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doi: <https://doi.org/10.57709/1357698>

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FUNCTIONAL SUBSTRATES OF SOCIAL ODOR PROCESSING WITHIN THE
CORTICOMEDIAL AMYGDALA: IMPLICATIONS FOR REPRODUCTIVE BEHAVIOR IN
MALE SYRIAN HAMSTERS

by

PAMELA M. MARAS

Under the direction of Aras Petrulis

ABSTRACT

Adaptive reproductive behavior requires the ability to recognize and approach possible mating partners in the environment. Syrian hamsters (*Mesocricetus auratus*) provide a useful animal model by which to study the neural processing of sexual signals, as mate recognition in this species relies almost exclusively on the perception of social odors. In the laboratory, male hamsters prefer to investigate female odors compared to male odors, and this opposite-sex odor preference provides a sensitive measure of the underlying neural processing of sexual stimuli. In addition to chemosensory cues, reproductive behavior in hamsters also requires sufficient levels of circulating gonadal steroid hormones, which reflect the reproductive state of the animal. These

chemosensory and hormone signals are processed within an interconnected network of ventral forebrain nuclei, and within this network, the posteromedial cortical amygdala (PMCo) and medial amygdala (MA) are the only nuclei that both receive substantial chemosensory input and are also highly sensitive to steroid hormones. Although a large body of evidence suggests that the MA is critical for generating attraction to sexual odors, the specific role of the PMCo in regulating odor-guided aspects of male reproductive behavior has never been directly tested. Furthermore, detailed analyses of the MA suggest that separate, but interconnected sub-regions within this nucleus process odors differently. Specifically, the anterior MA (MeA) receives the majority of chemosensory input and responds to a variety of social odors, whereas the posterodorsal MA (MePD) receives less chemosensory input but contains the vast majority of steroid receptors. In order to further elucidate how the PMCo and/or MA process sexual odors, this dissertation addressed the following research questions: (1) Is the PMCo required for the expression of either opposite-sex odor preferences or male copulatory behavior? (2) Are functional interactions between MeA and MePD required for the expression of opposite-sex odor preferences? (3) How do MeA and MePD regulate odor responses within the MePD and MeA, respectively? (4) Are odor and/or hormone cues conveyed directly between MeA and MePD? Together, these experiments provide a comprehensive analysis of the functional and neuroanatomical substrates by which the brain processes sexual odors and generates appropriate behavioral responses to these stimuli.

INDEX WORDS: Hamster, Olfaction, Odor preference, Copulatory behavior, Medial amygdala, Posteromedial cortical amygdala

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PAMELA M. MARAS

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

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2010

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May 2010

DEDICATION

I would like to dedicate this Dissertation to *Ashley*. I could never have done this without you.

Thank you for taking the stress out of my life and making me so happy every day. You know I do, even though you will never admit it in song.

ACKNOWLEDGEMENTS

I would like to thank the many people who have provided guidance and support throughout my graduate education.

First and foremost is my mentor, *Aras Petrulis*, who has provided an extremely supportive environment for me to develop as a scientist, and from whom I have learned so much about creating a successful and happy lab. I have thoroughly enjoyed working, thinking, and writing with Aras, and I am especially proud to be his first PhD student.

I would also like to acknowledge the other fantastic members of my dissertation committee, *Tim Bartness*, *Anne Murphy*, and *Walt Wilczynski*. Deepest thanks to Tim Bartness for his overwhelming support. His faith in my abilities continues to motivate me. Anne Murphy has continually gone out of her way not only to help me with certain techniques, but also to provide career advice and support. She provides an extra mentor to so many students, and we are all very lucky to have her on our side. I would like to thank Walt Wilczynski for his service during both my qualifying exam and dissertation process. He has provided a unique point of view to my work and has helped me to conceptualize social communication in a broader context.

I would also like to thank my undergraduate faculty mentors from Florida State University, *Dr. James Smith* and *Dr. Frank Johnson*, who are responsible for getting me excited about neuroscience. I am very grateful to them.

I would like to thank the members of the Petrulis lab: *Laura Been*, *Marc Badura*, *Dr. Christine Lewis*, *Luis Martinez*, and *Fidel*. Thank you all for the friendship and support, and for putting up with my incessant need to organize. We have the best darn lab environment in the history of the second floor of Kell Hall, and I am sincerely sad to be leaving. Sorry about all those heaters. I

also wish to acknowledge the wonderful undergraduate students that I have had the pleasure of mentoring, in particular: *Don Bearden, Larry Burrell, Laurie Hale, Nina King, Anaam Mohammed, and Victoria Smith*. I hope you learned something.

I would not have made it through this crazy process without the support of some great friends, including: *Marc Badura, Laura Been, Kelli Duncan, Desiree Krebs-Kraft, Stacie Lin-Taylor, Varenka Lorenzi, Joe Normandin, and Victoria Smith*. Laura, you are the best mini-me ever and so much more. I promise to keep in touch.

Finally, I would like to acknowledge the love and support provided by my parents and family. *Mom and Dad*, thank you for always cheering me on and being so proud, even though none of your friends understand what it is I actually do. *Barb and Fred*, thank you for welcoming me into your family and giving me so much encouragement. *Jerry, Matt, Sarah, and Beckie*, thank you for always supporting your crazy, dorky little sister.

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CHAPTER 1: Introduction

Overview

Adaptive reproductive behavior requires integrating external signals about available mates in the environment with internal signals about an individual's own reproductive state (Wingfield *et al.*, 1997). Indeed, reproductive fitness is optimal when the expression of reproductive behaviors is restricted toward appropriate mating partners (ie. conspecific, opposite-sex) and also coincides with the maximum fecundity of the individual (Wingfield *et al.*, 1997; Gowaty & Hubbell, 2009). The attempt to mate when these conditions are not met would not only be a costly waste of energetic resources, but may even have severe consequences for an individual's immediate survival (Groning & Hochkirch, 2008; Gowaty & Hubbell, 2009). In rodents (Brennan, 2004; Keverne, 2004), as well as many other species (Johnston, 1983; Rodriguez, 2004), mate recognition relies heavily on the perception of chemical signals released by conspecifics, whereas reproductive state is tightly linked to circulating levels of gonadal steroid hormones (Gomes & VanDemark, 1974). The mechanisms by which the brain integrates these external and internal signals remain poorly understood, although the expression of reproductive behavior in rodents involves an extended network of ventral forebrain nuclei that process chemosensory and/or steroid hormone cues (Wood, 1997; 1998). Within this circuit, the posteromedial cortical amygdala (PMCo) and medial amygdala (MA) are the only nuclei that both receive substantial direct chemosensory input and are also highly sensitive to steroid hormones (Wood, 1997; 1998), suggesting a unique role for these nuclei in the integration of chemosensory and hormone signals. The current review will therefore focus on the roles of the PMCo and MA in processing sexual odors and generating reproductive behavior in rodents, with an emphasis on the neural mechanisms of sexual odor processing in male Syrian hamsters.

Chemosensory regulation of rodent reproductive behavior

As in many mammalian species (Johnston, 1983; Rodriguez, 2004), social communication in rodents involves the active release and detection of chemical signals (Johnston, 1990; Brennan, 2004; Keverne, 2004). Social odors convey a wide range of information about the sender, including species, sex, kinship, and even social or reproductive status (Johnston, 1983; Rich & Hurst, 1999; Hurst & Beynon, 2004; Brennan & Kendrick, 2006; Arakawa *et al.*, 2008). In the contexts of reproduction, females of many rodent species advertise their sexual receptivity through active scent-marking behaviors, and these sexual odors are highly attractive to conspecific males (Johnston, 1983; Coquelin, 1992; Matochik *et al.*, 1992). For example, female Syrian hamsters display a highly stereotyped vaginal marking behavior, during which the female lowers and thrusts her pelvis, depositing vaginal secretion onto the substrate (Johnston, 1979; Been & Petrulis, 2007). These vaginal secretions serve as a potent chemical attractor for male hamsters and are sufficient to stimulate the expression of male copulatory behaviors (Johnston, 1974; 1975; Johnston & Kwan, 1984; Petrulis & Johnston, 1995). Male hamsters are attracted to other components of female odors as well (Johnston, 1986) and display robust preferences to approach and investigate female odors compared to male odors, referred to as an opposite-sex odor preference (Johnston, 1981; Steel, 1982; Maras & Petrulis, 2006; Ballard & Wood, 2007).

In rodents, social odors are processed by two, anatomically distinct chemosensory systems, the main and accessory olfactory systems (Meredith, 1991; Restrepo *et al.*, 2004). Sensory receptors of the main olfactory system, located in the main olfactory epithelium, respond best to low molecular weight, volatile components of social odors (Meredith, 1991; Menco & Morrison, 2003; Ma, 2007). In contrast, sensory receptors of the accessory olfactory

system, located in the vomeronasal organ, are thought to process high molecular weight, non-volatile components of social odors (Meredith, 1991; Keverne, 1999; Halpern & Martinez-Marcos, 2003; Rodriguez, 2004). Together, these chemosensory systems regulate the expression of most rodent social behaviors, including male reproductive behaviors (Hull *et al.*, 2002; Keverne, 2004; Restrepo *et al.*, 2004; Keller *et al.*, 2009).

In particular, the expression of reproductive behavior in male Syrian hamsters relies heavily on chemosensory processing. Either olfactory bulbectomy (Murphy & Schneider, 1970) or simultaneous deafferentation of the main and accessory olfactory systems (Powers & Winans, 1975) completely eliminates the expression of copulatory behavior in male hamsters. In contrast to rats (Larsson, 1975), previous sexual experience does not mitigate these deficits in hamsters (Murphy & Schneider, 1970). Chemosensory processing is also required for a male hamster's attraction to approach and investigate female odors (Powers *et al.*, 1979), as well as the pre-copulatory anogenital investigation of a receptive female (Murphy & Schneider, 1970; Devor & Murphy, 1973; Powers & Winans, 1975). Thus, both the appetitive and consummatory aspects of male reproductive behavior in hamsters require on the chemosensory detection of sexual odors.

Social odors are processed initially within the main and accessory olfactory bulbs (MOB and AOB, respectively), which send projections to the ventral forebrain primarily via the lateral olfactory tract (Figure 1.1) (Kratskin & Belluzzi, 2003; Lin *et al.*, 2005). MOB efferents travel to a broad network of secondary olfactory nuclei, including the olfactory tubercle, piriform and entorhinal cortices, anterior and posterolateral cortical amygdala, and MA (Scalia & Winans, 1975; Cleland & Linster, 2003). In contrast to these wide projections, the AOB sends limited projections specifically to MA and PMCo, as well as some modest projections to the bed nucleus of the stria terminalis (BNST) (Scalia & Winans, 1975; Pro-Sistiaga *et al.*, 2007). Social odor

information reaches downstream forebrain nuclei, such as the medial preoptic area (MPOA), either directly from MA or indirectly through the BNST (Wood, 1997). Disruption of the flow of chemosensory information at any point in this circuit disrupts the expression of male reproductive behavior in many rodent species, including hamsters (Murphy & Schneider, 1970; Devor, 1973; Macrides *et al.*, 1976; Lehman *et al.*, 1980; Lehman & Winans, 1982; Lehman *et al.*, 1983; Powers *et al.*, 1987).

Chemosensory-hormone interactions

In addition to chemosensory cues, male reproductive behavior is also regulated by internal signals of reproductive state via changes in circulating levels of gonadal steroid hormones (Beyer *et al.*, 1976; Morin & Zucker, 1978; Hull *et al.*, 2002). In hamsters, testosterone and its primary metabolites, estradiol and dihydrotestosterone, are critical not only for the expression of male copulatory behavior (Morin & Zucker, 1978; Powers *et al.*, 1985), but also for male's attraction to investigate female odors (Steel, 1982; Powers & Bergondy, 1983; Powers *et al.*, 1985; Petrulis & Johnston, 1995). Given the importance of both chemosensory and hormone cues for the expression of reproductive behavior in rodents, it is perhaps not surprising that these signals are processed by largely overlapping neural circuits. In fact, dense populations of steroid receptor-containing neurons are found within many of the ventral forebrain nuclei that receive either direct or indirect chemosensory input (Wood, 1997; Figure 1.1). Specifically, androgen receptor (AR) and estrogen receptor (ER) containing neurons are found within the MA, PMCo, BNST, and MPOA (Doherty & Sheridan, 1981; Simerly *et al.*, 1990; Wood *et al.*, 1992; Li *et al.*, 1993). This overlap of chemosensory and hormone-sensitive nuclei highlights the potential for odor-hormone integration throughout the ventral forebrain mating circuit.

In addition to these anatomical data, there is substantial evidence for functional interactions between chemosensory and steroid hormones systems in male hamsters. Exposure to female odors rapidly increases circulating levels of testosterone in male hamsters (Macrides *et al.*, 1974; Pfeiffer & Johnston, 1992). This increase in testosterone relies on chemosensory processing via the vomeronasal organ (Pfeiffer & Johnston, 1994), and likely involves the activation of GnRH-expressing cells within the MPOA (Meredith & Fernandez-Fewell, 1994). Furthermore, gonadal hormones are critical for male hamster's attraction to female odors; castrated male hamsters display low levels of investigation of female odors, and testosterone treatment fully restores this behavior (Steel, 1982; Powers & Bergondy, 1983; Powers *et al.*, 1985; Petrulis & Johnston, 1995). These data suggest that chemosensory processing can modulate gonadal physiology, and vice versa. Within the brain, hormones appear to act within specific forebrain nuclei to generate attraction to sexual odors, as unilateral testosterone implants into either the MPOA/BNST or MA increase anogenital investigation in castrated male hamsters (Wada *et al.*, 1990; Wood & Newman, 1995c; Wood, 1996; Wood & Williams, 2001). Unilateral olfactory bulbectomy ipsilateral to the steroid implant, however, prevents this increase (Wood & Newman, 1995b; Wood & Coolen, 1997), suggesting that attraction to sexual odors requires the neural integration of chemosensory and hormone cues. Although the mechanisms of chemosensory-hormone integration remain unclear, they likely involve brain areas that process both types of information (Wood & Coolen, 1997).

Candidate sites for chemosensory-hormone integration

Within the ventral forebrain, the PMCo and MA are the only nuclei that both receive substantial chemosensory input and are also highly sensitive to gonadal steroid hormones (Wood, 1997). The PMCo and MA receive direct projections from the AOB and together,

constitute the main components of the “vomeronasal amygdala” (Scalia & Winans, 1975; Kevetter & Winans, 1981a; Pro-Sistiaga *et al.*, 2007). The MA receives direct projections from the MOB (Scalia & Winans, 1975; Pro-Sistiaga *et al.*, 2007), and both nuclei share extensive indirect connections with secondary nuclei of the main olfactory system, including the anterior cortical and posterolateral cortical nuclei of the amygdala (Kevetter & Winans, 1981b). In male hamsters, neurons within PMCo and MA display increases in Fos expression following exposure to female odors (Fiber *et al.*, 1993; Kollack-Walker & Newman, 1997; Fewell & Meredith, 2002), indicating that these areas are activated during processing of sexually relevant odors. Finally, both PMCo and MA contain dense populations of steroid receptor-containing neurons (Doherty & Sheridan, 1981; Simerly *et al.*, 1990; Wood *et al.*, 1992), and many of these steroid-sensitive neurons are activated during mating (Wood & Newman, 1993). These data indicate that the PMCo and/or MA mediate odor-guided aspects of male reproductive behavior.

PMCo. Despite the anatomical evidence detailed above, few studies have addressed the role of the PMCo in regulating reproductive behavior. In hamsters, males with large lesions of the corticomедial amygdala display deficits in male copulatory behavior (Lehman *et al.*, 1983). As these lesions damaged several nuclei, the specific role of the PMCo in regulating copulatory behavior cannot be determined from this study. Using more discrete lesions, Romero and colleagues demonstrated that the PMCo is required for the preference to investigate intact males compared to castrated males in female rats (Romero *et al.*, 1990), indicating that the PMCo is critical for generating attraction to sexually relevant odors. Finally, in male hamsters, neurons within PMCo display elevated levels of Fos expression following either copulatory behavior or exposure to female odors (Kollack & Newman, 1992; Kollack-Walker & Newman, 1995; Fernandez-Fewell & Meredith, 1998; Fewell & Meredith, 2002). Although these data suggest

that the PMCo is involved in processing sexual odors and regulating male reproductive behavior, this hypothesis has never been directly tested. Consequently, the goal of Chapter 2 will be to assess the effects of specific lesions of PMCo on opposite-sex odor preferences, as well as the expression of copulatory behavior, in male hamsters.

MA. Relative to PMCo, much more is known about the role of the MA in processing social odors and regulating various aspects of social behavior. Increases in immediate early gene expression within the MA have been reported following exposure to many different types of social odors (Fiber *et al.*, 1993; Coolen *et al.*, 1997; Dielenberg *et al.*, 2001; Day *et al.*, 2004; Meredith & Westberry, 2004; Choi *et al.*, 2005; Kiyokawa *et al.*, 2005), suggesting that MA plays a significant role in social odor processing. In fact, the MA is critical for the expression of several odor-guided social behaviors, including maternal (Numan *et al.*, 1993; Keller *et al.*, 2004), aggressive (Koolhaas *et al.*, 1980; Luiten *et al.*, 1985; Potegal *et al.*, 1996a), and defensive behaviors (Dielenberg & McGregor, 2001; Li *et al.*, 2004). Regarding reproductive behavior, lesions of MA reduce or eliminate the expression of male copulatory behaviors in many rodent species (Lehman *et al.*, 1980; Lehman & Winans, 1982; Kondo, 1992; Stark *et al.*, 1998; Heeb & Yahr, 2000; Kondo & Sachs, 2002). In male hamsters, MA lesions completely eliminate male copulatory behavior and dramatically decrease the pre-copulatory investigation of the receptive female (Lehman *et al.*, 1980; Lehman & Winans, 1982). MA also mediates behavioral responses to sexual odors outside of the copulatory sequence, as MA lesions eliminate the preference to investigate opposite-sex odors in male (Maras & Petrulis, 2006) and female (Petrulis & Johnston, 1999) hamsters. Similarly, MA lesions in male rats eliminate the preference to investigate odors from estrus females compared to ovariectomized females (Kondo & Sachs, 2002), as well as non-contact penile erections in response to female odors (Kondo *et*

al., 1998). Taken together, these data highlight a critical role for the MA in processing social odors and generating appropriate behavioral responses to these stimuli in a variety of behavioral contexts, including reproduction.

Functionally distinct sub-regions within MA

Although MA receives both chemosensory and hormonal information, several lines of evidence suggest that these signals are processed by separate sub-regions of the MA (Wood, 1997). Specifically, the anterior MA (MeA) receives substantial chemosensory input from both main and accessory olfactory systems, whereas the posterodorsal MA (MePD) receives only limited chemosensory input, primarily from the accessory olfactory system (Scalia & Winans, 1975; Kevetter & Winans, 1981a; b; Lehman & Winans, 1982; Coolen & Wood, 1998). Data from several immediate early gene studies indicate that these differences in chemosensory input correspond to functional differences in how MeA and MePD process social odors; whereas the MeA is activated by a wide variety of social odors, including conspecific and heterospecific odors, the MePD is activated only in response to conspecific odors (Day *et al.*, 2004; Meredith & Westberry, 2004; Kiyokawa *et al.*, 2005; delBarco-Trillo *et al.*, 2009; Samuelsen & Meredith, 2009). Furthermore, the MeA responds equally to presentations of opposite- and same-sex odors, whereas the MePD preferentially responds to opposite-sex odors (Samuelsen & Meredith, 2009). These data suggest that MeA is involved in processing many categories of social odors, whereas the MePD is limited to processing sexually relevant (opposite-sex conspecific) odors.

In contrast to the processing of chemosensory information, the processing of hormonal cues within the MA occurs primarily within the MePD. Indeed, the vast majority of AR- and ER-expressing cells within MA are located within MePD (Doherty & Sheridan, 1981; Simerly *et al.*,

1990; Wood *et al.*, 1992). Moreover, testosterone implants into MePD, but not MeA, facilitate male copulatory behavior in castrated male hamsters (Wood & Newman, 1995c), suggesting that MePD is in fact more responsive to steroid hormones than MeA. Interestingly, the anatomical separation of chemosensory and hormone processing that is observed within MA is also observed within sub-regions of the BNST, MPOA and hypothalamus (Wood, 1997), and this parallel processing of chemosensory and hormone signals may therefore represent a fundamental principle of the ventral forebrain network.

Results from several lesion studies highlight important functional differences between the MeA and MePD in regulating the output of male reproductive behavior. Whereas MeA lesions completely eliminate male hamster copulatory behavior (Lehman *et al.*, 1980), similar to deficits observed following bilateral destruction of the olfactory bulbs (Murphy & Schneider, 1970), males with MePD lesions still mate, but display alterations in the temporal pattern of the copulatory sequence (Lehman *et al.*, 1983). More recently, our laboratory has shown that MeA and MePD mediate distinct aspects of opposite-sex odor preference (Maras & Petrulis, 2006). Specifically, we tested males with lesions of either MeA or MePD for their (1) preference to investigate female odors or male odors when presented simultaneously and (2) attraction to investigate each sexual odor when presented opposite clean (neutral) odors. Although lesions of either MeA or MePD eliminate the preference to investigate opposite-sex odors, these lesions are associated with qualitatively different patterns of odor investigation (Figure 1.2). Males with MePD lesions fail to investigate female odors longer than clean odors, indicating a decreased attraction to investigate opposite-sex odors. In contrast, males with MeA lesions remain highly attracted to female odors, but are *also* highly (and inappropriately) attracted to male odors. This pattern of results suggests that the MePD is critical for generating motivation to investigate

opposite-sex odors, whereas MeA evaluates the sexual relevance of odor stimuli and directs investigation specifically toward opposite-sex odors.

Taken together, these studies indicate that MeA and MePD differentially process chemosensory and hormone signals and consequently, regulate distinct aspects of reproductive behavior. Given the substantial reciprocal connections between MeA and MePD (Gomez & Newman, 1992; Coolen & Wood, 1998), we hypothesize that these sub-regions interact during the processing of social odors (Figure 1.3). We predict that MeA functions as a chemosensory filter to identify the sexual relevance of odor stimuli and regulate odor responses within MePD. Specifically, MeA may enhance responses to *opposite-sex* odors, but suppress responses to *same-sex* odors, within MePD. Furthermore, we hypothesize that MePD provides positive feedback onto MeA during the processing of sexually relevant odors, such that MePD normally enhances responses to *opposite-sex* odors within MeA. Together, these sub-regions can modulate behavioral responses to different categories of social odors through projections to downstream nuclei, such as the BNST and MPOA (Kevetter & Winans, 1981a; Gomez & Newman, 1992; Coolen & Wood, 1998). Importantly, if this model is correct, then we expect that (a) disrupting the interactions between MeA and MePD will eliminate the preference to investigate opposite-sex odors (Chapter 3) and (b) odor responses within MeA and MePD will be altered in the absence of MePD or MeA, respectively (Chapter 4). Finally, if the connections between MeA and MePD provide a mechanism for chemosensory-hormone integration, then we expect that odor and hormone cues will be conveyed directly between these sub-regions (Chapter 5).

Goals of Dissertation

The fundamental goal of this research is to identify the functional substrates by which the brain recognizes sexual signals in the environment and generates appropriate reproductive responses to these stimuli. Using Syrian hamsters as a model species, this dissertation combines the use of behavioral, lesion, and neuroanatomical techniques to provide a comprehensive analysis of how the corticomedial amygdala processes sexual odors and regulates odor-guided aspects of reproductive behavior. Specifically, we addressed the following research questions: (1) Is the PMCo required for the expression of either opposite-sex odor preferences or copulatory behavior? (2) Are functional interactions between MeA and MePD required for the expression of opposite-sex odor preferences? (3) How do MeA or MePD regulate odor responses within the MePD or MeA, respectively? (4) Are odor and/or hormone cues conveyed directly between MeA and MePD? Together, these results fill a critical gap in our knowledge regarding fundamental mechanisms by which the brain processes sexually relevant stimuli and identify possible substrates for the integration of chemosensory and hormone signals that is required for male reproductive behavior in rodents. Although these mechanisms are critical for odor processing specifically related to reproductive behaviors, they are also likely important for appropriate behavioral output in a variety of social contexts.

Chapter 1 Figures

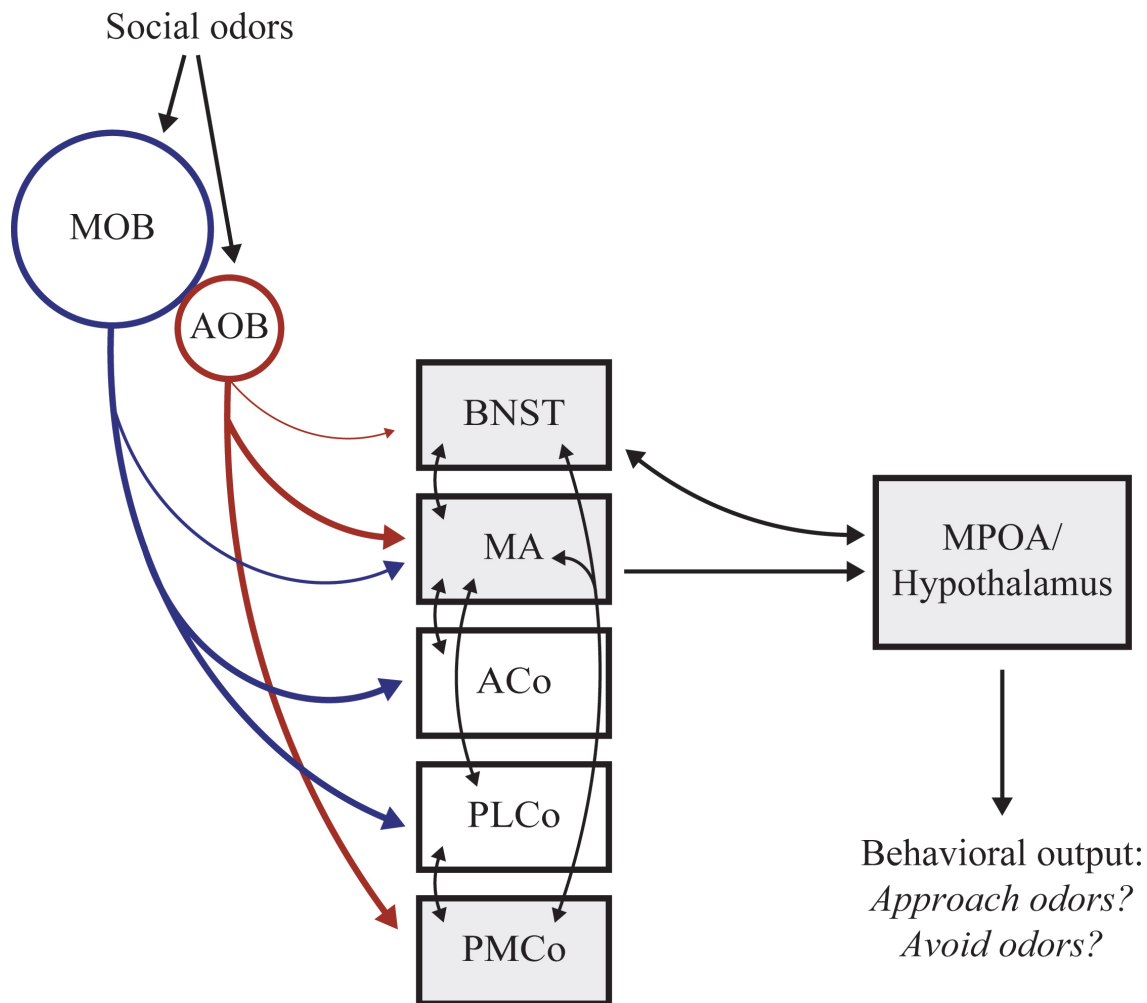


Figure 1.1 Flow of chemosensory information through the corticomedial amygdala. The olfactory bulbs transmit social odor information to several nuclei within the ventral forebrain. The medial and cortical nuclei of the amygdala receive the majority of direct chemosensory input and convey this information to downstream forebrain nuclei to regulate behavioral responses to social odors. Several of these nuclei also express gonadal steroid receptors (indicated by gray boxes), suggesting that chemosensory and hormone signals are processed via overlapping neural circuits. In particular, the PMCo and MA are the only nuclei that both receive substantial direct chemosensory input and also contain steroid receptors. ACo, anterior cortical amygdala; AOB, accessory olfactory bulbs, BNST, bed nucleus of the stria terminalis, MA, medial amygdala, MOB, main olfactory bulbs, MPOA, medial preoptic area; PLCo, posterolateral cortical amygdala; PMCo, posteromedial cortical amygdala.

