldwell et al., 1994; Floody et al., 1998; Whitman and Albers, 1995). Prior lesion studies examining the role of hypothalamic areas in vaginal marking also did not distinguish between MPOA-AH and BNST (Malsbury et al., 1977). It is possible that these brain areas work together as a functional unit to regulate vaginal marking, as MPOA-AH and BNST are densely interconnected (Simerly and Swanson, 1986; Wood and Swann, 2005). Both areas receive chemosensory input relevant for vaginal marking behavior via connections with the medial amygdala (ME) (Coolen and Wood, 1998; Gomez and Newman, 1992; Wood and Swann, 2005), and project to midbrain areas important for motoric aspects of vaginal marking, including the periaqueductal gray (Been and Petrulis, 2007). Finally, both MPOA-AH and BNST bind estradiol and express OT (Krieger et al., 1976; Whitman and Albers, 1998). Alternatively, the results of the present experiments are consistent with the conclusion that BNST exclusively mediates the observed effects of OTA. BNST overlays much of the rostral-caudal extent of MPOA-AH; thus, OTA injected in MPOA-AH may have spread back up to BNST, and any effects attributed to MPOA-AH may instead represent effects of OTA in BNST. This conclusion is further supported by the presence of OTR in BNST, but not MPOA-AH, of Syrian hamsters (Dubois-Dauphin et al., 1992).

The underlying neural mechanisms whereby OTA inhibits male-odor induced vaginal marking are not clear. Failure of antibodies generated against OTR to consistently label neurons in rodent brain tissue has limited the characterization of OTR-bearing neurons in MPOA-AH or BNST (unpublished observations). Although the neural mechanisms whereby OT regulates the processing of vaginal marking-relevant signals within these brain areas has not been examined, evidence from behavioral studies suggests that OT may interact with the progesterone system to regulate reproductive behaviors. In hamsters and rats, OT can substitute for progesterone treatment in inducing lordosis in ovariectomized, estradiol-primed females, and OTA can block the facilitation of lordosis by progesterone treatment (Caldwell et al., 1989, 1994; Whitman and Albers, 1995). These results raise the possibility that in the current study, OTA may have inhibited vaginal marking by blocking the downstream effects of progesterone. This seems unlikely, as proestrous female hamsters have relatively low levels of serum progesterone (Saidapur and Greenwald, 1978) and the surge in progesterone levels that occurs just prior to the onset of behavioral receptivity appears to inhibit vaginal marking (Johnston, 1977; Takahashi and Lisk, 1983; Takahashi et al., 1985). In contrast, estradiol treatment facilitates vaginal marking in response to males, either when administered systemically (Floody, 2002; Lisk and Nachtigall, 1988; Malsbury et al., 1977) or when implanted directly into MPOA-AH (Takahashi and Lisk, 1987; Takahashi et al., 1985). Given that OT and OTR expression in MPOA-AH and BNST are enhanced by estradiol in rats (Jirikowski et al., 1988; Patchev et al., 1993), it seems likely that OT may mediate the effects of estradiol on vaginal marking. More research is required, however, to determine the mechanisms whereby OT acting downstream of estradiol specifically enhances the vaginal marking response to male odors.

Although the decrease in vaginal marking following OTA injection was statistically significant in both Experiment 1 and Experiment 2, the magnitude of the decrease was not large (Experiment 1: 31%; Experiment 2: 22%). This may be due to the specific procedures employed in this study. For all experiments, subjects were injected unilaterally with 200 nl of OTA (900 μ M) and tested 30 minutes following

injection. When administered unilaterally, this volume and concentration of OTA effectively stimulates aggression in female hamsters 30 minutes, but not immediately, following injection (Harmon et al., 2002a). OTA may be working through different mechanisms to modulate aggression and vaginal marking; therefore, bilateral injections or other alterations to the timing, dose, or volume of injections may be necessary for OTA in MPOA-AH or BNST to maximally inhibit vaginal marking behavior.

Flank marking behavior

In contrast to vaginal marking, injections of OTA in MPOA or BNST had no effect on the number of flank marks in response to male, clean, or female odors. Although it has been reported that OT injections in MPOA-AH can induce flank marking in male hamsters (Albers et al., 1986; Ferris et al., 1984; Harmon et al., 2002b), in females this effect is dependent on the presence of a familiar, subordinate partner (Harmon et al., 2002b). In the current study, however, subjects were only tested with odors from unfamiliar stimulus animals. Interestingly, there were also no differences in flank marking to male, female, or clean odors, irrespective of drug condition. Female hamsters flank mark at higher levels in response to same-, rather than opposite-sex stimuli (Johnston, 1977); however, this effect is not consistently observed in proestrous female hamsters (Petrulis and Johnston, 1999; Petrulis et al., 1999). Therefore, it is likely that a significant effect of odor condition on flank marking was not observed in the current study due to testing females only on the cycle day of proestrus.

In contrast to the present results suggesting that the normal expression of vaginal marking is reliant on OT signaling, flank marking appears to depend more critically on the related neuropeptide arginine vasopressin (AVP). For example, AVP injections into MPOA-AH strongly stimulate flank marking in both male and female hamsters (Ferris et al., 1984), whereas administration of V1aR antagonists block flank marking induced by AVP, as well as by stimuli present in conspecific home cages (Albers et al., 1986; Ferris et al., 1985). In addition, these behaviors appear to be important for different aspects of intraspecific communication. Vaginal marking is important for mate solicitation, and therefore is most

prevalent in response to males or their odors, whereas flank marking appears to function in the establishment and maintenance of social hierarchies and territorial advertisement, and is most strongly induced by same-sex odors (Petrulis, 2009). There is a high degree of overlap, however, both in the brain areas that regulate vaginal and flank marking, as well as in the expression patterns of OT and AVP within these areas (Albers and Bamshad, 1998; Keverne and Curley, 2004; Petrulis, 2009). Given that OTA exhibits some level of V1aR antagonism (Manning et al., 1995), and V1aR expression has been confirmed in both MPOA-AH and BNST of Syrian hamsters (Caldwell and Albers, 2004), the possibility exists that the effects observed in the present study are the result of V1aR antagonism by OTA. This seems unlikely given that OTA had no effect on flank marking, a behavior that depends critically on V1aR signaling. Furthermore, preliminary studies examining the effects of a specific V1aR antagonist, Manning compound, in MPOA-AH or BNST found no effects of this drug on vaginal marking (unpublished observations), suggesting that the effects of OTA on vaginal marking are likely mediated by OTRs.

Role of OT within MPOA-AH or BNST in social odor investigation

In the present study, OTA decreased vaginal marking in response to male odors without affecting social odor investigation levels. Subjects were prevented from directly contacting the odor source in all social odor investigation tests, whereas during scent-marking tests, subjects were allowed direct contact with odor stimuli. Therefore, OTA may decrease social odor investigation but only in situations when direct contact with the odor stimulus is allowed. This is in agreement with data from mice, wherein some deficits in odor preference following manipulation of chemosensory structures are only evident in tests wherein direct contact with the odor source is allowed (Keller et al., 2006; Martinez-Ricos et al., 2008). Other studies examining the effects of OT in MPOA-AH on socio-sexual behaviors with strong chemosensory components such as ultrasonic vocalizations in hamsters (Floody et al., 1998) and lamb acceptance in ewes (Kendrick et al., 1992) did not control contact with the odor source, so it is not known if direct contact is necessary for OT to modulate these behavioral responses. Alternatively, OTA

in MPOA-AH or BNST may specifically target the male odor-induced component of vaginal marking without generally affecting male odor investigation, suggesting that separate neural pathways regulate these two behaviors. This is supported by previous work in hamsters, as females with lesions of the vomeronasal organ fail to vaginal mark more to male vs. female odors but show normal patterns of preferential investigation of male odors, whereas ME lesions eliminate opposite-sex odor preference without affecting preferential marking in response to male odors (Johnston, 1992; Petrulis and Johnston, 1999; Petrulis et al., 1999).

Conclusion

Together, these results suggest that OT within MPOA-AH and/or BNST facilitates vaginal marking in female Syrian hamsters. This is in agreement with the role of OT in other sociosexual behaviors, and suggests that the general role of OT is to facilitate sociosexual interactions (Insel, 1992). Importantly, the present study found that the effects of OTA are specific to the male odor-induced component of vaginal marking, indicating that OT in these areas can modulate the behavioral response to chemosensory cues important for social behaviors.

2.6 Acknowledgements

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Chapter 2 Tables

Table 2.1 Summary of behavioral measures from Experiment 1

Mean (\pm SEM) number of flank marks and quadrant entries by female hamsters during 15-minute scent-marking tests to male ($n = 11$), clean ($n = 10$), or female ($n = 9$) cage odors, following injection of OTA or vehicle. There were no significant effects of drug or odor condition on flank marks or quadrant entries, all $p > .05$.

	Number of flank marks			Number of quadrant entries		
	Male	Clean	Female	Male	Clean	Female
Drug						
Vehicle	3.2 \pm 1.4	3.3 \pm 1.5	4.6 \pm 1.7	79.6 \pm 9.9	107.6 \pm 18.5	99.2 \pm 7.2
OTA	2.5 \pm 0.7	2.3 \pm 0.7	8.0 \pm 2.0	72.2 \pm 5.8	101.5 \pm 15.7	100.4 \pm 8.2

Table 2.2 Summary of behavioral measures from Experiment 2

Mean (\pm SEM) number of flank marks and quadrant entries by female hamsters during 15-minute scent-marking tests to male odors, following injection of OTA or vehicle into either MPOA-AH ($n = 10$) or BNST ($n = 10$). OTA injections significantly decreased the number of vaginal marks compared to vehicle injections irrespective of brain area, $p = .023$. There were no significant effects of drug or brain area on the number of flank marks or quadrant entries, all $p > .05$.

	Number of vaginal marks	Number of flank marks	Number of quadrant entries
MPOA-AH			
Vehicle	31.0 \pm 3.0	9.4 \pm 3.1	92.2 \pm 9.4
OTA	25.5 \pm 4.1	8.7 \pm 4.4	76.6 \pm 7.6
BNST			
Vehicle	23.7 \pm 4.0	6.3 \pm 2.3	88.6 \pm 5.9
OTA	17.1 \pm 3.1	5.7 \pm 2.3	87.7 \pm 9.2

Table 2.3 Summary of behavioral measures from clean odor tests (Experiment 3)

Median (\pm IQR) number of midline crosses and duration (in seconds) of odor box investigation by female hamsters during 10-minute clean odor tests preceding assignment to either OTA ($n = 16$) or vehicle ($n = 11$) groups. Subjects investigated the left and right boxes more than the center box, $p < .001$. There were no significant effects of drug or odor condition on the number of midline crosses, $p > .05$.

Group assignment	Number of midline crosses	Investigation duration		
		Left	Center	Right
Vehicle	33.0 \pm 13.0	29.0 \pm 17.2	13.3 \pm 12.7	22.3 \pm 7.6
OTA	35.5 \pm 8.8	23.6 \pm 17.3	13.4 \pm 12.4	29.0 \pm 9.2

Chapter 2 Figures

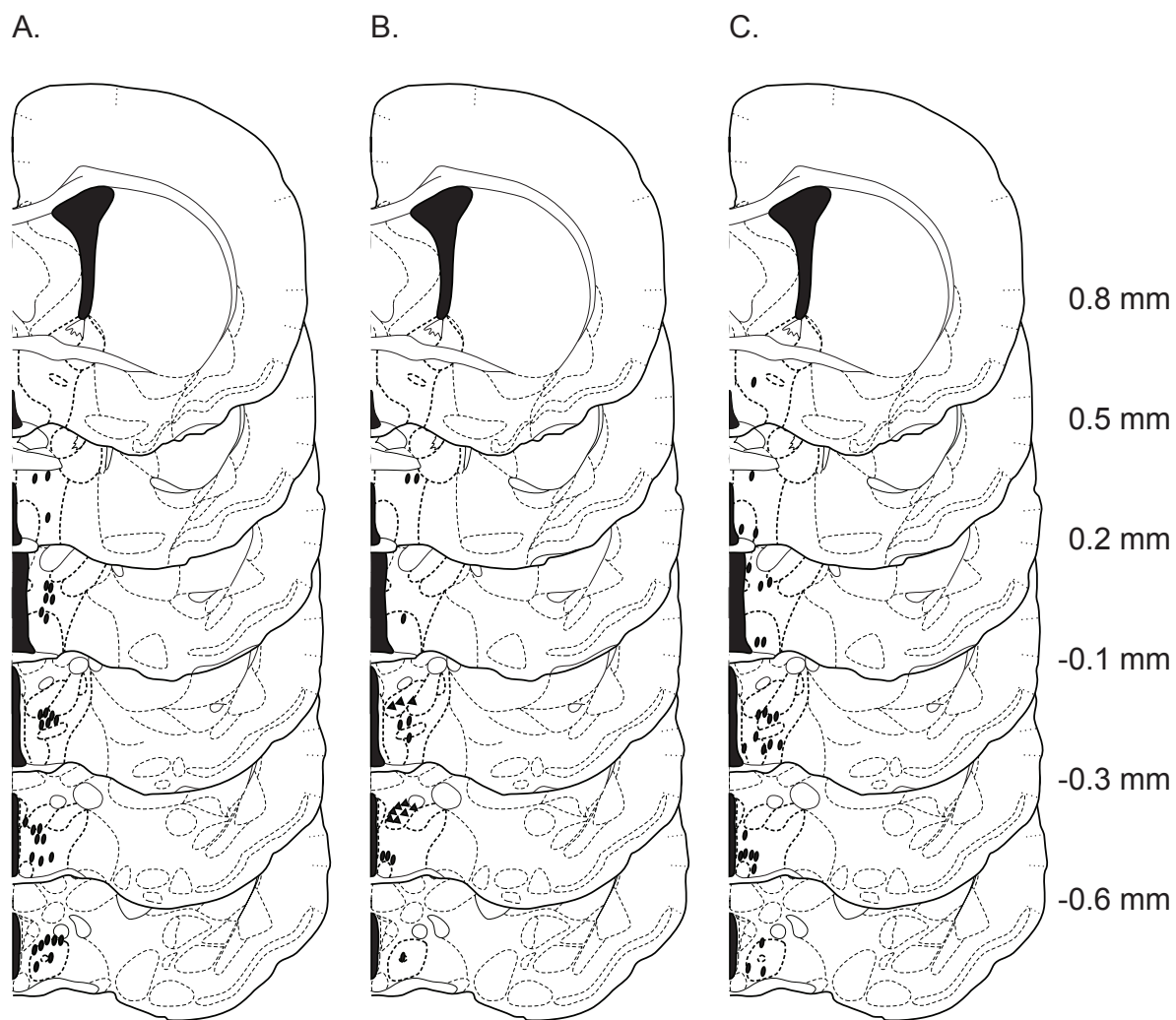


Figure 2.1 Histological verification of injection sites

Coronal sections through the rostral-caudal extent of the hamster brain of microinjection sites for Experiment 1 (A), Experiment 2 (B), and Experiment 3 (C), located within MPOA-AH and BNST. Ovals represent microinjections that terminated within MPOA-AH, whereas triangles represent microinjections that terminated within BNST.

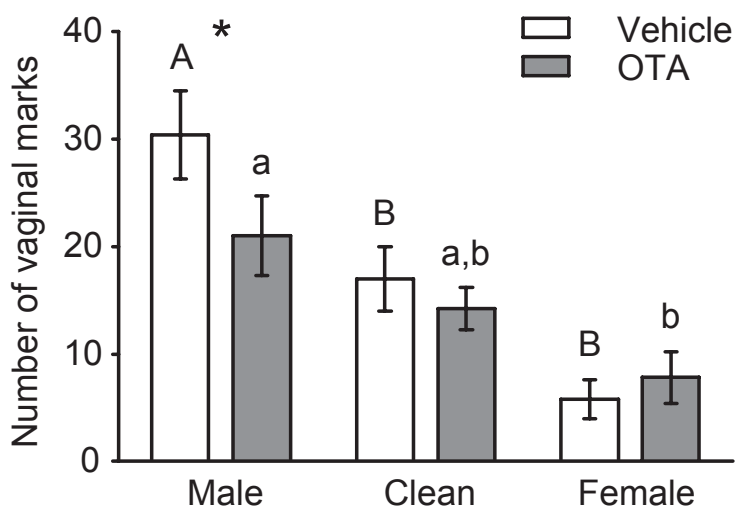


Figure 2.2 Number of vaginal marks during scent-marking tests

OTA injected into MPOA-AH decreased vaginal marks to male, but not female or clean, odors, compared to vehicle injections. Following vehicle injections, subjects vaginal marked more to male than to female or clean odors. In contrast, OTA-injected subjects only marked more to male odors than to female odors, as marking to clean odors was intermediate to both male and female odors. * represents significant difference between OTA and vehicle injected subjects in the male odor condition, $p = .007$. Dissimilar letters within each letter type (upper- and lowercase) represent significant mean differences following *Tukey's* post-hoc comparisons examining the simple main effect of odor within OTA and vehicle conditions.

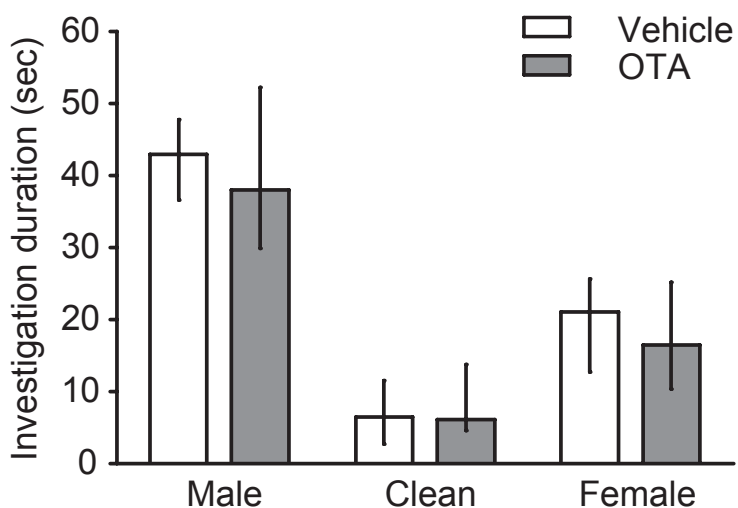


Figure 2.3 Odor box investigation during preference tests

Subjects investigated male odors more than either female or clean odors, irrespective of drug (OTA or vehicle) injected into MPOA-AH ($p < .05$ with *Bonferroni* correction). Investigation duration times were adjusted to account for pre-existing biases in investigation due to odor box positioning, as described in the *Data Analysis* section. Graphed data represent unadjusted investigation times.

CHAPTER 3: THE BED NUCLEUS OF THE STRIA TERMINALIS IS CRITICAL FOR SEXUAL SOLICITATION, BUT NOT OPPOSITE-SEX ODOR PREFERENCE, IN FEMALE SYRIAN HAMSTERS

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3.1 Abstract

Successful reproduction in vertebrates depends critically upon a suite of precopulatory behaviors that occur prior to mating. In Syrian hamsters (*Mesocricetus auratus*), these behaviors include vaginal scent marking and preferential investigation of male odors. The neural regulation of vaginal marking and opposite-sex odor preference likely involves an interconnected set of steroid-sensitive nuclei that includes the medial amygdala (MA), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA). For example, lesions of MA eliminate opposite-sex odor preference and reduce overall levels of vaginal marking, whereas lesions of MPOA decrease vaginal marking in response to male odors. Although BNST is densely interconnected with both MA and MPOA, little is known about the role of BNST in female precopulatory behaviors. To address this question, females received either bilateral, excitotoxic lesions of BNST (BNST-X) or sham lesions (SHAM), and were tested for scent marking and for investigatory responses to male and female odors. Whereas SHAM females vaginal marked more to male odors than female odors on two days of the estrous cycle, BNST-X females marked at equivalent levels to both odors. This deficit is not due to alterations in social odor investigation, as both BNST-X and

SHAM females investigated male odors more than female odors. Finally, BNST lesions did not generally disrupt the cyclic changes in reproductive behaviors that occur across the estrous cycle. Taken together, these results demonstrate that BNST is critical for the normal expression of solicitational behaviors by females in response to male odor stimuli.

3.2 Introduction

Successful reproduction in most vertebrates depends critically on precopulatory or solicitation behaviors that occur prior to mating (Beach, 1976). Although these behaviors can take many species-specific forms, they all serve to facilitate or coordinate mating behavior. Precopulatory behaviors are therefore particularly important for mating coordination in species where the sexes live in isolation from each other, such as Syrian hamsters (Gattermann et al., 2001; Lisk et al., 1983; Pfaff et al., 2008). In this species, females engage in several forms of precopulatory behaviors that include vaginal scent marking and the preference to investigate opposite-sex odors. Vaginal marking is a highly stereotyped behavior typified by the female lowering her perineum to the substrate and depositing a small amount of vaginal secretion as she moves forward (Johnston, 1977). Vaginal marks likely serve to advertise a female's location to males, as females vaginal mark to form a path linking the male and female's nesting areas when tested in a large, semi-natural environment (Lisk et al., 1983), and males vigorously investigate and follow these marks (Johnston and Schmidt, 1979; Johnston, 1974; Kwan and Johnston, 1980; Lisk et al., 1983).

The expression of vaginal marking relies on the perception of odor cues from conspecifics, as females vaginal mark at much higher levels in response to males or their odors compared to odors from other females (Johnston, 1977; Maras and Petrulis, 2008; Martinez et al., 2010; Petrulis and Johnston, 1999; Petrulis et al., 1999). In hamsters as well as other vertebrates, social odors are processed by an interconnected set of forebrain neural structures that includes the olfactory bulbs, the medial amygdala (MA), and the bed nucleus of the stria terminalis (BNST) (Wood, 1998). Some of these structures have

been implicated in the regulation of female precopulatory behavior. For example, disruption of the main olfactory system decreases overall levels of vaginal marking and ultrasonic vocalizations (Johnston, 1992), whereas disruption of the accessory olfactory system eliminates preferential vaginal marking to male odors (Petrulis et al., 1999). Input from the main and accessory olfactory systems converge on MA (Coolen and Wood, 1998), and bilateral electrolytic lesions centered on MA eliminate opposite-sex odor preference and decrease vaginal marking (Petrulis and Johnston, 1999; Takahashi and Gladstone, 1988). Finally, electrolytic lesions that target the entire corticomedial amygdala decrease ultrasonic vocalizations by females in response to males (Kirn and Floody, 1985).

In contrast to MA, less is known about the role of BNST in the regulation of precopulatory behaviors. BNST receives input from the olfactory system both directly (Davis et al., 1978), as well as indirectly via MA and other amygdaloid nuclei (Coolen and Wood, 1998; Wood and Swann, 2005) and in male hamsters, lesions of BNST eliminate opposite-sex odor preference (Been and Petrulis, 2010b). In female hamsters, large bilateral electrolytic lesions of the medial preoptic area (MPOA) that also damage BNST decrease vaginal marking in response to males (Malsbury et al., 1977). Furthermore, injection of a selective oxytocin receptor antagonist into BNST of female hamsters decreases vaginal marking to male odors, without affecting marking to female odors or the preference to investigate opposite-sex odors (Martinez et al., 2010). This effect, however, cannot be specifically attributed to BNST, as injections into the underlying MPOA also were effective in decreasing marking to male odors. In order to gain a more complete understanding of the role of BNST in precopulatory behaviors, we examined the effects of site-specific, excitotoxic lesions of BNST on vaginal marking, as well as investigatory responses to male and female odors. Based on previous data, we predicted that BNST lesions would disrupt both preferential vaginal marking and odor investigation. As BNST is a steroid-responsive brain area (Krieger et al., 1976; Li et al., 1993) and vaginal marking varies with changes in circulating gonadal steroids (Takahashi and Lisk, 1983) we tested females for scent marking on multiple days of the estrous cycle

(diestrous day 2 and proestrus), to determine if BNST mediates the effects of circulating gonadal steroids on vaginal marking.

3.3 Methods

Experimental design

Subjects were first assessed for levels of vaginal marking to male odors in order to screen out animals that failed to mark at sufficiently high levels (see below) (Figure 3.1A). Subjects then received either bilateral, excitotoxic lesions of BNST or sham surgeries. Following recovery, subjects underwent a series of behavioral tests. First, subjects were tested for their investigatory responses to male and female odors (Odor investigation tests). After an initial habituation test (Clean test), subjects were tested both when direct contact with the odor stimuli was prevented (Non-contact test) and when it was allowed (Contact test). Second, subjects were tested for scent marking responses to male or female stimuli on two days of the estrous cycle, diestrous day 2 and proestrus. Finally, to verify that BNST lesions had not disrupted the ability of females to display copulatory behavior, subjects were tested during behavioral estrus for receptive sexual responses to a sexually experienced male.

Subjects

Adult female Syrian hamsters (*Mesocricetus auratus*) were purchased from Harlan Laboratories (Indianapolis, IL, USA) at approximately 7-8 weeks of age. In addition to experimental subjects, a separate group of unrelated adult male and female Syrian hamsters was purchased from Harlan Laboratories to serve as stimulus animals. Animals were either individually housed (experimental subjects) or group housed (3-4 same-sex animals per cage; stimulus animals) in solid-bottom polycarbonate cages containing corncob bedding and cotton nesting material (Nestlets; Ancare, Bellmore, NY). Subjects and stimulus animals were maintained on a reversed light cycle (16:8 light:dark; lights out at 10 am), with all behavior testing occurring during the first four hours of the dark portion of the light cycle. Food and water were

available *ad libitum*. Animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and approved by the Georgia State University Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Estrous cycle monitoring

Prior to behavioral testing, adult subjects were examined daily for eight consecutive days to determine stage of the estrous cycle. Subjects were gently restrained while vaginal secretion was manually extruded using a disposable probe, and the consistency of the secretion was examined for stringy consistency indicative of behavioral estrus (Orsini, 1961). Once behavioral estrus was identified, the two cycle days prior to estrus were defined as diestrous day 2 and proestrus, respectively (Johnston, 1977). Estrous cycles were also monitored for eight days following surgery to ensure that this procedure had not disrupted cyclicity. In all cases, 'day' refers to the dark phase of the light cycle.

Surgery

At two to three months of age, subjects were assigned to either a BNST lesion group (BNST-X; $n = 28$) or a sham lesion group (SHAM; $n = 14$). A matched random assignment procedure was used, such that initial levels of vaginal marking in response to male odors on proestrus were equivalent across both experimental conditions (see below). Subjects were first deeply anesthetized with 2-3% isoflurane gas (Baxter, Deerfield, IL) in an oxygen (70%) and nitrous oxide (30%) mixture, and then placed within a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with ear- and incisor-bars positioned such that the top of the skull was level. Following a midline scalp incision, the skin and underlying temporal muscles were retracted to expose the skull. A hand-operated drill was then used to make holes in the skull in order to expose dura. For BNST-X subjects, the excitotoxin *N*-methyl-D-aspartic acid (20 mg/ml, 20 nl per injection site; Sigma, St. Louis, MO) was injected bilaterally via a Hamilton microinjection syringe (701R

10 μ l syringe; Hamilton, Reno, NV) under stereotaxic control. Subjects in the BNST-X group received two injections/side, one targeting the posterointermediate BNST (anterior-posterior, +1.65; medial-lateral, \pm 1.65; dorsal-ventral, -5.80) and the other the posteromedial BNST (anterior-posterior, +1.65; medial-lateral, \pm 1.05; dorsal-ventral, -5.90). The anterior-posterior and medial-lateral measurements were made relative to bregma, whereas dorsal-ventral measurements were made relative to dura, according to published anatomical plates of the Syrian hamster brain (Morin and Wood, 2001). The excitotoxin was expelled over the course of one minute, and the syringe needle was then left in place for an additional nine minutes to allow sufficient time for the injection volume to disperse from the syringe tip.

Sham surgeries were conducted in a similar manner to lesion surgeries except that no excitotoxin was infused into the target sites. Immediately prior to completion of all surgeries, the skull holes were sealed with bone wax and the incision closed with wound staples. Subjects were then injected with an analgesic agent (5 mg/kg; Ketofen, Fort Dodge Animal Health, Fort Dodge, IA). All subjects were allowed to recover for at least 14 days prior to post-operative behavioral testing.

Scent-marking tests

Odor stimulus and apparatus

Stimuli for scent-marking tests consisted of vacated cages (43 x 22 x 20 cm) that had previously been occupied by male or female stimulus animals for four days (Figure 3.1B). Estrous cycles of stimulus females were not monitored; however, given that each cage contained at least three females, and the occupation period comprised the complete four-day estrous cycle, it is likely that cage stimuli were representative of multiple cycle days. Approximately one to four hours prior to use, a researcher blind to the experimental condition of subjects prepared the stimulus cages as follows: Stimulus animals were removed from their cages along with any food pellets and caked urine present in the corncob bedding, the soiled cotton nesting material was distributed evenly across the bottom of the cage, and a 40 cm x

19 cm x 0.4 cm perforated Plexiglas plate was placed over the bedding just prior to testing. The surface of the plate was painted with black non-toxic chalkboard paint (Rust-oleum, Vernon Hills, IL) such that the plate was divided into four painted quadrants separated by an unpainted area in the shape of a cross. This plate facilitated the observation and quantification of vaginal marking behavior by elevating the subject out of the bedding material; in addition, the divisions on the plate provided a means for quantifying locomotor activity throughout the course of scent-marking tests.

Testing protocol

During each ten-minute scent-marking test, females were placed within a soiled stimulus cage and the number of vaginal and flank marks were scored using a hand counter by a researcher blind to experimental condition of the subjects. Similar to vaginal marking, flank marking is a discrete, stereotyped behavior that is differentially expressed in response to conspecific odors (Johnston, 1977). In contrast to vaginal marking, however, flank marking occurs more frequently in response to female than to male odors, and appears to function predominantly in territorial advertisement (Johnston, 1985). A flank mark was scored each time the female moved forward while maintaining contact between the flank region and the side of the stimulus cage. A vaginal mark was scored each time the female moved forward with tail deflected upwards while maintaining contact between the perineum and the underlying substrate. Tests were also recorded using a digital camcorder and videos were scored for the number of quadrant entries by researchers blind to the experimental conditions of the subjects, with an inter-rater reliability of 90% or greater. Entry into a quadrant was scored whenever greater than 50% of the body mass of the subject crossed from one quadrant into another. At the completion of each scent-marking test, the female was removed from the stimulus cage and returned to its home cage. Stimulus cages were not reused; rather, only one female was tested per stimulus cage.

Prior to surgical procedures, subjects were initially screened for vaginal marking responses to male odors (Figure 1A). Subjects that failed to mark at least 5 times during a ten-minute test ($n = 2$) were

removed from the study. After recovering from surgery, subjects were tested for scent marking responses to male or female odors, on both diestrous day 2 and proestrus. The order of testing was counterbalanced across subjects.

Odor investigation tests

Stimuli

Stimuli for odor investigation tests were collected from the cages of group-housed same-sex stimulus animals by researchers blind to the experimental condition of the subjects. During collection, approximately 50 ml of soiled corn cob bedding and 12 g of soiled cotton nesting material were placed into a one-quart re-sealable plastic collection bag. In addition, a total of three separate damp gauze pads (10 x 10 cm) were used to wipe down the walls of the cage, the anogenital region, and the bilateral flank glands of odor donors, and these pads were included in the collection bag. Vaginal secretion from odor donor females was collected onto an additional gauze pad by gently palpating the vaginal area with a disposable probe, and included in female odor stimuli collection bags. Hamsters investigate female odors collected on different days of the estrous cycle at relatively equivalent levels (Johnston, 1980); therefore, female stimuli (vaginal secretion, cage stimuli, and body odors) were collected irrespective of cycle day of odor donors. Clean odor stimuli consisted of 10 ml of clean corn cob bedding, four grams of clean cotton nesting material, and one clean cotton gauze pad. Once collected, all odor stimuli were stored at 4°C until 30 minutes prior to use.

