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Dissociated Functional Pathways for Appetitive and Consummatory Reproductive Behaviors in Male Syrian Hamsters (Mesocricetus auratus)

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DISSOCIATED FUNCTIONAL PATHWAYS FOR APPETITIVE AND CONSUMMATORY REPRODUCTIVE BEHAVIORS IN MALE SYRIAN HAMSTERS (*MESOCRICETUS AURATUS*)

by

LAURA BEEN

Under the Direction of Aras Petrulis, Ph.D.

ABSTRACT

In many species, including Syrian hamsters, male reproductive behavior depends on the perception of odor cues from conspecifics in the environment. Volatile odor cues are processed primarily by the main olfactory system, whereas non-volatile cues are processed primarily by the accessory olfactory system. Together, these two chemosensory systems mediate appetitive reproductive behaviors, such as attraction to female odors, and consummatory reproductive behaviors, such as copulation, in male Syrian hamsters. Main and accessory olfactory information are first integrated in the medial amygdala (MA), a limbic nucleus that is critical for the expression of reproductive behav-

iors. MA is densely interconnected with other ventral forebrain nuclei that receive chemosensory information and are sensitive to steroid hormones. Specifically, several lines of evidence suggest that MA may generate behavioral responses to socio-sexual odors via functional connections with the posterior bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA). It is unknown, however, how these three nuclei act as functional circuit to adaptively regulate appetitive and consummatory reproductive behaviors. Therefore, the overarching goal of this dissertation was to determine how BNST and MPOA function, both uniquely and as a circuit with MA, to generate attraction to female odors and copulatory behaviors in male Syrian hamsters. We found that BNST is required for attraction to female odors, but not for copulation, in sexually-naïve males. In contrast, MPOA is required for both attraction to female odors and for copulation in sexually-naïve males. Surprisingly, prior sexual experience mitigated the requirement of BNST and MPOA for these behaviors. Next, we found that MA preferentially transmits female odor information to BNST and to MPOA, whereas BNST relays female and male odor information equivalently to MPOA. Finally, we found that the functional connections between MA and BNST are required for attraction to female odors but not for copulation, whereas the functional connections between MA and MPOA are required for copulation but not for attraction to female odors. Ultimately, these data may uncover a fundamental mechanism by which this ventral forebrain circuit regulates appetitive and consummatory reproductive behaviors across many species and modalities.

INDEX WORDS: Medial amygdala, Bed nucleus of the stria terminalis, Medial preoptic area, Odor preference, Copulation, Olfaction, Reproduction, Pheromone

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ACo, anterior cortical nucleus of the amygdala

AHA, amygdalohippocampal area

AGI, anogenital investigation

AH, anterior hypothalamus

AOB, accessory olfactory bulbs

AOS, accessory olfactory system

A-P, anterior-posterior

AR, androgen receptors

AVP, vasopressin

BLA, basolateral amygdala

BNST, posterior bed nucleus of the stria terminalis

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

BNSTpi, posterointermediate bed nucleus of the stria terminalis

BNSTpl, posterolateral bed nucleus of the stria terminalis

BNSTpm, posteromedial bed nucleus of the stria terminalis

BNSTpc, posterior caudal bed nucleus of the stria terminalis

CeA, central nucleus of the amygdala

CTB, cholera toxin B

CTF, central tegmental field

CONTRA, two unilateral lesions in contralateral hemispheres

DA, dopamine

D-V, dorsal-ventral

E, ejaculations

ER, estrogen receptors

EXP, sexually-experienced

HBI, head and body investigation

I, intromissions

I-E, intromissions to ejaculation

IEG, immediate early gene

INAH, interstitial nucleus of the hypothalamus

IPSI, two unilateral lesions in ipsilateral hemispheres

LI, long intromissions

M, mounts

MA, medial amygdala

MA-BNST-X, disconnect lesion of the medial amygdala and posterior bed nucleus of the stria terminalis

MA-MPOA-X, disconnect lesion of the medial amygdala and medial preoptic area

ME, mount efficiency

MeA, anterior medial amygdala

MeP, posterior medial amygdala

M-L, medial-lateral

MOB, main olfactory bulbs

MOS, main olfactory system

MPN, medial preoptic nucleus

MPNmag, magnocellular medial preoptic nucleus

MPOA, medial preoptic area

MPOA-X, lesion of the medial preoptic area

NAc, nucleus accumbens

NeuN, Neuronal Nuclei protein

nPGi, nucleus paragigantocellularis

NVE, sexually-naïve

OT, oxytocin

PAG, periaqueductal gray

PBS, phosphate buffered saline

PEI, post-ejaculatory interval

PMCo, posteromedial cortical nucleus of the amygdala

PMV, ventral premammillary nucleus

PVH, paraventricular nucleus of the hypothalamus

SG, self-grooming

SHAM, sham lesion surgery

TnA, nucleus taeniae

VMH, ventromedial hypothalamus

VNO, vomeronasal organ

5-HT, serotonin

CHAPTER 1:

Introduction

OVERVIEW

Many animals, including rodents, use chemical signals as the primary means of conveying social information between individuals (Brennan and Keverne 2004; Zufall and Leinders-Zufall 2007). Volatile odor cues are processed primarily by the main olfactory system (MOS), whereas non-volatile cues are processed primarily by the accessory olfactory system (AOS) (Breer 2003). In Syrian hamsters, these two systems have been shown to mediate appetitive aspects of male reproductive behavior, such as attraction to female odors (Johnston 1974; Johnston 1975; Johnston and Kwan 1984; Petrulis and Johnston 1995), as well as consummatory aspects of reproductive behavior, such as copulation (Murphy and Schneider 1970; Powers and Winans 1975). MOS and AOS information is first integrated in the medial amygdala (MA), which is critical for the appropriate expression of reproductive behavior in male hamsters (Newman 1999). Several lines of evidence suggest that MA may influence reproductive behavior via its connections with the posterior bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) (Wood 1997). How these three nuclei work together to regulate different aspects of reproductive behavior, however, is poorly understood. Therefore, the overarching goal of this dissertation was to determine how BNST and MPOA function, both uniquely and as a circuit with MA, to regulate appetitive and consummatory reproductive behaviors in male hamsters. We answered this question across four chapters using functional, behavioral, and neuroanatomical approaches.

CHEMICAL COMMUNICATION IN RODENTS

Many animals use chemical signals as the primary means of conveying information between individuals (Brennan and Keverne 2004; Zufall and Leinders-Zufall

2007). In rodents, as in many mammalian species, odors can convey a broad range of social information, including the sex, individual identity, ownership, competitive ability, health, and reproductive status of the individual (Johnston 1990; Hurst and Beynon 2004; Brennan and Kendrick 2006). These social odors are processed by two anatomically distinct chemosensory systems: the main olfactory system (MOS), which processes primarily volatile odors, and the accessory olfactory system (AOS), which processes primarily non-volatile cues (Meredith 1991; Restrepo et al. 2004). In the MOS, low-molecular weight odorants dissolve in the mucus lining of the nasal passages. These cues are detected by receptors on olfactory sensory neurons located in the main olfactory epithelium, which, in turn, send axonal projections to the main olfactory bulb (MOB). Mitral cells in the MOB send efferent projections to the cerebrum via the lateral olfactory tract, and synapse on the anterior olfactory nucleus, the olfactory tubercle, the piriform and entorhinal cortices, and the corticomedial amygdala (Meredith 1991; Kelliher 2007). In contrast, in the AOS, higher molecular weight odorants are detected by receptors on sensory neurons located in a specialized sensory organ called the vomeronasal organ. Vomeronasal sensory neurons send axonal projections to mitral cells in the accessory olfactory bulb (AOB), which synapse on the bed nucleus of the olfactory tract, the corticomedial amygdala and the bed nucleus of the stria terminalis (Meredith 1991; Halpern and Martinez-Marcos 2003). Together, the MOS and AOS regulate the expression of most rodent social behaviors, including male reproductive behaviors (Meredith 1991; Keverne 2004; Hull and Dominguez 2007; Baum and Kelliher 2009; Keller et al. 2009).

Chemical communication is particularly important for solitary species, in which social odors can be the only means of synchronizing reproduction between sexes. For

example, male and female Syrian hamsters maintain large, non-overlapping territories in nature (Johnston 1990; Gattermann et al. 2008) and rely on the mutual perception of species-specific scent marks to come together for mating (Pfaff et al. 2008). It is perhaps not surprising then that removal of the olfactory bulbs (Murphy and Schneider 1970) or simultaneous deafferentation of the main and accessory olfactory systems (Powers and Winans 1975) eliminates mating behavior in sexually-naïve and sexually-experienced males. This is not the case, however, for social species, such as rats, in which social or sexual experience can prevent the elimination of mating behavior following olfactory bulbectomy (Larsson 1975). Furthermore, MOS and AOS are required for male hamsters' attraction to female hamster vaginal secretion alone (Powers et al. 1979) and for the stereotyped investigation of a receptive female's anogenital region during copulation (Devor and Murphy 1973; Powers and Winans 1975). Together, these data suggest that MOS and AOS information are critical for the appropriate expression of reproductive behaviors in male hamsters.

APPETITIVE AND CONSUMMATORY REPRODUCTIVE BEHAVIORS

In rodents and other non-human animals, male sexual behavior can be dissociated into two phases: the appetitive phase, which refers to a variety of behaviors that reflect courtship, sexual attraction, and/or sexual motivation, and the consummatory phase, which refers to the less variable behavioral sequence of copulation itself (Beach 1976; Everitt 1990; Ball and Balthazart 2008). In male hamsters (Bunnell et al. 1977; Arteaga-Silva et al. 2005; Hull and Dominguez 2007) and other rodents (Dewsbury 1969; Thornton et al. 1991; Hull and Dominguez 2007), the consummatory phase of re-

productive behavior involves a highly stereotyped sequence of mating bouts comprised of mounts, intromissions (penile insertions), and ejaculations. These mating bouts are followed by a post-ejaculatory period characterized by auto-grooming of the genitals and low levels of activity, after which, additional copulatory bouts can commence (Hull and Dominguez 2007). Unlike other rodents, male hamsters display an additional mating behavior termed “long intromissions,” during which males do not quickly dismount the female following vaginal penetration, but instead display a repetitive thrusting pattern (Bunnell *et al.* 1977). The expression of long intromissions is associated with the onset of sexual satiety in hamsters (Bunnell *et al.* 1977; Parfitt and Newman 1998). These fixed behavioral patterns comprise the consummatory phase of male sexual behavior in hamsters.

In comparison, the appetitive phase of reproductive behavior in male rodents involves the approach and investigation of female odors with the goal of bringing the male in contact with a receptive female. Male hamsters, like other rodents, are strongly attracted to female odors, particularly those found in females’ vaginal secretions (Johnston 1974; Johnston 1975; Johnston and Kwan 1984; Petrulis and Johnston 1995), and display a robust preference to investigate female odors more than male odors (Landauer et al. 1977; Steel 1982; Ballard and Wood 2007). Unlike other rodents, male hamsters do not require prior sexual experience to show this preference for opposite-sex odors (Maras and Petrulis 2006; Ballard and Wood 2007; Maras and Petrulis 2008; Been and Petrulis 2010a; Been and Petrulis 2010b), suggesting that attraction to female odors is an unconditioned appetitive response in male hamsters. Because the appetitive phase of reproductive behavior is more variable than the stereotyped se-

quence of copulatory behaviors, it can be difficult to accurately measure in a laboratory setting. Previous studies have used second-order conditioning tasks, such as training a male to press a lever for a stimulus previously paired with a receptive female, to measure sexual motivation (Everitt and Stacey 1987; Everitt et al. 1989). This can be problematic, however, as the incentive value of the unconditioned stimulus can vary between species (Coppola and O'Connell 1988) and second-order conditioning tasks do not necessarily recruit the same neural circuitry required for unconditioned appetitive sexual behaviors (Kippin et al. 2003). Tests that directly measure unconditioned appetitive responses, such as searching a bi-level chamber for a receptive female (Pfaus and Phillips 1991) or three-choice odor preference tests (Been and Petrulis 2010a; Been and Petrulis 2010b) are therefore better suited for testing the requirement of neural substrates for appetitive aspects of reproductive behavior.

SYRIAN HAMSTERS AS A MODEL SPECIES

Syrian hamsters provide a tractable model for studying the neural regulation of socio-sexual behaviors because their social interactions are simple, stereotyped, and, unlike several other well-studied laboratory species (e.g. rats, rhesus monkeys), depend *critically* on the perception of a limited set of chemosensory cues from conspecifics (Murphy and Schneider 1970). In hamsters, social odor cues are processed primarily in the limbic system (Wood 1998), which processes social information in a wide variety of species and in a manner that is independent of sensory modality. For example, homologous limbic nuclei in several avian species have been implicated in the behavioral and neural response to both visual and auditory social cues (Alger et al. 2009; Goodson et

al. 2009; Svec et al. 2009). Similarly, in non-human primates, limbic nuclei process visual cues from conspecifics, including those that are required for facial and emotional recognition (Leonard et al. 1985). Perhaps most importantly, neuroimaging studies in humans suggest that irregularities within limbic nuclei are correlated with psychopathological conditions that disrupt social processing in several different sensory modalities (Acosta and Pearl 2004; McCloskey et al. 2005; Aguilar et al. 2008; Drevets et al. 2008). Thus, our model can yield new and important information about how the brain processes social information at a more refined level of analysis and in a manner that can be generalized to a variety of species and conditions.

MA AND ODOR-GUIDED REPRODUCTIVE BEHAVIORS

Several lines of evidence suggest that a particular limbic nucleus, MA, is a critical node in the neural pathway that modulates odor-guided reproductive behaviors. First, MA receives social odor information directly from the main and accessory olfactory bulbs, as well as indirectly from other brain areas innervated by the olfactory bulbs (Lehman and Winans 1982; Coolen and Wood 1998). MA neurons are activated during exposure to a variety of social odors and during all social behaviors (Fernandez-Fewell and Meredith 1994), suggesting they play a significant role in processing social information. In addition to receiving social odor information, MA also contains dense populations of steroid receptor-containing neurons (Doherty and Sheridan 1981; Wood et al. 1992) that modulate social behavior. Specifically, unilateral testosterone implants into MA facilitate mating behavior in castrated male hamsters (Wood and Newman 1995c), suggesting testosterone within MA may modulate reproductive behavior. Functionally,

MA has been implicated as a major regulator of odor-guided social behavior. Lesion studies have consistently shown that MA is required for appropriate socio-sexual behavior in hamsters (Newman 1999; Petrulis and Johnston 1999), rats (Kondo 1992; Kondo et al. 1997; Stark et al. 1998), and gerbils (Heeb and Yahr 2000). Specifically, in male hamsters, large electrolytic lesions of MA eliminate copulatory behavior and reduce anogenital investigation of a receptive female (Lehman et al. 1980). Previous work in our laboratory has demonstrated that lesions of the anterior or posterior subnuclei of MA eliminate odor preference in male hamsters (Maras and Petrulis 2006). How MA interacts with other ventral forebrain nuclei to regulate odor-guided social behaviors, however, is poorly understood.

BNST AND ODOR-GUIDED REPRODUCTIVE BEHAVIORS

One mechanism by which MA may regulate odor-guided social behaviors is via its connections with BNST. Like MA, BNST receives social odor information, both directly from the accessory olfactory bulbs (Davis et al. 1978) and indirectly via the corticomedial amygdala (Scalia and Winans 1975; Gomez and Newman 1992). Specifically, BNST is densely interconnected with MA (Wood and Swann 2005), such that main and accessory olfactory information that converges on MA can terminate at BNST or continue to MPOA (Newman 1999). In addition to receiving social odor information, BNST contains dense populations of steroid receptor-containing neurons (Wood *et al.* 1992; Li et al. 1993) that are activated during social behaviors (Wood and Newman 1993). BNST has also been implicated in the hormonal modulation of social behavior, as unilateral testosterone implants into BNST facilitate mating behavior in castrated male hamsters

(Wood and Newman 1995c). Finally, evidence from lesion studies suggests that BNST may be critically involved in generating the motivation to investigate opposite-sex odors. For example, lesions of BNST delay copulation in rats (Valcourt and Sachs 1979) and gerbils (Finn and Yahr 2005) and decrease the frequency of rats' penile erections in response to remote odor cues from estrous females (Liu et al. 1997b). In male hamsters, large electrolytic lesions including BNST reduce investigation of female odor cues and reduce anogenital investigation during copulation (Powers et al. 1987). Whether BNST and MA play redundant or unique roles in the modulation of odor-guided social behaviors is, however, unknown.

MPOA AND ODOR-GUIDED REPRODUCTIVE BEHAVIORS

It is likely that MA and/or BNST exert their effects on social behavior via their connections to MPOA. Indeed, MA and BNST are both densely connected to MPOA (Wood and Swann 2005) and disrupting these connections delays the copulatory sequence in male hamsters (Lehman et al. 1983) and eliminates copulation in male rats (Kondo and Arai 1995) and gerbils (Sayag et al. 1994). Furthermore, castration increases the absolute refractory period of neurons in the stria terminalis that project to the MPOA (Kendrick and Drewett 1979), suggesting that hormones acting on MA and/or BNST can directly affect neural transmission within MPOA. MPOA is itself a critical regulator of social behavior and has been implicated in the control of a wide range of behaviors, including parental behavior (Numan 1997), social communication (Albers et al. 2002), aggression (Veening et al. 2005), social defeat (Kollack-Walker et al. 1997), and, most relevantly, odor-guided reproductive behaviors (Wood 1998). In every species

studied to date, permanent or reversible lesions of MPOA reduce or eliminate sexual behavior (Klaric and Hendricks 1986; Liu *et al.* 1997b; Hull et al. 2002; Swann et al. 2003), whereas stimulation of MPOA can enhance sexual behavior (Malsbury 1971; Paredes et al. 1990; Rodriguez-Manzo et al. 2000). Furthermore, lesions of MPOA reduce or reverse preference for opposite-sex odors in rats and ferrets (Kindon et al. 1996; Paredes et al. 1998; Hurtazo et al. 2008), suggesting MPOA, like MA and BNST, is also critical for the appropriate behavioral response to social odors.

GOALS OF DISSERTATION

Based on the evidence reviewed above, it is clear that MA, BNST, and MPOA each play a critical role in odor-guided reproductive behaviors. It is not known, however, how BNST and MPOA individually regulate odor-guided reproductive behaviors and whether functional interactions between MA, BNST, and/or MPOA are required to generate the appropriate behavioral response to socio-sexual odors. Therefore, the overarching goal of this research was to determine how MA, BNST, and MPOA function, both uniquely and as a circuit, to adaptively regulate odor preference and copulatory behaviors in male Syrian hamsters. We combined functional, behavioral, and neuroanatomical approaches to address the following questions:

1) Is BNST critical for odor-guided reproductive behaviors?

BNST receives social odor information, both directly from the olfactory bulbs (Scalia and Winans 1975) and indirectly from MA (Wood and Swann 2005). The role of BNST is odor-guided social behaviors, however, is poorly understood. Therefore, we

used discrete, excitotoxic lesions of BNST in combination with detailed behavioral analysis to test the hypothesis that BNST is critical for generating the appropriate behavioral response to social odors (Chapter 2). In addition, as several lines of evidence suggest that previous sexual experience can alter neural and behavioral responses to social odors (Dewsbury 1969; Fewell and Meredith 2002; Kelliher and Baum 2002; Meisel and Mullins 2006), these experiments were conducted in both sexually-naïve and sexually-experienced males.

2) Is MPOA critical for odor-guided reproductive behaviors?

Although the role of MPOA in male copulatory behavior has been extensively studied (Hull and Dominguez 2007), the function of MPOA in appetitive aspects of reproductive behavior, such as the approach and investigation of social odors, is not well understood. Therefore, we used discrete, excitotoxic lesions of MPOA in combination with detailed behavioral analysis to test the hypothesis that MPOA is critical for generating the appropriate behavioral response to social odors (Chapter 3). As in Chapter 2, these hypotheses will be tested in both sexually-naïve and sexually-experienced males.

3) Is sexual odor information directly relayed between MA, BNST, and MPOA?

Social odor information processed in MA may be relayed directly to MPOA without passing through BNST or terminate in BNST before continuing on to MPOA (Newman 1999). Therefore, we used immediate early gene expression in combination with neuroanatomical tract tracing to determine whether sex-specific odor information is directly relayed from MA to BNST and/or MPOA, as well as from BNST to MPOA (Chapter 4). In addition, as gonadal steroid hormones modulate neural and behavioral

responses to social odors (Fiber and Swann 1996; Kelliher et al. 1998), the hormone-sensitivity of MA and BNST afferents were also quantified.

4) Are connections between MA and BNST/MPOA critical for odor-guided reproductive behaviors?

The results of Chapters 2 and 3, together with previous research in our laboratory, demonstrated that MA, BNST, and MPOA are each individually required for the appropriate behavioral response to social odors (Maras and Petrulis 2006; Been and Petrulis 2010a; Been and Petrulis 2010b). Furthermore, the results of Chapter 4 demonstrated that MA relays sex-specific odor information from MA to BNST and MPOA (Been and Petrulis 2011). We therefore hypothesized that the connections between MA and BNST/MPOA are required for the appropriate behavioral response to social odors. We tested this hypothesis using the asymmetrical lesion technique to functionally disconnect MA from BNST or MPOA (Chapter 5).

The answer to these questions may uncover a fundamental mechanism by which this ventral forebrain circuit regulates socio-sexual behaviors across a wide variety of species and modalities.

CHAPTER 2:

The role of the posterior bed nucleus of the stria terminalis in appetitive and consummatory reproductive behaviors depends on odor volatility and sexual experience in male Syrian hamsters

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ABSTRACT

In Syrian hamsters (*Mesocricetus auratus*), the expression of reproductive behavior requires the perception of social odors. The behavioral response to these odors is mediated by a network of ventral forebrain nuclei, including the posterior bed nucleus of the stria terminalis (BNST). Previous studies have tested the role of BNST in reproductive behavior, but the use of large, fiber-damaging lesions in these studies make it difficult to attribute post-lesion deficits to BNST specifically. Thus, the current study used discrete, excitotoxic lesions of BNST to test the role of BNST in opposite-sex odor preference and copulatory behavior in both sexually-naïve and sexually-experienced males. Lesions of BNST decreased sexually-naïve males' investigation of volatile female odors, resulting in an elimination of opposite-sex odor preference. This elimination of preference was not due to a sensory deficit, as males with BNST lesions were able to discriminate between odors. When, however, subjects were given sexual experience prior to BNST lesions, their preference for volatile opposite-sex odors remained intact post-lesion. Similarly, when sexually-naïve or sexually-experienced subjects were allowed to contact the social odors during the preference test, lesions of BNST decreased males' investigation of female odors, but did not eliminate preference for opposite-sex odors, regardless of sexual experience. Finally, lesions of BNST delayed the copulatory sequence in sexually-naïve, but not sexually-experienced, males such that they took longer to mount, intromit, ejaculate, and display long intromissions. Together, these results demonstrate that BNST plays a unique and critical role in both appetitive and consummatory aspects of male reproductive behaviors.

INTRODUCTION

In many rodent species, including Syrian hamsters, male reproductive behavior depends critically on the perception of odor cues from conspecifics (Johnston 1990). Volatile odor cues are processed primarily by the main olfactory system (MOS), whereas non-volatile cues are processed primarily by the accessory olfactory system (AOS) (Breer 2003). Together, these two systems have been shown to mediate both appetitive and consummatory aspects of reproductive behavior in Syrian hamsters, including males' attraction to vaginal secretions (Powers *et al.* 1979) and copulatory behavior (Murphy and Schneider 1970; Powers and Winans 1975).

The behavioral response to these social odors is mediated by a network of ventral forebrain nuclei that receive chemosensory information and are sensitive to steroid hormones (Wood and Coolen 1997). In particular, several lines of evidence suggest the posterior bed nucleus of the stria terminalis (BNST) may regulate males' attraction to opposite-sex odors. First, BNST receives social odor information, both directly from the accessory olfactory bulbs (Davis *et al.* 1978) and indirectly via the corticomedial amygdala (Scalia and Winans 1975; Gomez and Newman 1992). Specifically, BNST is densely interconnected with the medial amygdala (MA) (Wood and Swann 2005), such that main and accessory olfactory information that converges on MA can terminate at BNST or continue to the medial preoptic area (MPOA) (Gomez and Newman 1992). In addition to receiving social odor information, BNST also processes steroid hormone cues that are required for reproductive behavior (Wood *et al.* 1992; Li *et al.* 1993; Wood and Newman 1993). Evidence from lesion studies suggests BNST may be involved in generating the motivation to investigate opposite-sex odors. For example, lesions in-

cluding BNST disrupt the copulatory sequence (Valcourt and Sachs 1979; Claro et al. 1995; Liu *et al.* 1997b; Finn and Yahr 2005) and decrease the frequency of penile erections in response to remote odor cues from females in rats (Liu *et al.* 1997b). In male hamsters, large electrolytic lesions including BNST reduce investigation of female odors when presented alone or during copulation, and also eliminate copulation in some males (Powers *et al.* 1987).

Although previous studies have implicated BNST in the regulation of male reproductive behavior, the use of large and/or non-fiber-sparing lesions makes it difficult to attribute these effects to BNST itself, as BNST is a heterogeneous structure that is bordered by multiple fiber tracts that relay information to other nuclei critical for reproductive behavior, such as MPOA (Wood and Swann 2005). The interpretation of these studies is further complicated by variability in the manner of stimulus presentation and differences in the sexual experience of the subjects. Thus, the following experiments used site-specific, excitotoxic lesions of BNST to comprehensively test the role of this region in generating the appropriate behavioral response to volatile and non-volatile social odors in both sexually-naïve and sexually-experienced males. We hypothesized BNST primarily mediates males' attraction to female odors rather than copulatory behavior. We therefore predicted lesions of BNST would eliminate males' preference for female odors and decrease investigation of females during mating, but leave non-chemoinvestigatory aspects of copulation intact.

MATERIALS AND METHODS

Experimental Design

The goal of the following experiments was to test the role of BNST in social odor investigation and copulatory behavior in male hamsters. Exposure to female odors causes an increase in circulating testosterone levels in male hamsters (Macrides et al. 1974; Pfeiffer and Johnston 1992) and it is possible that lesions of BNST may interfere with this surge. Thus, in order to equalize steroid hormone levels across experimental groups, all subjects were gonadectomized and maintained on physiological levels of exogenous testosterone for the duration of the experiment. Following bilateral, excitotoxic lesions of BNST or sham lesion surgeries, subjects underwent a series of behavioral tests: first, subjects were tested for their preference to investigate female odors over male odors (*Odor Preference*). To determine if any effects of BNST lesions on odor investigation depend on the volatility of the odor cues being processed, Odor Preference tests were conducted under conditions that either prevented contact with the odor sources (*Non-contact*; volatile odors only) or allowed contact with the odor sources (*Contact*; volatile and non-volatile odors). Second, in order to determine if a lack of preference in these tests was due to an inability to discriminate between odor stimuli, a separate group of subjects were tested for their ability to discriminate between social and non-social odor sources using a habituation-dishabituation task (*Odor Discrimination*). Lastly, to determine if lesions of BNST disrupt male copulatory behavior, subjects' response to a receptive stimulus female (*Copulatory Behavior Test*) was assessed. As converging lines of evidence suggest that previous sexual experience can alter neural and behavioral responses to social odors (Dewsbury 1969; Fewell and Meredith 2002;

Kelliher and Baum 2002; Meisel and Mullins 2006), we tested the effects of BNST lesions on both sexually-naïve and sexually-experienced males' behavior.

Animals

Experimental subjects were adult (3 to 6 months old) male Syrian hamsters (*Mesocricetus auratus*) purchased from Charles River Laboratories (Wilmington, MA, USA). A separate group of unrelated adult male and female hamsters served as odor stimulus donors for behavior tests. A third group of ovariectomized and hormone-primed adult female hamsters were used as stimulus females for copulatory behavior tests. Experimental subjects and copulatory stimulus females were single-housed, whereas odor donors were group-housed (three to four animals per cage), in solid-bottom Plexiglas cages (36 cm x 30 cm x 16 cm). All subjects were maintained on a reversed 14 hour light/ 10 hour dark photoperiod, and food and water were available *ad libitum*. All 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.

Sexual Experience

Following gonadectomy (see below), experimental subjects were randomly assigned to one of two experimental conditions: sexually-experienced (EXP; n = 40) or sexually-naïve (NVE; n = 36). EXP males were given weekly sexual experiences for three consecutive weeks. Subjects were placed into a clear, Plexiglas testing area (50 cm x 25 cm x 30 cm) for five minutes prior to the addition of a receptive stimulus female.

An angled mirror was placed below the testing arena to provide a view of the ventral surface of the animals. Encounters lasted for 30 minutes, or until the animals engaged in aggressive behavior, at which point the stimulus female was removed. The second and third encounters were video-recorded and the male's behavior was later scored using the Observer for Windows, version 5.0 (Noldus Information Technology, Wageningen, The Netherlands). Males who failed to copulate on at least two of the three encounters (n = 7) were eliminated from the study. To control for possible effects of experimenter handling on future behavior, NVE males were transported to the same behavioral testing room and placed into an empty cage for 30 minutes once a week for three consecutive weeks.

Surgery

All surgeries were performed under 2% isoflurane gas anesthesia vaporized in 100% oxygen (gonadectomy) or a 70:30% oxygen/nitrous oxide mixture (stereotaxic surgery). To minimize post-operative pain, ketoprofen (5 mg/kg subcutaneously, Henry Schein, Melville, NY, USA) was administered intra-operatively.

Gonadectomy and Hormone Implant. One to two weeks prior to lesion surgery, subjects' testes were bilaterally removed via a bilateral midline abdominal incision and cauterization of the ductus deferens and blood vessels. Silastic capsules (i.d. 1.57 mm, o.d. 2.41 mm, Dow Corning, Midland, MI, USA) packed with 20 mm length of crystalline testosterone (Sigma, St. Louis, MO, USA) were implanted subcutaneously between the scapulae immediately following gonadectomy. Vicryl suture (size 4-0, Ethicon, Somerville, NJ, USA) and wound clips were used to close the smooth muscle and skin incisions, respectively.

Stimulus females for copulatory experiences and behavior testing were ovariectomized at least two weeks prior to use. Following bilateral flank incisions, the ovaries were removed via cauterization of the uterine horn and blood vessels. Silastic capsules (i.d. 1.57 mm, o.d. 2.41 mm, Dow Corning, Midland, MI, USA) packed with 5 mm length of crystalline estradiol (Sigma, St. Louis, MO, USA) were implanted subcutaneously between the scapulae immediately following gonadectomy. Vicryl suture (size 4-0, Ethicon, Somerville, NJ, USA) and wound clips were used to close the smooth muscle and skin incisions, respectively. To induce behavioral receptivity, stimulus females were injected subcutaneously with 0.15 ml of progesterone dissolved in sesame oil (2.5 mg/ml, Sigma, St. Louis, MO, USA) 4 hours prior to copulatory behavior tests.

Excitotoxic Lesion. NVE and EXP subjects were randomly assigned to either a BNST lesion (BNST-X; NVE n = 26, EXP n = 25) or a sham lesion surgery (SHAM; NVE n = 8, EXP n = 10) group. Anesthetized subjects were secured in the stereotaxic apparatus such that their skull was level in the anterior-posterior (A-P) and medial-lateral (M-L) planes. Following a midline scalp incision, the skin and temporal muscles were retracted to expose the skull and a hand-operated drill was used expose dura. All A-P and M-L measurements were taken in mm relative to bregma and all dorsal-ventral (D-V) measurements were taken in mm relative to dura. Excitotoxic lesions were made by lowering a microinjection syringe (701R 10 µl syringe, Hamilton, Reno, NV, USA) under stereotaxic control into BNST and injecting N-methyl-D-aspartic acid (NMDA, 20 mg/ml; 20 nl per injection, Sigma, St. Louis, MO, USA) into two bilateral sites targeting the posterointermediate BNST (BNSTpi; A-P: - 1.85, M-L: ± 1.65, D-V: - 5.8) and posteromedial BNST (BNSTpm; A-P: - 1.85, M-L: ± 1.05, D-V: - 5.9), respectively. To minimize

the flow of excitotoxin up the syringe tract, the syringe was left in place for 10 minutes after each injection.

Sham surgeries were identical to lesion surgeries with two exceptions: 1) the microinjection syringe was lowered to 1 mm above the target injection site and 2) no excitotoxin was infused into the target injection sites. After all surgeries, skull holes were sealed using bone wax and incisions were closed with wound clips. Subjects were allowed to recover for at least two weeks prior to behavioral testing.

Behavioral Testing

All behavior testing took place during the first six hours of the dark phase and under light illumination.

Odor Stimuli. Social odor stimuli used for Odor Preference and Odor Discrimination tests were collected from cages of group-housed, same-sexed odor donors that had not been changed for at least four days prior to odor collection. Each social odor stimulus consisted of soiled cotton bedding (2 Nestlets, 12 g, ANCARE, Bellmore, NY, USA), soiled corncob litter (50 ml, Bed-o-cob, The Andersons, Maumee, OH, USA) and one damp cotton gauze pad that was used to wipe the inner walls of the cage. In addition, a damp gauze pad was used to wipe two of the cage resident's anogenital region and bilateral flank glands 10 times each. For female odor stimuli, vaginal secretion was also collected onto a gauze pad by gently palpating the vaginal area of one female with a disposable probe, and was added to each odor stimulus. Clean odor stimuli consisted of clean cotton bedding (2 Nestlets), clean corncob litter (50 ml), and clean cotton gauze pads (2). For non-social odor discrimination tests, odor stimuli consisted of 0.10 g (three

beads) of an artificial, multi-component odorant (baby powder or strawberry, International Flavors & Fragrances, Inc., NY, USA) mixed with clean odor stimuli.

For Contact tests, additional odors were collected directly onto glass microscope slides (25 mm X 75 mm X 1 mm) by rubbing a clean slide along an odor donor's flank and anogenital regions. All odor slides contained samples from two individual odor donors (collected separately onto each end of the slide). For female odor slides, a sample of vaginal secretion was also collected onto the same slide. All odor stimuli were stored in airtight containers at 4°C until 20 minutes before use. Odor stimuli older than one month were discarded and no subject was tested with the same odor stimulus more than once.

Odor Preference. A three-choice test was used to measure odor preference. Subjects were placed into a glass aquarium (50 cm x 25 cm x 30 cm) with opaque walls. Three acrylic odor presentation boxes (8 cm x 8 cm x 8 cm) were affixed to one of the short walls of the aquarium such that only the front and top surfaces of each box were accessible. Each odor presentation box had 7-mm holes drilled along the front surface that allowed volatile odors to pass, but prevented contact with the odor sources. During testing, a single odor stimulus (see above) was placed into each of the three odor presentation boxes. Additionally, a line bisecting the available floor space was drawn parallel to the short walls so that general activity levels (as measured by total number of line crosses) could be assessed during the test. The top of the aquarium was secured with a clear Plexiglas top to allow for overhead video recording of the subject's behavior. All surfaces of the aquarium and odor containers were thoroughly cleaned with 70% alcohol and allowed to dry between subjects.

Subjects were tested in a series of three tests in the 3-choice apparatus, each separated by 24 hours: Clean, Non-Contact preference, and Contact preference. At the beginning of each test, a subject was placed into the testing arena and then allowed ten minutes to freely explore the apparatus. For all tests, investigation of the odor stimulus was coded when the subject made contact with, or directed its nose within 1 cm of, the perforated front surface of the odor container and/or odor slide. For Clean tests, clean odor stimuli were placed into each of the three odor containers. These tests were used to acclimate the subjects to the testing arena, as well as to obtain baseline levels of activity in the absence of social odor stimuli. For subsequent preference tests, female and male odor stimuli were placed into each of the two outer odor containers, and clean odor stimuli were placed into the center odor container. The side on which each social odor was placed (left or right) was alternated between consecutive subjects. Non-Contact and Contact tests were identical except that during Contact tests, a single odor slide matching the type of odor stimulus in that container (female, male, clean) was secured to the center of the front surface of each odor presentation box.

Video recordings of all tests were digitized onto a computer and scored using the Observer for Windows, version 5.0 (Noldus Information Technology, Wageningen, The Netherlands). All observers were blind to the condition of the subject, and different observers reached at least a 90% inter-observer reliability score prior to coding behavior.

Odor Discrimination. In order to determine if the lack of preference for non-contact odors observed in NVE subjects (see Results) was due to an inability to discriminate between odor stimuli, a separate group of NVE males (BNST-X n = 12, SHAM n = 8) were tested for their ability to discriminate between volatile odors using a habituation-

dishabituation task. The habituation-dishabituation task involves repeated presentations of the same odor source followed by a test presentation of a novel odor source. A decrease in investigation during the repeated presentations indicates a perception of the odors as being the same or familiar. An increase in investigation of the novel odor compared to the last presentation of the habituated odor indicates an ability to discriminate between the two odors (Johnston 1993; Baum and Keverne 2002). The testing sequence consisted of four, 3-minute presentations of repeated odors (habituation) followed by a fifth, 3-minute presentation of a novel odor (dishabituation). Five-minute inter-trial intervals separated each odor presentation. Odor stimuli were presented in modified 50 ml polypropylene collection tubes, with 0.5-cm holes drilled 1 cm apart along the surface of the tube. Wire mesh lined the inner surface of the odor container to prevent contact with the odor stimulus. Odor containers were placed into the center of subjects' home cage and investigation was measured using a stopwatch. Odor containers were cleaned with 70% alcohol and allowed to dry between subjects.

Under these testing parameters, male hamsters consistently display a lack of habituation to repeated presentations of female odors (Maras and Petrulis 2006) and so all subjects were tested using male odors as the habituation stimuli and female odors as the dishabituation stimuli for social odor discrimination tests. Subjects were presented with different male odor sources on each of the habituation trials so that subjects were habituated to the sexual identity of the repeated odor, rather than to the individual identity of odor donors. In addition, in order to determine if lesion-induced deficits were specific to social odors, rather than a general deficit in odor processing, subjects were test-

ed for their discrimination of two complex, artificial odors (strawberry and baby powder; see above).

Copulatory Behavior. Copulatory behavior test procedures were identical to those used to provide sexual experience to EXP males (see above). Tests were video-recorded and later scored using Observer for Windows, version 5.0 (Noldus Information Technology, Wageningen, The Netherlands). The total number and latencies (from on-set of test) of several behavioral measures were scored: mounts (M), intromissions (I), ejaculations (E), and long intromissions (LI). LI are distinguished from I in that males do not quickly dismount the female following vaginal penetration, but instead display a re-petitive thrusting pattern (Bunnell *et al.* 1977). Importantly, the expression of LI is asso-ciated with the onset of sexual satiety in Syrian hamsters (Bunnell *et al.* 1977; Parfitt and Newman 1998). In addition, the total durations of time the male spent investigating the female's anogenital region (AGI), investigating the female's head or body region (HBI), and self-grooming (SG) were also scored. Finally, several derived measures of copulatory behavior were also analyzed: post-ejaculatory interval (PEI; latency to dis-play a mount or intromission after each ejaculation, the number of intromissions to reach each ejaculation (I-E), and mounting efficiency (ME; the total number of intromis-sions divided by the total number of mounts + intromissions).

Histology and Lesion Verification

Following the last behavioral test, subjects were injected with an overdose of so-dium pentobarbital (100 mg/kg; Sleep Away, Ft. Dodge, IA, USA) and transcardially per-fused with 200 ml of 0.1M phosphate-buffered saline (PBS, pH 7.4) followed by 200 ml of paraformaldehyde (4%). Brains were post-fixed in paraformaldehyde (4%) overnight

and then cryoprotected for 48 hours in 30% sucrose in PBS solution. Coronal sections (30-µm) of brain tissue were sectioned using a cryostat (-20°C) and stored in cryoprotectant until immunohistochemical localization of Neuronal Nuclei protein (NeuN, see below). Additionally, in order to further delineate lesion damage from fiber tracts (which are not readily detected by NeuN), every third section was mounted onto glass slides using a 1% gelatin mounting solution and stained for Nissl material with cresyl violet (Sigma, St. Louis, MO, USA). Nissl- and NeuN-stained sections were examined under a light microscope for the location and extent of lesion damage as compared with published hamster neuroanatomical plates (Morin and Wood 2001), and the minimum and maximum extents of lesion damage were traced onto anatomical plates using Adobe Illustrator CS 11.0 software.

Immunohistochemistry. Free-floating sections were removed from cryoprotectant, rinsed thoroughly in PBS, and then incubated in a monoclonal antibody against NeuN (1:30,000, Millipore MAB377, Billerica, MA, USA) in PBS with 0.4% Triton-X-100 for 48 hours at 4°C. After rinsing in PBS, sections were incubated in biotinylated secondary antibody (Rabbit Anti-Mouse, 1:600, Jackson Immunoresearch Laboratories 315-065-003, West Grove, PA, USA) in PBS with 0.4% Triton-X-100 (Sigma, St. Louis, MO, USA) for one hour at room temperature, rinsed in PBS, then incubated in an avidin-biotin complex (1:200, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for one hour at room temperature. Sections were then rinsed in PBS and reacted in a nickel-enhanced 3, 3′-diaminobenzidine tetrahydrochloride (DAB) solution (2 mg DAB plus 250 mg Nickel (II) Sulfate with 8.3 µl 3% H_2O_2 per 10ml of 175 mM sodium

acetate, Sigma, St. Louis, MO, USA) to yield a blue-black reaction product. After 15 minutes, sections were rinsed in PBS to stop the chromagen reaction.

Blood Collection and Radioimmunoassay

Blood samples were collected from the inferior vena cava after anesthesia and immediately prior to perfusion and stored in vacutainer collection tubes (4-mL draw, red/gray, VWR, West Chester, PA, USA) on ice until centrifugation. Samples were centrifuged at 2500 revolutions per minute at 4°C for 20 minutes and serum was stored in 200-μl aliquots at -20°C until assay. Testosterone levels were measured by radioimmunoassay kits from Diagnostics System Laboratories (DSL 4000, Beckman Coulter, Brea, CA, USA) with a sensitivity range of 0.05 to 22.92 ng/mL and an interassay variance of 6%, previously validated for hamster serum (Cooper et al. 2000). The mean testosterone levels (ng/nl) for experimental subjects were: NVE BNST-X = 4.25 ± .58; NVE SHAM = 3.027 ± .36; EXP BNST-X = 3.78 ± .70; EXP SHAM = 4.193 ± .66. There was no difference in testosterone levels between lesion groups for NVE or EXP males (NVE $t_{18} = 0.51$, $P = 0.61$; EXP, $t_{18} = 1.69$, $P = 0.11$).

Data Analysis

All data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) for Windows and significance was determined as $p < .05$. To establish investigatory preferences for each type of 3-choice test (Clean, Non-Contact Preference, Contact Preference), 2 (Experimental group: BNST-X, SHAM) X 3 (Odor) mixed-design ANOVAs were performed. Significant interactions were explored using simple effects analysis and pairwise comparisons with Bonferroni alpha adjustments. Furthermore, separate one-way ANOVAs were used to compare the levels of investigation of each stimulus directly

across experimental groups. To identify differences in general motor activity, additional one-way ANOVAs were used to compare the total number of midline crosses across experimental groups for the Clean test.

For the habituation-dishabituation data, data were split by experimental group, and paired *t*-tests (2-tailed, with Bonferroni alpha adjustments, $\alpha_{FW} = .05$) were used to detect both (1) a habituation to the repeated presentations of odors (odor 1 vs. odor 4) and (2) a dishabituation to the presentation of the test odor (odor 4 vs. Test).