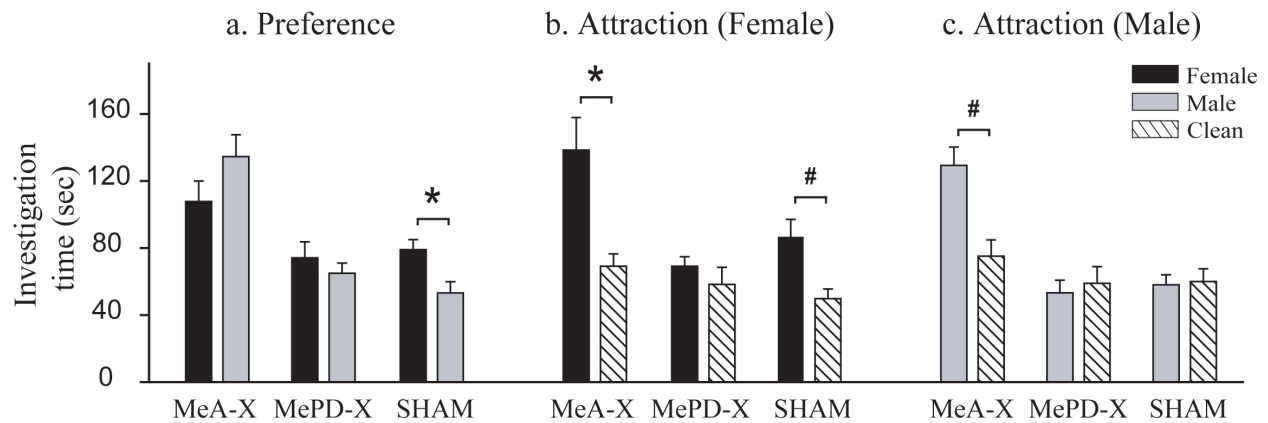


Figure 1.2 MeA and MePD regulate distinct aspects of odor preference. Mean (\pm SEM) investigation durations for males with lesions of MeA (MeA-X), lesions of MePD (MePD-X), or sham lesions (SHAM) during a series of three Y-maze tests: (a) female odors vs. male odors (b) female odors vs. clean odors (c) male odors vs. clean odors. Lesions of either MeA or MePD eliminate the preference to investigate female odors over male odors (a). MePD lesions decrease attraction to female odors (b), whereas MeA lesions increase attraction to investigate male odors (c). * $p < .01$; # $p < .05$ compared to investigation times of other odor stimulus.

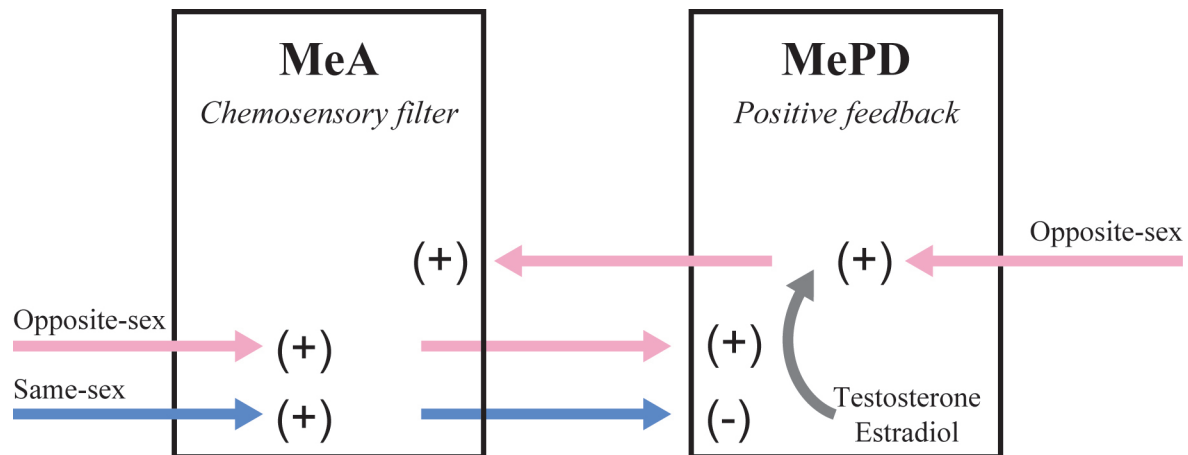


Figure 1.3 Proposed model of MeA-MePD interaction during social odor processing. We hypothesize that the MeA functions as a chemosensory filter to regulate odor responses within the MePD, such that it enhances responses to opposite-sex odors, but suppresses responses to same-sex odors, within MePD. In contrast, we hypothesize that the MePD provides feedback onto MeA regarding sexually relevant odors, such that it enhances processing of specifically opposite-sex odors within MeA. Pink and blue lines represent opposite-sex and same-sex odors, respectively. (+) enhancement or (-) suppression of odor responses.

CHAPTER 2: The Posteromedial Cortical Amygdala Regulates Copulatory Behavior, but not Sexual Odor Preference, in the Male Syrian Hamster (*Mesocricetus auratus*)

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Previously Published in Neuroscience 156(3): 425-35 (2008).

Abstract

In rodent species, the expression of reproductive behavior relies heavily on the perception of social odors, as well as the presence of circulating steroid hormones. In the Syrian hamster, chemosensory and hormonal cues are processed within an interconnected network of ventral forebrain nuclei that regulates many aspects of social behavior. Within this network, the posteromedial cortical amygdala (PMCo) receives direct projections from the accessory olfactory bulbs and contains a dense population of steroid receptor-containing neurons. Consequently, the PMCo may be important for generating odor-guided aspects of reproductive behavior, yet little is known regarding the role of this nucleus in regulating these behaviors. Thus, the present study tested male hamsters with site-specific electrolytic lesions of the PMCo for their (a) sexual odor preference in a Y-maze apparatus (b) sexual odor discrimination in a habituation-dishabituation task and (c) copulatory behavior when paired with a sexually receptive female. PMCo-lesioned males preferred to investigate female odors over male odors and were able to discriminate between these odor sources. However, PMCo lesions were associated with several alterations in the male copulatory pattern. First, PMCo-lesioned males displayed increased investigation of the female's non-anogenital region, suggesting that the PMCo may be involved in directing appropriate chemosensory investigation during mating. Second, PMCo lesions altered the temporal pattern of the mating sequence, as PMCo-lesioned males took longer than Sham-lesioned males to reach sexual satiety, as indicated by the delayed expression of long intromissions. This delayed onset of satiety was associated with an increased number of ejaculations compared to Sham-lesioned males. Importantly, these data provide the first direct evidence for a functional role of the PMCo in regulating male reproductive behavior.

Introduction

In many rodent species, including the Syrian hamster, male reproductive behavior relies heavily on the perception of sexual odors (Hull et al., 2002). Male hamsters are highly attracted to female odors, and these chemosignals stimulate the expression of copulatory behaviors (Johnston, 1974; 1975; 1986) via both the main and accessory olfactory systems (Murphy & Schneider, 1970; Winans & Powers, 1977; Meredith, 1991; Restrepo *et al.*, 2004). In addition to chemosensory processing, male hamster sexual behavior also requires the presence of circulating gonadal steroid hormones (Morin & Zucker, 1978; Powers & Bergondy, 1983; Powers *et al.*, 1985; Petrulis & Johnston, 1995). Consequently, the expression of male reproductive behavior in the hamster involves the integration of chemosensory and hormonal cues (Wood, 1998), and this integration likely occurs within the extended network of ventral forebrain nuclei known to regulate mating behavior (Wood & Newman, 1995b; Wood & Coolen, 1997).

Previous research has identified several critical brain areas within this network, including the medial preoptic areas (MPOA), bed nucleus of the stria terminalis (BNST) and medial amygdala (MA), which regulate various aspects of male reproductive behavior (Lehman *et al.*, 1980; Lehman *et al.*, 1983; Powers *et al.*, 1987; Maras & Petrulis, 2006). Although the posteromedial cortical nucleus of the amygdala (PMCo) is also part of the ventral forebrain circuit (Wood, 1997), the function of this nucleus in guiding male sexual behavior remains largely unknown. However, several lines of evidence suggest that it may be involved in reproductive behavior. First, the PMCo has reciprocal connections with BNST and MA (Kevetter & Winans, 1981a; Gomez & Newman, 1992; Coolen & Wood, 1998; Wood & Swann, 2005), as well as strong connections with chemosensory circuitry. Specifically, the PMCo receives direct projections from the accessory olfactory bulbs (Scalia & Winans, 1975) and indirect connections

from the main olfactory bulbs via the anterior and posterolateral cortical nuclei of the amygdala (Kevetter & Winans, 1981b). Second, the PMCo is sensitive to gonadal steroids, as it contains dense populations of steroid receptor-containing neurons (Simerly *et al.*, 1990; Wood *et al.*, 1992; Wood & Newman, 1993; Shughrue *et al.*, 1997). In rats, this nucleus is sexually dimorphic (Vinader-Caerols *et al.*, 1998) and is masculinized by perinatal estradiol treatment (Vinader-Caerols *et al.*, 2000). Finally, in male hamsters, neurons within the PMCo display elevated levels of c-fos expression, an indirect measure of neuronal activity, following either copulatory behavior or exposure to female odors (Kollack & Newman, 1992; Wood & Newman, 1993; Kollack-Walker & Newman, 1995; Fernandez-Fewell & Meredith, 1998; Fewell & Meredith, 2002).

Together, these data indicate that the PMCo may function to regulate odor-guided aspects of male reproductive behavior, yet to our knowledge, this hypothesis has never been directly tested. Consequently, we assessed the effects of electrolytic lesions of the PMCo on both appetitive and consummatory aspects of reproductive behavior in male Syrian hamsters. Males were tested first for their preference to investigate female odors over male odors in a Y-maze apparatus, as well as for their ability to discriminate between these odors in a habituation-dishabituation task. Males were then tested for their copulatory behavior to satiety when paired with a receptive female. Due to the PMCo's substantial projections from the accessory olfactory bulbs (Scalia & Winans, 1975), we hypothesized that this nucleus would regulate male reproductive behavior primarily through its processing of vomeronasal information. In male hamsters, the role of the vomeronasal organ in regulating sexual behavior changes with sexual experience. Specifically, although the vomeronasal organ is critical for the expression of sexual behavior in naïve males, the main olfactory system can compensate for a lack of vomeronasal

processing in sexually experienced males (Meredith, 1986). As the current study is an initial attempt to identify a functional role for the PMCo in regulating these behaviors, we wanted experimental conditions that specifically rely on accessory olfactory processing and therefore used sexually naïve male subjects. Our results show that the PMCo regulates distinct aspects of male hamster copulatory behavior, although this nucleus is not critical for the expression of sexual odor preferences.

Materials and Methods

Animals

All animals in this study were Syrian hamsters (*Mesocricetus auratus*) purchased from Charles River Laboratory at three weeks of age and singly-housed until the age of behavioral testing (3-6 months). Subjects were sexually naïve males that had been gonadectomized and implanted subcutaneously with testosterone Silastic capsules prior to lesion surgery (see below). Ovariectomized, hormone-primed female hamsters served as stimulus animals for the copulatory behavior tests (see below). A separate group of gonadally-intact male and female hamsters were used to provide social odor stimuli. Subjects were unrelated to, and had no previous contact with, stimulus females or odor donors. All animals were housed in solid-bottom Plexiglas cages (36 cm X 30 cm X 16 cm) and maintained on a reversed 14-h light/ 10-h dark photoperiod (lights off/on at 9 am/7 pm). Food and water were available *ad libitum*. All animal 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.

Surgical procedures

Gonadectomy and testosterone implants in male subjects. In male hamsters, exposure to female odors causes a rapid increase in serum testosterone levels (Macrides *et al.*, 1974; Pfeiffer & Johnston, 1992), and it is possible that lesions of the PMCo may alter this testosterone surge. Thus, in order to equalize steroid hormone levels between experimental groups, all subjects were gonadectomized and provided with physiological levels of exogenous testosterone (Maras & Petrulis, 2006). Males were anesthetized with 1-2% isoflurane (mixed with 100% oxygen). Following a midline abdominal incision, testicles were bilaterally removed via cauterization of the ductus deferens and blood vessels. Immediately following gonadectomy, males were given chronic testosterone (Sigma Chemical Co., St. Louis, MO, USA) replacement via a 20 mm Silastic capsule (i.d. 1.57 mm, o.d. 2.41 mm, Dow Corning, Midland, MI) that was implanted subcutaneously between the scapulae.

Ovariectomy and hormone priming of stimulus females. Stimulus females for copulatory behavior tests were anesthetized with 1-2% isoflurane (mixed with 100% oxygen) and ovariectomized via bilateral flank incisions. Immediately following ovariectomy, stimulus females were given chronic estradiol (Sigma Chemical Co., St. Louis, MO, USA) treatment via a 5 mm Silastic capsule (i.d. 1.57 mm, o.d. 2.41 mm, Dow Corning, Midland, MI, USA) that was implanted subcutaneously between the scapulae. Females were allowed at least two weeks for recovery prior to being used as stimulus animals in copulatory behavior tests. To induce behavioral receptivity, females were given a subcutaneous injection of 0.25 mg of progesterone (dissolved in sesame oil, 2.5 mg/ml) (Sigma Chemical Co., St. Louis, MO, USA) four hours prior to copulatory behavior tests.

Electrolytic lesions. One to two weeks following gonadectomy, male subjects were randomly assigned to either PMCo lesion (PMCoX, n = 22) or sham lesion (SHAM, n = 7) group. Subjects were anesthetized with 1-2% isoflurane (mixed with 7:3 oxygen: nitrous oxide) and positioned in a stereotaxic apparatus so that the skull was flat. The temporal muscles were retracted from the skull and small holes were drilled to expose the dura. Bilateral electrolytic lesions were made using a platinum/iridium electrode (0.25 mm diameter, 0.45 mm uninsulated tip, Frederick Haer & Co., Bowdoinham, ME, USA) and by passing 1 mA of anodal current from a lesion-making device (Ugo Basile, Comerio, VA, Italy). As the PMCo extends over 2 mm in the rostral-caudal direction, and the size, shape and location of the nucleus varies along its length, we used a combination of multiple small lesions in order to generate maximal damage of the PMCo while limiting collateral damage to nearby nuclei. Therefore, each PMCoX male received a total of five bilateral penetrations, and the current duration varied across penetrations (Table 2.1). Sham surgeries were identical to lesion surgeries except that the electrode was lowered 1.5 mm above the target coordinate and no current was passed. Gel foam (Pharmacia & Upjohn Co., Kalamazoo, MI, USA) was used to pack the holes, and the incision was closed with wound clips.

Behavioral testing

To determine the role of the PMCo in generating responses to sexual odors, as well as male copulatory behavior, subjects were given a series of behavioral tests beginning 2 to 3 weeks after lesion surgery (Figure 2.1). First, subjects were tested for their preference to investigate female odors over male odors in a Y-maze apparatus (*Sexual odor preference*). Subjects were then tested for their ability to discriminate between these odor sources in habituation-dishabituation task (*Sexual odor discrimination*). Finally, subjects were tested for their

copulatory behavior when paired with a sexually receptive female (*Male copulatory behavior*). All testing was done during the first four hours of the dark phase of the photoperiod and under dim light.

Odor Stimuli. Male and female odor stimuli used for sexual odor preference and sexual odor discrimination tests were collected from cages that had housed a single odor donor and had not been changed for 10-13 days. Odor stimuli consisted of 12 g of soiled cotton bedding (4 Nestlets, ANCARE, Bellmore, NY); 50 ml of soiled corncob litter; one damp cotton gauze pad that was used to wipe along the inner walls of the odor donor cage; and an additional damp gauze pad that was used to wipe the odor donor's bilateral flank and anogenital regions. For female odor stimuli, vaginal secretion was collected onto an additional gauze pad by inducing an estrous donor female into lordosis and gently palpating the vaginal area with a disposable probe. Clean odor stimuli consisted of unsoiled components identical to those of the social odor stimuli. All odor stimuli were stored in plastic bags at 4°C until 30 minutes prior to use. Odor samples older than one month were discarded, and care was taken to ensure that subjects were not tested with the same individual's odor more than once. Clean latex gloves were worn while collecting odor samples to prevent contamination of odor cues. To conserve stimulus odors, each odor was used for two consecutive sexual odor preference or discrimination tests.

Sexual odor preference. Subjects were tested for their preference to investigate female over male odor stimuli when presented in a Y-maze apparatus (Maras & Petrulis, 2006). The Y-maze consisted of a stem arm (61 cm long) and two side arms (68 cm long). All arms of the maze were 10 cm wide, with walls 10 cm high. Each side arm had a stimulus chamber (20 cm long) at its distal end, in which odor stimuli (see above) were placed. Stimulus chambers had perforated doors that allowed airflow, but prevented contact with the odor stimuli. Thus, subjects

were exposed to only the volatile components of the odor stimuli. A start chamber (20 cm long), with a removable, perforated door, was located at the distal end of the stem arm. An electric fan located behind the start chamber pulled air from the stimulus chambers through the entire length of the Y-maze (airflow rate of 2.0 km/hr, measured at the start box). The top of the Y-maze was secured with a clear Plexiglas top to allow for overhead video recording of the subject's behavior.

Subjects were tested in a sequence of two Y-maze tests, separated by 24 hours. First, to habituate the subjects to the Y-maze and obtain baseline behavioral data, subjects were tested with clean odor stimuli in both stimulus chambers of the Y-maze (Clean). Subjects were then tested for their sexual odor preference by placing male and female odors in opposite stimulus chambers (Preference). For all tests, subjects were placed in the start chamber for one minute, after which, the door was removed and subjects were allowed nine minutes to explore the Y-maze. All surfaces of the Y-maze were thoroughly cleaned with 50% alcohol and allowed to dry between subjects.

Video recordings of Y-maze tests were digitized onto a computer and scored using the Observer for Windows, version 5.0 (Noldus Information Technology B.V., Wageningen, The Netherlands). All observers were blind to the condition of the subject, and different observers reached at least an 85% inter-observer reliability score prior to coding behavior. Both the time spent investigating the stimulus chambers and the numbers of entries into each arm of the Y-maze were scored. Investigation of the stimulus chamber was coded when the subject made contact with, or directed its nose within 1 cm of, the stimulus chamber door. Arm entry was coded when the front half of the subject's body crossed into that arm.

Sexual odor discrimination. A habituation-dishabituation model was used to test discrimination between male and female odors. This approach 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 & 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 (test). Five-minute inter-trial intervals separated each odor presentation. As we have previously shown that male hamsters do not consistently habituate to repeated presentations of female odors (Maras & Petrulis, 2006), all subjects were tested using male odors as the habituation stimuli and female odors as the test stimuli. Subjects were presented with a different male's odor 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 an odor donor.

Odor stimuli were presented in modified 50 ml polypropylene collection tubes, with $\frac{1}{2}$ 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. Thus, subjects were exposed to only the volatile components of the odor stimuli during these tests. Odor containers were placed in the center of the subject's home cage and investigation was scored when the subject's nose contacted, or came within 1 cm of, the odor container. Total investigation times were measured using a stopwatch. Odor containers were cleaned with 50% alcohol and allowed to air dry for 24 hours prior to re-use.

Male copulatory behavior. Subjects were tested for their copulatory behavior when paired with a receptive stimulus female hamster in a clear, Plexiglas arena. An angled mirror was placed under the testing arena to provide a view of the ventral surface of the animals (in addition to the side view). Males were placed into the empty testing arena for five minutes prior to the addition of the stimulus female. Copulatory tests lasted 30 minutes, at which time the stimulus female was removed.

Copulatory behavior tests were video-recorded and the male's behavior was later scored using the Observer for Windows, version 5.0 (Noldus Information Technology B.V., Wageningen, The Netherlands). The total number and latencies (from test onset) of several behavioral measures were scored: mounts without intromissions (mounts), mounts with intromissions (intromissions), ejaculations, and long intromissions. Long intromissions are distinguished from regular intromissions in that the male does not quickly dismount the female following vaginal penetration, but instead displays a repetitive thrusting pattern (Bunnell *et al.*, 1977; Parfitt & Newman, 1998). Importantly, the expression of long intromissions is associated with the onset of sexual satiety in this species (Bunnell *et al.*, 1977; Parfitt & Newman, 1998). In addition, the total durations of time the male spent investigating the female's anogenital region, investigating the female's head or body region (non-anogenital) and self-grooming were also scored. Finally, several derived measures of copulatory behavior were also analyzed: Post-ejaculatory interval (latency to display a mount or intromission after each ejaculation), the number of intromissions to reach each ejaculation, and mounting efficiency (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 (Nembutal, 100 mg/kg) and transcardially perfused with 200 ml of 0.1M phosphate-buffered saline (PBS, pH 7.4) followed by 200 ml of phosphate-buffered formalin (10%). Brains were post-fixed in phosphate-buffered formalin (10%) overnight and then cryoprotected for 48-72 hours in 30% sucrose in PBS solution. Coronal sections (40- μ m) of brain tissue were sectioned on a cryostat (-20°C) and stored in PBS until mounting. Every third section was mounted onto glass slides using a 1% gelatin mounting solution and stained with cresyl violet.

Sections were examined under a light microscope for the location and extent of lesion damage as compared with published hamster neuroanatomical plates (Morin & Wood, 2001). Brain sections from subjects with minimum- and maximum-sized lesions were captured at 5X magnification by a Zeiss AxioCam using Axiovision 4.0 software (2002). These lesions were traced onto anatomical plates using Adobe Illustrator CS 11.0 software (2003).

Blood collection and radioimmunoassay

Blood samples were collected from the inferior vena cava immediately prior to perfusion and stored in vacutainer collection tubes (VWR, West Chester, PA., 4 ml draw, red/gray) on ice until centrifuging. Samples were centrifuged at 3200 rpms, at 4°C for 20 minutes and serum was stored in 200 μ l 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 0.05-22.92 ng/ml and an inter-assay variance of 6%, previously validated for hamster serum (Cooper et al., 2000).

Data analysis

All data were analyzed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA) for Windows and are reported as mean \pm SEM. To establish investigatory preferences in each Y-maze test (Clean, Preference), 2 (Lesion group: PMCoX, SHAM) X 2 (Stimulus; Clean test: left, right; Preference: female, male odor) ANOVAs were performed. In addition, independent *t*-tests were used to detect group differences in the total number of arm entries made during each Y-maze test.

For the sexual odor discrimination tests, data were analyzed using a 2 (Lesion group) X 5 (Odor presentation: Male 1-4, Female) ANOVA. For post-hoc analysis, pairwise comparisons with Bonferroni corrections were used to compare investigation times between Male 1 vs. Male 4 and Male 4 vs. Female presentations.

Group differences in all copulatory measures were detected using independent *t*-tests. Furthermore, to detect changes in post-ejaculatory intervals or the number of intromissions to reach each ejaculation across the duration of the copulatory test, separate 2 (Lesion group) X 2 (First, Last ejaculatory series) ANOVAs were performed.

Results

Lesion verification

Males were included in the PMCoX lesion group ($n = 11$) only if they had extensive bilateral damage of the PMCo. Specifically, all males in the PMCoX group had at least 50% bilateral damage that included the middle three sections of the PMCo (Figure 2.2). In four of these males, damage extended into the rostral sections of the PMCo, whereas in seven subjects, damage extended into caudal PMCo. However, there were no differences in either the preference

or copulatory behavior between males with rostral or caudal spread of lesion damage (all $p > .05$). Males were excluded from the PMCoX group if there was any damage to the posterior medial amygdala ($n = 2$) or if there was substantial sparing of the PMCo ($n = 9$).

In addition to damage of the PMCo, a subset of PMCoX males also had minimal damage ($< 10\%$ in any section) to adjacent nuclei, including the posterior basomedial (BMP, $n = 5$), posterior basolateral ($n = 6$) and posterolateral cortical amygdala ($n = 6$), and the amygdalopiriform transition area ($n = 3$). Importantly, damage to these regions was mostly unilateral and never complete. All PMCoX males also had some lesion damage to the amygdalohippocampal area (AHi). In four males, this damage was minimal ($< 10\%$), whereas in seven males, damage to the AHi was moderate ($\leq 50\%$). There were no differences in either the preference or copulatory behavior between males with minimal or moderate damage to the AHi (all $p > .05$).

Behavioral measures

Sexual odor preference. In the Clean test, there were no significant differences between investigation levels of the two sides of the Y-maze, $F(1, 16) = 1.139, p > .05$, or between experimental groups, $F(1, 16) = .228, p > .05$; there was also no significant interaction between these factors, $F(1, 45) = .001, p > .05$ (Table 2.2). Furthermore, when the investigation times were summed for the left and right arms, PMCoX and SHAM males did not differ in their total duration of investigation of the clean stimulus chambers, $t(16) = .478, p > .05$. Levels of activity, as measured by the total number of arm entries, were also not different between PMCoX and SHAM males, $t(16) = 1.088, p > .05$ (Table 2.2).

In the Preference test, subjects investigated female odors longer than male odors, $F(1, 16) = 7.760, p < .05$, with no difference in investigation between experimental groups, $F(1, 16) = .029, p > .05$, or significant interaction between odor stimulus and experimental group, $F(1, 16) = .019, p > .05$ (Figure 2.3a). In addition, PMCoX and SHAM males spent similar amounts of time investigating female odors, $t(16) = -.054, p > .05$, and male odors, $t(16) = -.248, p > .05$.

Sexual odor discrimination. There was a significant difference in investigation times across stimulus presentations, $F(4, 16) = 32.359, p < .05$ (Figure 2.3b). There was no significant difference between lesion groups, $F(1, 16) = 2.908, p > .05$, nor was there a significant interaction between stimulus presentation and lesion group, $F(4, 16) = 2.294, p > .05$. Post-hoc pairwise comparisons detected significant differences in investigation times between the first and fourth presentations of male odors, $t(16) = 10.120, p < .05$, as well as between the fourth presentation of the male odor and the test presentation of the female odor, $t(16) = -11.151, p < .05$.

Male copulatory behavior. When tested with a sexually receptive female, all male subjects ejaculated and all, except one PMCoX male, reached sexual satiety, as indicated by the expression of long intromissions. However, PMCoX and SHAM males did differ in the expression of several aspects of the male copulatory sequence.

PMCoX males investigated the female's non-anogenital region significantly longer than SHAM males, $t(16) = -2.309, p < .05$, although groups did not differ in their duration of anogenital investigation, $t(16) = -1.218, p > .05$ (Figure 2.4). PMCoX males also displayed less self-grooming than SHAM males, $t(16) = 2.953, p < .05$ (PMCoX = 454 ± 29.0 seconds; SHAM = 580 ± 28.2 seconds).