For tests involving direct contact with odors, additional stimuli were collected directly onto glass microscope slides (25 x 75 x 1 mm). Stimulus animals were gently restrained while clean slides were rubbed along the flank and anogenital regions. For male odor slides, one half of the slide contained flank gland secretions and the other half contained anogenital secretions. For female odor slides, the slide was divided into three sections rather than two, with the center section containing a sample of vaginal

secretion collected from a stimulus female using a disposable probe. In all cases, individual slides contained stimuli from multiple animals, as each type of stimuli collected onto a slide was derived from a different stimulus animal. This was done to minimize the impact of individual differences in stimulus quality on subsequent investigatory behavior by experimental subjects during testing. Following collection, slides containing same-sex stimuli were stored together in airtight containers at 4°C until 30 minutes prior to use. All collected odor stimuli were stored for up to one month and discarded if not used within that time.

Apparatus and testing protocol

Odor investigation tests were conducted in a three-choice odor investigation apparatus (Figure 3.1C) by a researcher blind to the experimental condition of the subjects. This apparatus consisted of a modified 51 cm x 25.5 cm x 30.5 cm glass aquarium with opaque paper lining the exterior of the four vertical glass walls and the glass floor. A black line drawn parallel to the short axis of the apparatus bisected the available floor space, allowing activity levels to be quantified. Three 8 cm square acrylic odor containers were attached along the inside of one short wall of the apparatus. Each odor container had a perforated door to allow subjects to investigate the volatile components of the stimuli without allowing direct access to the contents of the container. The top of the apparatus was secured with a clear Plexiglas lid, allowing for overhead video recording of the subject throughout the behavioral test.

Subjects were tested on three separate occasions in the three-choice odor investigation apparatus (Clean test, Non-contact test, and Contact test) (Figure 3.1A). Testing occurred across three consecutive estrous cycles, with each test occurring on the cycle day of estrus. Following recovery from stereotaxic surgery, subjects were first tested with clean odor stimuli in each of the three odor containers. This test served to habituate the subjects to the testing protocol. Subjects were then tested in the Non-contact test followed by the Contact test. During these tests, one of the two outer odor containers contained male odor stimuli, the other outer container contained female odor stimuli, and the center con-

tainer contained clean odor stimuli. This pattern of odor container placement was designed to maximize the discriminability of male and female odors. For the Contact test, a single slide containing stimuli sex-matched to the contents of the odor container (male, female, or clean) was affixed to the center of the exposed, perforated side of the odor container, such that the long axis of the slide was parallel to the floor of the apparatus.

Subjects were allowed to freely explore the apparatus for ten minutes, and upon completion of the test, subjects were removed, returned to their home cages, and the apparatus was cleaned thoroughly with 70% ethanol. Odor containers were emptied and cleaned with 70% ethanol. Prior to testing with another subject, fresh odor stimuli were added to the odor containers and the containers were replaced within the apparatus. The left/right positioning of the male and female odor stimuli containers was counterbalanced across subjects.

All behavior tests were recorded using a digital camcorder and videos from each subject were then scored using the Observer for Windows, version 9.0 (Noldus Information Technology B.V., Wageningen, the Netherlands). Researchers blind to the experimental conditions of the subjects scored the videos, with an inter-rater reliability of at least 90%. Within each test, the number of midline crosses and the duration of investigation of each of the three odor containers were scored. Investigation was scored whenever the snout of a subject came within one cm of the perforated front panel of an odor box. Entry into a compartment was scored whenever greater than 50% of the body mass of the subject crossed from one compartment into another.

Lordosis tests

Subjects were tested for the latency and expression of sexual receptivity (lordosis) on the predicted day of behavioral estrus (Figure 3.1A) by a researcher blind to the experimental condition of the subjects. Lordosis is a posture female hamsters assume to allow copulation with males, and is identified as the appearance of a prolonged immobile posture, an elevated rump, and a level/concave spine

(Tiefer, 1970). During lordosis tests, subjects were placed in the home cage of a sexually experienced male and the two animals were allowed to freely interact. Stimulus males were removed from the cage once subjects displayed lordosis (typically following anogenital investigation of the female by the male), and so were not allowed to mount the female. The latency to assume lordosis (measured from the time the subject was introduced into the male's cage) and duration of lordosis were scored live using hand-held stopwatches. Tests were concluded when the female exited the lordosis posture, or when ten minutes had elapsed.

Histology and lesion verification

Upon completion of behavioral testing, subjects were administered a lethal dose of sodium pentobarbital (0.2 ml, i.p.; SleepAway, Fort Dodge Animal Health, Fort Dodge, IA), and transcardially perfused with 200 ml of 0.1 M phosphate buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde (overnight) followed by 20% sucrose in PBS (1-2 days). Coronal sections (30 μ m) were taken using a cryostat and stored in cryoprotectant at -20°C until processed for immunohistochemistry for neuronal nuclei protein (NeuN), a protein specific to neurons (Mullen et al., 1992). The extent of lesion damage was then determined by a researcher blind to the experimental condition of the subjects, via examination of NeuN-stained tissue under a light microscope and comparison against published neuroanatomical plates of the Syrian hamster brain (Morin and Wood, 2001).

Immunohistochemistry

Free-floating coronal sections were removed from cryoprotectant, rinsed in PBS, and then incubated in a solution containing PBS, 1% Triton-X (Sigma, St. Louis, MO), and 1:30,000 monoclonal mouse anti-NeuN antibody (MAB377; Millipore, Billerica, MA) for 18 hours at room temperature. After rinsing in PBS, tissue sections were incubated in a solution of PBS, 0.4% Triton-X, and 1:600 biotinylated rabbit

anti-mouse secondary antibody (315-065-003, Jackson ImmunoResearch Laboratories, West Grove, PA) for one hour at room temperature, rinsed in PBS, and then incubated in a solution of PBS, 0.4% Triton-X, and 1:200 avidin-biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for one hour at room temperature. Tissue sections were then rinsed in PBS followed by 0.175 M sodium acetate, and reacted in a nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride solution (2 mg 3,3'-diaminobenzidine tetrahydrochloride plus 250 mg nickel (II) sulfate with 8.3 μ L 3% H₂O₂ per 10 mL of 0.175 M sodium acetate; Sigma, St. Louis, MO) for 15 minutes at room temperature. Tissue sections were then rinsed in 0.175 M sodium acetate followed by PBS in order to terminate the chromagen reaction.

Data analysis

All data were analyzed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL). Data were first examined to determine if the assumptions of parametric statistical tests were met. When assumptions were violated, an applicable non-parametric alternative was employed. For all statistical tests, results were considered to be statistically significant if $p < .05$.

Behavioral measures from scent-marking tests (number of vaginal and flank marks and number of quadrant entries) were first examined using mixed-design 2 X 2 X 2 ANOVAs with experimental group (BNST-X or SHAM) as a between-subjects factor, and odor stimulus (male or female) and cycle day (diestrus day 2, proestrus) as within-subjects factors. Statistically significant two-way interactions were further examined for the effect of odor within each lesion group using paired-samples *t*-tests. The number of quadrant entries was compared across experimental group using independent-samples *t*-tests.

For the Clean odor test, investigation durations were examined using mixed-design 2 X 3 ANOVAs with experimental group (BNST-X or SHAM) as a between-subjects factor and odor box position (left, center, right) as a within-subjects factor. Significant main effects of odor box position were further examined using Friedman tests followed by Wilcoxon sign-ranks tests with Bonferroni correction. For

the Non-contact and Contact odor tests, investigation durations were examined using mixed-design 2 X 2 ANOVAs with experimental group (BNST-X or SHAM) as a between-subjects factor and odor stimulus (male or female) as a within-subjects factor. Significant main effects of odor were further examined using Wilcoxon sign-ranks tests. Within each the odor investigation tests, odor box investigation times and the number of compartment entries were compared across experimental group using Mann-Whitney *U* tests. Lastly, behaviors measured during copulatory tests (latency to assume lordosis, duration of lordosis) were compared across experimental group using Mann-Whitney *U* tests.

3.4 Results

Lesion reconstruction

Subjects were included in the BNST-X group if bilateral damage comprised at least 50% damage to BNST on at least two consecutive stereotaxic planes (Figure 3.2A) (Morin and Wood, 2001). All subjects included in the BNST-X group sustained substantial bilateral damage to BNST at the level of the most caudal pole of the anterior commissure (0.5 mm anterior to Bregma, $n = 17$) (Figure 3.2C). Furthermore, most subjects in this group also sustained bilateral damage to more posterior levels of BNST (Bregma +0.2 mm, $n = 14$; Bregma -0.1 mm, $n = 1$). A few BNST-X subjects also had bilateral damage at more rostral levels of BNST (Bregma +0.8 mm, $n = 3$).

Subjects that received NMDA injections but did not exhibit significant bilateral damage to BNST on two consecutive stereotaxic planes were not included in the BNST-X group. Specifically, these subjects all had partial (less than 50%), bilateral damage to BNST (Partial Lesion, $n = 15$) restricted within the same rostral-caudal extent of BNST as BNST-X subjects. Importantly, these subjects did not differ significantly from SHAM subjects on any measure of vaginal marking or odor investigation ($p > .05$ for all comparisons).

In both BNST-X and Partial Lesion subjects, minimal and predominantly unilateral damage to surrounding nuclei was occasionally observed (defined as less than 20% of the nuclei on any stereotaxic plane) (Morin and Wood, 2001). This damage was seen in the ventrolateral septum ($n = 9$), the medial preoptic area ($n = 3$), the medial preoptic nucleus ($n = 1$), the ventral pallidum ($n = 3$), the globus pallidus ($n = 1$), and the caudate putamen ($n = 1$). No differences were observed in any behaviors between subjects with damage to any of these nuclei compared to BNST-X and Partial Lesion subjects. Although needle tracts were typically visible in the brains of SHAM subjects (Figure 3.2B), no significant damage to any brain structure was observed in these subjects.

Scent-marking tests

Preferential vaginal marking to male vs. female odors was eliminated in females with bilateral lesions of BNST (Figure 3.3). As expected (see Introduction), females vaginal marked more on proestrus compared to diestrus day 2, $F(1,39) = 52.925$, $p < .001$, and more to male odors than to female odors, $F(1,39) = 38.516$, $p < .001$. The interaction of lesion group by odor condition, however, was statistically significant, $F(1,25) = 10.160$, $p < .01$, indicating that the effect of odor condition was driven predominantly by SHAM females marking more to male odors on both diestrus day 2, $t(9) = 4.80$, $p < .01$, and proestrus, $t(9) = 3.94$, $p < .01$. In contrast, BNST-X females vaginal marked at equivalent levels to male and female odors on both cycle days (diestrus day 2: $t(16) = 1.11$, $p = .28$; proestrus: $t(16) = .518$, $p = .61$). This loss of preferential vaginal marking to male odors did not reflect an overall decrease in vaginal marking levels in BNST-X females, as the total number of vaginal marks (summed across cycle days and odor conditions) did not differ between BNST-X and SHAM females, $F(1,25) = .132$, $p = .72$. BNST-X and SHAM females also did not differ in the number of line crosses during scent-marking tests, in response to either male, $t(23) = -.88$, $p = .39$, or female, $t(21) = -1.28$, $p = .22$, odors. Similar to SHAM females, vaginal marking in the Partial Lesion group was significantly higher to male compared to female odors on both diestrus day 2 and proestrus (Table 3.1).

There were no significant differences in flank marking behavior between BNST-X and SHAM females (Table 3.2). Although females flank marked more in response to female odors compared to male odors, $F(1,39) = 8.74, p < .01$, the interaction of odor condition by lesion group was not significant, $F(2,39) = 1.11, p = .34$. BNST-X and SHAM females also did not differ in the overall number of flank marks, $F(1,25) = .71, p = .41$.

Odor investigation tests

Clean test

Subjects differentially investigated the three odor boxes when clean cage materials were presented in each box, $\chi^2(2) = 30.25, p < .001$ (data not shown). Specifically, females investigated the center box less than either the left box, $z = -4.26, p < .001$, or the right box, $z = -4.23, p < .001$. Importantly, the investigation times of the two outer boxes were not significantly different, $z = -.49, p = .63$. Furthermore, there were no significant differences in odor box investigation (left box: $z = -.82, p = .412$; center box: $z = -.59, p = .56$; right box: $z = -.176, p = .86$) or midline crosses, $z = -.53, p = .60$, between BNST-X and SHAM females.

Non-contact test

Irrespective of lesion condition, females differed in their investigation of male and female cage stimuli when direct contact with the odor source was prevented, $z = -3.87, p < .001$ (Figure 3.4A). Both SHAM ($z = -2.70, p < .01$) and BNST-X ($z = -2.79, p < .01$) females investigated male odors more than female odors. The main effect of lesion, $z = -.264, p = .79$, and the lesion by odor stimulus interaction, $F(1,24) = .64, p = .43$, were not significant. Similar to SHAM and BNST-X females, Partial Lesion subjects also investigated male odors more than female odors (Table 3.1).

Contact test

Both SHAM and BNST-X females investigated the odor stimuli for differing amounts of time when contact with the odor stimuli was allowed, $z = -4.18$, $p < .001$ (Figure 3.4B). Females in both conditions investigated male odors more female odors (SHAM: $z = -2.80$, $p < .01$; BNST-X: $z = -3.15$, $p < .01$). There was no significant main effect of lesion group, $z = -.30$, $p = .76$, or significant lesion by odor stimulus interaction, $F(1,24) = 1.18$, $p = .29$. This pattern of results was also seen in females in the Partial Lesion group (Table 3.1).

Lordosis tests

All females exhibited lordosis on the predicted day of behavioral estrus in response to a stimulus male. BNST-X and SHAM females did not differ in either the latency, $z = -.40$, $p = .69$, or duration, $z = -.18$, $p = .86$, of their lordotic response to males (Table 2).

3.5 Discussion

We show here, for the first time, that discrete, bilateral lesions of BNST specifically disrupt the expression of female sexual solicitation to conspecific stimuli, as evidenced by the elimination of preferential vaginal marking to male odors compared to female odors in BNST-X females. This loss of preferential vaginal marking cannot be attributed to a deficit in social odor investigation, as BNST-X females investigated male odors more than female odors both when direct contact with the odor source was allowed and when it was prevented. BNST-X females also displayed the normal pattern of elevated flank marking to female odors, and had no deficits in the expression of sexual receptivity. Together, these results support our hypothesis that BNST is required for the normal expression of odor-guided precopulatory behaviors in female Syrian hamsters.

Role of BNST in sexual solicitation

The finding that lesions of BNST eliminate preferential vaginal marking to male odors is consistent with a previous study demonstrating that bilateral electrolytic lesions targeted at MPOA, that also damaged BNST, decrease vaginal marking to males (Malsbury et al., 1977). BNST receives substantial input from the olfactory bulbs, predominantly via connections with corticomедial amygdaloid nuclei such as MA (Wood and Swann, 2005), and the normal pattern of vaginal marking responses to conspecifics is severely disrupted following damage to these olfactory systems (Johnston, 1992; Petrulis et al., 1999). Lesions of MA, however, do not eliminate preferential vaginal marking to male odors, but rather result in an overall decrease in vaginal marking regardless of odor condition (Petrulis and Johnston, 1999). These data suggest, therefore, that MA may process chemosensory information important for supporting overall levels of vaginal marking to conspecific odors, whereas information critical for preferential vaginal marking may be processed by BNST independent of MA, perhaps involving the direct connections between AOB and BNST (Davis et al., 1978; Fan and Luo, 2009).

Although the neurochemical mechanisms underlying the regulation of preferential vaginal marking by BNST have not been extensively examined, there is some evidence to suggest that the neuropeptide oxytocin may be involved. BNST appears to be sensitive to oxytocin, given that it contains both oxytocin-immunoreactive fibers (Whitman and Albers, 1998) and binding sites for oxytocin (Dubois-Dauphin et al., 1992), and neurons in BNST exhibit excitatory responses following application of oxytocin (Ingram et al., 1990). In a recent study we found that unilateral injections of a specific oxytocin receptor antagonist into BNST decrease vaginal marking to male odors, without affecting marking to female odors or preferential investigation of male odors (Martinez et al., 2010). Although these data suggest that oxytocin normally acts in BNST to support male odor-guided vaginal marking without more generally affecting investigatory responses to male odors, it is important to note that BNST is also important for other precopulatory behaviors in females such as ultrasonic vocalizations (Kirn and Floody, 1985). Indeed, injec-

tions of oxytocin into MPOA increase ultrasonic vocalizations to males (Floody et al., 1998). Further research is needed, therefore, in order to clarify the role of oxytocin in BNST across the full range of female precopulatory behaviors.

In addition to regulation by odor signals from conspecifics, the expression of vaginal marking also varies in response to changes in circulating gonadal steroids. Vaginal marking levels increase across the estrous cycle and peak on proestrus (Johnston, 1977; Takahashi and Lisk, 1983). In the present study, BNST-X females vaginal marked more on proestrus compared to diestrous day 2, indicating that BNST lesions did not disrupt the normal cyclic pattern of vaginal marking. This was surprising, given that estradiol receptors are expressed in many neurons throughout BNST (Li et al., 1993), and that that overall levels of vaginal marking appear to be mediated by estradiol acting within several hypothalamic structures that are interconnected with BNST (Takahashi and Lisk, 1985, 1987; Takahashi et al., 1985). Taken together, the evidence suggests that the neural circuit mediating the effects of cyclic changes in estradiol on precopulatory and other reproductive behaviors does not include BNST. This conclusion is consistent with our observation that all BNST-X females, like SHAM females, were sexually receptive on the predicted day of behavioral estrous.

Role of BNST in social odor investigation

Females with BNST lesions showed the normal preference to investigate male odors more than female odors, indicating that BNST is not critical for the normal expression of opposite-sex odor preference in females. These results were unexpected, given that in male hamsters, similar lesions of BNST completely eliminate opposite-sex odor preference (Been and Petrulis, 2010b), and larger, electrolytic lesions of BNST decrease investigation of female hamster vaginal secretion (Powers et al., 1987). Moreover, both males and females require a functional MA, the major input structure for BNST, for odor preference (Maras and Petrulis, 2006; Petrulis and Johnston, 1999), and both sexes show similar neural responses to opposite-sex odors in MA (DelBarco-Trillo et al., 2009; Maras and Petrulis, 2010a; Meredith

and Westberry, 2004) and BNST (Hosokawa and Chiba, 2007; Maras and Petrulis, 2010a). This suggests that with the exception of MA, a fundamental sex difference may exist in the neural architecture regulating opposite-sex odor preference. This is congruent with evidence that the sexes differ in their requirement for VNO, the primary sensory organ of the accessory olfactory system, in processing of opposite-sex odors (Keller et al., 2006; Pankevich et al., 2004, 2006).

The intact opposite-sex odor preference in females with BNST lesions suggests that their deficits in preferential vaginal marking cannot be attributed to an inability to discriminate between male and female odors. It also argues for the existence of partially separate and independent neural circuits regulating different forms of precopulatory behaviors. Sexual identity information critical for preferential vaginal marking may be processed by a neural circuit that includes VNO/AOB and BNST, as BNST receives direct input from AOB (Davis et al., 1978) and lesions of either VNO (Petrulis et al., 1999) or BNST (present study) eliminate the normal pattern of elevated marking to males without eliminating social odor preference. Despite its essential role in supporting overall levels of vaginal marking, MA is not critical for preferential vaginal marking (Petrulis and Johnston, 1999) and may be more important for regulating opposite-sex odor preference in females via a BNST-independent pathway.

Role of BNST in flank marking

In contrast to vaginal marking, the normal pattern of elevated flank marking to same-sex odors (Johnston, 1977; Petrulis, 2009) was not disrupted in females with BNST lesions. Although the role of BNST in flank marking has not been extensively examined, lesions of the lateral septum (LS) that also result in BNST damage decrease flank marking by males in response to male odors (Ferris et al., 1990). Furthermore, injections of arginine vasopressin (AVP), a compound known to induce high levels of flank marking when injected into specific brain areas (Ferris et al., 1984), also induce flank marking when injected into the LS/BNST of males (Irvin et al., 1990). Given the present data, it is likely that LS, rather

than BNST, is the critical site for regulating flank marking via its connections with MPOA and MA (Coolen and Wood, 1998; Ferris et al., 1990).

Anatomical considerations

Lesions of animals included in the BNST-X group included damage to the anterior and/or posterior divisions of BNST. Therefore, it is difficult to assign the observed behavioral deficits to particular subdivisions of BNST. There are known anatomical and functional differences between the anterior and posterior divisions (Dong et al., 2001). For example, the posterior division of BNST is more heavily interconnected with MA, and has been implicated in the control of reproductive behaviors (Been and Petrulis, 2010b; Wood, 1998). Although it also receives some input from MA, the anterior division is more strongly interconnected with the central nucleus of the amygdala and appears to be involved in anxiety and stress responses (Davis and Shi, 1999). Given this evidence, it seems likely that deficits in vaginal marking observed in the present study were the result of posterior, rather than anterior, BNST damage. No correlation was observed, however, between the anterior-posterior positioning of lesions and loss of preferential marking, indicating that damage throughout this axis may be sufficient to disrupt preferential marking to males.

Conclusion

The finding that lesions of BNST eliminate preferential vaginal marking to male odors is in general agreement with a role for BNST in the regulation of odor-guided precopulatory behaviors. Specifically, BNST may process odor signals derived from primary olfactory structures to ultimately generate a preferential behavioral response to opposite-sex odors. In female hamsters, this area appears to play a rather specific role in preferential vaginal marking, whereas in males it is critical for preferential odor investigation. These data suggest that the neural circuitry regulating precopulatory behaviors are not only differentiated between types of female precopulatory behaviors such as vaginal marking and oppo-

site-sex odor preference, but that this circuit is also differentiated between the sexes. Further studies are needed to understand the underlying neural mechanisms whereby BNST regulates context-appropriate precopulatory responses in both males and females.

3.6 Acknowledgements

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Chapter 3 Tables

Table 3.1 Odor preference and vaginal marking of partial lesion subjects

Partial Lesion (see Introduction) females vaginal marked more in response to male compared to female odors on diestrous day 2 and proestrus. These females also investigated male odors more than the female in both the Non-contact and Contact tests. [#]*p* < .05 and **p* < .01 for male vs. female within each test. All data are mean ± SEM.

Vaginal marking				Non-contact odor preference		Contact odor preference	
Diestrous day 2		Proestrous		Male	Female	Male	Female
Male	Female	Male	Female	Male	Female	Male	Female
7.0 ± 1.9 [#]	3.6 ± 1.0	15.7 ± 2.1*	8.1 ± 1.8	47.9 ± 3.7*	25.2 ± 5.1	64.4 ± 6.2*	26.7 ± 2.7

Table 3.2 Flank marking and lordosis of SHAM and BNST-X subjects

During scent marking tests, females flank marked more to female odors than to male odors, $p < .01$. The interaction of odor by lesion group was not significant, $p = .34$. BNST-X females did not significantly differ from SHAM females in lordosis latency or duration, all $p > .05$. Data are presented as mean \pm SEM.

	Number of flank marks		Lordosis latency	Lordosis duration
	Male	Female		
SHAM	13.4 \pm 3.0	17.2 \pm 4.6	49.2 \pm 17.3	16.9 \pm 3.1
BNST-X	7.8 \pm 1.6	16.1 \pm 3.2	45.6 \pm 9.6	17.7 \pm 2.0

Chapter 3 Figures

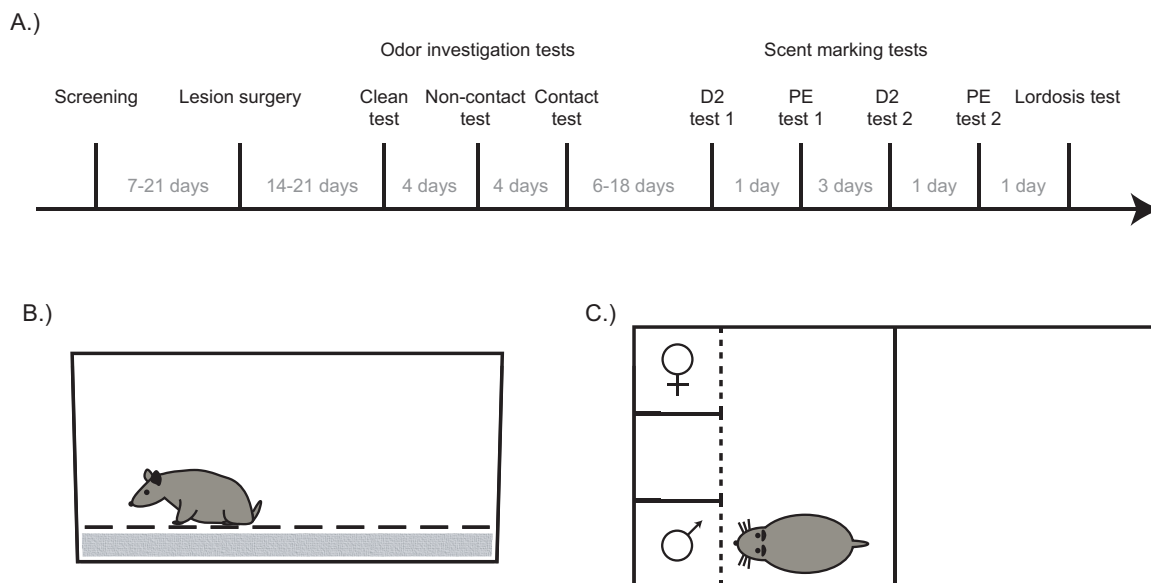


Figure 3.1 Timeline and testing apparatus

A. Timeline of behavioral testing and surgeries in experiment. D2=diestrus day 2; PE=proestrus. B. Side view of testing apparatus for Scent-marking tests. C. Top-down view of apparatus used for Odor investigation tests.

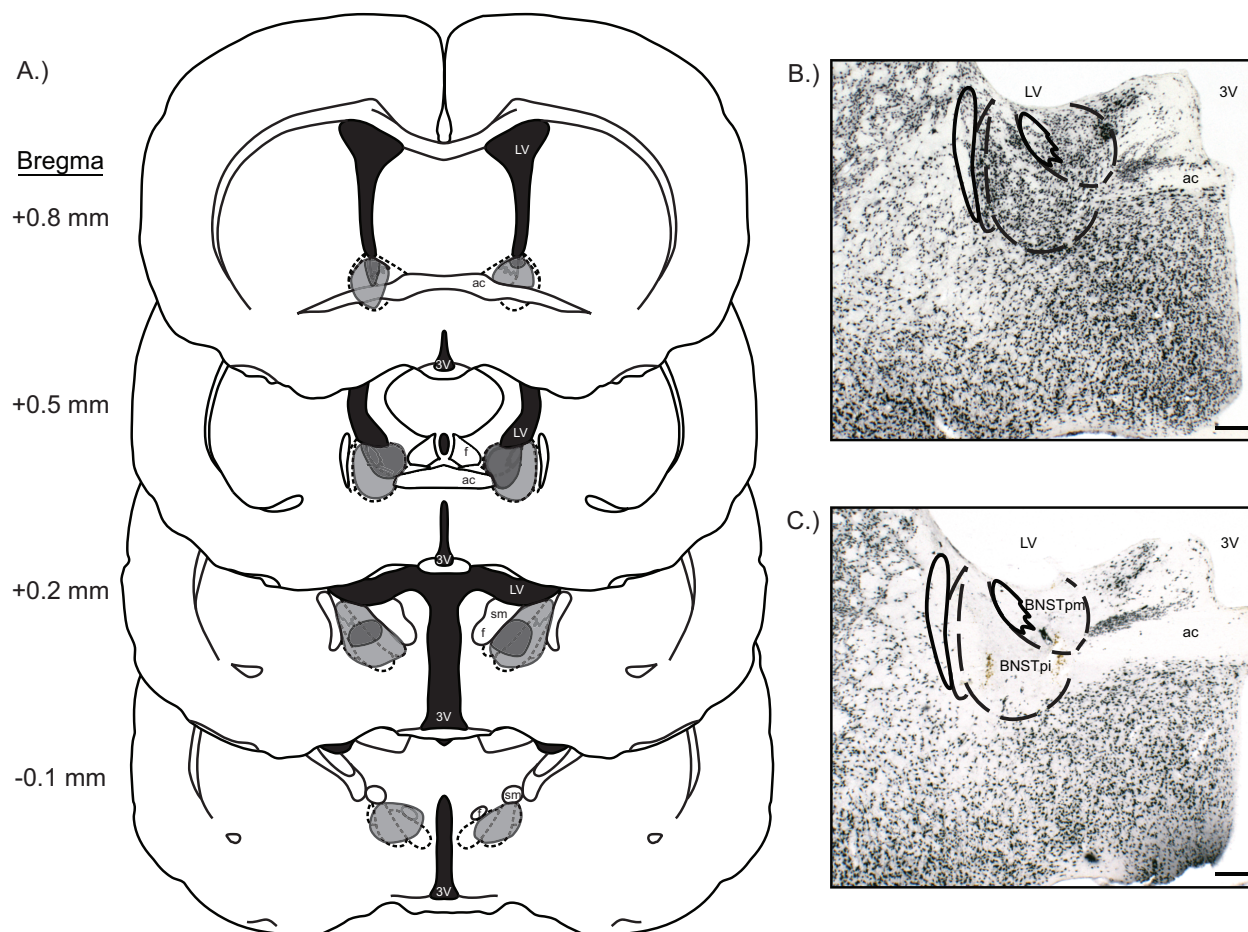


Figure 3.2 Lesion reconstruction

A. Coronal sections depicting largest (light gray) and smallest (dark gray) excitotoxic lesion damage in bed nucleus of the stria terminalis of female hamsters included in the BNST-X group. Sections are organized from anterior (top) to posterior (bottom), all relative to bregma. Neuronal damage was visualized following immunohistochemistry for NeuN for both (B) SHAM and (C) BNST-X subjects. In all cases, damage depicted on atlas plates that comprised less than 50% of BNST was derived from females with greater than 50% damage to BNST on two other consecutive plates (see Results section). Scale bar = 200 μ m. 3V=third ventricle; LV=lateral ventricle; ac=anterior commissure; sm=stria medullaris; f=fornix.

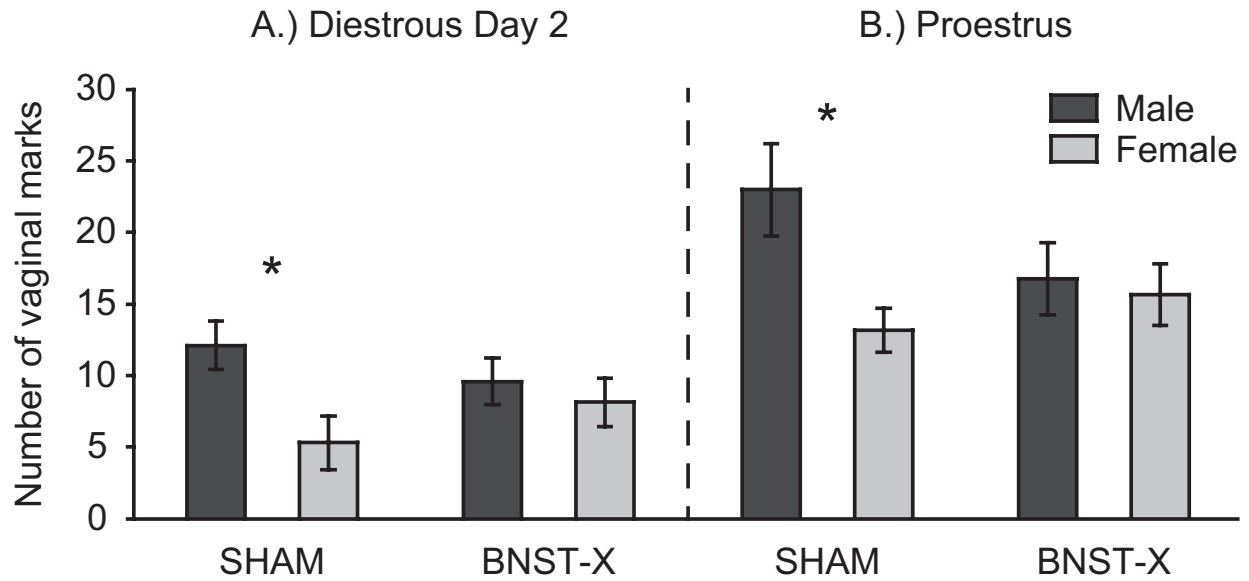


Figure 3.3 Mean (\pm SEM) number of vaginal marks to male and female odors

SHAM, but not BNST-X, females preferentially marked at higher levels to male odors compared to female odors on both (A) diestrus day 2, and (B) proestrus. * $p < .01$, male vs. female odor condition.