Many copulatory behavior tests were terminated before 30 minutes because the male and female engaged in aggressive behavior. Thus, only the first 20 minutes of each copulatory test were analyzed to eliminate variability caused by differences in test duration. Group differences in all copulatory measures were detected using one-way ANOVAs. Additionally, separate 2 x 2 (lesion group x ejaculatory series) ANOVAS were used to detect changes in post-ejaculatory intervals or the number of intromissions to reach each ejaculation in the copulatory tests.

RESULTS

Lesion Reconstruction

Subjects were included in the BNST-X lesion group (NVE *n* = 12; EXP *n* = 12) only if they had extensive bilateral damage to BNST, defined as at least 60% bilateral damage to BNSTpi and BNSTpm in at least two stereotaxic planes of section (Figure 2.1a; (Morin and Wood 2001). All subjects included in the BNST-X lesion group sustained significant damage to the largest part of BNST (Bregma 0.2 mm), including BNSTpi and BNSTpm, as well as the posterolateral BNST (BNSTpl; Figure 2.1c). Most

subjects (*n* = 21) also sustained significant damage to more rostral and anterior (Bregma 0.5 mm) parts of BNSTpi and BNSTpm. Ten subjects had bilateral lesion damage to more caudal and posterior BNST (Bregma 0.1 mm); of these subjects, half had bilateral damage of both BNSTpi and BNSTpm and half had bilateral damage to BNSTpm only. No subjects included in the BNST-X group sustained damage to the most caudal and ventral level of BNST (Bregma -0.3 mm).

Subjects were excluded from the BNST-X group if their lesions extended significantly outside of BNST or failed to damage a significant portion of BNST. Specifically, one group of subjects had partial (less than 60% damage in two stereotaxic planes of section), bilateral damage to BNST (NVE *n* = 3; EXP *n* = 3) and another separate group of subjects had primarily unilateral damage to BNST (NVE *n* = 6; EXP *n* = 1). Analyses indicated that these subjects' behavior did not differ significantly from SHAM subjects' behavior in Odor Preference or Copulatory Behavior tests (see Results). Only needle tracts were visible in most SHAM males (Figure 2.1b; NVE *n* = 8; EXP *n* = 8); three SHAM males also had unilateral cortical damage. These subjects did not differ in behavior from SHAM subjects without cortical damage and were retained in the analysis.

Some subjects included in the BNST-X group sustained minimal or unilateral damage to other adjacent nuclei, defined as less than 20% damage at only one stereotaxic plane of section of the nucleus (Morin and Wood 2001). This included nuclei within the anterior BNST, such as the anterointermediate BNST (*n* = 8), anteromedial BNST (*n* = 6), and anteroventral BNST (*n* = 10). Outside of the BNST, partial or unilateral damage was sustained to sub-cortical nuclei including the ventral lateral septum (*n* = 6), paraventricular hypothalamic nucleus (*n* = 8), and globus pallidus (*n* = 4). Finally, par-

tial or unilateral damage also occurred in thalamic nuclei, such as the anterodorsal thalamic nucleus (n = 3), anteroventral thalamic nucleus (n = 1), ventrolateral anteroventral thalamic nucleus (n = 1), reuniens thalamic nucleus (n = 2), and reticular thalamic nucleus (n = 3). Two males also sustained unilateral damage to overlying cortex. There was no difference in behavior across subjects with unilateral, bilateral, or no damage to any of the adjacent nuclei.

Behavioral Measures

Odor Preference

Clean Test. In the Clean test, both NVE and EXP subjects investigated the three stimulus containers equally (NVE $F_{2,23}$ = 3.37, P = 0.14; EXP $F_{2,35}$ = 2.69, P = 0.23). There were also no differences in the total number of midline crosses (NVE t_{18} = 1.48, P = 0.15; EXP t_{18} = 1.35, P = 0.47) or total duration of investigation of the three odor containers (NVE t_{18} = .641, P = 0.08; EXP t_{18} =.861, P = 0.13) between BNST-X and SHAM males.

Non-Contact Preference Test. Lesions of BNST decreased NVE males' investigation of volatile female odors, resulting in an elimination of opposite-sex odor preference. There was a significant interaction between lesion group and the duration of investigation of the three odor stimuli ($F_{2,54}$ = 3.51, P = 0.03). Whereas SHAM males investigated female odors longer than male odors (t_7 = 5.00, P < 0.01) and investigated both female (t_7 = 6.31, P < 0.01) and male (t_7 = 2.65, P = 0.03) odors more than clean odors, BNST-X males spent equivalent amounts of time investigating male and female odors (t_{11} = 0.53, P = 0.60), and did not investigate either female (t_{11} = 1.51, P = 0.16) or male (t_{11} = 2.07, P = 0.06) odors more than clean odors. In addition, BNST-X males

spent significantly less time investigating female odors ($F_{1,19}$ = 8.51, P < 0.01), and significantly more time investigating clean odors ($F_{1,19}$ = 4.79, P < 0.01), than did SHAM males (Fig 2.2a).

In contrast to NVE subjects, lesions of BNST did not decrease EXP males' investigation of volatile female odors. In EXP males, there was a significant main effect of odor stimulus ($F_{2,53}$ = 21.652, P < 0.01), but no interaction between lesion group and the duration of investigation of the three odor stimuli. Both SHAM and BNST-X males spent more time investigating female odors than male odors (SHAM t_7= 2.64, P = 0.03; BNST-X t_{11} = 2.24, P = 0.04) and investigated female odors more than clean odors (SHAM t_7 = 2.79, P = 0.03; BNST-X t_{11} = 5.04, P < 0.01). BNST-X males also spent significantly more time investigating male odors than clean odors (t_{11} = 5.71, P < 0.01), although this was not the case for SHAM males (t_7 = 1.371, P = 0.21; Fig 2.2b).

In both NVE and EXP males, neither unilateral nor partial, bilateral lesions of BNST disrupted males' preference to investigate opposite-sex odors in the Non-Contact test (Table 2.1a). Although the small sample sizes of these groups precluded them from statistical analysis, examination of the means confirmed that, like SHAM males, males with incomplete damage to BNST investigated female odors more than they did male or clean odors.

Contact Preference Test. Lesions of BNST decreased both NVE and EXP males' investigation of female odors in the Contact test, but did not eliminate opposite-sex odor preference. In NVE males, there was a significant interaction between lesion group and the duration of investigation of the three odor stimuli ($F_{2,54}$ = 6.43, P = 0.03). Whereas SHAM males spent significantly more time investigating female odors than male odors

(t_7 = 3.86, P = 0.01), and investigated both female (t_7 = 4.28, P < 0.01) and male (t_7 = 2.40, P = 0.04) odors more than clean odors, BNST-X males spent more time investigating female odors than male odors (t_{11} = 2.49, P = 0.03) or clean odors (t_{11} = 2.99, P = 0.01), but did not spend more time investigating male odors than clean odors (t_{11} = 2.05, P = 0.06). In addition, BNST-X males spent significantly less time investigating female odors ($F_{1,19}$ = 5.28, P = 0.03), and significantly more time investigating clean odors ($F_{1,19}$ = 5.55, P = 0.03), than did SHAM males (Figure 2.3a).

In EXP males, there was a significant interaction between lesion group and the duration of investigation of the three odor stimuli ($F_{2,53}$ = 8.71, P < 0.01). Both SHAM and BNST-X males spent significantly more time investigating female odors than male odors (SHAM t_7 = 9.91, P < 0.01; BNST-X t_{11} = 6.55, P < 0.01) and spent more time investigating both female (SHAM t_7 = 11.37, P < 0.01; BNST-X t_{11} = 8.20, P < 0.01) and male (SHAM t_7 = 3.33, P = 0.01; BNST-X t_{11} = 3.21, P < 0.01) odors than clean odors. BNST-X males, however, spent significantly less time investigating female odors than did SHAM males ($F_{1,19}$ = 12.21, P < .01; Figure 2.3b).

In both NVE and EXP males, neither unilateral nor partial, bilateral lesions of BNST disrupted males' preference to investigate opposite-sex odors in the Contact test (Table 2.1b). Although the small sample sizes of these groups precluded them from statistical analysis, examination of the means confirmed that, like SHAM males, males with incomplete damage to BNST investigated female odors more than they did male or clean odors.

Odor Discrimination

Social Odors. NVE BNST-X and SHAM males habituated to repeated presentations of different male odors, as indicated by decreased investigation of the male odor on the fourth trial compared to the first trial (BNST-X t_{13} = 2.99, P < 0.01; SHAM t_8 = 3.61, P < 0.01). Importantly, both lesion groups also dishabituated to a novel female odor, as indicated by an increased investigation of the female odor compared to the last presentation of the habituated male odor (BNST-X t_{13} = 4.62, P < 0.01; SHAM t_8 = 5.21, P < 0.01; Figure 2.4a).

Non-Social Odors. Both lesion groups habituated to repeated presentations of the non-social odors, as indicated by a decreased investigation on the fourth trial compared to the first trial (BNST-X t_{11}= 4.12, P < 0.01; SHAM t_8 = 3.16, P < 0.01). Importantly, both experimental groups also dishabituated to a novel non-social odor, as indicated by an increased investigation of the test odor compared to the last presentation of the habituated odor (BNST-X t_{11} = 3.05, P < 0.01; SHAM t_8 = 3.028, P < 0.01; Figure 2.4b).

Copulatory Behavior

All NVE and EXP males ejaculated and all except two BNST-X males reached sexual satiety, as indicated by the expression of long intromissions. In NVE males, however, lesions of BNST disrupted several aspects of copulatory behavior.

In NVE subjects, the total number of mounts, intromissions, ejaculations, and long intromissions did not differ between BNST-X and SHAM males (Table 2.2a; all P > 0.05). However, BNST-X males took significantly longer than SHAM males did to mount ($F_{1,19}$ = 5.29, P = 0.03), intromit ($F_{1,19}$ = 6.12, P = 0.02), ejaculate ($F_{1,19}$ = 11.47,P =

0.04), and display long intromissions ($F_{1,17}$ = 4.42, P = 0.05; Figure 2.5). BNST-X males also had a significantly longer latency to investigate the females' anogenital region ($F_{1,19}$ = 8.01, P = 0.01), although the total duration of anogenital investigation, head-body investigation, or self-grooming did not differ between lesion groups (Table 2.2b; all P > 0.05). These increased latencies were not simply due to a delay in the initiation of mating, as the latency to display mounts, intromissions, ejaculations, and long intromissions remained significantly longer in BNST-X males compared to SHAM males when data was normalized for the initiation of anogenital investigation (data not shown). NVE BNST-X males did not differ from SHAM males in post-ejaculatory interval, intromissions-to-ejaculation, or mount efficiency (Table 2.2c; all P > 0.05).

In EXP subjects, lesions of BNST did not disrupt any measure of copulatory behavior. BNST-X males did not differ from SHAM males in their latencies to mount ($F_{1,16}$ = 0.01, P = 0.95), intromit ($F_{1,16}$ = 0.51, P = 0.49), ejaculate ($F_{1,16}$ = 0.55, P = 0.46), or display long intromissions ($F_{1,15}$ = 2.06, P = 0.18; Figure 2.5), nor did they differ in the total number (Table 2.2a) or durations (Table 2.2b) of any of the behaviors measured (all P > 0.05). There was, however, a non-significant trend to spend less time investigating the anogenital region in BNST-X males than SHAM males ($F_{1,16}$ = 3.56, P = 0.07). EXP BNST-X males also did not differ from SHAM males in any of the derived measures of sexual behavior (Table 2.2c; all P > 0.05).

In both NVE and EXP males, neither unilateral nor partial, bilateral lesions of BNST disrupted any measure of copulatory behavior. Although the small sample sizes of these groups precluded them from statistical analysis, examination of the means con-

firmed that males with incomplete damage to BNST did not differ from SHAM males in their total number (Table 2.3a) or latencies to display (Table 2.3b) any mating event.

DISCUSSION

These results demonstrate for the first time that BNST specifically mediates both appetitive and consummatory aspects of male reproductive behavior. Unlike previous studies in which lesions extended significantly outside of BNST and/or were created with fiber-damaging techniques, the current study demonstrates that discrete, excitotoxic lesions of BNST decreased males' investigation of volatile female odors, resulting in an elimination of opposite-sex odor preference and supporting our prediction that BNST mediates males' attraction to female odors. This lack of preference was not due to an inability to discriminate between volatile odors, as BNST-X males performed at levels comparable to SHAM males on social and non-social odor discrimination tasks. When, however, subjects were given sexual experience prior to BNST lesions, their preference for volatile opposite-sex odors remained intact post-lesion. Similarly, when sexually-naïve or sexually-experienced subjects were allowed to contact the social odors during the preference test, thus providing access to both volatile and non-volatile social odors, BNST lesions did not eliminate preference for opposite-sex odors. Lesions of BNST did, however, decrease males' investigation of female odors in this test, regardless of sexual experience. Finally, lesions of BNST delayed the copulatory sequence in sexually-naïve, but not sexually-experienced, male hamsters such that they took longer to mount, intromit, ejaculate, and display long intromissions compared to SHAM males.

These results suggest that, contrary to our prediction, BNST also mediates non-chemoinvestigatory aspects of copulatory behavior in sexually-naïve males.

Role of BNST in Opposite-Sex Odor Preference

The finding that lesions of BNST decreased males' investigation of volatile and non-volatile female odors is consistent with data from previous lesion studies that suggest that BNST may mediate males' attraction to female odors. Specifically, in male rats, radiofrequency lesions including BNST decrease non-contact erections in response to volatile cues from estrous females (Liu *et al.* 1997b). Similarly, in male hamsters, large, electrolytic lesions including BNST reduce investigation of female hamster vaginal secretion (Powers *et al.* 1987). Importantly, our results suggest that the chemoinvestigatory deficits observed in these previous studies can be attributed to BNST damage specifically, rather than damage to other parts of BNST or to MPOA deafferentation. Interestingly, lesions of BNST did not decrease investigation of female odors during the social odor discrimination test. It is therefore possible that the novelty of a placing a foreign object in a subject's home cage during this task may override any deficits in attraction to female odors (Johnston 1981).

Role of Odor Volatility. The current results suggest that the importance of BNST for the appropriate investigation of female odors depends on the volatility of the odor cues available. When only the volatile components of odors are present, as would be the case when animals are detecting odors from a distance, the odors are processed primarily by the main olfactory system (MOS) (Meredith 1991; Sanchez-Andrade and Kendrick 2009). BNST does not receive any direct projections from the main olfactory bulbs, so MOS information must be transmitted to BNST indirectly from other cortico-

medial amygdala structures, such as MA (Scalia and Winans 1975). The elimination of preference for volatile, opposite-sex odors by BNST lesions suggests that interactions between the MOS and BNST are critical for the appropriate investigation of volatile social odors. In contrast, non-volatile components of odors, such as those that would be available when animals are in direct contact with conspecifics or their odors, are processed primarily by the accessory olfactory system (AOS) (Keverne 2004; Keller *et al.* 2009). BNST receives both a small, direct projection from the accessory olfactory bulbs and indirect AOS information from MA and other corticomedial amygdala structures (Scalia and Winans 1975), making it well-situated for regulating investigation of non-volatile social odors. Furthermore, BNST sends projections back to AOB in mice (Fan and Luo 2009), providing an additional substrate for the regulation of non-volatile odor investigation. Our finding that lesions of BNST decreased investigation of female odors when contact was allowed suggests that AOS connections with BNST modulate levels of attraction to non-volatile female odors. However, lesions of BNST did not eliminate preference for non-volatile opposite-sex odors, suggesting that other connections, such as those between MA and MPOA, are sufficient to maintain preference under these conditions.

Role of Sexual Experience. Our results demonstrate that prior sexual experience can compensate for BNST lesion-induced deficits in the appropriate investigation of volatile odors, but does not rescue behavioral deficits when odors can be contacted. This suggests that sexual experience can change the type of sensory information required for appropriate behavioral responses, a phenomenon that has been demonstrated in several species and contexts. For example, removal of the vomeronasal organ (VNO)

eliminates mating in sexually-naïve, but not sexually-experienced, male hamsters (Meredith 1986). Similarly, combined lesions of the MOS and AOS decrease male hamsters investigation of female vaginal secretion and eliminate the characteristic surge in plasma testosterone in response to a female (or her odors) in sexually-naïve, but not sexually-experienced male hamsters (Pfeiffer and Johnston 1994) One neural mechanism by which sexual experience may alter how volatile social odor information is processed is experience-dependent changes in synaptic strength. Given its position in the social behavior circuit (Wood 1997), BNST may function in sexually-naïve animals to increase the strength of odor signals relayed from MA to MPOA in order to ensure an appropriate behavioral output. Sexual experience may increase synaptic connectivity/strength between MA and MPOA such that BNST is no longer required for the appropriate behavioral response to social odors (Hosokawa and Chiba 2005).

Role of BNST in Copulatory Behavior

Although nearly all subjects with BNST lesions copulated until sexual satiety, lesions of BNST increased the latencies to several mating events in sexually-naïve males, thus replicating and extending a previous report of copulatory delays in sexually-naïve male rats with BNSTpm lesions (Claro *et al.* 1995). In the current study, these copulatory delays were not due to a general delay in the initiation of copulation (that would delay all subsequent mating events), as latencies to mount, intromit, ejaculate, and the onset of long intromissions remained significantly longer than those for SHAM males even after the data was corrected for the initiation of anogenital investigation. These deficits also cannot be attributed to a deficit in chemosensory investigation during copulation, as the total duration of anogenital investigation did not differ between BNST-X and SHAM

males and male hamsters do not require odor stimulation once copulation has been initiated (Devor and Murphy 1973). It is therefore likely that the post-lesion copulatory deficits in this study are due to a general decrease in sexual motivation or arousal, consistent with our finding that BNST lesions decrease the motivation to investigate female odors.

In contrast to sexually-naïve males, sexually-experienced males with lesions of BNST did not differ from SHAM males on any measure of copulatory behavior. This directly challenges previous reports that large, electrolytic lesions of BNST reduce the duration of anogenital investigation during copulation and eliminate copulation in a subset of sexually-experienced male hamsters (Powers *et al.* 1987). Similarly, our results run counter to studies in sexually-experienced male rats, in which BNST lesions increase the number of mounts, intromissions (Claro *et al.* 1995), I-E, and PEI (Valcourt and Sachs 1979), and decrease the total number of intromissions and ejaculations (Liu *et al.* 1997b). The differences between our results and those of previous studies may be explained by our use of excitotoxic lesions compared to the use of large and/or fiber-damaging lesions by previous investigators. That is, the copulatory deficits in previous studies were not due to damage to BNST itself, but resulted from disruption of social odor information transfer through the stria terminalis, the major fiber pathway between MA, BNST, and MPOA. Indeed, lesions or knife cuts of the stria terminalis produce copulatory deficits in sexually-experienced male hamsters (Lehman *et al.* 1983) and rats (Kondo and Yamanouchi 1995). Similarly, asymmetrical lesions that functionally disconnect MA from MPOA severely disrupt copulatory behavior in sexually-experienced male rats (Kondo and Arai 1995), suggesting that the connections between MA and MPOA

are critical for copulation. Together, this evidence suggests that previous reports of BNST lesion-induced copulatory deficits in sexually-experienced males were due to deafferentation of MPOA rather than damage to BNST itself.

Alternatively, previous reports of BNST lesion-induced deficits in copulatory behavior in sexually-experienced males may be due to lesion damage extending to anterior BNST and the most ventral portion of BNST. The ventral BNST in particular may be critical for male copulatory behavior, as excitotoxic lesions of this area alone eliminate mating in sexually-experienced male rats (Finn and Yahr 2005). Similarly, asymmetrical lesions that functionally disconnect ventral BNST from the sexually dimorphic area of the hypothalamus eliminate mating in sexually-experienced male gerbils (Sayag *et al.* 1994). In the current study, however, we were unable to damage the most rostral areas of BNST and ventral BNST simultaneously without incurring substantial damage to adjacent posterior nuclei, including MPOA. It is therefore possible that additional damage to ventral BNST may account for the deficits in copulatory behavior observed in sexually-experienced males in previous studies and we would predict that lesions of this area alone may disrupt copulation in male hamsters.

Role of Sub-Nuclei within BNST

In this study, lesion damage was primarily restricted to BNSTpm, BNSTpi, and BNSTpl. Given the known differences in odor-processing and connectivity within these subnuclei, it is likely that they contributed differentially to the observed deficits in reproductive behavior. For example, BNSTpm contains dense populations of steroid receptor-containing neurons that are activated during copulatory behavior in male hamsters (Kollack and Newman 1992). In contrast, BNSTpi contains fewer steroid receptors and

shows increased Fos expression in response to both reproductive odors and agonistic encounters in male hamsters (Kollack-Walker and Newman 1995), as well as exposure to predator odors in male rats (Dielenberg et al. 2001). Furthermore, the connections of BNSTpm are restricted principally to other steroid-responsive regions within the limbic system, whereas BNSTpi has more extensive connections throughout the limbic system and related areas (Wood and Swann 2005). These functional and anatomical dissociations suggest that damage to BNSTpm may cause deficits in males' motivation to approach and interact with sexual odors, whereas damage to BNSTpi may cause deficits in categorizing all social odors. BNSTpl also sustained significant damage in most BNST-X males, raising the possibility that the post-lesion deficits observed in the current study were due to BNSTpl damage. Unlike BNSTpi and BNSTpm, BNSTpl is connected primarily with neural areas that are involved in motivated behaviors other than reproduction (i.e. basolateral and central amygdala, lateral hypothalamus) (Wood and Swann 2005), making it unlikely that BNSTpl contributed to the observed deficits in reproductive behavior. Future studies would need to delineate which of these anatomically separate and functionally dissociated subnuclei is most critical for the different aspects of male reproductive behavior.

Conclusion

Together, these results suggest that BNST plays a unique and critical role in the mediation of male reproductive behavior. Our findings demonstrate that BNST mediates the approach and investigation of volatile opposite-sex odors in sexually-naïve males, but plays a more modulatory role when these odors are directly contacted or after prior sexual experience. Despite confirming one aspect of previous findings, we found no

support for the idea that BNST alone is critical for copulatory behavior in sexually-experienced males. Indeed, BNST appears to be important for the timing of the copulatory sequence in sexually-naïve males only, suggesting previous reports of lesion-induced deficits in sexually-experienced males may be due to disruption of social odor information transfer between MA and MPOA or to more ventral BNST damage. Ongoing studies will determine whether the functional connections between MA, BNST, and MPOA are critical for the appropriate behavioral response to social odors.

ACKNOWLEDGEMENTS

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CHAPTER 2 FIGURES

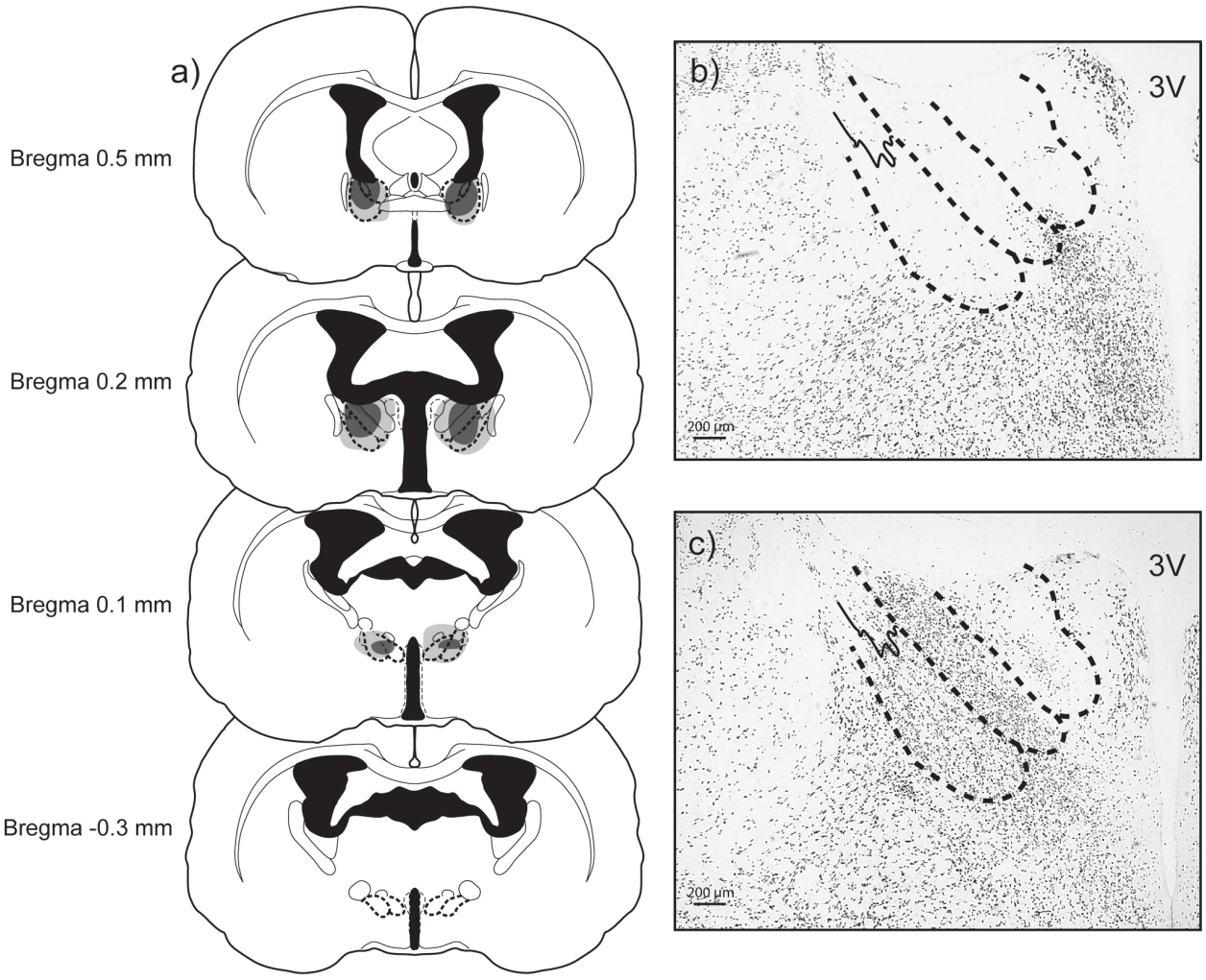

Figure 2.1: Lesion Reconstruction. a) Coronal sections through rostral to caudal extent of BNST showing largest (light gray) and smallest (dark gray) lesion included in the BNST-X group. Immunohistochemical localization of NeuN protein was used to visualize cell loss in males with excitotoxic lesions (b) compared to males with SHAM lesions (c).

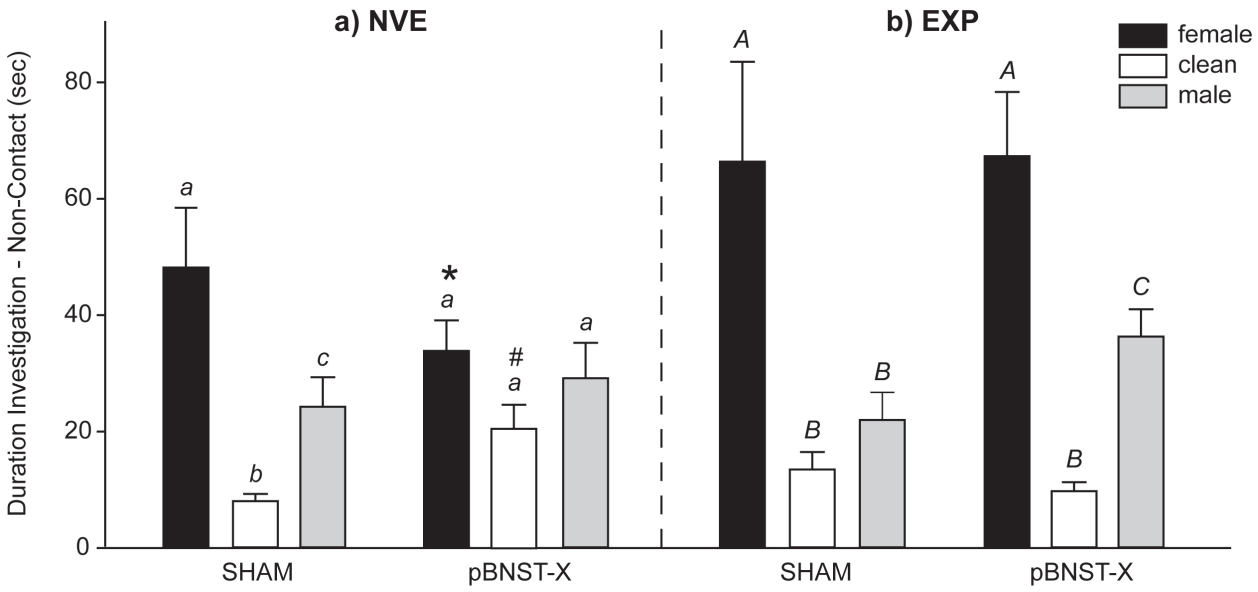

Figure 2.2: Investigation Times for Non-Contact Preference Test. a) In NVE males, lesions of pBNST eliminated preference for opposite-sex odors, whereas b) preference for opposite-sex odors remained intact in EXP males. Dissimilar letters indicate significant differences in investigation duration within lesion group, $p < 0.05$. * and # indicate significant differences in investigation duration between lesion groups, $p < 0.05$. Data expressed as mean ± standard error of means.

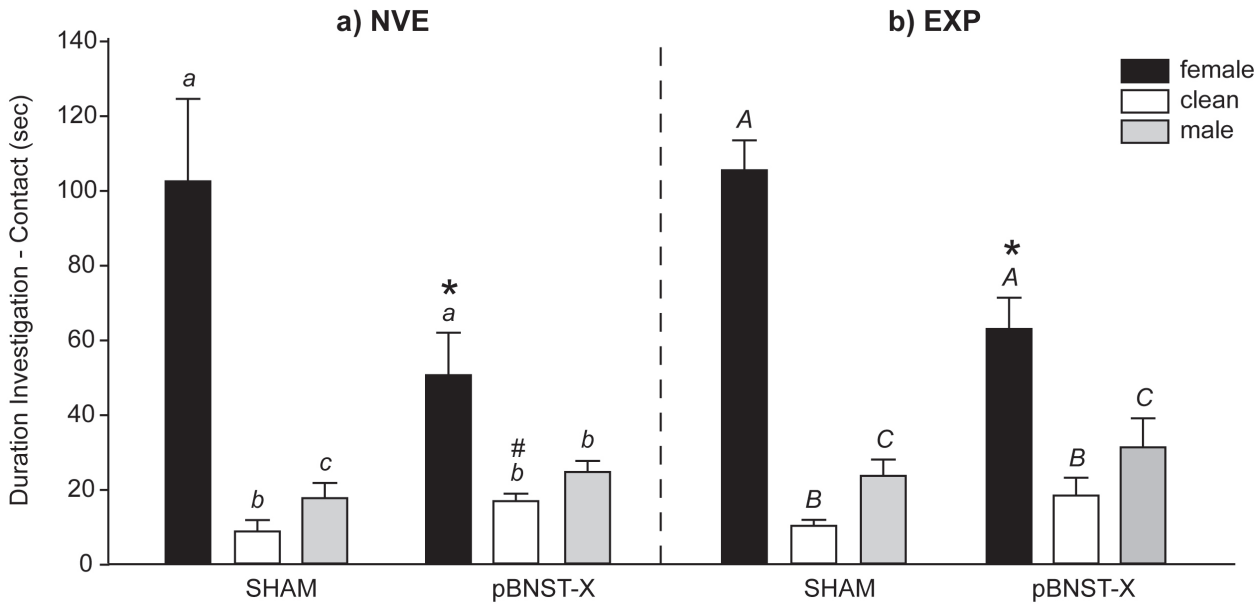

Figure 2.3: Investigation Times for Contact Preference. Lesions of pBNST significantly decreased males' investigation of female odors in a) NVE and b) EXP males, but preference for opposite-sex odors remained intact for both groups. Dissimilar letters indicate significant differences in investigation duration within group, $p < 0.05$. * and # indicate significant differences in investigation duration between groups, $p < 0.05$. Data expressed as mean ± standard error of means.

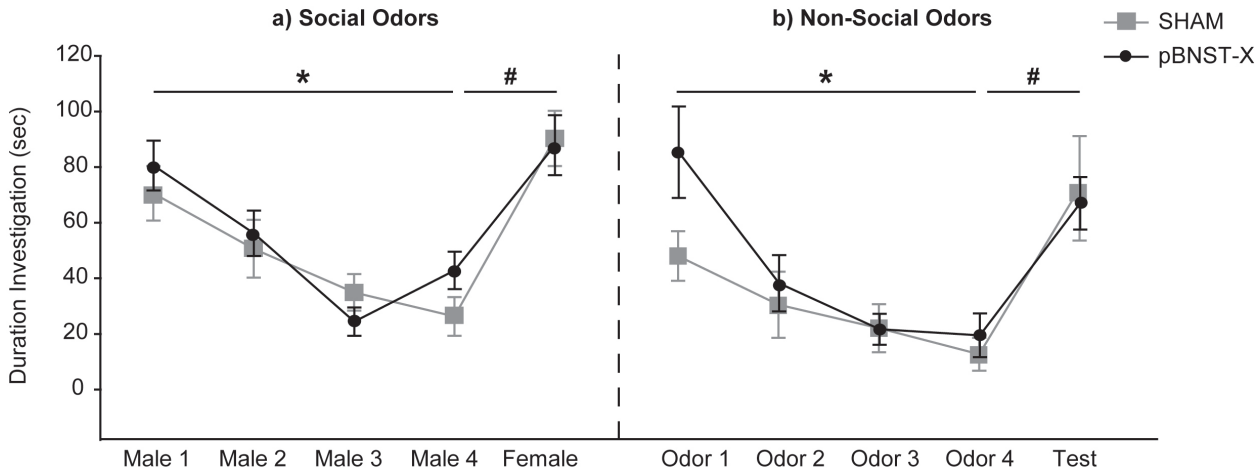

Figure 2.4: Investigation Times for Odor Discriminiation Test. a) Both SHAM and pBNST-X NVE males a) habituated to repeated presentations of male odors and dishabituated to a novel female odor and b) habituated to strawberry or baby powder odors and dishabituated to a novel non-social odor. * indicates a significant decrease between the first and fourth presentations, # indicates a significant increase between the fourth and fifth presentations. Data expressed as mean ± standard error of means.

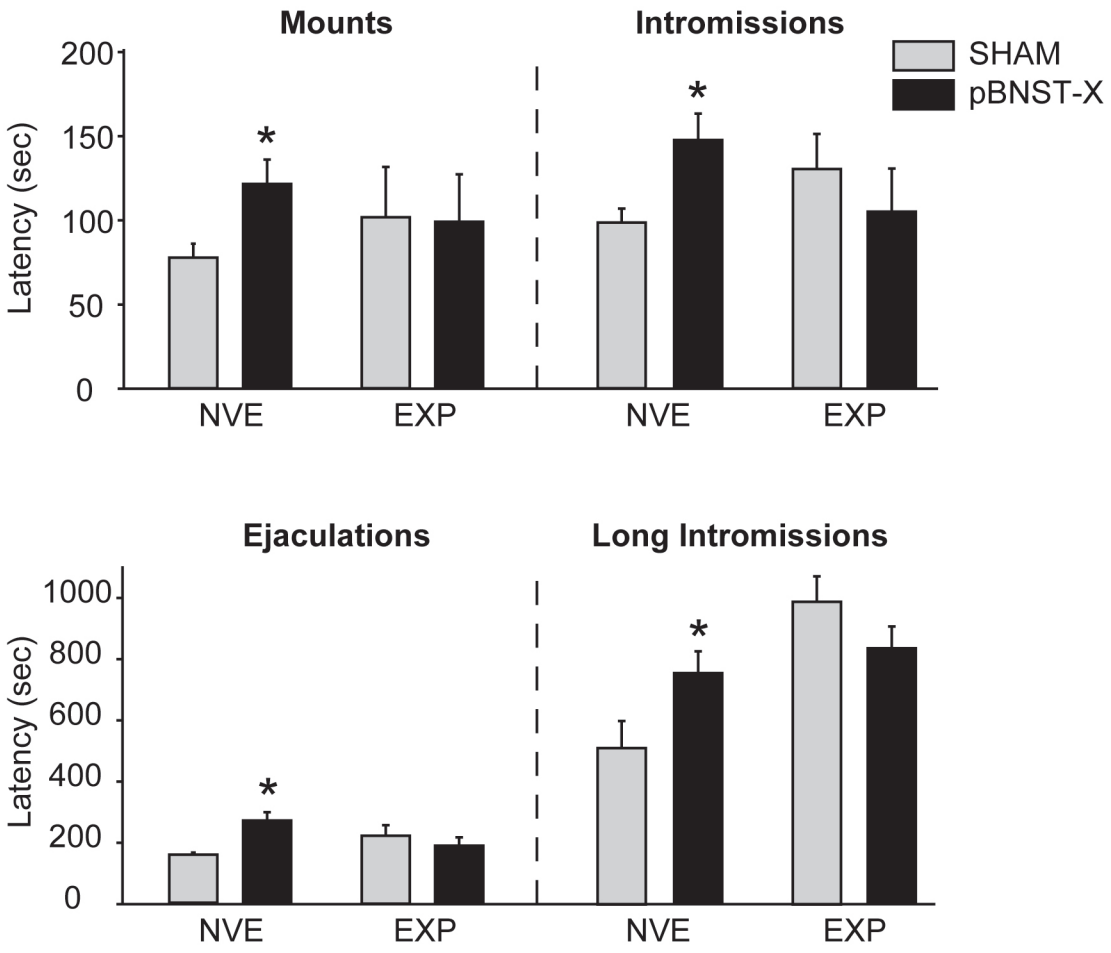

Figure 2.5: Latencies to Mating Events. In NVE subjects, pBNST-X males had significantly longer latencies to display mounts, intromissions, ejaculations, and long intromissions than SHAM males. In EXP subjects, pBNST-X and SHAM males did not differ in their latency to any mating event. * indicates significant differences between lesion groups, $p < 0.05$. Data expressed as mean ± standard error of means.

CHAPTER 2 TABLES

Table 2.1: Summary of odor preference measures from males with incomplete lesion damage to BNST. Unilateral or bilateral, partial lesions of BNST do not disrupt NVE or EXP males' preference to investigate female odors more than male or clean odors in a) Non-Contact or b) Contact tests. Data expressed as mean ± standard error of means.

	a) Non-Contact			b) Contact		
NVE	Female	Male	Clean	Female	Male	Clean
Unilateral (*n* = 6)	42.31 ± 9.07	20.84 ± 3.91	12.33 ± 5.10	59.42 ± 11.55	20.13 ± 2.68	13.13 ± 2.93
Partial (*n* = 3)	47.76 ± 5.53	17.4 ± 6.68	6.77 ± 1.52	57.61 ± 4.14	21.39 ± 10.4	6.04 ± 3.46
EXP						
Unilateral (*n* = 1)	42.86	7.74	5.06	88.83	32.41	24.13
Partial (*n* = 3)	57.83 ± 15.95	20.87 ± 6.43	7.12 ± 1.15	65.26 ± 9.27	16.43 ± 2.90	13.61 ± 3.63

Table 2.2: Total number, duration, and derived measures of mating events. In NVE and EXP subjects, pBNST-X males did not differ from SHAM males in the a) total number or b) duration of any mating event, nor did they differ in any c) derived measure of copulation. Data expressed as mean ± standard error of means.

	NVE		EXP	
a) Total Number	**SHAM**	**BNST-X**	**SHAM**	**BNST-X**
M	46.87 ± 2.93	42.75 ± 4.59	63.57 ± 3.33	66.60 ± 2.28
I	21.63 ± 1.53	20.00 ± 3.20	34.02 ± 2.05	33.58 ± 1.69
E	5.50 ± 0.50	5.83 ± 0.92	8.18 ± 1.34	9.67 ± 0.62
LI	11.00 ± 0.50	5.92 ± 1.40	7.56 ± 2.28	7.78 ± 1.46
b) Total Duration				
AGI	102.13 ± 19.92	115.05 ± 13.7	105.50 ± 18.29	60.10 ± 14.68
HBI	228.88 ± 30.05	237.08 ± 46.4	135.80 ± 33.98	79.20 ± 13.23
SG	188.59 ± 41.80	237.08 ± 46.4	350.70 ± 50.50	379.30 ± 22.85
c) Derived Measures				
PEI	39.61 ± 4.71	37.81 ± 6.23	41.32 ± 5.63	38.81 ± 4.74
I-E	4.21 ± 1.11	4.89 ± 0.62	3.29 ± 1.03	4.16 ± 0.98
ME	0.32 ± 0.04	0.32 ± 0.06	0.34 ± 0.07	0.33 ± 0.09

Table 2.3: Summary of copulatory behavior measures from males with incomplete lesion damage to BNST. Unilateral or bilateral, partial lesions of BNST do not disrupt the a) total number or b) latency to express any mating event in NVE and EXP males. Data expressed as mean ± standard error of means.

	NVE			EXP		
	SHAM	**Unilateral**	**Partial**	**SHAM**	**Unilateral**	**Partial**
	(*n* = 8)	(*n* = 6)	(*n* = 3)	(*n* = 8)	(*n* = 1)	(*n* = 3)
a) Total Number						
M	46.87 ± 2.93	52.50 ± 4.47	61.66 ± 3.47	65.22 ± 3.33	60.0	71.66 ± 7.31
I	21.62 ± 2.77	25.00 ± 3.13	29.33 ± 2.78	34.02 ± 2.05	25.0	42.66 ± 6.01
E	5.5 ± 0.50	7.16 ± 0.69	10.00 ± 1.08	8.18 ± 1.34	6.0	8.66 ± 1.20
LI	11.25 ± 2.77	9.50 ± 2.93	7.00 ± 2.15	7.56 ± 2.28	14.0	8.00 ± 0.81
b) Latencies (s)						
M	77.80 ± 8.29	123.92 ± 35.45	84.30 ± 23.76	101.80 ± 39.99	86.33	99.18 ± 29.6
I	98.74 ± 8.26	147.97 ± 32.78	100.33 ± 26.46	130.60 ± 34.92	104.42	117.11 ± 28.29
E	158.63 ± 6.49	173.31 ± 58	180.07 ± 20.35	223.00 ± 30.88	220.25	246.14 ± 75.68
LI	512.88 ± 87.99	649.70 ± 82.19	714.97 ± 68.28	990.60 ± 70.45	784.02	936. 62 ± 55.34

CHAPTER 3:

The role of the medial preoptic area in appetitive and consummatory reproductive behaviors depends on odor volatility and sexual experience in male Syrian hamsters

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ABSTRACT

In Syrian hamsters (*Mesocricetus auratus*), the expression of reproductive behavior requires the perception and discrimination of sexual odors. The behavioral response to these odors is mediated by a network of ventral forebrain nuclei, including the medial preoptic area (MPOA). The role of MPOA in male copulatory behavior has been well-studied, but less is known about the role of MPOA in appetitive aspects of male reproductive behavior. Furthermore, many previous studies that examined the role of MPOA in reproductive behavior have used large lesions that damaged other nuclei near MPOA or fibers of passage within MPOA, making it difficult to attribute post-lesion deficits in reproductive behavior to MPOA specifically. Thus, the current study used discrete, excitotoxic lesions of MPOA to test the role of this nucleus in opposite-sex odor preference and copulatory behavior in both sexually-naïve and sexually-experienced males. Lesions of MPOA eliminated preference for volatile, opposite-sex odors in sexually-naïve, but not sexually-experienced, males. When, however, males were allowed to contact the sexual odors, preference for female odors remained intact. Surprisingly, lesions of MPOA caused severe copulatory deficits only in sexually-naïve males, suggesting previous reports of copulatory deficits following MPOA lesions in sexually-experienced males were not due to damage to MPOA itself. Together, these results demonstrate that the role of MPOA in appetitive and consummatory aspects of reproductive behavior depends on the type of access to sexual odors and the sexual experience of the male.