Although PMCoX and SHAM males displayed equal numbers of mounts, $t(16) = .093, p > .05$, and intromissions, $t(16) = -.626, p > .05$, PMCoX males displayed more ejaculations, $t(16) = -3.320, p < .05$, and less long intromissions, $t(16) = 2.831, p < .05$, compared to SHAM males (Figure 2.5a). PMCoX males also took slightly longer than SHAM males to express mounts, intromissions and ejaculations from test onset, although these differences were not statistically significant (all $p > .05$; Figure 2.5b). PMCoX males, however, took significantly longer than SHAM males to express long intromissions, $t(16) = -2.634, p < .05$ (Figure 2.5b).

Because the latencies to initiate mating (display the first mount) were slightly different between experimental groups, and this difference could affect the latencies to display subsequent mating behaviors, we also analyzed the latencies to display intromissions, ejaculations, and long intromissions after correcting for the latency to first mount (Figure 2.5c). After mating began, PMCoX and SHAM males had comparable latencies to display intromissions, $t(16) = -.329, p > .05$, and ejaculations, $t(16) = -.314, p > .05$. PMCoX males, however, still took longer than SHAM males to display long intromissions, $t(16) = -2.314, p < .05$.

PMCoX and SHAM males did not differ in the duration of their first post-ejaculatory interval, $t(16) = .075, p > .05$. In both PMCoX and SHAM males, post-ejaculatory interval durations increased across the ejaculatory series, $F(1,16) = 26.155, p < .05$, and there was no difference between groups in the pattern of this increase, $F(1,16) = 2.789, p > .05$. PMCoX and SHAM males also did not differ in the number of intromissions to reach the first ejaculation, $t(16) = .358, p > .05$. In both groups, fewer intromissions were required to reach the last ejaculation compared to the first ejaculation, $F(1,16) = 26.807, p < .05$, and there was no difference between groups in the pattern of this decline, $F(1,16) = .309, p > .05$. Finally, PMCoX

and SHAM males were comparable in their mounting efficiency, $t(16) = -.407, p > .05$. Table 2.3 summarizes these derived measures of male copulatory behavior.

Males with damage primarily outside the PMCo. For an additional comparison, we also analyzed the copulatory behavior of a subset of males that were excluded from the PMCoX group (Non-PMCoX, $n = 6$). In this subset, males had less than 20% damage of the PMCo and moderate to substantial damage of the AHi and/or BMP. Importantly, males with damage primarily outside the PMCo did not differ from SHAM males in any of the copulatory behavior measures analyzed (all $p > .05$, Table 2.4).

Testosterone assay

There was no difference in testosterone levels (ng/ml) between PMCoX and SHAM males, $F(1,16) = .006, p > .05$ (PMCoX = 5.972 ± 0.699 ; SHAM = 5.880 ± 1.043). The ranges of testosterone levels in both groups (PMCoX = 2.82 – 8.05 ng/ml; SHAM = 2.66 – 7.70 ng/ml) were within the physiological range reported for this species (Moore et al., 2004).

Discussion

The present results demonstrate that the PMCo regulates two distinct aspects of the mating sequence in male Syrian hamsters. First, the PMCo may be involved in directing appropriate chemosensory investigation during mating, as males with lesions of the PMCo displayed increased investigation of the female's non-anogenital region compared to SHAM males. Second, the PMCo may regulate sexual satiety, as PMCo-lesioned males took longer than SHAM males to display long intromissions, an indication of the onset of sexual satiety in this species (Bunnell *et al.*, 1977; Parfitt & Newman, 1998). This delayed onset of satiety was associated with both a decreased number of long intromissions and an increased number of

ejaculations, compared to SHAM males. In contrast to these effects on copulatory behavior, males with lesions of the PMCo preferred to investigate female odors over male odors, as did SHAM males, and were able to discriminate between male and female odors in a habituation-dishabituation task.

Electrolytic lesion technique

This study used multiple, small electrolytic lesions to generate discrete damage targeted at the PMCo. One limitation of this technique is that damage is not restricted to neuronal cell bodies but also includes fibers of passage. Consequently, it is possible that PMCo lesions disrupted anatomical connections of nearby brain areas. However, the primary fiber tracts associated with the PMCo are the accessory olfactory bulb efferents traveling along the ventral surface of the brain to the PMCo itself (Kevetter & Winans, 1981a; Kemppainen *et al.*, 2002). As the PMCo is the most caudal target of these fibers (Scalia & Winans, 1975), damage to the ventral surface does not disconnect the accessory olfactory bulb from other brain areas. Furthermore, males with lesion damage primarily to nuclei outside the PMCo, including the AHi and/or BMP, displayed copulatory behavior similar to that of SHAM males, suggesting that the behavioral deficits observed in PMCo-lesioned males do not simply reflect a disconnection of nearby brain areas. The use of excitotoxins for making lesions would reduce many of these concerns, as they spare fibers of passage, but we have found that they do not produce reliable, controllable lesion damage in this nucleus (unpublished observations).

The role of the PMCo in male copulatory behavior

Unlike lesions of the MPOA or MA, which eliminate male copulatory behavior in many rodent species (Lehman *et al.*, 1980; Powers *et al.*, 1987; Kondo, 1992; Paredes & Baum, 1997;

Heeb & Yahr, 2000), PMCo-lesioned males displayed all components of the mating sequence. PMCo lesions were associated, however, with critical alterations in the pattern of mating behavior, and these changes can be partitioned into two functional categories: inappropriate direction of chemosensory investigation and delayed onset of sexual satiety.

Modulation of chemosensory investigation. Male copulatory behavior in the Syrian hamster is characterized by intense chemosensory investigation of the female throughout the mating sequence (Bunnell et al., 1977), and this investigation is critical for stimulating and maintaining other aspects of male copulatory behavior (Johnston, 1975; 1986). Most investigation is targeted toward the female's anogenital region (Bunnell *et al.*, 1977; Kwan & Johnston, 1980), which contains the highly attractive vaginal secretion, although other regions are also attractive to males (Johnston, 1986).

In the present study, males with PMCo lesions displayed an over-investigation of the female's non-anogenital region, even though these males displayed normal levels of anogenital investigation. These results suggest that the PMCo is not required for attraction to, or investigation of, the female's anogenital region. Indeed, other chemo-responsive areas, such as the MA or BNST, mediate this aspect of mating behavior (Lehman *et al.*, 1980; Powers *et al.*, 1987). The PMCo may instead be critical for limiting extraneous investigation of relatively less appropriate areas of the female during mating. Non-anogenital odors, such as those produced by the flank, Harderian, and ear glands, provide different kinds of social information (Johnston & Rasmussen, 1984; Johnston, 1990) and thus may normally compete with the anogenital region for the male's attention when investigating a female. Our data suggest that the PMCo normally functions to inhibit investigation of these non-anogenital odors. Interestingly, PMCo-lesioned males did not display increased olfactory investigation during any of the other behavioral tests

(sexual odor preference or discrimination), indicating that this effect on investigation may be specific to the mating context, rather than reflecting general over investigatory behavior. Future studies are needed to determine whether the PMCo regulates non-anogenital investigation in other social contexts, such as agonistic encounters, during which investigation of non-anogenital odors may be more critical.

This proposed role of the PMCo in directing investigation behavior is supported by the PMCo's strong chemosensory inputs. Indeed, the PMCo is reciprocally connected with the accessory olfactory bulbs (Scalia & Winans, 1975; Kevetter & Winans, 1981a; Canteras *et al.*, 1992) and also has substantial indirect connections with the main olfactory system (Kevetter & Winans, 1981b). In hamsters, PMCo neurons display increases in c-fos expression following exposure to female vaginal secretion (Fewell & Meredith, 2002), although it is currently unclear whether the PMCo displays similar c-fos responses to other sources of female odors or whether these responses are seen in other species. Interestingly, male hamsters with damage of the stria terminalis (ST) display similar increases in non-anogenital investigation of the female during mating tests (Lehman *et al.*, 1983). As many PMCo projections to the BNST travel via the ST (Kevetter & Winans, 1981a; Canteras *et al.*, 1992; Wood & Swann, 2005), this particular effect of ST lesions may be due to a disconnection of the PMCo from the BNST.

Timing of sexual satiety. PMCo lesions were associated with shifts in the temporal pattern of the mating sequence. Specifically, males with PMCo lesions took longer to express long intromissions, and displayed an increased number of ejaculations, compared to SHAM males. As long intromissions and ejaculations are interdependent behaviors, it is difficult to define the underlying neural mechanism that is altered by PMCo lesions. There are, however, several possible interpretations of this pattern of results.

First, PMCo lesions may increase the expression of ejaculations independently of their effect on long intromissions. Thus, the PMCo may function as a central inhibitor of ejaculations. As the PMCo does not project to hypothalamic or brainstem nuclei known to stimulate ejaculations (Kevetter & Winans, 1981a; Canteras *et al.*, 1992; Normandin & Murphy, 2008), a direct modulation of ejaculations by the PMCo is unlikely. It remains possible, however, that the PMCo may indirectly modulate these ejaculation centers via its connections with the MA and BNST (Kevetter & Winans, 1981a; Canteras *et al.*, 1992).

Second, PMCo lesions may decrease neural processing of sensory feedback from the penis. This decreased sensory processing could, in turn, cause lesioned males to require more ejaculations to reach satiety. Ejaculation-related sensory information from the penis appears to be processed via lumbar spinothalamic cells that project to the parvocellular subparafascicular nucleus of the thalamus (Coolen *et al.*, 2004). Although other areas of the ventral forebrain, such as the MPOA BNST, and MA, receive projections from the SPFP (Coolen *et al.*, 1998; Coolen & Wood, 1998; Greco *et al.*, 1998a; Heeb & Yahr, 2001), it is currently unclear whether this sensory information is projected directly or indirectly to the PMCo. Future studies are therefore needed to determine whether the observed effects of PMCo lesions are due to decreased processing of penile sensory information.

Finally, we propose that the PMCo may function as a central regulator of sexual satiety. In this view, the increased ejaculations observed in PMCo-lesioned males may have occurred simply as a result of the delayed onset of satiety. The concept of a central regulator of sexual satiety has been proposed previously for the posterodorsal region of the MA, in which c-fos expression correlates specifically with the expression of long intromissions (Parfitt & Newman, 1998), and lesions of this nucleus have also been reported to delay sexual satiety (Parfitt *et al.*,

1996). Although c-fos expression within the PMCo has not been analyzed using this sexual satiety paradigm, reciprocal connections between the PMCo and MA (Kevetter & Winans, 1981a; Canteras *et al.*, 1992; Coolen & Wood, 1998; Wood & Swann, 2005) suggest a possible functional relationship between these brain areas. Consistent with this interpretation, the PMCo contains a dense population of μ -opioid receptors, which are known to regulate the expression of sexual satiety in rats (Miller & Baum, 1987; Rodriguez-Manzo & Fernandez-Guasti, 1995) and hamsters (Wu & Noble, 1986). Thus, this population of neurons within the PMCo may mediate the effects of endogenous opioids on the expression of sexual satiety. Although additional studies are needed to confirm the specific role of the PMCo, our data provide initial evidence that this nucleus regulates the timing of sexual satiety.

As males in this study were sexually naïve, the effects of PMCo lesions on copulatory behavior may be specific to the first sexual encounter. Indeed, many rodent species display improvements in mating behavior after sexual experience (Dewsbury, 1969; Fleming & Kucera, 1991; Phelps *et al.*, 1998; Hull *et al.*, 2002). However, it is important to note that both PMCo-lesioned and SHAM males were sexually naïve and would have similar, if any, mating problems. Furthermore, most of the changes in copulatory behavior resulting from sexual experience are related to mating efficiency (ie. number of mounts, ratio of mounts to intromissions, etc.) (Hull *et al.*, 2002). PMCo-lesioned and SHAM males, however, did not differ in any of these efficiency parameters, making it unlikely that the observed deficits were due solely to sexual inexperience. Nevertheless, the effects of PMCo lesions may be different in sexually experienced males, and future studies are needed to directly test whether sexual experience can compensate for the deficits associated with PMCo lesions.

The role of the PMCo in sexual odor preference

In contrast to its role in regulating male copulatory behavior, our results suggest that the PMCo is not critical for generating sexual odor preferences. In fact, males with lesions of the PMCo displayed robust preferences to investigate female odors over male odors when presented in a Y-maze and were able to discriminate between these odor sources in a habituation-dishabituation task. These results show that, although the PMCo receives substantial chemosensory input (Scalia & Winans, 1975; Kevetter & Winans, 1981b), other structures mediate attraction to opposite-sex odors. Specifically, we have previously demonstrated the role of the MA in regulating opposite-sex odor preferences in male (Maras & Petrulis, 2006) and female hamsters (Petrulis & Johnston, 1999). The present results also demonstrate that the elimination of sexual odor preferences observed following MA damage (Maras & Petrulis, 2006) was not simply due to a disconnection of the more caudal PMCo from the accessory olfactory bulbs.

Our finding that PMCo does not regulate odor preferences differs from what has been observed in a previous lesion study in female rats. Specifically, lesions of the PMCo in female rats eliminate the preference to spend time near intact males compared to castrated males (Romero et al., 1990). There are several critical differences between the previous study (Romero et al., 1990) and the current one that may explain this discrepancy. First, there may be significant sex and/or species differences in the neural regulation of odor preferences. Second, the study using female rats (Romero et al., 1990) examined a different type of odor preference (within opposite-sex, intact vs. castrated) compared to the current study (opposite- vs. same-sex). It is therefore possible that distinct brain areas regulate these qualitatively different types of odor preference.

Conclusions

The present study provides the first direct evidence for a functional role of the PMCo in regulating male reproductive behavior. Specifically, the PMCo regulates two distinct aspects of male copulatory behavior in the Syrian hamster: directing chemosensory investigation of the female and regulating the onset of sexual satiety. Importantly, the PMCo is part of an interconnected network of ventral forebrain nuclei that regulates many aspects of rodent social behavior (Wood, 1997). We hypothesize that the PMCo affects male copulatory behavior primarily through its modulation of other nuclei, such as the BNST and MA, within this circuit. Future studies are needed to address the nature of the connections among these brain regions that may contribute to the regulation of male reproductive behavior.

Acknowledgements

We would like to thank Mary Karom for performing all radioimmunoassays for this study. 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 2 Tables

Table 2.1 Surgical coordinates for electrolytic lesions of the PMCo. PMCo lesions were generated using a combination of 5 bilateral coordinates (total of 10 penetrations/animal). All coordinates are in millimeters. Anterior-posterior (AP) and medial-lateral (ML) coordinates are relative to bregma, whereas dorsal-ventral (DV) coordinates are relative to the level of dura at each coordinate. Seconds indicate the duration of anodal current (1mA) passed at each coordinate.

AP	ML	DV	Seconds
+ 1.00	± 2.75	– 8.05	5
+ 1.40	± 3.45	– 8.30	5
+ 1.85	± 3.50	– 7.95	9
+ 1.85	± 4.00	– 8.10	12
+ 2.20	± 3.75	– 7.95	6

Table 2.2 Summary of behavioral measures from Clean Y-maze tests. Both PMCoX and SHAM males investigated the left and right stimulus sides equally. There were no differences in general activity levels or total investigation levels between SHAM and PMCoX males. All investigation times are in seconds.

	Total number of arm entries	Investigation time (left)	Investigation time (right)
SHAM	21.3 ± 2.8	53.8 ± 8.1	60.6 ± 9.9
PMCoX	19.1 ± 1.5	50.2 ± 8.9	64.2 ± 10.1

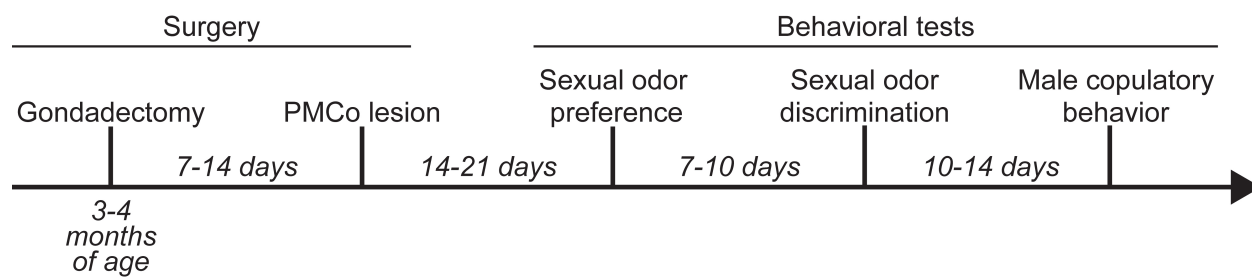
Table 2.3 Summary of derived behavioral measures from male copulatory tests. In both PMCoX and SHAM males, the post-ejaculatory intervals increased, whereas the number of intromissions to ejaculation decreased, across the first to last ejaculatory series within the copulatory test, * $p < .05$ compared to first ejaculatory series within each experimental group. Mounting efficiencies were not different between PMCoX and SHAM males.

	Post-ejaculatory interval		Number of intromissions to ejaculation		Mounting efficiency
	First	Last	First	Last	
SHAM	30.6 ± 4.6	47.7 ± 5.4*	7.3 ± 1.1	2.6 ± 0.6*	.59 ± .06
PMCoX	30.2 ± 4.0	55.6 ± 4.9*	6.7 ± 1.2	2.9 ± 0.6*	.62 ± .04

Table 2.4 Comparison of copulatory behavior between SHAM males and males with damage primarily outside the PMCo (Non-PMCoX). The copulatory behavior of males with damage primarily outside the PMCo (Non-PMCoX, $n = 6$) was compared to that of SHAM males. In Non-PMCoX males, damage targeted the amygdalohippocampal area (AHi) and posterior basomedial amygdala (BMP). Non-PMCoX males did not differ from SHAM males in any of the measures of copulatory behavior analyzed (all $p > .05$). Investigation times and latencies are in seconds.

Investigation times	SHAM	Non-PMCoX
Anogenital	210.2 \pm 10.2	256.4 \pm 34.9
Non-anogenital	132.8 \pm 24.2	149.2 \pm 24.4
Self-groom	580.0 \pm 28.2	547.0 \pm 32.2

Mating events	Number		Latency	
	SHAM	Non-PMCoX	SHAM	Non-PMCoX
Mount	16.6 \pm 3.1	15.3 \pm 3.0	150.8 \pm 25.5	286.7 \pm 94.4
Intromission	20.1 \pm 3.4	28.7 \pm 3.9	280.3 \pm 58.6	340.2 \pm 75.8
Ejaculation	5.5 \pm 0.4	5.7 \pm 0.4	417.1 \pm 48.1	453.4 \pm 73.5
Long intromission	18.0 \pm 1.1	15.2 \pm 1.7	783.5 \pm 83.5	876.3 \pm 76.9

Chapter 2 Figures**Figure 2.1 Timeline of surgeries and behavioral testing**

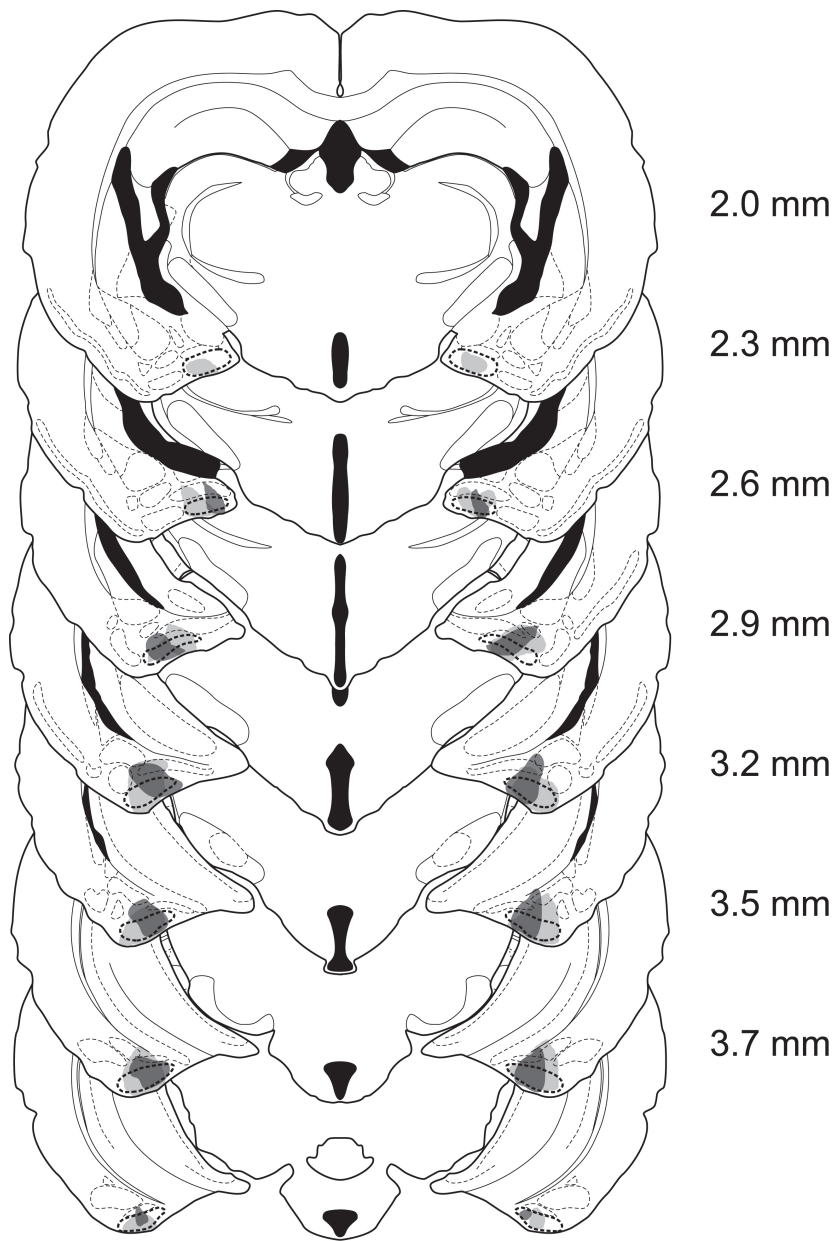


Figure 2.2 Lesion reconstruction. Reconstruction of the largest (light gray) and smallest (dark gray) lesions in PMCoX males. Sections proceed from anterior (top) to posterior (bottom) levels, with the numbers representing the distance posterior to bregma.

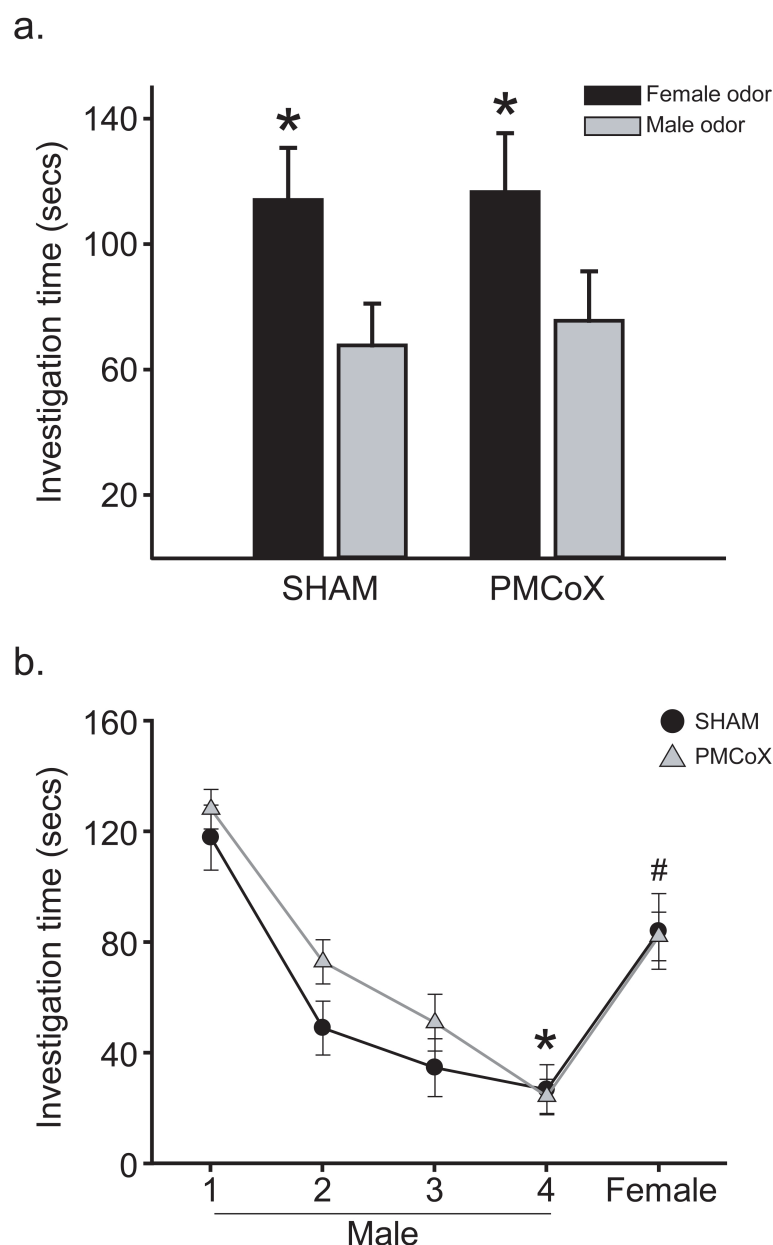


Figure 2.3 Behavioral results from sexual odor preference and discrimination tests. (a) Odor investigation times from the sexual odor preference test in the Y-maze. Both PMCoX ($n = 11$) and SHAM ($n = 7$) males preferred to investigate female odors longer than male odors, * $p < .05$ relative to investigation of male odor. (b) Odor investigation times during the sexual odor discrimination test. Both groups displayed decreased investigation during the fourth presentation of the male odor compared to the first presentation of the male odor, * $p < .05$. Both groups also displayed increased investigation during the test presentation of the female odor compared to the fourth presentation of the male odor, # $p < .05$.

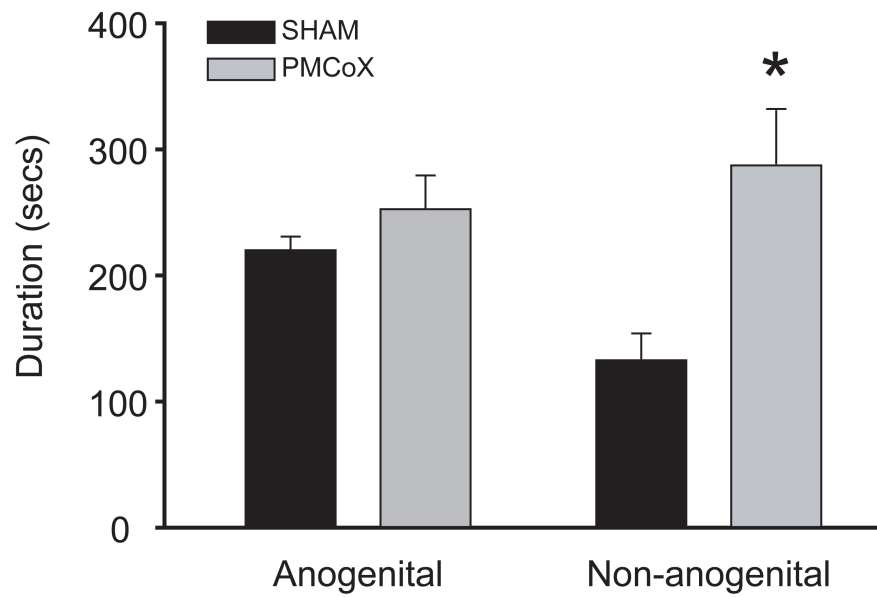


Figure 2.4 Investigation behaviors during male copulatory tests. Although groups did not differ in the duration of anogenital investigation, PMCoX males ($n = 11$) increased investigation of the female's non-anogenital region compared to SHAM males ($n = 7$). * $p < .05$ relative to SHAM group.