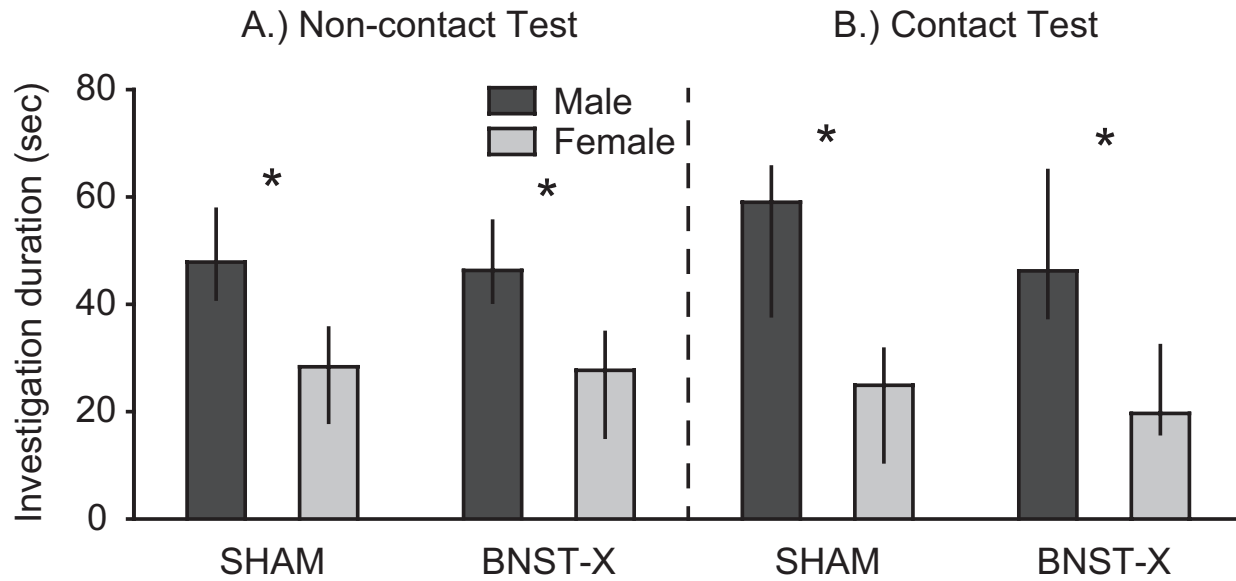


Figure 3.4 Median (\pm IQR) duration of odor investigation

Subjects investigated male odors more than female odors, irrespective of lesion condition, in both the (A) Non-contact and (B) Contact tests. * $p < .01$, male vs. female within each test.

**CHAPTER 4: THE MEDIAL PREOPTIC AREA IS NECESSARY FOR SEXUAL ODOR PREFERENCE, BUT NOT
SEXUAL SOLICITATION, IN FEMALE SYRIAN HAMSTERS**

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4.1 Abstract

Precopulatory behaviors that are preferentially directed towards opposite-sex conspecifics are critical for successful reproduction, particularly in species wherein the sexes live in isolation, such as Syrian hamsters (*Mesocricetus auratus*). In females, these behaviors include sexual odor preference and vaginal scent marking. The neural regulation of precopulatory behaviors is thought to involve a network of forebrain areas that includes the medial amygdala (MA), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA). Although MA and BNST are necessary for sexual odor preference and preferential vaginal marking to male odors, respectively, the role of MPOA in odor-guided female precopulatory behaviors is not well understood. To address this issue, female Syrian hamsters with bilateral, excitotoxic lesions of MPOA (MPOA-X) or sham lesions (SHAM) were tested for sexual odor investigation, scent marking, and lordosis. MPOA-X females did not investigate male odors more than female odors in an odor preference test, indicating that MPOA may be necessary for normal sexual odor preference in female hamsters. This loss of preference cannot be attributed to a sensory deficit, since MPOA-X females successfully discriminated male odors from female odors during an odor

discrimination test. Surprisingly, no deficits in vaginal scent marking were observed in MPOA-X females, although these females did exhibit decreased overall levels of flank marking compared to SHAM females. Finally, all MPOA-X females exhibited lordosis appropriately. These results suggest that MPOA plays a critical role in the neural regulation of certain aspects of odor-guided precopulatory behaviors in female Syrian hamsters.

4.2 Introduction

Precopulatory behaviors that aid in the identification and localization of potential mating partners are an important component of reproductive behavior for most mammals (Beach, 1976). For species typified by non-cohabitating sexes such as Syrian hamsters (*Mesocricetus auratus*), these behaviors are essential for successful mating (Gattermann et al., 2001; Pfaff et al., 2008). Female Syrian hamsters engage in a number of different precopulatory or solicitational behaviors, including vaginal marking (a stereotyped scent marking behavior resulting in deposition of vaginal secretion) and preferential approach towards, and investigation of, opposite-sex odors (Petrulis, 2009). Although both vaginal marking and opposite-sex odor preference are behavioral responses that are preferentially directed towards male compared to female odors (Johnston, 1977; Martinez and Petrulis, 2011; Martinez et al., 2010; Petrulis and Johnston, 1999; Petrulis et al., 1999), odor preference is more clearly linked to the identification and localization of potential mating partners, whereas vaginal marking plays a key role in attracting mates. Indeed, the deposited secretion is highly attractive to male hamsters (Johnston and Schmidt, 1979; Johnston, 1974; Kwan and Johnston, 1980), and females deposit these marks in such a way as to direct the male to her nesting area (Lisk et al., 1983).

The expression of both sexual odor preference and vaginal marking depends critically on an interconnected set of brain areas that are more broadly involved in processing conspecific odor information (Petrulis, 2009). These areas include the medial amygdala (MA), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA) (Wood, 1998). Odor information detected by

the main and accessory olfactory systems is initially processed by MA and relayed to MPOA, either directly or via BNST (Coolen and Wood, 1998; Wood and Swann, 2005). Not surprisingly, neurons in these brain areas exhibit selective activation to opposite- vs. same-sex odors in both male and female hamsters (DelBarco-Trillo et al., 2009; Maras and Petrulis, 2010a). These areas also appear to play specific, functional roles in female precopulatory behaviors. For example, lesions of MA eliminate preferential investigation of male vs. female odors and reduce overall levels of vaginal marking by female hamsters, but do not eliminate preferential vaginal marking in response to male odors (Petrulis and Johnston, 1999). In contrast, females with lesions of BNST do not vaginal mark preferentially to male odors, but do display a normal preference to investigate male odors more than female odors (Martinez and Petrulis, 2011). These data suggest that although BNST may be a critical component of the neural circuit regulating vaginal marking responses to sexual odors, it is not necessary for the expression of sexual odor preference; therefore, odor information relevant for sexual odor preference processed by MA likely bypasses BNST and continues to other limbic/hypothalamic areas connected to MA, such as MPOA.

Although there is substantial evidence suggesting MPOA is broadly involved in regulating sexual behavior in rodents (Hull and Dominguez, 2007; Sakuma, 2008), its specific role in odor-guided female precopulatory behaviors is not clear. In rats, radiofrequency lesions of MPOA decrease the amount of solicitational behaviors towards, and time spent with, a sexually-experienced male, and disrupt females' preference for intact compared to castrated male rat odors (Xiao et al., 2005). Furthermore, excitotoxic lesions of MPOA decrease the preference of female rats to spend time with intact males compared to estrous females (Guarraci and Clark, 2006). However, it should be noted that other researchers have found no effects of electrolytic lesions of MPOA on sexual odor/partner preference in female rats (Paredes et al., 1998) or ferrets (Robarts and Baum, 2007). Although comparable data for the role of MPOA in sexual odor preference in female hamsters is not available, this area does appear to be involved in other precopulatory behaviors that can be induced by opposite-sex odors. Indeed, large elec-

trolytic lesions of MPOA that also damaged BNST decrease vaginal marking during interactions with males (Malsbury et al., 1977), and radiofrequency lesions of MPOA decrease ultrasonic vocalizations by females following exposure to male hamsters (Floody, 1989).

A significant limitation of previous studies examining the role of MPOA in odor-guided precopulatory behaviors is the lack of specificity in disrupting MPOA vs. nearby areas such as BNST. This is a critical issue given that these areas are highly interconnected and share similar patterns of connectivity with other brain areas that regulate precopulatory behaviors (Coolen and Wood, 1998; Simerly and Swanson, 1986, 1988; Wood and Swann, 2005). As mentioned above, we have recently utilized discrete, excitotoxic lesions in order to determine the role of BNST in preferential vaginal marking and sexual odor investigation (Martinez and Petrulis, 2011); however, it is not known if MPOA plays either a comparable or dissociable role from that of BNST in the regulation of these behaviors. In order to address this issue, we administered excitotoxic lesions of MPOA to female Syrian hamsters and measured effects of lesions on sexual odor investigation and scent-marking responses. Given that specific lesions of MPOA disrupt sexual odor preference in male hamsters (Been and Petrulis, 2010b), we hypothesized that MPOA would be necessary for the normal expression of preferential investigation of male odors by females. Furthermore, given the previously observed effects of MPOA/BNST disruption (Malsbury et al., 1977), and considering that implants of estradiol specifically into MPOA increase the expression of vaginal marking (Takahashi and Lisk, 1987; Takahashi et al., 1985), we hypothesized that MPOA would also be necessary for maintaining overall levels of vaginal marking.

4.3 Methods

Overview of design

Subjects were initially screened for adequate levels of vaginal marking to male odors (> 5 marks/10 min), and then received either bilateral, excitotoxic lesions of MPOA or sham surgeries. Fol-

lowing recovery, subjects underwent a series of behavioral tests. First, subjects were tested for their investigatory responses to male and female odors (Odor investigation tests). This consisted of an initial test to familiarize subjects with the testing apparatus (Clean test), followed by a volatile odor preference test utilizing conspecific odor stimuli (Preference test). Second, subjects were tested for their ability to discriminate the sexual identity of odor stimuli using a habituation-discrimination task (Odor discrimination test). Subjects were then tested for scent-marking responses to male or female stimuli on two days of the estrous cycle, diestrous day 2 and proestrus. Finally, to verify that MPOA lesions had not disrupted the ability of females to display copulatory behavior, subjects were tested during behavioral estrus for receptive sexual responses to a sexually experienced male.

Subjects

Adult female Syrian hamsters (*Mesocricetus auratus*) were purchased from Harlan Laboratories (Indianapolis, IL, USA) at approximately 7-9 weeks of age. In addition to experimental subjects, a separate group of unrelated adult male and female Syrian hamsters was purchased from Harlan Laboratories to serve as stimulus animals. Animals were either individually housed (experimental subjects) or group housed (3-4 same-sex animals per cage; stimulus animals) in solid-bottom polycarbonate cages containing corncob bedding and cotton nesting material (Nestlets; Ancare, Bellmore, NY). Subjects and stimulus animals were maintained on a reversed light cycle (14:10 light:dark; lights out at 10 am), with all behavior testing occurring during the first four hours of the dark portion of the light cycle. Food and water were available *ad libitum*. Animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and approved by the Georgia State University Institutional Animal Care and Use Committee. It should be noted that none of the survivable manipulations utilized in the present study resulted in animal mortality; furthermore, that all efforts were made to minimize the total number of animals used and their suffering.

Estrous cycle monitoring

Prior to screening for sufficient vaginal marking levels, subjects were examined daily for eight consecutive days in order to determine their stage of the estrous cycle. Subjects were gently restrained while vaginal secretion was manually extruded using a disposable probe, and the consistency of the secretion was examined for stringy consistency indicative of behavioral estrus (Orsini, 1961). Once the day of behavioral estrus was identified, the two cycle days prior to estrus were defined as diestrous day 2 and proestrus, respectively (Johnston, 1977). Estrous cycles were also monitored for eight days following surgery to ensure that this procedure had not disrupted cyclicity. Finally, in order to verify that estrous cycle stage had been properly inferred from cyclic changes in vaginal secretion consistency, females were tested for sexual receptivity in response to a male prior to the conclusion of behavioral testing (see Lordosis test below). In all cases, 'day' refers to the dark phase of the light cycle.

Surgery

At two to three months of age, subjects were assigned to either a MPOA lesion group (MPOA-X) or a sham lesion group (SHAM). A matched random assignment procedure was used, such that initial levels of vaginal marking in response to male odors on proestrus were equivalent across subjects assigned to the MPOA-X and SHAM groups (see below). Subjects were first anesthetized with 2-3% isoflurane gas (Baxter, Deerfield, IL) in an oxygen (70%) and nitrous oxide (30%) mixture, and then placed within a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with ear- and incisor-bars positioned such that the top of the skull was level. Following a midline scalp incision, the skin and underlying temporal muscles were retracted to expose the skull. A hand-operated drill was then used to make holes in the skull in order to expose dura. For MPOA-X subjects, the excitotoxin *N*-methyl-D-aspartic acid (20 mg/ml, 25 nl per injection site; Sigma, St. Louis, MO) was injected bilaterally via a Hamilton microinjection syringe (701R 10 μ l syringe; Hamilton, Reno, NV) under stereotaxic control. A single injection of excitotoxin was made per hemisphere, using the following coordinates: Anterior-posterior, +1.7 mm (rela-

tive to bregma); medial-lateral, ± 0.6 mm (relative to bregma); dorsal-ventral, -7.4 mm (relative to dura) using published anatomical plates of the Syrian hamster brain (Morin and Wood, 2001). The excitotoxin was expelled over the course of one minute, and the syringe needle was then left in place for an additional nine minutes to allow sufficient time for the injection volume to disperse from the syringe tip. Sham surgeries were conducted in a similar manner to lesion surgeries except that no liquid was infused into the target sites.

Immediately prior to completion of all surgeries, the skull holes were sealed with bone wax and the incision closed with wound staples. Subjects were then injected with an analgesic agent (5 mg/kg; Ketofen, Fort Dodge Animal Health, Fort Dodge, IA). All subjects were allowed to recover for at least 14 days prior to post-operative behavioral testing.

Scent-marking tests

Odor stimulus and apparatus

Stimuli for scent-marking tests consisted of vacated cages (43 x 22 x 20 cm) previously occupied by male or female stimulus animals for four days. Estrous cycles of stimulus females were not monitored; however, given that each cage contained at least three females, and the occupation period comprised the complete four-day estrous cycle, it is likely that female cage stimuli was a composite of multiple cycle days. Approximately one to four hours prior to use, a researcher blind to the experimental condition of subjects prepared the stimulus cages as follows: Stimulus animals were removed from their cages along with any food pellets and caked urine present in the corncob bedding, the soiled cotton nesting material was distributed evenly across the bottom of the cage, and a 40 cm x 19 cm x 0.4 cm perforated Plexiglas plate was placed over the bedding just prior to testing. This plate was centered within the cage such that bedding within three cm of the outer walls of the stimulus cages remained directly accessible to subjects. The surface of the plate was painted with black non-toxic chalkboard

paint (Rust-oleum, Vernon Hills, IL), thereby dividing the plate into four painted quadrants separated by an unpainted area in the shape of a cross. This plate aided observation and quantification of vaginal marking behavior by elevating the subject out of the bedding material; in addition, the divisions on the plate provided a means for quantifying locomotor activity during scent-marking tests.

Testing protocol

During each ten-minute scent-marking test, females were placed within a soiled stimulus cage and the number of vaginal and flank marks were scored using a hand counter by a researcher blind to experimental condition of the subjects. Vaginal marking and flank marking are discrete, stereotyped behaviors that are differentially expressed in response to conspecific odors (Johnston, 1977). In contrast to vaginal marking, however, flank marking occurs more frequently in response to female than to male odors, and appears to function predominantly in territorial advertisement (Johnston, 1985). A flank mark was scored each time the female moved forward while maintaining contact between the flank region and the side of the stimulus cage. A vaginal mark was scored each time the female moved forward with tail deflected upwards while maintaining contact between the perineum and the underlying substrate (Been et al., 2012). Tests were also recorded using a digital camcorder and videos were scored for the number of quadrant entries by researchers blind to the experimental conditions of the subjects, with an inter-rater reliability of 90% or greater. Entry into a quadrant was scored whenever greater than 50% of the body mass of the subject crossed from one quadrant into another. At the completion of each scent-marking test, the female was removed from the stimulus cage and returned to its home cage. Stimulus cages were not reused; rather, only one female was tested per stimulus cage.

Odor investigation tests

Stimuli

Stimuli for odor investigation tests were collected from the cages of group-housed same-sex stimulus animals by researchers blind to the experimental condition of the subjects. During collection, approximately 50 ml of soiled corncob bedding and 12 g of soiled cotton nesting material were placed into a one-quart re-sealable plastic collection bag. In addition, a total of three separate damp gauze pads (10 x 10 cm) were used to wipe down the walls of the cage, the anogenital region, and the bilateral flank glands of odor donors, and these pads were included in the collection bag. Vaginal secretion from odor donor females was collected onto an additional gauze pad by gently palpating the vaginal area with a disposable probe, and included in female odor stimuli collection bags. Hamsters investigate female odors collected on different days of the estrous cycle at relatively equivalent levels (Johnston, 1980); therefore, female stimuli (vaginal secretion, cage stimuli, and body odors) were collected irrespective of cycle day of odor donors. Clean odor stimuli consisted of 10 ml of clean corncob bedding, four grams of clean cotton nesting material, and one clean cotton gauze pad. Once collected, all odor stimuli were stored at 4°C until 30 minutes prior to use.

Apparatus and testing protocol

Odor investigation tests were conducted in a three-choice odor investigation apparatus by a researcher blind to the experimental condition of the subjects. This apparatus consisted of a modified 51 cm x 25.5 cm x 30.5 cm glass aquarium with opaque paper lining the exterior of the four vertical glass walls and the glass floor. A black line drawn parallel to the short axis of the apparatus bisected the available floor space, allowing activity levels to be quantified. Three 8 cm square acrylic odor containers were attached along the inside of one short wall of the apparatus. Each odor container had a perforated door to allow subjects to investigate the volatile components of the stimuli without allowing direct access to

the contents of the container. The top of the apparatus was secured with a clear Plexiglas lid, allowing for overhead video recording of the subject throughout the behavioral test.

Subjects were tested on two separate occasions in the three-choice odor investigation apparatus (Clean test and Preference test). Testing occurred across two consecutive estrous cycles, with each test occurring on the cycle day of estrus. Following recovery from stereotaxic surgery, subjects were first tested with clean odor stimuli in each of the three odor containers. This test served to habituate the subjects to the testing protocol. Subjects were then tested in the Preference test. During this test, one of the two outer odor containers contained male odor stimuli, the other outer container contained female odor stimuli, and the center container contained clean odor stimuli. This pattern of odor container placement was designed to maximize the discriminability of male and female odors; however, given that the position of the box containing clean odor stimuli was not alternated during testing, investigatory behavior towards the center box was not included in behavioral analyses for the Preference test.

Subjects were allowed to freely explore the apparatus for ten minutes, and upon completion of the test, subjects were removed, returned to their home cages, and the apparatus was cleaned thoroughly with 70% ethanol. Odor containers were emptied and cleaned with 70% ethanol. Prior to testing with another subject, fresh odor stimuli were added to the odor containers and the containers were replaced within the apparatus. The left/right positioning of the male and female odor stimuli containers was counterbalanced across subjects.

All behavior tests were recorded using a digital camcorder and videos from each subject were then scored using the Observer for Windows, version 9.0 (Noldus Information Technology B.V., Wageningen, the Netherlands). Researchers blind to the experimental conditions of the subjects scored the videos, with an inter-rater reliability of at least 90%. Within each test, the number of midline crosses and the duration of investigation of each of the three odor containers were scored. Investigation was scored whenever the snout of a subject came within one cm of the perforated front panel of an odor box.

Odor discrimination test

Following the completion of odor investigation tests, subjects were tested for their ability to discriminate the sexual identity of conspecific odors. This was accomplished using a habituation-discrimination task, wherein an initial odor type is presented repeatedly, followed by a final presentation of a second (different) odor type. A decrease in investigation across repeated presentations of the initial odor type (habituation) is expected if the subject recognizes the odors as similar, whereas an increase in investigation during presentation of the second odor type indicates that the subject recognizes this odor as different from the initial odor type (discrimination) (Johnston, 1993).

Subjects were tested for odor discrimination on the day of estrus, by a researcher blind to the experimental condition of the subjects. The testing procedure consisted of four consecutive three-minute presentations of female odor stimuli, followed by a final three-minute presentation of male odor stimuli. Presentations were separated by a five-minute inter-trial interval. All subjects were tested using female odors as the habituation odor, as it has been our experience that normal female hamsters do not readily habituate to repeated presentations of male odors. In order to ensure that subjects were habituating to the sexual, rather than individual, identities of stimulus females, each of the four presentations of female stimuli were derived from unique odor donor cages. Stimuli were collected, stored, and presented in containers as previously described for odor investigation tests. Odor containers were affixed to an inner wall of the subject's home cage, in order to prevent the subject from moving the odor container throughout the cage. Investigation was scored whenever the snout of a subject came within one cm of the perforated front panel of the odor container.

Lordosis test

Subjects were tested for the expression of sexual receptivity (lordosis) on the predicted day of behavioral estrus by a researcher blind to the experimental condition of the subjects. Lordosis is a posture female hamsters assume to allow copulation with males, and is identified as the appearance of a

prolonged immobile posture, an elevated rump, and a level/concave spine (Tiefer, 1970). During lordosis tests, subjects were placed in the home cage of a sexually experienced male and the two animals were allowed to freely interact. Stimulus males were removed from the cage once subjects displayed lordosis (typically following anogenital investigation of the female by the male), but before any mounting could occur. The latency to assume lordosis was defined as the length of time from when the female was first introduced into the male's cage until she first exhibited the lordosis posture, whereas the duration of lordosis was defined as the length of time from when the female initiated the posture until she resumed locomotor activity (e.g., walking, lateral head/body movements). Both lordosis latency and duration were scored live using hand-held stopwatches. Tests were concluded when the female exited the lordosis posture, or when ten minutes had elapsed.

Histology and lesion verification

Upon completion of behavioral testing, subjects were administered a lethal dose of sodium pentobarbital (0.2 ml, i.p.; SleepAway, Fort Dodge Animal Health, Fort Dodge, IA), and transcardially perfused with 200 ml of 0.1 M phosphate buffered saline (PBS) followed by 200 ml of 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Brains were removed and post-fixed in 10% neutral buffered formalin (overnight) followed by 20% sucrose in PBS (1-2 days). Coronal sections (30 μ m) were taken using a cryostat and stored in cryoprotectant at -20°C until processed for immunohistochemistry for neuronal nuclei protein (NeuN), a protein specific to neurons (Mullen et al., 1992). The extent of lesion damage was then determined by a researcher blind to the experimental condition of the subjects, via examination of NeuN-stained tissue under a light microscope and comparison against published neuro-anatomical plates of the Syrian hamster brain (Morin and Wood, 2001).

Immunohistochemistry

Free-floating coronal sections were removed from cryoprotectant, rinsed in PBS, and then incubated in a solution containing PBS, 0.4% Triton-X (Sigma, St. Louis, MO), and 1:20,000 monoclonal mouse anti-NeuN antibody (MAB377; Millipore, Billerica, MA) for ~18 hours at room temperature. After rinsing in PBS, tissue sections were incubated in a solution of PBS, 0.4% Triton-X, and 1:600 biotinylated horse anti-mouse secondary antibody (BA-2000; Vector Laboratories, Burlingame, CA) for one hour at room temperature, rinsed in PBS, and then incubated in a solution of PBS, 0.4% Triton-X, and 1:200 avidin-biotin complex (Vectastain Elite ABC kit; Vector Laboratories) for one hour at room temperature. Tissue sections were then rinsed in PBS followed by 0.175 M sodium acetate, and reacted in a nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride solution (2 mg 3,3'-diaminobenzidine tetrahydrochloride plus 250 mg nickel (II) sulfate with 8.3 μ L 3% hydrogen peroxide per 10 mL of 0.175 M sodium acetate; Sigma, St. Louis, MO) for 15 minutes at room temperature. Tissue sections were then rinsed in 0.175 M sodium acetate followed by PBS in order to terminate the chromagen reaction.

Data analysis

All data were analyzed using SPSS for Windows, version 18.0 (SPSS Inc., Chicago, IL). Data were first examined to determine if the assumptions of parametric statistical tests were met. When assumptions were violated, attempts were first made to normalize the distributions using applicable data transformations (Osborne and Overbay, 2004; Sheskin, 2000); however, for the duration of investigation of male/female odors in the odor preference test, these transformations were unsuccessful. In this case the deletion of an extreme outlier (> 3 SD above the mean) was necessary. For all statistical tests, results were considered to be statistically significant if $p < .05$.

For odor investigation tests, odor box investigation durations were subjected to a mixed design 2 X 2 ANOVA with lesion (MPOA-X vs. SHAM) as an independent factor and odor box (clean test: left vs. right; preference test: male vs. female) as a repeated factor. A significant interaction was further exam-

ined for the effect of odor within each lesion group via separate dependent-samples *t*-tests. The total number of crossings between sections of the apparatus was compared across lesion groups using independent-samples *t*-tests. For odor discrimination tests, the log-transformed duration of odor box investigation was subjected to a mixed-design 2 X 3 ANOVA with lesion (MPOA-X vs. SHAM) as an independent factor, odor (first female odor vs. fourth female odor vs. first male odor) as a repeated factor. The main effect of odor was further examined via dependent-samples *t*-tests with Bonferroni correction for multiple comparisons.

Behavioral measures from scent-marking tests (number of vaginal and flank marks) were analyzed via 2 X 2 X 2 mixed-design ANOVAs, with lesion (MPOA-X vs. SHAM) as an independent factor, and cycle day (D2 vs. PE) and odor (male vs. female) as repeated factors. Statistically significant two-way interactions were further examined for the effect of odor within each cycle day via separate dependent-samples *t*-tests. Finally, behaviors measured during lordosis tests (latency to assume lordosis, duration of lordosis) were compared across lesion groups using independent samples *t*-tests.

4.4 Results

Lesion reconstruction

Subjects were included in the MPOA-X group ($n = 9$) if bilateral damage to approximately 60% of MPOA was observed on two consecutive plates of the Syrian hamster atlas (Figure 4.1) (Morin and Wood, 2001). Although some MPOA-X females sustained bilateral damage at more anterior levels of MPOA (0.5 mm anterior to bregma, $n = 4$), every subject in this group sustained substantial bilateral damage to MPOA at the level of the juncture of the lateral and third ventricles (0.2 mm anterior to bregma, $n = 9$). Furthermore, most MPOA-X females sustained bilateral damage at more posterior levels of MPOA (-0.1 mm posterior to bregma, $n = 7$). Finally, one MPOA-X subject had significant bilateral damage to the anterior hypothalamus (AH; bregma -0.3 mm, $n = 1$). It is important to note that subjects

included in the MPOA-X group also sustained minor damage to BNST (less than 25% bilaterally on any single atlas plate).

Subjects that received excitotoxin injections resulting in less than 60% bilateral damage to MPOA on at least two consecutive atlas plates were excluded from the MPOA-X group. In these females, MPOA damage was restricted to the same rostral-caudal extent as in MPOA-X females. Females with partial MPOA lesions were further divided into two groups based on the extent of concurrent BNST damage: Partial MPOA-X (less than 25% bilateral damage to BNST on any atlas plate; $n = 19$), and Partial BNST/MPOA-X (greater than 25% bilateral damage to BNST on any atlas plate; $n = 18$). In order to determine if this concurrent damage to BNST (in the absence of more substantial MPOA damage) was sufficient to drive behavioral effects, both partial lesion groups were directly compared to shams for all measures of odor investigation, scent marking and copulatory behavior. Neither Partial MPOA-X nor Partial BNST/MPOA-X females differed from SHAMs on any measure (all $p > .05$); therefore, data from partial lesion groups were not included in any comparisons of MPOA-X vs. SHAM females (Table 4.1). Although needle tracts were typically visible in the brains of SHAMs, no significant damage was observed to any brain areas in these females.

Odor investigation tests

Clean test

Females did not differentially investigate the left and right boxes when both boxes contained clean cage stimuli, $F(1,23) = 2.00$, $p = .17$, $\eta_p^2 = .08$, indicating that females were not biased in their investigation across the two outer boxes. Furthermore, there was no evidence to suggest that there were any differences in odor box investigation between the two lesion groups, as neither the main effect of lesion ($F(1,23) = 0.22$, $p = .64$, $\eta_p^2 = .01$) nor the odor box by lesion interaction ($F(1,23) = 0.59$, $p = .45$, η_p^2

= .03) were statistically significant. Finally, locomotor activity was equivalent across both lesion groups, $t(23) = 1.51, p = .14, d = .65$.

Odor preference test

Preferential investigation of male vs. female odors was disrupted in females with lesions of MPOA (Figure 4.2). Overall, females exhibited a significant preference for male vs. female odors, $F(1,22) = 21.18, p < .001, \eta_p^2 = .49$; however, the odor box by lesion interaction was significant, $F(1,22) = 5.50, p = .03, \eta_p^2 = .20$, indicating that this effect was not consistent across lesion groups. Whereas SHAM females investigated male odors more than female odors, $t(14) = 7.09, p < .001, d = 1.83$, MPOA-X females investigated male and female odors equivalently, $t(8) = 1.12, p = .30, d = .37$. This loss of odor preference cannot be specifically attributed to changes in the investigatory responses to either the male or female odor boxes, as there were no significant differences for MPOA-X vs. SHAM females in time spent investigating male ($t(22) = 0.19, p = .85, d = .08$) or female ($t(22) = -1.89, p = .07, d = .70$) odors.

Odor discrimination test

Females with lesions of MPOA were able to discriminate male odors from female odors in the odor discrimination test (Figure 4.3). Irrespective of lesion group, females differentially investigated the presented odors, $F(2,44) = 21.40, p < .001, \eta_p^2 = .49$. Specifically, females successfully habituated to repeated presentation of female odors, $t(23) = 3.72, p < .05, d = 1.01$, and discriminated female odors from male odors, $t(23) = -5.71, p < .05, d = 1.64$. The interaction of odor by lesion group was not significant, $F(2,44) = 1.24, p = .30, \eta_p^2 = .05$, indicating that the effect of odor was consistent across the two lesion groups.

Scent-marking tests

Vaginal marking

There were no significant effects of MPOA lesions on vaginal marking responses to male or female odors (Figure 4.4). Overall, females vaginal marked more on proestrus compared to diestrus day 2, $F(1,23) = 79.79$, $p < .001$, $\eta_p^2 = .78$, and more in response to male odors compared to female odors, $F(1,23) = 17.97$, $p < .001$, $\eta_p^2 = .44$. This effect of odor was not consistent across cycle days, $F(1,23) = 6.81$, $p = .02$, $\eta_p^2 = .23$. Specifically, females vaginal marked more to male odors compared to female odors only on proestrus, $t(24) = 4.73$, $p < .001$, $d = .95$, not diestrus day 2, $t(24) = 1.46$, $p = .15$, $d = .30$. Overall levels of vaginal marking did not differ across lesion groups, $F(1,23) = 1.77$, $p = .20$, $\eta_p^2 = .07$, and the effects of cycle day and odor condition on vaginal marking (as described above) did not differ across lesion groups (cycle day x lesion: $F(1,23) = .03$, $p = .87$, $\eta_p^2 = .001$; odor x lesion: $F(1,23) = 2.84$, $p = .11$, $\eta_p^2 = .11$).