INTRODUCTION

In many rodent species, including Syrian hamsters, male reproductive behavior depends critically on the perception of odor cues from the environment (Johnston 1990). Volatile odor cues are processed primarily by the main olfactory system (MOS), whereas non-volatile odor cues are processed primarily by the accessory olfactory system (AOS) (Breer 2003). Together, these two systems have been shown to mediate both appetitive and consummatory aspects of reproductive behavior in Syrian hamsters, including males' attraction to female vaginal secretion (Powers *et al.* 1979) and copulatory behavior (Murphy and Schneider 1970; Powers and Winans 1975).

The behavioral response to social odors in mediated by a network of ventral forebrain nuclei including the medial amygdala (MA), posterior bed nucleus of the stria terminalis (BNST), and medial preoptic area (MPOA) (Wood 1997). Specifically, MA and BNST are both densely connected to MPOA (Wood and Swann 2005) and several lines of evidence suggest that main and accessory olfactory information processed in MA and BNST must reach MPOA in order to elicit the appropriate behavioral response (Lehman *et al.* 1983; Sayag *et al.* 1994; Kondo and Arai 1995). Indeed, lesions of MPOA eliminate copulation in male Syrian hamsters (Powers *et al.* 1987; Floody 1989) and reduce or eliminate copulatory behavior in nearly every species studied, (Bean et al. 1981; Balthazart and Surlemont 1990b; Kingston and Crews 1994; Liu *et al.* 1997b), whereas stimulation of MPOA can enhance copulation (Malsbury 1971; Paredes *et al.* 1990; Rodriguez-Manzo *et al.* 2000).

The role of MPOA in appetitive aspects of reproductive behavior, such as the approach and investigation of social odors, is less well understood. Electrolytic lesions of

MPOA do not impair male Syrian hamsters' investigation of female hamster vaginal secretion when presented alone or during copulation (Powers *et al.* 1987). When, however, subjects are given the choice between two simultaneously presented odors, lesions of MPOA produce differing results. For example, electrolytic lesions including MPOA eliminate male rats' preference to investigate bedding from estrous females over anestrous female bedding (Hurtazo and Paredes 2005). Similarly, lidocaine injections targeting MPOA reduce the amount of time male rats spend near an estrous female that they cannot contact, although they continue to investigate the female more than a simultaneously presented male (Hurtazo *et al.* 2008). In contrast, lesions including MPOA reverse male ferrets' preference to approach an estrous female over a male (Paredes and Baum 1995) and this reversal of preference is strengthened when the stimulus animals cannot be contacted (Kindon *et al.* 1996). Together, these studies suggest that volatile odor cues may be the critical stimulus for MPOA-mediated attraction to, and preference for, opposite-sex conspecifics, but the role of MPOA in opposite-sex odor preference has never been directly tested.

Therefore, the following experiments used site-specific, excitotoxic lesions to test the role of MPOA in generating the appropriate behavioral response to volatile and non-volatile social odors. We hypothesized that MPOA is required for male hamsters' attraction to volatile female odor cues, but is not critical for attraction to non-volatile opposite-sex odors. If so, then lesions of MPOA should eliminate males' preference for opposite-sex odors when they cannot contact the odor stimuli, but preference should remain intact when contact is allowed. Furthermore, as small lesions of the sexually dimorphic nucleus of MPOA have produced different behavioral results depending on the sexual

experience of the subjects (Arendash and Gorski 1983; De Jonge et al. 1989), we tested the effects of MPOA lesions in both sexually-naïve and sexually-experienced males.

MATERIALS AND METHODS

The goal of the following experiments was to test the role of MPOA in sexual odor investigation in male hamsters. Exposure to female odors causes an increase in circulating testosterone levels in male hamsters (Macrides *et al.* 1974; Pfeiffer and Johnston 1992) and it is possible that lesions of MPOA may interfere with this surge. Thus, in order to equalize steroid hormone levels across experimental groups, all subjects were gonadectomized and maintained on physiological levels of exogenous testosterone for the duration of the experiment. Following bilateral, excitotoxic lesions of MPOA or sham lesion surgeries, sexually-naïve and sexually-experienced subjects underwent a series of behavioral tests: first, subjects were tested for their preference to investigate female odors over male odors (*Odor Preference*). To determine if any effects of MPOA lesions on odor investigation depend on the volatility of the odor cues being processed, Odor Preference tests were conducted under conditions that either prevented contact with the odor sources (*Non-contact*; volatile odors only) or allowed contact with the odor sources (*Contact*; volatile and non-volatile odors). Second, in order to determine if a lack of preference in these tests was due to an inability to discriminate between odor stimuli subjects were tested for their ability to discriminate between social odor sources using a habituation-dishabituation task (*Odor Discrimination*). Lastly, as a positive control, subjects' sexual behavior in response to a receptive stimulus female (*Copulatory Behavior Test*) was assessed.

Animals

Experimental subjects were adult (3 to 6 months old) male Syrian hamsters (*Mesocricetus auratus*) purchased from Charles River Laboratories (Wilmington, MA, USA). A separate group of unrelated adult male and female hamsters served as odor stimulus donors for behavior tests. A third group of ovariectomized and hormone-primed adult female hamsters were used as stimulus females for copulatory behavior tests. Experimental subjects and copulatory stimulus females were single-housed, whereas odor donors were group-housed (three to four animals per cage), in solid-bottom Plexiglas cages (36 cm x 30 cm x 16 cm). All subjects were maintained on a reversed 14 hour light/ 10 hour dark photoperiod, and food and water were available *ad libitum*. All 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.

Sexual Experience

Following gonadectomy (see 2.3.1), experimental subjects were randomly assigned to one of two experimental conditions: sexually-experienced (EXP; n = 30) or sexually-naïve (NVE; n = 32). EXP males were given weekly sexual experiences for three consecutive weeks. Subjects were placed into a clear, Plexiglas testing area (50 cm x 25 cm x 30 cm) for five minutes prior to the addition of a receptive stimulus female. An angled mirror was placed below the testing arena to provide a view of the ventral surface of the animals. Encounters lasted for 30 minutes, or until the animals engaged

in aggressive behavior, at which point the stimulus female was removed. The second and third encounters were video-recorded and the male's behavior was later scored using the Observer for Windows, version 9.0 (Noldus Information Technology, Wageningen, The Netherlands). Males who failed to copulate on at least two of the three encounters (n = 4) were eliminated from the study. To control for possible effects of experimenter handling on future behavior, NVE males were transported to the same behavioral testing room and placed into an empty cage for 30 minutes once a week for three consecutive weeks.

Surgery

All surgeries were performed under 2% isoflurane gas anesthesia vaporized in 100% oxygen (gonadectomy) or a 70:30% oxygen/nitrous oxide mixture (stereotaxic surgery). To minimize post-operative pain, ketoprofen (5 mg/kg subcutaneously, Henry Schein, Melville, NY, USA) was administered intra-operatively.

Gonadectomy and Hormone Implant. One to two weeks prior to lesion surgery, subjects' testes were bilaterally removed via a bilateral midline abdominal incision and cauterization of the ductus deferens and blood vessels. Silastic capsules (i.d. 1.57 mm, o.d. 2.41 mm, Dow Corning, Midland, MI, USA) packed with 20 mm length of crystalline testosterone (Sigma, St. Louis, MO, USA) were implanted subcutaneously between the scapulae immediately following gonadectomy. Vicryl suture (size 4-0, Ethicon, Somerville, NJ, USA) and wound clips were used to close the smooth muscle and skin incisions, respectively.

Stimulus females for copulatory experiences and behavior testing were ovariectomized at least two weeks prior to use. Following bilateral flank incisions, the ovaries

were removed via cauterization of the uterine horn and blood vessels. Silastic capsules (i.d. 1.57 mm, o.d. 2.41 mm, Dow Corning, Midland, MI, USA) packed with 5 mm length of crystalline estradiol (Sigma, St. Louis, MO, USA) were implanted subcutaneously between the scapulae immediately following gonadectomy. Vicryl suture and wound clips were used to close the smooth muscle and skin incisions, respectively. To induce behavioral receptivity, stimulus females were injected subcutaneously with 0.15 ml of progesterone dissolved in sesame oil (2.5 mg/ml, Sigma, St. Louis, MO, USA) 4 hours prior to copulatory behavior tests.

Excitotoxic Lesion. NVE and EXP subjects were randomly assigned to either a MPOA lesion (MPOA-X; NVE n = 22, EXP n = 20) or a sham lesion surgery (SHAM; NVE n = 8, EXP n = 10) group. Anesthetized subjects were secured in the stereotaxic apparatus such that their skull was level in the anterior-posterior (A-P) and medial-lateral (M-L) planes. Following a midline scalp incision, the skin and temporal muscles were retracted to expose the skull and a hand-operated drill was used expose dura. All A-P and M-L measurements were taken in mm relative to bregma and all dorsal-ventral (D-V) measurements were taken in mm relative to dura. Excitotoxic lesions were made by lowering a microinjection syringe (701R 10 µl syringe, Hamilton, Reno, NV, USA) under stereotaxic control (Microinjection Unit, Model 5002, David Kopf Instruments, Tujunga, CA, USA) into bilateral sites targeting MPOA (A-P: - 2.0, M-L: ± 0.7, D-V: - 7.0) and injecting N-methyl-D-aspartic acid (NMDA, 20 mg/ml; 20 nl per injection, Sigma, St. Louis, MO, USA). To minimize the flow of excitotoxin up the syringe tract, the syringe was left in place for 10 minutes after each injection.

Sham surgeries were identical to lesion surgeries with two exceptions: 1) the microinjection syringe was lowered to 1 mm above the target injection site and 2) no excitotoxin was infused into the target injection sites. After all surgeries, skull holes were sealed using bone wax and incisions were closed with wound clips. Subjects were allowed to recover for at least two weeks prior to behavioral testing.

Behavioral Testing

All behavior testing took place during the first six hours of the dark phase and under light illumination.

Odor Stimuli. Sexual odor stimuli used for Odor Preference and Odor Discrimination tests were collected from cages of group-housed, same-sexed odor donors that had not been changed for four days prior to odor collection. Each sexual odor stimulus consisted of soiled cotton bedding (2 Nestlets, 12 g, ANCARE, Bellmore, NY), soiled corncob litter (50 ml, Bed-o-cob, The Andersons, Maumee, OH, USA) and one damp cotton gauze pad that was used to wipe the inner walls of the cage. In addition, a damp gauze pad was used to wipe two of the cage resident's anogenital region and bilateral flank glands 10 times each. For female odor stimuli, vaginal secretion was also collected onto a gauze pad by gently palpating the vaginal area of a female with a disposable probe, and was added to each odor stimulus. Clean odor stimuli consisted of clean cotton bedding (2 Nestlets), clean corncob litter (50 ml), and clean cotton gauze pads (2).

For Contact tests, additional sexual odors were collected directly onto glass microscope slides (25 mm X 75 mm X 1 mm) by rubbing a clean slide along an odor donor's flank and anogenital regions. All odor slides contained samples from two individual odor donors (collected separately onto each end of the slide). For female odor slides, a

sample of vaginal secretion was also collected onto the same slide. All odor stimuli were stored in airtight containers at 4°C until 20 minutes before use. Odor stimuli older than one month were discarded and no subject was tested with the same odor stimulus more than once.

Odor Preference. A three-choice test was used to measure odor preference. Subjects were placed into a glass aquarium (50 cm x 25 cm x 30 cm) with opaque walls. Three acrylic odor presentation boxes (8 cm x 8 cm x 8 cm) were lined up on the floor of the aquarium such that the left and right sides of the center box touched one side of each of the lateral boxes. The backs of the three boxes were then affixed to one of the short walls of the aquarium; thus, only the front and top surfaces of each box were accessible. Each odor presentation box had 7-mm holes drilled along the front surface that allowed volatile odors to pass, but prevented contact with the odor sources. During testing, a single odor stimulus (see above) was placed into each of the three odor presentation boxes. Additionally, a line bisecting the available floor space was drawn parallel to the short walls so that general activity levels (as measured by total number of line crosses) could be assessed during the test. The top of the aquarium was secured with a clear Plexiglas top to allow for overhead video recording of the subject's behavior. All surfaces of the aquarium and odor boxes were thoroughly cleaned with 70% alcohol and allowed to dry between subjects.

Subjects were tested in a series of three tests in the 3-choice apparatus, each separated by 24 hours: Clean, Non-Contact preference, and Contact preference. At the beginning of each test, a subject was placed into the testing arena and then allowed ten minutes to freely explore the apparatus. For all tests, investigation of the odor stimulus

was coded when the subject made contact with, or directed its nose within 1 cm of, the perforated front surface of the odor box and/or odor slide. For Clean tests, clean odor stimuli were placed into each of the three odor boxes. These tests were used to acclimate the subjects to the testing arena, as well as to obtain baseline levels of activity in the absence of sexual odor stimuli. For subsequent preference tests, female and male odor stimuli were placed into each of the two outer odor boxes, and clean odor stimuli were placed into the center odor box. The side on which each sexual odor was placed (left or right) was alternated between consecutive subjects. Non-Contact and Contact tests were identical except that during Contact tests, a single odor slide matching the type of odor stimulus in that container (female, male, clean) was secured to the center of the front surface of each odor presentation box.

Video recordings of all tests were digitized onto a computer and scored using the Observer for Windows, version 9.0. All observers were blind to the condition of the subject, and different observers reached at least a 90% inter-observer reliability score prior to coding behavior.

Odor Discrimination. In order to determine if deficits observed in Non-Contact Odor Preference tests (see section 3.3) could be due to an inability to discriminate between odor stimuli, all males were tested for their ability to discriminate between volatile odors using a habituation-dishabituation test. The habituation-dishabituation test involves repeated presentations of the same odor source followed by a test presentation of a novel odor source. A decrease in investigation during the repeated presentations indicates a perception of the odors as being the same or familiar. An increase in investigation of the novel odor compared to the last presentation of the habituated odor indi-

cates an ability to discriminate between the two odors (Johnston 1993; Baum and Keverne 2002). The testing sequence consisted of four, 3-minute presentations of repeated odors (habituation) followed by a fifth, 3-minute presentation of a novel odor (dishabituation). Five-minute inter-trial intervals separated each odor presentation. Odor stimuli were presented in the same odor presentation boxes used for the Odor Preference tests. Odor presentation boxes were affixed to one of the short walls of subjects' home cage and investigation was measured using a stopwatch. Odor containers were cleaned with 70% alcohol and allowed to dry between subjects. Subjects were presented with different odor sources on each of the habituation trials so that subjects were habituated to the sexual identity of the repeated odor, rather than to the individual identity of odor donors. Under these testing parameters, male hamsters consistently display a lack of habituation to repeated presentations of female odors (Maras and Petrulis 2006) and so all subjects were tested using male odors as the habituation stimuli and female odors as the dishabituation stimuli for social odor discrimination tests.

Copulatory Behavior. Copulatory behavior test procedures were identical to those used to provide sexual experience to EXP males. Tests were video-recorded and later scored using Observer for Windows, version 9.0. The total number and latencies (from onset of test) of several behavioral measures were scored: mounts (M), intromissions (I), ejaculations (E), and long intromissions (LI). LI are distinguished from I in that males do not quickly dismount the female following vaginal penetration, but instead display a repetitive thrusting pattern (Bunnell *et al.* 1977). Importantly, the expression of LI is associated with the onset of sexual satiety in Syrian hamsters (Bunnell *et al.* 1977; Parfitt and Newman 1998). In addition, the total durations of time the male spent investigating

the female's anogenital region (AGI), investigating the female's head or body region (HBI), and self-grooming (SG) were also scored. Finally, several derived measures of copulatory behavior were also analyzed: post-ejaculatory interval (PEI; latency to display a mount or intromission after each ejaculation), the number of intromissions to reach each ejaculation (I-E), and mounting efficiency (ME; the total number of intromissions divided by the total number of mounts + intromissions).

Histology and Lesion Verification

Following the last behavioral test, subjects were injected with an overdose of sodium pentobarbital (100 mg/kg; Sleep Away, Ft. Dodge, IA, USA) and transcardially perfused with 200 ml of 0.1M phosphate-buffered saline (PBS, pH 7.4) followed by 200 ml of paraformaldehyde (4%). Brains were post-fixed in paraformaldehyde (4%) overnight and then cryoprotected for 48 hours in 30% sucrose in PBS solution. Coronal sections (30-µm) of brain tissue were sectioned using a cryostat (-20°C) and stored in cryoprotectant until immunohistochemical localization of Neuronal Nuclei protein (NeuN, see below). Additionally, in order to further delineate lesion damage from fiber tracts (which are not readily detected by NeuN), every third section was mounted onto glass slides using a 1% gelatin mounting solution and stained for Nissl material with cresyl violet. Nissl- and NeuN-stained sections were examined under a light microscope for the location and extent of lesion damage as compared with published hamster neuroanatomical plates (Morin and Wood 2001), and the minimum and maximum extents of lesion damage were traced onto anatomical plates using Adobe Illustrator CS 11.0 software.

Immunohistochemistry. Free-floating sections were removed from cryoprotectant, rinsed thoroughly in PBS, and then incubated in a monoclonal antibody against NeuN in

PBS with 0.4% Triton-X-100 (1:30,000, Millipore, Billerica, MA, USA) for 48 hours at 4°C. After rinsing in PBS, sections were incubated in biotinylated secondary antibody (1:600, Jackson Immunoresearch Laboratories, West Grove, PA, USA) in PBS with 0.4% Triton-X-100 for one hour at room temperature, rinsed in PBS, then incubated in an avidin-biotin complex (1:200, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for one hour at room temperature. Sections were then rinsed in PBS and reacted in a nickel-enhanced 3, 3′-diaminobenzidine tetrahydrochloride (DAB) solution (2 mg DAB plus 250 mg Nickel (II) Sulfate with 8.3 µl 3% H_2O_2 per 10ml of 175 mM sodium acetate) to yield a blue-black reaction product. After 15 minutes, sections were rinsed in PBS to stop the chromagen reaction.

Blood Collection and Radioimmunoassay

Blood samples were collected from the inferior vena cava after anesthesia and immediately prior to perfusion and stored in vacutainer collection tubes (4-ml draw, red/gray, VWR, West Chester, PA, USA) on ice until centrifugation. Samples were centrifuged at 2500 revolutions per minute at 4°C for 20 minutes and serum was stored in 200-µl aliquots at -20°C until assay. Testosterone levels (ng/ml) were measured by radioimmunoassay kits from Diagnostics System Laboratories (DSL 4000 Testosterone) with a sensitivity range of 0.05 to 22.92 ng/ml and an interassay variance of 8%, previously validated for hamster serum (Cooper *et al.* 2000). The mean testosterone levels (± standard errors) for experimental subjects were: NVE MPOA-X = 3.525 ± 0.403; NVE SHAM = 4.241 ± 0.527; EXP MPOA-X = 3.240 ± 0.573; EXP SHAM = 3.471 ± 0.541. There was no difference in testosterone levels between lesion groups for NVE or EXP males (NVE $t(18) = 1.009$, $P = 0.327$; EXP $t(16) = 0.286$, $P = 0.778$).

Role of Endocrine Status on Copulatory Behavior

In order to equalize steroid hormone levels across experimental groups, all subjects were gonadectomized and maintained on physiological levels of exogenous testosterone for the duration of the experiment. However, in order to confirm that lesions of MPOA do not produce differing results in gonadally-intact versus hormone-replaced subjects, the copulatory behavior of a small group (n = 6) of gonadally-intact males with lesions of MPOA were compared to the copulatory behavior of a subset (n = 7) of hormone-replaced experimental subjects. The mean number of mounts ($t(11) = 1.076$, $P = 0.315$) intromissions ($t(11) = 2.761$, $P = 0.476$), and ejaculations ($t(11) = 1.091$, $P = 0.344$) did not differ between gonadally-intact and hormone-replaced subjects with lesions of MPOA.

Data Analysis

All data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for Windows and significance was determined as $P < .05$. To establish investigatory preferences for each type of 3-choice test (Clean, Non-Contact Preference, Contact Preference), 2 (Experimental group: MPOA-X, SHAM) X 3 (Odor) mixed-design ANOVAs were performed. Significant interactions were explored using simple effects analysis and pair-wise comparisons with Bonferroni alpha adjustments. Furthermore, separate one-way ANOVAs were used to compare the levels of investigation of each stimulus directly across experimental groups. To identify differences in general motor activity, additional one-way ANOVAs were used to compare the total number of midline crosses across experimental groups for the Clean test.

For the habituation-dishabituation data, data were split by experimental group, and paired *t*-tests (2-tailed, with Bonferroni alpha adjustments) were used to detect both (1) a habituation to the repeated presentations of odors (male 1 vs. male 4) and (2) a dishabituation to the presentation of the test odor (male 4 vs. female).

Many copulatory behavior tests were terminated before 30 minutes because the male and female engaged in aggressive behavior. Thus, only the first 20 minutes of each copulatory test were analyzed to eliminate variability caused by differences in test duration. Group differences in most copulatory measures were detected using one-way ANOVAs, whereas group differences in the proportion of animals displaying copulatory measures were detected using *z*-tests for independent proportions. Additionally, separate 2 x 2 (lesion group x ejaculatory series) ANOVAS were used to detect changes in post-ejaculatory intervals or the number of intromissions to reach each ejaculation in the copulatory tests.

RESULTS

Lesion Reconstruction

Subjects were included in the MPOA-X lesion group (NVE *n* = 11; EXP *n* = 10) only if they had extensive bilateral damage to MPOA, defined as at least 60% bilateral damage to MPOA in at least two stereotaxic planes of section (Figure 3.1a, (Morin and Wood 2001). All subjects included in the MPOA-X group sustained significant damage to MPOA, including the medial preoptic nucleus (MPN), below the most caudal extent of the anterior commissure (Bregma 0.5 mm). Most subjects (NVE *n* = 9; EXP *n* = 8) also sustained significant damage to MPOA, including MPN, at the level where the lateral

and third ventricles fuse (Figure 3.1b; Bregma 0.2 mm). Some subjects (NVE *n* = 5; EXP *n* = 4) had damage to the most caudal level of MPOA, including MPN and the magnocellular medial preoptic nucleus (MPNmag) (Bregma -0.1 mm). Subjects with MPNmag damage did not differ from subjects without MPNmag damage on any behavioral measure and were therefore collapsed into the MPOA-X lesion group. Fewer subjects (NVE *n* = 2; EXP *n* = 3) sustained damage to the more rostral aspects of MPOA, including MPN (Bregma 0.8 mm).

Some subjects included in the MPOA-X group sustained minimal or unilateral damage to other adjacent nuclei, defined as less than 20% damage at only one stereotaxic plane of section of the nucleus (Morin and Wood 2001). These included the parastriatal nucleus (*n* = 3), lateral preoptic area (*n* = 11), and periventricular hypothalamic nucleus (*n* = 10). Eight males also sustained damage to the most rostral level of the anterior hypothalamus (Bregma -0.3 mm). There was no difference in behavior across subjects with minor unilateral, minor bilateral, or no damage to any of the adjacent nuclei. Only needle tracts were visible in SHAM males (NVE *n* = 8; EXP *n* = 7).

Subjects were excluded from the MPOA-X lesion group if their lesions extended significantly outside of MPOA. Specifically, four subjects (NVE *n* =1; EXP *n* = 3) were excluded from the lesion group because they sustained significant damage to the most caudal levels of the posterior bed nucleus of the stria terminalis (BNSTpc; Figure 3.1c, Bregma - 0.3). In addition, subjects were excluded from the MPOA-X lesion group if their lesions failed to damage a significant portion of MPOA. Specifically, four NVE subjects sustained partial (less than 60% damage in two stereotaxic planes of section), bilateral damage to MPOA and three NVE subjects had primarily unilateral damage to

MPOA. Although the small sample sizes of these groups precluded them from statistical analysis, data from subjects with lesions that extended significantly outside of MPOA or that failed to significantly damage MPOA were examined to determine the specificity of MPOA lesions on odor preference and copulatory behaviors (see below).

Odor Preference

Clean Test. In the Clean test, both NVE and EXP subjects investigated the center odor presentation box less than the left (NVE $t(18) = 5.203$, $P < .001$; EXP $t(16) = 7.287$, $P < .001$) or right (NVE $t(18) = 6.493$, $P < .001$; EXP $t(16) = 5.529$, $P < .001$) odor presentation boxes; there was no difference in time spent investigating the left and right boxes (NVE $t(18) = .227$, $P = .823$; EXP $t(16) = 1.067$, $P = .302$). Thus, although there was a general bias to investigate the outside boxes, there was no difference in this bias across experimental groups, and more importantly, there was no preference to investigate either one of the boxes used to present social odors. There was also no difference in the total number of midline crosses between MPOA-X and SHAM males (NVE $F(1,17) = .678$, $P = .422$; EXP $F(1,15) = .376$, $P = .549$), indicating similar levels of activity across experimental groups.

Non-Contact Preference Test. Lesions of MPOA decreased NVE males' investigation of volatile female odors, resulting in an elimination of opposite-sex odor preference (Figure 3.2a). There was a significant interaction between lesion group and the duration of investigation of the three odor stimuli ($F(2,48) = 5.085$, $P = .010$). Whereas SHAM males investigated female odors more than male odors ($t(7) = 4.444$, $P = .004$) and investigated both female odors ($t(7) = 4.689$, $P = .003$) and male odors ($t(7) = 3.860$, $P = .008$) more than clean odors, MPOA-X males spent equivalent amounts of

time investigating female and male odors ($t(10) = 1.128$, $P = .286$), although they investigated both female odors ($t(10) = 2.415$, $P = .036$) and male odors ($t(10) = 5.830$, $P < .001$) more than clean odors. In addition, MPOA-X males spent significantly less time investigating female odors than did SHAM males ($F(1,17) = 5.917$, $P = .027$).

In contrast to NVE subjects, EXP males with lesions of MPOA did not display a deficit in their preference to investigate volatile opposite-sex odors (Figure 3.2b). There was a significant main effect of odor stimulus ($F(2,45) = 46.352$, $P < .001$), but no interaction between lesion group and the duration of investigation for the three odor stimuli. Both SHAM and MPOA-X males spent significantly more time investigating female odors than male odors (SHAM $t(6) = 7.011$, $P < .001$; MPOA-X $t(9) = 3.864$, $P = .004$), and investigated both female odors (SHAM $t(6) = 7.897$, $P < .001$; MPOA-X $t(9) = 5.538$, $P < .001$) and male odors (SHAM $t(6) = 3.123$, $P = .020$; MPOA-X $t(9) = 4.383$, $P = .002$) more than clean odors.

NVE subjects with unilateral or bilateral, partial damage to MPOA spent more time investigating female odors than male odors in the Non-Contact Preference Test. Interestingly, NVE and EXP subjects with damage that extended into BNSTpc did not investigate female odors more than male odors in the Non-Contact Preference Test (Table 3.1a).

Contact Preference Test. Lesions of MPOA did not affect NVE or EXP males' preference for opposite-sex odors when contact with the odor stimuli was allowed. In NVE males, there was a significant main effect of odor stimulus ($F(2,48) = 41.891$, $P < .001$), but no interaction between lesion group and the duration of investigation of the three odor stimuli (Figure 3.3a). Both SHAM and MPOA-X males spent significantly

more time investigating female odors than male odors (SHAM $t(7) = 5.032$, $P = .002$; MPOA-X $t(10) = 3.330$, $P = .008$) and investigated both female odors (SHAM $t(7) = 6.585$, $P < .001$; MPOA-X $t(10) = 5.792$, $P < .001$) and male odors (SHAM $t(7) = 6.227$, $P < .001$; MPOA-X ($t(10) = 3.698$, $P = .004$) more than clean odors.

In EXP males, there was also a significant main effect of odor stimulus ($F(2,45) = 98.968$, $P < .001$), but no interaction between lesion group and the duration of investigation of the three odor stimuli (Figure 3.3b). Both SHAM and MPOA-X males spent significantly more time investigating female odors than male odors (SHAM $t(6) = 4.535$, $P = .004$; MPOA-X $t(9) = 7.526$, $P < .001$) and investigated both female odors (SHAM $t(6) = 11.243$, $P < .001$; MPOA-X $t(9) = 9.114$, $P < .001$) and male odors (SHAM $t(6) = 2.476$, $P = .048$; MPOA-X ($t(9) = 6.801$, $P < .001$) more than clean odors.

NVE subjects with unilateral or bilateral, partial damage to MPOA spent more time investigating female odors than male odors in the Non-Contact Preference Test. Similarly, NVE and EXP subjects with damage that extended into BNSTpc spent more time investigating female odors than male odors in the Non-Contact Preference Test (Table 3.1b).

Odor Discrimination

NVE SHAM and MPOA-X males habituated to repeated presentations of different male odors, as indicated by decreased investigation of the male odor on the fourth trial compared to the first trial (SHAM $t(7) = 2.104$, $P = .052$; MPOA-X $t(10) = 2.551$, $P = .031$). Importantly, both lesion groups also dishabituated to a novel female odor, as indicated by an increased investigation of the female odor compared to the last presenta-

tion of the habituated male odor (SHAM $t(7) = 4.425$, $P = .003$; MPOA-X $t(10) = 2.966$, $P = .016$) (Figure 3.4).

Copulatory Behavior

In NVE males, lesions of MPOA caused severe deficits in copulatory behavior. Whereas all SHAM males displayed mounts, intromissions, and ejaculations, only four out of eleven MPOA-X males mounted, one intromitted, and none achieved ejaculation. As a result of this, the total number of mating events for MPOA-X males did not follow a normal distribution; therefore the proportion of subjects displaying each mating event was compared between groups instead. The proportion of males displaying mounts ($z = 2.839$, $P = .004$), intromissions ($z = 3.918$, $P < .001$), ejaculations ($z = 4.358$, $P < .001$), ectopic mounts ($z = 2.839$, $P < .004$), and long intromissions ($z = 2.639$, $P = .008$) was significantly different between SHAM and MPOA-X groups (Figure 3.5a). Although SHAM and MPOA-X males did not differ in their total duration of anogenital investigation ($F(1,16) = .398$, $P = .537$) or head/body investigation ($F(1,16) = .015$, $P = .904$), MPOA-X spent significantly less time self-grooming than SHAM males ($F(1,16)$ 15.195, $P = .001$) (Figure 3.6a). Derived measures of copulatory behavior were not calculated for NVE males, as the majority of subjects failed to intromit or ejaculate.

In contrast to NVE males, EXP males with lesions of MPOA displayed relatively normal copulatory behavior. All SHAM and MPOA-X males mounted, intromitted, and ejaculated, and the proportion of subjects displaying these behaviors did not differ between groups (Figure 3.5b). Furthermore, the proportion of subjects displaying ectopic mounts ($z = 0.485$, $P = .627$) and long intromissions ($z = 0.290$, $P = .772$) did not differ between SHAM and MPOA-X males (Figure 3.5b), nor did the total duration of anogeni-

tal investigation ($F(1,14) = 2.753$, $P = .119$), head/body investigation ($F(1,14) = .240$, $P = .632$), and self-grooming ($F(1,14) = .930$, $P = .351$) (Figure 3.6b).

There were, however, subtle differences in the temporal pattern of mating between EXP SHAM and MPOA-X males. MPOA-X males took significantly longer to begin anogenital investigation (Figure 3.7a) ($F(1,14) = 8.401$, $P = .012$) and displayed significantly longer ejaculation latencies ($F(1,14) = 5.339$, $P = .037$) than did SHAM males (Figure 3.7c). These differences remained significant even after data was corrected for the latency to begin anogenital investigation. In contrast, the latencies to first mount ($F(1,14) = 2.288$, $P = .153$) and first intromission ($F(1,14) = 2.812$, $P = .116$) did not differ between SHAM and MPOA-X males (Figure 3.7b), nor did latency to display long intromissions ($F(1,4) = 1.522$, $P = .285$) (Figure 3.7c). Finally, EXP MPOA-X males did not differ from SHAM males in any derived measure of copulatory behavior (Table 3.2).

NVE subjects with unilateral or bilateral, partial damage to MPOA performed at levels comparable to SHAM males on all measures of copulatory behavior (Table 3.3a). In contrast, subjects with damage that extended into BNSTpc were severely impaired in their copulatory behavior. Only one EXP male with BNSTpc damage displayed a single bout of mounting that resulted in one intromission, whereas all other males with BNSTpc damage (NVE and EXP) failed to mount, intromit, or ejaculate (Table 3.3b).

DISCUSSION

Summary

This study is the first to comprehensively test the role of MPOA in the processing of distal and proximate sexual odor cues and copulatory behavior in both sexually-naïve and sexually-experienced males. We found that excitotoxic lesions specific to MPOA eliminate preference for volatile opposite-sex odors in sexually-naïve male Syrian hamsters. Importantly, this lack of preference was not due to an inability to discriminate between odors, as MPOA-X males, like SHAM males, could discriminate between volatile male and female odors in a habituation-dishabituation test. Preference for opposite-sex odors remained intact, however, when subjects were allowed to contact the sexual odors, supporting our prediction that MPOA is more critical for the initial approach and investigation of volatile female odors, rather than close, intensive investigation of non-volatile female odors. In addition, subjects with sexual experience were unimpaired in their preference for opposite-sex odors under both stimulus conditions. Surprisingly, lesions of MPOA severely compromised copulatory behavior only in sexually-naïve males, conflicting with previous reports of copulatory deficits following electrolytic lesions of MPOA in sexually-experienced male hamsters (Powers *et al.* 1987; Floody 1989).

Role of MPOA in opposite-sex odor preference

The finding that lesions of MPOA eliminate preference for volatile opposite-sex odors in male hamsters is congruent with previous studies that suggest MPOA mediates males' unconditioned anticipatory responding to a female or her odors. In male rats, for example, large, electrolytic lesions that include MPOA decrease preference for estrous

female bedding over non-estrous female bedding (Hurtazo and Paredes 2005), preference to interact with a female over a male (Paredes *et al.* 1998), and pursuit of females (Paredes et al. 1993). Similarly, temporary inactivation of MPOA using lidocaine decreases the amount of time male rats spend near a female in an incentive motivation test where subjects can choose to spend time near a female or male stimulus animal that they cannot contact (Hurtazo *et al.* 2008). In male ferrets, electrolytic (Kindon *et al.* 1996) or excitotoxic (Paredes and Baum 1995) lesions that include MPOA reverse the normal preference for approaching and interacting with females over males. Finally, MPOA also mediates anticipatory responses to females in other comparative animal models, including anogenital investigation, tongue-flicking, and anticipatory erections in male marmosets (Lloyd and Dixson 1988), preference to view females over males in male Japanese Quails (Balthazart et al. 1998a), and pre-copulatory courtship behaviors in male garter snakes (Friedman and Crews 1985a). Together, these data suggest that MPOA mediates males' anticipatory responses to female cues in a variety of conditions, sensory modalities, and species.

Role of Odor Volatility

The results of the current study also suggest the importance of MPOA for male attraction to female odors depends on the volatility of the odor cues available. When only volatile odors are present, as would be the case when animals are detecting odors from a distance, they are processed primarily by MOS. The primary source of MOS information to the MPOA is via afferents from MA, a structure that receives both direct and indirect input from the main olfactory bulbs (Scalia and Winans 1975). As with lesions of MPOA in the current study, lesions of MA eliminate preference for volatile, op-

posite-sex odors (Maras and Petrulis 2006), suggesting that interactions between MA and MPOA are critical for the appropriate investigation of volatile odors. It is also possible that both MA and BNST must interact with MPOA to regulate volatile odor investigation, as excitotoxic lesions of BNST also eliminate preference for volatile, opposite-sex odors (Been and Petrulis 2010a).

The present results confirm a previous report of MPOA lesions not disrupting male hamsters' investigation of directly-contacted female odors (Powers *et al.* 1987).This suggests that when non-volatile odors are present, and the AOS is additionally recruited (Keller *et al.* 2009), nuclei that receive direct AOS projections, such as MA and BNST (Scalia and Winans 1975), do not modulate direct odor investigation via MPOA. Instead, connections between MA and/or BNST and other nuclei, such as the ventromedial hypothalamus (VMH), ventral premammillary nucleus (PMV), or the nucleus accumbens (NAc) may mediate males' preference for directly-contacted opposite-sex odors. This idea is supported by the fact that lesions of either MA or BNST decrease male hamsters' investigation of female odors when contact is allowed (Lehman *et al.* 1980; Been and Petrulis 2010a). Furthermore, in male hamsters, VMH, PMV, and NAc express Fos protein following exposure to female odors that they can contact (Kollack-Walker and Newman 1995) and lesions of VMH disrupt female ferrets' preference for male odors that they can contact (Robarts and Baum 2007).

Role of MPOA in copulatory behavior

Lesions of MPOA caused significant impairments in copulatory behavior in sexually-naïve male hamsters, but only caused subtle deficits in sexually-experienced males. This finding differs notably from previous reports of large, electrolytic MPOA le-

sions causing severe copulatory deficits in sexually-experienced male hamsters (Powers *et al.* 1987; Floody 1989). Unlike previous studies, subjects in the present experiment were excluded from the MPOA-X lesion group if lesion damage extended into BNSTpc. This is an important distinction, as damage to this region may cause severe disruption of male copulatory behavior. In the current study, for example, both sexually-naïve and sexually-experienced males with lesion damage that extended into BNSTpc (and therefore excluded from the MPOA-X group) had severe deficits in copulatory behavior. Furthermore, only large, electrolytic lesions of BNST cause severe mating deficits in male hamsters, whereas smaller or excitotoxic lesions that do not damage BNSTpc cause more subtle copulatory deficits (Been and Petrulis 2010a). Similarly, lesions of the sexually-dimorphic nucleus of MPOA in male rats, which do not damage BNSTpc, only cause severe deficits in copulatory behavior in sexually-naïve males (De Jonge *et al.* 1989); copulatory deficits in sexually-experienced males with similar lesions are subtle and temporary (Arendash and Gorski 1983). Finally, disconnecting the sexually-dimorphic area of the MPOA from the caudal part of the medial BNST severely disrupts copulation in sexually-experienced male gerbils (Sayag *et al.* 1994). It is therefore possible that BNSTpc, and not MPOA itself, is critical for copulatory behavior in sexually-experienced males.

The current study also differs from previous reports in that the lesions made by Powers and colleagues (1987) were electrolytic and therefore were not limited to neurons, but also damaged fibers of passage. Therefore, it is possible that the copulatory deficits observed in the previous study were not due to damage to MPOA itself, but rather resulted from the disruption of social odor information from MA and/or BNST that

passes through MPOA to other hypothalamic nuclei critical for male reproductive behavior. In addition, whereas the subjects in the current study were gonadectomized and maintained on exogenous testosterone, the subjects in Powers *et. al*. were gonadally-intact. It is therefore possible that differences in post-lesion copulatory behavior were due not to lesion size or technique, but rather to the endocrine status of the subjects. This seems unlikely, however, as we demonstrated that gonadally-intact males are indistinguishable from castrated and hormone-replaced males in their copulatory behavior. Finally, MPOA is a heterogeneous structure and so it is possible that damage to additional structures within or near MPOA would have led to greater deficits in sexually-experienced males. For example, PVH, which did not sustain significant, bilateral damage in the current study, may be important for copulatory reflexes. Although radiofrequency lesions of PVH increase ejaculation latencies in sexually-experienced male rats (Liu et al. 1997a), it seems unlikely that PVH is critical for regulating copulation, as smaller, excitotoxic lesions of PVH do not impair copulation in sexually-experienced male rats (Liu *et al.* 1997a).

Role of Sexual Experience

Our results demonstrate that prior sexual experience can compensate for MPOA lesion-induced deficits in the appropriate investigation of volatile social odors and deficits in copulatory behavior, suggesting that sexual experience changes how female reproductive cues are processed centrally. Sexual experience may lead to a more distributed processing of female odor and/or other sensory cues such that MPOA becomes redundant and is no longer required for the appropriate behavioral response to female cues. This type of experience-dependent plasticity may result from associations be-

tween volatile odors and copulatory cues learned during sexual experience. In male rats, for example, exposure to estrous female odors increases immediate early gene expression in a circuit that includes MPOA, whereas exposure to an artificial odor that has previously been paired with a sexually-receptive female induces immediate early gene expression in a different neural pathway that includes the NAc (Kippin *et al.* 2003). As such, MPOA may be required for the appropriate behavioral response to a female or her odors in sexually-naïve males, but following sexual experience, the same stimuli may elicit a conditioned behavioral response that is mediated by NAc and associated circuitry (Pfaus et al. 2001). Of the cues available during copulation, non-volatile odor cues may be the most critical for the formation of associations with the attractive properties of volatile female odor cues. In fact, allowing male hamsters contact with volatile and non-volatile female odors (and not sexually experience *per se*) is sufficient to rescue the deficits in copulatory behavior following VNO removal (Westberry and Meredith 2003). Although male hamsters do not require sexual experience to show a preference for volatile, opposite-sex odors (Maras and Petrulis 2006; Ballard and Wood 2007), our finding that lesions of MPOA only eliminate preference in the non-contact condition suggests that attraction to a combination of non-volatile and volatile female odors is more robust than attraction to just volatile opposite-sex odors. If a learned association with non-volatile female odors (as would occur either during close investigation or copulation) is required to strengthen preference for opposite-sex volatile odors, then it is not surprising that volatile odor preference is more susceptible to lesion-induced deficits in sexually-naïve males than in sexually-experienced males, as these males have not had contact with a female or with non-volatile female odors.

Conclusions

Although previous lesion studies have examined the role of MPOA in reproductive behavior, this study is the first to comprehensively test the role of MPOA in both appetitive and consummatory reproductive behavior while directly addressing the effects of sexual experience and odor volatility. Together, these results demonstrate that MPOA mediates preference for volatile female odors and copulatory behavior only in sexually-naïve male Syrian hamsters. In contrast, we found no support for previous reports that lesions of MPOA cause severe copulatory deficits in sexually-experienced male hamsters and suggest that these deficits are more likely mediated by damage to BNSTpc. Although the lesions in the current study are specific to MPOA, it would be valuable to further delineate the role of subnuclei within MPOA. Indeed, Ball and Balthazart (Balthazart and Ball 2007) have suggested that the rostral MPOA may be more important for appetitive reproductive behavior whereas the caudal MPOA may be more critical for consummatory reproductive behaviors. Ultimately, a more detailed analysis of the functional microstructure of MPOA is needed to identify specific neural regulators of appetitive and consummatory reproductive behaviors.

ACKNOWLEDGEMENTS

We would like to thank Mary Karom for performing the testosterone radioimmunoassays for this study. We would also like to thank Shelease Johnson and Nina King for their assistance in collecting behavioral data. This work was supported by NIH grant MH072930 to A.P. and in part by the Center for Behavioral Neuroscience under the STC program of the NSF, under agreement IBN 9876754.