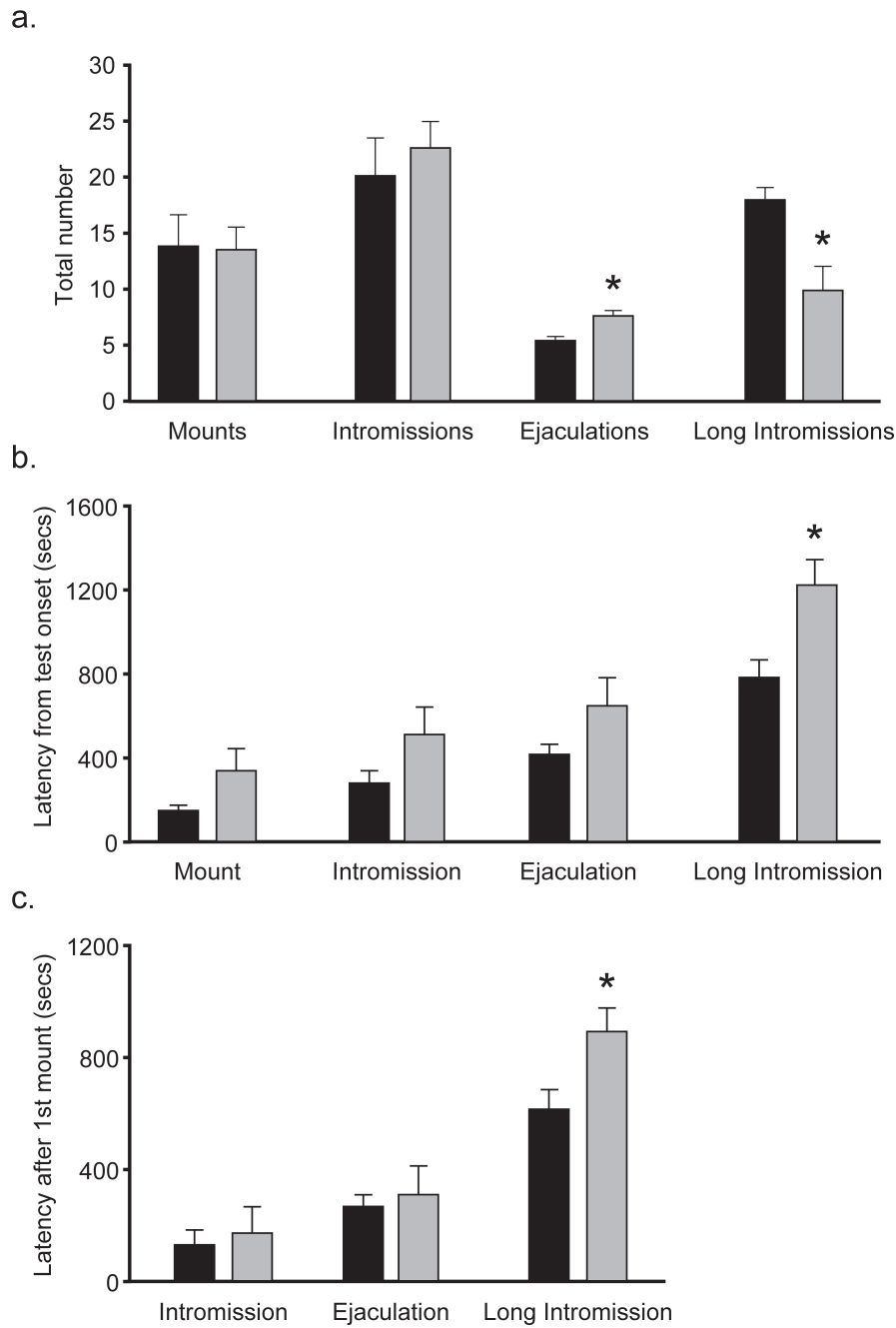


Figure 2.5 Summary of mating events during the male copulatory behavior test. (a)

Although groups displayed equal numbers of mounts and intromissions, PMCoX males ($n = 11$) displayed more ejaculations, and less long intromissions, compared to SHAM males ($n = 7$). (b) PMCoX males took longer (from test onset) than SHAM males to display long intromissions. (c) After mating began, PMCoX males still took longer than SHAM males to display long intromissions. All * $p < .05$, relative to SHAM group.

CHAPTER 3: Lesions that Functionally Disconnect the Anterior and Posterodorsal Subregions of the Medial Amygdala Eliminate Opposite-sex Odor Preference in Male Syrian Hamsters (*Mesocricetus auratus*)

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Previously Published in Neuroscience 65(4):1052-62 (2010).

Abstract

In many rodent species, such as Syrian hamsters, reproductive behavior requires neural integration of chemosensory information and steroid hormone cues. The medial amygdala processes both of these signals through anatomically distinct sub-regions; the anterior region (MeA) receives substantial chemosensory input, but contains few steroid receptor-labeled neurons, whereas the posterodorsal region (MePD) receives less chemosensory input, but contains a dense population of steroid receptors. Importantly, these sub-regions have considerable reciprocal connections, and the goal of this experiment was therefore to determine whether interactions between MeA and MePD are required for male hamsters' preference to investigate female over male odors. To functionally disconnect MeA and MePD, males received unilateral lesions of MeA and MePD within opposite brain hemispheres. Control males received either unilateral lesions of MeA and MePD within the same hemisphere or sham surgery. Odor preferences were measured using a 3-choice apparatus, which simultaneously presented female, male and clean odor stimuli; all tests were done under conditions that either prevented or allowed contact with the odor sources. Under non-contact conditions, males with asymmetrical lesions investigated female and male odors equally, whereas males in both control groups preferred to investigate female odors. Under contact conditions, all groups investigated female odors longer than male odors, although males with asymmetrical lesions displayed decreased investigation of female odors compared to sham males. These data suggest that MeA-MePD interactions are critical for processing primarily the volatile components of social odors and highlight the importance of input from the main olfactory system to these nuclei in the regulation of reproductive behavior. More broadly, these results support the role of the medial amygdala in

integrating chemosensory and hormone information, a process that may underlie social odor processing in a variety of behavioral contexts.

Introduction

In many rodent species, including Syrian hamsters, social behavior relies heavily on the perception of chemosignals released from conspecifics (Johnston, 1983; Hull *et al.*, 2002; Beauchamp & Yamazaki, 2003). In the context of reproductive behavior, male hamsters are highly attracted to female odors (Murphy, 1973; Johnston, 1974; Landauer *et al.*, 1977) and these chemosignals serve as the primary signal to initiate male copulatory behavior (Murphy, 1973; Johnston, 1975; 1986). Social odors are processed by two, anatomically distinct chemosensory systems; sensory receptors of the main olfactory system (MOS), located in the main olfactory epithelium, respond best to low molecular weight, volatile components of social odors, whereas sensory receptors of the accessory olfactory system (AOS), located in the vomeronasal organ, are thought to process high molecular weight, non-volatile components of social odors (Meredith, 1991; Restrepo *et al.*, 2004; Keller *et al.*, 2009). Together, these systems regulate most aspects of rodent social behavior, including the attraction to, and preference for, opposite-sex odors (Murphy & Schneider, 1970; Rowe & Edwards, 1972; Powers *et al.*, 1979; Edwards *et al.*, 1990; Keverne, 2004; Keller *et al.*, 2009).

In addition to chemosensory cues, male reproductive behavior is also regulated by internal signals of reproductive state via changes in circulating levels of gonadal steroid hormones (Beyer *et al.*, 1976; Morin & Zucker, 1978; Hull *et al.*, 2002). In hamsters, testosterone and its primary metabolites, estradiol and dihydrotestosterone, are critical not only for the expression of male copulatory behavior (Morin & Zucker, 1978; Powers *et al.*, 1985), but

also for male's attraction to investigate female odors (Steel, 1982; Powers & Bergondy, 1983; Powers *et al.*, 1985; Petrulis & Johnston, 1995). Consequently, the expression of reproductive behavior in male hamsters involves the neural integration of chemosensory and hormonal cues (Wood, 1998).

The medial amygdala (MA) has been suggested as one candidate site for integrating chemosensory and hormonal cues, as it receives both types of information (Wood, 1998). Functionally, the MA plays a critical role in odor-guided reproductive behaviors in many rodent species, including hamsters (Lehman *et al.*, 1980; Petrulis & Johnston, 1999; Maras & Petrulis, 2006), rats (Kondo, 1992; Kondo *et al.*, 1997; Stark *et al.*, 1998), and gerbils (Heeb & Yahr, 2000). Detailed analysis of the distinct sub-regions within MA, however, suggests that chemosensory and hormonal signals are processed separately within this nucleus. Indeed, the anterior MA (MeA) has extensive connections with both the MOS and AOS, via direct projections from the olfactory bulbs, as well as indirect projections through secondary chemosensory structures (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Lehman & Winans, 1982; Coolen & Wood, 1998; Kang *et al.*, 2009). Although the posterodorsal MA (MePD) receives some input from the accessory olfactory bulbs, these projections are less substantial than compared to MeA, and the MePD has much more limited connections with the MOS (in particular the secondary nuclei of the MOS) than compared to MeA (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Lehman & Winans, 1982; Coolen & Wood, 1998). The processing of steroid hormone information also appears to be separated within MA, as the vast majority of steroid receptor-containing neurons are localized specifically within MePD, not MeA (Doherty & Sheridan, 1981; Wood *et al.*, 1992; Wood & Newman, 1993).

In addition to these anatomical data, several lines of evidence suggest functional differences between MeA and MePD. For example, lesions of MeA completely eliminate male hamster copulatory behavior (Lehman et al., 1980) similar to deficits observed following destruction of the olfactory bulbs (Murphy & Schneider, 1970), whereas lesions restricted to MePD only alter the temporal pattern of the male copulatory sequence (Lehman et al., 1983). More recently, we have shown that, although both MeA and MePD are critical for the preference to investigate opposite-sex odors in male hamsters, these sub-regions regulate distinct aspects of social odor investigation (Maras & Petrulis, 2006). Specifically, MeA appears to function as a chemosensory filter to identify or categorize the sexual/social relevance of odors in the environment, whereas MePD may be critical for generating attraction specifically to opposite-sex odors (Maras & Petrulis, 2006). These behavioral findings are supported by several immediate-early gene studies that find that neurons within MeA respond to a wide variety of social odors, whereas neurons within MePD respond specifically to sexually relevant odors (Day *et al.*, 2004; Meredith & Westberry, 2004; Kiyokawa *et al.*, 2005; delBarco-Trillo *et al.*, 2009; Samuelsen & Meredith, 2009).

Taken together, these data suggest that MeA and MePD differentially process chemosensory and steroid hormone cues. Critically, substantial reciprocal fibers connect MeA and MePD (Gomez & Newman, 1992; Coolen & Wood, 1998), providing a substrate for the neural integration of these cues. We therefore hypothesized that interactions between MeA and MePD are *required* for appropriate behavioral responses to social odors in male hamsters. To test this hypothesis, we compared opposite-sex odor preferences displayed by males in which MeA and MePD connections were either disrupted or intact. As the connections between MeA and MePD do not constitute a discrete, identifiable pathway (Coolen & Wood, 1998), we used an

asymmetrical lesion technique, in which males received a combination of unilateral lesions of MeA and MePD in *opposite* brain hemispheres, to disconnect these nuclei. This technique takes advantage of three important facts: (1) the MeA and MePD each regulate distinct aspects of odor processing (Wood, 1997; Meredith & Westberry, 2004; Maras & Petrulis, 2006), (2) the connections between MeA and MePD are almost exclusively ipsilateral (Gomez & Newman, 1992; Coolen & Wood, 1998), and (3) unilateral lesions of MeA or MePD by themselves do not affect opposite-sex odor preferences in male hamsters (Maras & Petrulis, 2006). Thus, the asymmetrical lesion technique (hereby referred to as a “functional disconnection”) leaves each nucleus sufficiently intact to generate behavior, but prevents these nuclei from communicating with each other. These results provide the first evidence that the interactions between chemosensory and steroid-sensitive sub-regions of the MA are indeed critical for the attraction to sexual odors and further support the concept of MA as a critical node in the regulation of odor-guided aspects of social behavior (Newman, 1999).

Materials and Methods

Animals

Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratory (Wilmington, MA) at eight weeks of age. Subjects were sexually naïve males (4 – 6 months old, 120 – 150g) that had been singly housed upon arrival. A separate group of male and female hamsters (3 – 8 months old) were used to provide social odor stimuli. Subjects were unrelated to, and had no previous contact with, these odor donor animals. All animals were housed in solid-bottom Plexiglas cages (36 cm X 30 cm X 16 cm) and were maintained on a reversed 14-h light/10-h dark photoperiod (lights off/on at 9 am/7 pm). Food and water were available *ad libitum*.

All animal 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.

Surgical procedures

Male subjects were randomly assigned to one of three experimental groups. One group of males received asymmetrical electrolytic lesions of MeA and MePD (i.e. unilateral lesion of MeA combined with a unilateral lesion of MePD in the opposite hemisphere of the brain; ASYM $n = 35$). Seventeen of these males received MeA lesions in the left hemisphere, whereas 18 of these males received MeA lesions in the right hemisphere. To control for the effects of unilateral lesions of MeA and MePD, a second group of males received unilateral electrolytic lesions of MeA or MePD within the same hemisphere of the brain (UNI, total $n = 14$; left $n = 7$, right $n = 7$). Thus, ASYM and UNI males differed only in the functional connection between MeA and MePD. Finally, a third group of males received sham lesion surgery (SHAM, $n = 12$), in which there was no damage to MeA or MePD.

All males were anesthetized with 2% isoflurane anesthesia and placed into a stereotaxic apparatus so that the skull was flat. The temporal muscles were retracted from the skull and small holes were drilled to expose the surface of the brain. We used a combination of multiple small electrolytic lesions in order to generate maximal damage of MeA or MePD (Maras & Petrulis, 2006), while limiting collateral damage to nearby nuclei or major fiber tracts (Table 3.1). Electrolytic lesions were made by lowering a platinum/iridium electrode (0.25 mm diameter, 0.45 mm uninsulated tip, Frederick Haer & Co., Bowdoinham, ME) under stereotaxic

control and passing anodal current from a lesion-making device (Ugo Basile, Comerio, VA, Italy). Sham lesions were identical to electrolytic lesions except that the electrode was lowered 1.5 mm above each target coordinate (MeA and MePD), and no current was passed. Half of SHAM males received sham penetrations in opposite hemispheres of the brain (similar to ASYM males), whereas the other half received sham penetrations in the same hemisphere of the brain (similar to UNI males). Gel foam (Pharmacia & Upjohn Co., Kalamazoo, MI) was used to pack the holes in the skull, and the incision was closed with wound clips.

Behavioral testing

To determine whether interactions between MeA and MePD are critical for generating appropriate responses to social odors, subjects were given a series of behavioral tests beginning two weeks after lesion surgery. First, subjects were tested for their preference to investigate female odors over male odors in a 3-choice preference apparatus (*Odor preference*). In order to determine if any lack of preference in these tests was due to an inability to discriminate between female and male odor stimuli, a sub-set of males were then tested for their ability to discriminate between these odor sources using a habituation-dishabituation task (*Odor discrimination*). Furthermore, to determine if any effects of disconnecting MeA and MePD on odor investigation depend on the source of chemosensory input (MOS or AOS), all tests were done under conditions that either prevented contact with the odor sources (volatile odors only, stimulation of MOS) or allowed contact with the odor sources (volatile and non-volatile odors, stimulation of MOS and AOS).

Odor stimuli. Male and female odor stimuli were collected from cages that had housed 3 – 4 male or female hamsters and that had not had bedding changed for 3 – 4 days. Odor stimuli

consisted of 12 g of soiled cotton bedding (4 Nestlets, ANCARE, Bellmore, NY); 50 ml of soiled corncob litter (Bed-o-cob, The Andersons, Maumee, OH); one damp cotton gauze pad that was used to wipe the inner walls of the odor donor cage; and an additional damp gauze pad that was used to wipe the odor donor's flank and anogenital regions. For female odor stimuli, vaginal secretion was collected onto an additional gauze pad by gently palpating the vaginal area of a donor female with a disposable probe. 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), and female odor slides also contained a sample of vaginal secretion that was collected as described above. All odor stimuli were kept in airtight containers at 4°C until 30 minutes prior to use. Clean odor stimuli consisted of unsoiled components identical to those of the social odor stimuli.

For presentation of the odor stimuli, a single odor sample was placed into an acrylic odor container box (3 in X 3 in X 3 in), which had 7-mm holes drilled along the front surface that allowed volatile odors to pass, but prevented contact with the odor sources. For Contact tests only, in addition to the odors placed inside the odor containers, a single odor slide was secured to the front surface of each odor container, matching the type of odor stimulus (female, male, clean) for that container. 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 or odor slide.

Odor preference. Subjects were tested for their preference to investigate female odors over male odors using a 3-choice preference test. The testing apparatus consisted of a rectangular glass aquarium (20 in X 10 in X 12 in), with three odor containers secured along the bottom edge

of one of the short walls of the aquarium. During testing, a single odor stimulus (see above) was placed into each of the three odor containers. A midline was drawn down the center of the aquarium (parallel to short walls) in order to divide the aquarium into two areas (with or without odor containers). The top of the aquarium was secured with a clear Plexiglas top to allow for overhead video recording of the subject's behavior.

Subjects were tested in a series of four tests in the 3-choice apparatus, separated by 24 hours: Non-Contact Clean, Non-Contact Preference, Contact Clean, and Contact Preference. Non-Contact and Contact tests were identical, except that during Contact tests, odor slides were secured to the front surface of each of the odor containers (see above). For Clean tests, clean odor stimuli were placed into each of the three odor containers. These tests were used to habituate the subjects to the testing arena, as well as to obtain baseline behavioral data. For subsequent Preference tests, female or 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.

At the beginning of each test, a subject was placed into the area of the aquarium without odor containers and then allowed ten minutes to explore the apparatus. All surfaces of the aquarium and odor containers were thoroughly cleaned with 70% alcohol and allowed to dry between subjects. Video recordings of all tests were digitized onto a computer and scored using the Observer for Windows, version 5.0 (Noldus Information Technology B.V., 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. Both the time spent investigating each odor container and the number of times the subject crossed the midline of the aquarium were scored.

Odor discrimination. Following the final preference test, a sub-set of subjects (ASYM $n = 7$; UNI $n = 6$; SHAM $n = 7$) was tested for their ability to discriminate between female and male odors using a habituation-dishabituation task (Maras & Petrulis, 2008b). This approach 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 & 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. Under these testing parameters, male hamsters consistently display a lack of habituation to repeated presentations of female odors (Maras & Petrulis, 2006), and so all subjects were tested using male odors as the habituation stimuli and female odors as the dishabituation stimuli. 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.

Subjects were tested for their odor discrimination under two stimulus conditions, separated by 24 hours: Non-Contact (without slides) and Contact (with slides). Odor stimuli were presented in odor containers identical to those used in 3-choice preference apparatus. For discrimination tests, however, odor containers were presented inside the subject's home cage by securing the odor container onto an inside wall of the cage (with electrical tape on the back of the odor container). Investigation was measured live using a stopwatch, and odor containers were cleaned with 70% alcohol and allowed to dry between subjects.

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 phosphate-buffered formalin (10%). Brains were post-fixed in phosphate-buffered formalin (10%) overnight and then cryoprotected for 48 hours in 30% sucrose in PBS solution. Coronal sections (30- μ m) of brain tissue were sectioned using a cryostat (-20°C) and stored in PBS until mounting. Every third section was mounted onto glass slides using a 1% gelatin mounting solution and stained with cresyl violet. Sections were examined under a light microscope for the location and extent of lesion damage as compared with published hamster neuroanatomical plates (Morin & Wood, 2001), and the minimum and maximum extents of lesion damage were traced onto anatomical plates using Adobe Illustrator CS 11.0 software (2003).

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 (Non-Contact Clean, Non-Contact Preference, Contact Clean, Contact Preference), separate 3 (Experimental group: ASYM, UNI, SHAM) X 3 (Odor containers) mixed-design ANOVAs were performed. Significant interactions were explored using simple effects analysis and pair-wise comparisons with Bonferroni alpha adjustments ($\alpha_{FW} = .05$). 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 both Clean tests (Non-Contact and Contact).

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 male odors (Male 1 vs. Male 4) and (2) a dishabituation to the presentation of the female odor (Male 4 vs. Female).

Results

Lesion verification

Males were included in the ASYM lesion group ($n = 15$) or the UNI lesion group ($n = 12$) only if they had at least 60% damage of MeA and MePD, respectively, within at least two atlas plates (Morin & Wood, 2001; Figure 3.1). In the ASYM group, males were excluded from the analyses if there was substantial sparing of MeA and/or MePD (MeA $n = 5$; MePD $n = 5$; both $n = 3$) or if there was any bilateral damage of MeA or MePD ($n = 3$). Importantly, males were also excluded if damage from MeA lesions spread to the ventral surface of the brain ($n = 4$), as such damage may disrupt accessory olfactory bulb efferents passing to more caudal nuclei. In the UNI group, two males were excluded from the analysis because of substantial spread of lesion damage to the central nucleus of the amygdala. Importantly, the range of estimated total MeA-MePD damage was comparable between ASYM and UNI males (ASYM: 59 – 85%; UNI: 61 – 89%).

In both ASYM and UNI males, lesion damage was primarily restricted to MeA and MePD (Figure 3.1). Within MeA, most males had damage of both the dorsal and ventral regions (ASYM $n = 12$; UNI $n = 10$). Within MePD, damage extended to the caudal region in most

males (ASYM $n = 13$; UNI $n = 9$). In addition to damage of MeA-MePD, a sub-set of males had minor (less than 10%) damage of adjacent nuclei, including the posteroventral medial amygdala, (ASYM $n = 5$; UNI $n = 3$), intercalated nucleus of the amygdala (ASYM $n = 5$; UNI $n = 4$), and the amygdalohippocampal area (ASY $n = 4$; UNI $n = 2$). Importantly, damage to adjacent nuclei was minimal (Figure 3.1), and there were no differences in either the preference or discrimination behavior of males with or without damage to adjacent nuclei.

Odor preference

Clean tests. In the Non-Contact condition, all experimental groups investigated the center container less than the left container, $t(38) = 7.305, p < .001$, and the center container less than the right container $t(38) = 5.813, p < .001$, although there was no difference between the investigation durations of the left and right containers (Table 3.2). Thus, although there was a general bias to investigate the outside containers, there was no difference in this bias across experimental groups, and more importantly, there was no preference for either one of the outside containers that were used to present social odors. In the Contact condition, all subjects investigated the three stimulus containers equally (Table 3.2). In both the Non-Contact and Contact conditions, there were no differences in the total number of midline crosses or the total duration of investigation across experimental groups (Table 3.2).

Preference tests. For both Non-Contact and Contact Preference tests, Mauchly's tests indicated that the assumption of sphericity had been violated, and analyses were therefore corrected using Greehouse-Geisser estimates of sphericity (Non-Contact $\epsilon = .814$; Contact $\epsilon = .596$). In the Non-Contact Preference test, there was a significant interaction between experimental group and investigation durations across the three odor stimuli, $F(3.25, 58.5) =$

16.703, $p < .001$. Whereas both SHAM and UNI males preferred to investigate female odors over male odors (SHAM $t(11) = 8.290$, $p < .001$; UNI $t(11) = 8.452$, $p < .001$), ASYM males investigated female odors and male odors equally, $t(14) = 1.215$, $p = .244$ (Figure 3.2A).

Although all groups investigated female odors longer than clean odors (SHAM $t(11) = 10.225$, $p < .001$; UNI $t(11) = 10.515$, $p < .001$; ASYM $t(14) = 4.263$, $p = .001$) and male odors longer than clean odors (SHAM $t(11) = 5.116$, $p < .001$; UNI $t(11) = 3.853$, $p = .003$; ASYM $t(14) = 6.440$, $p < .001$), ASYM males displayed decreased investigation of the female odor, $F(2, 36) = 13.999$, $p < .001$, and increased investigation of the male odor, $F(2, 36) = 7.157$, $p = .002$, than compared to SHAM and UNI males.

In the Contact Preference test, there was a significant main effect of stimulus, $F(1.19, 42.9) = 156.417$, $p < .001$, but there was no significant interaction between experimental group and investigation durations across the odor stimuli (Figure 3.2B). Indeed, all experimental groups investigated female odors longer than male odors, $t(38) = 7.336$, $p < .001$, female odors longer than clean odors, $t(38) = 15.763$, $p < .001$, and male odors longer than clean odors, $t(38) = 15.266$, $p < .001$ (Figure 3.2B). There was a significant difference, however, in the duration of investigation of the female odor across experimental groups, $F(2, 36) = 3.400$, $p = .041$; ASYM males investigated the female odor less than compared to SHAM males, whereas UNI males investigated the female odor at levels that were not different from either SHAM or ASYM males (Figure 3.2B).

Odor discrimination

In the Non-Contact Discrimination test (Figure 3.3A), all experimental groups habituated to repeated presentations of different male odors, as indicated by a decreased investigation of the

male odor on the fourth trial compared to the first trial (SHAM $t(6) = 6.307, p < .001$; UNI $t(5) = 7.252, p = .001$; ASYM $t(6) = 6.221, p = .002$). Importantly, all experimental groups also discriminated between the male odor and female odor, as indicated by an increased investigation of the test female odor compared to the last presentation of the habituated male odor (SHAM $t(6) = 3.978, p = .005$; UNI $t(5) = 3.874, p = .006$; ASYM $t(6) = 10.341, p < .001$). There were no significant differences among the groups in their investigation levels during any of the odor presentations.

In the Contact Discrimination test (Figure 3.3B), all experimental groups again habituated to repeated presentations of different male odors (SHAM $t(6) = 9.576, p < .001$; UNI $t(5) = 6.446, p = .001$; ASYM $t(6) = 8.670, p < .001$) and also discriminated between the sequential presentations of male and female odors (SHAM $t(6) = 6.786, p < .001$; UNI $t(5) = 4.982, p = .008$; ASYM $t(6) = 5.300, p = .003$). Furthermore, in the Contact condition, ASYM males investigated the female odor less than compared to both SHAM and UNI males, $F(2, 19) = 4.399, p = .029$, similar to what was observed in both the Non-Contact and Contact Preference tests.

Discussion

The results demonstrate that a functional connection between MeA and MePD is critical for the preference to investigate the volatile components of opposite-sex odors. Indeed, males that had MeA and MePD functionally disconnected by asymmetrical lesions investigated female and male odors equally when contact with the odor sources was prevented. In contrast, control males that had the same amount of total MA damage, but in which the connections between MeA and MePD remained intact in one hemisphere, displayed a strong preference to investigate

female over male volatile odors, similar to control males. The lack of preference displayed by males with asymmetrical lesions was associated with both a decreased investigation of female odors and an increased investigation of male odors, suggesting an inappropriate direction of investigatory behavior.