Flank marking

Lesions of MPOA disrupted the expression of flank marking by females (Figure 4.5). Although females in both groups flank marked more in response to female odors compared to male odors, $F(1,23) = 22.90$, $p < .001$, $\eta_p^2 = .50$, the overall levels of flank marking were lower in MPOA-X females compared to SHAM females, $F(1,23) = 9.91$, $p = .005$, $\eta_p^2 = .30$. There was no evidence to suggest, however, that MPOA-X females differed from SHAM females in their ability to appropriately target flank marking responses to female odors, given that the interaction of odor by lesion group was not significant, $F(1,23) = 3.63$, $p = .07$, $\eta_p^2 = .14$. Finally, overall levels of flank marking were consistent across both cycle days, $F(1,23) = 2.18$, $p = .15$, $\eta_p^2 = .09$, and the interaction of cycle day by lesion group was not significant, $F(1,23) = .52$, $p = .48$, $\eta_p^2 = .02$.

Lordosis test

All females exhibited lordosis in response to a stimulus male on the predicted day of behavioral estrus. MPOA-X and SHAM females did not differ in latency to exhibit the latency to express lordosis, $t(23) = -.59, p = .56, d = .23$, or the duration of the lordotic response, $t(23) = -1.19, p = .25, d = .44$.

4.5 Discussion

This study provides the first evidence that bilateral lesions of MPOA disrupt sexual odor preference in female Syrian hamsters. MPOA-X females were able to differentially respond to male vs. female odors during the odor discrimination test, suggesting that the deficits observed in the odor preference test likely result from altered motivation to investigate conspecific odors, rather than an inability to distinguish male odors from female odors. MPOA-X females also displayed decreased overall levels of flank marking; however, no deficits in vaginal scent marking or copulatory behavior were observed in these females. Taken together, these results provide partial support for our hypothesis that MPOA is critical for the normal expression of odor-guided precopulatory behaviors in female Syrian hamsters.

Role of MPOA in sexual odor investigation

Our finding that females with MPOA lesions fail to investigate male odors more than female odors is in agreement with previous studies that have identified MPOA as a critical area in regulating appetitive sexual behavior in females. For example, lesions of MPOA in female rats decrease the expression of solicitational movements to a stimulus male (Hoshina et al., 1994; Whitney, 1986) and increase the latency to return to the male during paced mating tests (Guarraci et al., 2004). Furthermore, lesions of MPOA essentially eliminate the preference for intact vs. castrated male odors in female rats (Xiao et al., 2005), as well as the preference to spend time with intact males vs. estrous females when contact with stimulus animals was allowed (Guarraci and Clark, 2006). These data are not consistent, however, with findings of other studies that did not find an effect of MPOA lesions on partner preference involv-

ing direct contact with stimulus animals in female rats (Paredes et al., 1998), or in preference tests in female ferrets that either prevented or allowed direct contact with stimulus animals (Robarts and Baum, 2007). Given that the differences in the results of these studies cannot be easily attributed to the manner of stimulus presentation, the species tested, or the size and placement of MPOA lesions, it seems likely that MPOA plays a highly nuanced role in sexual odor preference in females. This stands in contrast to males, wherein MPOA has been found to be essential for the normal expression of sexual odor preference in several different species across multiple testing protocols (Been and Petrulis, 2010b; Hurtazo and Paredes, 2005; Paredes and Baum, 1995; Paredes et al., 1998). It may be other hypothalamic nuclei play a role in female sexual odor preference that is directly comparable to that of MPOA in males; however, with the exception of the established role of the ventromedial hypothalamus in female ferret odor/partner preference (Robarts and Baum, 2007), these data are presently lacking.

The disruption of odor preference observed in MPOA-X females provides support for a model wherein MPOA is a component of a larger neural circuit that regulates sexual odor preference in female hamsters. Sexual odor information reaches MPOA indirectly, predominantly via connections with MA and BNST (Wood, 1998). In females, lesions of MA completely eliminate sexual odor preference (Petrulis and Johnston, 1999), whereas lesions of BNST do not affect this behavior (Martinez and Petrulis, 2011). Therefore, it may be that sexual odor information relevant for odor preference is transmitted directly from MA to MPOA (bypassing BNST), and that these areas act together as a functional unit in the regulation of sexual odor preference in females. This is an intriguing possibility, given that in males, functional connections between MA and BNST, rather than MA and MPOA, are required for sexual odor preference (Been and Petrulis, 2012).

The underlying neurochemical mechanisms that mediate the effects of MPOA on investigatory responses to sexual odors in females are not well understood. Neurons in MPOA express receptors for estradiol and progesterone (Du et al., 1996; Li et al., 1993), and gonadal steroids strongly regulate other

precopulatory behaviors that are disrupted following MPOA lesions, such as ultrasonic vocalizations (Floody, 1989; Floody et al., 1979). Although there is evidence in rats to suggest that estrogen alone or estrogen plus progesterone treatment may enhance the preference for male stimuli in females (Clark et al., 2004; Xiao et al., 2005), this does not appear to be the case in either mice (Moncho-Bogani et al., 2004) or hamsters (Eidson et al., 2007). Indeed, female hamsters display a robust preference for male odors across the estrous cycle (Eidson et al., 2007; Martinez and Petrulis, 2011; Martinez et al., 2010) and following ovariectomy (Eidson et al., 2007). Therefore, it seems unlikely that either estradiol or progesterone acts within MPOA to critically regulate the expression of sexual odor preference in female hamsters.

Role of MPOA in scent marking

It was surprising to observe no deficit in vaginal marking in MPOA-X females. Previously, Malsbury et al. (1977) reported that females with large, electrolytic lesions of MPOA vaginal mark less in response to a stimulus male compared to sham-operated controls. There are several important differences between the two studies that may explain the differences in findings. First, the lesions of MPOA reported by Malsbury and colleagues were fairly large and appear to have substantially damaged BNST at anterior as well as posterior levels. We have previously reported that fairly discrete, excitotoxic lesions of BNST decrease vaginal marking to male odors (Martinez and Petrulis, 2011); this raises the possibility that the effects of MPOA lesions on vaginal marking reported by Malsbury and colleagues were mediated by BNST, not MPOA. Furthermore, the method whereby the MPOA was lesioned differed across the two studies, with the present study employing excitotoxic lesions and the study by Malsbury and colleagues employing electrolytic or radiofrequency lesions. Although all of these lesion types destroy cell bodies within the targeted area, electrolytic and radiofrequency lesions also result in damage to fibers of passage (Jarrard, 2002). Given that projection fibers from other brain areas such as the lateral septum pass through MPOA, and that disruption of these fibers has behavioral consequences that

are independent of direct damage to neuronal cell bodies within MPOA (Hoshina et al., 1994), it may be that differences in lesion effects between the two studies are due to differential damage to fibers of passage. Finally, the present study also differs from that of Malsbury and colleagues in the manner whereby females were exposed to male stimuli. In the present study, scent-marking tests were conducted in vacated cages that had previously contained males, as females that are allowed to directly interact with males can engage in behaviors that are incompatible with the expression of vaginal marking, such as lordosis. Indeed, MPOA-lesioned females in the study by Malsbury et al. (1977) did express lordosis to the male stimulus animal, thereby reducing the total amount of time those females were free to engage in locomotor-dependent activities such as vaginal marking.

It has also been reported previously that implants of estradiol directly into MPOA increase the expression of vaginal marking in ovariectomized females (Takahashi and Lisk, 1987; Takahashi et al., 1985). This suggests that the changes in vaginal marking that occur across the estrous cycle (Johnston, 1977) may at least be partially mediated by MPOA. It should be noted, however, that implants in MPOA were equally effective at inducing vaginal marking compared to implants in VMH, AH, combined implants in MPOA-VMH, and combined implants in AH-VMH (Takahashi and Lisk, 1987; Takahashi et al., 1985). These data suggest that estradiol's effects on vaginal marking are distributed across multiple brain areas, and therefore areas other than MPOA may have been sufficient to drive the normal levels of vaginal marking we observed in MPOA-X females.

In contrast to MPOA, BNST appears to be a critical component of the neural circuit regulating the odor-guided aspects of vaginal marking (Martinez and Petrulis, 2011). Given the present findings that MPOA is required for the normal expression of sexual odor preference, it would appear that there is a clear disassociation between the neural circuitry that regulates these two forms of odor-guided pre-copulatory behaviors in females. This dissociation may allow for these two behaviors to be differentially regulated by internal or external factors. Indeed, vaginal marking is disrupted following either ovariec-

tomy or blockade of oxytocin receptors, whereas sexual odor preference is normally expressed despite these manipulations (Eidson et al., 2007; Martinez et al., 2010; Takahashi and Lisk, 1983).

The overall decrease in flank marking observed in MPOA-X females suggests that MPOA may be critical for the normal expression of this behavior to conspecific signals. This conclusion is in general agreement with a previous study that reported that females with MPOA or AH lesions flank mark at significantly lower levels in response to a female stimulus animal (Hammond and Rowe, 1976). This effect may be mediated by the neuropeptide arginine vasopressin (AVP), as injections of AVP into MPOA induce flank marking in both male and female hamsters (Albers and Ferris, 1985; Albers et al., 1996), and at least in males, injections of a vasopressin 1a receptor antagonist into this brain area decreases the expression of flank marking induced by same-sex stimuli (Ferris et al., 1985). In addition to MPOA, the neural circuit that regulates flank marking in female hamsters likely includes MA, but not BNST. Lesions of MA completely abolish flank marking to either female or male odors (Petrulis and Johnston, 1999), whereas lesions of BNST do not appear to alter flank-marking responses in females (Martinez and Petrulis, 2011). This would seem to suggest that the expression of flank marking is regulated in a manner similar to sexual odor preference, with MA and MPOA (but not BNST) operating to generate appropriate responses to conspecific odors.

Anatomical considerations

Nearly all females included in the MPOA-X group exhibited some damage to BNST, raising the possibility that the behavioral effects observed in the present study could be attributed to BNST rather than MPOA. This seems unlikely, given that the effects observed in MPOA-X females in the present study are very distinct from the effects observed in BNST-X females that we have reported previously (Martinez and Petrulis, 2011). Therefore, damage to BNST alone is not sufficient to produce the unique set of behavioral effects we observed in the present study. It may be, however, that combined damage to both MPOA and BNST were responsible for the effects we observed here. This is also unlikely to be the case,

given that females with more substantial damage to BNST in addition to lesser damage to MPOA were analyzed as a separate group (Partial BNST/MPOA-X), and these females did not differ significantly from SHAM females on any behavioral measure.

Conclusion

The finding that MPOA lesions disrupt preferential investigation of male odors indicates that this area is a critical component of the neural circuitry regulating sexual odor preference in female hamsters. This area is not, however, required for the preferential expression of vaginal marking to male odors. These results complement and extend our previous finding that BNST is critical for preferential vaginal marking, but not sexual odor preference. Therefore, it appears that there is a clear dissociation in the neural regulation of these two behaviors in female Syrian hamsters. Further research is needed to fully clarify the functional relevance of this dissociation, as well as to determine the underlying neurochemical mechanisms whereby MPOA and BNST regulate odor-guided precopulatory behaviors in females.

4.6 Acknowledgements

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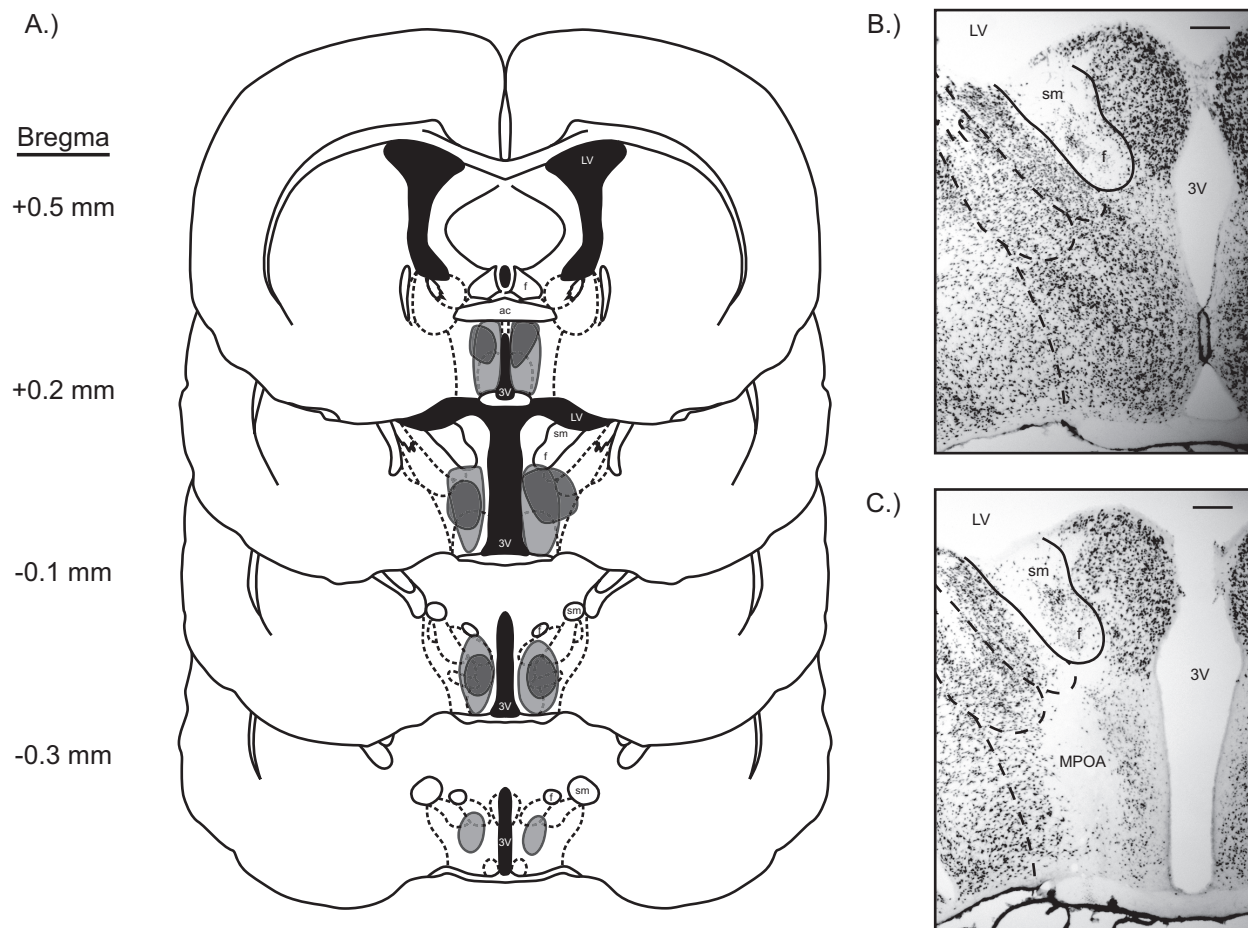
Chapter 4 Table

Table 4.1 Odor preference and scent-marking test results for partial lesion subjects

Females with partial lesions that predominantly damaged MPOA (Partial MPOA-X) or damaged both BNST and MPOA (Partial BNST-X/MPOA-X) investigated male odors more than female odors in the odor preference test. Females with partial lesions also vaginal marked more to male odors compared to female odors on proestrus, but not diestrus day 2, and flank marked more to female odors compared to male odor on proestrus (both groups) and diestrus day 2 (Partial BNST-X/MPOA-X only). Data represented as mean \pm standard error of the mean. * = $p < .05$; # = $p < .01$ (responses to male vs. female odors within each partial lesion group, for each behavioral test).

	Odor Preference		Vaginal Marking				Flank Marking			
	Male	Female	Diestrous Day 2		Proestrus		Diestrous Day 2		Proestrus	
			Male	Female	Male	Female	Male	Female	Male	Female
Partial MPOA-X	84 \pm 11	46 \pm 10*	9 \pm 2	6 \pm 2	19 \pm 3	13 \pm 2*	13 \pm 3	22 \pm 3	11 \pm 2	26 \pm 4#
Partial BNST-X/ MPOA-X	88 \pm 10	41 \pm 5#	5 \pm 1	5 \pm 1	19 \pm 2	11 \pm 2#	6 \pm 1	19 \pm 4#	10 \pm 2	16 \pm 3*

Chapter 4 Figures

**Figure 4.1 Lesion reconstruction**

A. Coronal sections depicting largest (light gray) and smallest (dark gray) excitotoxic lesion damage in the medial preoptic area of female hamsters included in the MPOA-X group. Sections are organized from anterior (top) to posterior (bottom), all relative to bregma. Lesion size was well distributed between the depicted extremes, with the exception of +0.5 mm (seven females had lesions resembling the larger extreme) and -0.2 mm (damage depicted is from a single female). In all cases, damage depicted on a particular atlas comprising less than 60% of MPOA was derived from females with greater than 60% damage to MPOA on two other consecutive plates (see Results section). Neuronal damage was visualized following immunohistochemistry for NeuN for both (B) SHAM and (C) MPOA-X subjects. Scale bar = 200 μ m. 3V=third ventricle; LV=lateral ventricle; ac=anterior commissure; sm=stria medullaris; f=fornix.

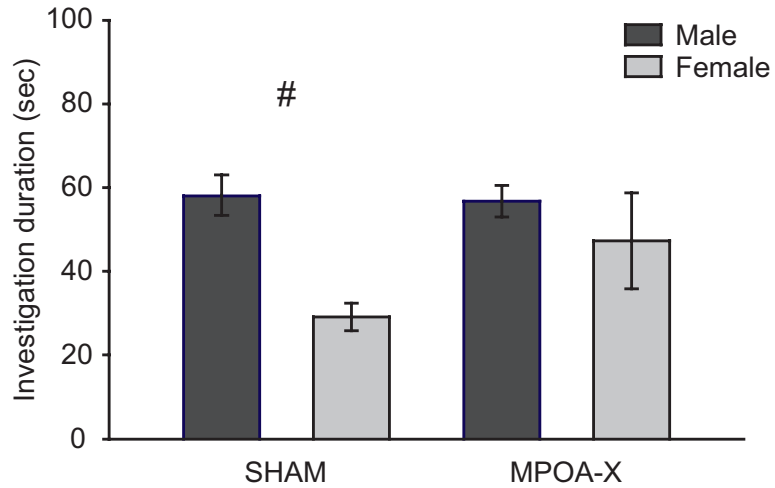


Figure 4.2 Mean (\pm SEM) duration of odor investigation (odor preference test)

In contrast to SHAM females, MPOA-X females failed to investigate male odors significantly more than female odors. # $p < .01$, duration of male odor investigation vs. female odor investigation within the SHAM lesion group.

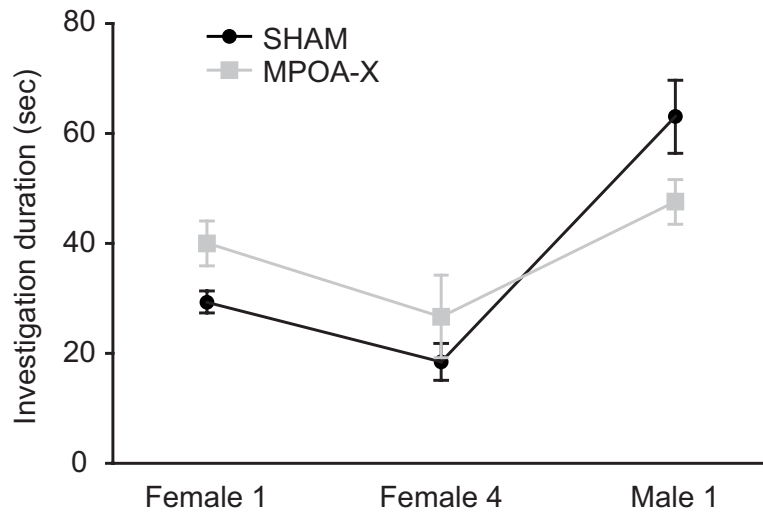


Figure 4.3 Mean (\pm SEM) duration of odor investigation (odor discrimination test)

Females successfully habituated to repeated presentations of female odors and discriminated male odors from female odors. This response to odors did not differ across lesion groups.

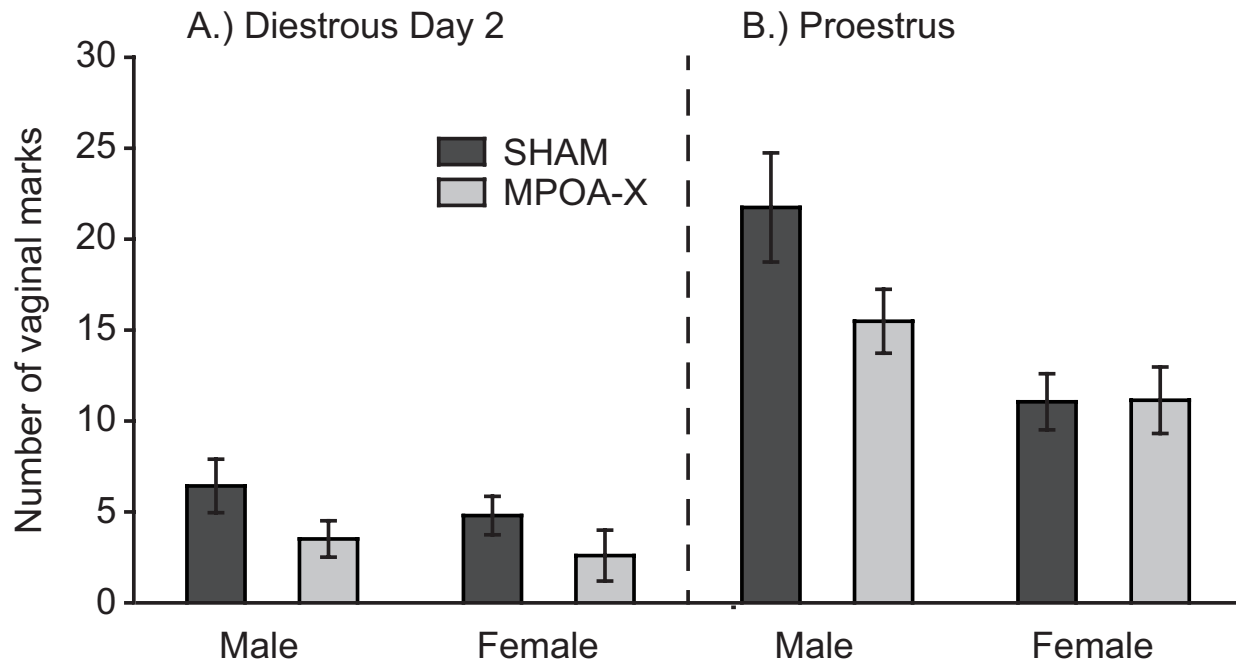


Figure 4.4 Mean (\pm SEM) number of vaginal marks

Females vaginal marked more on (B) proestrus compared to (A) diestrous day 2, and more in response to male odors compared to female odors (only on proestrus). These effects did not differ across lesion groups.

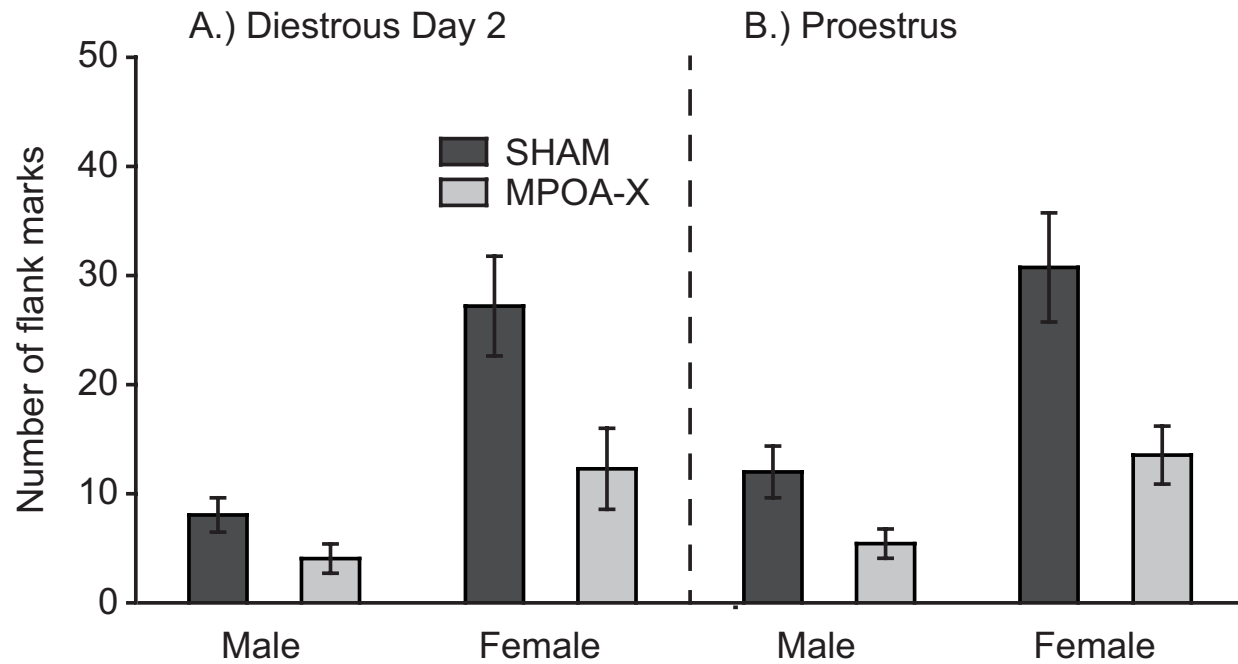


Figure 4.5 Mean (\pm SEM) number of flank marks

Although both MPOA-X and SHAM females flank marked at higher levels to female compared to male odors on (A) diestrus day 2 and (B) proestrus, overall levels of flank marking were lower in MPOA-X females compared to SHAM females.

**CHAPTER 5: ENDOGENOUS OXYTOCIN IS NECESSARY FOR PREFERENTIAL FOS EXPRESSION TO MALE
ODORS IN THE BED NUCLEUS OF THE STRIA TERMINALIS IN FEMALE SYRIAN HAMSTERS**

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5.1 Abstract

Successful reproduction in mammals depends on solicitational behaviors that enhance the probability of encountering potential mates. In female Syrian hamsters, one such behavior is vaginal scent marking. Recent evidence suggests that the neuropeptide oxytocin (OT) may be critical for regulating this behavior. Blockade of OT receptors in the bed nucleus of the stria terminalis (BNST) or the medial preoptic area (MPOA) decreases vaginal marking responses to male odors; lesion data suggest that BNST, rather than MPOA, mediates this effect. However, how OT interacts with sexual odor processing to drive preferential solicitation is not known. To address this issue, intact female Syrian hamsters were exposed to male or female odors and their brains processed for immunohistochemistry for Fos, a marker of recent neuronal activation, and OT. Additional females were injected intracerebroventricularly (ICV) with an oxytocin receptor antagonist (OTA) or vehicle, and then tested for vaginal marking and Fos responses to sexual odors. Colocalization of OT and Fos in the paraventricular nucleus of the hypothalamus (PVH) was unchanged following exposure to male odors, but decreased following exposure to fe-

male odors. Furthermore, ICV injections of OTA, but not a vasopressin 1a receptor antagonist, decreased vaginal marking to male odors. OTA injections also decreased Fos expression to male odors in BNST, but not in MPOA. These data suggest that preferential solicitation of males depends both on suppression of OT neurons in PVH by stimuli from inappropriate (female) targets, and OT-dependent facilitation of neuronal activity in BNST in response to stimuli from appropriate (male) targets.

5.2 Introduction

Precopulatory behaviors that facilitate the identification and localization of potential mating partners are an important component of sexual behavior for most mammals (Beach, 1976). For species wherein the sexes do not typically cohabit as adults, such as Syrian hamsters (*Mesocricetus auratus*), these behaviors are particularly critical for mating success (Gattermann et al., 2001; Pfaff et al., 2008). Female Syrian hamsters engage in a number of different precopulatory or solicitational behaviors, including vaginal scent marking (Petrulis, 2009). This behavior deposits vaginal secretion such that 'trails' are formed along the ground as the animal scent marks throughout its environment (Lisk et al., 1983). Although females will vaginal mark in response to various types of stimuli, this behavior is preferentially directed towards males or their odors (Johnston, 1977; Martinez and Petrulis, 2011; Petrulis and Johnston, 1999; Petrulis et al., 1999), suggesting that vaginal marking may be particularly important for mate attraction. Indeed, the deposited vaginal secretion is highly attractive to male hamsters (Johnston and Schmidt, 1979; Johnston, 1974; Kwan and Johnston, 1980), and females deposit these marks in such a way as to direct the male to her nesting area (Lisk et al., 1983).

The expression of vaginal marking depends critically on the processing of sexual odor information by chemosensory-responsive brain areas. In most mammalian species, including Syrian hamsters, chemosensory signals from conspecifics are initially detected and processed by two distinct but interconnected systems, the main and accessory olfactory systems (Keller et al., 2009). Disruption of inputs to the main (Johnston, 1992) or accessory (Petrulis et al., 1999) olfactory bulbs, or ablation of the

olfactory bulbs themselves (Kairys et al., 1980), decreases vaginal marking to males or their odors. Sexual odor information is transmitted from the olfactory bulbs to areas throughout the ventral forebrain, including the medial amygdala (MA), the posterior division of the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA) (Coolen and Wood, 1998; Davis et al., 1978; Gomez and Newman, 1992; Wood and Swann, 2005). These areas also appear to be important for regulating the expression of vaginal marking. Lesions centered on MA decrease overall levels of vaginal marking (Petruilis and Johnston, 1999), whereas lesions of BNST specifically decrease vaginal marking in response to male, but not female odors (Martinez and Petruilis, 2011). In contrast, lesions of MPOA do not affect the expression of vaginal marking, although this area is important for other forms of precopulatory behaviors in female hamsters (Floody, 1989; Martinez and Petruilis, 2013).

The underlying neurochemical mechanisms whereby the ventral forebrain regulates precopulatory responses in females are not well understood. However, there is a growing body of evidence to suggest that the neuropeptide oxytocin may be involved. Oxytocin is a nine amino acid peptide that is produced and released from neurosecretory cells located in the paraventricular (PVH) and supraoptic (SON) nuclei of the hypothalamus. Within the brain, oxytocin acts to broadly facilitate a range of prosocial responses, including social recognition, maternal behavior, and sexual behavior (Lee et al., 2009). In hamsters and rats, oxytocin infused directly into MPOA facilitates the expression of sexual receptivity (Caldwell et al., 1989; Whitman and Albers, 1995), whereas MPOA injections of an oxytocin receptor antagonist (OTA) decreases receptivity (Caldwell et al., 1994; Whitman and Albers, 1995). Intracerebroventricular (ICV) injections of OTA decrease both sexual solicitation and sexual receptivity in female rats (Witt and Insel, 1991), but this effect on solicitation was not replicated following OTA injections directly into MPOA (Caldwell et al., 1994). This suggests that areas other than MPOA in the ventral forebrain mediate the effects of oxytocin on sexual solicitation. The bed nucleus of the stria terminalis (BNST) is one such candidate area. We have found that injections of OTA into either MPOA or BNST decreased

vaginal marking to male, but not female odors, and this effect was greater following injections into BNST (Martinez et al., 2010). Furthermore, binding sites for oxytocin are present in BNST, but not MPOA, in hamsters (Dubois-Dauphin et al., 1992). Considered together, it seems likely that oxytocin acts within BNST, rather than MPOA, to regulate vaginal marking; however, the underlying mechanisms whereby oxytocin influences sexual odor processing within BNST are not known.