CHAPTER 3 FIGURES

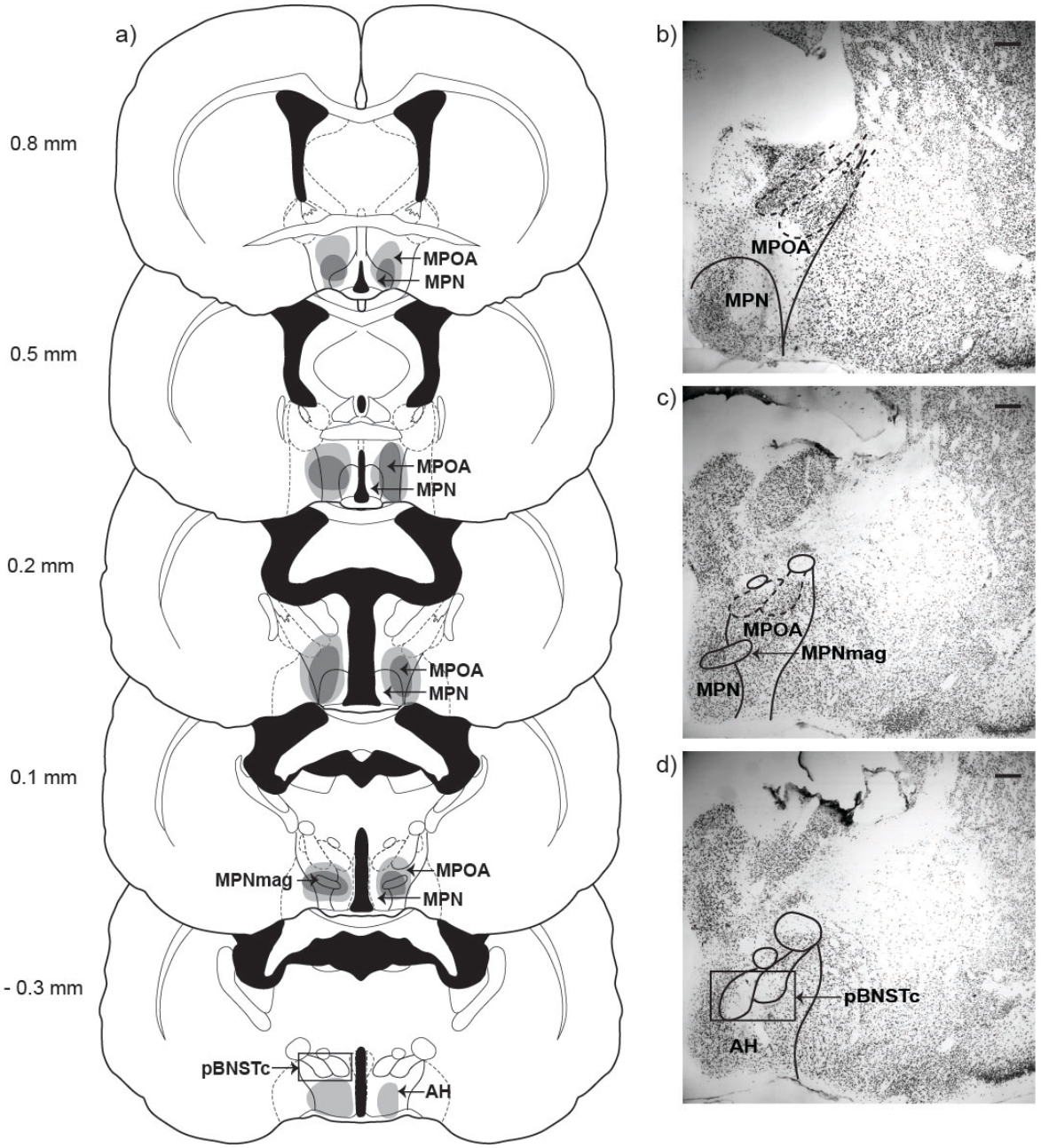

Figure 3.1: Lesion Reconstruction**.** a) Coronal sections through rostral to caudal extent of MPOA showing largest (light gray) and smallest (dark gray) lesions included in MPOA-X group. Immunohistochemical localization of neuronal nuclei (NeuN) protein was used to visualize cell loss in males with b) excitotoxic lesions of MPOA; some males also sustained damage to c) the most caudal portions of the posterior bed nucleus of the stria terminalis (pBNSTc) and were excluded from the MPOA-X lesion group. Measurements in mm relative to bregma, scale bars = 200 μm

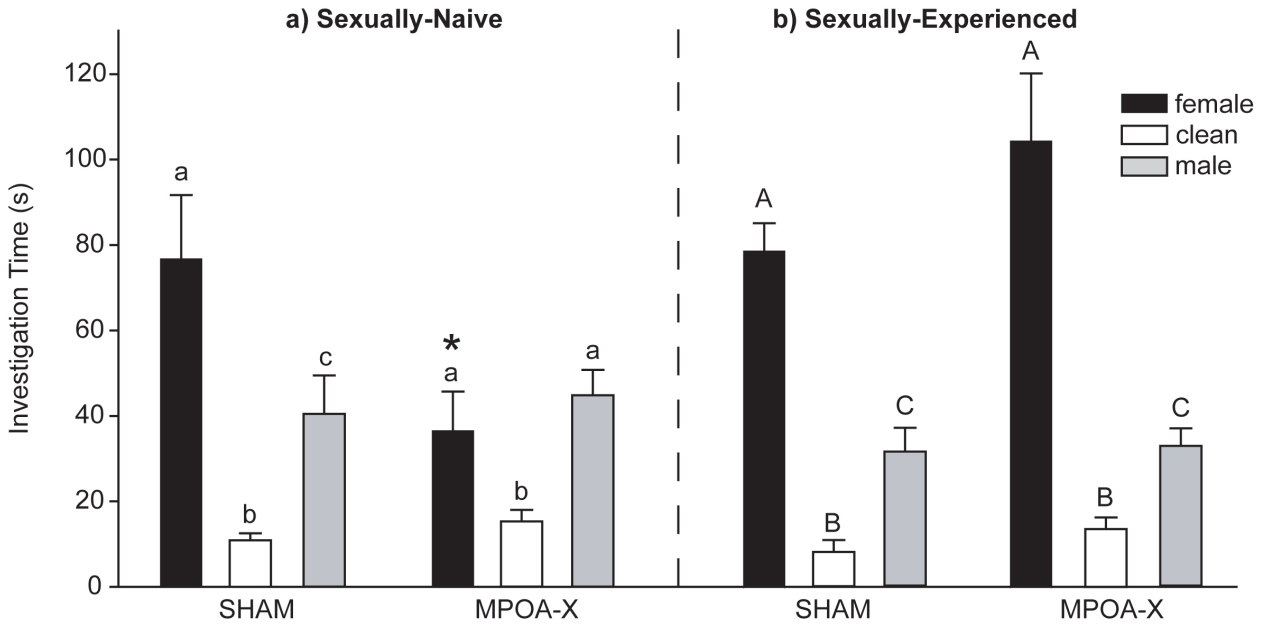

Figure 3.2: Investigation Times for Non-Contact Preference Test. a) In sexually-naïve males, lesions of MPOA eliminated preference for opposite-sex odors, whereas b) preference for opposite-sex odors remained intact in sexually-experienced males. Dissimilar letters indicate significant differences in investigation duration within lesion group, $P < 0.05$. * indicates significant differences in investigation duration between lesion groups, $P < 0.05$. Data expressed as means ± standard error of means.

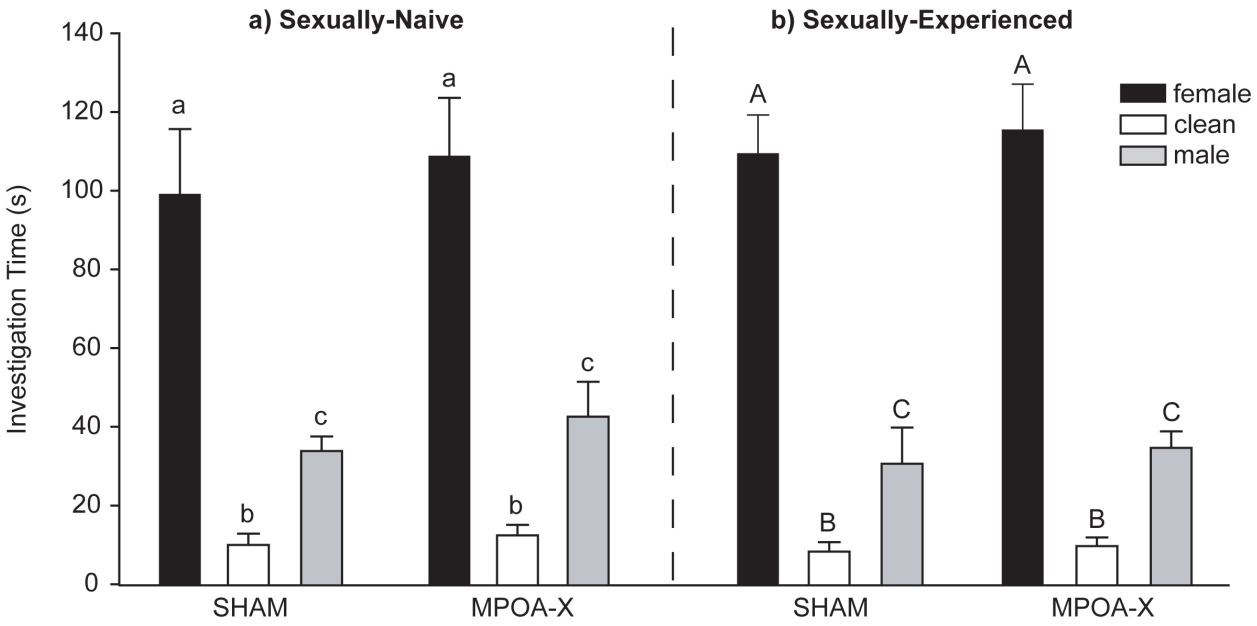

Figure 3.3: Investigation Times for Contact Preference Test. In a) sexually-naïve or b) sexually-experienced males, lesions of MPOA did not affect preference for opposite sex odors. Dissimilar letters indicate significant differences in investigation duration within lesion group, $P < 0.05$. Data expressed as means ± standard error of means.

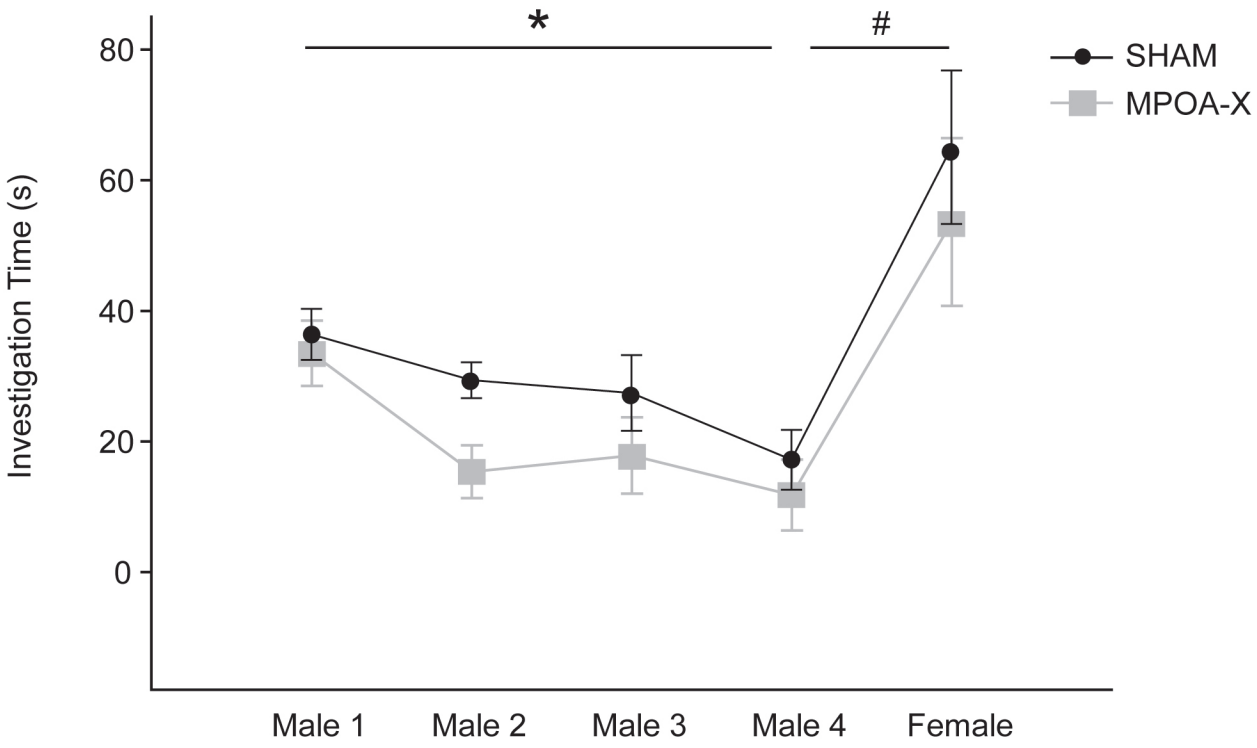

Figure 3.4: Investigation Times for Odor Discrimination Test. Sexually-naïve SHAM and MPOA-X males both habituated their investigation to repeated presentations of male odors and increased their investigation to a subsequently presented female odor. * indicates a significant decrease between Male 1 and Male 4, $P \leq 0.05$, # indicates a significant increase between Male 4 and Female, $P < 0.05$. Data expressed as means ± standard error of means.

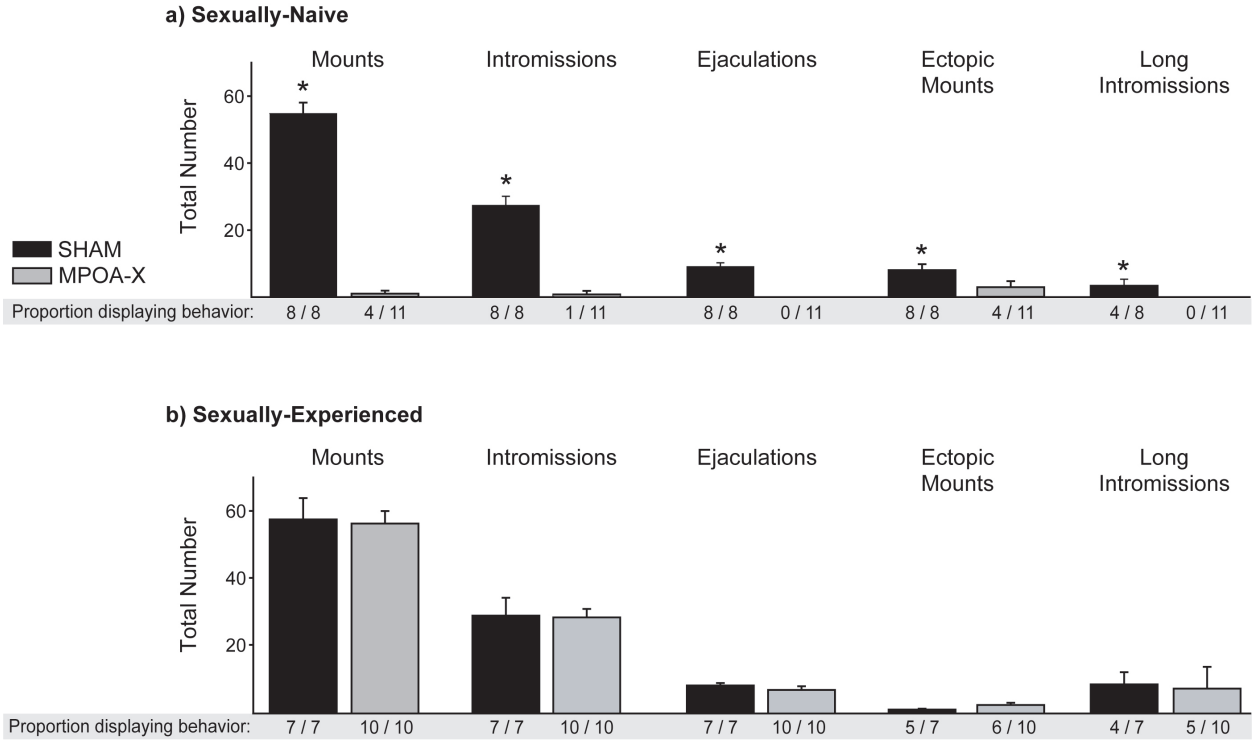

Figure 3.5: Total number of Mating Events in NVE and EXP males. In sexually-naïve males, the proportion of MPOA-X males displaying mounts, intromissions, ejaculations, ectopic mounts, and long intromissions was significantly less than in SHAM males. b) In EXP males, the proportion of subjects displaying any mating event did not differ between SHAM and MPOA-X males. * indicates significant differences in proportions, $P < 0.05$. Data expressed as means ± standard error of means.

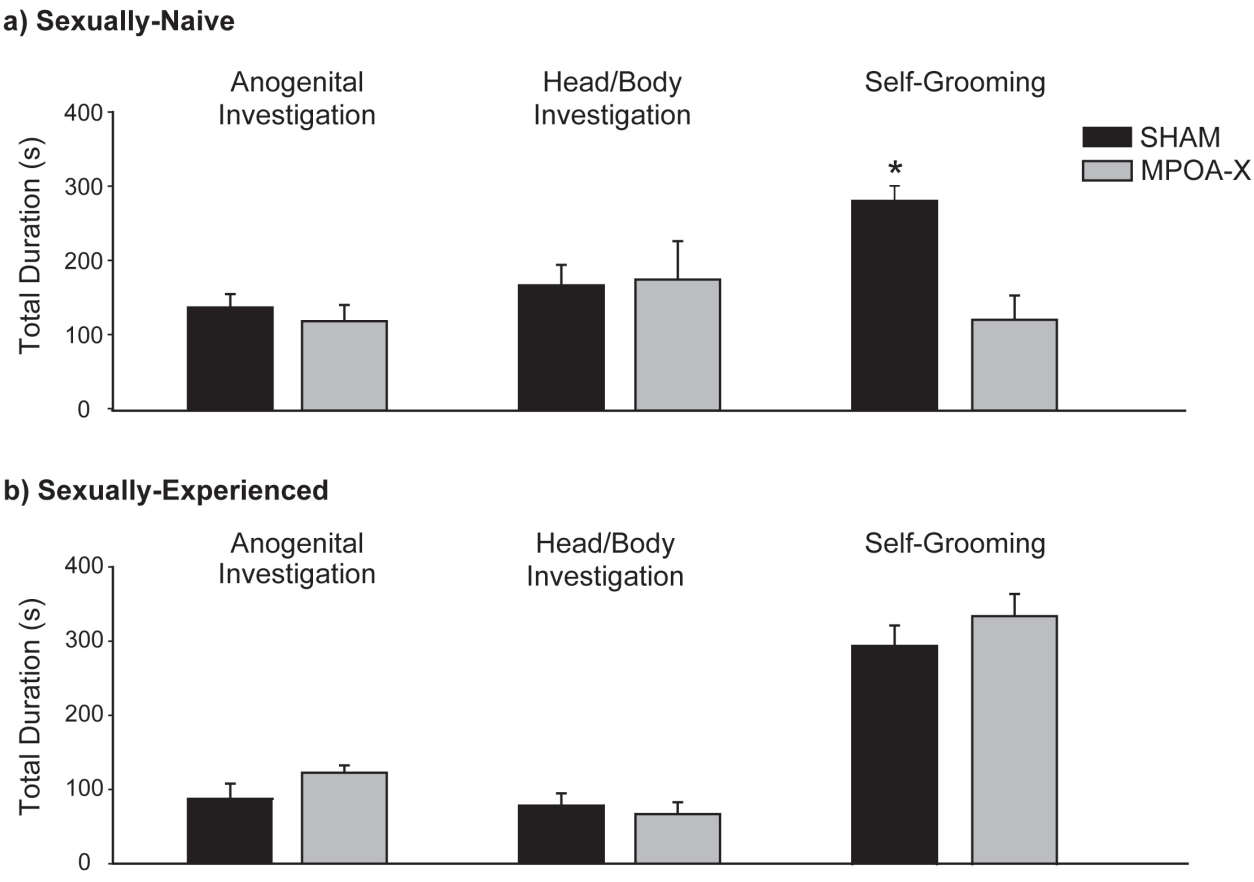

Figure 3.6: Total Durations of Mating Events in NVE and EXP males. a) In sexually-naïve males, the total duration of anogenital and head/body investigation did not differ between SHAM and MPOA-X males, but MPOA-X males spent significantly less time self-grooming than did SHAM males. b) In sexually-experienced males, the total duration of anogenital investigation, head/body investigation, and self-grooming did not differ between SHAM and MPOA-X males. * indicates significant differences between lesion groups, $P < .05$. Data expressed as means ± standard error of means.

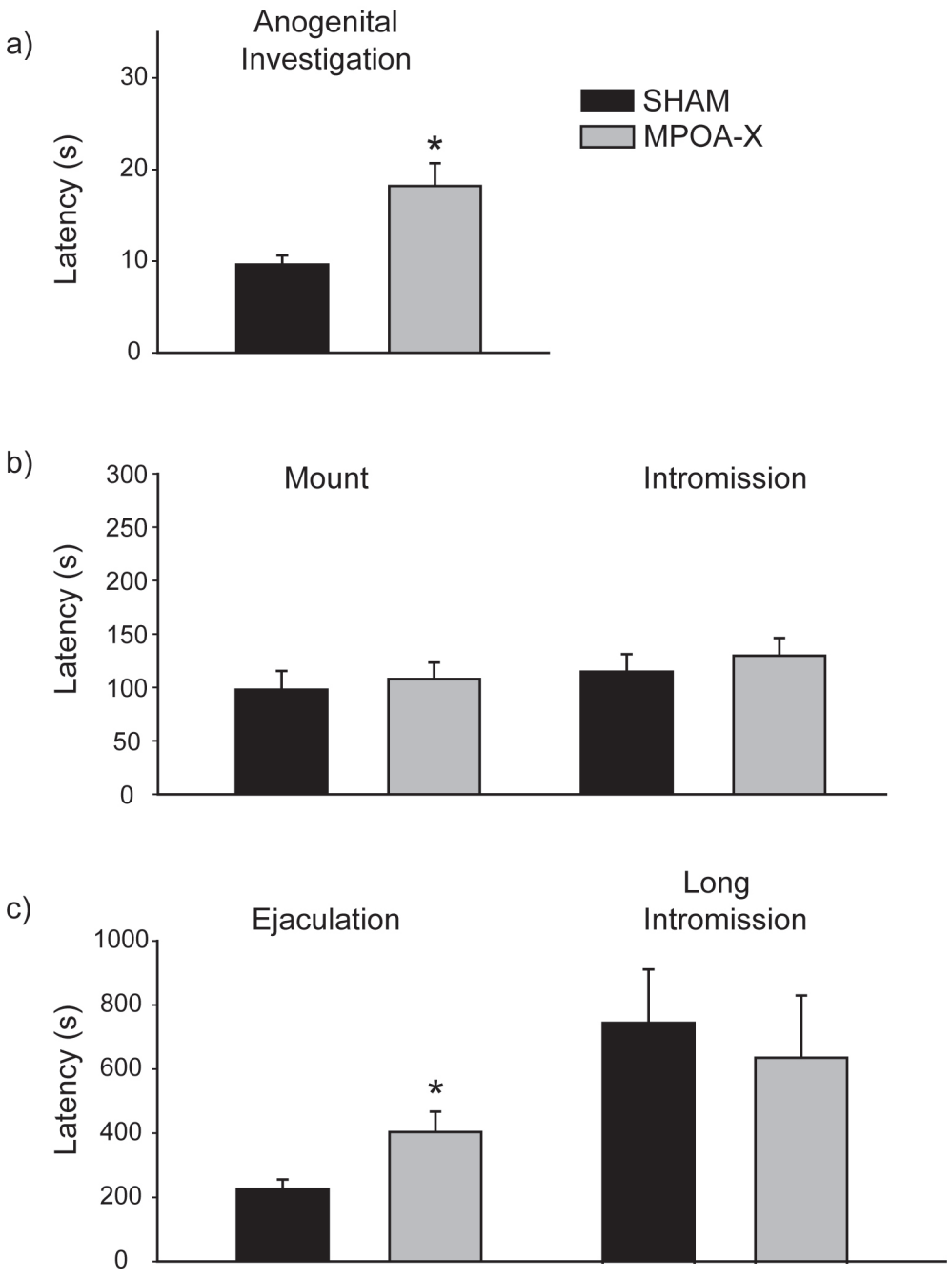

Figure 3.7: Latencies to Mating Events in EXP males. Lesions of MPOA increased the latency to a) begin anogenital investigation and c) ejaculate in sexually-experienced males, although the latencies to display mounts, intromissions, and long intromissions did not differ between SHAM and MPOA-X males, * indicates $P < 0.05$. Data expressed as means ± standard error of means.

CHAPTER 3 TABLES

Table 3.1: Summary of Odor Preference measures from males excluded from MPOA-X lesion group. Unilateral or bilateral, partial lesions of MPOA do not disrupt sexually-naïve (NVE) or sexually-experienced (EXP) males' preference to investigate female odors more than male or clean odors in a) Non-Contact or b) Contact Preference tests. In contrast, lesions that extend into pBNSTc eliminate preference for opposite-sex odors in the Non-Contact test only. Data expressed as mean ± standard error of means.

	a) Non-Contact			**b) Contact**		
	Female	Male	Clean	Female	Male	Clean
Unilateral NVE (*n* = 3)	77.76 ± 13.70	36.89 ± 12.40	10.11 ± 4.22	61.80 ± 12.19	25.88 ± 3.6	7.7 ± 2.83
Partial NVE (*n* = 4)	99.63 ± 10.09	49.86 ± 5.95	11.67 ± 2.12	110.80 ± 6.56	59.66 ± 9.33	20.54 ± 1.13
pBNSTc NVE (*n* = 1)	67.62	64.75	22.12	126.48	42.51	20.20
pBNSTc EXP (*n* = 4)	43.66 ± 5.64	58.60 ± 32.95	10.79 ± 2.21	91.98 ± 5.43	33.54 ± 9.13	13.12 ± 5.46

Table 3.2: Derived measures of mating events for EXP males. In sexually-experienced subjects, MPOA-X males did not differ from SHAM males in any derived measure of copulation. Data expressed as mean ± standard error of means.

	SHAM	**MPOA-X**
Derived Measures		
PEI	41.43 ± 4.87	39.12 ± 5.66
I-E	9.33 ± 1.68	8.88 ± 1.20
ME	0.32 ± 0.02	0.33 ± 0.01

Table 3.3: Total number of mating events in males excluded from MPOA-X lesion group. Unilateral or bilateral, partial lesions of MPOA do not disrupt a) sexually-naïve (NVE) or b) sexually-experienced (EXP) males' total number of mounts, intromissions, ejaculations, or long intromissions. In contrast, lesions extending into pBNSTc cause copulatory deficits in a) NVE and b) EXP males. Data expressed as mean ± standard error of means.

	Mating Events			
	M	**I**	**E**	**LI**
a) NVE				
Unilateral (*n* = 3)	44.33 ± 7.96	18.00 ± 1.52	6.66 ± 0.88	6.33 ± 1.85
Partial (*n* = 4)	54. 25 ± 10.06	31.75 ± 6.70	6.75 ± 0.85	5.50 ± 0.35
pBNSTc (*n* = 1)	0	0	0	0
b) EXP				
pBNSTc (*n* = 3)	1.66 ± 1.66	0.33 ± 0.33	0	0

CHAPTER 4:

Chemosensory and hormone information are relayed directly between the medial amygdala, posterior bed nucleus of the stria terminalis, and medial preoptic area in male Syrian hamsters

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ABSTRACT

In many rodent species, including Syrian hamsters, the expression of appropriate social behavior depends critically on the perception and identification of conspecific odors. The behavioral response to these odors is mediated by a network of steroid-sensitive ventral forebrain nuclei including the medial amygdala (MA), posterior bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA). Although it is well-known that MA, BNST, and MPOA are densely interconnected and each uniquely modulates odor-guided social behaviors, the degree to which conspecific odor information and steroid hormone cues are directly relayed between these nuclei is unknown. To answer this question, we injected the retrograde tracer, cholera toxin B (CTB), into the BNST or MPOA of male subjects and identified whether retrogradely-labeled cells in MA and BNST 1) expressed immediate early genes (IEGs) following exposure to male and/or female odors or 2) expressed androgen receptor (AR). Although few retrogradely-labeled cells co-localized with IEGs, a higher percentage of BNST- and MPOA-projecting cells in the posterior MA (MeP) expressed IEGs in response to female odors than to male odors. The percentage of retrogradely-labeled cells that expressed IEGs did not, however, differ between and female- and male- odor-exposed groups in the anterior MA (MeA), posterointermediate BNST (BNSTpi), or posteromedial BNST (BNSTpm). Many retrogradely-labeled cells co-localized with AR, and a higher percentage of retrogradely-labeled MeP and BNSTpm cells expressed AR than retrogradely-labeled MeA and BNSTpi cells, respectively. Together, these data demonstrate that MA, BNST, and MPOA interact as a functional circuit to process sex-specific odor cues and hormone information in male Syrian hamsters.

INTRODUCTION

In many rodent species, including Syrian hamsters (*Mesocricetus auratus*), appropriate behavior towards conspecifics depends critically on the perception of odor cues from the environment (Johnston 1990; Baum and Kelliher 2009). Indeed, the detection and correct identification of sex-specific chemosignals is required for both appetitive and consummatory aspects of reproductive behavior in Syrian hamsters, including males' attraction to female vaginal secretion (Powers *et al.* 1979) and copulation (Murphy and Schneider 1970; Powers and Winans 1975). These chemosignals are processed by a network of ventral forebrain nuclei including the medial amygdala (MA), posterior bed nucleus of the stria terminalis (BNST), and medial preoptic area (MPOA) (Wood 1997). Specifically, MA and BNST both receive chemosensory input (Scalia and Winans 1975) and are densely interconnected to each other and to MPOA (Scalia and Winans 1975; Gomez and Newman 1992; Coolen and Wood 1998; Wood and Swann 2005). As such, main and accessory olfactory information that converges on MA can terminate at BNST or continue directly to MPOA (Gomez and Newman 1992; Coolen and Wood 1998), whereas accessory olfactory information received by BNST can be relayed to MPOA (Gomez and Newman 1992; Wood and Swann 2005).

In addition to these anatomical relationships, functional data suggest that MA, BNST, and MPOA all process the chemosignals required for the appropriate behavioral response to conspecifics. Exposure to female odors or copulation increases the expression of immediate early genes (IEGs) in the MA, BNST, and MPOA of male hamsters (Fiber et al. 1993; Fernandez-Fewell and Meredith 1994; Kollack-Walker and Newman 1997) and other species (Robertson et al. 1991; Baum and Everitt 1992; Heeb and Yahr

1996; Wang et al. 1997), suggesting that neurons in these nuclei are activated by sex-specific odor stimuli. Furthermore, lesions of MA (Maras and Petrulis 2006) and MPOA (Been and Petrulis 2010b) eliminate preference for volatile opposite-sex odors in male hamsters and severely impair copulatory behavior in hamsters (Lehman *et al.* 1980; Been and Petrulis 2010b) and other species (De Jonge *et al.* 1989; Kondo 1992; Heeb and Yahr 2000). Similarly, lesions of BNST eliminate volatile opposite-sex odor preference in male hamsters (Been and Petrulis 2010a), but cause more subtle, temporal deficits in copulatory behavior in male hamsters and other species (Claro *et al.* 1995; Liu *et al.* 1997b; Been and Petrulis 2010a). Together, these data suggest MA, BNST, and MPOA each play a unique and critical role in regulating chemosensory-dependent behaviors.

Conspecific chemosignals must also be integrated with internal indicators of an animal's own endocrine status in order to generate the appropriate behavioral response. Several lines of evidence suggest that this integration of chemosensory and hormone cues takes place within MA, BNST, and MPOA. Each of these three nuclei is sensitive to steroid hormones (Wood *et al.* 1992; Wood and Newman 1995a) and site-specific implants of testosterone into either MA or BNST/MPOA restore reproductive behavior in castrated male hamsters (Wood and Newman 1995c). Furthermore, removal of the olfactory bulb ipsilateral to the testosterone implant prevents this restoration of copulatory behavior, suggesting that chemosensory and hormonal cues are integrated within MA (Wood and Coolen 1997) and BNST/MPOA (Wood and Newman 1995b), and that this integration is required for the expression of reproductive behavior.

Despite anatomical and functional data suggesting that MA, BNST, and MPOA are individually required for chemosensory and hormonal modulation of behavior, how these three nuclei interact as a functional circuit to process these cues remains largely unknown. We hypothesized that sex-specific odor information and hormone information are directly relayed between MA, BNST, and MPOA. To test this hypothesis, we injected a retrograde tracer into either MPOA or BNST of male hamsters and subsequently identified whether retrogradely-labeled cells within MA and/or BNST (1) were activated following exposure to sex-specific odors, using IEG expression as an indirect marker of neuronal activation (Pfaus and Heeb 1997), or (2) expressed androgen receptor (AR). These anatomical data add to the growing body of literature that suggests that MA, BNST, and MPOA interact as a functional circuit to process both sex-specific odor cues and hormone information in male Syrian hamsters.

MATERIALS AND METHODS

Animals

Adult male Syrian hamsters *(Mesocricetus auratus)* were purchased from Harlan Laboratories (Prattville, AL, USA) at eight weeks of age. Upon arrival, subjects were singly-housed and remained so for the duration of the study. All subjects (n = 64) were gonadally-intact and sexually-naïve. A separate group of group-housed (3-4 same-sex animals per cage), gonadally-intact, adult male and female hamsters (n = 80) were used to provide odor stimuli. Subjects were unrelated to, and had no previous contact with, these odor donor animals.

Subjects and odor donors were housed in solid-bottom, polycarbonate cages (325 mm x 470 mm x 205 mm) with corncob litter (The Andersons, Maumee, OH, USA) and cotton bedding material (Ancare, Bellmore, NY, USA). Cages were self-contained in an exhaust-vented carousel caging system (OptiRat System, Animal Care Systems, Centennial, CO, USA). All animals were maintained on a reversed 16-h light/8-h dark photoperiod (lights off/on at 10 AM/6 PM). Food and water were available *ad libitum*. All experiments 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 were 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.

Retrograde Tracer Injections

To visualize the cell bodies that project to MPOA and BNST, the retrograde tracer cholera toxin B (CTB) was deposited unilaterally into either MPOA (*n* = 24) or BNST (*n* = 20). CTB (List Biological Laboratories, Campbell, CA, USA) was prepared as a 0.5% solution in 0.1 M phosphate-buffered saline (PBS; pH 7.4) according to the manufacturer's instructions. Subjects were anesthetized with 2% isoflurane (Butler Schein Animal Health, Dublin, OH, USA) vaporized in a 70:30% oxygen/nitrous oxide mixture and were secured in the stereotaxic apparatus such that their skull was level in the anterior-posterior (A-P) and medial-lateral (M-L) planes. Following a midline scalp incision, the skin and temporal muscles were retracted to expose the skull and a hand-operated drill was used expose dura. All A-P and M-L measurements were taken in mm relative to bregma and all dorsal-ventral (D-V) measurements were taken in mm relative to dura.

30 nl of CTB was deposited over a 1 minute period by lowering a microinjection syringe (701R 10 µl syringe, Hamilton, Reno, NV, USA) under stereotaxic control (Microinjection Unit, Model 5002, David Kopf Instruments, Tujunga, CA, USA) into either MPOA (A-P: + 2.0 mm; M-L: ± 0.6 mm; D-V: -7.1 mm) or BNST (A-P: + 1.85 mm; M-L: ± 1.35 mm; D-V: -5.9 mm). Injections were alternated between the right and left hemispheres between subjects. To minimize the flow of CTB up the needle tract, the syringe was left in place for 15 minutes after each injection. After injections, skull holes were sealed using bone wax and incisions were closed with wound clips. To minimize post-operative pain, ketoprofen (5 mg/kg subcutaneously, Henry Schein, Melville, NY, USA) was administered intra-operatively.

IEG Induction

All males were sacrificed seven days following tracer injection to allow for sufficient transport of CTB (Vercelli et al. 2000). During this time, males were handled extensively and habituated to the testing room. On the day of sacrifice, males were brought into the testing room and allowed to sit undisturbed for at least 1 hour prior to stimulus exposure. To determine if BNST- and/or MPOA-projecting neurons respond to same-sex and/or opposite-sex odors, a sub-set of injected males was exposed to either female (BNST n = 8; MPOA n = 10) or male (BNST n = 7; MPOA n = 8) odors. Sex-specific odor exposure was accomplished by placing subjects into a vacated odor-donor cage that had housed 3-4 same-sex animals and had not been changed for 4 days prior to use. In this way, subjects were exposed to a heterogeneous odor stimulus that contained volatile and non-volatile chemosignals (including those from the soiled litter, bedding, and cage walls) and reflected the composite sexual identity of the odor, rather

than the individual identity of a single animal. Furthermore, as these cages had not been changed for four days, female stimulus cages included odors from across the entire estrous cycle, including behavioral estrus. To provide a measure of baseline activation, a third group of injected males was transferred to a clean cage (BNST *n* = 5; MPOA *n* = 6). Males were left undisturbed in the stimulus cage for 70 minutes, at which time they were injected with an overdose of sodium pentobarbital (100 mg/kg; Sleep Away, Fort Dodge, IA, USA) and allowed to reach a deep level of anesthesia (additional 10 minutes) prior to perfusion. This survival time is within the time range for peak IEG induction (60-90 min; (Herdegen and Leah 1998) and has been previously used for odor stimulus-induced expression of Fos (Fiber and Swann 1996; Delville et al. 2000; Swann et al. 2001) and EGR-1 (Lai et al. 2004; Lai et al. 2005) in Syrian hamsters.

Histology and Immunohistochemistry

Males were transcardially perfused with 200 ml of 0.1 M PBS (pH 7.4), followed by 200 ml of 4% paraformaldehyde in phosphate buffer (pH 7.2). Brains were immediately removed and post-fixed in 4% paraformaldehyde overnight (4° C) and then cryoprotected for 48 hours in 30% sucrose in PBS. In order to distinguish between left and right brain hemispheres, an angle was cut into the right dorsal cortex of each brain. Coronal sections (30-µm) of brain tissue were sectioned on a cryostat (-20°C), collected in a 1:6 series, and stored in cryoprotectant until immunohistochemical processing.

To determine whether BNST and/or MPOA afferents respond to male and/or female odor stimuli, one series of tissue was processed for immunohistochemical localization of Fos and CTB. To determine whether BNST and/or MPOA afferents are androgen-sensitive, a separate series of tissue was stained for AR and CTB. Finally, because

we observed low levels of co-localization of Fos and CTB (see Results), a subset of female odor-exposed subjects' tissue (MPOA-injected *n* = 3; BNST-injected *n* = 3) was also processed for immunohistochemical localization of a different IEG, EGR-1, and CTB, in order to confirm that Fos is an accurate marker of neuronal activation in MPOA- and BNST-projecting cells.

Procedures for double-labeling Fos/CTB, AR/CTB, and EGR-1/CTB were identical except for the specific primary antibodies used. Sections were removed from cryoprotectant and rinsed thoroughly in PBS. To reduce endogenous peroxidase activity, tissue sections were incubated in 0.5% hydrogen peroxide in PBS for 15 minutes. After several rinses, sections were then incubated in the appropriate primary antibody to Fos, AR, or EGR-1 in 0.4% Triton-X 100 for 24 hours at room temperature. The primary antibody for Fos was a rabbit polyclonal antibody (1: 20,000, sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) generated against a peptide mapping the N-terminus of the human c-Fos. The primary antibody for AR was a rabbit polyclonal antibody (1: 3,000, sc-816, Santa Cruz Biotechnology) generated against a peptide mapping the N-terminus of the human AR. The primary antibody for EGR-1 was a rabbit polyclonal antibody (1: 20,000, sc-110, Santa Cruz Biotechnology) generated against a peptide mapping the C-terminus of the human EGR-1. The specificity of these primary antibodies has been previously confirmed (Kollack-Walker and Newman 1997; Creutz and Kritzer 2004; Lai *et al.* 2004)

After incubation in primary antibody, sections were rinsed in PBS and then incubated for 1 hour in anti-rabbit biotinylated secondary antibody (1:600 Jackson ImmunoResearch, West Grove, PA, USA) in PBS with 0.4% Triton-X 100. Sections were

rinsed again in PBS and then incubated for 1 hour in avidin-biotin complex (4.5 μl each of A and B reagents/ml PBS with 0.4% Triton-X 100, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). After rinsing in PBS and then in 0.175 M sodium acetate, sections were incubated in 3,3'-diaminobenzidine HCl (0.2 mg/ml, Sigma) and hydrogen peroxide (0.83 μl/ml, Sigma) in a nickel-sulfate solution (25 mg/ml, Sigma) for 20 minutes, yielding a blue-black product. The reaction was stopped by rinsing sections in sodium acetate. Immediately following visualization of Fos, AR, and/or EGR-1, tissue sections were incubated in the primary antibody to CTB in PBS with 0.4% Triton-X 100 for 24 hours at room temperature. The primary antibody to CTB was a goat polyclonal antibody (1: 80,000, List Biological Laboratories, Campbell, CA, USA) generated against the CTB subunit. Procedures for labeling CTB were identical to those described above except that the biotinylated secondary antibody anti-goat (Vector Laboratories, Burlingame, CA) and CTB was visualized using 3,3'-diaminobenzidine HCl (0.2 mg/ml, Sigma) and hydrogen peroxide (0.83 μl/ml, Sigma) in Tris Buffer (pH 7.2), yielding a brown reaction product. Stained tissue sections were mounted onto subbed glass slides and allowed to air-dry overnight. Slides were then dehydrated in alcohols, cleared in xylenes, and coverslipped using Permount (Fisher Scientific).

Data Analysis

A single researcher that was blind to the stimulus condition of the animal conducted all analyses. Tissue was first examined for the placement and spread of CTB injections. Only those subjects with CTB deposition restricted to the target region were analyzed. As projections between MA, BNST, and MPOA are primarily unilateral (Coolen and Wood 1998; Wood and Swann 2005), Fos/CTB, AR/CTB, and EGR-1/CTB

was counted in the hemisphere ipsilateral to the injection. Furthermore, as sexual odor information flows primarily from MA and/or BNST to MPOA (Wood 1997), cells were counted in both MA and BNST in males with MPOA injections, whereas in males with BNST injections, cells were only counted in MA. Sections were examined using a Nikon Eclipse E800 Microscope with a QImaging digital camera attached. Counting domains were generated by projecting the microscopic field (10x) onto a computer screen using iVision software (Atlanta, GA, USA). For data presentation, photomicrographs were taken in iVision and processed using Adobe Photoshop CS2 Version 9.0 (San Jose, CA, USA) only to enhance tone, brightness, and contrast.

Within MA, two sections of the anterior medial amygdala (MeA, Figure 4.1A and 1B) and two sections of the posterior medial amygdala (MeP, Figure 4.1C and 4.1D) were counted. Within BNST, two sections of the posterior intermediate BNST (BNSTpi) and posterointermediate BNST (BNSTpm) were counted (Figure 4.11E and 1.1F). Fos-positive (Fos+), AR-positive (AR+), and EGR-1-positive (EGR-1+) cells were identified as having dark, blue-black nuclear staining, whereas CTB-positive (CTB+) cells were identified as having brown, cytoplasmic staining that filled the shape of the cell. Fos/CTB-positive (Fos/CTB+), AR/CTB-positive (AR/CTB+), and EGR-1/CTB-positive (EGR-1/CTB+) cells were identified as having a dark, blue-black nucleus surrounded by brown cytoplasmic staining. The total numbers of Fos+, AR+, EGR-1+, Fos/CTB+, AR/CTB+, and EGR-1/CTB+ cells (two sections per domain) were calculated separately and were divided by the total area analyzed for each region (MeA and MeP = 1.298 mm^2, BNSTpi and BNSTpm = 0.649 mm^2) to reflect the density of immunoreactive cells in cells/mm^2. The sum of the densities of double-labeled cells (Fos/CTB+ or AR/CTB+)

was then divided by the sum of the densities of single-labeled cells (CTB+, Fos+, or AR+) in order to generate the percentages of single-labeled cells that were double-labeled for each brain region.

SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for all data analyses and significance was determined as $P < 0.05$. Unless otherwise noted, all data are reported as mean ± SEM. To identify differences in the densities of single- or double-labeled cells across stimulus conditions (male, female, clean), separate one-way ANOVAs were used for each brain area (MeA, MeP, BNSTpi, BNSTpm) followed by Tukey's-B post hoc tests. To compare the densities of single- and double-labeled cells between brain areas (MeA vs. MeP and BNSTpi vs. BNSTpm), data were combined across odor stimulus conditions and compared using separate independent *t*-tests. Finally, *z*-tests for independent proportions with Bonferroni corrections for multiple comparisons were used to identify differences in the percentages of double-labeled cells between odor conditions (Fos co-localization experiment) and brain areas (AR co-localization experiment).

RESULTS

CTB deposition and retrograde labeling

Males were included in the MPOA or BNST injection groups only if dark CTB staining was observed around the deposition site within at least two atlas plates of section for MPOA (Figure 4.2A) and BNST (Figure 4.2B), respectively (Morin and Wood 2001). Males were excluded from analysis if the injection was misplaced (MPOA $n = 4$; BNST $n = 5$). Within the MPOA-injected group, misplaced injections resulted in CTB

deposition rostral to MPOA, into BNST (*n* = 2), or caudal to MPOA, into the anterior hypothalamus (*n* = 2). Within the BNST-injected group, misplaced injections resulted in CTB deposition dorsal to BNST, into the lateral ventricles (*n* = 4) or the lateral septum (*n* = 1). These inclusion and exclusion criteria resulted in a final total of 16 MPOA-injected males (Female odor condition *n* = 6; Male odor condition *n* = 6; Clean odor condition *n* = 4) and 15 BNST-injected males (Female odor condition *n* = 6; Male odor condition *n* = 5; Clean odor condition *n* = 4).

The largest and smallest extent of CTB deposition in MPOA and BNST are shown in Figure 4.2C and 4.2D, respectively. In all subjects included in the MPOA-injected group, CTB was deposited into the mid-rostral MPOA, including the medial preoptic nucleus (MPN), below the most caudal extent of the anterior commissure (Bregma 0.5 mm). In most subjects, CTB was also deposited into MPOA at the level where the lateral and third ventricles fuse (*n* = 14; Bregma 0.2 mm) as well as the most caudal level of MPOA, including MPN and the magnocellular medial preoptic nucleus (*n* = 12; Bregma -0.1 mm). In fewer subjects, CTB deposition spread into the more rostral aspects of MPOA, including MPN, (*n* = 8; Bregma 0.8 mm). In all subjects in the BNST-injected group, CTB was deposited into the mid-rostral BNST (Bregma 0.2 mm), including BNSTpi and BNSTpm, as well as the posterolateral BNST. In most subjects (*n* = 13), CTB was also deposited into more rostral and anterior (Bregma 0.5 mm) parts of BNSTpi and BNSTpm and into the more caudal and ventral level of BNSTpi and BNSTpm (*n* = 12; Bregma – 0.1 to -0.3 mm). There was no difference in the overall pattern of retrograde labeling between males with the smallest and largest injections, although larger injections were associated with higher densities of retrograde labeling.

Furthermore, within each injection group, the placement and spread of CTB deposition were comparable across the different odor stimulus conditions.

In the current experiments, we only quantified retrograde labeling in MA (MPOA- and BNST-injected) and BNST (MPOA-injected only). However, CTB+ cells were also observed within several previously identified afferents of MPOA, including the anterior cortical amygdala, amygdalohippocampal area, lateral septum, and ventromedial hypothalamus (Simerly and Swanson 1986; Wang and Swann 2006), and within several previously identified afferents of BNST, including the basolateral amygdala, posterior medial cortical amygdala, lateral septum, and ventromedial hypothalamus (Wood and Swann 2005).

Co-localization of CTB and Fos

MPOA-projecting cells. Overall, we observed low levels of co-localization of CTB and Fos in MA (Figure 4.3A) following injection of CTB into MPOA. The densities of CTB+, Fos+, and Fos/CTB+ cells for MPOA-projecting cells are shown in Table 4.1. MeP had significantly more retrogradely-labeled cells than MeA ($t(10)= 2.78$, $P = 0.02$), whereas the density of retrogradely-labeled cells did not differ between BNSTpm and BNSTpi ($t(10)= 1.94$, $P = 0.08$). In MeA and MeP, the density of Fos+ cells did not differ between female odor-exposed and male odor-exposed subjects (MeA: $F(2,13) = 13.78$, $P = 0.001$; MeP: $F(2,13) = 15.45$ $P < 0.001$), although exposure to either female (MeA: $P = 0.002$, MeP: $P = 0.001$) or male (MeA: $P = 0.001$, MeP: $P = 0.001$) odors significantly increased the densities of Fos+ cells above clean odor-exposed (i.e. baseline) densities. Similarly, the density of Fos+ cells did not differ between female odor-exposed and male odor-exposed subjects in BNSTpi ($F(2,13) = 5.56$, $P = 0.02$) or BNSTpm ($F(2,13)$

= 5.78, P = 0.02). In both BNSTpi and BNSTpm, exposure to both female (BNSTpi: P = 0.014; BNSTpm: P = 0.03) and male (BNSTpi: P = 0.02; BNSTpm: P = 0.01) odors increased the density of Fos+ cells above baseline levels. The densities of Fos/CTB+ cells did not differ between subjects exposed to female, male, or clean odors in MeA (F(2,13) = 0.21, P = 0.81), MeP (F(2,13) = 1.08, P = 0.37), BNSTpi (F(2,13) = 1.08, P = 0.37), and BNSTpm (F(2,13) = 1.42, P = 0.28).

Figure 4.4A depicts the percentage of CTB+ cells that were also Fos+ in response to each odor exposure condition for MPOA-projecting cells. In MeA, the percentage of CTB+ cells that were double-labeled with Fos following exposure to female odors (18.45%) and male odors (14.56%) did not differ from each other (z = 1.62, P = 0.05), but both were significantly higher than the percentage of CTB+ cells that were also Fos+ following exposure to clean odors (8.67%; female z = 3.10, male z = 4.37, both P < 0.001). In contrast, the percentage of CTB+ cells in MeP that were also Fos+ following exposure to female odors (15.84%) was significantly higher than the percentage of CTB+ cells that also expressed Fos following exposure to male odors (11.58%; z = 2.36, P = 0.009). Furthermore, the percentage of CTB+ cells in MeP that were Fos+ following exposure to either female (z = 5.05, P < 0.001) or male (z = 3.17, P < 0.001) odors was significantly higher than the percentage of CTB+ cells that were Fos+ following clean odor exposure (7.10%).

As in MA, we observed low levels of co-localization of CTB and Fos in BNST (Figure 4.3B) following injection of CTB into MPOA. In BNSTpi and BNSTpm, the percentages of CTB+ cells that were Fos + following female (BNSTpi: 12.73%; BNSTpm: 11.09%) and male (BNSTpi: 11.69%; BNSTpm: 8.19%) odor exposure did not differ

from each other (BNSTpi: $z = 0.36$, $P = 0.36$; BNSTpm: $z = 1.44$, $P = 0.07$). The percentages of CTB+ cells that were Fos+ following exposure to male (BNSTpi: $z = 4.40$; BNSTpm: $z = 4.13$, both $P < 0.001$) and female (BNSTpi: $z = 4.09$; BNSTpm: $z = 3.15$, both $P < 0.001$) odors, however, were higher than the percentages of CTB+ cells that were also Fos+ following exposure to clean odors in BNSTpi (1.53%) and BNSTpm (1.85%) (Figure 4.4A).

Figure 4.4B depicts the percentage of Fos+ cells responding to each odor exposure condition that were also CTB+ in MPOA-injected males. In MeA, the percentage of Fos+ cells following exposure to female odors that were also CTB+ (4.76%) was significantly greater than the percentage of Fos+ cells following exposure to male odors that were also CTB+ (3.40%; $z = -2.15$, $P = 0.01$). The percentages of Fos+ cells following exposure to either female or male odors that were CTB+, however, were significantly lower than the percentage of Fos+ following exposure to clean odors that were CTB+ (10.38%; $z = -6.35$ and 4.57, both $P < 0.001$). Similarly, in MeP, the percentage of Fos+ cells following exposure to female odors that were CTB+ (7.49%) was significantly higher than the percentage of Fos+ cells following exposure to male odors that were CTB+ (5.52%; $z = -2.23$, $P = 0.01$) and the percentages of Fos+ cells following exposure to either female or male odors that were CTB + were significantly lower than the percentage of Fos+ following exposure to clean odors that were CTB+ (21.07%; $z = -8.70$ and -6.92, both $P < 0.001$).

In contrast, in BNSTpi and BNSTpm, there was no difference in the percentage of Fos+ cells that were CTB+ between female odor-exposed (BNSTpi: 8.21%; BNSTpm: 10.56%) and male odor-exposed (BNSTpi: 7.76%; BNSTpm: 10.69%) groups (BNSTpi:

z = -0.22, P = 0.41; BNSTpm: z = 0.05, P = 0.47), nor did the percentage of Fos+ cells that were CTB+ differ between the clean odor-exposed group (BNSTpi: 18.75%; BNSTpm: 18.18%) and either the female odor-exposed group (BNSTpi: z = -1.46, P = 0.07; BNSTpm: z = -1.09, P = 0.14) or the male odor-exposed group (BNSTpi: z = -1.58, P = 0.06; BNSTpm: z = -1.10, P = 0.13) (Figure 4.4B).

BNST-projecting cells. The densities of CTB+, Fos+, and Fos/CTB+ cells for BNST-projecting cells are shown in Table 4.2. There was no difference in the density of retrogradely-labeled cells between MeA and MeP (t(9) = 1.71, P = 0.21). In MeA, the densities of Fos+ cells were greater following exposure to either female odors (P = 0.001) or male odors (P = 0.003) compared to the density of Fos+ cells following exposure to clean odors (F(2,12) = 14.14, P = 0.001), although there was no difference between the female odor-exposed and male odor-exposed groups (P = 0.72). In MeP, however, the densities of Fos+ cells differed significantly between all three odor-exposure conditions (F(2,12) = 94.37, P < 0.001). Exposure to either female (P < 0.001) or male (P = 0.003) odors increased the density of Fos+ cells over exposure to clean odors, and the density of Fos+ cells was significantly greater following exposure to female odors than it was following exposure to male odors (P < 0.001). The densities of Fos/CTB+ did not differ following exposure to any of the three odor conditions in MeA (F(2,12)= 0.57, P = 0.58) or MeP (F(2,12) = 0.03, P = 0.97).

In MeA, the percentage of CTB+ cells that were double-labeled with Fos following exposure to female odors (15.70%) and male odors (16.69%) did not differ from each other (z = 0.46, P = 0.32), although exposure to both female (z = 3.61, P < 0.001) and male (z = 4.10, P < 0.001) odors increased the percentage of CTB+ cells that were

Fos+ over the percentage of CTB+ that were Fos+ following exposure to clean odors (9.34%) (Figure 4.5A). In contrast, the percentage of CTB+ cells that were Fos+ in MeP following exposure to female odors (15.95%) was significantly higher than the percentage of CTB+ cells that expressed Fos+ following exposure to male odors (12.33%; z = -2.14, P = 0.016). Furthermore, the percentages of CTB+ cells that were Fos+ in MeP following exposure to either female (z = 4.82, P < 0.001) or male (z = 2.70, P = 0.003) odors were significantly higher than the percentage of CTB+ cells that were Fos+ following clean odor exposure (8.59%) (Figure 4.5A).

In MeA, the percentage of Fos+ cells following exposure to female odors that were also CTB+ (7.50%) was significantly greater than the percentage of Fos+ cells following exposure to male odors that were CTB+ (5.3%; z = 2.38, P = 0.008), although the percentage of Fos+ cells following exposure to either female odors (z = -11.16, P < 0.001) or male odors (z = -8.57, P < 0.001) that were also CTB+ was significantly lower than the percentage of double-labeled cells following exposure to clean odors (24.28%) (Figure 4.5B). Similarly, in MeP, the percentage of Fos+ cells following exposure to female odors that were CTB+ (13.44%) was significantly higher than the percentage of Fos+ cells following exposure to male odors that were CTB+ (6.52%; z = 5.95, P < 0.001) and the percentages of Fos+ cells following exposure to either female odors (z = -10.93, P < 0.001) or male odors (z = -4.86, P < 0.001) that were CTB+ were significantly lower than the percentage of Fos+ following exposure to clean odors that were CTB+ (25%) (Figure 4.5B).

Co-localization of CTB and EGR-1

The overall pattern of labeling observed in tissue double-labeled for EGR-1 and CTB was very similar to that of tissue double-labeled for Fos and CTB, suggesting that the low levels of CTB/Fos co-localization were not specific to Fos, but rather, may be a veridical feature of the IEG response (Figure 4.3C). The densities of CTB+, EGR-1+ and EGR-1/CTB+ cells following exposure to female odors are shown in Table 4.3. In MPOA-projecting cells (Table 4.3A), the densities of EGR-1+ cells did not differ from the densities of Fos+ cells in MeA ($t(7) = 0.76$, $P = 0.47$), MeP ($t(7) = 0.53$, $P = 0.61$) , BNSTpi ($t(7) = 0.09$, $P = 0.93$), or BNSTpm ($t(7) = 0.67$, $P = 0.53$). Furthermore, the densities of EGR-1/CTB+ cells did not differ from the densities of Fos/EGR-1+ cells in MeA ($t(7) = 0.79$, $P = 0.45$), MeP ($t(7) = 1.74$, $P = 0.12$), BNSTpi ($t(7) = 0.87$, $P = 0.41$), or BNSTpm ($t(7) = 0.09$, $P = 0.92$). Similarly, in BNST-projecting cells (Table 4.3B), the densities of EGR-1+ and EGR-1/CTB+ did not differ from the densities of Fos+ or Fos/EGR-1+ cells in MeA (single-labeled: $t(7) = 0.09$, $P = 0.93$; double-labeled: $t(7) = 0.24$, $P = 0.82$) or MeP (single-labeled: $t(7) = 2.46$, $P = 0.04$; double-labeled: $t(7) = 0.06$, $P = 0.57$).

Co-localization of CTB and AR

MPOA-projecting cells. The densities of CTB+, AR+, and AR/CTB+ cells for MPOA-projecting cells are shown in Table 4.4A. There were no differences in the densities of cells single- or double-labeled with AR across odor exposure conditions so data was collapsed across odor conditions. MeP had significantly more retrogradely-labeled cells than MeA ($t(20)= 5.09$, $P < 0.001$) and BNSTpm had significantly more retrogradely-labeled cells than BNSTpi ($t(20)= 8.28$, $P < 0.001$). Similarly, MeP had more AR+

($t(20)= 2.35$, $P = 0.03$) and AR/CTB+ ($t(20)= 3.50$, $P = 0.002$) cells than MeA, and BNSTpm had more AR+ ($t(20)= 13.21$, $P < 0.001$) and AR/CTB+ ($t(20)= 9.32$, $P < 0.001$) cells than BNSTpi.

We observed high levels of co-localization of CTB and AR within both MA (Figure 4.6A) and BNST (Figure 4.6B). The percentage of CTB+ cells that were AR+ was significantly higher in MeP (90.63%) than in MeA (75%; $z = -11.67$, $P < 0.001$). Similarly, the percentage of CTB+ cells that were AR+ was significantly higher in BNSTpm (88.01%) than in BNSTpi (67.71%; $z = -8.32$, $P < 0.001$) (Figure 4.7A). The percentage of AR+ cells that were CTB+ was significantly higher in MeP (38.60%) than in MeA (29.71%; $z = -7.57$, $P < 0.001$) as well as significantly higher in BNSTpm (45.89%) than in BNSTpi (23.92%; $z = -3.03$, $P = 0.001$) (Figure 4.7B).

BNST-projecting cells. The densities of CTB+, AR+, and AR/CTB+ cells for BNST-projecting cells are shown in Table 4.4B. Again, there were no differences in the densities of cells single- or double-labeled with AR across odor exposure conditions so data was collapsed odor conditions. MeP had significantly more retrogradely labeled cells than MeA ($t(22) = 2.92$, $P = 0.007$). Furthermore, the densities of AR+ ($t(22) = 5.21$, $P < 0.001$) and AR/CTB+ ($t(22) = 5.21$, $P < 0.001$) cells were significantly higher in MeP than in MeA. The percentage of CTB+ cells that were AR + was significantly higher in MeP (87.38%) than in MeA (68.37%; $z = -15.35$, $P < 0.001$) (Figure 4.8A). There was no difference, however, in the percentage of AR+ cells that were CTB+ between MeA (45.89%) and MeP (45.48%; $z = -0.33$, $P = 0.37$) (Figure 4.8B).

DISCUSSION

Methodological Considerations

Previous studies have used CTB to label afferents of MA (Coolen and Wood 1998; Maras and Petrulis 2010a) and BNST (Wood and Swann 2005) in hamsters, and we observed a pattern of retrograde labeling similar to previous reports. By combining CTB retrograde tracing with immunohistochemical markers for IEGs and AR, we extended these previous findings by characterizing the nature of odor-responsive and hormone-sensitive MA and BNST projection neurons.

IEGs have been widely used as indirect markers of neuronal activation in response to discrete stimulus presentations, including odor stimuli (Pfaus and Heeb 1997). Although we observed low levels of co-localization of Fos and CTB within cells of MA and BNST, the total number of cells expressing Fos were similar to previous reports of Fos expression following odor stimulus exposure (Fiber *et al.* 1993; Fernandez-Fewell and Meredith 1994; Kollack-Walker and Newman 1997). Furthermore, we observed low levels of Fos expression in the clean control group, suggesting that the co-localization of Fos and CTB observed in males exposed to sexual odors likely reflects specific activation of MA and BNST afferents in response to sexual odor stimuli, rather than constitutive expression. The overall numbers of cells participating in coding social odors, however, are likely to be underestimated by IEG expression, as it primarily reflects the cumulative number of neurons activated via excitatory, NMDA-receptor mediated inputs and is therefore not a sensitive measure of inhibitory processing or rapid, non-NMDA receptor-mediated excitation (Flavell and Greenberg 2008).

It is also possible that we would observe a different pattern of IEG activity and/or co-localization with CTB in sexually-experienced males than we do in the current, sexually-naïve subjects. Indeed, previous research has demonstrated that sexual experience can alter male hamsters' neural (Kollack-Walker and Newman 1997; Fewell and Meredith 2002) and behavioral (Been and Petrulis 2010a; Been and Petrulis 2010b) responses to females and/or their odors. Unlike other rodent species, however, male hamsters do not require sexual experience to show a preference for (or discriminate between) female vs. male odors (Maras and Petrulis 2006; Ballard and Wood 2007; Been and Petrulis 2010a; Been and Petrulis 2010b). If the pattern of neural activation observed in the current study mediates this opposite-sex odor preference, then we might expect to see a similar, or more pronounced, difference between female and male odor-responsive projection cells in sexually-experienced males.

MA afferents relay sex-specific odor information to BNST and MPOA

The current results provide the first direct evidence that opposite-sex odors activate a higher percentage of MA afferents than do same-sex odors. Specifically, within MeP, female odors activated a higher percentage of neurons that project to either MPOA or BNST than did male odors. Furthermore, within both MeA and MeP, a higher percentage of female odor-responsive neurons than male odor-responsive neurons projected to BNST and/or MPOA. Together, these data suggest that male hamsters' preference for opposite-sex odors may rely on this differential projection of female- versus male-responsive neurons, particularly from MeP to MPOA and/or BNST. This finding is consistent with previous studies demonstrating that lesions of MA eliminate opposite-sex odor preference in hamsters (Maras and Petrulis 2006), and cause deficits in other

odor-guided reproductive behaviors in hamsters (Lehman *et al.* 1980; Petrulis and Johnston 1999), rats (Kondo 1992; Kondo *et al.* 1997) and gerbils (Heeb and Yahr 2000). Furthermore, functionally disconnecting MA from MPOA using asymmetrical pathway lesions disrupts copulation in male rats (Kondo and Arai 1995) and gerbils (Heeb and Yahr 2000), suggesting MA projections to MPOA may be particularly critical for reproductive behavior. Future studies will determine whether connections between MA and MPOA/BNST are similarly required for the appropriate behavioral response to sex-specific chemosignals in hamsters.

Although exposure to either female or male odors consistently activated more cells than exposure to clean control odors, the mean densities of MA neurons expressing Fos did not consistently differ between female and male odor-exposed groups. Exposure to female and male odors activated equivalent numbers of neurons in MeA, as is typical in studies that directly compare Fos induction following exposure to different categories of social odors (Meredith and Westberry 2004; Samuelsen and Meredith 2009; Maras and Petrulis 2010a; Maras and Petrulis 2010b). In MeP, however, female odors did not consistently activate more cells than male odors, unlike previous reports (Meredith and Westberry 2004; Samuelsen and Meredith 2009; Maras and Petrulis 2010b). This difference may be explained by the fact that MeP is a heterogeneous nucleus (Canteras et al. 1992) and subtle differences in the placement of counting domains within and between studies may therefore contribute to variability in the total number of Fos-immunoreactive cells counted in this area.

The percentage of MA cells that were activated by female or male odors and projected to MPOA and/or BNST was also lower than the percentage of cells that were ac-

tivated by clean odors and projected to MPOA and/or BNST. One possible explanation for this finding is that the cells activated by clean odors comprise a portion of the cells activated by female and/or male odors. That is, it is possible that some cells may express Fos in response to female, male, and/or clean odors. If this is case, then it is likely that the small numbers of cells in MA and BNST that respond to clean odors also respond to female and/or male odors, and therefore encode chemoinvestigatory behavior rather than stimulus identity. Unfortunately, conventional IEG methods cannot distinguish whether the same cells are activated by different categories of sexual odor stimuli.

The percentage of MA cells that project to MPOA and are also activated by female odor stimuli in the current study (16-18%) is markedly lower than the percentage of MA-MPOA projecting cells activated by copulation (60-65%) in rats (Coolen et al. 1998; Greco et al. 1998). These lower levels of activation likely reflect the fact that presentation of female odors alone provides only part of the total sensory input to MA during copulatory behavior (Kollack-Walker and Newman 1997). As such, the immediate early gene response in the current study likely corresponds to the perception and identification of female odors. The large number of retrogradely-labeled cells that did not express Fos in response to female odor stimuli may therefore represent neurons that require the additional sensory input received during copulatory behavior to be activated. Conversely, the large number of odor-responsive cells that were not retrogradely-labeled may represent MA cells that project to other brain areas important for odor-guided reproductive behaviors, such as the ventromedial hypothalamus or nucleus accumbens (Heeb and Yahr 1996; Kippin *et al.* 2003; Robarts and Baum 2007), or to brain

areas important for other, non-reproductive, odor-guided behaviors (Blanchard et al. 2005; Choi et al. 2005).

BNST afferents relay nonspecific sexual odor information to MPOA

As in MA, exposure to either female or male odors consistently activated more BNST cells than exposure to clean control odors, although the mean densities of MA neurons expressing Fos did not differ between female and male odor-exposed groups. In contrast to MA afferents, however, the sex-specificity of odor information was lost at the population level of BNST projections to MPOA. The percentage of BNST cells that project to MPOA and were also activated in response to female odor stimuli (11-13%) is lower than the percentage MA afferents to MPOA that were also activated by female odors in the current study, and far lower than the percentage of BNST cells that project to MPOA and are also activated by copulation (55-60%) in rats (Coolen *et al.* 1998; Greco *et al.* 1998). These results are consistent with previous studies that suggest BNST may play a less critical role than MA in generating the behavioral response to sex-specific odors. Whereas lesions of MA decrease female odor-induced Fos expression in BNST and MPOA (Maras and Petrulis 2010b), lesions of BNST do not reduce Fos expression in MPOA or other downstream brain areas (Been and Petrulis 2008). Similarly, lesions of the vomeronasal organ in male hamsters do not decrease female odor-induced Fos expression in BNST, suggesting that BNST may only be involved in the response to volatile female odors, or may mediate integrative or motoric components of chemoinvestigation, rather than investigation of directly-contacted female odors specifically (Fernandez-Fewell and Meredith 1994). Finally, whereas MA lesions cause severe deficits in male copulatory behavior (Lehman *et al.* 1980; Kondo 1992), lesions

of BNST alter the temporal sequence of copulation but do not severely impair mating (Valcourt and Sachs 1979; Claro *et al.* 1995; Liu *et al.* 1997b; Been and Petrulis 2010a).

Hormone Information is directly conveyed between MA, BNST, and MPOA

As in many rodent species (Moffatt 2003), appropriate levels of gonadal hormones are critical for male hamsters' attraction to female odors (Steel 1982; Powers and Bergondy 1983; Powers et al. 1985; Petrulis and Johnston 1995), suggesting hormones modulate neural processing of conspecific odors (Fiber and Swann 1996; Kelliher *et al.* 1998). In agreement with previous reports, we observed higher densities of cells expressing AR in MeP and BNSTpm than in MeA and BNSTpi, respectively (Wood *et al.* 1992; Wood and Newman 1999), and we have extended these findings by demonstrating that this pattern is preserved with regard to the number of AR-sensitive neurons that project within this circuit. Interestingly, both the percentage of AR-containing cells that project to MPOA and the percentage of MPOA-projecting cells that contain AR are greater in MeP and BNSTpm than in MeA and BNSTpi, respectively. In contrast, only the percentage of BNST-projecting cells that contain AR, but not the percentage of AR-containing neurons that project to BNST, is greater MeP than in MeA. These data suggest that MPOA may be the primary target of hormonal information from MeP and BNSTpm, whereas MeP projections to BNST may play a secondary role in the hormonal modulation of neural processing.

It is unknown whether MPOA- and BNST-projecting neurons that express Fos in response to male and female odors are a subset of the MPOA- and BNST-projecting neurons that express AR. In male hamsters, approximately 40% of MA neurons and 47% of BNST neurons that express Fos following copulation also express AR (Wood

and Newman 1993). Similarly, in rats, 89% of MA neurons that express Fos in response to copulation also express AR, and of those neurons 82% also project to MPOA (Greco *et al.* 1998). Although we observed lower percentages of cells double-labeled with Fos/CTB following exposure to sexual odors than others have observed following copulation, these previous studies suggest that we might expect to see a substantial amount of overlap between Fos/CTB+ and AR/CTB+ cells in MA and BNST, likely to a proportionally smaller degree.

Although in the current study we only examined the expression of AR in MA, BNST, and MPOA, these nuclei also contain abundant estrogen receptor (ER)-containing cells (Wood *et al.* 1992; Wood and Newman 1995a). Unfortunately, the only antibody that consistently labels ER in hamster brain tissue is the H222 antibody (Li *et al.* 1993; Wood and Newman 1995a; Mangels et al. 1998; Boers et al. 1999), and this antibody labels unbound ER, precluding its use in any studies that require subjects to maintain normal levels of steroid hormones (Blaustein 1993). It is worth noting, however, that ERs may mediate of the effects of steroid hormones on males' behavioral response to conspecifics odors (Wood 1996). Although systemic estradiol administration, either alone or in combination with dihydrotestosterone, does not restore levels of female odor investigation in castrated males as effectively as systemic testosterone administration (Powers and Bergondy 1983), the aromatization of testosterone into estradiol may play a role in the identification and investigation of female odors in male hamsters. Indeed, administration of testosterone propionate, which can be aromatized, restores males' preference for female odors in castrated males (Gregory et al. 1975). Furthermore, inhibiting aromatase activity decreases olfactory investigation of a female

and, at high doses, eliminates males' ability to discriminate between individual females' vaginal secretions, whereas subsequent treatment with estradiol can reverse these deficits (Steel and Hutchison 1987; Steel and Hutchison 1988). In hamsters, cells in MA and BNST often co-express AR and ER, such that cells expressing only ER constitute a small proportion (12-18%) of the total population of AR- and ER-expressing cells within these nuclei (Wood and Newman 1995a). These data would suggest that the current findings about AR-containing cells may generalize to ER-containing cells, and we would therefore expect to observe similar proportions of ER/CTB double-labeling as were observed for AR/CTB double-labeling.

Summary and Conclusions

This study demonstrates that sex-specific odor information and steroid hormone cues are directly relayed between MA, BNST, and MPOA in male Syrian hamsters. As few MPOA- or BNST-projecting cells expressed IEGs in response to female or male odors, the present results show that sparse and discrete populations of cells send male and female chemosignals to BNST and MPOA. Sex-specific odor information may be carried primarily by MeP, as IEG expression in this region is greater to female odors than to male odors in MPOA- and BNST-projecting cells, whereas female and male odors induced equivalent levels of IEGs within retrogradely-labeled MeA, BNSTpi, and BNSTpm cells (Figure 4.9A). We observed high levels of co-localization of AR with cells that project to MPOA or BNST, and these hormone-sensitive cells also followed a distinct pattern. Specifically, a higher percentage of retrogradely-labeled MeP and BNSTpm cells also expressed AR than retrogradely-labeled MeA and BNSTpi cells, respectively (Figure 4.9B). Together, these data demonstrate that MA, BNST, and MPOA

interact to transmit chemosensory and hormonal information within this ventral forebrain circuit. These odor-responsive and steroid-sensitive projections may provide the neural basis for the appropriate behavioral response to conspecific odors.

ACKNOWLEDGEMENTS

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CHAPTER 4 FIGURES

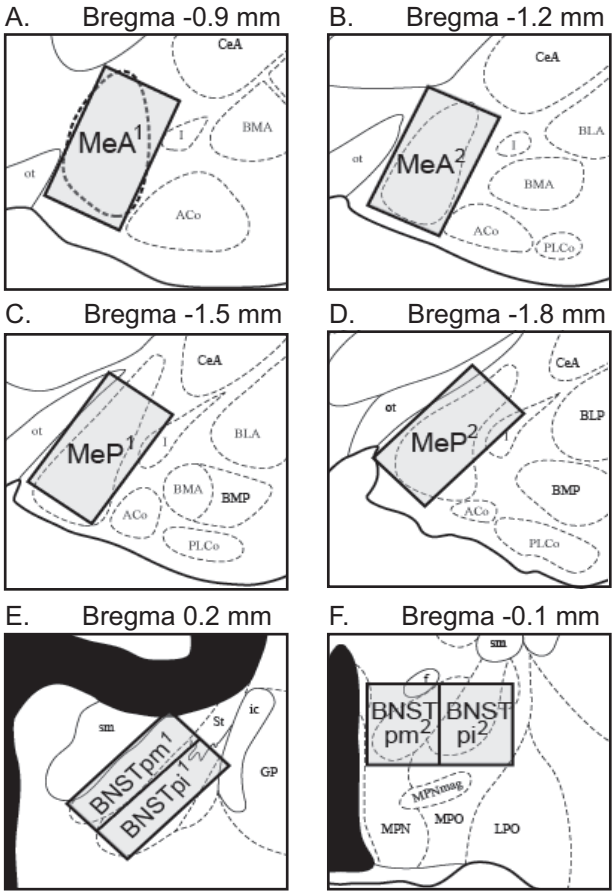

Figure 4.1: Counting domains for analysis of cholera toxin B (CTB), immediate early genes (Fos, EGR-1) and androgen receptor (AR). The total number of single- and double-labeled cells within the specified counting domains (grey rectangles) were counted and summed across two sections for the anterior medial amygdala (MeA; A and B), posterior medial amygdala (MeP; C and D), and posterointermediate and posteromedial bed nucleus of the stria terminalis (BNSTpi and BNSTpm; E and F). Illustration modified from hamster brain atlas (Morin and Wood, 2001). ACo, anterior cortical amygdala; BLA, basolateral amygdala; BLP, posterior basolateral amygdala; BMA, basomedial amygdala; CeA, central amygdala; f, fornix; GP, globus pallidus; I, intercalated amygdala; ic, internal capsule; LPO, lateral preoptic area; ot, optic tract; MPN, medial preoptic nucleus; MPO, medial preoptic area; PLCo posterolateral cortical amygdala; sm, stria medullaris; St, stria terminalis.

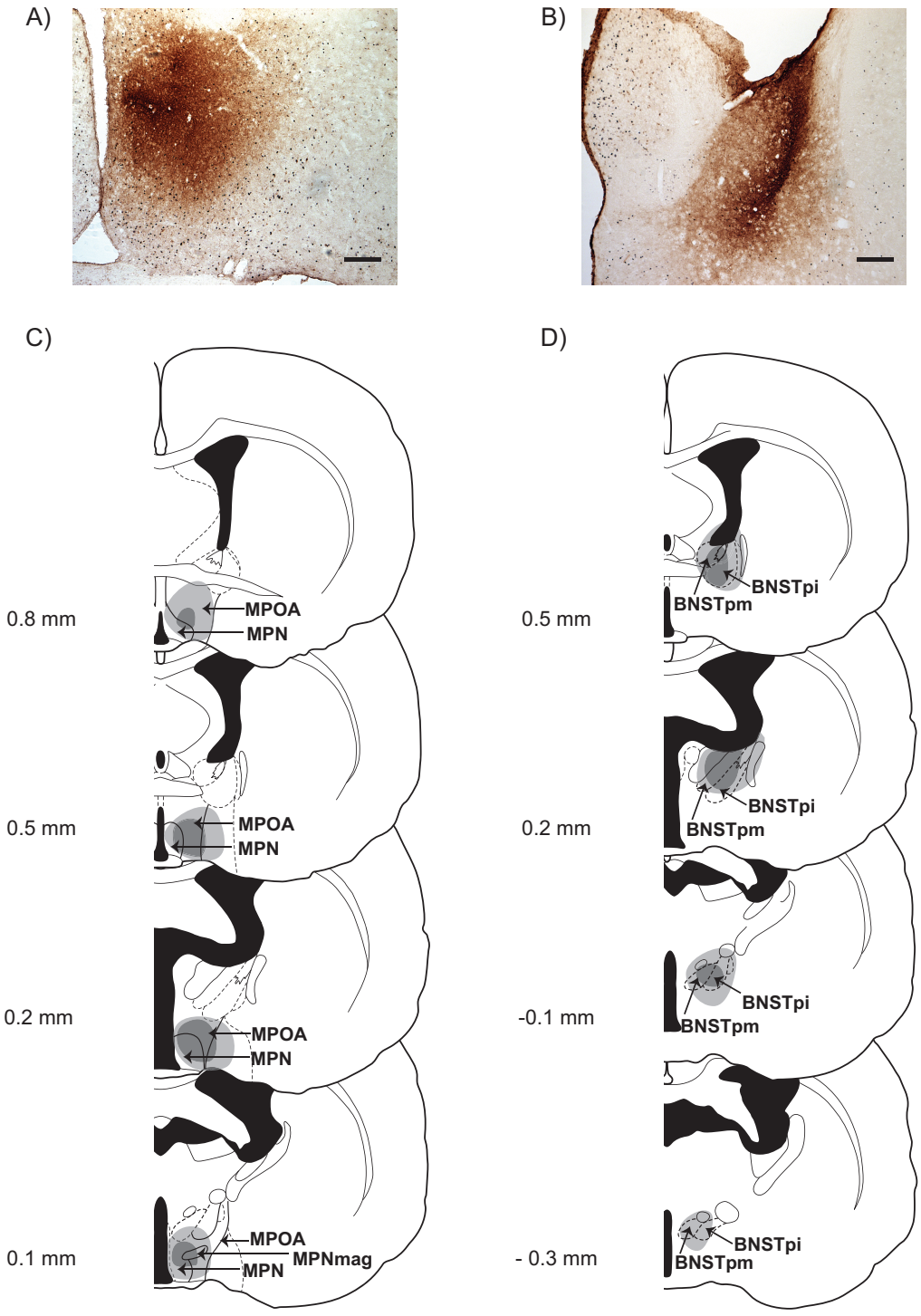

Figure 4.2: Verification of cholera toxin B (CTB) injections. Photomicrographs depict typical deposition of CTB in A) the medial preoptic area (MPOA) and B) the posterior bed nucleus of the stria terminalis (BNST). Reconstruction of largest (light grey) and smallest (dark grey) CTB injections (numbers represent distance posterior to bregma) through the rostral-caudal extent of (C) MPOA and (D) BNST. Scale bars = 100 µm.

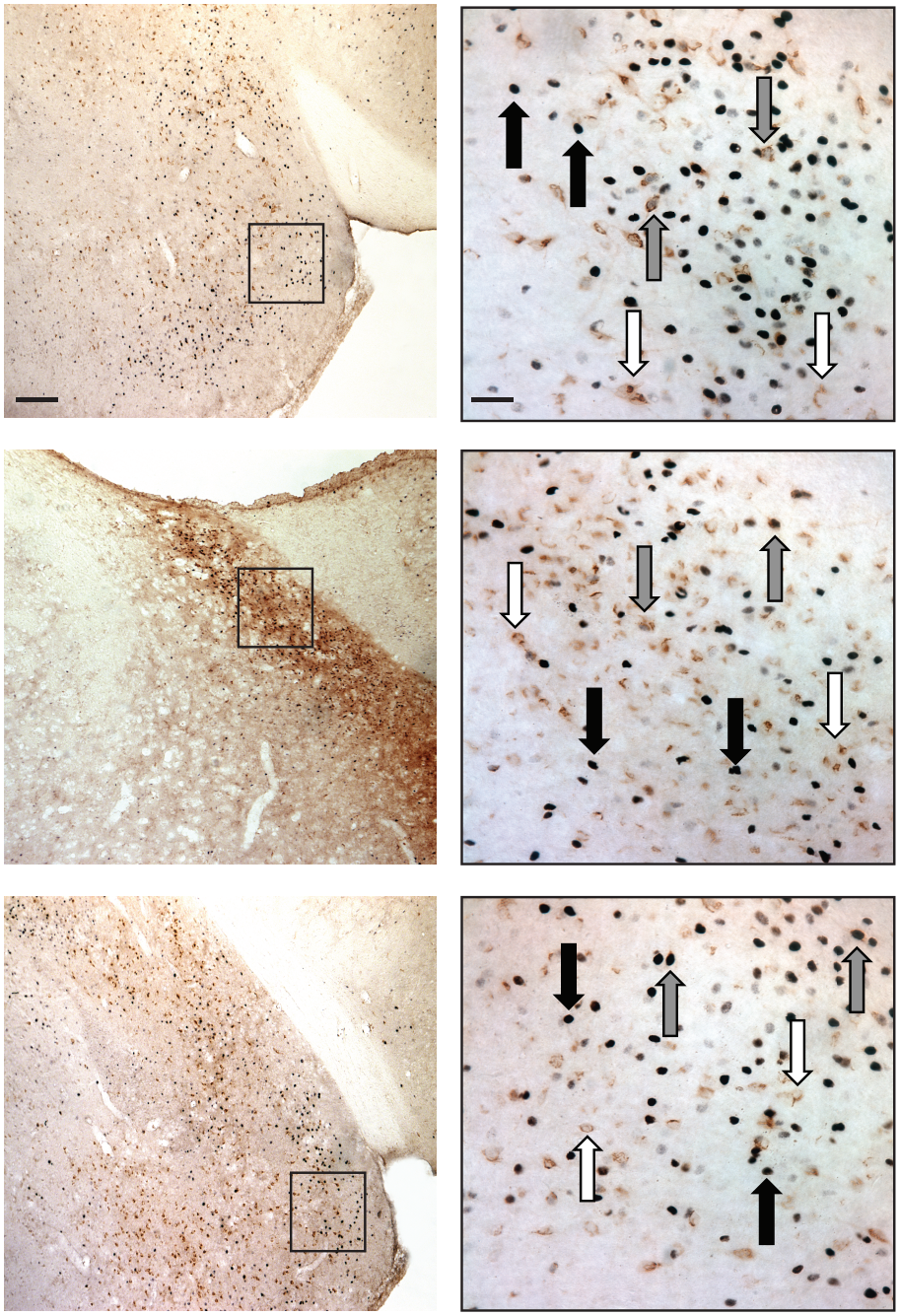

Figure 4.3: Co-localization of cholera toxin B (CTB) and immediate early genes. Photomicrographs from representative sections immunoreactive for CTB and Fos in A) the medial amygdala (MA) and B) the posterior bed nucleus of the stria terminalis (BNST), and C) from a representative section immunoreactive for CTB and EGR-1 in MA. Images to right provide higher magnifications of areas included in the boxes. Cells with brown cytoplasmic staining (white arrows) are CTB+, cells with black nuclear staining (black arrows) are Fos+ (A and B) or EGR-1+ (C), and cells with black nuclear staining surrounded by brown cytoplasmic staining (grey arrows) are Fos/CTB+ (A and B) or EGR-1/CTB+ (C). Scale bars = 100 μm and 25 μm for lower and higher magnifications, respectively.