When contact with the odor sources was allowed, all males preferred to investigate female odors over male odors. Thus, our results suggest that the importance of MeA-MePD interactions depends on the type of odor cues available in the environment and consequently, which sensory system is activated (MOS or AOS). When only volatile components of social odors are available, as would be the case when animals are approaching odors from a distance, these odors primarily stimulate sensory receptors of the MOS (Meredith, 1991; Restrepo et al., 2004; Keller et al., 2009). Destruction of the main olfactory epithelium, via intranasal zinc sulfate infusions, disrupts the ability to detect the volatile components of female vaginal secretion in male hamsters (Powers & Winans, 1973) and eliminates the preference to investigate the volatile components of sexual odors in male and female mice (Keller *et al.*, 2006b; a). Compared to MeA, MePD receives relatively few projections from the MOS and therefore relies heavily on MeA to receive main olfactory input (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Coolen & Wood, 1998). Thus, when the primary chemosensory input is from the MOS, functional interactions between the MeA and MePD are absolutely critical for the processing and recognition of social odor information.

However, when non-volatile components are available, as would be the case when animals are in direct contact with odor sources or interacting with conspecifics, the AOS is stimulated by the additional non-volatile cues (Meredith, 1991; Restrepo et al., 2004). Although the AOS is not needed for sex discrimination of volatile odors, the AOS is critical for the

preference to investigate sexual odors when direct contact with the odors is allowed (Petrulis et al., 1999; Pankevich et al., 2004; Keller et al., 2006c; Pankevich et al., 2006). As the MePD does receive accessory olfactory input independently of the MeA (Scalia & Winans, 1975; Kevetter & Winans, 1981a), our results suggest that this additional input is sufficient for the accurate processing of odor information in the absence of MeA-MePD interactions. Nevertheless, the fact that males with asymmetrical lesions displayed decreased investigation of female odors when contact with the odor sources was allowed suggests that interactions between MeA and MePD may still modulate levels of attraction to sexual odors processed by the AOS.

The lack of preference observed in males with asymmetrical lesions cannot be explained by basic sensory deficits, as all males discriminated between male and female odors during the habituation-dishabituation tasks. We have previously shown that neither MeA nor MePD are required for discrimination between female and male odors (Maras & Petrulis, 2006), and chemosensory structures outside of MA, such as the piriform and entorhinal cortices, are likely sufficient to make these basic types of odor discrimination (Petrulis & Eichenbaum, 2003). Furthermore, during the Clean tests, there were no differences across experimental groups in the levels of investigation or numbers of line crosses, suggesting that deficits in preference were not due to changes in baseline investigation or general motor ability, respectively.

Electrolytic lesion technique

This study used multiple, small electrolytic lesions to generate specific damage of MeA and MePD. One limitation of this technique, however, is that damage is not restricted to neuronal cell bodies but also includes fibers of passage. Of particular concern for MA lesions are the accessory olfactory bulb (AOB) efferents located ventral to MeA and the stria terminalis located

caudal to MePD (Scalia & Winans, 1975; Lehman *et al.*, 1983). To reduce the possibility that behavioral deficits could be due to damage of these fiber tracts, we used strict exclusion criteria to eliminate males from the analyses in which there was any lesion damage of AOB efferents or the stria terminalis. As a result, males included in both lesion groups (asymmetrical and unilateral) had highly homologous patterns of lesion damage, and damage was restricted to MeA and MePD nuclei. The use of excitotoxins for making lesions would eliminate these concerns, as they spare fibers of passage. We have found, however, that excitotoxins do not produce reliable and controllable lesion damage of MA (P.M. Maras and A.P. Petrulis, unpublished observations), perhaps due to the neuroprotective effects of estradiol receptor activation within this nucleus (Suzuki *et al.*, 2006).

Differential roles of MeA and MePD

Data from both anatomical and functional studies suggest that MeA and MePD have distinct roles in regulating odor processing. Specifically, we propose that MeA functions as a chemosensory filter to identify the category (species, sex) of social odors. Indeed, MeA receives direct projections from both the main and accessory olfactory bulbs, as well as projections from the piriform cortex, anterior cortical amygdala and posterolateral cortical amygdala (MOS nuclei) and posteromedial cortical amygdala (AOS nuclei) (Scalia & Winans, 1975; Kevetter & Winans, 1981a; b; Lehman & Winans, 1982; Coolen & Wood, 1998). Functionally, male hamsters with bilateral lesions of MeA do not prefer to investigate opposite-sex odors and display inappropriately high levels of investigation toward male odors (Maras & Petrulis, 2006), suggesting that in the absence of MeA, males treat male and female odors as equally attractive. This interpretation is supported by the finding that MeA neurons show increases in immediate early gene expression, a marker of excitatory neuronal activity, following different kinds of

social behaviors (Kollack-Walker & Newman, 1995), as well as exposure to many types of conspecific and heterospecific odors (Meredith & Westberry, 2004; Kiyokawa *et al.*, 2005; delBarco-Trillo *et al.*, 2009; Samuelsen & Meredith, 2009), including predator odors (Day *et al.*, 2004).

Compared to MeA, MePD receives relatively less input from AOS and has much more limited connections with secondary structures of the MOS (Scalia & Winans, 1975; Kevetter & Winans, 1981b), although there is now evidence that MePD receives some direct projections from the main olfactory bulbs in mice (Kang *et al.*, 2009). Given the dense population of steroid receptor-containing neurons within MePD (Doherty & Sheridan, 1981; Wood *et al.*, 1992; Wood & Newman, 1993), this nucleus may function to enhance processing of sexually relevant odors specifically when the animal is reproductively active (i.e. when sufficient levels of gonadal hormones are present). In fact, steroid hormones are required for male attraction to female chemosignals in a variety of rodent species (Carr *et al.*, 1966; Powers & Bergondy, 1983; Bean *et al.*, 1986; Ferkin & Gorman, 1992), and implants of testosterone or estradiol directly into MePD can facilitate many aspects of reproductive behavior in gonadectomized male hamsters (Wood & Newman, 1995c; Wood, 1996) and rats (Bialy & Sachs, 2002; Huddleston *et al.*, 2003). When MePD is damaged, male hamsters display decreased attraction to investigate female odors in a Y-maze apparatus (Maras & Petrulis, 2006), as well as decreased anogenital investigation of a receptive female (Lehman *et al.*, 1983). Similarly, MePD lesions eliminate the expression of non-contact penile erections displayed by male rats exposed to volatile odors from an estrous female (Kondo *et al.*, 1998; Kondo & Sachs, 2002), consistent with a pattern of decreased arousal in response to sexual cues.

Models of MeA-MePD interaction

The current findings provide strong evidence for the importance of interactions between MeA and MePD during social odor processing, and there are several possible ways by which these nuclei may integrate chemosensory and steroid information. First, the role of MeA may be to convey information about the social and/or sexual relevance of odor stimuli directly to MePD. Indeed, MePD receives the majority of its chemosensory input from MeA (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Coolen & Wood, 1998), and several immediate early gene studies describe a pattern of odor responses that becomes more specific from anterior to posterior levels of MA. Specifically, at the level of MeA, neurons respond to a broad variety of social odors, whereas at the level of MePD, neurons respond selectively to opposite-sex odors (Day *et al.*, 2004; Meredith & Westberry, 2004; Kiyokawa *et al.*, 2005; delBarco-Trillo *et al.*, 2009; Samuelsen & Meredith, 2009). The MePD may function to enhance processing specifically of opposite-sex odors and then convey this odor-specific response to downstream nuclei such as the BNST and/or MPOA (Gomez & Newman, 1992; Canteras *et al.*, 1995). This model describes a unidirectional flow of odor information from MeA to MePD and would suggest that MePD provides the primary output of MA. A unidirectional model is in fact supported by recent experiments in our laboratory that measured odor-induced Fos expression in males with unilateral lesions of either MeA or MePD; MeA-lesions decrease odor-induced Fos expression within MePD, but MePD -lesions have no effect on Fos expression within MeA (Maras & Petrulis, 2008a).

Alternatively, MeA and MePD may interact reciprocally to process social odor information. Similar to the model above, MeA provides the primary information about odor stimuli to MePD but, in this reciprocal model, MePD provides feedback onto MeA to enhance

processing of opposite-sex odors (Coolen & Wood, 1998). This model suggests that MeA provides the primary output to BNST and/or MPOA (Gomez & Newman, 1992; Canteras *et al.*, 1995) and that MeA is critical for appropriately directing social behavior. In support of this model, MeA serves as the primary MA-output for the regulation of copulatory behavior in male hamsters; lesions of MeA not only abolish mating, but also eliminate anogenital investigation of a receptive female (Lehman *et al.*, 1980), whereas lesions of MePD only alter the temporal sequence of mating behaviors and decrease levels of anogenital investigation (Lehman *et al.*, 1983). Furthermore, lesions of MeA, but not MePD, alter odor-induced Fos expression within BNST and MPOA (Maras & Petrulis, 2008a), supporting a model in which MeA serves as the primary chemosensory output to downstream nuclei.

In addition to direct interactions between MeA and MePD, it is possible that MeA may indirectly modulate odor processing within MePD via feedback interactions with AOB. Indeed, MeA neurons directly project onto mitral/tufted cells of AOB (Gomez & Newman, 1992; Martel & Baum, 2009), and this feedback may be critical for the selectivity of odor responses within AOB, perhaps via modulation of inhibitory processing (Hendrickson *et al.*, 2008). This model would suggest that, in the absence of MeA, the output from AOB to MePD would be sufficiently degraded such that MePD incorrectly processes social odor cues. The possible importance of MeA-AOB interactions is highlighted by recent studies showing the activation of AOB-projecting neurons within MeA specifically in response to volatile, opposite-sex urinary odors in female mice (Martel & Baum, 2009).

Finally, it is possible that chemosensory and hormonal cues are integrated throughout the extended forebrain network. Indeed, the anatomical separation of chemosensory and hormone processing that is observed within MA is maintained throughout bed nucleus of the stria

terminalis (BNST) and medial preoptic area (MPOA), as well as other downstream structures (Wood, 1998). The posterior medial zone of the BNST and the medial subdivisions of the MPOA contain high concentrations of steroid receptor-containing neurons (Doherty & Sheridan, 1981; Wood *et al.*, 1992; Li *et al.*, 1993) and are strongly linked to the MePD (Gomez & Newman, 1992; Coolen & Wood, 1998), whereas the posterior intermediate zone of the BNST and the lateral subdivisions of the MPOA contain relatively fewer steroid receptor-containing neurons (Doherty & Sheridan, 1981; Wood *et al.*, 1992; Li *et al.*, 1993) and are preferentially connected with the MeA (Gomez & Newman, 1992; Coolen & Wood, 1998). Given the reciprocal connections within MA, BNST, and MPOA (Simerly & Swanson, 1986; 1988; Coolen & Wood, 1998; Wood & Swann, 2005), the integration of chemosensory and hormonal cues may occur at any or all of these levels of processing and may reflect a mechanism for recurrent modification of social odor signals within the ventral forebrain circuit (Wood, 1997).

Concluding remarks

Interactions between chemosensory and hormone-sensitive forebrain nuclei may represent a basic mechanism by which the brain integrates information about social cues in the environment with hormonal indices of reproductive state (Wood, 1998; Newman, 1999). The connections between MeA and MePD may therefore provide a neural substrate for the refinement of odor responses and regulation of social behaviors in a dynamic internal and external environment. Although the current findings suggest that MeA-MePD interactions are critical for odor processing related to reproductive contexts, these interactions are likely important for the appropriate expression of other social behaviors, including agonistic, defensive and maternal behaviors (Luiten *et al.*, 1985; Numan & Sheehan, 1997; Newman, 1999; Dielenberg & McGregor, 2001). Future studies that identify the chemical phenotype of odor-

responsive neurons within MA, as well as the nature of the connections between MeA and MePD and their downstream targets, will be critical for our understanding of the fundamental mechanisms by which the brain processes social odor information and regulates the output of behavior in a variety of social contexts.

Acknowledgements

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 Tables

Table 3.1 Coordinates for electrolytic lesions of MeA or MePD. Electrolytic lesions of MeA or MePD were generated using a combination of 2 or 3 small lesions, respectively. All coordinates are in millimeters. Anterior-posterior (AP) and medial-lateral (ML) coordinates are relative to bregma, whereas dorsal-ventral (DV) coordinates are relative to dura at each coordinate. Seconds indicate the duration of anodal current (1mA) passed at each coordinate.

Site	MeA		MePD		
	1	2	1	2	3
AP	– 0.60	– 0.30	+ 0.50	+ 0.70	+ 0.95
ML	± 2.45	± 2.50	± 2.85	± 2.80	± 2.75
DV	– 7.45	– 7.50	– 7.20	– 7.30	– 7.25
Seconds	11	10	5	6	6

Table 3.2 Summary of behavioral measures from Clean tests in the 3-choice apparatus. The mean (\pm SEM) total number of midline crosses and investigation durations (seconds) of the three stimulus containers during Clean tests (SHAM $n = 12$; UNI $n = 12$; ASYM $n = 15$). * indicates significant differences between center container and both left and right containers using pairwise comparisons, $p < .05$, $\alpha_{FW} = .05$.

		Investigation duration		
		Left	Center	Right
<i>Non-Contact</i>				
SHAM	27.1 ± 2.5	23.0 ± 4.1	13.1 ± 2.2*	25.2 ± 4.5
UNI	26.8 ± 2.1	27.8 ± 3.6	11.7 ± 1.8*	22.4 ± 3.7
ASYM	28.5 ± 1.6	28.2 ± 4.8	14.2 ± 1.6*	23.0 ± 4.2
<i>Contact</i>				
SHAM	29.2 ± 3.5	25.2 ± 2.0	21.0 ± 4.4	23.4 ± 3.1
UNI	25.3 ± 1.5	21.0 ± 1.7	19.1 ± 3.4	24.7 ± 3.2
ASYM	28.6 ± 2.6	24.2 ± 3.0	22.1 ± 3.8	26.5 ± 3.9

Chapter 3 Figures

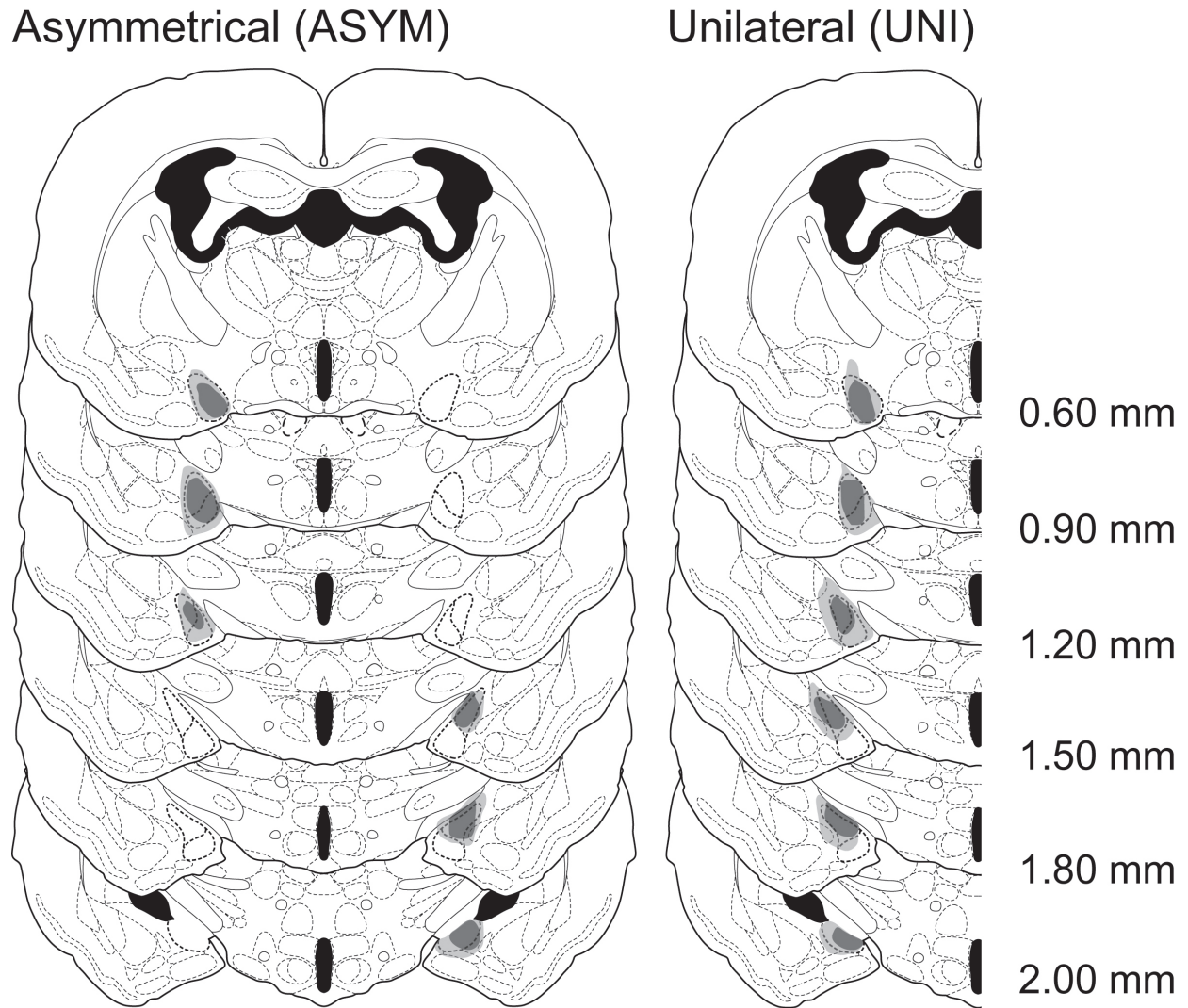
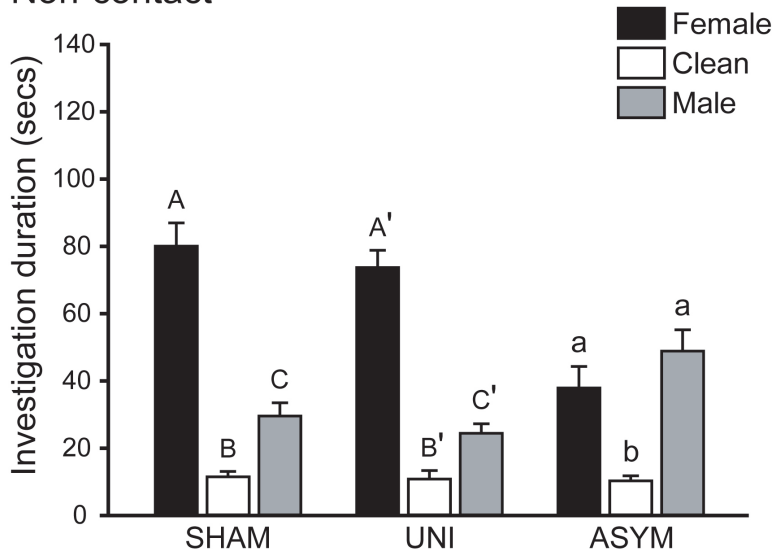


Figure 3.1 Lesion reconstruction. Drawings of the largest (light gray) and smallest (dark gray) extent of lesion damage in males with asymmetrical (ASYM; $n = 15$) or unilateral (UNI; $n = 12$) lesions of MeA and MePD. Sections proceed from anterior (top) to posterior (bottom) levels, with the numbers representing the distance posterior to bregma. The side (left or right) of the MeA and/or MePD lesion was randomly assigned within each lesion group.

A. Non-contact



B. Contact

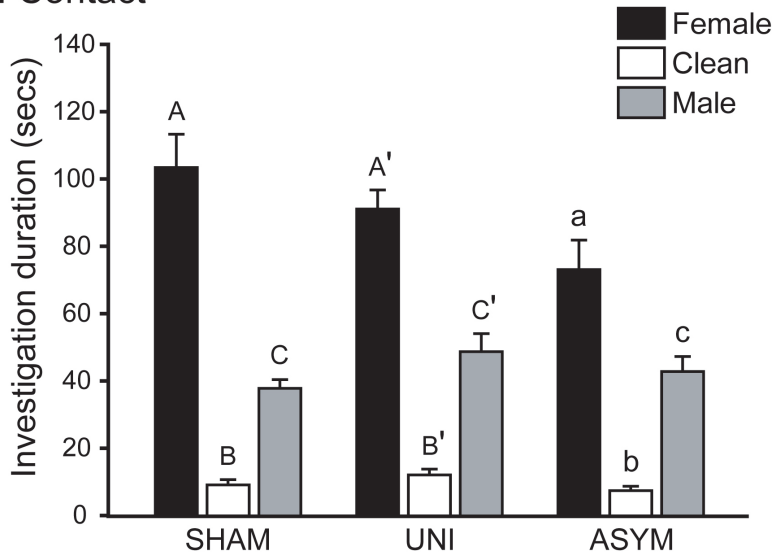
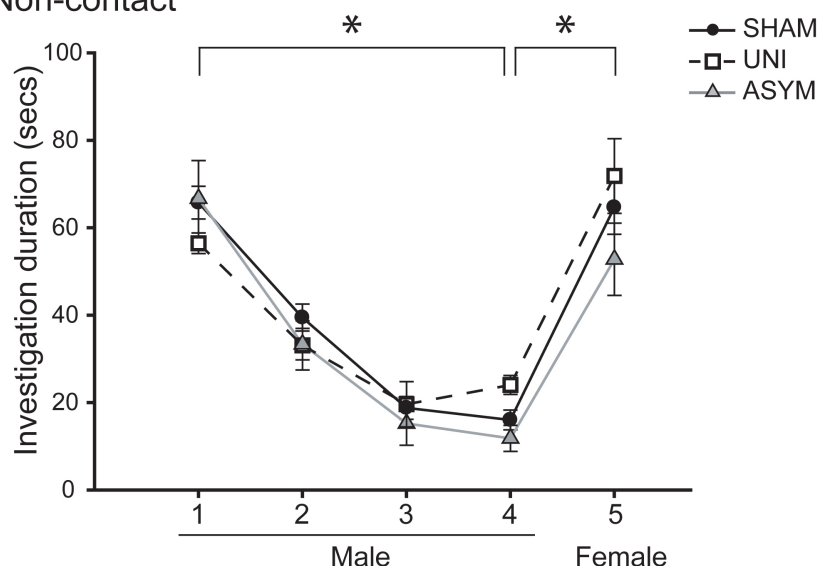


Figure 3.2 Summary of results from Odor Preference tests. Mean (\pm SEM) durations of odor investigation during Odor Preference tests when contact with odor sources was either prevented (A) or allowed (B) (SHAM $n = 12$; UNI $n = 12$; ASYM $n = 15$). Each letter type (uppercase, primed, lowercase) represents post-hoc comparisons within experimental group, and identical letters represent homologous means within each group ($p > .05$, pairwise comparisons with $\alpha_{fw} = .05$). When contact with the odor sources was prevented, ASYM males investigated female and male odors equally, whereas SHAM and UNI males preferred to investigate female odors. When contact with the odor sources was allowed, all groups preferred to investigate female odors over male odors. In both stimulus conditions, all groups investigated the female and male odors longer than clean odors.

A. Non-contact



B. Contact

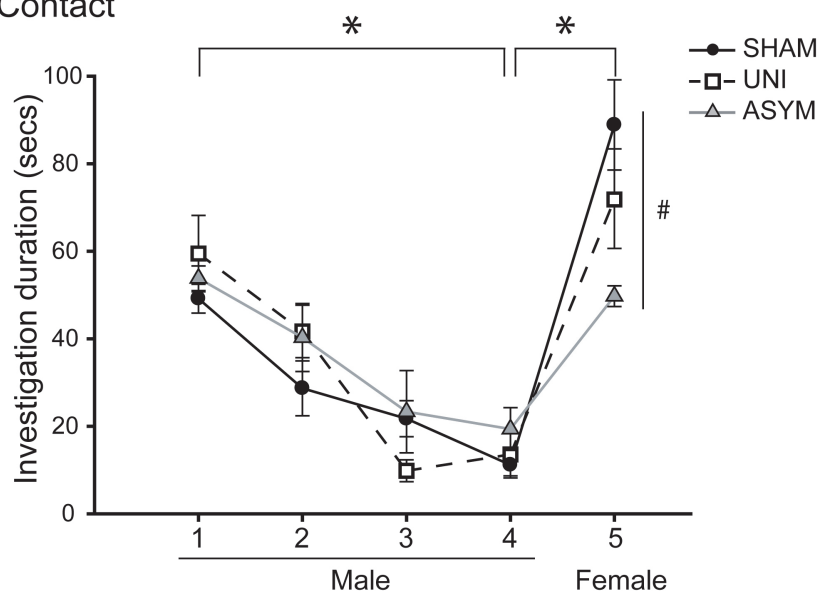


Figure 3.3 Summary of results from Odor Discrimination tests. Mean (\pm SEM) durations of investigation during the habituation-dishabituation tests when contact with the odor sources was either prevented (A) or allowed (B) (SHAM $n = 7$; UNI $n = 6$; ASYM $n = 7$). All groups displayed decreased investigation during the fourth presentation of the male odor compared to the first presentation of the male odor ($* p < .05$ with $\alpha_{fw} = .05$). All groups also displayed increased investigation during the presentation of the female odor compared to the fourth presentation of the male odor ($* p < .05$ with $\alpha_{fw} = .05$). When contact with the odor sources was allowed, ASYM males investigated the female odor for less time than compared to SHAM and UNI males ($\# p < .05$, Tukey's B post-hoc comparisons).

**CHAPTER 4: The Anterior Medial Amygdala Transmits Sexual Odor Information to the
Posterior Medial Amygdala and Related Forebrain Nuclei**

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In press, European Journal of Neuroscience (2010).

Abstract

In Syrian hamsters, reproductive behavior relies on the perception of chemical signals released from conspecifics. The medial amygdala (MA) processes sexual odors through functionally distinct, but interconnected sub-regions; the anterior sub-region (MeA) appears to function as a chemosensory filter to distinguish between opposite-sex and same-sex odors, whereas the posterodorsal region (MePD) is critical for generating attraction specifically to opposite-sex odors. To identify how these sub-regions interact during odor processing, we measured odor-induced Fos expression, an indirect marker of neuronal activation, in the absence of either MeA or MePD processing. In Experiment 1, electrolytic lesions of MeA decreased Fos expression throughout the posterior MEA in male hamsters exposed to either female or male odors, whereas MePD lesions had no effect on Fos expression within MeA. These results indicate that MeA normally enhances processing of sexual odors within MePD and that this interaction is primarily unidirectional. Furthermore, lesions of MeA, but not MePD, decreased Fos expression within several connected forebrain nuclei, suggesting that MeA provides the primary excitatory output of MA during sexual odor processing. In Experiment 2, we observed a similar pattern of decreased Fos expression using fiber-sparing, NMDA lesions of MeA, suggesting that the decreases in Fos expression were not due exclusively to damage of passing fibers. Taken together, these results provide the first direct test of how the different sub-regions within MEA interact during odor processing and highlight the role of MeA for transmitting sexual odor information to the posterior MA, as well as to related forebrain nuclei.

Introduction

In many rodent species, including Syrian hamsters, social behavior relies on the perception of chemical signals released from conspecifics (Johnston, 1983). In the context of reproduction, male hamsters are highly attracted to female odors, and these odors serve as the primary signal to initiate male copulatory behavior (Murphy, 1973; Johnston, 1975; 1986). A network of ventral forebrain nuclei processes sexual odors and generates appropriate behavioral responses to these stimuli (Wood, 1997; Newman, 1999).