Across many mammalian species, including sheep, mice, rats, and hamsters, expression of immediate early genes (IEGs; an indirect marker of recent neuronal activation (Pfaus and Heeb, 1997)) throughout the ventral forebrain is increased in females when exposed to male stimuli (Bennett et al., 2002; DelBarco-Trillo et al., 2009; Gelez and Fabre-Nys, 2006; Kang et al., 2009). Furthermore, stimulation/extracellular recordings in these areas in awake, behaving females provide additional evidence in support of a link between neural activity in these areas and both copulatory and precopulatory responses (Hoshina et al., 1994; Kato and Sakuma, 2000; Rose, 1990). Therefore, we hypothesized that oxytocin interacts with sexual odor processing in the ventral forebrain to ultimately drive preferential expression of solicitational responses toward appropriate targets. This may occur through two non-exclusive mechanisms: (1) oxytocinergic cells in ventral forebrain areas may be preferentially activated in response to male vs. female stimuli, and (2) in ventral forebrain areas that regulate the preferential expression of vaginal marking, such as BNST, endogenous oxytocin may preferentially facilitate neural activity in response to male vs. female odors. Consequently, we predicted that more oxytocinergic cells in MPOA, BNST, PVH, and MA would co-express Fos, the protein product of the IEG *c-fos*, in females exposed to male stimuli compared to female stimuli (Experiment 1). Furthermore, we predicted that ICV injections of OTA would selectively decrease vaginal marking to male stimuli (Experiment 2), similar to effects of OTA following direct injections into MPOA or BNST (Martinez et al., 2010). Finally, given that BNST, but MPOA or MA, is required for the preferential expression of vaginal marking to male stimuli (Martinez and Petrulis, 2011, 2013; Petrulis and Johnston, 1999), we predicted that ICV injections of OTA would

selectively decrease Fos expression to male stimuli in BNST, without affecting preferential Fos expression in MPOA or MA (Experiment 3).

5.3 Methods

Overview of experimental design

In Experiment 1, females were exposed to male, female, or clean cage stimuli on behavioral proestrus, sacrificed, and had their brains processed for double-label immunohistochemistry (IHC) for oxytocin and Fos (Figure 5.1A). In Experiment 2, females were implanted with unilateral guide cannulae directed at the lateral cerebral ventricles (Figure 5.1B). These females were then tested for scent-marking responses to male or female stimuli on three consecutive proestrous days. One hour prior to each test, females received ICV injections of either OTA or a vasopressin 1a receptor antagonist (V1aA) at the following doses: 0, 90, or 900 μ M of OTA in 2 μ l saline vehicle (OTA group); 0, 900, or 9000 μ M V1aA in 2 μ l saline vehicle (V1aA group). Finally, in Experiment 3, females were implanted with unilateral guide cannulae and exposed to male or female stimuli on proestrus (Figure 5.1C). One hour prior to exposure, these females were injected ICV with either 0 or 900 μ M OTA in 2 μ l saline vehicle. Females were sacrificed following exposure to stimuli and brain tissue processed for single-label IHC for Fos.

Subjects

Adult Syrian hamsters (*Mesocricetus auratus*) were purchased from Harlan Laboratories (Indianapolis, IL, USA) at approximately 7-11 weeks of age. In addition to female hamsters used as experimental subjects, a separate group of unrelated male and female hamsters served as stimulus animals. Animals were either individually housed (experimental subjects) or group housed (stimulus animals; 3-4 animals per cage) in solid-bottom polycarbonate cages containing corncob bedding and cotton nesting material (Nestlets; Ancare, Bellmore, NY). Hamsters were maintained on a reversed light cycle (14:10 light:dark; lights out at 10 am), with all behavior testing occurring during the first four hours of the dark portion of

the light cycle. Food and water were available *ad libitum*. Animal procedures were carried out in accordance with the Eighth Edition of the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council, 2011) and approved by the Georgia State University Institutional Animal Care and Use Committee. All efforts were made to minimize the total number of animals used and their suffering.

Estrous cycle monitoring

Prior to stimulus exposure or behavioral testing, subjects were examined daily for eight consecutive days in order to determine their stage of the estrous cycle. Subjects were gently restrained while vaginal secretion was manually extruded using a disposable probe, and the consistency of the secretion was examined for stringy consistency indicative of behavioral estrus (Orsini, 1961). Once the day of behavioral estrus was identified, the two cycle days prior to estrus were defined as diestrous day 2 and proestrus, respectively (Johnston, 1977). It has been our experience that this technique for determining estrous cycle stage correctly predicts the day of behavioral receptivity with perfect accuracy (Martinez and Petrusis, 2011, 2013).

Stimulus exposure

On the predicted day of proestrus (see Estrous cycle monitoring), subjects were exposed to male, female, or clean cage stimuli. Proestrus was chosen for stimulus exposures because females exhibit the highest overall levels of vaginal marking on this cycle day (Johnston, 1977; Martinez and Petrusis, 2011, 2013). Subjects were handled for at least eight consecutive days prior to exposure. Stimuli consisted of vacated cages (43 x 22 x 20 cm) previously occupied by male or female stimulus animals for four days, or clean cages containing unsoiled bedding. Estrous cycles of stimulus females were not monitored; however, given that each cage contained at least three females, and the occupation period comprised the complete four-day estrous cycle, it is likely that female cage stimuli were a composite of mul-

multiple cycle days. Approximately one to four hours prior to use, a researcher blind to the experimental condition of subjects prepared the stimulus cages as follows: Stimulus animals were removed from their cages along with any food pellets and caked urine present in the corncob bedding, the soiled cotton nesting material was distributed evenly across the bottom of the cage, and a 40 cm x 19 cm x 0.4 cm perforated Plexiglas plate was placed over the bedding just prior to testing. This plate was centered within the cage such that bedding within three cm of the outer walls of the stimulus cages remained directly accessible to subjects. The surface of the plate was painted with black non-toxic chalkboard paint (Rust-oleum, Vernon Hills, IL) such that it divided the plate into four painted quadrants separated by unpainted areas. We have previously used this technique to facilitate the scoring of vaginal/flank marking and locomotor activity (Martinez and Petruilis, 2011, 2013; Martinez et al., 2010). Although scent marking was only quantified in Experiment 2 and Experiment 3, these plates were utilized in all experiments to maintain consistency in odor presentation procedures.

Experiment 1: Co-localization of oxytocin and Fos following exposure to sexual odors

Perfusion and IHC

Following a 70 min stimulus exposure period, subjects were transcardially perfused with 200 ml of 0.1 M phosphate buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in PBS. Brains were removed and post-fixed in 4% paraformaldehyde in PBS overnight followed by 20% sucrose in PBS (1-2 days). Coronal sections (30 μ m) were taken using a cryostat and stored in cryoprotectant at -20°C until processed for double-label IHC for oxytocin and Fos.

Free-floating coronal sections were removed from cryoprotectant, rinsed in PBS, and then incubated in 0.3% hydrogen peroxide in PBS for 15 min in order to quench any endogenous peroxidase activity. After rinsing in PBS, sections were incubated in a solution containing PBS, 0.4% Triton-X (Sigma, St. Louis, MO), and 1:20,000 polyclonal rabbit anti-cFos antibody (SC-52; Santa Cruz Biotechnology, Santa

Cruz, CA) for 18 h at room temperature. After rinsing in PBS, tissue sections were incubated in a solution of PBS, 0.4% Triton-X, and 1:600 biotinylated goat anti-rabbit secondary antibody (111-065-003; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature, rinsed in PBS, and then incubated in a solution of PBS, 0.4% Triton-X, and 1:200 avidin-biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Tissue sections were rinsed in PBS followed by 0.175 M sodium acetate, and reacted in a nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride solution (2 mg 3,3'-diaminobenzidine tetrahydrochloride plus 250 mg nickel (II) sulfate with 8.3 μ L 3% hydrogen peroxide per 10 mL of 0.175 M sodium acetate; Sigma, St. Louis, MO) for 15 minutes at room temperature, resulting in a purple-black nuclear stain. Tissue sections were rinsed again in 0.175 M sodium acetate followed by PBS in order to terminate the chromagen reaction. Sections then underwent additional processing to label for oxytocin. Briefly, sections were incubated in 3% normal horse serum (S-2000; Vector Laboratories) in PBS for 1h. Sections were placed in a solution containing PBS, 0.3% Triton-X, and 1:10,000 monoclonal mouse anti-oxytocin (MAB 5296; Millipore, Temecula, CA) for 48 h at 4°C. Tissue sections were subsequently processed as described above for Fos, with the following exceptions: the secondary antibody was 1:600 horse anti-mouse (BA2000; Vector Laboratories), 3,3'-diaminobenzidine tetrahydrochloride in Tris buffer was utilized for the chromagen reaction (resulting in a brown, cell body stain) and Tris buffer was used to rinse the tissue both prior to and following the chromagen reaction. At the completion of IHC processing, tissue sections were mounted on subbed slides and dried overnight. Slides were then dehydrated through an ethanol series, cleared with xylene (Fisher Scientific, Pittsburgh, PA), and coverslipped using Permount (Fisher Scientific).

Quantification

A single researcher blind to the experimental condition of the subjects counted both single- and double-labeled cells. Slides were examined using a QImaging camera connected to a Nikon E800 light microscope. Images were projected onto a computer running iVision software (v. 4.0.10) at 10X magnifi-

cation. Photomicrographs of representative tissue labeling were processed using Adobe Photoshop CS5 (Adobe, San Jose, CA) for brightness, contrast, and tone. Counting domains were fitted to the following brain areas: the posteromedial (BNSTpm) and posterointermediate (BNSTpi) subdivisions of the bed nucleus of the stria terminalis, the medial preoptic area (MPOA), the paraventricular nucleus of the hypothalamus (PVH), and the anterior and posterior divisions of MA (MeA and MeP, respectively) (Figure 5.2). For each brain area, counting domains were fitted to both hemispheres on two consecutive atlas plates. Cells positive for Fos (Fos+) were identified by purple-black nuclear staining, whereas cells positive for oxytocin (OT+) were identified by brown cell body staining. Cells positive for both oxytocin and Fos (OT/Fos+) were identified by the presence of both a purple-black nucleus and a brown cell body. Counts of single- and double-labeled cells were summed across hemispheres and atlas plates for each brain area, and then divided by the total area of the counting domains in order to generate cell densities (labeled cells per mm²).

Experiment 2: Effects of ICV injections of OTA on scent-marking responses to sexual odors

Surgery

At two to three months of age, subjects were unilaterally implanted with guide cannulae. Subjects were first anesthetized with 2-3% isoflurane gas (Butler Schein, Dublin, OH) in an oxygen (80%) and nitrous oxide (20%) mixture, and then placed within a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with ear- and incisor-bars positioned such that the top of the skull was level. Following a midline scalp incision, the skin and underlying temporal muscles were retracted to expose the skull. A hand-operated drill was then used to make a hole in the skull in order to expose dura. Each subject was then fitted with a single 3-mm 26-gauge guide cannula (Plastics One, Roanoke, VA) directed at the lateral cerebral ventricle at the level of BNST, using the following coordinates: Anterior-posterior, +1.40 mm (relative to bregma); medial-lateral, ±1.45 mm (relative to bregma); dorsal-ventral, -2.00 mm (relative to du-

ra). The placement of the guide cannula in the left or right hemisphere was alternated across all subjects. Stereotaxic coordinates were derived from published anatomical plates for the Syrian hamster brain (Morin and Wood, 2001). Injection cannulae were cut to extend an additional 1 mm beyond the end of guide cannulae, thereby penetrating into the lateral ventricle. Guide cannulae were secured to the skull with dental cement and skull screws, and immediately prior to the completion of the surgical procedure, subjects were injected subcutaneously with an analgesic agent (Ketofen; Fort Dodge Animal Health, Fort Dodge, IA). All subjects were allowed to recover for at least 10 days prior to behavioral testing.

Drugs

OTA (des-Gly-NH₂, d(CH₂)₅ [Tyr(Me)², Thr⁴] OVT; Bachem, Torrance, CA) and V1aA (d(CH₂)₅ [Tyr(Me)², Dab⁵] AVP; generous gift of Dr. Maurice Manning, University of Toledo, Toledo, OH) were diluted in sterile saline and stored in aliquots of 30 μl at -20°C until use. OTA was placed into solution at 0, 90, and 900 μM whereas V1aA was placed into solution at 0, 900, and 9000 μM. Both OTA and V1aA are selective antagonists for their targeted receptor subtypes (oxytocin receptors (OTRs) and vasopressin receptors (V1aRs), respectively). Indeed, OTA exhibits a 17-fold greater potency for antagonizing OTRs versus V1aRs, whereas V1aA exhibits essentially no antagonism for OTRs (Manning et al., 2008). All drug injections occurred one hour prior to behavior testing/stimulus exposure. Subjects were injected with either 2 μl of OTA (OTA group) or 2 μl of V1aA (V1aA group). Within each group, subjects were injected on three consecutive proestrous cycle days with one of the three doses of drug described above, such that each female was tested once following injection of each drug dose. OTA and V1aA were delivered to the lateral ventricle via an injection cannula connected via polyethylene tubing to a 10 μl Hamilton syringe driven by a Harvard Apparatus infusion pump (PHD 2000; Holliston, MA). Subjects were allowed to freely explore a temporary holding cage for 90 s while the drug was injected, and then for an additional 60 s post-injection to allow the drug to diffuse away from the tip of the injection cannula.

One hour following injections, subjects were exposed to stimuli present in male or female cages for 10 min. Marking was scored manually by a researcher blind to the experimental condition of the subjects. Vaginal marking and flank marking are discrete, stereotyped behaviors that are differentially expressed in response to conspecific odors (Johnston, 1977). A vaginal mark was scored each time the female moved forward with tail deflected upwards while maintaining contact between the perineum and the underlying substrate (Been et al., 2012). A flank mark was scored each time the female moved forward while maintaining contact between the flank region and the side of the stimulus cage. Tests were also recorded using a digital camcorder and videos were scored for the number of quadrant entries by researchers blind to the experimental conditions of the subjects, with an inter-rater reliability of 90% or greater. Entry into a quadrant was scored whenever greater than 50% of the body mass of the female crossed from one quadrant into another. At the completion of the 10 min exposure, subjects were returned to their home cages.

Histology

Subjects were administered a lethal dose of sodium pentobarbital (0.2 ml, i.p.; Beuthanasia-D, Merck Animal Health, Summit, NJ) following their final stimulus exposure, and then injected via cannulae with 200 nl of India ink. Brains were removed, post-fixed in 10% neutral buffered formalin, and 50 μ m sections were taken using a cryostat. Sections were counterstained with cresyl violet and examined under light microscopy for ink penetration into the lateral cerebral ventricle. The site of penetration was plotted using anatomical plates of the Syrian hamster brain (Morin and Wood, 2001). In all cases, the injection needle penetrated into the lateral cerebral ventricle. Therefore, data from all subjects were analyzed.

Experiment 3: Effects of OTA injected ICV on sexual odor-induced Fos expression

Surgery and drugs

Subjects were implanted with guide cannula and injected as described for Experiment 2, with the following exceptions: (1) only OTA was injected, and (2) subjects received only a single injection of either 0 or 900 μM OTA. As in Experiment 2, scent-marking responses were quantified during the first 10 min of stimulus exposure; however, subjects were allowed to remain in the stimulus cage for an additional 60 min.

Perfusion and histology

Following the 70 min stimulus exposure, subjects were sacrificed, perfused, and brain tissue processed and quantified as described in Experiment 1, with the following exceptions: (1) tissue was only processed for Fos immunoreactivity, and (2) only the number of Fos+ cells were quantified, and only in BNSTpi, BNSTpm, MPOA, MeA, and MeP.

Data analysis

All data were analyzed using SPSS for Macintosh, version 19.0 (SPSS Inc., Chicago, IL). Data were first examined to determine if the assumptions of parametric statistical tests were met. When assumptions were violated, distributions were normalized using applicable data transformations (Osborne and Overbay, 2004; Sheskin, 2000). For all statistical tests, results were considered to be statistically significant if $p < .05$.

In Experiment 1, the density of Fos+ cells and proportion of OT+/Fos+ cells within each brain area was compared across stimulus groups (male, female, or clean) using one-way ANOVAs. Significant main effects were followed by post-hoc pairwise comparisons using Tukey's *HSD*.

In Experiment 2, the number of vaginal and flank marks exhibited by females within each drug group was subjected to a mixed-design factorial ANOVA, with stimulus (male or female) as an independ-

ent factor and drug concentration (OTA: 0, 90, or 900 μM ; V1aA: 0, 900, or 9000 μM) as a repeated factor. Significant interactions were followed by pairwise comparisons of the different drug concentrations within each stimulus condition, with Bonferroni corrections for multiple comparisons.

In Experiment 3, the density of Fos+ cells within each brain area was subjected to a factorial ANOVA with stimulus (male or female) and drug concentration (0 or 900 μM OTA) as independent factors. Significant interactions were further examined for the effect of drug concentration within each stimulus condition using independent-samples *t*-tests. In addition, the strength and significance of the linear relationship between the density of Fos+ cells in each brain area and the following measures was determined using Pearson's product-moment correlations: (1) the overall number of vaginal marks, (2) the number of vaginal marks in response to male odors only, and (3) the residual variance in overall vaginal marking not explained by stimulus type.

5.4 Results

Experiment 1

Throughout most areas examined, a significant increase in Fos expression was observed in females exposed to sexual odor stimuli (male or female cages) compared to stimuli present in clean cages. The total densities of Fos+ cells within each brain area are presented in Table 5.1. Within MeA, MeP, BNSTpm, BNSTpi, and MPOA, the density of Fos+ cells differed across the three stimulus conditions (BNSTpm: $F(2,17) = 9.49, p = .002$; BNSTpi: $F(2,17) = 9.70, p = .002$; MPOA: $F(2,17) = 12.97, p < .001$; MeA: $F(2,17) = 4.95, p = .02$; MeP: $F(2,17) = 19.20, p < .001$). Tukey's *HSD* post-hoc comparisons revealed that the density of Fos+ cells was higher in response to male vs. clean stimuli in BNSTpm, BNSTpi, MPOA, MeA, and MeP; higher in response to male vs. female stimuli in BNSTpi, and MPOA, and MeP; and higher in response to female vs. clean stimuli in BNSTpm (all $p < .05$). In contrast, the density of Fos+ cells did not differ across stimulus conditions within PVH ($F(2,17) = 1.76, p = .20$).

We observed few double-labeled OT+/Fos+ cells within BNSTpm, BNSTpi, or MeP; therefore, we only quantified the proportion of OT+/Fos+ cells within PVH, MPOA, and MeA (Figure 5.3). Within PVH, the proportion of OT/Fos+ cells was higher in response to male vs. female stimuli ($z = 5.16, p < .05$) and higher in response to clean vs. female stimuli ($z = 4.13, p < .05$) (Figure 5.4). There was no difference in the proportion of OT/Fos+ cells in response to male vs. clean stimuli ($z = 1.05, p > .05$). Furthermore, there were no differences across stimulus conditions in the proportion of OT+/Fos+ cells within MPOA (male vs. female: $z = 0.50, p > .05$; female vs. clean: $z = 1.16, p > .05$; male vs. clean: $z = 0.74, p > .05$) or MeA (male vs. female: $z = 0.76, p > .05$; female vs. clean: $z = 0.83, p > .05$; male vs. clean: $z = 1.60, p > .05$).

Experiment 2

ICV injections of OTA decreased vaginal marking to male stimuli (Figure 5.5A). In the OTA group, overall number of vaginal marks was higher in response to male vs. female stimuli ($F(1,16) = 6.52, p = .02$). In addition, the overall number of vaginal marks also differed across the three drug doses ($F(2,32) = 7.59, p = .002$). This effect of OTA dose on vaginal marking differed across the different stimulus conditions ($F(2,32) = 3.73, p = .04$). In the male stimulus condition, the higher dose of OTA (900 μ M) significantly decreased vaginal marking compared to saline injections ($t(8) = 3.54, p < .05$). There were no differences in the number of vaginal marks between injections of the lower dose of OTA (90 μ M) and saline ($t(8) = 2.45, p > .05$) or between the 900 μ M and 90 μ M doses ($t(8) = 1.08, p > .05$). In the female stimulus condition, injections of OTA had no effect on vaginal marking responses (900 μ M vs. saline: $t(8) = 1.32, p > .05$; 90 μ M vs. saline: $t(8) = .47, p > .05$; 900 μ M vs. 90 μ M: $t(8) = .79, p > .05$).

ICV injections of V1aA did not mimic the effects of OTA on vaginal marking (Figure 5.5B). Overall vaginal marking levels tended to be higher in response to male vs. female stimuli, although this difference was not statistically significant ($F(1,17) = 4.21, p = .06$). Overall levels of vaginal marking did not

differ across the different drug doses ($F(2,34) = 0.47, p = .63$), and the drug x stimulus interaction was not significant ($F(2,34) = 0.51, p = .61$).

There were no significant effects of ICV injections of OTA or V1aA on flank marking behavior (Table 5.2). Females flank marked at similar levels in response to male and female stimuli (OTA group: $F(1,16) = 2.10, p = .17$; V1aA group: $F(1,17) = 2.38, p = .14$), and equivalently across the different drug doses (OTA group: $F(2,32) = 0.03, p = .97$; V1aA group: $F(2,34) = .18, p = .84$). The drug by stimulus interaction was not significant for either group (OTA group: $F(2,34) = 2.62, p = .09$; V1aA group: $F(2,34) = 2.35, p = .11$).

Experiment 3

The expression of Fos following exposure to male stimuli was decreased in BNST following ICV injections of OTA. Representative photomicrographs of Fos immunostaining within BNSTpm, BNSTpi and MPOA are presented in Figure 5.6, and for MeA and MeP in Figure 5.7. Within BNSTpm, the overall density of Fos+ cells did not differ in response to male vs. female stimuli ($F(1,23) = .124, p = .73$) (Figure 5.8A). The overall density of Fos+ cells was, however, lower in females injected with OTA compared to those injected with saline vehicle ($F(1,23) = 7.12, p = .005$). This effect of OTA differed across the different stimulus conditions ($F(1,23) = 5.96, p = .02$). In females exposed to male stimuli, OTA significantly decreased the density of Fos+ cells compared to saline ($t(12) = 5.03, p < .001$). In contrast, there was no effect of OTA on Fos+ cell density in response to female stimuli ($t(11) = 0.13, p = .90$). A similar pattern was seen in BNSTpi (Figure 5.8B). The overall density of Fos+ cells was higher in females exposed to male stimuli vs. female stimuli ($F(1,23) = 9.67, p = .005$), and lower in females injected with OTA vs. saline ($F(1,23) = 12.94, p = .002$). There was a significant drug x stimulus interaction ($F(1,23) = 7.47, p = .01$). Specifically, OTA injections significantly decreased the density of Fos+ cells compared to saline injections in females exposed to male stimuli ($t(12) = 5.18, p < .001$), whereas females exposed to female stimuli exhibited equivalent Fos+ cell densities across drug conditions ($t(11) = 0.54, p = .60$).

In MPOA, MeA, and MeP, overall Fos+ cell densities were higher in response to male stimuli compared to female stimuli (MPOA: $F(1,23) = 12.45, p = .002$; MeA: $F(1,23) = 13.43, p = .001$; MeP: $F(1,23) = 13.03, p = .001$) (Figure 5.8C-E). In contrast to BNST, however, there were no overall effects of drug (MPOA: $F(1,23) = 0.19, p = .67$; MeA: $F(1,23) = 1.71, p = .20$; MeP: $F(1,23) = 0.58, p = .45$) or drug x stimulus interactions (MPOA: $F(1,23) = 0.86, p = .36$; MeA: $F(1,23) = 0.48, p = .50$; MeP: $F(1,23) = 0.87, p = .36$).

Linear relationships between the densities of Fos+ cells and the number of vaginal marks exhibited in the first 10 min of stimuli exposure were calculated for each brain area (Figure 5.9). Significant, positive correlations were found for BNSTpm, BNSTpi, and MPOA. In MeA and MeP, however, the linear relationships were not significant. In order to clarify whether the expression of Fos in BNSTpm, BNSTpi, and MPOA was more directly linked to the effects of OTA on vaginal marking, rather than the overall effect of stimulus type on vaginal marking, additional correlational analyses were performed. Specifically, the relationship between Fos expression and vaginal marking was examined as follows: (1) when looking at females in the male stimulus condition only (Figure 5.10A-C), or (2) when the effect of stimulus type on Fos expression and vaginal marking were first removed from the data (Figure 5.10D-F). When examining the male stimulus condition only, there were significant, positive correlations between the densities of Fos+ cells and the number of vaginal marks for BNSTpm and BNSTpi, but not MPOA. Likewise, Fos+ cell densities in BNSTpm and BNSTpi, but not MPOA, were significantly and positively correlated with vaginal marking when the effect of stimulus type was removed from vaginal marking data prior to examining the linear relationships.

5.5 Discussion

The results presented here demonstrate that the oxytocin system interacts with the processing of sexual odor stimuli to ultimately regulate the expression of one aspect of odor-guided precopulatory behaviors in females, vaginal marking. Specifically, we found that Fos expression by OT+ cells in PVH is

unchanged following exposure to stimuli that normally induce vaginal marking (male odors), but reduced following exposure to stimuli that normally inhibit vaginal marking (female odors). We also show that ICV injections of OTA decreased vaginal marking to male, but not female odors, as it does following MPOA/BNST injections (Martinez et al., 2010). This effect cannot be attributed to OTA acting exclusively at V1aRs, given that ICV injections of V1aA did not mimic the effects of OTA on vaginal marking. Finally, the same dose of OTA that was behaviorally effective when injected ICV also decreased Fos expression in BNST to male, but not to female, stimuli. This effect of OTA on Fos expression was not seen in MPOA or MA. Moreover, Fos expression in BNST was strongly correlated to the expression of vaginal marking by females. Considered together, these results suggest that endogenous oxytocin facilitates preferential vaginal marking to male odors via its effects on sexual odor processing within BNST.

Experiment 1: Co-localization of oxytocin and Fos following exposure to sexual odors

Our findings that Fos expression by OT+ cells in PVH was unchanged in response to male odors, but decreased in response to female odors, suggest that female odors may specifically suppress activation of OT+ cells in this area. This was unexpected, since blocking OTRs decreases vaginal marking responses to male odors without affecting responses to female odors, either when injected either ICV (Experiment 2) or directly into BNST/MPOA (Martinez et al., 2010). There are several potential explanations for these findings. It could be that Fos expression does not identify OT+ cells that were activated and subsequently released oxytocin in response to sexual odors. This is a possibility, since Fos expression is dependent on intracellular calcium signaling events associated with persistent, rather than transient, excitation (Lyons and West, 2011). However, given that Fos expression in OT+ cells is associated with dendritic release of oxytocin (Sabatier et al., 2003), and that dendritic release may be a primary mechanism whereby oxytocin reaches target brain areas (Bergquist and Ludwig, 2008; Ludwig and Leng, 2006), there is likely some connection in the present study between Fos expression in OT+ cells and oxytocin release. If true, our data suggest that baseline levels of oxytocin release within target areas are sufficient

for normal vaginal responses to male odors. Furthermore, the suppression of OT+ cells following exposure to female odors may be necessary to ensure minimal levels of solicitation towards same-sex conspecifics. This suppression would also enable females to express other behaviors directed towards same-sex conspecifics that would otherwise be inhibited by oxytocin, such as aggression (Harmon et al., 2002a).

The sparse and variable expression of OT+ cells in BNST prevented us from quantifying colocalization of oxytocin and Fos in this area. Across the two subdivisions of BNST, we found an average of 3.0 (BNSTpm) and 1.4 (BNSTpi) OT+ cells per female. Furthermore, no OT+ cells were identified in BNSTpm for 3 females, or in BNSTpi for 6 females. It was previously reported that some OT+ cells are present in BNST of female hamsters (Whitman and Albers, 1998), but that study did not quantify oxytocin immunoreactivity in BNST or any brain area. It is therefore difficult to determine if OT+ cells within BNST contribute to any behavioral effects of the oxytocin system observed in the present study. In addition to cell bodies, OT+ fibers are also found in BNST (Whitman and Albers, 1998), and OT+ fiber density in this area is substantially reduced following lesions of PVH (de Vries and Buijs, 1983). Therefore, the predominant source of oxytocin in BNST is likely distal (i.e., from PVH), rather than from OT+ cells within BNST.

The present study is the first to describe the pattern of Fos expression in ventral forebrain areas of female Syrian hamsters, following exposure to both male and female odors. The increased Fos expression in BNST, MPOA, and MA in response to opposite-sex odors are in agreement with previous data from female mice (Halem et al., 1999), rats (Bennett et al., 2002; Hosokawa and Chiba, 2007), and build upon those previously reported in female hamsters (DelBarco-Trillo et al., 2009). Male odors can therefore induce Fos expression throughout this circuit in a fairly conserved manner across species. The results of the present study also are in good agreement with previous studies examining Fos expression in male hamsters (Been and Petrulis, 2011; Maras and Petrulis, 2010a), and suggest that the same circuit

processes sexual odors in both males and female hamsters as it does in rats (Bennett et al., 2002; Hurta-zo and Paredes, 2005) and mice (Halem et al., 1999; Pankevich et al., 2003).

Experiment 2: Effects of ICV injections of OTA on scent-marking responses to sexual odors

OTA injected ICV decreased vaginal marking to male odors without affecting vaginal marking to female odors, as we expected based on our previous finding that injections of OTA directly into BNST or MPOA decrease vaginal marking to male, but not female odors (Martinez et al., 2010). The same dose of OTA (900 μ M) was behaviorally effective in both studies, and blockade of V1aRs was not sufficient to mimic the effects of OTA in either study. Given the high degree of similarity in behavioral effects across these studies, it seems likely that OTA injected ICV inhibited vaginal marking via blockade of OTRs in BNST.

Experiment 3: Effects of OTA injected ICV on sexual odor-induced Fos expression

Our findings that ICV injections of OTA selectively decreased Fos expression in BNST to male odors further support the conclusion that this brain area mediates the effects of endogenous oxytocin on precopulatory behaviors. This effect of OTA on Fos expression was seen in both subdivisions of BNST examined. Although anatomical data suggest that the two subdivisions may be differentially involved in processing steroid hormone (BNSTpm) and chemosensory (BNSTpi) signals (Been and Petrulis, 2011; Wood and Swann, 2005), both subdivisions of BNST are preferentially activated by opposite-sex odors in hamsters (Maras and Petrulis, 2010a). Considered in the context of our previous work wherein we failed to note any obvious differential involvement of BNST subdivisions in the regulation of vaginal marking (Martinez et al., 2010), it seems likely that BNSTpm and BNSTpi function together to mediate the reported neural and behavioral effects of endogenous oxytocin.

No effect of OTA on Fos expression was seen in other brain areas quantified, including MPOA. MPOA has previously been identified as a critical area mediating the effects of oxytocin system manipu-

lations on social and sexual behaviors in female hamsters (Floody et al., 1998; Harmon et al., 2002a, 2002b; Whitman and Albers, 1995). However, the available data do not support a role for MPOA in mediating the effects of oxytocin on vaginal marking. In our previous study targeting BNST or MPOA (Martinez et al., 2010), injections into either area were equally effective at decreasing vaginal marking. It is very likely that OTA was able to diffuse up the injection pathway into the overlying BNST during MPOA-targeted injections; this would not have occurred during BNST injections. Therefore, the most parsimonious explanation for the effects reported in that study is that OTA acted in BNST, not MPOA, to decrease vaginal marking. This conclusion is further supported by the presence of oxytocin binding sites in BNST, but not MPOA, of hamsters (Dubois-Dauphin et al., 1992), and our previous findings that discrete, excitotoxic lesions of BNST eliminate preferential vaginal marking to male odor stimuli (Martinez and Petrulis, 2011), whereas lesions of MPOA do not affect preferential marking (Martinez and Petrulis, 2013).