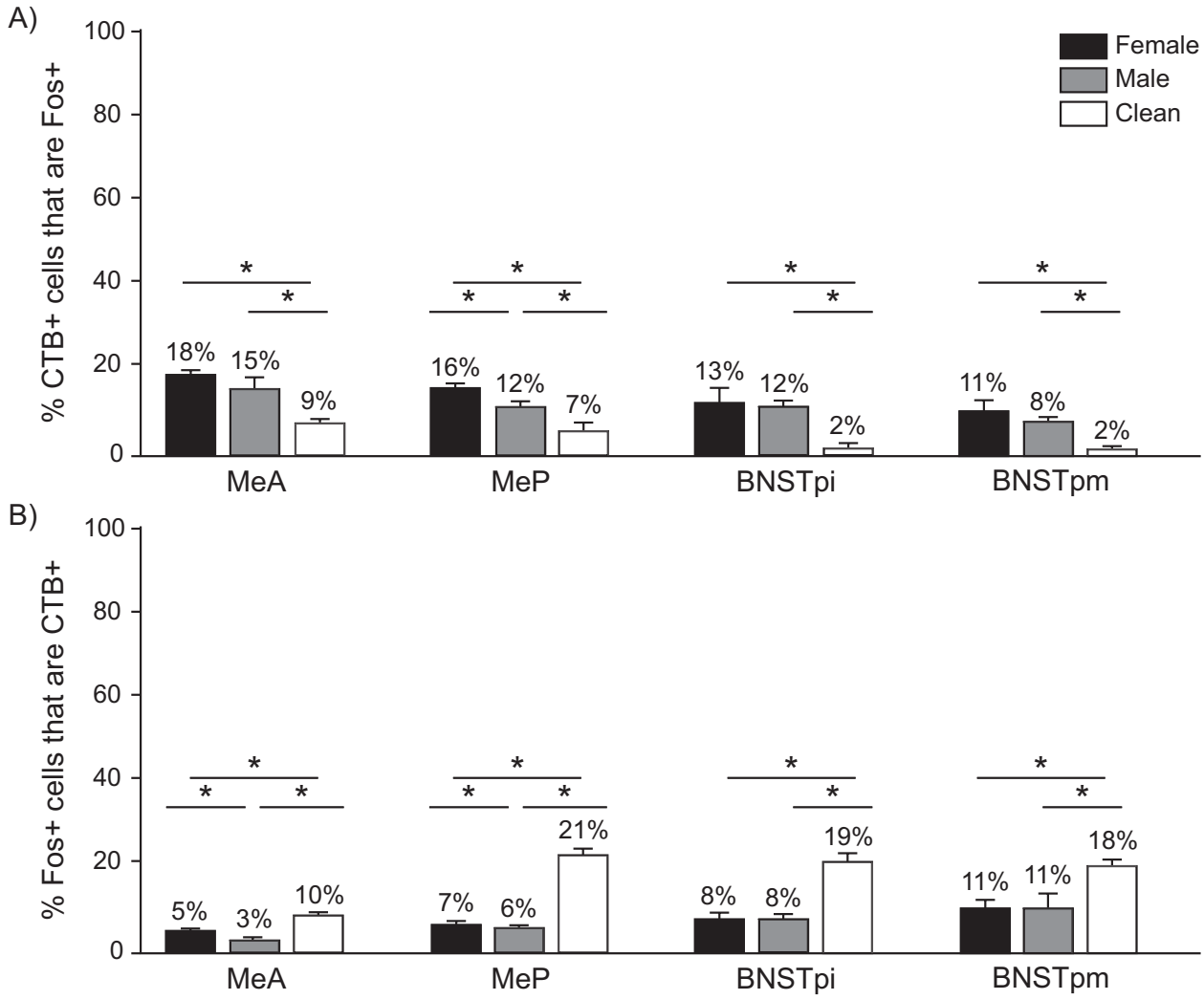

Figure 4.4: Percentages of Fos/CTB double-labeled cells in males with medial preoptic area (MPOA) injections. A) Total percentage of CTB+ cells that were also Fos+ in the anterior medial amygdala (MeA), posterior medial amygdala (MeP), posterointermediate bed nucleus of the stria terminalis (BNSTpi), and posteromedial bed nucleus of the stria terminalis (BNSTpm). B) Total percentage of Fos+ that were also CTB+ in MeA, MeP, BNSTpi, and BNSTpm. Error bars represent standard errors of mean group percentages, * reflects significant differences between brain areas, $P < 0.05$ (*z*-test for independent proportions, $\alpha_{fw} = 0.05$).

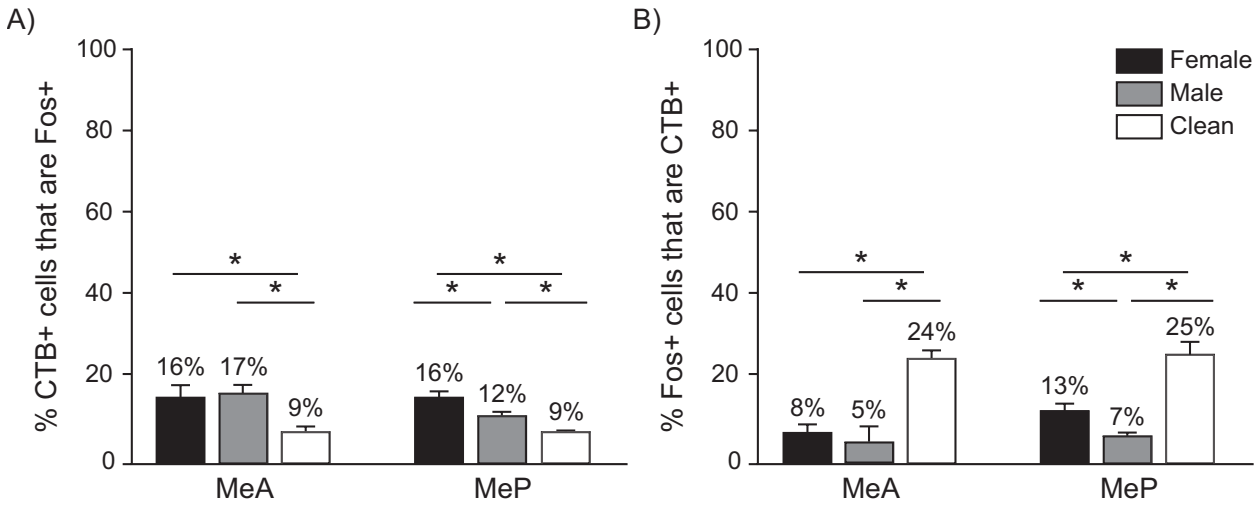

Figure 4.5: Percentages of Fos/CTB double-labeled cells in males with posterior bed nucleus of the stria terminalis (BNST) injections. A) Total percentage of CTB+ cells that were also Fos+ in the anterior medial amygdala (MeA) and posterior medial amygdala (MeP). B) Total percentage of Fos+ that were also CTB+ in MeA and MeP. Error bars represent standard errors of mean group percentages, * reflects significant differences between brain areas, $P < 0.05$ (*z*-test for independent proportions, $\alpha_{fw} = 0.05$).

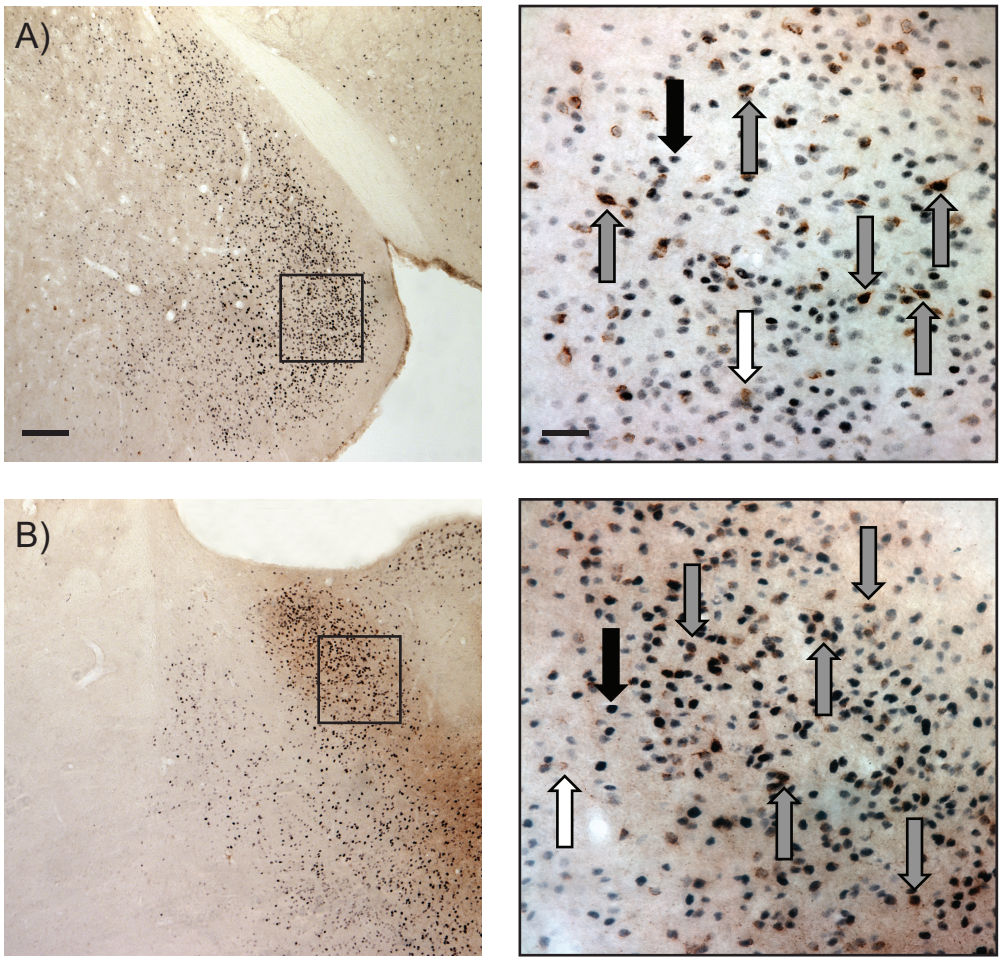

Figure 4.6: Co-localization of cholera toxin B (CTB) and androgen receptor (AR). Photomicrographs from representative sections immunoreactive for CTB and AR in A) the medial amygdala (MA) and B) the posterior bed nucleus of the stria terminalis (BNST). Images to right provide higher magnifications of areas included in the boxes. Cells with brown cytoplasmic staining (white arrows) are CTB+, cells with black nuclear staining (black arrows) are AR+, and cells with black nuclear staining surrounded by brown cytoplasmic staining (grey arrows) are AR/CTB+. Scale bars = 100 μm and 25 μm for lower and higher magnifications, respectively.

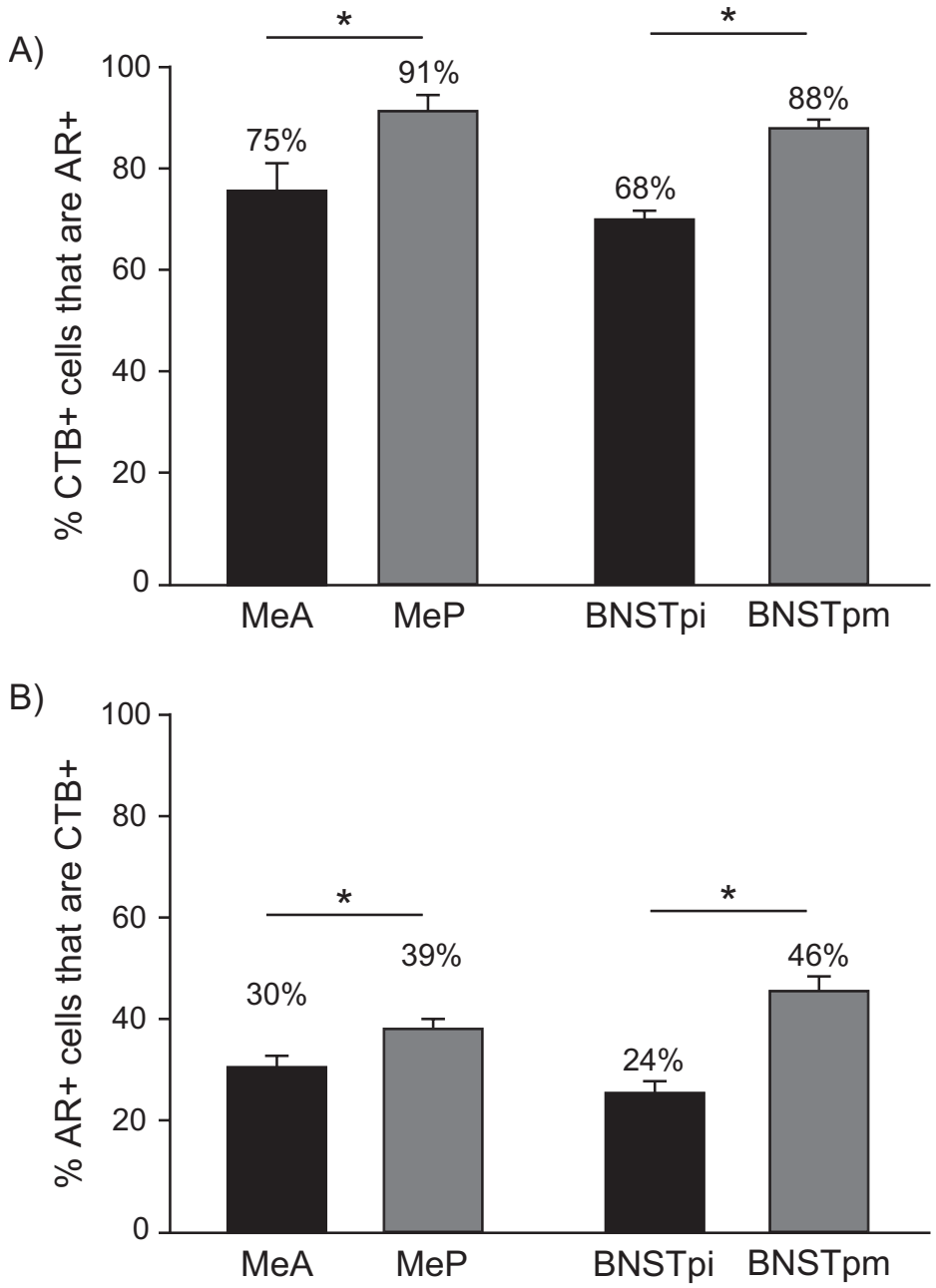

Figure 4.7: Percentages of AR/CTB double-labeled cells in males with medial preoptic area (MPOA) injections. A) Total percentage of CTB+ cells that were also AR+ in the anterior medial amygdala (MeA), posterior medial amygdala (MeP), posterointermediate bed nucleus of the stria terminalis (BNSTpi) and posteromedial bed nucleus of the stria terminalis (BNSTpm). B) Total percentage of AR+ cells that were also CTB+ in MeA, MeP, BNSTpi, and BNSTpm. Error bars represent standard errors of mean group percentages, * reflects significant differences between brain areas, $P < 0.05$ (*z*-test for independent proportions, $\alpha_{fw} = 0.05$).

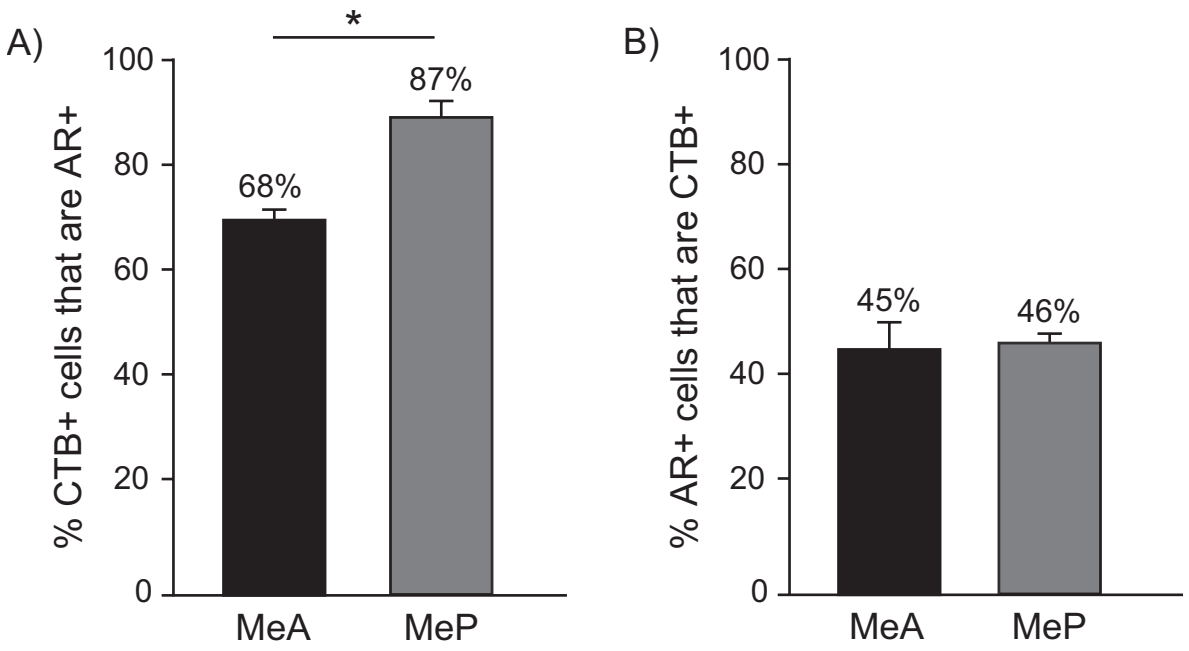

Figure 4.8: Percentages of AR/CTB double-labeled cells in males with posterior bed nucleus of the stria terminalis (BNST) injections. A) Total percentage of CTB+ cells that were also AR+ in the anterior medial amygdala (MeA) and posterior medial amygdala (MeP). B) Total percentage of AR+ cells that were also CTB+ in MeA and MeP. Error bars represent standard errors of mean group percentages, * reflects significant differences between brain areas, $P < .05$ (*z*-test for independent proportions, $\alpha_{fw} = 0.05$).

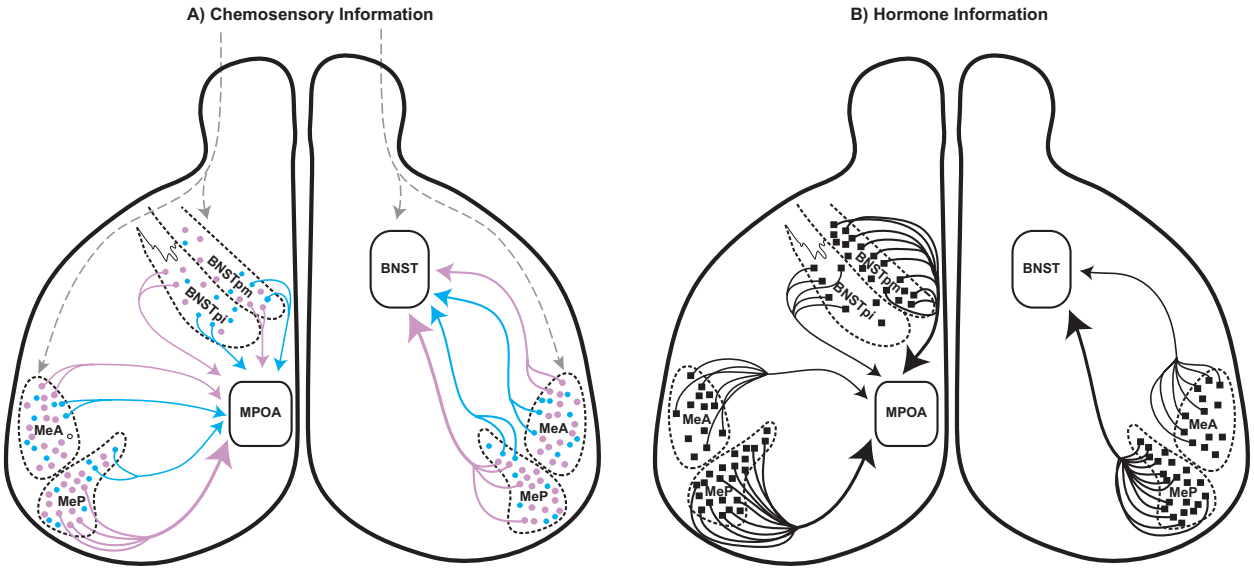

Figure 4.9: Summary of chemosensory and steroid-sensitive projections to medial pre-optic area (MPOA) and posterior bed nucleus of the stria terminalis (BNST). A) A low percentage of cells that respond to female odors (pink circles) or male odors (blue circles) project to MPOA (left) or BNST (right). In the posterior medial amygdala (MeP), however, a higher percentage of female-odor responsive cells project to BNST and/or MPOA than do male odor-responsive cells. B) A high percentage of androgen receptor (AR)-bearing cells (squares) project to MPOA (left) and BNST (right). Specifically, more AR+ cells in MeP than in the anterior medial amygdala (MeA) project to MPOA and/or BNST; similarly, more AR+ cells in the posteromedial BNST (BNSTpm) than in the posterointermediate BNST (BNSTpi) project to MPOA. Solid arrows indicate afferent projections; dashed arrows represent direct olfactory projections.

CHAPTER 4 TABLES

Table 4.1: Densities of CTB+, Fos+, and Fos/CTB+ cells in cells that project to the medial preoptic area (MPOA). Data reported as means ± standard errors of the means. * reflects significant differences between brain areas, dissimilar letters reflect significant differences between odor stimulus conditions (Tukey's-B post-hoc comparisons, $P < .05$). MeA, anterior medial amygdala; MeP, posterior medial amygdala; BNSTpi, posterointermediate bed nucleus of the stria terminalis; BNSTpm, posteromedial bed nucleus of the stria terminalis.

Total/mm^2	MeA	MeP	BNSTpi	BNSTpm
CTB+	73.86 ± 11.83	144.44 ± 22.46*	36.06 ± 3.20	95.37 ± 37.43
Fos+				
Female	238.06 ± 37.12^a	196.97 ± 6.46^a	101.39 ± 19.67^a	72.93 ± 5.39^a
Male	260.14 ± 17.75^a	200.18 ± 24.53^a	115.82 ± 18.06^a	111.26 ± 43.21^a
Clean	87.25 ± 21.40^b	50.27 ± 14.27^b	6.16 ± 1.54^b	2.15 ± 0.77^b
Fos/CTB+				
Female	13.10 ± 3.93^a	14.77 ± 1.13^a	8.32 ± 3.92^a	16.17 ± 8.26^a
Male	8.86 ± 2.79^a	11.04 ± 1.48^a	8.99 ± 2.88^a	7.70 ± 3.75^a
Clean	9.05 ± 2.44^a	10.59 ± 4.31^a	1.15 ± 0.77^a	0.77 ± 0.77^a

Table 4.2: Densities of CTB+, Fos+, and Fos/CTB+ cells that project to the posterior bed nucleus of the stria terminalis (BNST). Data reported as means ± standard errors of the means. Dissimilar letters reflect significant differences between odor stimulus conditions (Tukey's-B post-hoc comparisons, $P < .05$). MeA, anterior medial amygdala; MeP, posterior medial amygdala.

Total/mm^2	MeA	MeP
CTB+	101.44 ± 12.89	140.22 ± 16.12
Fos+		
Female	220.21 ± 15.5^a	249. 56 ± 12.56^a
Male	197.22 ± 28.42^a	138.29 ± 11.43^b
Clean	60.29 ± 15.49^b	68.27 ± 6.39^c
Fos/CTB+		
Female	11.81 ± 1.60^a	16.29 ± 5.25^a
Male	14.79 ± 3.35^a	19.97 ± 3.16^a
Clean	14.64 ± 7.07^a	17.07 ± 5.27^a

Table 4.3: Densities of CTB+, ERG-1+, and CTB/ERG-1+ cells following female odor exposure in cells the project to A) the medial preoptic area (MPOA) and B) the posterior bed nucleus of the stria terminalis (BNST). MeA, anterior medial amygdala; MeP, posterior medial amygdala; BNSTpi, posterointermediate bed nucleus of the stria terminalis; BNSTpm, posteromedial bed nucleus of the stria terminalis.

Total/mm^2	MeA	MeP	BNSTpi	BNSTpm
A) MPOA-projecting				
EGR-1+	195.61 ± 21.55	208.63 ± 30.53	98.46 ± 24.87	88.06 ± 33.84
EGR-1/CTB+	17.70 ± 1.18	11.16 ± 1.89	13.29 ± 4.72	12.37 ± 2.65
B) BNST-projecting				
EGR-1+	223.01 ± 30.17	197.58 ± 16.00		
EGR-1/CTB+	11.04 ± 3.34	11.56 ± 2.67		

Table 4.4: Densities of CTB+, AR+, and CTB/AR+ cells in cells that project to A) the medial preoptic area (MPOA) and B) the posterior bed nucleus of the stria terminalis (BNST). * reflects significant differences between brain areas (Independent *t*-tests, $P < .05$). MeA, anterior medial amygdala; MeP, posterior medial amygdala; BNSTpi, posterointermediate bed nucleus of the stria terminalis; BNSTpm, posteromedial bed nucleus of the stria terminalis.

Total/mm^2	MeA	MeP	BNSTpi	BNSTpm
A) MPOA-projecting				
CTB+	78.58 ± 1.63	192.60 ± 22.33*	9.75 ± 2.02	171.54 ± 19.43*
AR+	185.93 ± 39.82	306.63 ± 32.37*	93.47 ± 12.93	362.60 ± 15.74*
AR/CTB+	68.05 ± 3.33	167.69 ± 24.04*	2.56 ± 0.68	156.65 ± 16.52*
B) BNST-projecting				
CTB+	105.03 ± 5.54	197.57 ± 31.12*		
AR+	171.54 ± 25.44	376.24 ± 29.94*		
AR/CTB+	68.82 ± 6.42	172.64 ± 28.94*		

CHAPTER 5:

Dissociated functional pathways for appetitive and consummatory reproductive behaviors in male Syrian hamsters

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ABSTRACT

In many species, including Syrian hamsters, the generation of male reproductive behavior depends critically on the perception of female odor cues from conspecifics in the environment. The behavioral response to these odors is mediated by a network of ventral forebrain nuclei including the medial amygdala (MA), posterior bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA). Previous studies have demonstrated that each of these three nuclei is required for appropriate sexual behavior and that MA preferentially sends female odor information directly to BNST and MPOA. It is unknown, however, how the functional connections between MA and BNST and/or MPOA are organized to generate different aspects of reproductive behavior. Therefore, the following experiments used the asymmetrical pathway lesion technique to test the role of the functional connections between MA and BNST and/or MPOA in odor preference and copulatory behaviors. Lesions that functionally disconnected MA from MPOA eliminated copulatory behavior but did not affect odor preference. In contrast, lesions that functionally disconnected MA from BNST eliminated odor preference but did not affect copulatory behavior. These results therefore demonstrate a double dissociation in the functional connections required for attraction to sexual odors and copulation and, more broadly, suggest appetitive and consummatory reproductive behaviors are mediated by distinct neural pathways.

INTRODUCTION

In most animals, appropriate reproductive behavior depends critically on the perception of chemical cues from conspecifics in the environment (Johnston 1990; Baum and Kelliher 2009). Syrian hamsters (*Mesocricetus auratus*) are a prominent model

species for studying the neural regulation of chemosensory-guided reproductive behaviors because their reproductive behavior is well characterized, stereotyped, and depends *critically* on the perception of a limited set of chemosensory cues (Murphy and Schneider 1970; Landauer *et al.* 1977). Specifically, the correct detection, identification, and valuation of sex-specific odors are needed for both appetitive and consummatory aspects of reproductive behavior in male Syrian hamsters (Murphy and Schneider 1970; Powers and Winans 1975; Powers *et al.* 1979), as well as in other mammals (Rowe and Edwards 1972; Wysocki et al. 1982; Sachs et al. 1994). Sex-specific odors required for reproductive behaviors are processed by the main (MOB) and accessory (AOB) olfactory bulbs and subsequently integrated in the medial amygdala (MA) (Scalia and Winans 1975; Keller *et al.* 2009). MA plays a critical role in odor-guided reproductive behaviors, as neurons in MA are activated during exposure to female odors and copulatory behavior (Fernandez-Fewell and Meredith 1994), and lesions of MA impair sexual behavior in many rodent species (Kondo 1992; Kondo *et al.* 1997; Stark *et al.* 1998; Newman 1999; Petrulis and Johnston 1999; Heeb and Yahr 2000). Specifically, in male hamsters, large lesions of MA eliminate copulatory behavior and greatly reduce anogenital investigation of a receptive female (Lehman *et al.* 1980), and more targeted lesions of either the anterior or posterodorsal subnuclei of MA eliminate preference for volatile female odors (Maras and Petrulis 2006).

From MA, sexual odor information is conveyed to the posterior bed nucleus of the stria terminalis (BNST) and/or the medial preoptic area (MPOA) (Wood 1997). MOB and AOB input that converges on MA (Davis *et al.* 1978) can be directly sent to BNST and/or MPOA (Simerly and Swanson 1986; Gomez and Newman 1992; Coolen and

Wood 1998; Dong et al. 2001), while chemosensory information received by BNST (either directly from AOB or indirectly from MA) can also terminate at MPOA (Davis *et al.* 1978; Gomez and Newman 1992; Dong and Swanson 2004; Wood and Swann 2005). We have demonstrated that MA preferentially sends female odor information directly to BNST and MPOA, whereas BNST relays male and female odor information to MPOA without sexual specificity (Been and Petrulis 2011), suggesting direct MA connections to MPOA and BNST may be the most critical for odor-guided sexual behaviors. Like MA, BNST and MPOA are also critically involved in generating appropriate odor-guided reproductive behaviors. Lesions of MPOA eliminate preference for volatile opposite-sex odors (Been and Petrulis 2010b) and severely impair or eliminate copulatory behavior in male hamsters (Lehman *et al.* 1980) and nearly every vertebrate species studied to date (Hull *et al.* 2002). In comparison, lesions of BNST eliminate male hamsters' preference for volatile opposite-sex odors and decrease investigation of directly contacted female odors (Been and Petrulis 2010b), without causing dramatic changes in copulatory behavior in male hamsters and other rodents (Claro *et al.* 1995; Liu *et al.* 1997b; Been and Petrulis 2010b).

We therefore hypothesized that functional connections between MA and MPOA are required for both sexual odor preference and copulatory behavior, whereas functional connections between MA and BNST are only required for sexual odor preference and not copulation. To test these hypotheses, we used the asymmetrical pathway lesion technique to functionally disconnect MA from either MPOA (MA-MPOA-X) or BNST (MA-BNST-X). This technique takes advantage of the primarily ipsilateral connections between MA, BNST and MPOA (Kevetter and Winans 1981; Simerly and Swanson

1986; Wood and Swann 2005; Wang and Swann 2006) and the finding that unilateral lesions of MA (Maras and Petrulis 2006), BNST (Been and Petrulis 2010b), or MPOA (Been and Petrulis 2010b) do not disrupt odor preference or copulation in male Syrian hamsters. Thus, unilateral lesions of two of these nuclei in contralateral brain hemispheres (CONTRA) result in a *functional disconnection*, in which two nuclei within a hemisphere are prevented from communicating with each other, but a sufficient amount of each nucleus to generate behavior remains. In contrast, unilateral lesions of the two nuclei within the same hemisphere (IPSI) remove the same total volume of nuclei as in CONTRA males, but leave the functional within-hemisphere connections intact. We found that lesions that functionally disconnect MA from MPOA virtually eliminate copulation but have no effect on odor preference; in contrast, lesions that functionally disconnect MA from BNST eliminate volatile odor preference, but do not impact copulatory behavior. These results therefore demonstrate that the neural pathways mediating sexual odor preference and copulatory behavior are functionally and anatomically dissociated.

MATERIALS AND METHODS

Animals

Adult male Syrian hamsters (n = 120) were purchased from Harlan Laboratories (Prattville, AL, USA) at 110-120 g and singly housed. A separate set of group-housed (3-4 same-sex animals per cage), gonadally intact, adult male and female hamsters (Harlan Laboratories; n = 80) were used to provide odor stimuli. A third group of sexually experienced adult female hamsters (Harlan Laboratories; n = 20) were used as stimulus females for copulatory behavior tests. Animals were maintained on a reversed 16-h

light/8-h dark photoperiod (lights off/on at 10 AM/6 PM). Food and water were available *ad libitum*. All experiments 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 were 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.

Surgery

All surgeries were performed under 2% isoflurane gas anesthesia vaporized in 100% oxygen (gonadectomy) or a 70:30% oxygen/nitrous oxide mixture (stereotaxic surgery). To minimize post-operative pain, ketoprofen (5 mg/kg subcutaneously, Henry Schein, Melville, NY, USA) was administered intra-operatively.

Gonadectomy and Hormone Implant. Exposure to female odors causes an increase in circulating testosterone levels in male hamsters (Macrides *et al.* 1974; Pfeiffer and Johnston 1992) and it is possible that lesions of MA, BNST, and/or MPOA may interfere with this surge. Consequently, all subjects were gonadectomized and maintained on physiological levels of testosterone during the experiment (Been and Petrulis 2010a; Been and Petrulis 2010b). Copulatory stimulus females' ovaries were removed and estradiol capsules were implanted as described previously (Been and Petrulis 2010a; Been and Petrulis 2010b). To induce behavioral receptivity, stimulus females were injected subcutaneously with 0.15 ml of progesterone dissolved in sesame oil (2.5 mg/ml, Sigma, St. Louis, MO, USA) 4 hours prior to copulatory behavior tests.

Asymmetrical Pathway Lesions. Subjects were randomly assigned to CONTRA, IPSI, or SHAM lesion groups within MA-MPOA-X and MA-BNST-X experimental groups.

Excitotoxic lesions were made by lowering a microinjection syringe (701R 10 µl syringe, Hamilton, Reno, NV, USA) under stereotaxic control (Microinjection Unit, Model 5002, David Kopf Instruments, Tujunga, CA, USA) into target sites and injecting N-methyl-D-aspartic acid (NMDA, 20 mg/ml, Sigma, St. Louis, MO, USA) (Table 5.1). In sham surgeries, the injector was lowered to 1 mm above the target and no excitotoxin was injected. Subjects were allowed to recover from lesion surgery for at least one week prior to behavioral testing.

Behavioral Testing

All behavior testing took place during the first six hours of the dark phase and under red illumination. Video recordings of tests were scored using the Observer for Windows, Version 9.0 (Noldus Information Technology, Wageningen, The Netherlands).

Odor Preference. The stimuli and procedures for odor preference tests have been described in detail elsewhere (Been and Petrulis 2010a; Been and Petrulis 2010b). Briefly, flank and anogenital samples, soiled litter/bedding, and cage wall rubbings were collected from group-housed, same-sexed odor donor cages that had not been changed for four days prior to odor collection. Odor stimuli were therefore a heterogeneous and reflected the composite sexual identity of the odor, rather than the individual identity of a single animal. Subjects were tested in a series of three tests, each separated by 24 hours: Clean, Non-Contact preference, and Contact preference. During each test, subjects were placed into a testing aquarium containing three acrylic odor presentation boxes with holes drilled along the front surface that allowed volatile odors to pass, but prevented contact with the odor sources. For Clean tests, clean odor stimuli were placed into each of the three odor boxes. For Non-Contact and Contact tests, fe-

male and male odor stimuli were placed into each of the two outer odor boxes, and clean odor stimuli were placed into the center odor box. The side on which each sexual odor was placed (left or right) was alternated between consecutive subjects. Non-Contact and Contact tests were identical except that during Contact tests, a single odor slide matching the type of odor stimulus in that container (female, male, clean) was secured to the center of the front surface of each odor presentation box.

Copulatory Behavior. The procedures for copulatory behavior tests have been described in detail elsewhere (Been and Petrulis 2010a; Been and Petrulis 2010b). Briefly, subjects were placed into a clear testing arena with a receptive stimulus female for 20 minutes. An angled mirror was placed below the testing arena to provide a view of the ventral surface of the animals. The total number, durations, and latencies of mating events (anogenital investigation, mounts, intromissions, ejaculations, long intromissions) were scored.

Histology and Lesion Verification

Following the last test, subjects were injected with an overdose of sodium pentobarbital (100 mg/kg; Sleep Away, Ft. Dodge, IA, USA) and transcardially perfused with 200 ml of 0.1M phosphate-buffered saline (PBS, pH 7.4) followed by 200 ml of 10% neutral-buffered formalin (Richard Allan Scientific, Kalamazoo, MI, USA). Brains were post-fixed in 10% formalin overnight and then cryoprotected for at least 24 hours in 30% sucrose in PBS solution. Coronal sections (30-μm) of brain tissue were sectioned using a cryostat (-20°C) and stored in cryoprotectant until immunohistochemical localization of Neuronal Nuclei protein (NeuN, see (Been and Petrulis 2010a; Been and Petrulis 2010b) for immunohistochemistry procedures). NeuN-stained sections were examined

under a light microscope for the location and extent of lesion damage as compared with published hamster neuroanatomical plates (Morin and Wood 2001), and the minimum and maximum extents of lesion damage were traced onto anatomical plates using Adobe Illustrator CS 11.0 software.

Data Analysis

All data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for Windows and significance was determined as $P < 0.05$. To establish investigatory preferences, 3 X 3 (lesion condition x odor) mixed-design ANOVAs were performed separately for MA-MPOA-X and MA-BNST-X groups. Significant interactions were explored using simple effects analysis and pair-wise comparisons with Bonferroni alpha adjustments. Furthermore, separate one-way ANOVAs were used to compare the levels of investigation of each stimulus directly across experimental groups. Group differences in most copulatory measures were detected using one-way ANOVAs, whereas group differences in the proportion of animals displaying copulatory measures were detected using *z*-tests for independent proportions.

RESULTS

Lesion Reconstruction

Lesion damage to MA was centered at the juncture between the anterior and posterior subdivisions of MA (Bregma -1.35 mm, Figure 5.1C). All subjects included in the MA-MPOA-X (CONTRA $n = 12$; IPSI $n = 12$) or MA-BNST-X (CONTRA $n = 12$; IPSI $n = 14$) experimental groups sustained at least 60% damage in two atlas plates of the anterior MA (Bregma - 0.09 to -1.2) and two atlas plates of the posterior MA (Bregma -

1.5 mm to -1.8 mm). Most subjects sustained additional, albeit lesser (≤ 40%) damage to more anterior and posterior aspects of MA (Bregma - 0.06 mm and -2.0 mm, respectively).

Lesion damage to MPOA was centered below the most caudal extent of the anterior commissure (Bregma 0.5 mm, Figure 5.1A). All subjects included in the MA-MPOA-X lesion groups (CONTRA AND IPSI) sustained at least 60% damage to MPOA, including the medial preoptic nucleus (MPN) in at least two atlas plates between Bregma 0.8 mm and Bregma -0.1 mm. Many (CONTRA *n* = 7; IPSI *n* = 8) subjects had lesion damage that extended more caudally into the anterior hypothalamus (Bregma -0.3 mm).

Lesion damage to BNST was centered at the juncture where the lateral and third ventricles fuse (Bregma 0.2 mm, Figure 5.1B). All subjects included in the MA-BNST-X lesion groups (CONTRA AND IPSI) sustained at least 60% damage to posterointermediate BNST (BNSTpi), posteromedial BNST (BNSTpm), and posterolateral BNST (BNSTpl) in at least two atlas plates between Bregma 0.5 mm and Bregma -0.3 mm. Many subjects (CONTRA *n* = 5; IPSI *n* = 8) also sustained lesser (≥ 40%) damage to the anterior BNST.

Subjects were excluded from analysis if their lesions failed to damage a significant portion (≤ 60%) of the target nucleus or if their lesions damaged a significant portion (≥ 40%) of nuclei outside of the target nucleus. To control for non-specific effects of brain surgery, CONTRA and IPSI males were also compared to males that received a sham lesion surgery (SHAM). Only needle tracks were visible in all SHAM subjects (MA-MPOA-X *n* = 12; MA-BNST-X *n* = 11).

Odor Preference

Clean Test. In both MA-MPOA-X and MA-BNST-X experimental groups, there was a general bias to investigate the outside boxes more than the center box, but there was no bias towards either of the outside boxes that were used to present male and female odors in subsequent tests (SHAM $t(21) = -2.31$; IPSI $t(24) = -0.37$; CONTRA $t(22) = 0.28$, all $P > 0.05$). There was also no difference in the total number of midline crosses between lesion groups (MA-MPOA-X $F(1, 33) = 0.75$; MA-BNST-X, $F(1, 34) = 0.81$, both $P > 0.05$), indicating similar levels of activity.

Non-Contact preference test. In the MA-MPOA-X group, there was a significant main effect of odor stimulus ($F(1, 33) = 112.89$, $P < 0.001$), but no significant interaction between lesion group and investigation durations across the three odor stimuli (Figure 5.2A). Indeed, all experimental groups investigated female odors longer than male odors ($t(35) = 8.50$), female odors longer than clean odors ($t(35) = -13.02$), and male odors longer than clean odors ($t(35) = -7.61$) (all $P < 0.001$).

In contrast, in the MA-BNST-X group, there was a significant interaction between lesion group and investigation durations across the three odor stimuli ($F(1, 34) = 4.12$, $P = 0.02$; Figure 5.2B). Whereas SHAM and IPSI males spent significantly more time investigating female odors than male odors (SHAM $t(10) = 8.22$, $P < 0.001$; IPSI $t(13) = 4.04$, $P < 0.001$), males with CONTRA lesions spent equivalent amounts of time investigating female and male odors ($t(11) = 1.59$, $P = 0.14$). This elimination of preference was mediated by a decrease in investigation of female odors, as CONTRA males investigated female odors significantly less than SHAM males ($t(10) = 4.45$, $P < 0.001$) or IPSI males ($t(11) = 3.15$, $P = 0.009$). All lesion groups investigated female odors (SHAM

t(10) = -2.28, *P* = 0.04; IPSI *t*(13) = -6.35, *P* < 0.001; CONTRA *t*(11) = -8.71, *P* < 0.001) and male odors (SHAM *t*(10) = -1.13, *P* = 0.05; IPSI *t*(13) = -5.03, *P* < 0.001; CONTRA *t*(11) = -4.35, *P* < 0.001) more than clean odors.

Contact preference test. In the MA-MPOA-X and the MA-BNST-X experimental groups, there was there was a significant main effect of odor stimulus (MA-MPOA-X *F*(1, 33) = 112.56; MA-BNST-X *F*(1, 34) = 100.74 , both *P* < 0.001), but no significant interaction between lesion group and investigation durations across the three odor stimuli (Figures 5.2A and 5.2B). Indeed, all experimental groups investigated female odors longer than male odors (MA-MPOA-X *t*(35) = 10.16; MA-BNST-X *t*(36) = 7.68), female odors longer than clean odors (MA-MPOA-X *t*(35) = -11.27; MA-BNST-X *t*(36) = -12.81), and male odors longer than clean odors (MA-MPOA-X *t*(35) =-3.98; MA-BNST-X *t*(36) = -5.51) (all *P* < 0.001).

Copulatory Behavior

In the MA-MPOA-X experimental groups, males with CONTRA lesions displayed severe deficits in copulatory behavior compared to SHAM and IPSI males (Figure 5.4A). The proportion of males displaying mounts (*z* = 3.79), intromissions (*z* = 3.79), ejaculations (*z* = 3.79), and long intromissions (*z* = 3.79) was far lower in CONTRA males than in SHAM males (all *P* < 0.001), whereas the proportion of males displaying mounts, intromissions, ejaculations, and long intromissions did not differ between SHAM and IPSI lesion groups (all *P* > 0.05). CONTRA males did not differ from SHAM or IPSI males in the total duration of anogenital investigation (AGI) (*F*(2,35) = 0.145, *P* = 0.86).

In contrast, copulatory behavior measures did not differ within the MA-BNST-X experimental groups (Figure 5.4B). Indeed, the total number of mounts (*F*(2,36) = 0.97),

intromissions ($F(2,36) = 0.22$), ejaculations ($F(2,35) = 2.41$), and long intromissions ($F(2,29) = 0.06$) did not differ between SHAM, IPSI, and CONTRA males (all $P > 0.05$). The total duration of AGI ($F(2,36) = 2.19$, $P = 0.13$) also did not differ between lesion groups.

DISCUSSION

It is perhaps not surprising that these data support our hypothesis that the functional connections between MA and MPOA are required for copulatory behavior in hamsters, as lesions of either MA (Lehman *et al.* 1980; Kondo 1992; Heeb and Yahr 2000) or MPOA (Hull and Dominguez 2007; Been and Petrulis 2010b) alone severely impair copulation in male hamsters and other species, and functional disconnection of MA and MPOA eliminates copulation in rats (Kondo and Arai 1995) and gerbils (Kondo and Arai 1995; Heeb and Yahr 2000). Importantly, the current results extend these findings by demonstrating that functional connections between MA and MPOA cell groups are specific to copulation and can be dissociated from the functional connections required for pre-copulatory aspects of male sexual behavior. These data do not, however, support our hypothesis that the functional connections between MA and MPOA are required for odor preference, suggesting that the elimination of odor preference by bilateral MPOA lesions (Been and Petrulis 2010b) is mediated by a mechanism that is independent of MA. It is possible that, in addition to sexual odor information transmitted from MA to MPOA, direct AOB information transmitted from BNST to MPOA (Davis *et al.* 1978) is also sufficient to generate attraction to female odors. Indeed, redundancy of function within a network is a common mechanism to accomplish complex behavioral tasks and

also ensures that evolutionarily-important behaviors, such as reproduction, are not easily disrupted (Wood 1997).