Within this network, the medial amygdala (MA) has been identified as a critical node for processing odor information (Newman, 1999). Indeed, MA receives direct input from both main and accessory olfactory bulbs (MOB and AOB, respectively) (Scalia & Winans, 1975; Coolen & Wood, 1998) and conveys this chemosensory information to downstream forebrain nuclei to regulate the output of reproductive behavior (Gomez & Newman, 1992; Canteras *et al.*, 1995). Lesions of MA disrupt the expression of both the appetitive and consummatory aspects of male reproductive behavior in many rodent species (Lehman *et al.*, 1980; Kondo, 1992; Heeb & Yahr, 2000), although the mechanisms by which MA identifies different sexual odors remain poorly understood.

Detailed analysis of the distinct sub-regions within MA has provided important insights into how MA processes different social odors. In particular, the anterior MA (MeA) receives the majority of chemosensory input (Scalia & Winans, 1975; Kevetter & Winans, 1981b), and MeA neurons display increases in immediate early gene (IEG) expression, a marker of neuronal activation, in response to a wide variety of social odors, including both conspecific and heterospecific odors (Meredith & Westberry, 2004; delBarco-Trillo *et al.*, 2009; Samuelsen &

Meredith, 2009). Compared to MeA, the posterodorsal MA (MePD) receives relatively less chemosensory input (Scalia & Winans, 1975; Kevetter & Winans, 1981b) and displays a more restricted response to social odors; MePD neurons are only activated by conspecific odors and display higher levels of IEG expression in response to opposite-sex compared to same-sex odors (Meredith & Westberry, 2004; delBarco-Trillo *et al.*, 2009; Samuelson & Meredith, 2009).

Although both MeA and MePD are required for opposite-sex odor preferences in male hamsters, these sub-regions regulate distinct aspects of this behavior; MeA lesions increase investigation of same-sex odors, indicating an over-generalized odor response, whereas MePD lesions decrease investigation specifically to opposite-sex odors, indicating decreased attraction to sexually relevant odors (Maras & Petrulis, 2006). Importantly, MeA and MePD are reciprocally connected (Coolen & Wood, 1998), and interactions between these sub-regions are critical for the preference to investigate opposite-sex odors in male hamsters (Maras & Petrulis, 2009).

Several lines of evidence therefore suggest that MeA and MePD have different, but related roles in processing sexual odors. The goals of the current experiments were to identify: (1) how MeA and MePD interact during sexual odor processing and (2) the role of MeA and/or MePD in transmitting sexual odor information to downstream forebrain nuclei. To address these goals, we measured responses to opposite-sex or same-sex odors within the MA and related forebrain nuclei in the *absence* of either MeA or MePD processing.

Materials and Methods

Experimental design

We hypothesized that MeA and MePD interact during the processing of sexual odors, such that in the *absence* of either MeA or MePD, odor responses within the other sub-region

would be altered. To test this hypothesis, we exposed males that had unilateral lesions of either MeA or MePD to different sexual odors and then quantified the expression of the IEG Fos (Herdegen & Leah, 1998) throughout the MA. This design takes advantage of the primarily ipsilateral connections between the sub-regions of MA (Coolen & Wood, 1998). Levels of Fos expression were compared between nuclei ipsilateral and contralateral to the lesion side, thereby providing a within-subject control for non-specific variation in Fos expression. In order to identify how MeA and MePD convey sexual odor information to the rest of the ventral forebrain circuit, we also analyzed Fos expression within several forebrain nuclei that are known to process sexual odors in hamsters, including the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPOA), and posteromedial cortical amygdala (PMCo) (Fiber *et al.*, 1993; Kollack-Walker & Newman, 1997).

In Experiment 1, we exposed males with unilateral electrolytic lesions of either MeA or MePD to female odors, male odors, or their own cage odors (baseline) and found that lesions of MeA decreased Fos expression within several brain areas only following exposure to female or male odors. To confirm that these decreases were due specifically to a loss of MeA neurons, we exposed a separate group of males with fiber-sparing excitotoxic lesions of MeA to either female odors or male odors in Experiment 2. Furthermore, to confirm that decreases in Fos expression were not simply due to decreases in the total number of neurons within connected nuclei, we also quantified the number of neurons (labeled by the neuron-specific nuclear protein NeuN; Mullen *et al.*, 1992) within each nucleus analyzed for Fos (Experiment 2).

Animals

Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratory (Wilmington, MA). All subjects (total $n = 99$) were sexually naïve, gonadally intact males that were singly housed upon arrival (8 weeks of age) and remained singly housed for the duration of the study. A separate group of gonadally intact male and female hamsters (3 – 8 months old, $n = 80$) were used to provide sexual odor stimuli. Subjects were unrelated to, and had no previous contact with, these odor donor animals. All animals were housed in solid-bottom Plexiglas cages (36 cm X 30 cm X 16 cm) with corncob litter (the Andersons, Maumee, OH, USA) and cotton bedding material (Ancare, Bellmore, NY, USA) and were maintained on a reversed 14-h light/10-h dark photoperiod (lights off/on at 9 am/7 pm). Food and water were available *ad libitum*. All animal 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.

Lesion surgery

Subjects underwent lesion surgery at 3 – 5 months of age (120 – 150 g). Males were anesthetized with 2% isoflurane anesthesia and placed into a stereotaxic apparatus so that the skull was flat. The temporal muscles were retracted from the skull and small holes were drilled to expose the surface of the brain. Table 4.1 provides the stereotaxic coordinates used to generate electrolytic (Experiment 1) or excitotoxic (Experiment 2) lesions within MA. For both electrolytic and excitotoxic lesions, we combined multiple, small lesions in order to generate specific damage of the target region, while limiting damage to nearby nuclei or fiber tracts.

Electrolytic lesions of MeA or MePD (Experiment 1) were made by lowering a platinum/iridium electrode (0.25 mm diameter, 0.45 mm uninsulated tip, Frederick Haer & Co., Bowdoinham, ME) under stereotaxic control and passing anodal current (1mA) from a lesion-making device (Ugo Basile, Comerio, VA, Italy). Excitotoxic lesions of MeA (Experiment 2) were made by combining two, 10-nl microinjections (total of 20 nl) of the excitotoxin N-methyl-D-aspartate (NMDA, 20mg/ml, Sigma). Each microinjection occurred over a one-minute period, and the needle was left in place for ten minutes prior to removal from the brain.

All lesions were unilateral and the side of the brain (left or right) in which the lesion was placed was alternated between subjects. To provide a within-subject control for the effects of brain surgery on Fos expression, all males received sham lesions contralateral to the side of the lesion. Sham procedures were identical to lesion procedures, except that the electrode (Experiment 1) or injection needle (Experiment 2) was lowered 1.5 mm above the target coordinate and no current was passed (Experiment 1) or no solution was injected (Experiment 2).

Stimulus exposure and tissue collection

During a 2 – 3 week recovery period, subjects were extensively handled and habituated to the testing room. On the day of stimulus exposure, subjects were brought into the testing room and allowed to sit undisturbed for at least one hour prior to any manipulation. The procedures used for exposure to sexual odors in Experiments 1 and 2 were identical. Exposure to female or male odors consisted of placing a subject into a vacated female or male stimulus cage, respectively; subjects were therefore exposed to both the volatile and non-volatile components of sexual odors, including those from the soiled litter, bedding, and walls of the cage. To minimize differences in odor quality across individual odor donors, all stimulus cages housed 3 – 4 female

or male hamsters. These stimulus cages therefore provided a composite source of sexual (rather than individual) odors, and we have previously shown that male hamsters display a robust preference to investigate female compared to male odors collected from group-housed odor donors (Maras & Petrulis, 2010). Furthermore, to ensure equivalent levels of odor stimuli, all stimulus cages had not been changed for 4 days prior to use; thus, all female stimulus cages included sexual odors across the entire estrous cycle, including behavioral estrus. Finally, exposure to home cage odors was used to determine if MeA or MePD lesions altered baseline levels of activation and consisted of picking up a subject and placing him back into his home cage (Experiment 1 only).

Males were left undisturbed in the stimulus (or home) cage for exactly 60 minutes, upon which time they were injected with an overdose of sodium pentobarbital (100 mg/kg; Sleep Away, Ft. Dodge, IA, USA) and allowed to reach a deep level of anesthesia prior to perfusion (an additional 10 – 15 minutes). This survival time is within the time range for peak Fos protein expression (60 – 90 minutes; Herdegen & Leah, 1998) and has previously been used for the induction of Fos by odor stimuli in Syrian hamsters (Fiber & Swann, 1996; Delville *et al.*, 2000; Swann *et al.*, 2001; Lai *et al.*, 2004). Males were transcardially perfused with 200 ml of 0.1M phosphate-buffered saline (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) prior to being placed in 30% sucrose in PBS solution for 48 – 72 hours (4°C). In order to distinguish between left and right brain hemispheres, an angle was cut into the dorsal right cortex of each brain. Brains were sectioned coronally (30- μ m thickness) using a cryostat set at -20°C. Brain sections beginning at the posterior BNST and continuing

through the PMCo (Morin & Wood, 2001) were collected into a 1:4 series and stored in cryoprotectant-antifreeze solution (Watson *et al.*, 1986) until processing.

Immunohistochemistry

Free-floating tissue sections were stained for Fos (Experiments 1 and 2) or NeuN (Experiment 2) using identical standard immunohistochemical techniques (Hoffman *et al.*, 2008). All rinses and incubations were done with gentle agitation and at room temperature, unless otherwise stated. Sections were first removed from cryoprotectant and rinsed thoroughly in PBS (pH 7.4). To reduce endogenous peroxidase activity, tissue sections were incubated in 0.5% H₂O₂ in PBS for 15 minutes. After several rinses, sections were then incubated in the primary antibody in PBS with 0.4% Triton-X100 for 1 hour at room temperature, followed by 48 hours at 4°C. The primary antibody for Fos was a rabbit polyclonal antibody generated against the N-terminus of human c-Fos peptide (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and was used at a 1:20,000 concentration. The primary antibody for NeuN was a mouse monoclonal antibody (Millipore, Billerica, MA, USA) and was used at a concentration of 1:30,000. These Fos and NeuN primary antibodies have previously been validated for use in Syrian hamster brain tissue (Kollack-Walker & Newman, 1997; Lai *et al.*, 2004; Meredith & Westberry, 2004; Lindley *et al.*, 2008). After incubation in primary antibody, sections were rinsed in PBS and then incubated for 1 hour in either an anti-rabbit (Fos) or anti-mouse (NeuN) biotinylated secondary antibody (1:600; Jackson ImmunoResearch, West Grove, PA, USA) in PBS with 0.4% Triton-X100. 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 of PBS with 0.4% Triton-X100, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). After rinsing in PBS and then in 0.175M sodium acetate, sections were stained in nickel-sulfate (25mg/ml, Sigma), 3,3-diaminobenzidine

HCl (0.2mg/ml, Sigma) and H₂O₂ (0.83 µl/ml, Sigma) in sodium acetate solution for 15 minutes. The staining reaction was stopped by rinsing sections in sodium acetate followed by PBS. Stained 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 ©.

Quantification of immunoreactivity

Procedures for the quantification of Fos (Experiments 1 and 2) and NeuN (Experiment 2) immunoreactivity were identical. All quantification was done by a single researcher who was blind to both the lesion group and stimulus condition of the animal. Furthermore, in order to decrease the chance that the researcher could detect a pattern of staining within an individual subject's brain, we analyzed the left hemisphere of each brain area for all subjects separately, and prior to, analyzing the right hemisphere of each brain area for all subjects. Fos-immunoreactive (Fos-ir) or NeuN-immunoreactive (NeuN-ir) cells were identified as having dark, blue-black nuclear staining and were counted live using a handheld counter.

Counting domains for each of the forebrain nuclei (Figure 4.1) were generated by projecting the microscopic field onto a computer screen using Bioquant 2K software (Nashville, TN, USA). Each of the sub-regions within MA was analyzed separately. For MeA-X males, cells were counted throughout the posterior MA (MeP), including the rostral and caudal sections of MePD, as well as the posteroventral MA (MePV) (Figure 4.1D,E). For MePD-X males, cells were counted within the anteroventral and anterodorsal MA (MeAV and MeAD, respectively) (Figure 4.1C). Because lesion damage spread into MePV in a sub-set of MePD-X males (see RESULTS) the MePV was not analyzed in MePD-X males.

The BNST, MPOA, and PMCo were analyzed in both MeA-X and MePD-X males. For the BNST, separate cell counts were made for the posterointermediate (BNSTpi) and posteromedial (BNSTpm) regions (Figure 4.1A). For the MPOA (Figure 4.1A, B), the counting domain was centered within the medial preoptic nucleus and was placed at a 25° angle to capture the cluster of activated cells within the MPOA (Fernandez-Fewell & Meredith, 1994; Swann *et al.*, 2001). For MPOA (Figure 4.1A, B) and PMCo (Figure 4.1E, F), cells were counted at two separate planes of section, representing rostral and caudal levels of each nucleus, and the total number of immunoreactive cells for MPOA and PMCo reflects the sum of the two sections.

Photomicrographs were taken using a Synsys digital camera attached to a Nikon Eclipse E800 microscope (4X magnification). Images were captured using iVision (Atlanta, GA, USA) and processed using Adobe Photoshop CS2 Version 9.0 (San Jose, CA, USA) only to enhance brightness and contrast.

Data analysis

All data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) for Windows, and significance was determined at $p < .05$. To detect differences in the levels of Fos-ir (Experiments 1 and 2) or NeuN-ir (Experiment 2) between nuclei ipsilateral (IPSI) and contralateral (CONTRA) to lesion side, separate paired *t*-tests (2-tailed) were done for each nucleus analyzed (MeA-X, total of 7 nuclei; MePD-X, total of 6 nuclei). A multi-stage Bonferroni correction (Holm, 1979) with a familywise alpha of .05 was used to reduce Type I error due to multiple comparisons. A Pearson correlation matrix was used to determine the relationship between the estimated size of the lesion and the difference in Fos-ir between IPSI

and CONTRA nuclei (Difference score = number of Fos-ir cells within CONTRA – number of Fos-ir cells within IPSI) for each stimulus condition.

Specific experiments

Experiment 1

Males were randomly assigned to one of two lesion groups: unilateral electrolytic lesions of MeA (MeA-X, $n = 32$) or unilateral electrolytic lesions of MePD (MePD-X, $n = 28$). Males received lesions in either the left hemisphere (MeA-X, $n = 14$; MePD-X, $n = 14$) or the right hemisphere (MeA-X, $n = 18$; MePD-X, $n = 14$). To determine how MeA or MePD regulates processing of sexual odors, a sub-set of males from each lesion group was exposed to either female odors (FEM; MeA-X, $n = 14$; MePD-X $n = 12$) or male odors (MALE; MeA-X $n = 13$; MePD-X $n = 11$) as described above. Furthermore, to determine whether MeA or MePD regulates baseline levels of activation, an additional sub-set of males from each lesion group was handled and returned to their home cage (HOME; MeA-X $n = 5$; MePD-X $n = 5$). After collection of brain tissue, the first series of sections was stained with cresyl violet (Sigma) to determine the extent of lesion damage, and the second series of sections was processed for the immunohistochemical detection of Fos protein. Fos-ir was quantified as described above.

In addition to the main statistical analysis described above, we used the number of Fos-ir cells within nuclei contralateral to lesion side (control hemisphere) to detect overall patterns of odor-induced Fos expression throughout the ventral forebrain. There were no differences in the levels of Fos-ir within the contralateral hemispheres between MeA-X and MePD-X males (all $P > .05$), so lesion groups were collapsed for this analysis. The total numbers of Fos-ir cells across stimulus conditions (FEM, MALE, HOME) were compared using separate one-way ANOVAs

for each brain area. If a significant omnibus difference was detected across stimulus conditions, then Tukey's B post-hoc comparisons were used to identify homogenous means within each nucleus.

Experiment 2

Male subjects ($n = 39$) received unilateral NMDA-lesions of MeA (left, $n = 19$; right, $n = 20$). Given that electrolytic lesions of MeA had no effect on Fos expression in the HOME condition in Experiment 1, there was no reason to expect that NMDA-lesions of MeA would alter baseline levels of Fos expression. Thus, only the FEM ($n = 20$) or MALE ($n = 19$) odor conditions were included in Experiment 2. After brain tissue collection, the first and second series of sections were processed for immunohistochemical detection of NeuN and Fos protein, respectively. NeuN-ir and Fos-ir were quantified as described above.

Results: Experiment 1

Experiment 1

Lesion Reconstruction

Males were included in the MeA-X lesion group (total $n = 17$; FEM $n = 7$; MALE $n = 7$; HOME $n = 3$) or the MePD-X lesion group (total $n = 16$; FEM $n = 7$; MALE $n = 6$; HOME $n = 3$) only if they had at least 60% damage of MeA or MePD, respectively, within at least two atlas plates (Morin & Wood, 2001; Figure 4.2). The total extent of lesion damage for each subject was calculated by estimating the percent of damage at each atlas plate of section, summing these estimates, and then dividing by the total number of atlas plates for that nucleus. For MeA-X males, the extent of lesion damage ranged from 55 – 81% of MeA; for MePD-X males, the

extent of lesion damage ranged from 56 – 83% of MePD. Males were excluded from the analyses if damage spread into both sub-nuclei ($n = 8$) or if there was substantial sparing of the target region ($n = 11$). In addition, males were excluded from MeA-X group if lesion damage spread to the ventral surface of the brain ($n = 8$), as such damage may disrupt olfactory bulb efferents traveling to more caudal brain nuclei (Scalia & Winans, 1975).

Within the MeA-X lesion group, thirteen males had damage that included both MeAD and MeAV, whereas four males had damage restricted primarily to MeAD. Within the MePD-X lesion group, twelve males had damage that extended into caudal region of the MePD. In addition to damage of the target region, a sub-set of MeA-X and MePD-X males had minor (less than 10%) damage of adjacent nuclei. For MeA-X males, collateral damage included the substantia innominata ($n = 5$), intercalated nucleus of the amygdala ($n = 2$), and anterior cortical amygdala ($n = 1$). For MePD-X males, collateral damage included the MePV ($n = 8$), the intercalated nucleus of the amygdala ($n = 6$), and the amygdalohippocampal area ($n = 5$). In all cases, damage to adjacent nuclei was minimal (Figure 4.2), and Fos-ir did not differ between males with or without this minor collateral damage.

Fos expression

Social odor-induced Fos expression. Table 4.2 summarizes the results from the analysis of Fos-ir within nuclei contralateral to lesion side (control hemisphere). There was a significant difference across stimulus conditions (FEM, MALE, HOME) for every nucleus analyzed, and Tukey's B post-hoc comparisons revealed three distinct patterns of activation (Table 4.2). Within MeAD, $F_{2,30} = 12.78$, $P < .001$, and MeAV, $F_{2,30} = 13.51$, $P < .001$, both the FEM and MALE conditions resulted in increased Fos-ir compared to the HOME condition, and there was no

difference in the number of Fos-ir cells between FEM and MALE conditions. Within the MePV, $F_{2,30} = 20.43$, $P < .001$, and rostral MePD, $F_{2,30} = 37.96$, $P < .001$, both the FEM and MALE conditions again resulted in increased Fos-ir compared to the HOME condition, but in these nuclei, the FEM condition resulted in more Fos-ir cells than the MALE condition. Within the caudal MePD, $F_{2,30} = 53.57$, $P < .001$, PMCo, $F_{2,30} = 18.89$, $P < .001$, MPOA, $F_{2,30} = 16.23$, $P = .006$, BNSTpi, $F_{2,30} = 35.42$, $P < .001$, and BNSTpm, $F_{2,30} = 26.45$, $P < .001$, only the FEM condition resulted in increased Fos-ir compared to the HOME condition; there was no difference in the number of Fos-ir cells between MALE and HOME conditions.

MeA lesions and Fos expression. Lesions of MeA decreased odor-induced Fos expression within all sub-regions of the posterior MEA (Figure 4.3A, Figure 4.4). Within the rostral MePD, MeA lesions decreased the number of Fos-ir cells within IPSI compared to CONTRA hemispheres in both the FEM condition, $t_6 = 4.57$, $P = .004$, and the MALE condition, $t_6 = 4.12$, $P = .006$. Within the caudal MePD, $t_6 = 8.82$, $P < .001$, and MePV, $t_6 = 8.31$, $P < .001$, MeA lesions decreased the number of Fos-ir cells within IPSI compared to CONTRA hemispheres only in the FEM condition.

MeA lesions also decreased odor-induced Fos expression outside of the MEA (Figure 4.6). Within the MPOA, $t_6 = 2.79$, $P = .032$, BNSTpi, $t_6 = 3.89$, $P = .008$, and BNSTpm, $t_6 = 3.57$, $P = .012$, MeA lesions decreased the number of Fos-ir cells within IPSI compared to CONTRA hemispheres in the FEM condition (Figure 4.6A-C). Within the PMCo, MeA lesions decreased the number of Fos-ir cells within IPSI compared to CONTRA hemispheres in both the FEM condition, $t_6 = 5.28$, $P = .003$, and the MALE condition, $t_6 = 4.38$, $P = .005$ (Figure 4.6D). When males were exposed to handling procedures alone (HOME), MeA lesions did not alter the number of Fos-ir cells within any of the nuclei analyzed (all $P > .05$; Figure 4.6A-D).

Furthermore, there were no significant correlations between total size of MeA lesion and the difference in Fos-ir cells between IPSI and CONTRA hemispheres within any of the nuclei analyzed, although there was a trend for a positive correlation for the caudal MePD in the FEM condition ($r = .480$, $P = .07$).

MePD lesions and Fos expression. In contrast to MeA, lesions of MePD did not alter Fos expression within MeA (Figure 4.3B, Figure 4.5), nor within any of the other ventral forebrain nuclei analyzed (Figure 4.6). Indeed, for any of the stimulus conditions, the number of Fos-ir cells between nuclei IPSI and CONTRA to MePD lesion were not different within MeAV or MeAD (all $P > .05$; Figure 4.3B, Figure 4.5), or within BNSTpi, BNSTpm, MPOA, or PMCo (all $P > .05$; Figure 4.6). There were also no significant correlations between the size of MePD lesion and the difference in Fos-ir cells between IPSI and CONTRA hemispheres within any of the nuclei analyzed (all $P > .05$).

Results: Experiment 2

Lesion Reconstruction

Males were included in the MeA-X lesion group ($n = 14$; FEM $n = 7$, MALE $n = 7$) only if they had at least 60% damage of MeA, within at least two atlas plates (Morin & Wood, 2001; Figure 4.7). The extent of lesion damage ranged from 60 – 85% of the total MeA. Males were excluded from the analyses if damage spread into posterior MEA ($n = 14$) or if there was substantial sparing of MeA ($n = 11$). Ten males had damage that included both MeAD and MeAV, whereas four males had damage restricted primarily to MeAD. In addition to damage of MeA, a sub-set of males had minor (less than 10%) damage of adjacent nuclei, including the

substantia innominata ($n = 4$), intercalated nucleus of the amygdala ($n = 2$), and anterior cortical amygdala ($n = 2$).

MeA lesions and Fos expression

Similar to what was observed following electrolytic lesions of MeA, NMDA-lesions of MeA decreased odor-induced Fos expression within all sub-regions of the posterior MA (Figure 4.8A, Figure 4.9). In fact, NMDA-lesions of MeA decreased the number of Fos-ir cells within IPSI compared to CONTRA hemispheres in both FEM and MALE conditions within the rostral MePD (FEM: $t_6 = 14.45$, $P < .001$; MALE: $t_6 = 5.89$, $P = .001$), caudal MePD (FEM: $t_6 = 4.24$, $P = .004$; MALE: $t_6 = 5.16$, $P = .003$), and MePV (FEM: $t_6 = 5.95$, $P = .001$; MALE: $t_6 = 4.56$, $P = .004$).

Within the PMCo, NMDA-lesions of MeA decreased the number of Fos-ir cells within IPSI compared to CONTRA hemispheres in both the FEM condition, $t_6 = 7.43$, $P < .001$, and the MALE condition, $t_6 = 5.17$, $P = .002$ (Figure 4.8C). Within the BNSTpm and MPOA, NMDA-lesions of MeA decreased the numbers of Fos-ir cells within IPSI compared to CONTRA hemispheres only in the FEM condition, although these differences were not significant after alpha adjustments (BNSTpm: $t_6 = 2.55$, $P = .035$; MPN: $t_6 = 2.50$, $P = .047$; Figure 4.8B,C). NMDA-lesions of MeA did not significantly alter the number of Fos-ir cells within BNSTpi in either FEM or MALE conditions (all $P > .05$, Figure 4.8B).

MeA lesions and NeuN expression

In contrast to the effects on Fos-ir, NMDA-lesions of MeA did not alter the numbers of NeuN-ir cells within any of the nuclei analyzed (all $P > .05$; Figure 4.10).

Discussion

Several converging lines of evidence suggest that MeA and MePD have distinct, but related roles in social odor processing (Wood, 1998). To identify how each of these sub-regions regulates processing of sexual odors throughout MA, as well as within related forebrain nuclei, the current set of experiments analyzed responses to either opposite-sex or same-sex odors in the *absence* of either MeA or MePD processing. As a measure of neuronal activation, we quantified the expression of the IEG Fos, which is tightly linked to calcium influx/cAMP turnover and therefore generally reflects excitatory input (Sheng & Greenberg, 1990; Sheng *et al.*, 1990). Although Fos expression provides a useful snapshot of activated neurons in response to a stimulus, this technique does not necessarily reflect inhibitory synaptic transmission, nor can it inform us about the temporal dynamics of a neural response during ongoing behavior, such as the duration of firing or firing rate (Sheng & Greenberg, 1990; Pfaus & Heeb, 1997). Nevertheless, our results indicate that MeA normally enhances excitation of neurons throughout MeP in response to sexual odors, but that MePD does not regulate these odor responses within MeA. The MeA appears to also provide the primary excitatory output of the MA during sexual odor processing; lesions of MeA, but not MePD, decreased Fos expression within PMCO, BNST, and MPOA in response to opposite-sex and/or same-sex odors. Taken together, these results provide the first direct test of how the different sub-regions of MEA interact during odor processing and highlight the role of MeA for transmitting sexual odor information to MeP, as well as to other ventral forebrain nuclei.

Methodological considerations

We have previously found that electrolytic lesions provide the most reliable and controllable method for damaging the sub-regions within MA (Maras & Petrulis, 2006), and so we initially used this technique to generate specific lesions of MeA or MePD in Experiment 1. One limitation of this technique, however, is that damage is not restricted to cell bodies but also includes fibers of passage. However, results from Experiment 2 indicate that the decreases in Fos expression observed following electrolytic lesions of MeA were not due exclusively to damage of passing fibers; males with fiber-sparing, NMDA-lesions of MeA displayed patterns of decreased Fos expression that were largely consistent with those observed following electrolytic lesions of MeA. Furthermore, we observed no differences in the number of cells expressing NeuN between nuclei ipsilateral and contralateral to the MeA lesion (Experiment 2), suggesting that MeA lesions did not decrease Fos expression simply by decreasing the total number of neurons in those nuclei. Finally, neither lesions of MeA nor MePD altered Fos expression when males were exposed to handling procedures alone, indicating that these sub-regions do not regulate baseline levels of excitatory activation of nuclei within this circuit.