The finding that there were no effects of OTA on Fos expression in MA was surprising. MA expresses OTRs in hamsters as well as other species (Campbell et al., 2009; Dubois-Dauphin et al., 1992; Lee et al., 2008; Veinante and Freund-Mercier, 1997), and endogenous oxytocin acts within MA to modulate responses to conspecific odor stimuli. In male mice, injections of OTA into MA decreases investigation of odor stimuli from same-sex conspecifics (Arakawa et al., 2010), and ICV injections of OTA decrease Fos expression in MA following exposure to opposite-sex odors (Samuelson and Meredith, 2011). Considering the present findings, there likely are substantial species differences in the behavioral and neural effects of oxytocin acting within MA. In agreement with this conclusion, blockade of OTRs in MA disrupts individual recognition in male and female mice (Choleris et al., 2007; Ferguson et al., 2001; Samuelson and Meredith, 2011), whereas MA is not required for individual recognition in female hamsters (Petrulis and Johnston, 1999).

The expression of Fos in both subdivisions of BNST was strongly and positively correlated with vaginal marking, suggesting that there may be a functional relationship between IEG expression in BNST and the expression of vaginal marking. However, a positive correlation between Fos expression and vaginal marking was also seen for MPOA, an area that is not critical for vaginal marking (Martinez and Petrulis, 2013). Therefore, it may be that these relationships are spurious, driven by factors critical for the expression of both Fos expression and vaginal marking (i.e., odor stimulus). We found that the relationship between Fos expression in BNST and vaginal marking was independent of stimulus type, given that it persisted when examining the male stimulus condition only, as well as when statistically controlling for the effect of stimulus type on Fos expression and vaginal marking. This was not seen for MPOA, suggesting that Fos expression in BNST is more closely linked to the behavioral effects of OTA on vaginal marking.

The underlying mechanisms whereby OTA alters neural activity in BNST have not been thoroughly examined. Although it is possible that the effects observed in the present study are exclusively indirect (i.e., driven by effects of OTA in other brain areas that are connected, directly or indirectly, to BNST), this seems unlikely for several reasons. First, there was no effect of OTA on Fos expression in MA or MPOA, two areas that are highly interconnected with BNST (Coolen and Wood, 1998; Wood and Swann, 2005). Second, the behavioral effects of ICV-injected OTA are identical to those observed following direct injections in BNST, as described above. Finally, OTRs are present in BNST (Dubois-Dauphin et al., 1992), and *in vitro* application of oxytocin onto slices containing BNST results in persistent excitation of BNST neurons (Ingram et al., 1990). Consequently, endogenous oxytocin may act in BNST to increase excitability of neurons expressing OTRs. It would be important to determine if OTRs in BNST are preferentially expressed by male odor-responsive neurons; however, it has been the experience of our group (unpublished observations) and others (Dr. Larry Young; personal communication) that existing antibodies against OTR do not successfully label OTRs in rodent brain tissue.

Conclusion

Our results indicate that there are multiple mechanisms whereby the oxytocin system regulates solicitational responses towards males. Sexual odor information can modulate activity of oxytocinergic cells in PVH; furthermore, endogenous oxytocin is required for the normal pattern of cellular activity in BNST in response to sexual odors. These data complement and extend our previous findings that BNST, rather than MPOA, is critical for the preferential targeting of solicitational responses towards male stimuli, and that endogenous oxytocin acts within BNST to drive this effect. Further research is required to determine the precise intracellular mechanisms mediating the effects of oxytocin on solicitational responding in females.

5.6 Acknowledgements

The authors would like to thank Alica Helman, Marisa Levy, Emily Mobley, Jamin Peters, Alix Pijoux, Manal Tabbaa and July Tran for their technical assistance, and Dr. Anne Murphy for the generous donation of time on her microscope. This work was supported by NIH grant MH072930 to A. Petrusis, a Georgia State University dissertation grant and a Center for Neuromics grant to L. Martinez, and in part by the Center for Behavioral Neuroscience under the STC program of the NSF, under agreement IBN 9876754.

Chapter 5 Tables

Table 5.1 Fos expression during stimulus exposures (Experiment 1)

Mean (\pm SEM) number of Fos-positive cells per mm^2 , within each brain area (see Methods section for a detailed description of cell counting protocol). Dissimilar letters within a specific brain area indicate non-homologous means (Tukey's *HSD* post-hoc comparisons). BNSTpm, posteromedial subdivision of the bed nucleus of the stria terminalis; BNSTpi, posterointermediate subdivision of the bed nucleus of the stria terminalis; MPOA, medial preoptic area; MeA, anterior division of the medial amygdala; MeP, posterior division of the medial amygdala; PVH, paraventricular nucleus of the hypothalamus.

	Male	Female	Clean
BNSTpm	171.6 \pm 16.8 ^a	149.3 \pm 12.0 ^a	87.6 \pm 11.0 ^b
BNSTpi	154.8 \pm 17.5 ^a	102.6 \pm 8.9 ^b	75.8 \pm 9.1 ^b
MPOA	229.7 \pm 8.5 ^a	190.7 \pm 7.7 ^b	172.9 \pm 7.7 ^b
MeA	327.3 \pm 11.2 ^a	296.8 \pm 15.0 ^{a,b}	258.0 \pm 20.0 ^b
MeP	307.4 \pm 15.9 ^a	223.8 \pm 12.1 ^b	180.6 \pm 15.8 ^b
PVH	188.1 \pm 13.5 ^a	183.1 \pm 15.0 ^a	201.5 \pm 21.2 ^a

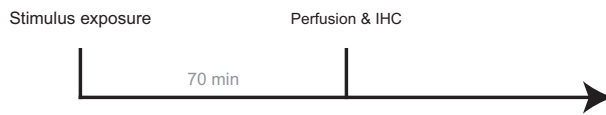
Table 5.2 Flank marking responses during stimulus exposures (Experiment 2)

Mean (\pm SEM) number of flank marks following ICV injections of an oxytocin receptor (OTA) or vasopressin 1a receptor (V1aA) antagonist. In both the OTA and V1aA groups, females flank marked at equivalent levels to male and female odors, and at equivalent levels across the different drug doses. Furthermore, drug \times stimulus interactions were not significant (all $p > .05$).

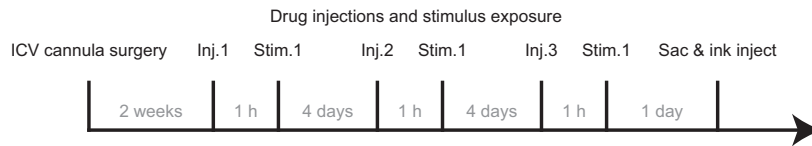
	Male	Female
OTA		
0 μ M	9.0 \pm 2.9	10.0 \pm 3.9
90 μ M	7.0 \pm 3.3	11.6 \pm 3.7
900 μ M	4.4 \pm 1.7	15.2 \pm 3.2
V1aA		
0 μ M	7.8 \pm 1.3	15.6 \pm 5.2
900 μ M	7.1 \pm 1.6	17.2 \pm 4.0
9000 μ M	11.3 \pm 2.4	14.1 \pm 3.5

Chapter 5 Figures

A.) Experiment 1



B.) Experiment 2



C.) Experiment 3

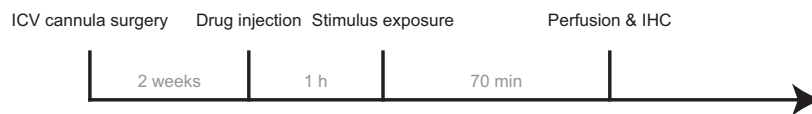


Figure 5.1 Timelines of experimental manipulations

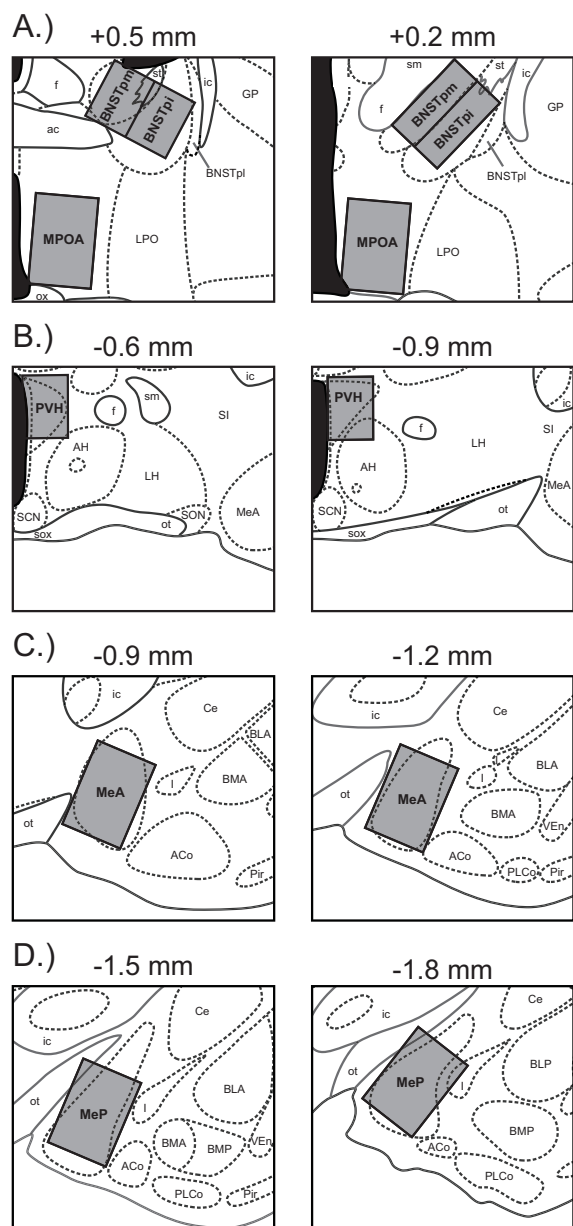


Figure 5.2 Counting domains for quantifying oxytocin- and Fos-positive cells

Domains (gray boxes) were fitted to the following areas: (A) the posteromedial (BNSTpm) and postero-intermediate (BNSTpi) subdivisions of the bed nucleus of the stria terminalis, and the medial preoptic area (MPOA), (B) the paraventricular nucleus of the hypothalamus (PVH), (C) the anterior division of the medial amygdala (MeA), and (D) the posterior division of the medial amygdala (MeP). Atlas plates are modified from Morin and Wood (2001), and arranged in distances relative to bregma. ac, anterior commissure; ACo, anterior cortical amygdaloid nucleus; AH, anterior hypothalamus; BLA, anterior division of the basolateral amygdaloid nucleus; BLP, posterior division of the basolateral amygdaloid nucleus; BMA, anterior division of the basomedial amygdaloid nucleus; BMP, posterior division of the basomedial amygdaloid nucleus; BNSTpl, posterolateral subdivision of the bed nucleus of the stria terminalis; Ce, central amygdaloid nucleus; f, fornix; GP, globus pallidus; I, intercalated nuclei of the amygdala; ic, internal capsule; LH, lateral hypothalamus; LPO, lateral preoptic area; ot, optic tract; ox, optic chiasm; Pir,

piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; SCN, superchiasmatic nucleus; SI, substantia innominata, sm, stria medullaris; SON, supraoptic nucleus; sox, supraoptic decussation; st, stria terminalis; VEn, ventral endopiriform nucleus.

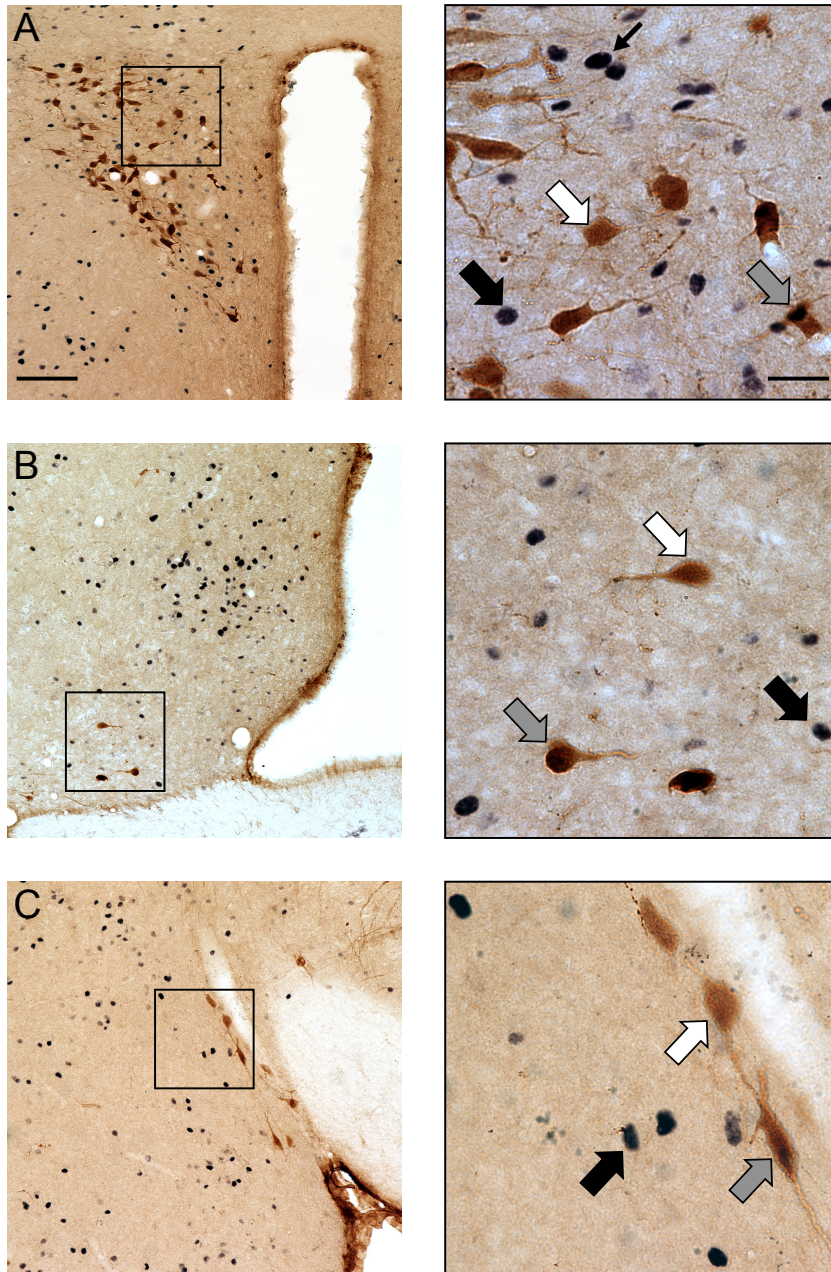


Figure 5.3 Immunohistochemical staining for oxytocin and Fos

Representative photomicrographs of double-label immunohistochemistry in sections depicting (A) the paraventricular nucleus of the hypothalamus (PVH), (B) the medial preoptic area (MPOA), and (C) the anterior division of the medial amygdala (MeA). A higher magnification (40X objective) of the areas highlighted within the black boxes is provided on the right of each lower magnification (10X objective) image. White arrows indicate oxytocin-immunoreactive cells (brown cytoplasmic staining). Black arrows indicate Fos-immunoreactive cells (black nuclear staining). Gray arrows indicate cells immunoreactive for both oxytocin and Fos (brown cytoplasmic staining with black nuclear staining). Scale bars = 100 μm (lower magnification) and 25 μm (higher magnification).

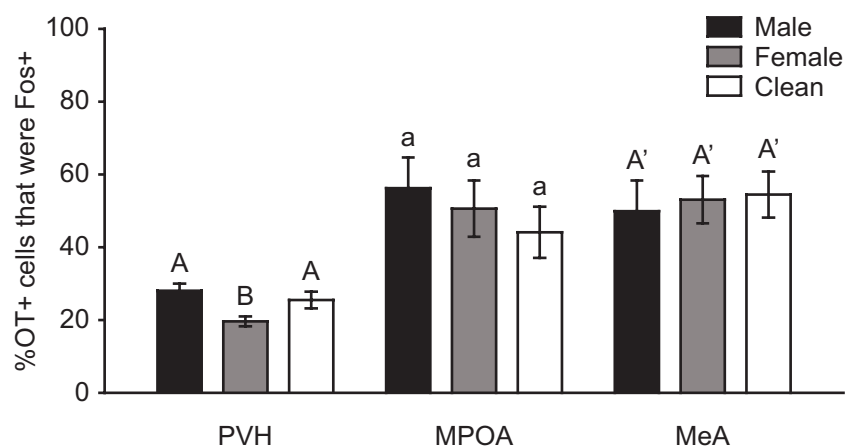


Figure 5.4 Mean (\pm SEM) percentages of oxytocin/Fos double-labeled cells following stimulus exposures

In the paraventricular nucleus of the hypothalamus (PVH), a lower percentage of oxytocin-positive (OT+) cells were positive for Fos (Fos+) following exposure to female odors as compared to either male or clean odors. No differences were observed in the percentage of OT+/Fos+ cells across stimulus conditions in either the medial preoptic area (MPOA) or the anterior division of the medial amygdala (MeA). Few oxytocin-positive cells were observed in the bed nucleus of the stria terminalis (BNST) or posterior medial amygdala (MeP), and so double-labeled cells in those areas were not counted. Within each area, dissimilar letters = nonhomologous means (Tukey's *HSD* post-hoc tests).

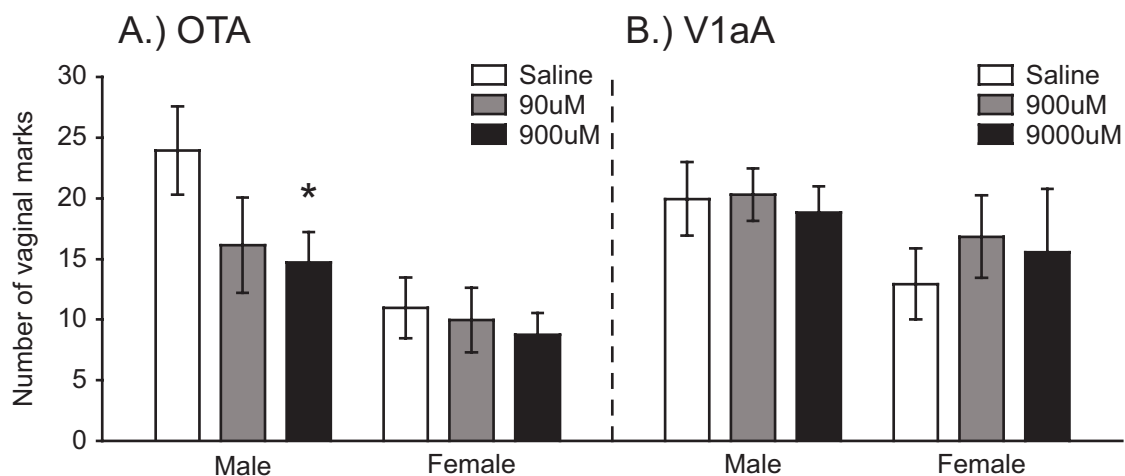


Figure 5.5 Mean (\pm SEM) number of vaginal marks following ICV drug injections

Mean (\pm SEM) number of vaginal marks following ICV drug injections. ICV injections of an oxytocin receptor antagonist (OTA) decreased vaginal marking in response to male odors, as compared to saline injections (A). OTA was most effective at the highest dose administered (900 μ M). There was no effect of OTA on vaginal marking in females exposed to female odors. Injections of a vasopressin 1a receptor antagonist (V1aA) did not affect vaginal marking in females exposed to either male or female odors (B). * $p < .05$ (Bonferroni correction), saline vs. 900 μ M OTA in the male stimulus condition.

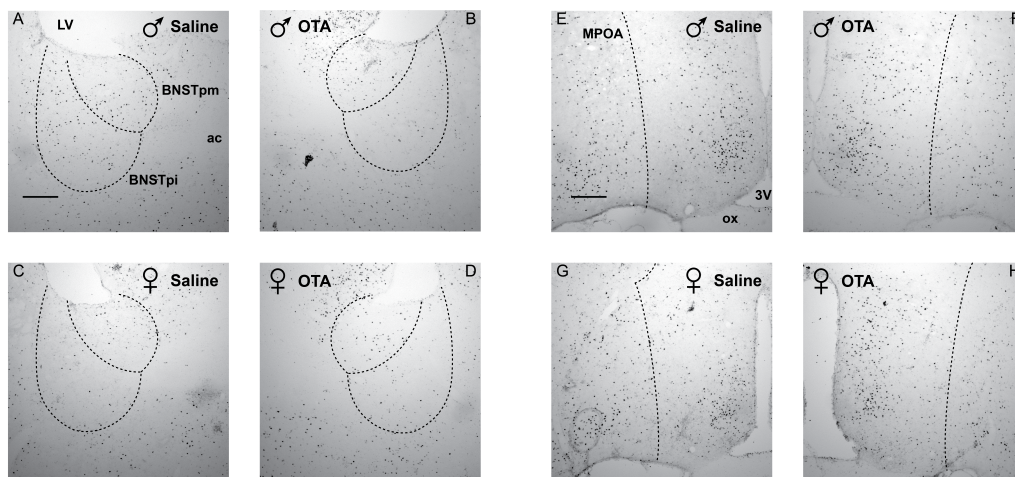


Figure 5.6 Immunohistochemical staining for Fos in the bed nucleus of the stria terminalis and the medial preoptic area

Representative photomicrographs of single-label immunohistochemistry for Fos in sections depicting (A-D) the posteromedial (BNSTpm) and posterointermediate (BNSTpi) subdivisions of the bed nucleus of the stria terminalis, and (E-H) the medial preoptic area (MPOA). Females were injected ICV with saline vehicle or an oxytocin receptor antagonist (OTA), and then exposed to male or female odors. Dotted lines represent borders of examined brain areas. 3V, third ventricle; ac, anterior commissure; ox, optic chiasm. Scale bar = 250 μ m.

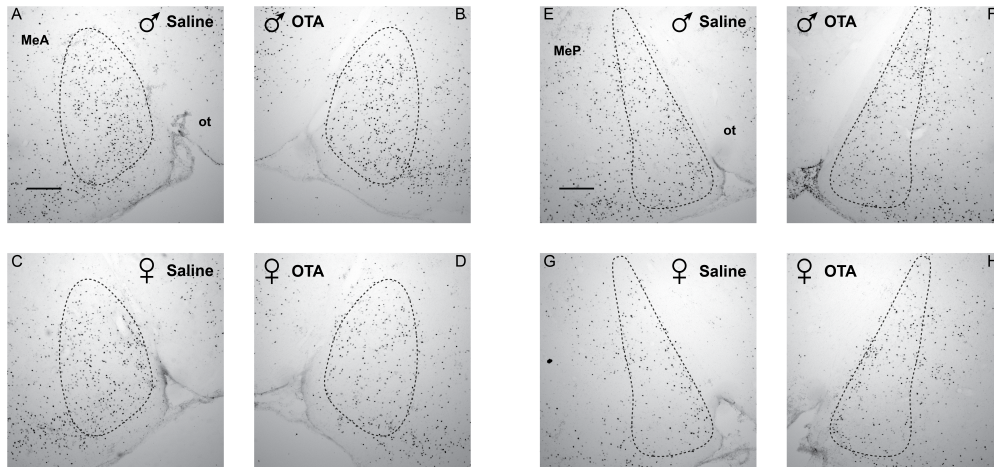


Figure 5.7 Immunohistochemical staining for Fos in the anterior and posterior divisions of the medial amygdala

Representative photomicrographs of single-label immunohistochemistry for Fos in sections depicting the (A-D) anterior and (E-H) posterior divisions of the medial amygdala (MeA and MeP, respectively). Females were injected ICV with saline vehicle or an oxytocin receptor antagonist (OTA), and then exposed to male or female odors. Dotted lines represent borders of examined brain areas. ot, optic tract. Scale bar = 250 μ m.

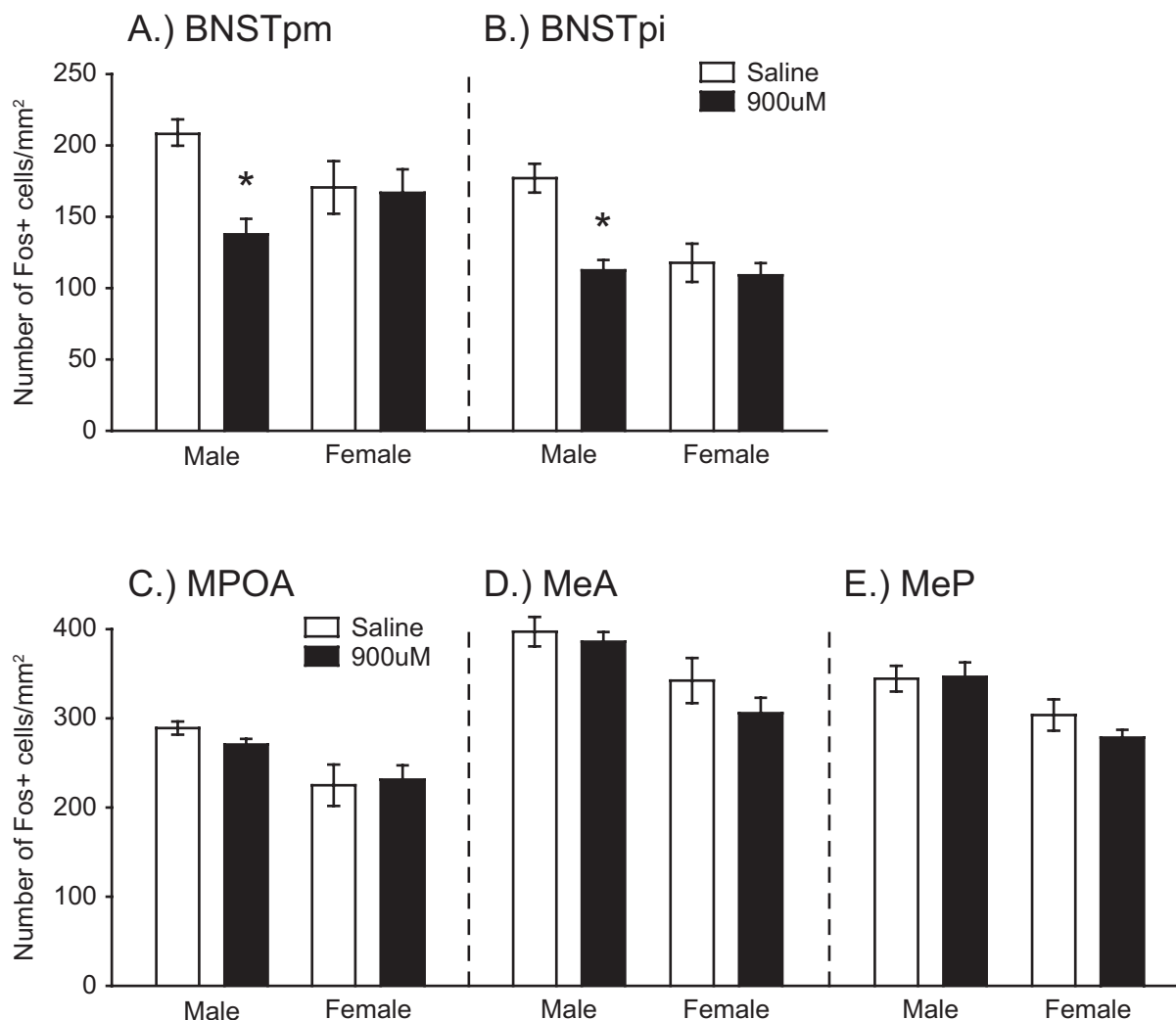


Figure 5.8 Mean (+/- SEM) densities of Fos-positive cells following ICV injections of OTA

Mean (+/- SEM) densities of Fos-positive cells following ICV injections of OTA. In females exposed to male odors, ICV injections of a selective oxytocin receptor antagonist (OTA) reduced the density of Fos-positive cells in both the posteromedial (BNSTpm) and posterointermediate (BNSTpi) subdivisions of the bed nucleus of the stria terminalis, as compared to saline injections (A, B). This effect was not seen in females exposed to female odors. Although there was an overall increase in the density of Fos-positive cells in the medial preoptic area (MPOA) and the anterior (MeA) and posterior (MeP) divisions of the medial amygdala in response to male vs. female odors, there was no effect of OTA on Fos expression within these areas (C, D). * $p < .05$, OTA vs. saline in the male odor condition.

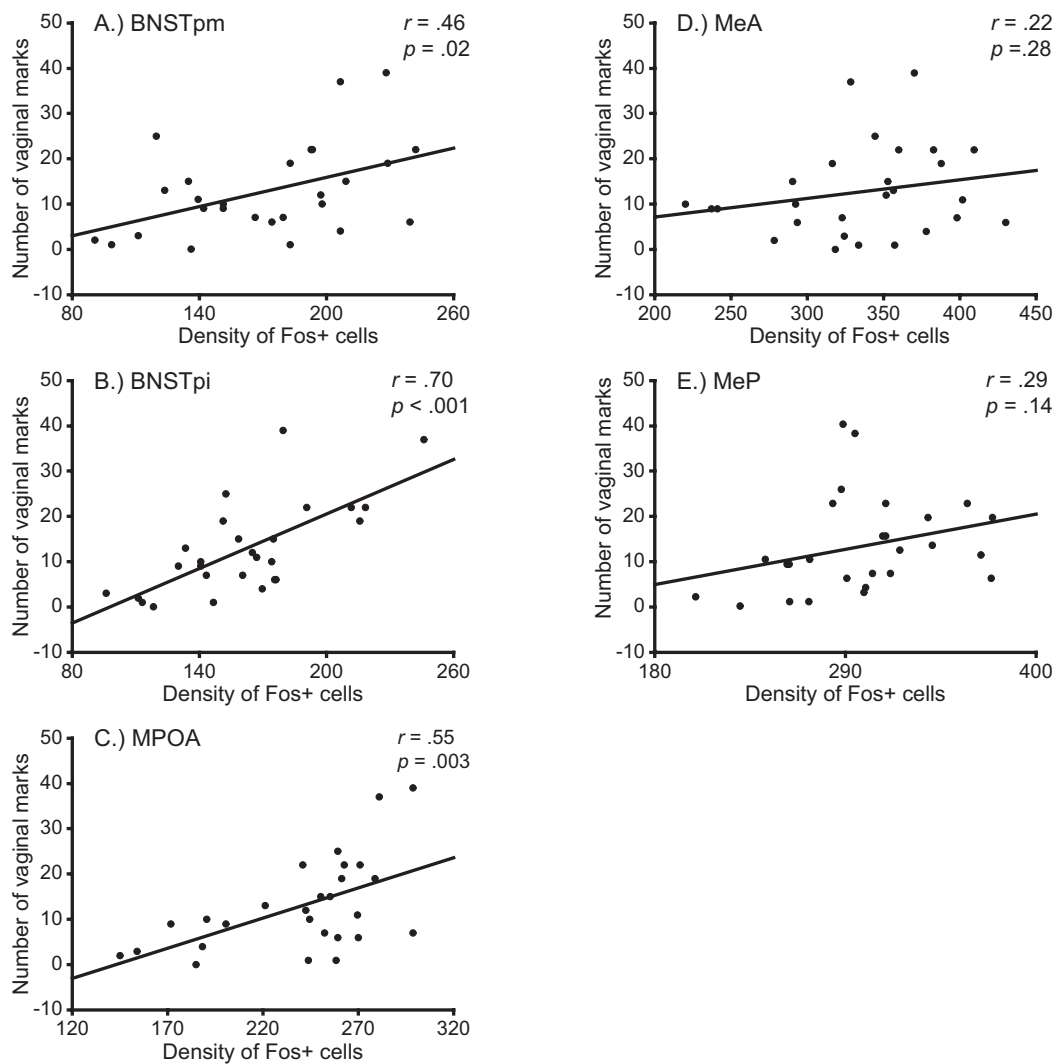


Figure 5.9 Relationships between the density of Fos-positive cells and the number of vaginal marks
 Irrespective of stimulus type (male or female), the density of Fos-positive cells was correlated with the number of vaginal marks for the (A) posteromedial (BNSTpm) and (B) posterointermediate (BNSTpi) subdivisions of the bed nucleus of the stria terminalis, and the (C) medial preoptic area (MPOA). The relationship between Fos expression and vaginal marking was not significant for either the (D) anterior (MeA) or (E) posterior (MeP) divisions of the medial amygdala.