The current results also support our hypothesis that the functional connections between MA and BNST are required for odor preference but not copulation. Several lines of evidence suggest that the functional connections between MA and BNST are well positioned to support the attraction to female odors. First, BNST preferentially receives female-specific odor information directly from MA (Been and Petrulis 2011) and lesions of the anterior MA decrease the c-fos response to female odors in BNST (Maras and Petrulis 2010b), suggesting MA provides sexually-relevant odor information to BNST. Second, lesions of BNST alone eliminate volatile odor preference in male hamsters (Been and Petrulis 2010a) and decrease non-contact erections in response to remote cues from estrous females in male rats (Liu *et al.* 1997b), supporting the idea that main olfactory information relayed from MA to BNST mediates attraction to volatile female odors. Third, BNST maintains reciprocal connections with AOB and MA (Scalia and Winans 1975; Wood and Swann 2005); the bidirectional nature of these connections may allow BNST to provide feedback to earlier stages of olfactory processing (Fan and Luo 2009). Finally, BNST is also reciprocally connected with the ventral striatum and ventral tegmental area in hamsters (Wood and Swann 2005), providing a potential substrate for the valuation of odor-stimuli and/or generating the motivation to approach and investigate sexual odors.

Although lesions that disconnected MA from BNST did not decrease investigation of directly-contacted (i.e. non-volatile) female odors in the current study, bilateral lesions of BNST decrease investigation of directly-contacted female odors (Been and Petrulis

2010a) and large lesions including BNST decrease anogenital investigation during copulation (Powers *et al.* 1987) in male hamsters, suggesting that BNST may mediate attraction to non-volatile female odors independently from MA. There are several alternative connections that could provide the substrate for generating preference for non-volatile odors. For example, preference for non-volatile female odors may be mediated by direct AOB projections to BNST (Davis *et al.* 1978), whereas preference for volatile female odors appears to be mediated by serial projections from MOB to MA to BNST. Alternatively, BNST connections with other nuclei important for chemosensory responding, such as the posteromedial cortical nucleus of the amygdala (PMCo) (Wood and Swann 2005), may mediate attraction to directly contacted female odors. Indeed, lesions of PMCo cause inappropriate investigation of females during copulatory behavior (Maras and Petrulis 2008). Finally, it is possible that the very sparse, contralateral projections between MA and BNST (Kevetter and Winans 1981; Wood and Swann 2005) are sufficient to maintain preference for female odors when contact is involved. To test this, future studies could combine functional disconnection of MA and BNST with lesions of the anterior commissure, thus preventing cross-hemisphere communication.

The presence of a dissociation between individual brain areas mediating the appetitive and consummatory aspects of reproductive behavior has been suggested before (Everitt 1990). However, previous studies have relied on second-order conditioning tasks, which measure arbitrary responses to learned associations between neutral stimuli and sexual reinforcers and do not necessarily recruit circuitry required for unconditioned sexual attraction (Kippin *et al.* 2003). For example, lesions of the basolateral amygdala (BLA) (Everitt *et al.* 1989), but not MPOA (Everitt and Stacey 1987), decrease

male rats' bar-pressing response for access to a receptive female. BLA is not, however, required for appetitive aspects of copulatory behavior in rats or hamsters (Lehman *et al.* 1980; McGregor and Herbert 1992), but is required for instrumental responding to other non-sexual conditioned stimuli (Cador et al. 1989), suggesting BLA may mediate appetitive responding to conditioned reinforcers generally, not to sexual stimuli specifically. Similarly, although attraction to female odors is an unconditioned response that does not require sexual experience in male hamsters (Maras and Petrulis 2006; Ballard and Wood 2007; Maras and Petrulis 2008; Been and Petrulis 2010a; Been and Petrulis 2010b; Maras and Petrulis 2010c), male hamsters will not bar-press for female hamster vaginal secretion (Coppola and O'Connell 1988), suggesting that natural appetitive behaviors are not always comparable with conditioned appetitive behaviors. The odor preference tests used in the current study model an unconditioned, appetitive response to female odors and therefore better test the neural substrates required for sexual attraction.

Finally, it is important to note that MA, BNST, and MPOA each consist of anatomically, hodologically, and functionally distinct sub-nuclei that may differentially contribute to reproductive behavior (Wood 1997). Indeed, data from our laboratory suggest that anterior and posterodorsal subnuclei within MA differentially regulate odor preference (Maras and Petrulis 2006), and that their interaction is required for appropriate investigation of female odors (Maras and Petrulis). Although the functional significance of BNST and/or MPOA subnuclei has not been directly tested in hamsters, studies in rats (Arendash and Gorski 1983; De Jonge *et al.* 1989; Claro *et al.* 1995), gerbils (Finn and Yahr 1994; Sayag *et al.* 1994; Heeb and Yahr 2000; Finn and Yahr 2005), and Japa-

nese quails (Balthazart and Ball 2007) lend support to the idea that subnuclei within MPOA may regulate distinct aspects of appetitive and consummatory reproductive behavior. A better understanding of the functional microcircuitry of MA, BNST, and MPOA is fundamental to the progress of research on the neural control of reproductive behavior and, ultimately, may demonstrate that the functional dissociation of the pathways required for appetitive and consummatory sexual behaviors is a common mechanism for organizing the neural control of reproductive behavior.

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CHAPTER 5 FIGURES

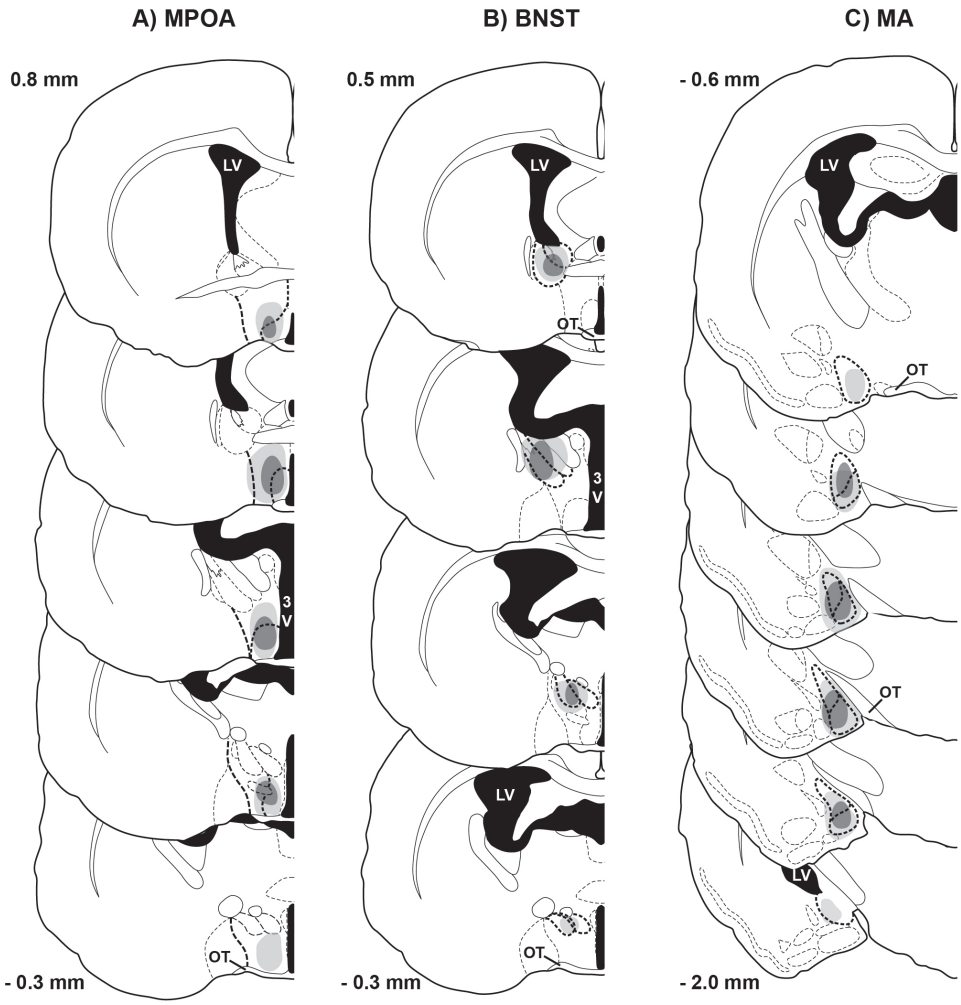

Figure 5.1: Lesion Reconstructions. Coronal sections through the rostral to caudal extent of A) MPOA, B) BNST, and C) MA showing the largest (light gray) and smallest (dark gray) unilateral lesions included in the IPSI and CONTRA groups. Measurements indicate mm relative to bregma, 3V, 3rd ventricle; LV, lateral ventricle; OT, optic tract.

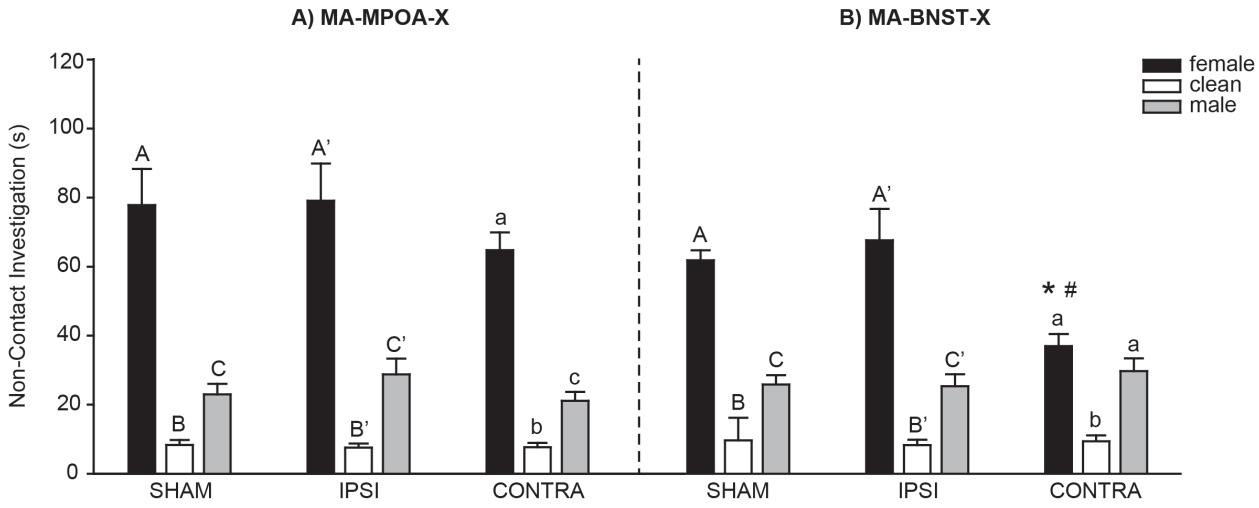

Figure 5.2: Non-Contact Odor Preference. A) In the MA-MPOA-X lesion group, SHAM (*n* = 12), IPSI (*n* = 12), and CONTRA (*n* = 12) males all investigated female odors more than male or clean odors. B) In the MA-BNST-X lesion group, SHAM (*n* = 11) and IPSI (*n* = 14) males investigated female odors more than male or clean odors, but CONTRA (*n* = 12) males investigate female and male odors equally. Furthermore, CONTRA males investigated female odors significantly less than SHAM or IPSI males. Data presented as mean ±SEM. Different letters indicate significant differences between odor conditions, * and # indicate significant differences between lesion groups ($P < 0.05$).

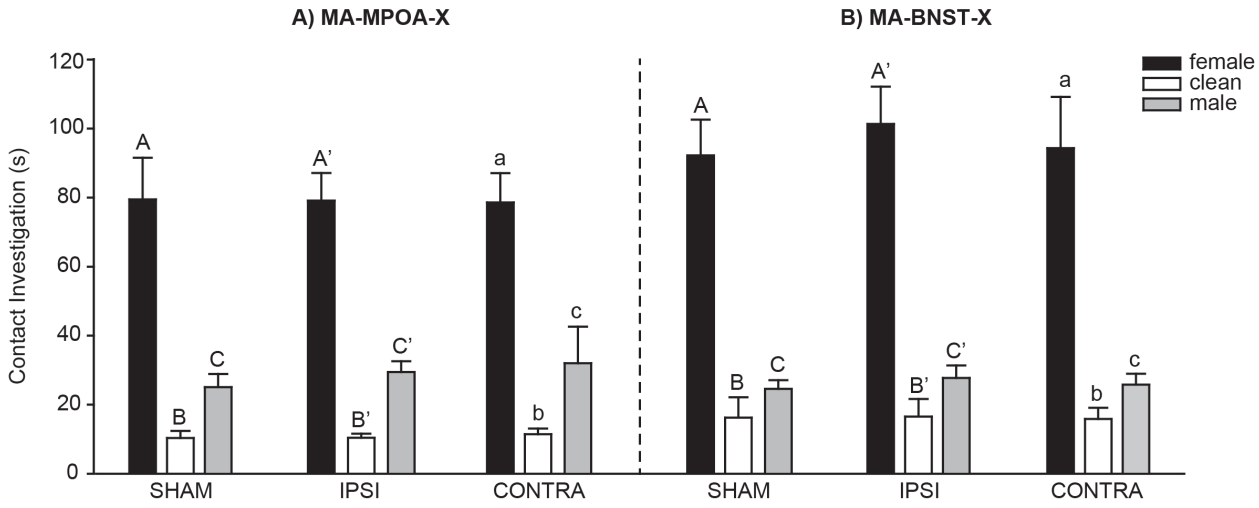

Figure 5.3: Contact Odor Preference. A) In the MA-MPOA-X lesion group, SHAM (*n* = 12), IPSI (*n* = 12), and CONTRA (*n* = 12) males all investigated female odors more than male or clean odors. B) Similarly, in the MA-BNST-X lesion group, SHAM (*n* = 11) and IPSI (*n* = 14) and CONTRA (*n* = 12) males all investigated female odors more than male or clean odors. Data presented as mean ±SEM. Different letters indicate significant differences between odor conditions ($P < 0.05$).

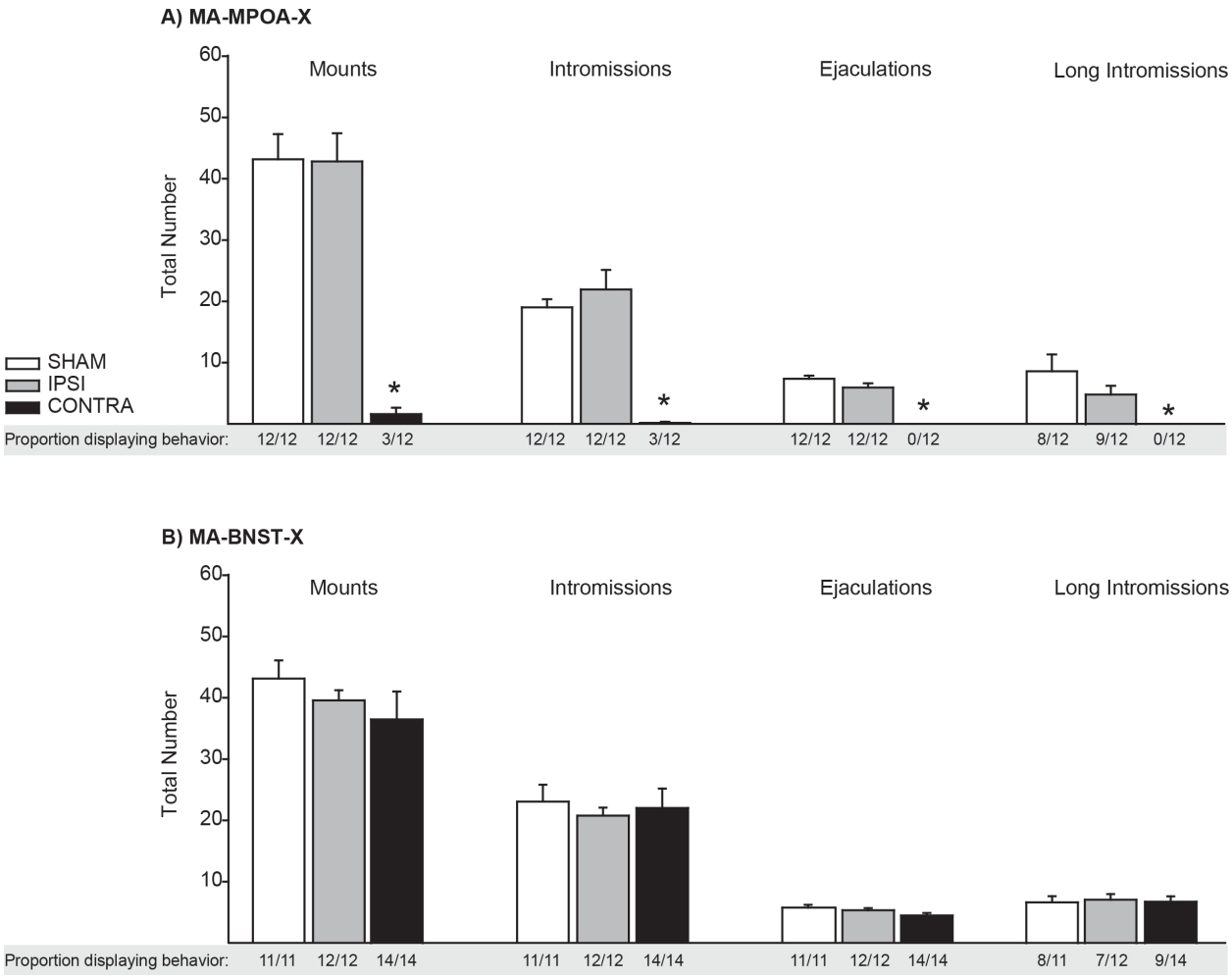

Figure 5.4: Copulatory Behavior. A) In the MA-MPOA-X lesion group, the proportion of CONTRA (*n* = 12) males displaying mounts, intromissions, ejaculations, or long intromissions was significantly lower than SHAM (*n* = 12) or IPSI (*n* = 12) males. B) In contrast, there was no difference between SHAM (*n* = 11), IPSI (*n* = 14), CONTRA (*n* = 12) males in the MA-BNST-X lesion group. Data presented as mean ±SEM. * indicates significant differences between lesion groups ($P < 0.05$).

CHAPTER 5 TABLES

Table 5.1: Stereotaxic coordinates and injection volumes. Unilateral, excitotoxic lesions were made by injecting N-methyl-D-aspartic acid (NMDA, 20 mg/ml) into sites targeting MA, BNST, or MPOA. Anterior-posterior (A-P) and medial-lateral (M-L) coordinates are relative to bregma, whereas dorsal ventral (D-V) coordinates are relative to dura.

	A-P (mm)	M-L (mm)	D-V (mm)	NMDA (nl)
MA	- 0.85	± 2.85	- 7.60	40
BNST	+ 1.85	± 1.35	- 5.90	30
MPOA	+ 2.00	± 0.60	- 7.10	40

CHAPTER 6:

GENERAL DISCUSSION

SUMMARY

In Syrian hamsters, like many rodent species, male reproductive behavior depends on the perception of odor cues from conspecifics in the environment (Johnston 1983; Brennan and Keverne 2004; Zufall and Leinders-Zufall 2007). Converging lines of evidence suggest that a neural circuit including the medial amygdala (MA), posterior bed nucleus of the stria terminalis (BNST), and medial preoptic area (MPOA) generates adaptive behavioral responses to socio-sexual odors. How these three nuclei work together to regulate appetitive and consummatory reproductive behaviors is, however, poorly understood. Therefore, the goal of this dissertation was to determine how BNST and MPOA act individually, and as functional circuit with MA, to generate attraction to female odors and copulatory behaviors in male Syrian hamsters. First, we demonstrated that BNST is required for generating attraction to female odors in both sexually-naïve and sexually-experienced males, as well as for the temporal modulation of copulatory behavior in sexually-naïve males (**Chapter 2**). Next, we found that MPOA is required for attraction to female odors and for copulation in sexually-naïve males, but surprisingly, does not play a role in either behavior in sexually-experienced males (**Chapter 3**). Third, we found that neurons in MA that project directly to BNST and/or MPOA are preferentially activated by female odors, whereas BNST neurons that project directly to MPOA respond equivalently to female and male odors. In addition, we demonstrated that steroid hormone information is preferentially relayed directly to other steroid-sensitive targets within this circuit (**Chapter 4**). Finally, we found that lesions that functionally disconnect MA from MPOA eliminate copulatory behavior but do not affect odor preference in sexually-naïve males. In contrast, lesions that functionally disconnect MA

from BNST eliminate odor preference but do not affect copulatory behavior in sexually-naïve males. These results therefore demonstrate a double dissociation in the functional connections required for attraction to sexual odors and for copulation in male hamsters (**Chapter 5**).

Taken together, these data suggest that distinct neural pathways mediate appetitive and consummatory aspects of reproductive behavior in male hamsters: female odor information processed in MA is preferentially relayed either A) to MPOA in order to generate copulatory behaviors or B) to BNST in order to generate the preferential approach and investigation of female odors. In the following discussion, we will review the functional, neuroanatomical, neurochemical, and experiential factors that influence how this circuit generates the appropriate behavioral responses to social odors, emphasizing elements that may contribute to the dissociated regulation of appetitive and consummatory reproductive behaviors. Furthermore, we will examine the roles of MA, BNST, and MPOA in other social behaviors and in other species, evaluating the evolutionary significance and clinical relevance of this functional circuit. Ultimately, we will conclude that the dissociation of the neural substrates required for appetitive and consummatory reproductive behaviors in male hamsters may reflect a fundamental principle of how the ventral forebrain is organized to regulate social and sexual behaviors.

FUNCTIONAL NEUROANATOMY OF MA, BNST, AND MPOA

Subnuclei in MA differentially regulate reproductive behaviors

MA, BNST, and MPOA are each comprised of multiple subnuclei that differ substantially with regard to their neuroanatomical and neurochemical environments (Wood

1997). Differences in the connectivity of these subnuclei to other brain areas, and in their sensitivity to steroid hormones, may contribute to the dissociated regulation of attraction to female odors and copulatory behavior in male hamsters (Figure 6.1). For example, MA is comprised of anterior (MeA) and posterior (MeP) subnuclei that are hodologically distinct from each other (Canteras et al. 1995). MeA receives direct and indirect projections from the main (MOB) and accessory (AOB) olfactory bulbs, whereas MeP receives only limited chemosensory input from AOB (Scalia and Winans 1975; Davis *et al.* 1978). MeA also has broader connections than MeP within the amygdala and throughout the basal forebrain (Scalia and Winans 1975; Kevetter and Winans 1981; Lehman and Winans 1982; Coolen and Wood 1998) (Figure 6.2A). These anatomical data suggest that MeA is better positioned than MeP to process and relay a wide variety of social odor stimuli. Therefore, MeA may play a role in regulating many odor-guided social behaviors, not just reproductive behaviors. This idea is supported by the finding that neurons in MeA are activated by many kinds of social odors (Day et al. 2004; Meredith and Westberry 2004) and by different types of social behaviors (Kollack-Walker and Newman 1995). Furthermore, in male hamsters, lesions of MeA eliminate preference for volatile opposite-sex odors by increasing investigation of both male and female odors (Maras and Petrulis 2006), suggesting a deficit in the ability to appropriately categorize social odors. This disruption in social odor categorization may be responsible for the severe deficits in appetitive and consummatory reproductive behaviors observed following lesions of MeA (Lehman *et al.* 1980).

Although it sends and receives fewer chemosensory projections than MeA, MeP contains vastly more steroid hormone receptor-bearing neurons than MeA (Doherty and

Sheridan 1981; Wood *et al.* 1992; Been and Petrulis 2011). Several lines of evidence suggest that MeP may generate appetitive and consummatory reproductive behaviors via the activation of these hormone-sensitive neurons. First, steroid hormones are required for appetitive and consummatory aspects of reproductive behavior in male hamsters (Steel 1982; Ballard and Wood 2007) and implants of testosterone or estradiol into MeP can reinstate reproductive behavior in gonadectomized males (Wood and Newman 1995c; Wood 1996). Furthermore, steroid-sensitive neurons in MeP are activated during mating (Wood and Newman 1993). Finally, lesions of MeP cause deficits in appetitive and consummatory reproductive behaviors in male hamsters (Lehman *et al.* 1983; Maras and Petrulis 2006) and eliminate appetitive aspects of reproductive behavior in male rats (Kondo et al. 1998; Kondo and Sachs 2002). These data therefore suggest that, unlike MeA, MeP may regulate reproductive behavior specifically via the activation of steroid-sensitive neurons.

It is important to note that MeA and MeP maintain strong, bidirectional connections with each other (Coolen and Wood 1998), thereby providing a substrate for MeA to transfer social odor information to MeP. Indeed, female and male odor-responsive cells in MeA project directly to MeP (Maras and Petrulis 2010a) and lesions of MeA decrease the immediate early gene response to social odors in MeP (Maras and Petrulis 2010b). Furthermore, lesions that disrupt the functional connections between MeA and MeP eliminate opposite-sex odor preference in male hamsters (Maras and Petrulis 2010c), suggesting that social odor information from MeA must reach MeP in order to elicit the appropriate behavioral response. It is unknown, however, if social odor-responsive neurons in MeA synapse directly onto steroid-sensitive neurons in MeP to regulate repro-

ductive behavior, or if the integration of chemosensory and hormone signals required for appropriate reproductive behaviors (Wood 1998) takes place downstream of MA.

Subnuclei in BNST differentially regulate reproductive behaviors

Like MA, BNST is also comprised of anatomically distinct, densely interconnected subnuclei: the posteromedial (BNSTpm), posterointermediate (BNSTpi) and posterolateral (BNSTpl) BNST (Wood and Swann 2005). Although BNSTpm receives a small, direct projection from AOB (Scalia and Winans 1975; Davis *et al.* 1978), most of the chemosensory information BNST receives is from MA. Specifically, MeA is densely and reciprocally connected to BNSTpi, whereas MeP is densely and reciprocally connected to BNSTpm (Kevetter and Winans 1981; Coolen and Wood 1998; Wood and Swann 2005) (Figure 6.1). In addition to receiving chemosensory input from MA, BNST also receives afferents from the anterior cortical nucleus of the amygdala (ACo) and posteromedial cortical nucleus of the amygdala (PMCo), as well as from the piriform, infralimbic, and insular cortices (Wood and Swann 2005) (Figure 6.2B). These numerous olfactory inputs provide several substrates by which BNST may regulate the appropriate behavioral responses to odors, including males' attraction to female odors. In contrast, BNSTpl is not connected to the corticomedial amygdala, but instead has extensive connections with other amygdala nuclei that are involved in non-reproductive motivated behaviors, such as the central (Ce) and basolateral (BLA) amygdala (Wood and Swann 2005); it therefore seems less likely that BNSTpl plays a strong role in mediating attraction to female odors.

The separation of chemosensory and hormone information is maintained in BNST subnuclei. As in MeA, few neurons in BNSTpi express steroid hormone receptors

(Doherty and Sheridan 1981; Wood *et al.* 1992; Been and Petrulis 2011), whereas BNSTpm, like MeP, contains dense populations of steroid hormone receptor-bearing neurons (Doherty and Sheridan 1981; Wood *et al.* 1992; Been and Petrulis 2011) that are activated during mating (Wood and Newman 1993). Furthermore, BNSTpi preferentially projects to the steroid-poor lateral aspects of MPOA, whereas BNSTpm maintains reciprocal connections with MPN and other steroid-sensitive hypothalamic nuclei (Wood and Swann 2005). Unlike MA subnuclei, which project unidirectionally to MPOA, BNSTpm maintains reciprocal connections with MPOA, thereby placing it in the unique position to exchange real-time feedback with both MA and MPOA as new odor information becomes available. These connections with BNSTpm therefore provide a potential substrate for the flexible updating of incoming odor information that would be required to execute complex appetitive reproductive behaviors, such as odor preference. The idea that BNSTpm in particular is critical for appetitive sexual behaviors is further supported by the finding that targeted lesions of BNSTpm in male rats decrease non-contact erections in response to volatile female odor cues (Liu *et al.* 1997b) and decrease anogenital investigation of females during copulation (Claro *et al.* 1995), but do not eliminate copulatory behaviors (Claro *et al.* 1995).

In addition to connectivity with other chemosensory and steroid-sensitive areas, all three BNST subnuclei are reciprocally connected to the ventral striatum (Wood and Swann 2005). Functional data in rats and hamsters suggest that connections between BNST and NAc may contribute to sexual attraction in males. First, exposure to female odors increases immediate early gene activity in NAc in male rats (Kippin *et al.* 2003), suggesting NAc cells are activated by female odors. Second, lesions of NAc eliminate

non-contact erections in response to remote cues from estrous females in sexually-naïve male rats (Kippin et al. 2004), suggesting that NAc plays a role in mediating appetitive aspects of reproductive behavior. Finally, lesions of BNST increase female odor-induced Fos expression in NAc (Been and Petrulis 2008), suggesting that BNST may send inhibitory projections to NAc. Given that the functional connections between MA and BNST are required for sexual odor preference (Chapter 5), it is possible that the appropriate investigation of sexual odors relies on serial connections from MA to BNST to NAc (Figure 6.3C). Furthermore, ventral striatum connections with brainstem motor areas may play a role in initiating the approach and investigation of sexual odors (Mogenson et al. 1980; Newman and Winans 1980). It is also worth noting that the strength of these BNST connections with NAc is unique to hamsters; in male rats, only the BNSTpl projects to the ventral striatum. It is possible that this interspecies difference in neuroanatomy may underlie interspecies differences in odor-guided reproductive behaviors, such as the requirement of sexual experience to display a preference for female odors in rats, but not in hamsters.

Subnuclei in MPOA differentially regulate reproductive behaviors

Despite interspecies variability in how the preoptic area is subdivided, it is generally agreed that it can be split into three interconnected zones: periventricular, medial, and lateral (Simerly et al. 1984). In hamsters, the periventricular zone consists of the para- and peri- ventricular nuclei, the medial zone consists of the median preoptic nucleus and MPN, and the lateral zone consists MPOA (Morin and Wood 2001). An additional subnucleus that is unique to hamsters, the magnocellular medial preoptic nucleus (MPNmag), straddles the border between the medial and lateral zones at the most cau-

dal aspects of MPN and MPOA (Swann *et al.* 2003). These preoptic subnuclei receive sparse cortical input; other than weak projections from the infralimbic and prelimbic cortices (Simerly and Swanson 1986; Wang and Swann 2006), all telencephalic input to MPOA originates from limbic nuclei. Specifically, MPN receives dense projections from MA and BNST (see above), as well as from ACo, PMCo, NAc, the amygdalohippocampal area (AHA), and the subiculum (Simerly and Swanson 1986; Wang and Swann 2006) (Figure 6.2).

Although MeA/BNSTpi preferentially project to the lateral zone of MPOA and MeP/BNSTpm preferentially project to the medial zone of MPOA, the separation of chemosensory and hormone information is less distinct at the level of preoptic subnuclei. In hamsters, the MPNmag has dense populations of steroid hormone receptor-bearing cells and also receives chemosensory information from ACo, MA, and BNST (Wang and Swann 2006). The integration of MOS/AOS information and hormone cues *within* this subnucleus, as opposed to between subnuclei in MA and BNST, may play a critical role in mediating appetitive reproductive behaviors. Indeed, following long-term castration, testosterone implants into MPOA restore anogenital investigation in males with contralateral, but not ipsilateral, olfactory bulbectomies, suggesting chemosensory and hormone cues must be integrated in MPOA to generate appetitive aspects of reproductive behavior (Wood and Newman 1995b). In addition, the direct projection from ACo to MPNmag provides an alternative pathway for MOS information to reach MPOA and may therefore reconcile the seemingly disparate findings that bilateral lesions of MPOA eliminate preference for volatile female odors (Been and Petrulis 2010b), but le-

sions that functionally disconnect MA and MPOA do not eliminate volatile odor preference (Chapter 5).

MPOA also has strong connections with areas in the midbrain and hindbrain that project directly to the spinal cord, including PAG, the nucleus paragigantocellularis (nPGi), and the central tegmental field (CTF) (Simerly and Swanson 1986; Simerly and Swanson 1988; Wang and Swann 2006) (Figure 6.2C). These projections have been shown to mediate the genital reflexes required for copulation in male rats. For example, stimulation of MPOA elicits the urethrogential reflex in male rats (Marson and McKenna 1994) and lesions of PAG prevent this stimulation-induced reflex (Marson 2004), suggesting that activating MPOA projections to PAG can overcome nPGi inhibition of genital reflexes in order to achieve erection and ejaculation during copulation (Marson et al. 1992; Normandin and Murphy 2011a). MPOA also receives input indirectly from all sensory systems and sends reciprocal connections back to those sources (Simerly and Swanson 1986), likely contributing to its critical role in male copulatory (Hull *et al.* 2002). Of particular relevance to the current findings, simultaneous unilateral lesions of MA and CTF reduce mating-induced Fos expression in the ipsilateral MPOA of male rats, whereas lesions of either area alone do not affect Fos expression (Baum and Everitt 1992). As such, the requirement of the functional connections between MA and MPOA for copulatory behavior (Chapter 5) may partially reflect MPOA's integration of chemosensory information from MA with genital somatosensory input from CTF in order to generate copulatory behaviors. Together, these data suggest that the regulation of consummatory reproductive behavior by MPOA likely depends on its projections to mid- and hindbrain nuclei important for somatosensory processing and genital reflexes.

NEUROCHEMICAL MODULATION OF MALE REPRODUCTIVE BEHAVIOR IN MA, BNST, AND MPOA

GABA and Glutamate

Although the distribution of GABAergic and glutamatergic cells in MA, BNST, and MPOA has not been mapped in hamsters, all three brain areas have dense populations of cells that express GABA and glutamate in rats (Ottersen and Storm-Mathisen 1984; McDonald 1996; Sagrillo and Selmanoff 1997; Stefanova 1998; Stefanova et al. 1998), mice (Ottersen and Storm-Mathisen 1984; Choi *et al.* 2005), and gerbils (Simmons and Yahr 2003; Simmons et al. 2011). In male rats, MA and BNST both send strong glutamatergic projections to MPOA (Kocsis et al. 2003) and glutamate activity in MPOA may be particularly important for consummatory aspects of reproductive behavior. For example, mating activates NMDA glutamate receptors in MPOA and this activation is necessary for the expression copulatory behavior (Dominguez et al. 2007). Furthermore, administration of glutamate into the MPOA of anesthetized rats increases penile erections (Giuliano et al. 1996) and extracellular glutamate increases in MPOA following ejaculation (Dominguez et al. 2006). Therefore, a simple model for how functional connections between MA and MPOA may mediate copulatory behavior is that female odors could activate excitatory projection neurons in MA and the resulting release of glutamate into MPOA could activate neurons that project to downstream targets required for genital reflexes (Figure 6.3A). In addition, genital somatosensory information obtained during copulation may feed back to MPOA to maintain glutamate levels throughout the copulatory sequence.

Surprisingly, there is minimal evidence available to suggest how GABA may regulate male reproductive behavior within this ventral forebrain circuit. In male gerbils, both glutamatergic and GABAergic neurons in MA and MPOA are activated by ejaculation (Simmons and Yahr 2003), suggesting that both excitatory and inhibitory transmission are required for copulatory behavior. Therefore, it is possible that male odors may activate GABAergic neurons in MA that either a) project directly to MPOA to inhibit the activation of downstream genital reflexes or b) act locally to inhibit excitatory projections to MPOA. This model of female odors exciting MA neurons and male odors inhibiting MA neurons may also carry over to the regulation of appetitive aspects of reproductive behavior. For example, excitatory (female odor-activated) and inhibitory (male odor-activated) projections from MA to BNST may drive preference for female odors (Figure 6.3A). These models are undoubtedly an oversimplification of the roles of GABA and glutamate in regulating reproductive behaviors, but it is difficult to make stronger predictions given the paucity of available data. A better understanding of the roles of these ubiquitous neurotransmitters in appetitive and consummatory aspects of male reproductive behavior is vital to progress in the field of behavioral neuroendocrinology.

Dopamine

There is a large body of evidence to suggest that dopamine (DA) plays a critical role in the ventral forebrain regulation of reproductive behaviors. In hamsters, MA, BNST, and MPOA all contain neurons that are immunoreactive for tyrosine hydroxylase (TH), the rate-limiting enzyme in the catecholamine biosynthetic pathway, and neurons in MeP, BNSTpm and PVH are immunoreactive for DA itself (Asmus et al. 1992; Asmus and Newman 1993; Asmus and Newman 1994). TH-immunoreactive neurons also co-

localize with AR, suggesting that steroid hormones may interact with catecholamines in this network to influence reproductive behaviors (Asmus and Newman 1993). In addition to local DA activity, MA, BNST, and MPOA are also connected with the mesolimbic, nigrostriatal, and incertohypothalamic DA pathways (see above). Hull and colleagues have proposed a model for the central control of male sexual behavior in which these three pathways work in concert to control both appetitive and consummatory aspects of reproductive behavior in male rats (Hull et al. 2004). In this model, increased DA in the mesolimbic system is important for sexual motivation and reinforcement, and increased DA in the incertohypothalamic system is important for genital reflexes, motor patterns of copulation, and possibly sexual motivation (Hull *et al.* 2004; Dominguez and Hull 2005). Our finding that MPOA is required for both appetitive and consummatory aspects of reproductive behavior (Been and Petrulis 2010b) is in agreement with this model. Furthermore, this model predicts that MA projections to BNST may mediate appetitive reproductive behaviors via connections with the mesolimbic DA pathway, whereas MA projections to MPOA may mediate consummatory reproductive behavior via connections with the incertohypothalamic system (Figure 6.3A).

MPOA in particular is a critical site for DA regulation of reproductive behaviors. Pharmacological studies have demonstrated that DA in MPOA is critical for copulatory behavior and genital reflexes in male rats (Hull et al. 1986; Pehek et al. 1988; Pehek et al. 1989). Similarly, microinjections of DA antagonists in MPOA reduce the percentage of times male rats choose a receptive female over a non-receptive female in an x-maze (Warner et al. 1991) and decrease males' anticipatory level changing in a bi-level mating chamber (Pfaus and Phillips 1991), suggesting that DA in MPOA is important for

appetitive aspects of reproductive behavior, such as sexual motivation. In fact, DA may be especially critical for chemosensory aspects of appetitive reproductive behaviors, as exposure to female odors increases MPOA DA activity in male rats (Hull et al. 1995) and hamsters (Schulz et al. 2003) and unilateral olfactory bulb removal prevents mating-induced increases in DA in male hamsters' ipsilateral MPOA (Triemstra et al. 2005). Importantly, MA connections to MPOA may mediate this effect, as stimulation of MA increases DA release in MPOA (Dominguez and Hull 2001), whereas lesions of MA decrease mating-induced increases in DA in MPOA (Dominguez et al. 2001). Furthermore, activating DA receptors in MPOA can restore copulation in male rats with MA lesions (Dominguez *et al.* 2001), suggesting that MA facilitates copulation by increasing DA in MPOA. As such, our finding that disconnecting MA from MPOA eliminates copulation (Chapter 5) may result from blocking MA-induced DA release in MPOA.

Serotonin

In contrast to DA, serotonin (5-HT) is generally regarded as inhibitory to male sexual behavior. Decreasing serotonergic activity in the brain facilitates copulatory behavior in male rats (Salis and Dewsbury 1971; Ahlenius et al. 1987; Kondo and Yamanouchi 1997) and mice (Rodriguez-Manzo et al. 2002), whereas increasing 5-HT in the brain impairs copulatory behavior in hamsters (Boscarino and Parfitt 2002) and rats (Larsson et al. 1978; Fernandez-Guasti et al. 1992). As is the case with DA, 5-HT may exert its effect on copulatory behavior via MPOA projections to mid- and hindbrain nuclei that mediate copulatory reflexes. This idea is supported by the finding that microinjections of 5-HT into MPOA impair copulation and penile reflexes in male rats (Fernandez-Guasti *et al.* 1992; Matsumoto et al. 1997). In addition, 5-HT-specific le-

sions of PAG, a source of serotonergic input to nPGi, facilitate genital reflexes in male rats (Normandin and Murphy 2011b) and most of the axons projecting from nPGi to the spinal cord also contain 5-HT (Marson and McKenna 1992). These data suggest that nPGi lesion-induced facilitation of copulatory behavior may be due to disrupting serotonergic transmission from PAG to nPGi (Marson *et al.* 1992; Normandin and Murphy 2011a). Given the finding that the functional connections between MA and MPOA are required for copulation in male hamsters (Chapter 5), it is possible that MA may project to separate populations of dopaminergic and serotonergic cells in MPOA to facilitate and inhibit sexual behavior, respectively, based on odor context (Figure 6.3B).

The role of 5-HT in appetitive aspects of reproductive behavior has not been investigated as extensively, but recent evidence from studies using transgenic mice suggests that 5-HT may also be important for the appropriate behavioral response to female odors (Liu et al. 2011). Unlike males with physiological levels of 5-HT, males in which a homozygous deletion of the *Lmx1b* allele prevents the development of 5-HT neurons do not have a preference for female odors. Similarly, transgenic males lacking tryptophan hydroxylase 2, the rate-limiting enzyme for the synthesis of 5-HT, also investigate female and male odors equivalently. Restoring 5-HT to wild-type levels in these tryptophan hydroxylase 2 knockouts, however, restores preference for female odors (Liu *et al.* 2011). Together, these data suggest that 5-HT is necessary and sufficient for opposite-sex odor preference. It is unknown, however, where in the brain 5-HT is acting to mediate this effect. It is possible that MA projections to BNST may play a role in 5-HT regulation of odor preference, as neurons in both MA and BNST receive projections

from the raphe nuclei (Coolen and Wood 1998; Wood and Swann 2005) and express receptors for 5-HT (Carrillo et al. 2010) (Figure 6.3B).

Vasopressin and Oxytocin

In many species, MA and BNST contain dense populations of vasopressin (AVP)-producing neurons that are sexually-dimorphic (De Vries and Panzica 2006), steroid-sensitive (DeVries et al. 1985; Miller et al. 1992), and innervate other forebrain regions important for socio-sexual behaviors (De Vries and Buijs 1983). Syrian hamsters, however, lack a homologous population of AVP-producing cells (Albers et al. 1991; Miller et al. 1999), suggesting AVP production in the extended amygdala does not play a large role in regulating reproductive behaviors in male hamsters. BNST and MPOA, however, contain cells that express receptors for AVP in hamsters (Dubois-Dauphin et al. 1990; Irvin et al. 1990; Young et al. 2000) and microinjection studies suggest AVP binding in these areas may regulate odor-guided social behaviors. For example, microinjections of AVP into MPOA (Ferris et al. 1984) and the LS/BNST axis (Irvin *et al.* 1990) trigger flank marking, whereas microinjections of AVP antagonists into MPOA inhibit flank marking (Ferris et al. 1985). MA, in contrast, does not appear to respond directly to AVP, as AVP receptor expression is weak in MA (Miller *et al.* 1992; Young *et al.* 2000) and blocking AVP receptors in MA does not prevent the formation of partner preference in prairie voles (Lim and Young 2004). Lesions of MA, however, decrease affiliative behavior towards females in male prairie voles (Kirkpatrick et al. 1994), suggesting that social odor information processed in MA may be relayed to AVP-sensitive cells in BNST and MPOA (as well as other AVP-sensitive forebrain sites) to regulate odor-guided social behaviors (Figure 6.3C).