Odor-induced Fos expression

Previous IEG studies have shown that neurons throughout MA are activated by social odors in several species (Fiber *et al.*, 1993; Heeb & Yahr, 1996; Coolen *et al.*, 1997; Kelliher *et al.*, 1998; Halem *et al.*, 1999; Sheehan *et al.*, 2000; Meredith & Westberry, 2004; Samuelsen & Meredith, 2009), and analysis of Fos expression within the contralateral (control) hemispheres of males that were exposed to different sexual odors (Experiment 1) confirmed these previous findings. Furthermore, our results suggest that the response patterns become more odor-

specific from rostral to caudal sub-regions of MA; neurons within MeA were activated equally in response to opposite-sex and same-sex odors, whereas those within the caudal MePD were activated only in response to opposite-sex odors. Neurons within the rostral MePD and MePV displayed an intermediate response pattern, as they responded significantly to presentations of either sexual odor, but were activated more by opposite-sex than by same-sex odors. Although previous studies have shown that caudal MePD neurons are activated during both sexual and aggressive encounters (Kollack-Walker & Newman, 1995; Veening *et al.*, 2005), our data suggest that, when only odors are presented, this sub-region is activated specifically by sexually relevant stimuli. Our results also suggest that MePV, which is primarily studied in the context of predator odor processing or defensive behavior (Dielenberg & McGregor, 2001; Canteras, 2002), may be involved in processing the sexual aspects of odor stimuli as well.

In addition to odor responses within MA, we found that several other forebrain nuclei also display odor-specific patterns of activation; neurons within BNSTpi, BNSTpm, MPOA, and PMCo displayed significant increases in Fos expression only following exposure to opposite-sex odors. These nuclei are part of an extended forebrain network that receives social odor information and regulates many aspects of social behavior, including reproduction (Wood, 1997; Veening *et al.*, 2005). Our results are consistent with previous IEG studies showing that these nuclei are activated during sexual behavior (Fiber *et al.*, 1993; Kollack-Walker & Newman, 1997; Halem *et al.*, 1999; Kelliher *et al.*, 1999), and further indicate that these nuclei respond specifically to sexually relevant odors.

MeA transmits sexual odor information to MeP

Our results suggest that MeA plays a substantial role in transmitting sexual odor information to the other sub-regions of MA, as MeA lesions consistently reduced the number of neurons activated by either opposite-sex or same-sex odors throughout the MeP. The importance of MeA for transmitting odor information is perhaps not surprising as this sub-region receives the majority of chemosensory input to this nucleus; MeA receives direct projections from both the MOB and AOB, as well as substantial input from secondary structures of the main olfactory system, including the anterior cortical nucleus, posterolateral cortical nucleus, piriform cortex, and endopiriform cortex (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Lehman & Winans, 1982; Coolen & Wood, 1998). Although AOB projects to MeP as well, these projections are less substantial than those innervating MeA (Scalia & Winans, 1975), and MeP has much more limited connections with the main olfactory system than compared to MeA (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Lehman & Winans, 1982; Coolen & Wood, 1998). Functionally, MeA appears to play a greater role in regulating odor-guided reproductive behaviors in male hamsters than MeP; MeA lesions completely eliminate the expression of copulatory behavior (Lehman *et al.*, 1980), similar to what is observed following destruction of the olfactory bulbs (Murphy & Schneider, 1970), but lesions of MeP only alter the temporal pattern of the copulatory sequence (Lehman *et al.*, 1983). Coupled with the dense projections from MeA to MeP (Coolen & Wood, 1998), our results suggest that MeA provides indirect chemosensory input to MeP and that this input is critical for generating the robust patterns of activation observed within MeP in response to sexual odors.

There are several possible mechanisms by which MeA may enhance activation of MeP neurons in response to sexual odors. First, these odors may activate excitatory neurons within

MeA, which then act directly on MeP neurons to increase their probability of firing. Although the MEA contains many GABAergic neurons (Mugnaini & Oertel, 1985; McDonald & Augustine, 1993; Stefanova, 1998), populations of glutamatergic neurons within this nucleus have been identified (Simmons & Yahr, 2003; Choi *et al.*, 2005; Bian *et al.*, 2008), and these excitatory neurons may be critical for transmitting sexual odor information from MeA to MeP. Alternatively, MeA may enhance activation of MeP neurons through changes in inhibitory processing. Specifically, the activation of GABAergic neurons within MeA may inhibit local inter-neurons, consequently releasing MeP neurons from inhibition. It is also possible that the transmission of odor information out of MeA involves both excitatory and inhibitory processes, depending on the nature of the stimulus. In fact, a recent study in male rats has shown that same-sex odors activate a higher percentage of GABAergic neurons within MA than do opposite-sex odors (Donato *et al.*, 2010), suggesting that different categories of sexual odors activate different populations of excitatory and inhibitory neurons. Finally, in addition to these direct interactions with MeP, MeA may indirectly enhance activation of MeP neurons via feedback onto AOB. MeA neurons directly project onto mitral/tufted cells of AOB (Gomez & Newman, 1992), and there is recent evidence in female mice that these MeA neurons are activated specifically by volatile components of opposite-sex urinary odors (Martel & Baum, 2009). If this feedback is critical for shaping response patterns within AOB, then MeA lesions may disrupt AOB output, consequently reducing activation of MeP neurons in response to sexual odors.

Importantly, MeA lesions never caused an *increase* in Fos expression, even when males were exposed to other male odors. These data indicate that MeA does not normally suppress the responses of MeP neurons to same-sex odors. This pattern of results may also reflect the fact that MeA provides the primary source of odor information to MeP, regardless of the sexual identity

of the stimulus. Thus, the sexual selectivity of MeP neurons, and in particular neurons within caudal MePD, may reflect the intrinsic responses properties of MePD neurons or may be due to inhibition from other nearby nuclei. For example, Meredith and Westberry (2004) suggest that the intercalated nucleus of the amygdala might provide an important source of inhibitory input into MeP during odor processing.

Although these experiments focused on the processing of different sexual odors (opposite-sex or same-sex), MeA may transmit other types of social odor information to MeP as well. Indeed, the MeA displays high levels of IEG expression following exposure to different types of heterospecific odors (Meredith & Westberry, 2004; Kiyokawa *et al.*, 2005; delBarco-Trillo *et al.*, 2009; Samuelsen & Meredith, 2009), including those from predators (Day *et al.*, 2004; Samuelsen & Meredith, 2009). Neurons within MePV also respond to predator odors (Dielenberg *et al.*, 2001; Staples *et al.*, 2008), and interactions between MeA and MePV may be especially critical for generating appropriate fear responses to predator odor stimuli. In fact, large lesions of the MA in rats (including both the anterior and posterior sub-regions) reduce the expression of fear responses to presentations of cat odors (Li *et al.*, 2004; Blanchard *et al.*, 2005). Future studies are needed to determine whether MeA transmits other biologically relevant aspects of odor information to the MeP outside of the context of reproduction.

MePD may not transmit sexual odor information to MeA

Although the MePD is not the primary chemosensory sub-region of MA, it does contain the majority of gonadal steroid receptor-containing neurons (Doherty & Sheridan, 1981; Wood *et al.*, 1992). The MePD may therefore function to enhance processing specifically of sexually relevant odors when the animal is reproductively active (i.e. when sufficient levels of gonadal

hormones are present). In fact, previous lesion studies have shown that MePD is critical for generating sexual arousal or attraction in response to odor stimuli. In male hamsters, lesions of MePD specifically decrease investigation of female odors (Maras & Petrulis, 2006) and decrease anogenital investigation of a receptive female (Lehman *et al.*, 1983). Similar lesions in male rats eliminate the expression of non-contact penile erections in response to volatile odors from an estrous female, a reflection of male sexual arousal (Kondo *et al.*, 1997; Kondo & Sachs, 2002).

The current results, however, indicate that MePD does not enhance MeA processing of sexual odors, as lesions of MePD had no effect on Fos expression within MeA following exposure to either opposite-sex or same-sex odors. The lack of effects from MePD lesions may indicate that MePD simply does not provide feedback onto MeA during odor processing. Given the robust levels of activation of MePD neurons in response to opposite-sex odors (Fiber *et al.*, 1993; Coolen *et al.*, 1997; Halem *et al.*, 1999; Meredith & Westberry, 2004), as well as the dense projections from MePD to MeA (Coolen & Wood, 1998), this interpretation seems unlikely. As previously mentioned, Fos expression does not reflect all types of synaptic input onto a cell, nor does it reflect the dynamic aspects of the firing properties of those neurons that do express Fos. Thus, it remains possible that MePD does modulate odor responses within MeA, perhaps through changes in GABAergic signaling or changes in the firing rate of MeA neurons, but that these types of modulation are not detectable by overall changes in Fos expression. Ongoing studies in our lab using electrophysiological recordings of MA neurons will provide a more complete analysis of the response properties of MA neurons and will address these possibilities.

MeA transmits sexual odor information throughout the ventral forebrain

In addition to transmitting odor information to MeP, the MeA also provides an important source of chemosensory input to several connected forebrain nuclei. Specifically, we found that MeA transmits both opposite-sex and same-sex odor information to the PMCo. During the male hamster copulatory sequence, the PMCo is important for restricting olfactory investigation specifically to the anogenital region of the female (Maras & Petrulis, 2008b), and our results suggest that this function may depend in part on chemosensory input from MeA.

Although MeA lesions consistently decreased Fos expression within BNST and MPOA in response to opposite-sex odors, these decreases were not as robust as those observed within the MeP and PMCo, as they did not reach statistical significance in Experiment 2. The smaller effect sizes within BNST/MPOA may indicate that these nuclei do not rely as heavily on MeA for chemosensory input. In fact, BNST does receive a small direct projection from the AOB (Davis *et al.*, 1978), and this input may provide an important source of chemosensory information to the BNST during odor processing. Nevertheless, the current results suggest that MeA transmits information about sexually relevant odors to BNST/MPOA, and this input may be critical, at least in part, for the activation of these downstream nuclei during odor processing.

Concluding remarks

As part of an extended network of ventral forebrain nuclei, the MA functions to process many types of social odors and is essential for generating appropriate behavioral responses to these stimuli (Newman, 1999). The current experiments provide important new insights into how the distinct sub-regions within MA interact during the processing of sexual odors and further describe how this information is conveyed throughout the forebrain network. The interactions

between these sub-regions may reflect a fundamental mechanism by which the brain processes social information and thus may have significant implications for understanding deficits in social processing that underlie many social disorders in humans (Anckarsater, 2006; Amaral *et al.*, 2008).

Acknowledgements

We would like to thank Anaam Mohammed for experimental assistance. 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 4 Tables

Table 4.1 Coordinates for lesion surgery in Experiments 1 and 2. In Experiment 1, lesions of MeA or MePD were generated using a combination of 2 or 3 small electrolytic lesions, respectively. Seconds indicate the duration of anodal current (1mA) passed at each coordinate. In Experiment 2, unilateral lesions of MeA were generated by combining 2 microinjections of NMDA (20mg/ml). Volume indicates the volume of NMDA (nl) injected at each coordinate. All coordinates are in millimeters. Anterior-posterior (AP) and medial-lateral (ML) coordinates are relative to bregma, whereas dorsal-ventral (DV) coordinates are relative to dura at each coordinate.

Experiment 1 (<i>Electrolytic</i>)					
	MeA		MePD		
Site	1	2	1	2	3
AP	– 0.60	– 0.30	+ 0.50	+ 0.70	+ 0.95
ML	± 2.45	± 2.50	± 2.85	± 2.80	± 2.75
DV	– 7.45	– 7.50	– 7.20	– 7.30	– 7.25
Duration	11	10	5	6	6

Experiment 2 (<i>Excitotoxic</i>)		
	MeA	
Site	1	2
AP	– 0.70	– 0.40
ML	± 2.55	± 2.60
DV	– 7.95	– 8.00
Volume (nl)	10	10

Table 4.2 Patterns of Fos expression within ventral forebrain nuclei in response to social odors. The mean (\pm SEM) number of Fos-ir cells within each forebrain nucleus for males exposed to female odors (FEM), male odors (MALE), or handled and returned to home cage (HOME). Letters represent homologous means within each nucleus (Tukey's-B post-hoc comparisons, $p < .05$).

Nucleus	FEM	MALE	HOME
MeAV	64.57 \pm 5.80 ^a	60.15 \pm 4.78 ^a	20.17 \pm 2.63 ^b
MeAD	55.36 \pm 5.29 ^a	48.77 \pm 3.56 ^a	17.83 \pm 1.70 ^b
MePV	58.79 \pm 2.47 ^a	39.31 \pm 3.53 ^b	17.33 \pm 2.47 ^c
rMePD	57.29 \pm 2.14 ^a	39.23 \pm 3.20 ^b	16.20 \pm 2.20 ^c
cMePD	52.62 \pm 4.05 ^a	13.77 \pm 1.98 ^b	7.80 \pm 2.18 ^b
PMCo	87.33 \pm 6.66 ^a	51.25 \pm 4.65 ^b	36.17 \pm 4.67 ^b
MPOA	86.62 \pm 11.32 ^a	51.46 \pm 8.05 ^b	36.50 \pm 4.62 ^b
BNSTpi	24.23 \pm 1.34 ^a	10.85 \pm 1.19 ^b	6.83 \pm 1.08 ^b
BNSTpm	30.15 \pm 2.48 ^a	14.15 \pm 1.36 ^b	9.67 \pm 1.67 ^b

Chapter 4 Figures

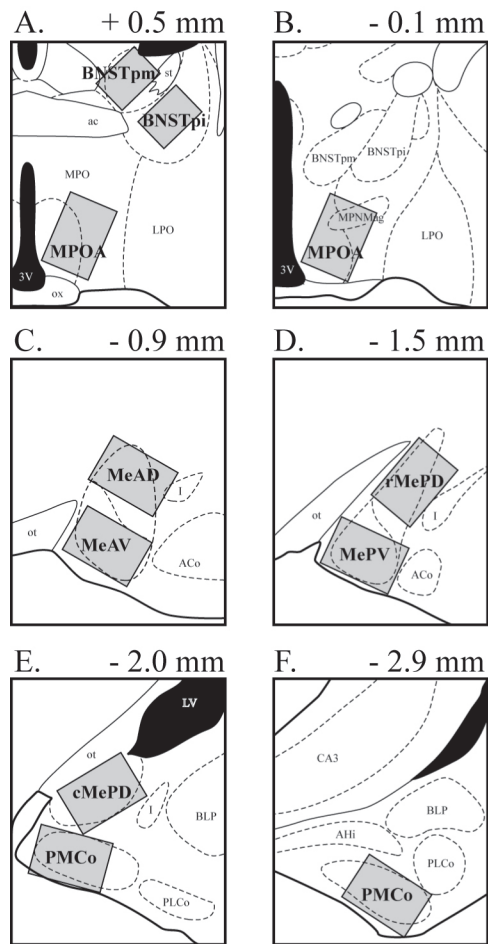


Figure 4.1 Counting domains for analysis of Fos-immunoreactivity. Illustration adapted and modified from hamster brain atlas of Morin and Wood (2001) showing placement of counting domains (gray rectangles) used in Experiments 1 and 2. Values given in millimeters indicate distance anterior (+) or posterior (-) to bregma. (A) BNSTpi, BNSTpm, and rostral section of MPOA (B) caudal section of MPOA (C) MeAD and MeAV (D) rMePD and MePV (E) cMePD and rostral section of PMCo (F) caudal section of PMCo. 3V, third ventricle; ac, anterior commissure; LV, lateral ventricle; ot, optic tract; st, stria terminalis.

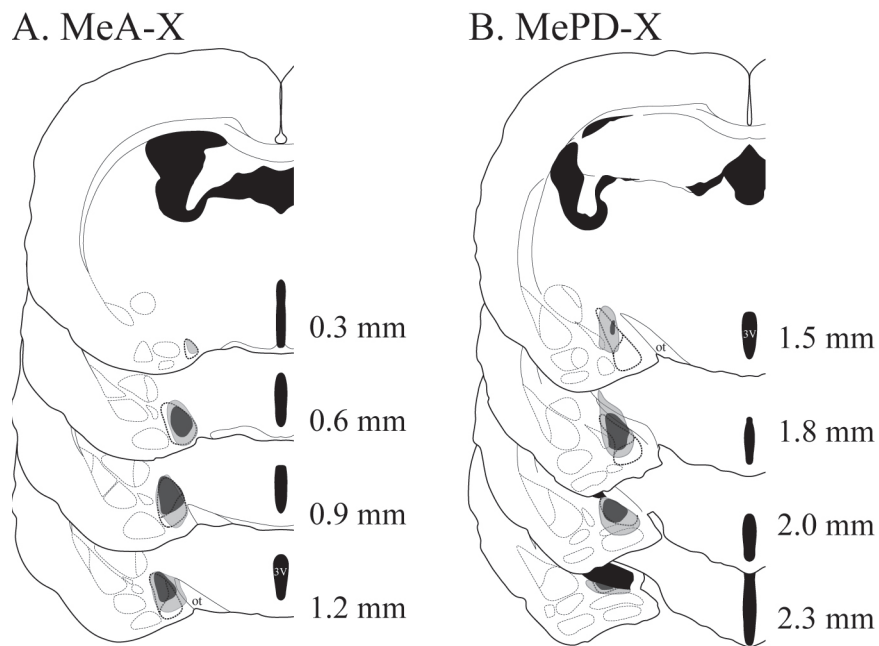


Figure 4.2 Lesion reconstruction for Experiment 1. Reconstruction of coronal sections of the largest (light gray) and smallest (dark gray) extents of damage in males with electrolytic lesions of (A) MeA or (B) MePD in Experiment 1. Sections proceed from anterior (top) to posterior (bottom) levels, with the numbers representing the distance posterior to bregma. ot, optic tract; 3V, third ventricle.

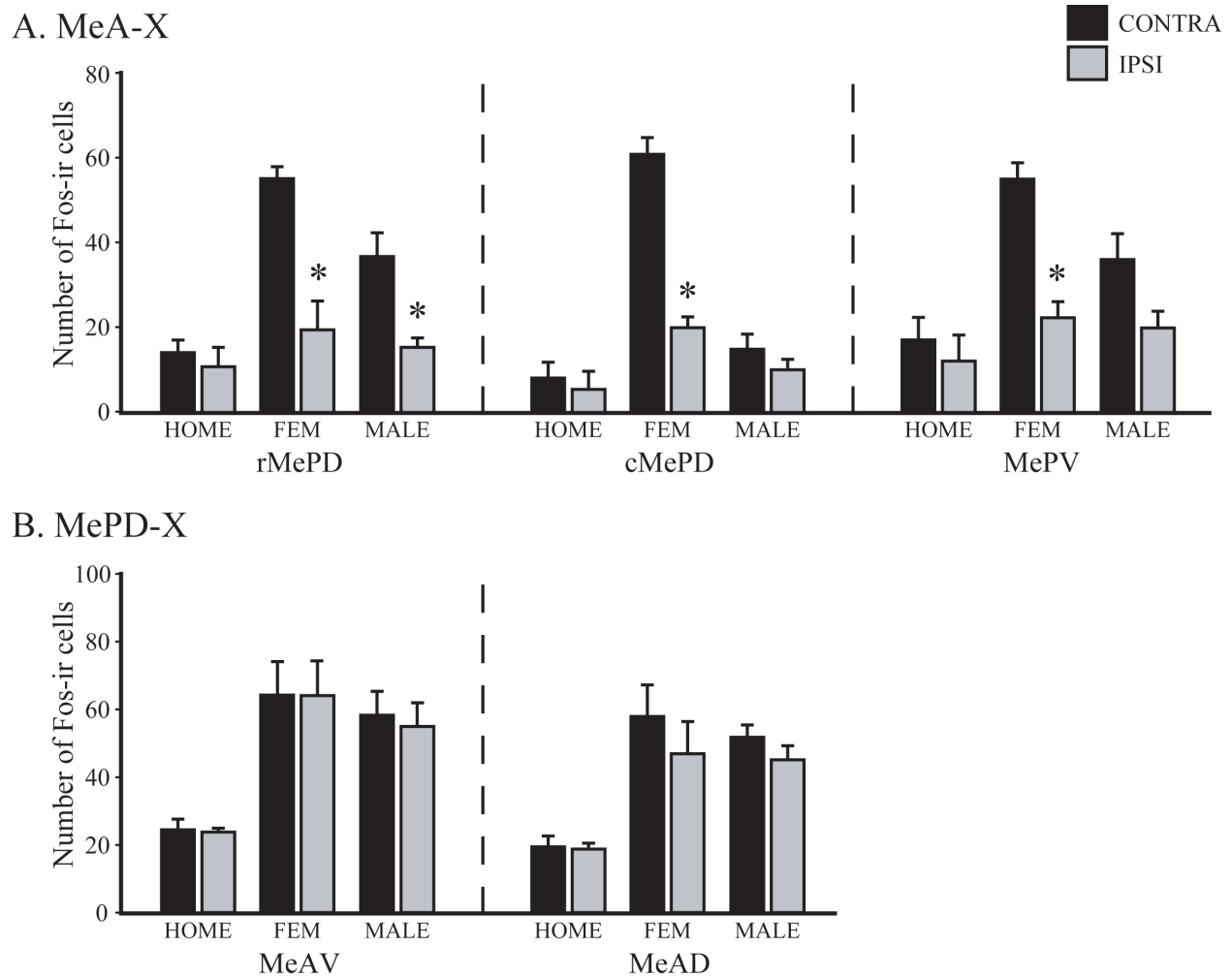


Figure 4.3 Summary of effects of MeA or MePD lesions on Fos-ir throughout the MA.

Effects of unilateral electrolytic lesions of either (A) MeA or (B) MePD on Fos-ir within MA nuclei ipsilateral (IPSI) and contralateral (CONTRA) to the lesion side of males that were exposed to female odors (FEM), male odors (MALE), or handled conditions (HOME) in Experiment 1. MeA lesions decreased odor-induced Fos-ir throughout the MeP, whereas MePD lesions had no effect on Fos-ir within MeA. * $p < .05$ ($\alpha_{fw} = .05$), compared to CONTRA hemisphere within each nucleus.

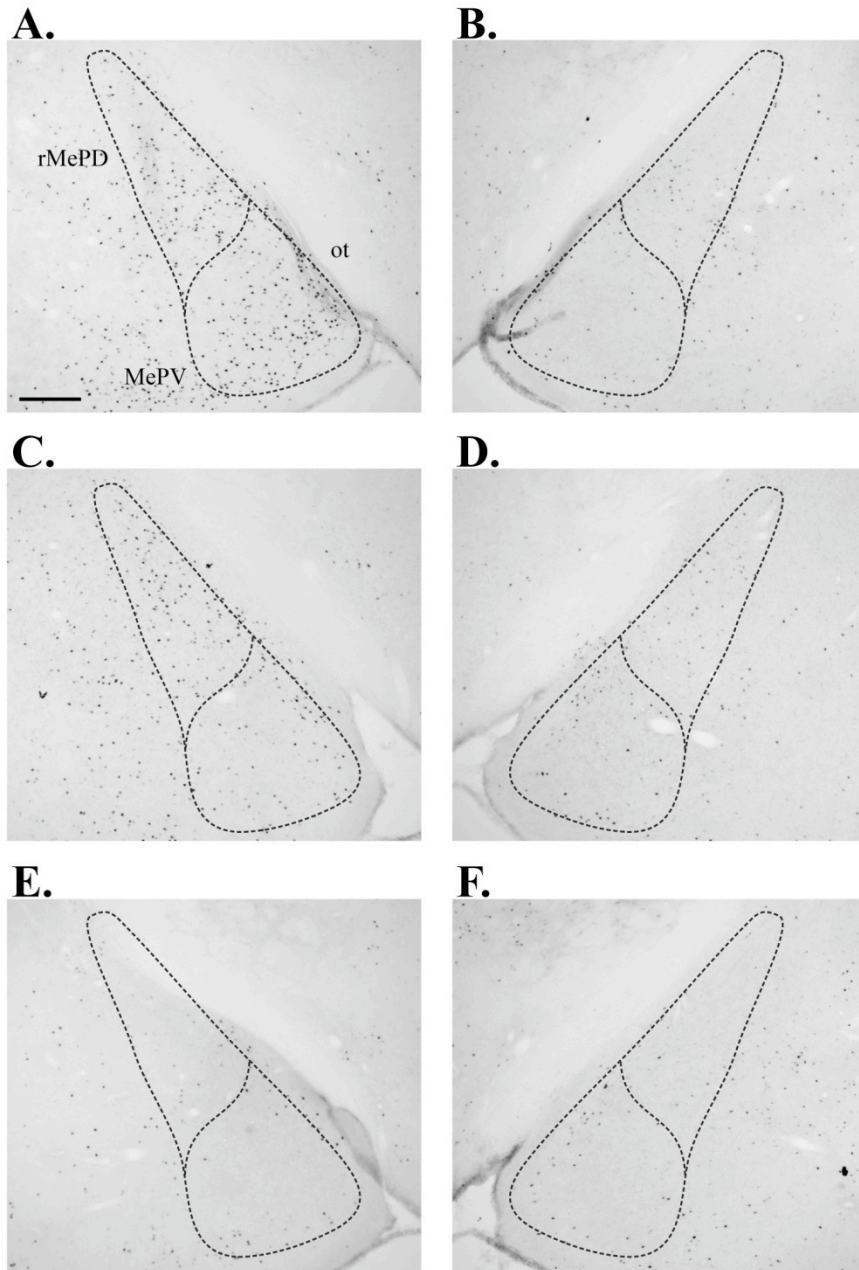


Figure 4.4 Representative photomicrographs of Fos-ir within MeP. Photomicrographs showing Fos-ir within MeP of males with electrolytic lesions of MeA exposed to (A, B) female odors, (C, D) male odors, or (E, F) handled conditions in Experiment 1. Left photomicrographs (A, C, E) show nuclei contralateral to MeA lesion. Right photomicrographs (B, D, F) show nuclei ipsilateral to MeA lesion. Dotted line denotes borders of rMePD and MePV. ot, optic tract. Scale bar = 250 μ m applies to A-F.