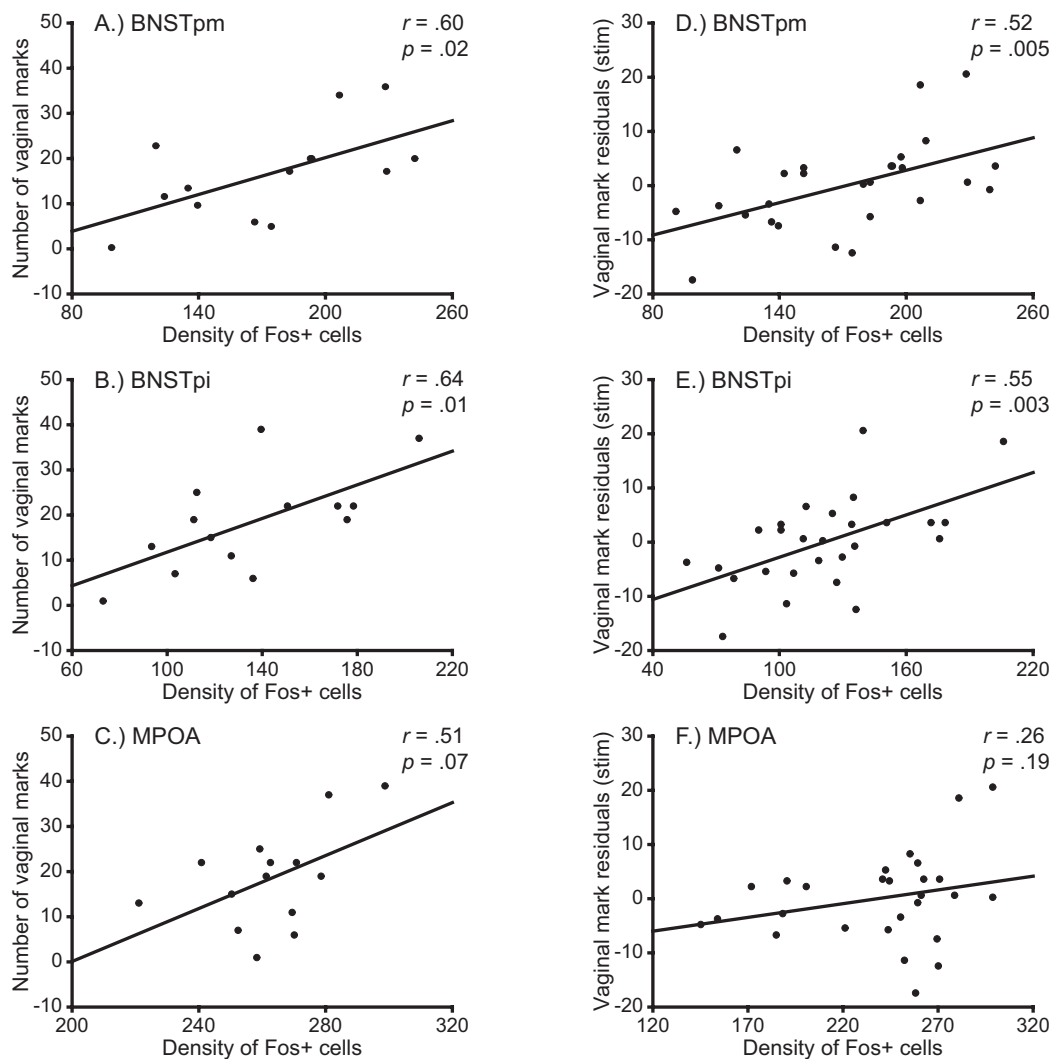


Figure 5.10 Relationships between the density of Fos-positive cells and the number of vaginal marks
 When examining responses to male odors only, the density of Fos-positive cells was positively correlated with the number of vaginal marks for the (A) posteromedial (BNSTpm) and (B) posterointermediate (BNSTpi) subdivisions of the bed nucleus of the stria terminalis, but not the (C) medial preoptic area (MPOA). This pattern of results was also seen when looking at overall responses across stimulus types, when controlling for the effect of stimulus type on Fos expression and vaginal marking (D-F).

CHAPTER 6: GENERAL DISCUSSION

6.1 Summary

The goal of this dissertation was to determine the functional and neurochemical mechanisms whereby two ventral forebrain areas, the bed nucleus of the stria terminalis (BNST) and the medial pre-optic area (MPOA), regulate the expression of odor-guided precopulatory behaviors in female hamsters. We found that endogenous oxytocin acting directly within these brain areas is necessary for the normally high levels of vaginal marking expressed in response to male odor stimuli (Chapter 2). This effect could not be attributed to the closely related vasopressin 1a receptor (V1aR) system, as blockade of V1aRs did not mimic the effects of oxytocin receptor (OTR) blockade. Furthermore, this effect was not due to a general deficit in odor-guided responses, since OTR blockade in these areas did not affect the normal preference to investigate opposite-sex stimuli. Next, we found that BNST is required for the preferential expression of vaginal marking in response to male vs. female stimuli, but not for opposite-sex odor preference (Chapter 3). In contrast, we found that MPOA is required for opposite-sex odor preference, but not for preferential vaginal marking to male odor stimuli (Chapter 4). Finally, we found that there are multiple mechanisms whereby the oxytocin system and sexual odor stimulus processing may interact within the ventral forebrain to regulate precopulatory behaviors (Chapter 5). Female odors suppressed activity in oxytocinergic neurons in the paraventricular nucleus of the hypothalamus (PVH), and central blockade of OTRs decreased vaginal marking, as well as activity in neurons in BNST in response to male odors. There was no effect of OTR blockade on neuronal activity in MPOA. Considered together, these results demonstrate that distinct neural circuits regulate two closely related aspects of precopulatory behaviors in females, and that oxytocin acts specifically within the sexual solicitation circuit to preferentially target this behavior towards opposite-sex conspecifics.

6.2 Functional Regulation of Female Sexual Behaviors

Precopulatory behaviors

Across species, females engage in a diverse range of precopulatory behaviors dedicated to locating and attracting the interest of potential mates (Beach, 1976). A conserved set of areas within the ventral forebrain, including the medial amygdala (MA), BNST, and MPOA, are involved in regulating these responses. Furthermore, this circuit appears to be more generally involved in regulating reproductive behaviors in males and females, as posited by Newman (1999). The specific roles of these brain areas in precopulatory behaviors will be described below, focusing specifically on regulation of behaviors within females (i.e., vaginal marking vs. opposite-sex odor preference), and between the sexes (opposite-sex odor preference in males vs. females).

Role of MA in female precopulatory behaviors

In mammals, chemosensory information critical for the expression of precopulatory behaviors is initially detected and processed by the main (MOS) and accessory (AOS) olfactory systems (Petrulis, 2009). These systems directly relay chemosensory information to MA, particularly to the anterior division of this brain area (MeA) (Davis et al., 1978; Kang et al., 2009). The posterior division of MA (MeP) receives less substantial direct olfactory input, but does receive indirect input via MeA, cortical amygdaloid structures, and olfactory cortex (Coolen and Wood, 1998). In contrast to MeA, MeP densely expresses steroid hormone receptors, including androgen, estrogen (ER), and progesterone (PR) receptors (Du et al., 1996; Li et al., 1993; Wood and Newman, 1999; Wood et al., 1992). Both MeA and MeP send projections to various limbic and hypothalamic brain areas, including the lateral septum (LS), BNST, MPOA, and the ventromedial hypothalamus (VMH) (Coolen and Wood, 1998; Gomez and Newman, 1992). One function of MA is to relay chemosensory relevant for precopulatory behaviors to downstream areas. Specifically, Fos (an immediate early gene induced by social stimuli (Pfaus and Heeb,

1997)) is preferentially induced in MA by opposite-sex odors in hamsters (DelBarco-Trillo et al., 2009; Maras and Petrulis, 2010a; Chapter 5), and at least in males, preferential Fos expression is disrupted in BNST and MPOA following MeA lesions (Maras and Petrulis, 2010a). Lesions centered on MA eliminate the preference for male odors in female hamsters, rats, and mice (DiBenedictis et al., 2012; Kondo and Sakuma, 2005; Petrulis and Johnston, 1999). Surprisingly, MA is not required for the preferential expression of vaginal marking to male odors (Petrulis and Johnston, 1999), suggesting that other brain areas may mediate this effect. In further support of this conclusion, Fos expression in MA was not correlated with the expression of vaginal marking (Chapter 5).

Although it is not critical for the odor-guided aspects of vaginal marking, MA is important for the overall expression of this behavior. Female hamsters exhibit a stable four day estrous cycle (Orsini, 1961), characterized by fluctuating levels of estrogen (E) and progesterone (P) (Baranczuk and Greenwald, 1973; Saidapur and Greenwald, 1978). Levels of vaginal marking also vary across the cycle (Johnston, 1977; Takahashi and Lisk, 1983); furthermore, vaginal marking is eliminated following ovariectomy (OVX) and can be reinstated in OVX females by systemic administration of E (Lisk and Nachtigall, 1988). Given that MA densely expresses ERs (Yamamoto et al., 2006; Zhang et al., 2002), this area could partially mediate the facilitative effects of E of vaginal marking. In agreement with this idea, lesions of MA decrease overall levels of vaginal marking (Petrulis and Johnston, 1999). These lesions also decrease ultrasonic vocalizations in female hamsters (Kirn and Floody, 1985), a behavior that is strongly regulated by circulating levels of E (Floody et al., 1979).

Role of MA in male precopulatory behavior

Both males and females prefer to investigate odors from opposite-sex conspecifics, thereby providing an ideal opportunity to examine whether the neural circuitry underlying this behavior is conserved across the sexes. There is substantial evidence implicating MA in the regulation of opposite-sex odor preference in males. Lesions of either MeA or MeP eliminate opposite-sex odor preference in male

hamsters (Maras and Petrulius, 2006), albeit via different mechanisms. Lesions of MeA result in equivalently high levels of investigation of both male and female odors, suggesting that MeA is critical for decreasing attraction towards inappropriate (male) stimuli. In contrast, males with MeP lesions investigate male and female odors at equivalent, low levels, suggesting that this subdivision may be critical for increasing attraction towards appropriate (female) stimuli. Integration between MeA and MeP is also required normal expression of opposite-sex odor preference in males, since lesions that functionally disconnect these two areas eliminate odor preference (Maras and Petrulius, 2010b). In male rats, however, lesions of MeA or MeP do not significantly impair investigatory preferences for estrous female odors (Kondo and Sachs, 2002), indicating that there are species differences in the precise role of MA in sexual odor preference in males. This is in contrast to females, wherein data more consistently support a role for MA in opposite-sex odor preference and attraction to male odors (DiBenedictis et al., 2012; Kondo and Sakuma, 2005; Petrulius and Johnston, 1999).

Role of BNST in female precopulatory behaviors

Similar to MA, BNST also receives direct input from AOS (Davis and Shi, 1999). Furthermore, BNST receives substantial indirect MOS and AOS input via its connections with corticomедial amygdaloid areas, including the anterior (ACo), posterolateral (PLCo), and posteromedial (PMCo) cortical amygdala, and MA (Wood and Swann, 2005). Olfactory input predominantly terminates within the posterointermediate subdivision of BNST (BNSTpi), whereas steroid hormone receptors are predominantly expressed in the posteromedial subdivision (BNSTpm). Both subdivisions of BNST are highly interconnected with MA and MPOA, and send projections to areas previously implicated in the motoric aspects of vaginal marking, including the midbrain periaqueductal gray (PAG) (Been and Petrulius, 2007; Wood and Swann, 2005). Based on these anatomical data, BNST appears ideally situated to integrate internal (hormonal and neurochemical) with external (chemosensory) factors relevant for odor-guided precopulatory behaviors, and to subsequently relay this information to downstream areas. In agreement with this model,

BNST is preferentially activated by opposite-sex odors in hamsters (Maras and Petrulis, 2010a; Chapter 5), and lesions of BNST eliminated preferential vaginal marking to male odors (Chapter 3). BNST lesions did not affect overall levels of vaginal marking, suggesting that this area does not mediate the permissive effects of gonadal steroids on solicitational responses.

Chemosensory information necessary for preferential vaginal marking may reach BNST via multiple mechanisms. As mentioned above, MA provides substantial olfactory input to BNST (both MOS and AOS); however, lesions of MA do not eliminate preferential marking to male odors (Petrulis and Johnston, 1999). These lesions also substantially damaged ACo, an area that provides MOS input to BNST. AOS input can reach BNST independent of MA via direct projections from the accessory olfactory bulbs (AOBs), as well as indirectly via PMCo (Davis et al., 1978; Wood and Swann, 2005). Removal of the vomeronasal organ (VNO) eliminates preferential vaginal marking to male odors (Petrulis et al., 1999), similar to our reports following BNST lesions (Chapter 3). Considered together, these data suggest that AOS, rather than MOS, information is critical for preferential vaginal marking to male odors, and that this information reaches BNST independently of MA.

If AOS input is required for preferential vaginal marking, then this behavior should be dependent on the detection and processing of non-volatile chemosignals. This is not the case, since females vaginal mark at elevated levels to male vs. female odors when access to non-volatiles is prevented by a perforated barrier (Maras and Petrulis, 2008). It is difficult to rectify these findings with those of Petrulis et al. (1999), given that odors processed by MOS (volatile or otherwise) were insufficient to induce preferential vaginal marking following VNO removal. VNO can detect some non-volatile odorants (Trinh and Storm, 2003), but it is unlikely that this applies to conspecific odors. Preferential activation of AOBs by opposite-sex volatile odors in mice is eliminated following lesions of the main olfactory epithelium (MOE) (Martel and Baum, 2007; Muroi et al., 2006), thereby implicating the centrifugal inputs to AOBs (Kang et al., 2009; Martel and Baum, 2007, 2009), rather than VNO, in driving this effect. Further re-

search is needed to determine if VNO can respond to volatile as well as non-volatile conspecific odors in female hamsters, and whether MOS or AOS input to BNST is required for preferential solicitational responses to male odors.

Role of BNST in male precopulatory behavior

In contrast to MA, BNST is not consistently required for opposite-sex odor preference across the sexes. BNST lesions eliminate opposite-sex odor preference in male hamsters (Been and Petrulis, 2010a), and lesions of BNST in male hamsters and rats also decrease anogenital investigation of females during sexual interactions (Claro et al., 1995; Powers et al., 1987). In female hamsters, however, opposite-sex odor preference is not disrupted following lesions of BNST (Chapter 3). Given that the two studies examining opposite-sex odor preference in male and female hamsters damaged comparable aspects of BNST, and utilized identical procedures for lesioning and testing, it seems likely that the difference in effects across the two studies represent true sex differences in the requirement of BNST for opposite-sex odor preference.

The mechanisms underlying this sex difference are not clear. As mentioned above, Fos expression in BNST is increased in response to opposite- vs. same-sex odor stimuli in both male and female hamsters (Maras and Petrulis, 2010a; Chapter 5), suggesting that sexual odor information relevant for odor preference reaches BNST in both sexes. An extensive description of the neuroanatomical features of BNST in hamsters, including afferent and efferent connections, is available for males, but not females (Wood and Swann, 2005); therefore it is not known if differential patterns of connectivity may underlie the differential role of BNST in odor preference. There is some evidence for neuroanatomical differences in BNST across the sexes in rats. The volume of BNST is larger in males compared to females (del Abril et al., 1987; Hines et al., 1992). Furthermore, a striking sex difference in the vasopressin system in BNST has been identified in this species. Males express substantially more vasopressin cells in BNST compared to females (van Leeuwen et al., 1985). This vasopressin system is androgen sensitive, as castration in

males substantially decreases the number of vasopressin cells in BNST (DeVries et al., 1985). A sexually-dimorphic, androgen-sensitive vasopressin system in BNST was subsequently identified in a number of other species (De Vries and Panzica, 2006), but is not present in Syrian hamsters (Albers et al., 1991; Bolborea et al., 2010; Ferris et al., 1995) and therefore is not sufficient to explain the sex difference in requirement of BNST for opposite-sex odor preference.

One key difference in the expression of opposite-sex odor preference in male vs. female hamsters is the role of steroid hormones in regulating this behavior. In males, castration (GDX) significantly decreases investigatory responses towards female hamster vaginal secretion (FHVS); testosterone replacement (GDX+T) restores this behavior to normal levels (Powers et al., 1985). This effect appears to be mediated by androgens rather than E, since the non-aromatizable androgen DHT, but not E, also restores normal investigatory responses towards FHVS. Similar results were found when looking at partner preference in male hamsters. Preference for the female is eliminated in GDX males, whereas GDX+T males display a significant preference (Ballard and Wood, 2007). In contrast, female hamsters maintain preference for male odors across different days of the estrous cycle, as well as following OVX (Eidson et al., 2007). This is not unique to female hamsters, as odor preference is also maintained in OVX mice (Moncho-Bogani et al., 2004). Therefore, BNST may specifically mediate the effects of androgens on sexual odor preference in males. In females, BNST would have no role in odor preference given that this behavior is not sensitive to gonadal steroids. In further support of this idea, there is a substantial sex difference in the expression of androgen receptors in BNST of hamsters, both in total cell counts and intensity of immunoreactivity (Wood and Newman, 1999). This sex difference is not seen for other areas that have a conserved role in regulating opposite-sex odor preference across the sexes, such as MA.

Role of MPOA in female precopulatory behaviors

The role of MPOA in female precopulatory behaviors has been firmly established across a number of different studies. In contrast to MA and BNST, MPOA exclusively receives indirect olfactory infor-

mation (Davis et al., 1978; Simerly and Swanson, 1986; Wang and Swann, 2006). MPOA is a heterogeneous brain area that can be divided into at least two divisions: a medial aspect including the median and medial preoptic nuclei, and a lateral aspect (Wang and Swann, 2006). Olfactory input from MeA, ACo, PMCo and BNSTpi is predominantly restricted to the more lateral aspects, whereas the more medial aspects of MPOA densely express steroid hormone receptors (Been and Petrulis, 2011; Du et al., 1996; Li et al., 1993) and receive input from steroid-sensitive brain areas such as BNSTpm and MeP (Wang and Swann, 2006). MPOA sends projections to areas in the midbrain important for the expression of precopulatory behaviors in female rodents, including PAG and the midbrain locomotor region (MLR) (Simerly and Swanson, 1988). The shared neuroanatomical phenotypes and patterns of connectivity between MPOA and BNST raise the possibility that these areas have similar functional roles in odor-guided precopulatory behaviors in females. MPOA is preferentially activated by opposite-sex odors in hamsters (Maras and Petrulis, 2010a; Chapter 5). In contrast to BNST, however, lesions of MPOA did not affect preferential vaginal marking to male odors (Chapter 4). Rather, these lesions disrupt opposite-sex odor preference both in female hamsters (Chapter 4) and rats (Xiao et al., 2005). MPOA lesions also decrease solicitational responses in female rats (Hoshina et al., 1994; Whitney, 1986), whereas BNST lesions do not affect this behavior (Guarraci et al., 2004). Considered together, these data support the conclusion that BNST and MPOA have clearly dissociated roles in regulating female precopulatory behaviors.

Although MPOA is not critical for the preferential expression of vaginal marking in response to male odors in female hamsters, overall levels of vaginal marking were somewhat reduced following lesions of MPOA (Chapter 4). MPOA may therefore partially mediate the facilitative effects of E on vaginal marking, similar to the proposed role for MA. MPOA expresses ERs (Li et al., 1993), and implants of E directly into MPOA facilitate the expression of vaginal marking in females (Takahashi and Lisk, 1987; Takahashi et al., 1985). The mechanisms whereby E may ultimately facilitate solicitational responses in hamsters are not known. In rats, neurons in MPOA that project to MLR respond to systemic E treatment

by increasing their firing rates (Takeo and Sakuma, 1995). The functional role of this projection in female solicitational behaviors remains to be determined.

Role of MPOA in male precopulatory behavior

MPOA, similar to MA, regulates the expression of odor preference in both males and females. In hamsters, lesions of MPOA eliminate opposite-sex odor preference in both sexes (Been and Petrulis, 2010b; Chapter 4). Although this role for MPOA is commonly seen in males of other species, including rats and ferrets (Paredes and Baum, 1995; Paredes et al., 1998), disparate results have been reported in females. MPOA is not critical for opposite-sex odor preference in female ferrets (Robarts and Baum, 2007). In female rats, lesions of MPOA have been reported to disrupt odor preference (Xiao et al., 2005), or have no effect on this behavior (Guarraci and Clark, 2006). These differing results cannot be readily explained by lesion type/placement, species tested, or hormonal state of females. This more nuanced role of MPOA in opposite-sex odor preference in females compared to males raises the possibility that other hypothalamic areas (e.g., VMH (Robarts and Baum, 2007)) are more critically involved in the expression of this behavior in females.

Summary and conclusions

The data presented above implicate separate and distinct neural pathways in the regulation of different forms of odor-guided precopulatory behaviors in female hamsters (i.e., vaginal marking and opposite-sex odor preference). The odor-guided aspects of vaginal marking are dependent on AOS input to BNST (Figure 6.1A). In contrast, opposite-sex odor preference is dependent on MOS input to MA that is then relayed to MPOA, via a pathway that is independent of BNST (Figure 6.1B).

Between males and females, separate neural pathways regulate the expression of opposite-sex odor preference. The circuitry in females is as described above. In males, sexual odor information is transmitted from MA to BNST (Figure 6C). Although MPOA is also a necessary component of this circuit,

functionally disconnecting MA from MPOA does not impair opposite-sex odor preference. MPOA may instead receive odor information critical for sexual odor preference via its connections with other olfactory areas.

Considered together, these data demonstrate that conspecific signals important for precopulatory behaviors are processed in a behavior- and sex-dependent manner. This distinction may be particularly critical to ensure that internal factors such as steroid hormones (testosterone in males vs. females) and neuropeptides (see Oxytocin and Social Behaviors below) can selectively modulate the expression of these behaviors.

Copulatory behavior

The neural circuitry that regulates precopulatory behaviors in females also regulates the expression of copulatory behaviors. In this section, I will briefly examine the functional roles of MA, BNST and MPOA in regulating lordosis (the stereotyped posture assumed by female rodents that allows copulatory access to the male (Tiefer, 1970)). For a review of other components of the copulatory behavior circuit, including VMH, see Kow and Pfaff (1998).

Role of MA

The expression of lordosis is substantially reduced following disruption of primary olfactory structures (Keller et al., 2006; Mackay-Sim and Rose, 1986; Rajendren et al., 1990; Thompson and Edwards, 1972), suggesting that chemosensory processing has an important role in this behavior. However, MA does not mediate the facilitative effects of odors on lordosis. In intact, estrous females, lesions of MA enhance lordosis in hamsters and rats (Polston and Erskine, 2001; Takahashi and Gladstone, 1988). These data suggest that MA is instead part of a lordosis inhibiting circuit that also includes LS and MPOA (Sakuma, 2008). However, lesions have been reported to have no substantial effects on lordosis in OVX, E+P-primed rats and hamsters (Guarraci et al., 2004; Kirn and Floody, 1985; Kondo and Sakuma,

2005), and inhibit the expression of lordosis in hormonally-primed mice (DiBenedictis et al., 2012). It is not clear why the effects of MA lesions vary so substantially between intact, estrous females and females that have been artificially induced into a comparable hormonal state. MA is important for several hormonal effects that ultimately facilitate the expression of lordosis, including the surge in prolactin that occurs prior to mating (Polston and Erskine, 2001). Therefore, it may be that MA lesions differentially affect these hormone systems depending on the gonadal status of females, and this may at least partly explain the differences in the reported effects of MA lesions on lordosis.

Role of BNST

The available data from female hamsters and rats suggest that BNST is not required for the expression of lordosis. Lesions centered on BNST (resulting in concurrent damage to LS) do not affect either lordosis duration or latency in OVX, E+P primed females (Kirn and Floody, 1985). More specific lesions of BNST also failed to affect lordosis induced by brief contact with a male (Chapter 3). Finally, lesions of BNST do not affect the expression of lordosis or paced mating in female rats (Guarraci et al., 2004). Fos expression in BNST is increased following mating in female rats and hamsters (Joppa et al., 1995; Polston and Erskine, 1995), indicating that this area may be important for behavioral plasticity associated with repeated mating experience. Female Syrian hamsters make small movements of their perineum while maintaining the lordosis posture, thereby facilitating intromissive access by the male (Noble, 1979, 1980). Sexual experience increases dopamine in the nucleus accumbens (NAc) (Kohlert and Meisel, 1999) and facilitates performance of these perineal movements (Bradley et al., 2005). Dopamine in NAc is in fact critical for the effects of sexual experience on perineal movements, since lesions of dopaminergic terminals in NAc block the effects of experience (Bradley et al., 2005). BNST is bidirectionally connected with NAc in hamsters (Wood and Swann, 2005), providing an anatomical substrate whereby sensory information associated with mating could interact with the mesolimbic dopamine reward system. Further research is needed to determine if BNST is required for the effects of repeated

sexual experience on copulatory responses in female hamsters, and if the connections with NAc are critical for mediating this effect.

Role of MPOA

In female rats and hamsters, MPOA normally inhibits the expression of lordosis. The effects of MPOA lesions in hamsters are most evident in OVX, E-primed females, a hormonal state associated with little or no expression of lordosis (Tiefer, 1970). MPOA lesions facilitate the expression of lordosis in these females, whereas no effect of MPOA lesions is seen in OVX, E+P primed or intact, estrous females (Floody, 1989; Chapter 4). In rats, MPOA lesions also facilitate lordosis when females receive sub-optimal hormone priming regimens (Hoshina et al., 1994; Whitney, 1986).

The mechanisms whereby MPOA inhibits lordosis in hamsters are not known. Subsets of neurons in MPOA are inhibited during the expression of lordosis in hamsters and rats (Kato and Sakuma, 2000; Rose, 1990), and stimulation of MPOA in rats disrupts the expression of lordosis (Hoshina et al., 1994; Takeo et al., 1993). The inhibitory effects of MPOA on lordosis in rats are likely mediated by projections from MPOA to the ventral tegmental area (VTA) (Hasegawa and Sakuma, 1990). This may also be true for hamsters, since VTA lesions inhibit lordosis (Lisciotto and DeBold, 1991; but see Floody and DeBold (2004)), and this area partially mediates the facilitative effects of P on this behavior (DeBold and Malsbury, 1989; Pleim et al., 1990).

Summary and conclusions

The data presented above demonstrate that the neural circuitry critical for the odor-guided aspects of precopulatory behaviors in females does not mediate the permissive effects of odors on copulatory behavior. Rather, this circuit generally functions to inhibit lordosis, a role that may be essential to allow chemosensory signals to stimulate precopulatory behaviors when the female is not engaging in lordosis. When given free access, females will mate with multiple males during a given period of sexual

receptivity (Brown et al., 1988; Huck et al., 1986). Indeed, females will actively pursue sexual contact with one male while aggressively rejecting a male she has already mated with (Huck et al., 1986). Even when limited to a single male, females will eject that male from her nest area after approximately one hour of mating interactions (Lisk et al., 1983). This suggests that females normally need to re-engage precopulatory responses and seek out a new mate even in the presence of an available mate. The precise roles of MA, BNST, and MPOA in this transition, however, are not known.

6.3 Oxytocin and Social Behaviors

A wealth of evidence exists describing the neurotransmitter systems within ventral forebrain systems that regulate social behaviors. Our data and that of others implicate the neuropeptide oxytocin in facilitating prosocial responses in animals. In this section, I will specifically focus on the role of oxytocin in female sociosexual behaviors, as well as the underlying mechanisms whereby oxytocin modulates responses to social stimuli.

Female precopulatory behaviors

Our finding that OTR blockade in BNST inhibited solicitational responses towards males (Chapter 2) suggests that endogenous oxytocin normally acts within this area to modulate chemosensory processing. In agreement with this mechanism of action, intracerebroventricular (ICV) injections of OTA decreased vaginal marking as well as Fos expression in BNST in response to males odors (Chapter 5). Modulation of chemosensory processing may be a primary mechanism whereby oxytocin regulates social behaviors in rodents. Genetic disruption of oxytocin (OTKO) or OTR (OTRKO) expression impairs the ability of mice to discriminate between familiar and unfamiliar conspecifics (Ferguson et al., 2000; Takayanagi et al., 2005; Wersinger et al., 2008). Fos expression in MA, BNST, and MPOA induced by conspecifics is also reduced in OTKOs, and ICV injections of OTA block social recognition and decrease Fos expression in MA to a variety of odorants, including odors from opposite-sex conspecifics and odors

from heterospecifics (predator and non-predator odors) (Samuelsen and Meredith, 2011). In contrast to the broad disruption of chemosensory processing seen in mice following oxytocin system disruption, oxytocin has a more specific role in female hamsters. Injections of OTA into BNST/MPOA disrupted preferential vaginal marking without affecting other odor-induced precopulatory responses (i.e., opposite-sex odor preference) (Chapter 2), and ICV injections of OTA disrupted Fos expression in BNST, but not MA or MPOA (Chapter 5).

Endogenous oxytocin may facilitate preferential solicitation of males via direct actions at OTRs on male odor-responsive neurons in BNST. OTRs are expressed in BNST of hamsters (Dubois-Dauphin et al., 1992), and in rats, oxytocin potentiates excitability of BNST neurons (Ingram et al., 1990). Both oxytocin binding and sensitivity to oxytocin in BNST are enhanced by treatment with E (Kremarik et al., 1995; Terenzi et al., 1999), thereby establishing baseline sensitivity of this area to oxytocin. If selective responses to male odors are strictly determined by OTR expression patterns, then OTRs should be preferentially expressed by male-odor responsive neurons in BNST, and further, OTA should decrease activation of OTR-expressing neurons in response to male, but not female, odors. These possibilities remain to be tested. Alternatively, if selectivity is determined by differential release of oxytocin, then release of oxytocin in BNST should vary in response to male vs. female odors. Although release oxytocin in BNST following exposure to conspecific odors has not been examined, male odors are not sufficient to induce oxytocin release above baseline in other brain areas in female voles (NAc) and sheep (VMH) (Kendrick et al., 1993; Ross et al., 2009a). PVH is the primary source of oxytocin input to BNST in hamsters (de Vries and Buijs, 1983), and we found that Fos expression by oxytocin cells in this area was equivalent in females exposed to either male or clean odors, but suppressed in response to female odors (Chapter 5). Basal levels of oxytocin may therefore be sufficient to support normal levels of vaginal marking to male odors, whereas normal levels of vaginal marking to female odors requires reduced levels of oxytocin.

Direct measures of oxytocin release in BNST following exposure to conspecific odors will be required to determine if this model of action is valid.

The specific facilitation of preferential vaginal marking, but not opposite-sex odor preference, by endogenous oxytocin in BNST provides further evidence for distinct neural circuits regulating different forms of odor-guided precopulatory behaviors. This difference in dependence on oxytocin may allow solicitation and investigatory responses to be independently modulated. As mentioned above, the expression of vaginal marking is sensitive to E (Lisk and Nachtigall, 1988), whereas sexual odor preference is not (Eidson et al., 2007). Furthermore, if oxytocin release from PVH is indeed reduced in response to female odors, then dependence of approach and investigatory responses on oxytocin would prevent females from dynamically responding to male odors with increased investigation following recent exposure to female odors.