Oxytocin (OT) in the ventral forebrain is also important for the appropriate behavioral response to social odors. In male mice, blocking OT receptors in MA prevents recognition of familiar females, whereas site-specific injections of OT into MA restores this recognition in OT-knockout males (Ferguson et al. 2001), suggesting that OT binding in MA is necessary and sufficient for social recognition. Furthermore, in male mice, central injections of OT antagonist block the immediate early gene response to female urine and other biological odors in MA (Samuelsen and Meredith 2011), suggesting OT influences the ability of MA to properly categorize sexual and social odors. OT also regulates odor-guided social behaviors in MPOA, as microinjections of OT in MPOA stimulate flank marking in male hamsters (Albers and Ferris 1985) and infusion of OT antagonist into either MPOA or BNST eliminates female hamsters' preferential scent-marking to opposite-sex odors (Martinez et al. 2010). Finally, although OT activity in MPOA is not necessary for the expression of male sexual behavior, microinjections of OT in MPOA stimulate anogenital investigation, decrease the latency to initiate copulation, and increase copulatory efficiency male rats, suggesting OT in MPOA is sufficient to facilitate appetitive and consummatory aspects of male reproductive behavior (Gil et al. 2011). Together, these data suggest that OT acts in MA, BNST, and MPOA to regulate chemosensory aspects of social and sexual behaviors (Figure 6.3C).

EXPERIENCE-DEPENDENT PLASTICITY AND REPRODUCTIVE BEHAVIORS

The finding that sexual experience can mitigate the necessity of BNST (Chapter 2) and MPOA (Chapter 3) for appetitive and consummatory aspects of reproductive behavior is consistent with previous research in hamsters suggesting that social and/or

sexual experience can alter the behavioral and physiological responses to sexual odors (Meredith 1986; Pfeiffer and Johnston 1994). One potential mechanism for these experience-dependent changes is that sexual experience may lead to a more distributed processing of sexual odors within the ventral forebrain. In support of this idea, exposing sexually-naïve male rats to estrous female odors increases immediate early gene expression in MA, but not in BNST or MPOA; in contrast, exposing sexually-experienced males to estrous female odors increases immediate early gene expression in MA, BNST, and MPOA (Hosokawa and Chiba 2005). Experience-dependent changes in social odor processing may result from the conditioning of female odors during copulatory experience. Indeed, exposing male rats to an artificial odor that was previously paired with a receptive female activates a different neural pathway than exposure to estrous female odors alone (Kippin *et al.* 2003). The fact that these two pathways overlap in NAc is perhaps not surprising, as evidence from other studies suggest that NAc may be particularly critical for plasticity following rewarding experiences, such as copulation. For example, sexual experience induces functional and morphological changes (Bradley et al. 2005; Pitchers et al. 2010a) and increases expression of the transcription factor Delta FosB (Meisel and Mullins 2006; Pitchers et al. 2010b) in NAc. Furthermore, overexpression of Delta FosB in NAc mimics the behavioral effects of sexual experience in female hamsters (Hedges et al. 2009). As such, plasticity in NAc may be a critical component of changes in brain-behavior relationships following sexual experience. It is possible that ventral forebrain projections to NAc (see above) may mediate the effects of sexual experience on the requirement of BNST and MPOA for odor preference and copulatory behavior.

An alternative mechanism by which sexual experience may alter the behavioral response to sexual odors is via adult neurogenesis. In female mice, exposure to opposite-sex odors enhances neurogenesis in the hippocampus and olfactory bulbs (Mak et al. 2007). Similarly, in male rats, acute or chronic sexual experience enhances adult neurogenesis in the hippocampus (Leuner et al. 2010). As such, sexual experience-induced neurogenesis in these areas may create new pathways to ventral forebrain targets that diminish the requirement of BNST and/or MPOA for the appropriate behavioral response to sexual odors. Interestingly, in the later study, acute sexual experience resulted in elevated glucocorticoid levels and limited neurogenesis, whereas chronic sexual experience did not elevate glucocorticoid levels and resulted in more widespread neurogenesis (Leuner *et al.* 2010). It is therefore possible that sexual experience-induced neurogenesis in the hippocampus results in increased inhibition of the HPA axis during sexual encounters. The subsequent decrease in binding of glucocorticoid receptors in MA, BNST, MPOA, and other glucocorticoid-sensitive forebrain targets (Ahima and Harlan 1990) may create a permissive environment for the regulation of reproductive behavior. Alternatively, sexual experienced-induced neurogenesis in the hippocampus may directly influence MA, BNST, or MPOA, which, in turn, may influence HPA axis responsivity via direct connections with PVH (see above). This idea is supported by the finding that lesions of MA (Dayas et al. 1999; Solomon et al. 2010), BNST (Choi et al. 2008), and MPOA (Viau and Meaney 1996) each alter HPA axis responses to stressors. Either way, sexual experience-induced neurogenesis may habituate the stress response to conspecific interaction in order to facilitate reproductive behavior in sexually-experienced males.

VENTRAL FOREBRAIN REGULATION OF OTHER SOCIAL BEHAVIORS

Maternal Behavior

Like reproductive behavior, maternal behavior can be divided into appetitive and consummatory phases: approach behaviors that help the female gain access to her pups can be conceptualized as appetitive maternal behaviors, whereas nursing behavior is considered the primary consummatory maternal behavior (Stolzenberg and Numan 2011). Evidence from lesion studies suggests that a neural circuit containing MA, BNST, and MPOA regulates maternal behavior. Specifically, lesions of MA facilitate the expression of maternal behaviors (Numan et al. 1993), whereas lesions of the ventral BNST or MPOA eliminate the expression of maternal behavior (Numan 1974; Numan et al. 1977). It is likely that MA sends pup-derived chemosignals to BNST, MPOA, and other hypothalamic areas in order to generate the appropriate behavioral response, as lesions of MA reduce pup-induced Fos expression limbic and hypothalamic nuclei including BNST and MPOA (Sheehan et al. 2001). Interestingly, the function of these projections can be altered by prior experience, as MA inhibits responding to pups in nulliparous females, but this inhibition is overcome in experienced mothers (Sheehan *et al.* 2001). This echoes the finding that prior sexual experience can mitigate the effect of BNST and MPOA lesions on appetitive and consummatory aspects of reproductive behavior (see Chapters 2 and 3) and it is possible that the same mechanism underlies the effect of experience on these behaviors. Finally, it has been suggested that separate populations of neurons in MPOA may differentially regulate appetitive and consummatory aspects of maternal behavior (Pereira and Morrell 2009), but the functional projections required for this dissociation are unknown.

Fear and Aggression

The neural substrates mediating the expression of male reproductive behavior also overlap substantially with those required for the expression of fear. Specifically, the neural circuits underlying behavioral models of conditioned fear, such as fear-potentiated startle (Davis and Shi 1999) and conditioned defeat (Huhman et al. 2003), have been well characterized. In these models, MA may serve as a gateway for olfactory information that plays a role in conditioned fear, but it is likely not involved in the expression of conditioned fear itself (Walker et al. 2005; Markham and Huhman 2008). Instead, other amygdala nuclei, such as CeA and BLA, mediate the expression of conditioned fear via connections to BNST (Jasnow et al. 2004). Although fear behavior cannot be dissociated into appetite and consummatory phases, the behavioral responses to fearful stimuli can be divided into immediate and long-term responses and the neural substrates underlying these two systems can be dissociated. Specifically, CeA mediates the rapid response to immediate threats, whereas BNST is involved in the slower response system that continues to influence behavior after the fearful stimulus has ended (Walker et al. 2003; Bangasser and Shors 2008; Markham et al. 2009). Like in reproductive behavior, these extended amygdala structures output to hypothalamic nuclei that regulate the expression of autonomic and motor signs of fear via connections to the midbrain and brainstem (Davis et al. 2010). In support of this idea, Fos expression is increased in the MPOA, PAG, raphe, and locus coeruleus of subordinate, but not dominant, male hamsters, following agonistic encounters (Kollack-Walker *et al.* 1997).

Similarly, MA may provide chemosensory input to ventral forebrain targets to mediate the expression of aggressive behaviors. In hamsters, electrical stimulation of

MA increases aggressive responses to intruders (Potegal et al. 1996b), whereas lesions of MA reduce aggression (Potegal et al. 1996a), suggesting that MA normally facilitates aggression. MPOA, in contrast, may not play a large role in the modulation of aggression, as aggressive behavior increases Fos expression in MA and BNST, but not in MPOA (Kollack-Walker and Newman 1995; Delville *et al.* 2000). Instead, MA and BNST may modulate aggressive behavior via projections to AH or VMH (Block et al. 1980). Specifically, separate populations of cells in MA converge on VMH to differentially regulate reproductive and defensive behaviors in mice (Choi *et al.* 2005). As such, the neural pathways mediating aggressive behaviors may rely on dissociated functional pathways from the extended amygdala to distinct hypothalamic targets.

Female Reproductive Behavior

The neuroanatomical, hormonal, and neurochemical environments that make the ventral forebrain well suited to regulate male reproductive behaviors also provide an appropriate environment for the neural regulation of female reproductive behaviors. Like in males, MA is critical for appetitive and consummatory aspects of female reproductive behavior, including the expression of odor preference (Petrulis and Johnston 1999; Kondo and Sakuma 2005; Dibenedictis et al. 2011) and lordosis (Kirn and Floody 1985; Dibenedictis *et al.* 2011). In female hamsters, immediate early gene activity increases in MA in response to opposite-sex odors (delBarco-Trillo et al. 2009) and mating (Shelley and Meisel 2005), consistent with the idea that MA is critical for the appropriate processing of sexual odor information. MA is also critical for neuroendocrine changes associated with female reproductive behavior, including pre-ovulatory prolactin surges (Polston and Erskine 2001) and the establishment of pseudopregnancy (Coopersmith et

al. 1996). The role of MPOA in odor preference and copulation in female hamsters is still under investigation (Martinez and Petrulis 2011b), but lesions of MPOA eliminate odor preference (Xiao et al. 2005; Guarraci and Clark 2006) and facilitate lordosis (Powers and Valenstein 1972; Takeo et al. 1993) in female rats, suggesting MPOA also regulates both appetitive and consummatory reproductive behaviors in females.

Unlike in males, however, lesions of BNST do not disrupt female hamsters' preference for opposite-sex odors, nor the expression of lordosis (Martinez and Petrulis 2011a). This sex difference in the requirement of BNST for odor preference may be explained by sex differences in the morphology of BNST, as BNST is larger and has more cells in males than in females (Noble and Alsum 1975; Bleier et al. 1982; Guillamon and Segovia 1997). Alternatively, this difference might be explained by differences in the behavioral ecology of male and female hamsters. In anticipation of receptivity, female hamsters deposit a trail of vaginal scent marks that lead to her burrow. Males must follow this trail in order to locate the receptive female and copulate with her. As such, attraction to female odors may be more important for successful reproduction in males, whereas appropriate scent marking may be more important for successful reproduction, and therefore a better measure of sexual motivation, in females. Indeed, preferential vaginal marking to opposite-sex odors is disrupted by lesions of MA (Petrulis and Johnston 1999) and BNST (Martinez and Petrulis 2011a) in female hamsters, suggesting that these two nuclei are involved in appetitive sexual behavior in females as well as males. Further studies in females are necessary to determine if the neural pathways for appetitive and consummatory aspects of reproductive behavior can be dissociated.

CONSERVATION OF NEURAL SUBSTRATES OF MALE REPODUCTIVE BEHAVIOR

Neural Control of Reproductive Behavior in Lower Vertebrates

Like in mammals, chemical communication is an important component of intersexual attraction in many species of amphibians and reptiles (Halpern and Martinez-Marcos 2003). Although few studies have examined the function of the extended amygdala in reproductive behavior in lower vertebrates, lesions of the posterior dorsal ventricular ridge, the amniote homologue of the mammalian amygdala (Striedter 1997), eliminate courtship displays in green anole lizards (Greenberg et al. 1984), suggesting that the amygdala is critical for reproductive behavior in reptiles as well as mammals. In contrast to the amygdala, the role of MPOA in reproduction has been well characterized in reptiles. Lesions of MPOA abolish male sexual behavior in lizards (Kingston and Crews 1994) and snakes (Friedman and Crews 1985b), whereas androgen implants into MPOA stimulate male sex behavior in these species (Crews and Morgentaler 1979; Friedman and Crews 1985a; Rozendaal and Crews 1989). Furthermore, copulatory behavior increases 2-deoxyglucose uptake in MPOA in male lizards (Rand and Crews 1994), suggesting MPOA is activated during copulation. Finally, in fishes, increased volume and neuron number in the MPOA is correlated with male-typical sexual behaviors. Specifically, intra- and intersexual dimorphisms in GnRH- and AVT-positive neurons in MPOA correlate with sexual maturation (Grober et al. 1994) and/or male transformation in sexually-plastic fishes (Bass and Grober 2001). These data suggest that, like in mammals, the amygdala and MPOA are required for appetitive and consummatory aspects of male sexual behavior in lower vertebrates.

Neural Control of Reproductive Behavior in Avian Species

Social odor processing in the ventral forebrain may modulate avian reproductive behaviors, but likely does not play a critical role. Indeed, occlusion of the naris decreases mating-induced Fos expression in the ventral forebrain, but does not disrupt mating behavior in Japanese quails (Balthazart and Taziaux 2009). Despite this difference in sensory primacy, however, the neural substrates that regulate reproductive behavior are strikingly similar in rodents and birds. In avian species, the homologue of MA is the nucleus taeniae (TnA) (Zeier and Karten 1971; Cheng et al. 1999). Like MA, TnA receives projections from the olfactory bulbs (Reiner and Karten 1985), projects to extended amygdala and hypothalamic targets (Cheng *et al.* 1999), and is sensitive to steroid hormones (Balthazart and Surlemont 1990a; Aste et al. 1998; Balthazart et al. 1998b). Similarly, functional studies indicate that TnA plays a role in both appetitive and consummatory aspects of reproductive behavior in Japanese quails (Thompson et al. 1998; Absil et al. 2002). Lesions of TnA also reduce social facilitation of feeding in male European starlings, suggesting that, like MA, TnA may modulate many social behaviors, not reproductive behavior specifically. BNST also regulates reproductive behaviors in birds, but unlike in hamsters, is more critical for consummatory than for appetitive aspects of reproductive behaviors in avian species. For example, in Japanese quails, lesions of BNST decrease mounting attempts and cloacal contact movements, but do not affect the amount of time males spend viewing a female through a window (Balthazart *et al.* 1998a). Studies using zebra finches as a model also suggest that BNST does not mediate appetitive aspects of reproductive behavior in birds, but instead may play a role in processing positively-valenced social stimuli that promote affiliate behaviors (Goodson

and Wang 2006; Goodson *et al.* 2009). Finally, detailed functional and anatomical studies in Japanese quail have demonstrated that large lesions including both the rostral MPOA and MPN eliminate appetitive and consummatory reproductive behaviors, whereas lesions of the caudal MPOA eliminate consummatory behaviors (Balthazart *et al.* 1998a). These data suggest that sub-regions in MPOA differentially regulate appetitive and consummatory aspects of avian reproductive behavior. It is possible that this is a common mechanism among vertebrates, but functional data regarding sub-regions of MPOA is limited in other species, including rodents.

Neural Control of Reproductive Behavior in Primates

As in birds, the importance of social odors for the expression of reproductive behaviors is debatable in primates (Michael and Keverne 1968; Wysocki and Preti 2004). Studies in both human and non-human primates, however, suggest that the extended amygdala and hypothalamus are important for processing social and sexual stimuli. Early studies in non-human primates found that bilateral removal of the temporal lobes resulted in hyper-sexuality and profound changes in emotional behavior (Bucy and Kluver 1955). More targeted lesions of the amygdala also cause deficits in social behaviors, including increased social affiliation, decreased anxiety, and reduced responses to threatening stimuli (Emery et al. 2001; Machado and Bachevalier 2008; Machado et al. 2008). Together, these data suggest that the amygdala is critical for generating the appropriate behavioral response to social cues in non-human primates. As in every other species studied to date (Hull *et al.* 2002), MPOA is also critical for reproductive behavior in non-human primates. Lesions of MPOA eliminate appetitive and consummatory aspects of male sexual behavior in marmosets (Lloyd and Dixson 1988) and macaques

(Slimp et al. 1978). Furthermore, functional imaging studies in macaques demonstrate activation of MPOA during exposure to odors from estrous females (Ferris et al. 2001), suggesting that MPOA may play a role in mediating the appropriate behavioral response to sexual odors in primates.

In humans, the amygdala, caudal BNST, and preoptic hypothalamus (also known as the interstitial nucleus of the anterior hypothalamus, INAH) are sexually dimorphic, with men having larger volume and cell density than women (Allen and Gorski 1990; Kruijver et al. 2000; Goldstein et al. 2001; Garcia-Falgueras and Swaab 2008). Consistent with the idea that MA processes many types of social stimuli, functional studies of the human amygdala point to its involvement in many social behaviors, including face processing, identification of emotion, social judgment, and threat detection (Adolphs 2003; Adolphs 2010), rather than having a specific role in reproductive behavior. In contrast, studies in people suggest that sexual dimorphisms in BNST and INAH may have functional implications for gender identity and sexual attraction. Specifically, in people who identify as transgendered, the volume and density of INAH and BNST more closely match the individual's self-identified gender than their biological gender (i.e. primary sex characteristics) (Kruijver *et al.* 2000; Garcia-Falgueras and Swaab 2008). That is, in male-to-female transsexuals, INAH and BNST display female-typical volumes and cell numbers, whereas in female-to-male transsexuals, INAH and BNST display male-typical volumes and cell numbers. It is possible that structural dimorphisms in MPOA may also have functional implications for sexual preference. For example, it has been reported that homosexual men have a smaller, female-typical volume in INAH (LeVay 1991). These data must be interpreted cautiously, however, as they have been difficult to repli-

cate (Byne et al. 2001), are based on small sample sizes, and deal with complex social constructs. Despite this caveat, homologs of BNST and MPOA may play a role in gender identity and/or sexual preference in humans. Ultimately, the similar requirement of MA, BNST, and MPOA for reproductive behavior in rodents, lower vertebrates, birds, and primates suggests that the role of these nuclei, and possibly the projections between them, may be conserved across a variety of species.

Clinical Relevance

Disorders that affect social behavior are highly prevalent, ranging from the approximately 2.4 million American adults (1.1% of the population) diagnosed with schizophrenia up to the 20.9 million American adults (9.5 % of the population) diagnosed with a mood disorder in a given year (NIMH 2008). In addition to decreasing the quality of life of affected individuals, disorders related to social behavior can negatively impact family members as well as the general public. For example, the lifetime cost of caring for a child with autism can range from 3.5 to 5 million dollars, and the United States spends nearly 90 billion dollars annually in autism-associated costs (ASA 2009). Although a large body of research has recognized that deficits in social processing contribute to the etiology of several psychopathological disorders, the basic mechanisms by which the brain processes social information remain largely unknown. A growing body of evidence, however, suggests that a common feature of these wide-ranging disorders is abnormalities within the limbic system (Byrum et al. 1997), a collection of brain nuclei critically involved in social and communicative behaviors (Anckarsater 2006). It is difficult, however, to use this information to predict who is at risk of developing psychopathology, or to preempt the disease process in affected individuals, because it is not

clear how the limbic system regulates social behavior in typically developing individuals. To investigate this question, we use an animal model in which relatively simple social interactions depend critically on the limbic system. The findings of this research provide important new information about how the limbic system processes social information to adaptively regulate behavior and can therefore point to targets for potential therapeutic intervention in clinical conditions where social behavior is disrupted.

CONCLUSIONS

The anatomical, hormonal, and neurochemical environments in which they reside contribute substantially to how MA, BNST, and MPOA regulate the expression of odor-guided reproductive behaviors. In addition, experiential and contextual factors can influence how these nuclei process social and sexual odors. Despite this complexity, MA, BNST, and MPOA are critical for generating adaptive responses to social and sexual cues in many species, suggesting that the function of these nuclei in regulating social behaviors is highly conserved. As such, the dissociation of the neural substrates required for appetitive and consummatory reproductive behaviors in male hamsters may reflect a fundamental principle of how the ventral forebrain is organized to regulate social and sexual behaviors.

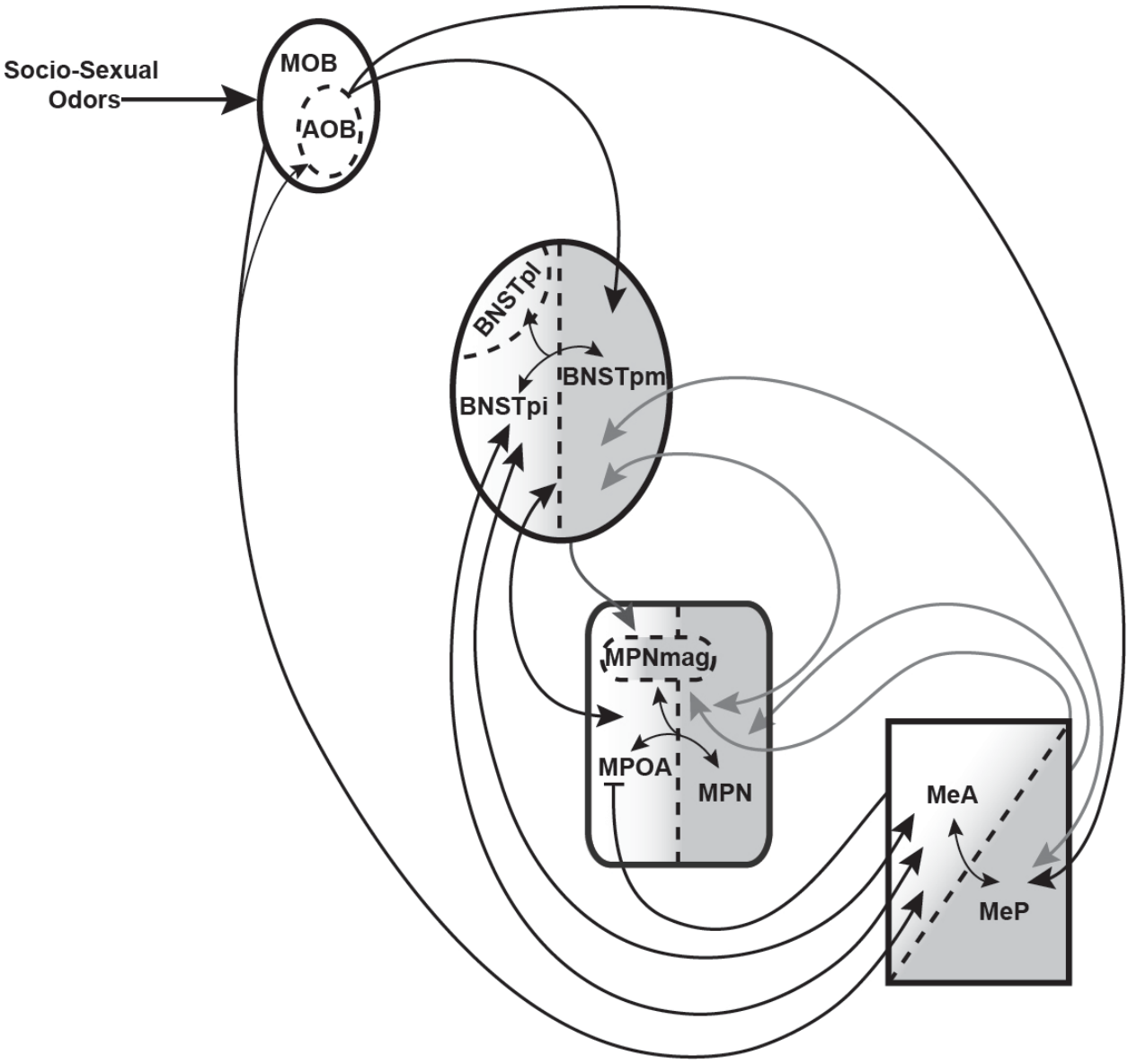

Figure 6.1: Subnuclei in MA, BNST, and MPOA process chemosensory and hormonal information. MA, MPOA, and BNST are comprised of interconnected subnuclei that differ in their connections to other brain areas and in their sensitivity to steroid hormones. In MA and BNST, chemosensory and hormone information are processed in distinct subnuclei and this dissociation may contribute to regulation of appetitive and consummatory aspects of reproductive behavior. In contrast, the separation of chemosensory and hormone information is less distinct in MPOA. Instead, the regulation of appetitive reproductive behaviors may rely on the convergence of chemosensory and hormone information within preoptic subnuclei, whereas consummatory reproductive behavior may rely on preoptic projections to mid- and hindbrain areas required for genital reflexes. Gradient represents hormone sensitivity, with darker areas representing higher expression of steroid receptors. Black arrows signify chemosensory projections and grey arrows signify projections between hormone-sensitive areas.

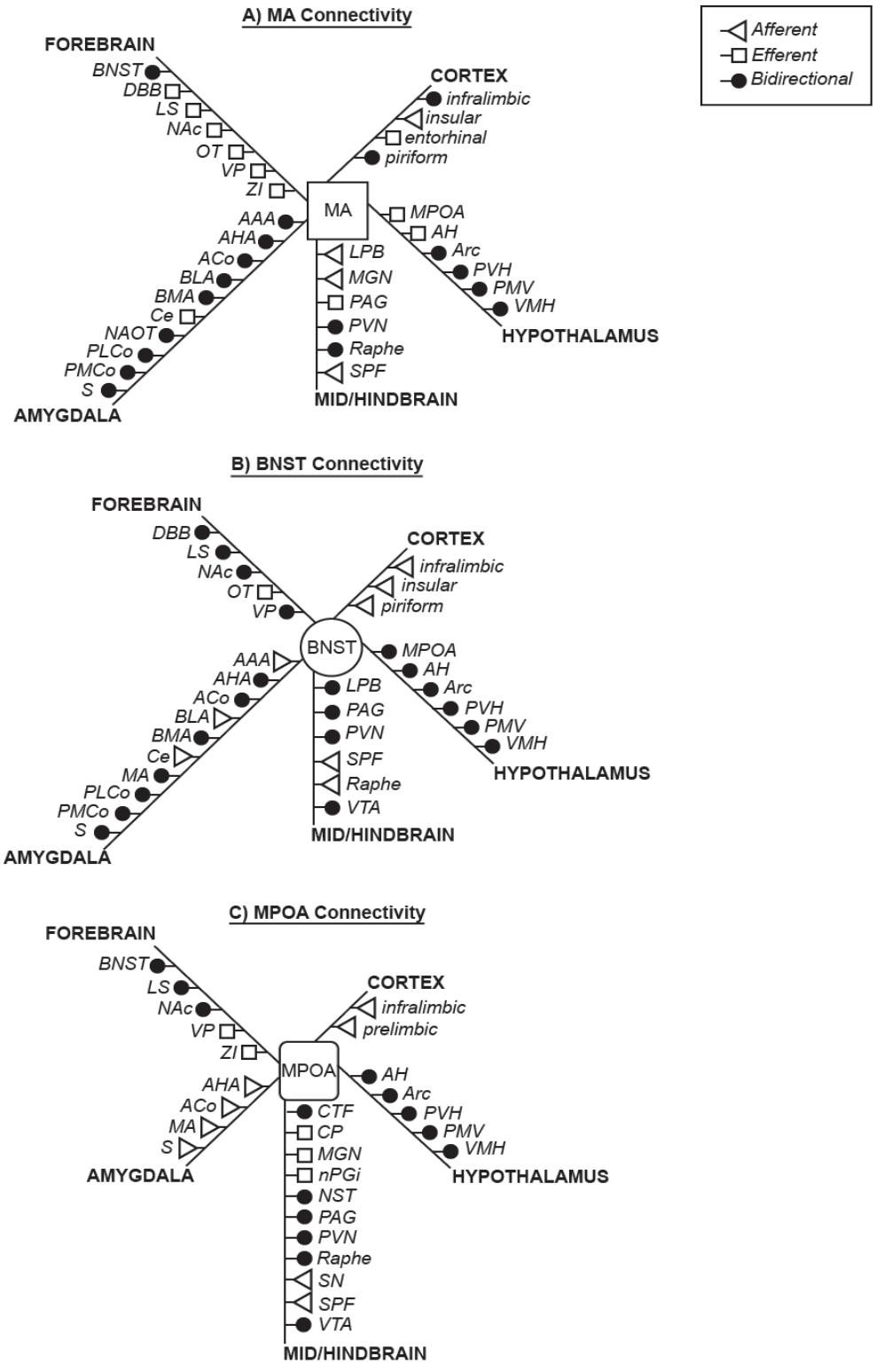

Figure 6.2: MA, BNST, and MPOA Connectivity with Other Brain Areas. Open triangles represent afferent projections, open squares represent efferent projections, and filled circles represent bidirectional projections. AAA, anterior amygdaloid area; AH, anterior

hypothalamus; AHA, amygdalo-hippocampal area; ACo, anterior cortical amygdala; Arc, arcuate nucleus; BLA, basolateral amygdala; BMA, basomedial amygdala; BNST, posterior bed nucleus of the stria terminalis; Ce, central amygdala; CTF, central tegmental field; CP, caudate putamen; DBB, diagonal band of broca; LPB, lateral parabrachial nucleus; LS, lateral septum; MPOA, medial preoptic area; MGN, medial geniculate nucleus; NAc, nucleus accumbens; NAOT, nucleus of the accessory olfactory tract; nPGi, nucleus paragigantocellularis; NST, nucleus of the solitary tract; PAG, periaqueductal gray; PLCo, posterolateral cortical amygdala; PMCo, posteromedial cortical amygdala; PVH, paraventricular hypothalamic nucleus; PMV, ventral premammillary hypothalamic nucleus; PVN, paraventricular nucleus of the thalamus; S, subiculum; SN, substantia nigra; SPF, subparafascicular nucleus; OT, olfactory tubercle; VMH, ventromedial hypothalamus; VP, ventral pallidum; VTA, ventral tegmental area; ZI, zona incerta

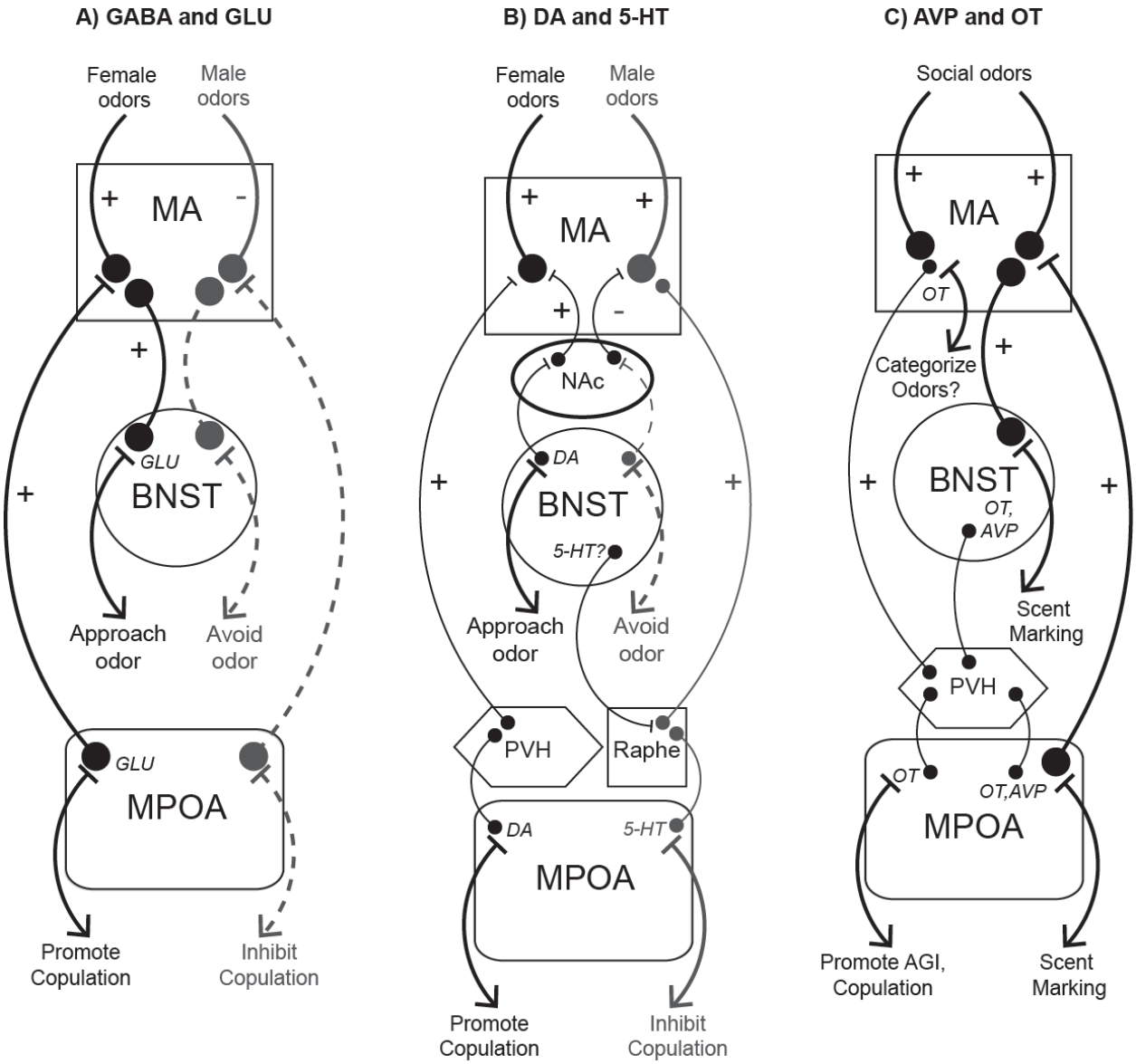

Figure 6.3: Possible Neurochemical Influences on Appetitive and Consummatory Reproductive Behaviors. **A)** Female odors may activate projection neurons in MA, stimulating glutamate (GLU) release into BNST and MPOA and promoting the generation of reproductive behaviors. In contrast, male odors may inhibit projection neurons in MA, preventing GLU release into BNST and MPOA and inhibiting the generation of reproductive behaviors. **B)** Female odors may activate MA projections to the mesolimbic dopamine (DA) pathway to mediate appetitive reproductive behaviors via BNST. Female odors may also activate MA projections to the incertohypothalamic DA pathway to mediate consummatory reproductive behaviors via MPOA. Male odors may activate MA projections to the raphe nuclei, stimulating serotonin (5-HT) release into MA, BNST, and

MPOA, and inhibiting reproductive behaviors. **C)** Social odor information processed in MA may be relayed to vasopressin (AVP)-sensitive cells in BNST and MPOA to regulate odor-guided social behaviors. MA may also relay social odor information to PVH to stimulate AVP and oxytocin (OT) release into MA, BNST, and/or MPOA.

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APPENDICIES

APPENDIX A: *Curriculum Vitae*

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EDUCATION

DEGREE: Bachelor of Arts
MAJORS: Biopsychology & Cognitive Sciences (with Honors)
English Language & Literature
ADVISOR: Jill Becker, Ph.D.
INSTITUTION: University of Michigan, Ann Arbor, MI (2000-2004)
THESIS TITLE: "Gonadal vs. chromosomal sex differences in dopamine neurotoxicity in mice"

DEGREE: Master of Arts
MAJOR: Psychology (Behavioral Neuroscience)
INSTITUTION: Georgia State University, Atlanta, GA (2005-2008)
THESIS TITLE: "The posterior bed nucleus of the stria terminalis mediates opposite-sex odor preference in male Syrian hamsters (*Mesocricetus auratus*)"

DEGREE: Doctor of Philosophy (in progress)
MAJOR: Neuroscience
ADVISOR: Aras Petrulis, Ph.D.
INSTITUTION: Georgia State University, Atlanta, GA (2005-present)
DISSERTATION TITLE: "Dissociated functional pathways for appetitive and consummatory reproductive behaviors in male Syrian hamsters (*Mesocricetus auratus*)"

Graduate School GPA: 3.99

AWARDS AND HONORS

University Honors, University of Michigan (2001- 2004)
Honors in Biopsychology and Cognitive Sciences, University of Michigan (2004)
Brains and Behavior Scholar Fellowship, Georgia State University (2005-present)
Atlanta Chapter of the Society for Neuroscience Poster Award (2006)
Travel Award to Keck Center for Behavioral Biology Annual Symposium (2010)
Travel Award to Society for Behavioral Neuroendocrinology Annual Meeting (2010)
Georgia State University Dissertation Grant (2011)
Society for Neuroscience Chapters Graduate Student Travel Award (2011)
NRSA Post-Doctoral Fellowship, Institutional Training Grant T32 DA07234 (2012)

PUBLICATIONS

- *Peer-Reviewed Primary Research Papers*

1. **Been, L.E.** and A. Petrulis (2010). Lesions of the posterior bed nucleus of the stria terminalis eliminate opposite-sex odor preference and delay copulation in male Syrian hamsters: role of odor volatility and sexual experience. *European Journal of Neuroscience*, 32(3): 483-93.

2. **Been, L.E.** and A. Petrulis (2010). The role of the medial preoptic area in appetitive and consummatory reproductive behaviors depends on sexual experience and odor volatility in male Syrian hamsters. *Neuroscience*, 170: 1120-1132.

3. **Been, L.E.** and A. Petrulis (2011). Chemosensory and hormone information are relayed directly between the medial amygdala, posterior bed nucleus of the stria terminalis, and medial preoptic area in male Syrian hamsters. *Hormones and Behavior*, 59(4): 536-548.

4. **Been, L.E.** and Petrulis, A. (2011). Dissociated functional pathways for appetitive and consummatory reproductive behaviors in male Syrian hamsters. *Submitted, Hormones and Behavior, October 2011.*

5. **Been, L.E.**, Bauman, J.B., Petrulis, A. and Chang, Y.H. (2011). X-ray kinematics of vaginal scent marking in female Syrian hamsters. *Submitted, Physiology and Behavior, October 2011.*

- Invited, Peer-Reviewed Book Chapters and Review Articles

1. **Been, L.** and A. Petrulis. The Neurobiology of Sexual Solicitation: Vaginal Marking in Female Syrian Hamsters. In: Hurst, J.L., Benyon, R. J., Roberts, S.C., and T. Wyatt (Eds) *Chemical Signals in Vertebrates XI*, New York, Springer, 2008.

POSTER PRESENTATIONS

1. **Been, L.E.** and A. Petrulis. The neurobiology of sexual solicitation: vaginal marking in female Syrian hamsters. Chemical Signals in Vertebrates XI, Chester, UK, 2006.

2. **Been, L.E.**, Murphy, A.Z. and Petrulis, A. The neurobiology of sexual solicitation: vaginal making in female Syrian hamsters. Society for Neuroscience, Atlanta, GA, 2006.

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4. **Been, L.E.**, Murphy, A.Z., Clancy, A.N. and Petrulis, A. Central nervous system neurons projecting to the vagina in Syrian hamsters: implications for vaginal marking. Society for Behavioral Neuroendocrinology, Pacific Grove, CA, 2007.

5. **Been, L.E.** and A. Petrulis. The role of the posterior bed nucleus of the stria terminalis in sexual odor preference in male Syrian hamsters. Brains and Behavior Annual Retreat, Atlanta, GA, 2008.

6. **Been, L.E.**, and A. Petrulis. The role of the posterior bed nucleus of the stria terminalis in sexual odor preference in male Syrian hamsters. Society for Behavioral Neuroendocrinology, Groningen, Netherlands, 2008.

7. **Been, L.E.** and A. Petrulis. The role of the posterior bed nucleus of the stria terminalis in opposite-sex odor preference and sexual odor processing. Society for Neuroscience, Washington, D.C., 2008.

8. **Been, L.E.** and A. Petrulis. Lesions of the posterior bed nucleus of the stria terminalis eliminate opposite-sex odor preference in sexually-naïve, but not sexually experienced, male Syrian hamsters." Society for Behavioral Neuroendocrinology, East Lansing, MI, 2009.

9. **Been, L.E.** and A. Petrulis. "The role of the medial preoptic area in opposite-sex odor preference in male Syrian hamsters." Society for Neuroscience, Chicago, IL, 2009.

10. **Been, L.E.** and A. Petrulis. "Reevaluating the role of the medial preoptic area in appetitive and consummatory aspects of reproductive behavior in male Syrian hamsters." Society for Behavioral Neuroendocrinology, Toronto, Canada, 2010.

11. **Been, L.E.** and A. Petrulis "Sexual odor-responsive cells in the medial amygdala and bed nucleus of the stria terminalis project to the medial preoptic area." Society for Neuroscience, San Diego, CA, 2010.

12. **Been, L.E.** and A. Petrulis. "Functionally disconnecting the medial amygdala from the medial preoptic area eliminates copulation but not odor preference in male hamsters." Society for Neuroscience, Washington, D.C., 2011.

INVITED ADDRESSES AND SYMPOSIA

1. “Dissociated Roles of the bed nucleus of the stria terminalis and medial preoptic area in appetitive and consummatory aspects of reproductive behavior,” Keck Center for Behavioral Biology Annual Symposium, Raleigh, NC, USA

2. Neural substrates of social odor processing in male Syrian hamsters,” Brains and Behavior Annual Retreat, Atlanta, GA, USA

TEACHING EXPERIENCE

- *Undergraduate Courses*
  Research Methods in Psychology, Lab Instructor (Spring 2009)

RESEARCH EXPERIENCE

POSITION: Undergraduate Research Assistant
ADVISOR: Jill Becker, Ph.D.
INSTITUTION: University of Michigan (2002-2004)
FIELD: Sex differences in neural and behavioral responses to psychomotor stimulants

POSITION: Graduate Research Assistant
ADVISOR: Aras Petrulis, Ph.D.
INSTITUTION: Georgia State University (2005-present)
FIELD: Neurobiology of social and reproductive behaviors

POSITION: Post-Doctoral Research Fellow
ADVISOR: Robert Meisel, Ph.D.
INSTITUTION: University of Minnesota (2012)
FIELD: Experience-dependent plasticity and motivated behaviors

PROFESSIONAL ORGANIZATIONS

Center for Behavioral Neuroscience
Brains and Behavior Program, Georgia State University
Society for Neuroscience
Society for Behavioral Neuroendocrinology
Society for Social Neuroscience

DEPARTMENTAL/UNIVERSITY SERVICE

Psychology Department
2007-2008, Graduate Program Committee Representative, Georgia State Graduate Association of Student Psychologists
2006-2007 Secretary, Georgia State Graduate Association of Student Psychologists

Neuroscience Institute
2009-2010, Student Coordinator, Neuroscience Institute Brown Bag Lunch (NIBBL)

Georgia State University
2011, mentor, undergraduate student, University Scholars
2010, mentor, undergraduate student, Research Practicum
2009, mentor, undergraduate student, Research Practicum
2009, teacher, Brain Awareness Month, Renfroe Middle School
2009, volunteer, Brains Rule! Neuroscience Expo at Zoo Atlanta
2008, mentor, undergraduate student, Research Practicum
2008, volunteer, Brains Rule! Neuroscience Expo at Zoo Atlanta
2008, T.A., Brain Awareness Month, Renfroe Middle School
2007, volunteer, Neuroscience Expo at Zoo Atlanta
2007, volunteer, Brain Awareness Month elementary school visits
2006 volunteer, Brains Rule! Neuroscience Expo at Zoo Atlanta
2006 mentor, undergraduate student, B.R.A.I.N. summer internship program
2006 volunteer, CBN Brain Balloon Project

RESEARCH INTERESTS

1) Neural substrates of motivated behaviors
2) Neural substrates of social information processing
3) Chemosensory and hormonal modulation of social and sexual behaviors
4) Sexual experience and neuroplasticity