Interactions with motivational systems

Vaginal marking is a behavioral indicator of sexual motivation in female hamsters, in that it is an active response that females perform to gain copulatory access to a male (Ågmo, 1999). Odor stimuli from males are sufficient to induce vaginal marking, and do not require conditioning through prior exposure to males (Maras and Petrulis, 2008). Since oxytocin itself is not rewarding in hamsters (Song et al., 2012), it is unlikely that oxytocin mediates the incentive properties of male odors to drive preferential vaginal marking. The mesolimbic dopamine system has classically been implicated in the motivated aspects of sexual behavior (Hull et al., 1999); however, recent studies have demonstrated that this system is not critical for mediating the rewarding properties of unconditioned incentive stimuli such as male odors. Specifically, male odors retain their incentive properties in chemically-naïve female mice following either systemic dopamine receptor blockade (Agustín-Pavón et al., 2007) or lesions of dopaminergic terminals in NAc (Martínez-Hernández et al., 2012).

The opioid system has also been implicated in mediating sexual reward (Paredes, 2009; Pfaus et al., 2012). Systemic blockade of opioid receptors following administration of naloxone eliminates conditioned place preference in female rats (Paredes and Martínez, 2001). This effect is also seen following injections of naloxone into several limbic areas, including MA and MPOA (García-Horsman et al., 2008). Female still preferentially vaginal mark to male odors following lesions of both of these areas, however, suggesting that male odors have some incentive properties that are independent of these areas. It is also unlikely that these effects are mediated by areas more classically associated with opioid-dependent reward such as VTA (Bals-Kubik et al., 1993), since opioids in this area engage the mesolimbic dopamine system (Shippenberg et al., 1993). Alternatively, opioids could act within BNST to mediate the incentive properties of male odors. BNST contains endogenous opioid cell bodies and fibers, and expresses mu-opioid receptors (Neal and Newman, 1989; Poulin et al., 2009). Although evidence for naturally rewarding stimuli is limited, endogenous opioids in BNST do mediate some of the rewarding effects of drugs of abuse (Walker et al., 2000).

Summary and conclusions

Our results demonstrate that oxytocin modulates stimulus processing within BNST to ultimately drive preferential solicitation of males (Figure 6.2). This may occur through multiple mechanisms. In one model of action (6.2A), baseline release of oxytocin from PVH is sufficient to activate OTRs and potentiate firing responses to male odors. This ultimately results in the normally high levels of vaginal marking seen in response to male odors. Following exposure to odors from other females, release of oxytocin from PVH is reduced, resulting in reduced activation of these neurons and the normally low levels of vaginal marking induced by female odors. In an alternative model of action (6.2B), OTRs are preferentially expressed by male-odor responsive neurons, resulting in potentiated firing responses to male odors and preferential vaginal marking responses to these stimuli. In either model, sexual odors may ultimately engage the endogenous opioid system to increase motivation to vaginal mark.

Although these models are characterized in terms of sexual solicitation, they can be applied to other social behaviors regulated by oxytocin in hamsters and other species. Indeed, the hypothesized decrease in PVH oxytocin release in response to female odors may be essential to prevent oxytocin from inhibiting behaviors normally elicited by potential rivals (see Aggressive behavior section below).

Female copulatory behaviors

One of the most well-studied behavioral effects of central oxytocin is the facilitation of female copulatory behavior (Witt, 1995; also see Introduction). The available research implicates two areas, MPOA and VMH, in mediating the effects of oxytocin. MPOA may be a more critical site of action, however, since the suppression of lordosis following injections of an oxytocin receptor antagonist (OTA) into MPOA is greater compared to injections into VMH (Caldwell et al., 1994; Whitman and Albers, 1995). Within MPOA, oxytocin interacts with gonadal steroids to regulate female copulatory behavior. Treatment with E induces oxytocin release in MPOA, and subsequent treatment with P further potentiates oxytocin release while also increasing oxytocin binding density in this area (Caldwell, 1992). Expression of PRs in MPOA is enhanced following E administration (Moguilewsky and Raynaud, 1979), a gene transcription-dependent event (Harrington et al., 2003; Kraus et al., 1994). When the genomic actions of E in MPOA are prevented through injections of E conjugated to bovine serum albumin, oxytocin is still able to facilitate sexual receptivity in this area (Caldwell and Moe, 1999). This is in contrast to the inability of P to facilitate sexual receptivity when OTA is injected prior to P (Caldwell et al., 1994). Considered together, this data suggests that E and P function together to increase oxytocin release and OTR expression in MPOA just prior to sexual receptivity, resulting in an oxytocin-dependent facilitation of lordosis.

The model of oxytocin and sexual receptivity described above is in agreement with data from rats, but it does not fit results in hamsters previously reported by Whitman and Albers (1995). In this species, MPOA lacks oxytocin binding sites (Dubois-Dauphin et al., 1992), despite the presence of oxytocin-containing fibers and varicosities in this area (Whitman and Albers, 1998). Although it is possible that

there are putative OTRs in this area that are not revealed through binding of an iodinated OTA ($[^{125}\text{I}]\text{OTA}$), this seems unlikely. OTRs in other brain areas are revealed by $[^{125}\text{I}]\text{OTA}$ (Dubois-Dauphin et al., 1992), the identical compound used by Whitman and Albers in its non-iodinated form (1995).

Alternatively, it may be that the effects of oxytocin and OTA in MPOA are mediated by V1aRs. V1aR is expressed throughout MPOA in hamsters (Caldwell and Albers, 2004; Young et al., 2000), and both oxytocin and OTA can activate V1aRs (Manning et al., 1989, 2008), albeit to a lesser extent than vasopressin or V1aA. Injections of vasopressin into MPOA of OVX, E-primed females induce lordosis in the absence of a male (Huhman and Albers, 1993), but V1aA injections into the anterior hypothalamus (AH) fail to inhibit lordosis in estrus females during interactions with other females (Gutzler, 2009). It is not known if V1aA affects lordosis when injected into MPOA rather than AH, or when females are tested for lordosis induced by males. As a result, it cannot be conclusively determined if the effects of oxytocin system manipulations on sexual receptivity in hamsters are at least partially mediated by V1aRs in MPOA.

Finally, areas other than MPOA may mediate the effects of oxytocin manipulations on sexual behavior in hamsters. BNST is located just dorsal to MPOA along its entire rostral-caudal extent (Morin and Wood, 2001) and as mentioned above, oxytocin binding sites are found throughout BNST in hamsters (Dubois-Dauphin et al., 1992). Our findings strongly implicate BNST, rather than MPOA, in mediating the effects of endogenous oxytocin on sexual solicitation. Specifically, OTA injections that terminated strictly within BNST were as effective at decreasing vaginal marking as more ventral injections into MPOA that passed through BNST (Chapter 2). Although BNST could also mediate the effects of oxytocin manipulations on sexual receptivity, lesions of this area do not impair lordosis in female hamsters (Kirn and Floody, 1985; Chapter 3).

Aggressive behavior

Oxytocin has an inhibitory role in aggression in both males and females. Male OTRKO mice are less aggressive in response to an intruder (Takayanagi et al., 2005), whereas ICV injections of oxytocin in male prairie voles increase aggression (Winslow et al., 1993). In female hamsters, oxytocin injected into MPOA decreases aggression towards intruders, whereas injections of OTA increase aggression (Harmon et al., 2002a). As mentioned above, hamsters lack OTRs in the hypothalamus, and as a result, other mechanisms may underlie these effects. Female rats are aggressive during pregnancy (Lonstein and Gammie, 2002), and oxytocin injections into BNST inhibit aggression during this period (Consiglio et al., 2005). The source of oxytocin mediating this effect is PVH, since lesions of PVH or injections of oxytocin antisense into PVH increase aggression (Giovenardi et al., 1998). Therefore, oxytocin may also act in BNST of female hamsters to inhibit aggression, and decreased release of oxytocin in response to rivals is necessary to allow high levels of aggression to occur. Alternatively, the effects of oxytocin on aggression in female hamsters could be mediated by V1aRs. Injections of V1aA into AH facilitate aggression in females (Gutzler et al., 2010), similar to the reported effects of OTA injections in MPOA (Harmon et al., 2002a).

Following aggressive encounters, hamsters form stable dominant-subordinate relationships exemplified by increased territorial scent marking (flank marking) by the dominant compared to the subordinate individual, and reduced aggressive encounters within the pair (Ferris et al., 1987). In contrast to the inhibitory effect of oxytocin on aggression, oxytocin injections into MPOA do not affect flank marking in socially-naïve hamsters (Ferris et al., 1984; Harmon et al., 2002b). However, oxytocin injected into this area can induce higher flank marking responses when dominant females are tested with their subordinate partner, an effect that is not mimicked by vasopressin (Harmon et al., 2002b). Despite differing sites of action, these results demonstrate that oxytocin has a conserved role in facilitating responses to familiar conspecifics in both hamsters and mice (Ferguson et al., 2001; Meredith and Westberry, 2004).

Maternal behavior

In addition to its essential role in the periphery mediating the milk-letdown reflex in post-partum females (Nishimori et al., 1996; Takayanagi et al., 2005; Young, W. S. et al., 1996), oxytocin can also act within the brain to facilitate maternal behavior. OTKO female mice show deficits in pup retrieval and the amount of time spent licking and grooming pups, and similar deficits in pup retrieval are seen in OTRKO females. Likewise, deficits in the onset and maintenance of maternal care are seen following ICV injections of OTA in post-partum rats (Pedersen and Boccia, 2003). Numerous brain areas show increased Fos during interactions with pups, including MPOA and BNST (Lonstein and De Vries, 2000), implicating these areas in mediating the effects of endogenous oxytocin on maternal behavior. Indeed, injections of OTA into MPOA block pup retrieval and decrease the time the mother spends huddling over pups (Pedersen et al., 1994). Injections of oxytocin into BNST do not affect pup retrieval (Consiglio et al., 2005), but this data is inconclusive, since ICV injections of oxytocin also fail to further facilitate maternal behavior in post-partum female rats (Fahrbach et al., 1985). Considering the preponderance of data suggesting that MPOA and BNST act as a functional unit in the regulation of maternal behavior (for a review, see Numan and Stolzenberg (2009)), it seems likely that BNST may mediate some effects of endogenous oxytocin on this behavior.

Oxytocin may at least partly influence maternal behavior through alterations in processing of offspring-related odors. Virgin female rats avoid pups and have no preference for pup odors, with attraction only developing around the time of parturition (Fleming and Luebke, 1981; Kinsley and Bridges, 1990). ICV oxytocin injections are sufficient to induce maternal behavior in these females (Pedersen, 1997), raising the possibility that oxytocin increases attraction to pups or their odors. A similar olfactory preference develops in ewes for amniotic fluid at the time of parturition (Levy et al., 1983). Amniotic fluid may be the critical olfactory stimulus for the selective bond that ewes form with their lambs, since removal of this fluid increases rejection behaviors by ewes (Alexander et al., 1986). Both MPOA and

BNST are functionally involved in establishing the ewe-lamb bond (Perrin et al., 2007), and oxytocin is released in these areas at the time of parturition (Kendrick et al., 1992). Resultingly, oxytocin may act in these areas to support imprinting of olfactory stimuli associated with familiar lambs in ewes. In support of this conclusion, oxytocin injected into MPOA decreases rejection behaviors towards an unfamiliar lamb (Kendrick et al., 1992).

Pair bonding

Prairie voles form lasting pair bonds exemplified by higher levels of social contact and sexual behavior within the pair, and increased aggression to towards conspecifics outside of the pair (Getz et al., 1981). Social contact for at least 24 hours is sufficient to induce pair bonding in this species (Williams et al., 1992). The distribution of oxytocin cells and fibers are fairly conserved across pair bonding and non pair-bonding species (Wang et al., 1996); in contrast, oxytocin binding patterns vary dramatically across these species, with monogamous vole species exhibiting higher relative binding in prelimbic cortex, NAc, BNST, and lateral amygdala, and polygamous vole species exhibiting higher binding in LS, VMH, and posterior cortical amygdala (Insel et al., 1991). These data suggest that oxytocin may be critical for pair bonding, and further, that species differences in oxytocin binding, rather than oxytocin expression, underlie differences in reproductive strategies across species. In the absence of mating, ICV injections of oxytocin induce a partner preference in female prairie voles, whereas injections of OTA blocked preference in mated females (Insel and Hulihan, 1995). In this study, injections of vasopressin or V1aA were not insufficient to modify partner preference in females. Oxytocin injected ICV has also been reported to facilitate partner preference formation in males, albeit at a higher dose than is required in females (Cho et al., 1999).

NAc has been identified as a specific site of action mediating the effects of oxytocin on pair bond formation. Oxytocin is released in NAc of female prairie voles during interactions with a male partner (Ross et al., 2009a), and injections of oxytocin into NAc induces pair bond formation in females (Liu and

Wang, 2003). OTRs in NAc likely mediate this effect, since OTA injections block oxytocin-induced pair bond formation. Furthermore, overexpression of OTRs in NAc facilitates pair bonding in female prairie voles by increasing the likelihood that females will form a partner preference and increasing the time spent with their partner (Ross et al., 2009b). Successful pair bonding in females is dependent on male chemosensory signals processed by AOS (Curtis et al., 2001); however, it seems unlikely that oxytocin acting within NAc directly modulates olfactory processing. Instead, oxytocin interacts with the meso- limbic reward pathway to induce partner preference and pair bonding. Specifically, oxytocin-induced pair bond formation is blocked by dopamine receptor antagonists in NAc, and dopamine-induced pair bond formation is blocked by OTA in this area (Liu and Wang, 2003).

Non-mammalian species

The homologs to oxytocin expressed by most non-mammalian vertebrates are isotocin and mesotocin. The limited data available suggests that these peptides have a relatively comparable role in social behaviors as oxytocin does in mammals. In this section I will examine data from fishes (isotocin) and birds (mesotocin), given that the isotocin/mesotocin systems have been predominantly studied within these classes of animals.

In fish, the distribution of isotocin neurons are restricted to MPOA, and these cells send projections to the pituitary as well as throughout the ventral forebrain (Saito et al., 2004). A putative isotocin receptor is expressed in the forebrain and midbrain of fishes (Hausmann et al., 1995; Lema, 2010). Although further specificity in brain expression patterns of isotocin receptors is lacking, MPOA has been identified as a potential site of action. In a monogamous species of cichlids, paternal care induces Fos expression in MPOA isotocin neurons, and intraperitoneal (IP) injections of OTA prevents the induction of paternal care normally seen following spawning (O'Connell et al., 2012). IP Injections of a nonselective V1aR/OTR antagonist (V1aA/OTA) does not disrupt either pair bond formation or maintenance in males of this species, despite decreasing affiliative behaviors expressed towards females (Oldfield and

Hofmann, 2011). Isotocin also affects social behaviors in non-monogamous fish species. In goldfish, IP injections of isotocin induce social approach (Thompson and Walton, 2004). In female midshipman, isotocin infusions onto isolated preparations of MPOA inhibit neural activity associated with aggressive vocalizations, whereas OTA infusions facilitate this activity (Goodson and Bass, 2000).

The distribution of mesotocin cells in bird species is somewhat intermediate between mammals and fishes, with cells localized to PVH, SON, and MPOA (Bons, 1980). The hypothalamic mesotocin system projects to ventral forebrain areas, including the avian homolog of MA (taenial nucleus of the amygdala), LS, MPOA, BNST, and VMH (Goodson et al., 2012), whereas expression of the avian OTR homolog (VT3; Gubrij et al., 2005) is restricted within the ventral forebrain to LS and VMH (Leung et al., 2011). Recent data from zebra finches, a monogamous songbird species, suggests that mesotocin regulates sociality in females. ICV injections of OTA interfere with pair bond formation by both sexes, albeit more substantially in females (Klatt and Goodson, 2013). These injections also decrease the time spent with a familiar conspecific, whereas mesotocin has the opposite effect (Goodson et al., 2009). There was no effect of ICV injections of vasotocin (the non-mammalian homolog of vasopressin) on time spent with familiar conspecifics, indicating that this effect was specific to mesotocin. Finally, mesotocin may be essential for maternal behavior in avian species, since injections of OTA into the third ventricle of female turkeys eliminates brooding of recently hatched young (Thayananuphat et al., 2011).

The limited data available in non-mammalian species suggest that the prosocial role of the oxytocin system is conserved across taxa. This is intriguing given the diversity of stimulus modalities used by non-mammalian species to communicate social information, and supports the conclusion that this system modulates social information processing, rather than information derived from a single sensory modality (e.g., olfaction in rodents).

Clinical implications

The ability to appropriately respond to social signals is critical to the health and well being of all animals that engage in social behaviors, including humans. This is readily evident when observing individuals with dysfunctions of social behavior such as autism spectrum disorders (ASD). Indeed, a core feature of ASD is abnormal or inappropriate behavioral responses in social contexts (Volkmar, 2011), often resulting in substantial distress for the affected individuals as well as those around them (Stokes and Kaur, 2005). Although ASD represents a societal problem of staggering proportions, affecting as many as 1 in 88 children (Baio, 2012) and costing approximately \$35 billion per year (Ganz, 2007), little progress has been made towards identifying effective treatments for ASD. However, there is a growing body of evidence that suggest that deficits in social information processing may underlie the behavioral abnormalities associated with ASD (Volkmar, 2011).

Given the role of the oxytocin system in social information processing in non-human animals, dysfunctions of this system may underlie abnormalities in social behaviors in humans. Polymorphisms at several locations on the OTR gene are associated with ASD core features (Campbell et al., 2011). Furthermore, polymorphisms have been identified in the gene for CD38, a protein that regulates oxytocin release, resulting in lower levels of oxytocin in ASD individuals with the mutated form of this gene (Higashida et al., 2012). Data from recent studies suggest that these changes in the oxytocin system may be targets for clinical intervention. Oxytocin treatment has been found to improve social information recall, social decision making, and eye contact in ASD adults (Andari et al., 2010; Hollander et al., 2007), as well as detection of emotion from facial expressions in ASD adolescents (Guastella et al., 2010).

6.4 Conclusion

The roles of MA, BNST, and MPOA in regulating preferential approach and solicitation of opposite-sex conspecifics are well conserved across species, and provide a useful model for understanding social information processing in the brain. Oxytocin acts within this circuit to facilitate preferential re-

sponses to appropriate targets. The mechanisms whereby this occurs in female Syrian hamsters can therefore provide insight into how human and non-human animals normally process this information to generate context-appropriate social responses.

Chapter 6 Figures

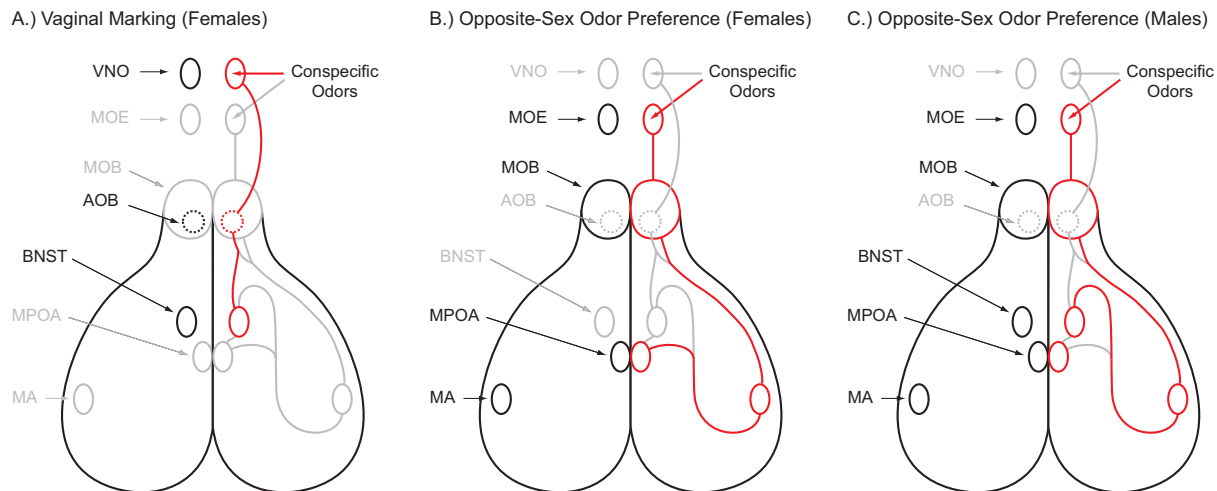
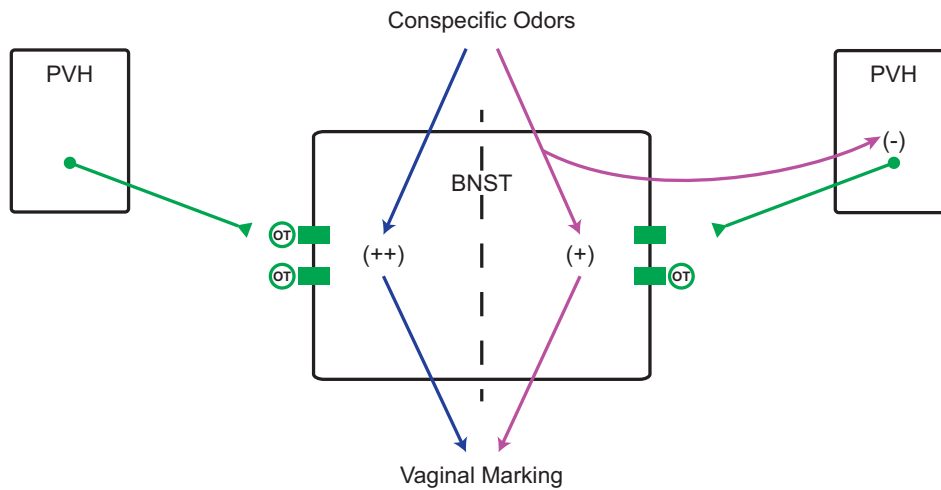


Figure 6.1 Functional pathways regulating odor-guided precopulatory behaviors

In hamsters, the pathway regulating preferential vaginal marking in females (A) requires chemosensory input from the vomeronasal organ (VNO) relayed through the accessory olfactory bulbs (AOB) to the bed nucleus of the stria terminalis (BNST). In contrast, the pathway regulating opposite-sex odor preference in females (B) requires chemosensory input from the main olfactory epithelium (MOE) relayed via the main olfactory bulbs (MOB) to the medial amygdala (MA), and ultimately the medial preoptic area (MPOA). The pathway regulating opposite-sex odor preference in males (C) is similar to that in females, with the notable exception that BNST and the connection between MA and BNST appear to be critical for this behavior in males.

A.)



B.)

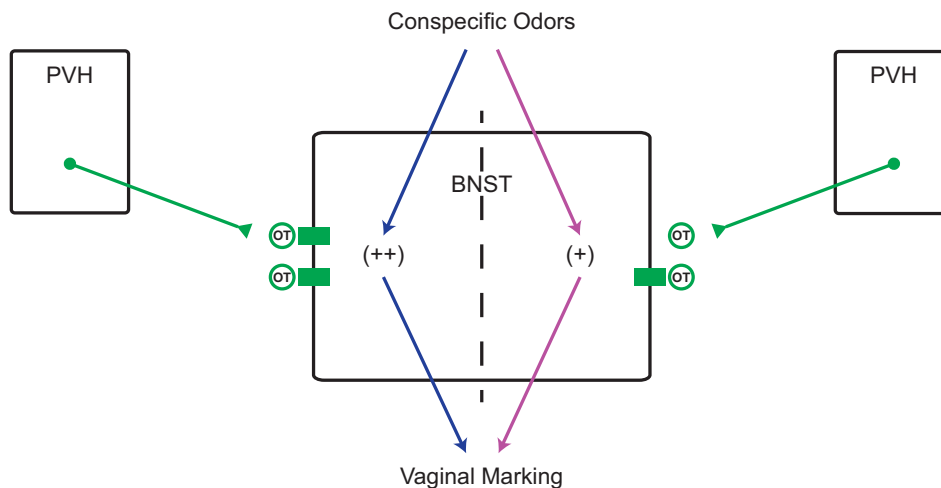


Figure 6.2 Regulation of preferential vaginal marking by oxytocin

Oxytocin could modulate sexual odor processing in BNST to drive preferential solicitation of males through multiple mechanisms. In one model of action (A), oxytocin is released at baseline levels in response to male odors, but suppressed in response to female odors, thereby resulting in enhanced facilitation of male odor-responsive neurons and preferential vaginal marking to male odors. In an alternative model of action (B), oxytocin receptors (OTRs) are expressed at higher levels on neurons that respond to male odors vs. female odors, resulting in selective facilitation of male odor-responsive neurons and preferential vaginal marking to male odors. Blue and pink represent sensory and motor signals induced by male odors or female odors, respectively. Green represents the oxytocin system (lines = fibers, circles = oxytocin released by fibers, and rectangles = OTRs). Positive (+) and negative (-) symbols represent facilitation and inhibition, respectively.

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INSTITUTION: University at Buffalo, Buffalo, NY (1998-2000, 2004-2006)

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MAJOR and ADVISOR(S): Neuroscience, Aras Petrulis (primary advisor), Elliott Albers (co-advisor)

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Fellowships, Awards, and Honors

GSU Dissertation Grant, Georgia State University, 2012-present

Honeycutt Fellow, Georgia State University, 2011-present

Center for Neuromics Student Research Award, Georgia State University, 2011

Travel Award, Society for Behavioral Neuroendocrinology Annual Meeting, Groningen, Netherlands, 2008

Brains & Behavior Program Fellow, Georgia State University, 2008-present

Center for Behavioral Neuroscience Scholar, Georgia State University, 2006-present

APA Diversity Program in Neuroscience Predoctoral Fellowship, Georgia State University, 2006-2008

Summer Program in Neuroscience, Ethics, and Survival Fellow, Marine Biological Laboratories, 2006

Feldman-Cohen Award for Distinguished Honors Achievement, University at Buffalo, 2006

McNair Research Award, University at Buffalo, 2006

Undergraduate Research and Scholarship Award of Distinction, University at Buffalo, 2006

Psychology Honors Program, University at Buffalo, 2005-2006

McNair Scholar, University at Buffalo, 2005-2006

McNair Summer Research Scholarship, University at Buffalo, 2005

Dean's List, University at Buffalo, 1998, 2004-2006

University Honors Scholar, University at Buffalo, 1998-2000

Minority Academic Achievement Program Scholar, University at Buffalo, 1998-2000

Publications

1. **Martinez, L.A.** & Petrulis, A. Endogenous oxytocin is required for Fos expression to male odors in the bed nucleus of the stria terminalis in female Syrian hamsters. Submitted to *Hormones and Behavior*, April 10th, 2013.
2. **Martinez, L.A.** & Petrulis, A. The medial preoptic area is necessary for sexual odor preference, but not sexual solicitation, in female Syrian hamsters. *Hormones and Behavior* (2013), 63(4): 606-614

3. **Martinez, L.A.** & Petrulis, A. The bed nucleus of the stria terminalis is critical for sexual solicitation, but not for opposite-sex odor preference, in female Syrian hamsters. *Hormones and Behavior* (2011), 60(5): 651-659.
4. **Martinez, L.A.**, Albers, H.E., & Petrulis, A. Blocking oxytocin receptors inhibits vaginal marking to male odors in female Syrian hamsters. *Physiology and Behavior* (2010), 101(5): 685-692.
5. Young, W.S., Wersinger, S.R., Caldwell, H.K., **Martinez, L.A.**, Gold, P., & Hu, S.B. Vasopressin 1a receptor knockout mice have a subtle olfactory deficit but normal aggression. *Genes, Brain and Behavior* (2007), 6(6): 540-551.

Presentations and Talks

1. **Martinez, L.A.** (April, 2013). *Neural regulation of sexual solicitation in female Syrian hamsters: Role of oxytocin*. PowerPoint presentation at the Brains and Behavior Annual Retreat, Georgia State University, Atlanta, G.A.
2. **Martinez, L.A.** & Petrulis, A. (October, 2012). *Ventral forebrain responses to sexual odors in female Syrian hamsters: Interactions with the central oxytocin system*. Poster presented at the Society for Neuroscience Annual Meeting 2012, New Orleans, L.A.
3. Petrulis, A., Helman, A., Tabbaa, M., & **Martinez, L.** (October, 2012). *Blocking GABA_A receptors in the bed nucleus of the stria terminalis disrupts sexual odor preference in male Syrian hamsters*. Poster presented at the Society for Neuroscience Annual Meeting 2012, New Orleans, L.A.
4. **Martinez, L.A.** & Petrulis, A. (November, 2011). *The role of the medial preoptic area in vaginal marking and opposite-sex odor preference in female Syrian hamsters*. Poster presented at the Society for Neuroscience Annual Meeting 2011, Washington, D.C.

5. **Martinez, L.A.** & Petrulis, A. (November, 2010). *The role of the posterior bed nucleus of the stria terminalis in vaginal marking and sexual odor preference in female Syrian hamsters*. Poster presented at the Society for Neuroscience Annual Meeting 2010, San Diego, CA.
6. **Martinez, L.A.** (May, 2010). *Neural regulation of mate solicitation in female Syrian hamsters: Role of oxytocin in the ventral forebrain*. PowerPoint presentation at the Neuroscience Institute Breakfast & Lecture Series, Georgia State University, Atlanta, GA.
7. **Martinez, L.A.** & Petrulis, A. (June, 2009). *Progesterone inhibits vaginal marking in ovariectomized, estradiol benzoate-primed female Syrian hamsters*. Poster presentation at the 13th annual meeting of the Society for Behavioral Neuroendocrinology, East Lansing, Michigan.
8. **Martinez, L.A.**, Petrulis, A., & Albers, E. (November, 2008). *Endogenous oxytocin in the medial preoptic-anterior hypothalamus facilitates vaginal marking and opposite-sex odor preference in female Syrian hamsters*. Poster presentation at the Society for Neuroscience annual meeting 2008, Washington, D.C.
9. **Martinez, L.A.**, Petrulis, A., & Albers, E. (July, 2008). *Oxytocin in the medial preoptic/anterior hypothalamus regulates vaginal marking in female Syrian hamsters*. Poster presentation at the 12th annual meeting of the Society for Behavioral Neuroendocrinology, Groningen, the Netherlands.
10. Caldwell, H.K., **Martinez, L.A.**, Hu, S., Young, W.S., & Wersinger, S.R. (June, 2006). *Vasopressin 1a Receptor Knockout Mice have Altered Circadian Rhythm and Olfaction*. Poster presentation at the Sixth International Congress of Neuroendocrinology, Pittsburgh, PA.

Instruction

Spring, 2012: PSYC 8420: Psychology Research Statistics II (Graduate Teaching Assistant)

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Spring, 2010: PSYC 3530: Advanced Research Design and Analysis (Graduate Teaching Assistant)

Fall, 2009: PSYC 8410: Psychology Research Statistics I (Graduate Teaching Assistant)

Spring, 2009: PSYC 8420: Psychology Research Statistics II (Graduate Teaching Assistant)

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Departmental and University Service

College of Arts and Sciences

Student Representative, GSU Life Sciences External Review Board Team Luncheon, August 28, 2012

Neuroscience Institute

Student Representative, Executive Committee, 2011-2012

Co-organizer, Neuroscience Institute Breakfast & Lecture Series, 2010-2011

Department of Psychology

Student Representative, PSYC 3530 Redesign Committee, 2011-2012

Student Coordinator, Graduate Student Recruitment Weekend, 2009

Program Representative, Graduate Association of Student Psychologists, 2007-2008

Center for Behavioral Neuroscience

University Representative, CBN Graduate Student Association, 2008-2009

Volunteer, Annual Neuroscience Exposition, 2007-2009

Student Mentoring

Graduate student mentor to one McNair Scholar, 2012-present

Graduate student mentor to two University Honors Scholars, 2011-present

Graduate student mentor to two University Honors Scholars, 2008-2010

Professional Organizations

Society for Neuroscience

Society for Behavioral Neuroendocrinology

Center for Behavioral Neuroscience, Atlanta, GA

Brains & Behavior Program, GSU, Atlanta, GA