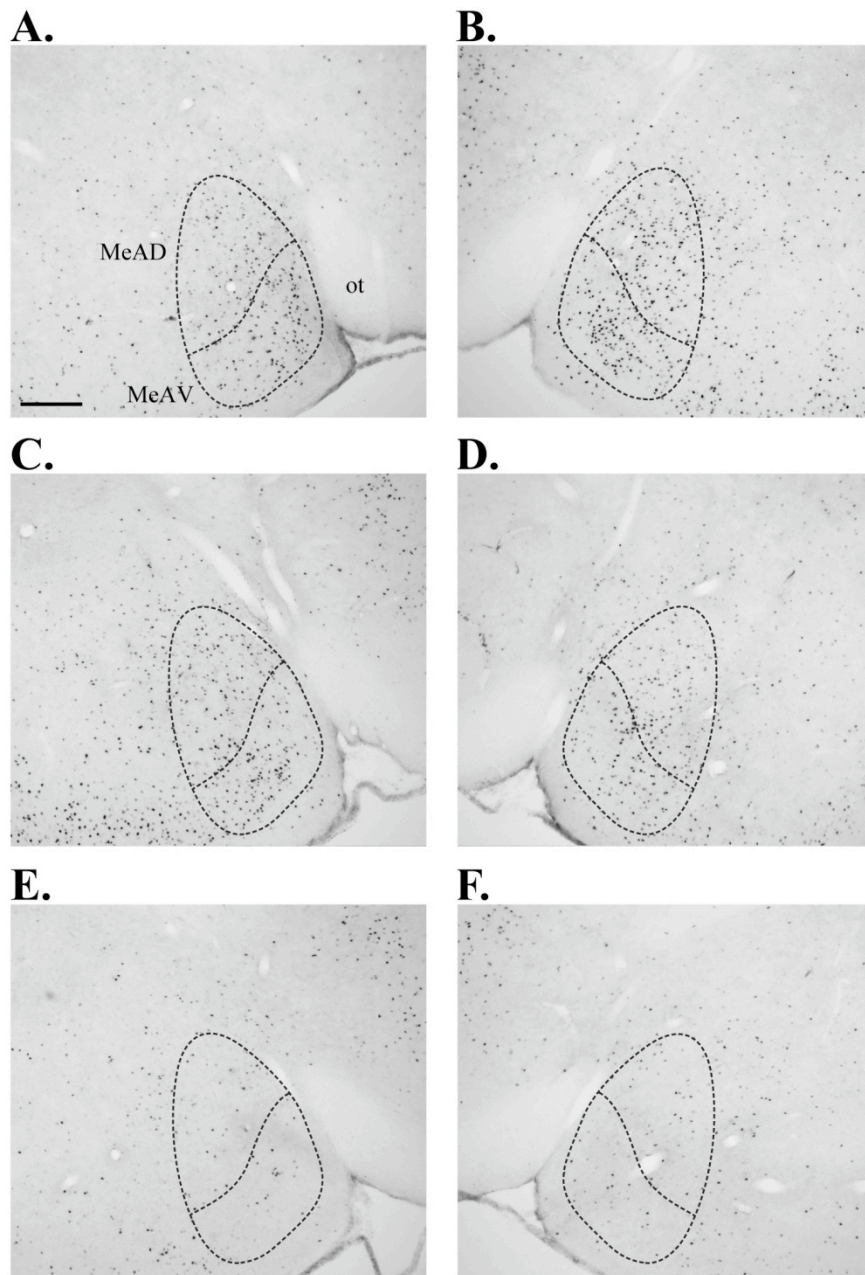


Figure 4.5 Representative photomicrographs of Fos-ir within MeA. Photomicrographs showing Fos-ir within MeA of males with electrolytic lesions of MePD exposed to (A, B) female odors, (C, D) male odors, or (E, F) handled conditions in Experiment 1. Left photomicrographs (A, C, E) show nuclei contralateral to MePD lesion. Right photomicrographs (B, D, F) show nuclei ipsilateral to MePD lesion. Dotted line denotes borders of MeAD and MeAV. ot, optic tract. Scale bar = 250 μ m applies to A-F.

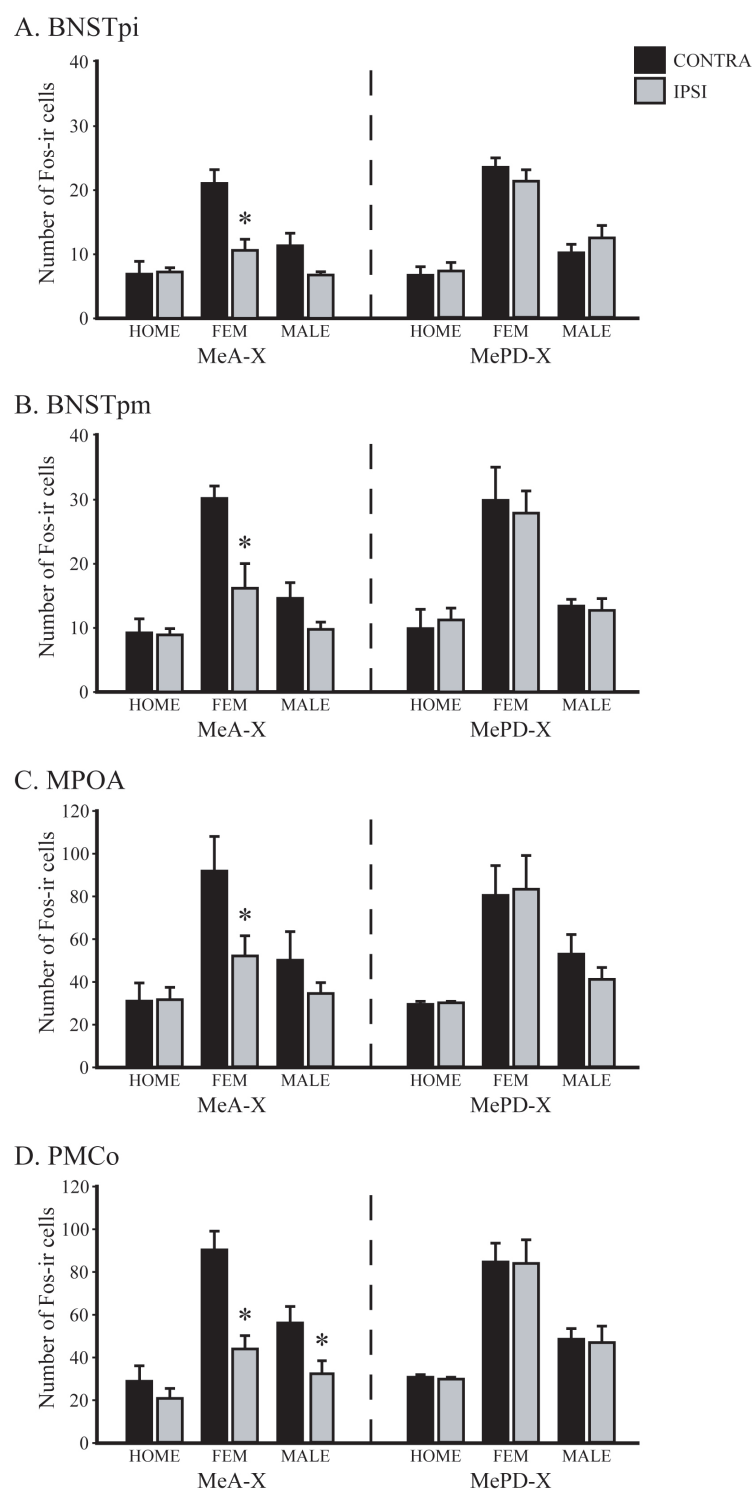


Figure 4.6 Summary of effects of MeA or MePD lesions on Fos-ir within ventral forebrain nuclei. Effects of unilateral electrolytic lesions of either MeA (left) or MePD (right) on the total number of Fos-ir cells within nuclei contralateral (CONTRA) and ipsilateral (IPSI) to the side of the lesion (Experiment 1). Males were exposed to female odors (FEM), male odors (MALE), or

handled conditions (HOME), and the total number of Fos-ir cells were counted within (A) BNSTpi (B) BNSTpm (C) MPOA and (D) PMCo. MeA lesions, but not MePD lesions, decreased odor-induced Fos-ir throughout the forebrain circuit. * $p < .05$ ($\alpha_{fw} = .05$), compared to CONTRA hemisphere within each nucleus.

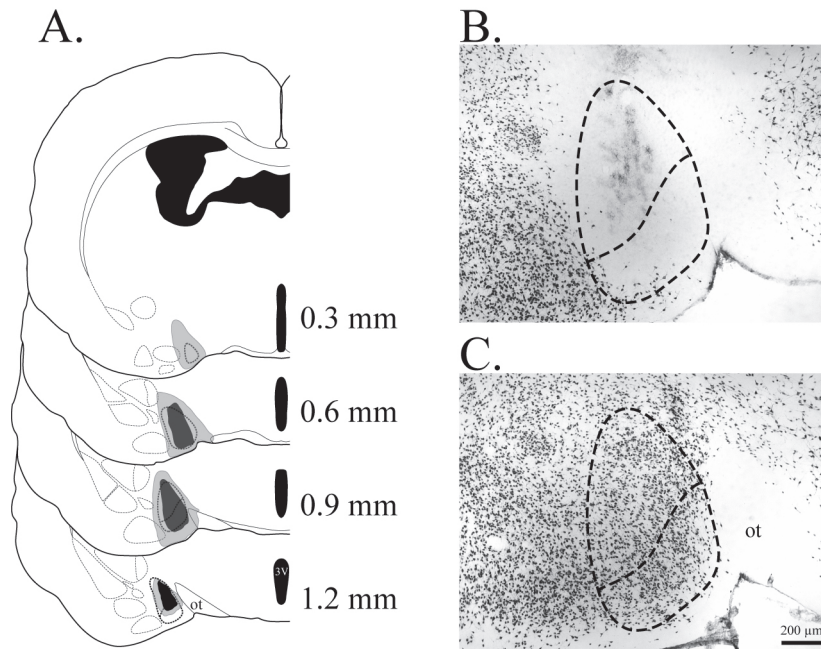


Figure 4.7 Lesion reconstruction for Experiment 2. (A) Reconstruction of coronal sections of the largest (light gray) and smallest (dark gray) extent of excitotoxic lesion damage of MeA in Experiment 2. Sections proceed from anterior (top) to posterior (bottom) levels, with the numbers representing the distance posterior to bregma. Photomicrographs of NeuN-ir within MeA showing (B) hemisphere with MeA lesion or (C) hemisphere contralateral to MeA lesion. Dotted line denotes borders of MeAD and MeAV. ot, optic tract. Scale bar = 200 μ m applies to B, C.

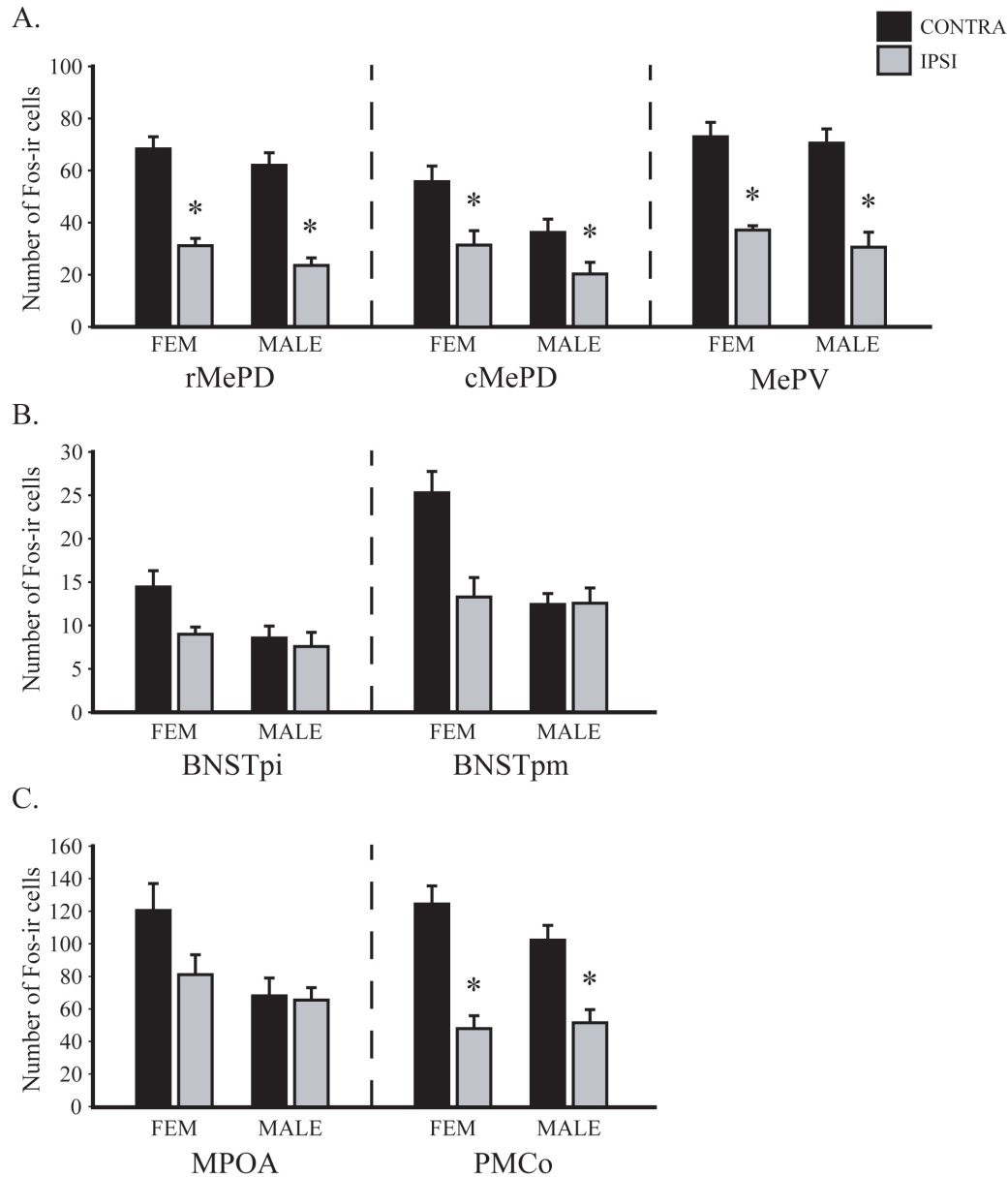


Figure 4.8 Summary of effects of excitotoxic MeA lesions of Fos-ir in Experiment 2. Effects of unilateral excitotoxic lesions of MeA on the total number of Fos-ir cells within nuclei contralateral (CONTRA) and ipsilateral (IPSI) to the side of the lesion in Experiment 2. Males were exposed to female odors (FEM) or male odors (MALE), and the total number of Fos-ir cells were counted within (A) rMePD, cMePD, MePV (B) BNSTpi, BNSTpm (C) MPOA, PMCo. MeA lesions significantly decreased odor-induced Fos-ir throughout MeP and PMCo. * $p < .05$ ($\alpha_{fw} = .05$), compared to CONTRA hemisphere within each nucleus.

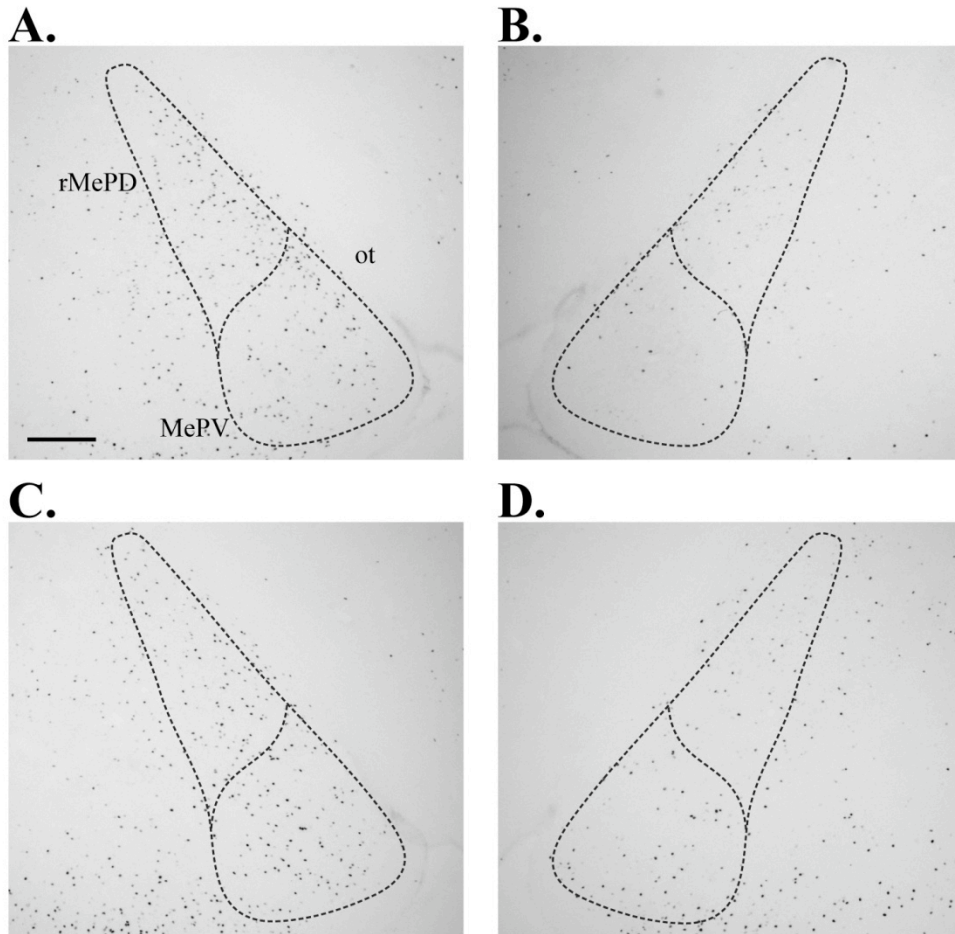


Figure 4.9 Representative photomicrographs of Fos-ir within MeP. Photomicrographs showing Fos-ir within MeP of males with excitotoxic lesions of MeA exposed to (A, B) female odors or (C, D) male odors in Experiment 2. Left photomicrographs (A, C) show nuclei contralateral to MeA lesion. Right photomicrographs (B, D) show nuclei ipsilateral to MeA lesion. Dotted line denotes borders of rMePD and MePV. ot, optic tract. Scale bar = 250 μ m applies to A-D.

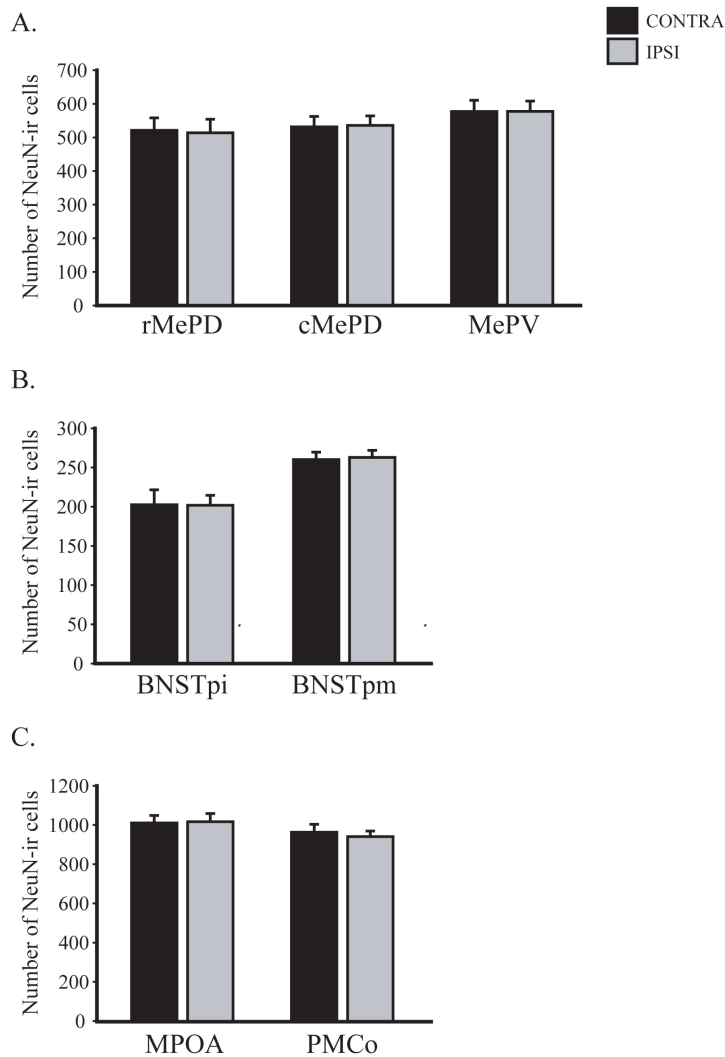


Figure 4.10 Summary of effects of excitotoxic MeA lesions on NeuN-ir. Effects of unilateral excitotoxic lesions of MeA on the total number of NeuN-ir cells within nuclei contralateral (CONTRA) and ipsilateral (IPSI) to the side of the lesion in Experiment 2. The total numbers of NeuN-ir cells were counted within (A) rMePD, cMePD, MePV (B) BNSTpi, BNSTpm (C) MPOA, PMCo. Excitotoxic lesions of MeA had no effect on the total number of neurons within any of the nuclei analyzed (all $p > .05$).

**CHAPTER 5: Anatomical Connections between the Anterior and Posterodorsal Sub-
regions of the Medial Amygdala: Integration of Odor and Hormone Signals**

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Submitted for Publication, Neuroscience (2010).

Abstract

In many rodent species, such as Syrian hamsters, reproductive behavior requires neural integration of chemosensory information and steroid hormone cues. The medial amygdala processes both of these signals through anatomically distinct sub-regions; the anterior region (MeA) receives substantial chemosensory input, but contains few steroid receptor-labeled neurons, whereas the posterodorsal region (MePD) receives less chemosensory input, but contains dense populations of androgen and estrogen receptors. Importantly, these sub-regions have considerable reciprocal connections, and previous studies in our lab have shown that functional interactions between MeA and MePD are required for the preference to investigate opposite-sex odors in male hamsters. We therefore hypothesized that chemosensory and hormone signals are conveyed *directly* between MeA and MePD. To test this hypothesis, we injected the retrograde tracer, cholera toxin B (CTB), into either MeA or MePD of male subjects and identified whether retrogradely labeled cells within MePD or MeA, respectively, expressed (1) Fos protein following exposure to female or male odors or (2) androgen receptors (AR). Approximately 36% of CTB-labeled cells within MeA (that project to MePD) also expressed Fos following exposure to either sexual odor, compared to the only 13% of CTB-labeled cells within MePD (that project to MeA) that also expressed odor-induced Fos. In contrast, 57% of CTB-labeled cells within MePD also contained AR, compared to the 28% of CTB-labeled cells within MeA that were double-labeled for AR/CTB. These results provide the first anatomical evidence that chemosensory and hormone cues are conveyed directly between MeA and MePD. Furthermore, these data suggest that chemosensory information is conveyed primarily from MeA to MePD, whereas hormone information is conveyed primarily from MePD to MeA. More broadly, the interactions between MeA and MePD may represent a basic mechanism by which

the brain integrates information about social cues in the environment with hormonal indices of reproductive state.

Introduction

Adaptive reproductive behavior requires integrating external signals about possible mates in the environment with internal signals of reproductive state (Wingfield *et al.*, 1997; Gowaty & Hubbell, 2009). In rodent species, including Syrian hamsters, sexual odors provide the primary signal for mate recognition (Johnston, 1983; 1990; Keverne, 2004); male hamsters are highly motivated to investigate female odors (Murphy, 1973; Johnston, 1974), and these chemosignals stimulate the expression male copulatory behavior (Murphy, 1973; Johnston, 1975). As in other vertebrates, internal signals of reproductive state in hamsters are conveyed through changes in circulating levels of gonadal steroid hormones, such as testosterone (Beyer *et al.*, 1976; Morin & Zucker, 1978; Hull & Dominguez, 2007). Testosterone and its primary metabolites, estradiol and dihydrotestosterone, are critical not only for the expression of male hamster copulatory behavior (Morin & Zucker, 1978; Powers *et al.*, 1985), but also for male's attraction to investigate female odors (Steel, 1982; Powers & Bergondy, 1983; Powers *et al.*, 1985; Petrulis & Johnston, 1995). Consequently, the expression of reproductive behavior in male hamsters involves the neural integration of chemosensory and hormonal cues (Wood, 1998).

The medial amygdala (MA) has been suggested as one candidate site for the integration of chemosensory and hormonal cues, as it receives both types of information (Wood, 1998). Lesions of MA disrupt the odor-guided aspects of reproductive behavior in many rodent species, including hamsters (Lehman *et al.*, 1980; Petrulis & Johnston, 1999; Maras & Petrulis, 2006), rats (Kondo, 1992; Kondo *et al.*, 1997; Stark *et al.*, 1998), and gerbils (Heeb & Yahr, 2000). The

MA also mediates hormonal control of reproductive behavior, as unilateral testosterone implants directly into MA facilitate the expression of copulatory behavior in castrated male hamsters (Wood & Newman, 1995c) and rats (Huddleston *et al.*, 2003; Huddleston *et al.*, 2006). Unilateral olfactory bulbectomy ipsilateral to the steroid implant, however, prevents this facilitation in male hamsters (Wood & Newman, 1995b; Wood & Coolen, 1997), suggesting that the processing of both chemosensory and steroid cues *within* MA is critical for the expression of reproductive behavior in male hamsters.

Detailed analysis of the MA suggests that chemosensory and hormone cues are processed within separate, but interconnected sub-regions of MA. In particular, the anterior MA (MeA) receives the majority of chemosensory input, as it has extensive connections with both the main and accessory olfactory systems (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Lehman & Winans, 1982; Coolen & Wood, 1998; Kang *et al.*, 2009). Although the posterodorsal MA (MePD) receives input from the olfactory bulbs, these projections are less substantial than compared to MeA, and the MePD also has much more limited connections with the main olfactory system compared to MeA (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Lehman & Winans, 1982; Coolen & Wood, 1998; Kang *et al.*, 2009). In contrast, the processing of steroid hormone information appears to be processed specifically within MePD, as the majority of steroid receptor-containing MA neurons are localized specifically within MePD rather than the MeA (Doherty & Sheridan, 1981; Wood *et al.*, 1992; Wood & Newman, 1993). This anatomical separation of chemosensory and hormone processing is maintained throughout much of the extended forebrain circuit; MeA and MePD preferentially project to chemosensory and steroid-sensitive sub-regions, respectively, of the bed nucleus of the stria terminalis and medial preoptic area (Gomez & Newman, 1992; Canteras *et al.*, 1995; Coolen & Wood, 1998).

Importantly, MeA and MePD share dense reciprocal connections (Coolen & Wood, 1998), and functional interactions between these sub-regions are critical the preference to investigate opposite-sex odors in male hamsters (Maras & Petrulis, 2010).

Taken together, these data indicate that although MeA and MePD process chemosensory and hormone signals differently, these signals are likely integrated at the level of the MA. We hypothesized that chemosensory and hormone signals are conveyed directly between MeA and MePD, such that odor-responsive neurons within MeA project to MePD, and hormone-sensitive neurons within MePD project to MeA. To test this hypothesis, we injected a retrograde tracer into either MeA or MePD of male hamsters and identified whether labeled cells within MePD or MeA, respectively, expressed (1) Fos protein (an indirect marker of neuronal activation) following exposure to sexual odors or (2) androgen receptors (AR). These anatomical data provide the first evidence that chemosensory and hormone cues are conveyed directly between MeA and MePD and identify a possible substrate for the neural integration of these signals within MA.

Materials and Methods

Animals

Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratory (Wilmington, MA) at eight weeks of age. All subjects (total $n = 45$) were gonadally intact, sexually naïve males (3 – 5 months old, 120 – 150g) that were singly housed upon arrival and remained singly housed for the duration of the study. A separate group of gonadally intact male and female hamsters (3 – 8 months old, $n = 80$) were used to provide sexual odor stimuli.

Subjects were unrelated to, and had no previous contact with, these odor donor animals. All

animals were housed in solid-bottom Plexiglas cages (36 cm X 30 cm X 16 cm) with corncob litter (the Andersons, Maumee, OH, USA) and cotton bedding material (Ancare, Bellmore, NY, USA) and were maintained on a reversed 14-h light/ 10-h dark photoperiod (lights off/on at 9 am/7 pm). Food and water were available *ad libitum*. All animal 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 injection

To visualize afferents of MeA or MePD (hereafter referred to as MeA-MePD projecting neurons), the retrograde tracer, cholera toxin B (CTB), was deposited into each sub-region (MeA $n = 23$; MePD $n = 22$). CTB (List Biological Laboratories, Campbell, CA, USA) was prepared as a 0.5% solution in 0.1M PBS (pH 7.5) according to manufacturer's instructions. For deposition of the tracer, males were anesthetized with 2% isoflurane anesthesia and placed into a stereotaxic apparatus so that the skull was flat. The temporal muscles were retracted from the skull, and small holes were drilled to expose the surface of the brain. CTB was loaded into a 10- μ l Hamilton microsyringe, which was lowered under stereotaxic control into either MeA (AP + 0.75 mm, ML \pm 2.60 mm, DV – 7.95 mm) or MePD (AP – 0.30 mm, ML \pm 2.80 mm, DV – 7.80 mm). For each sub-region, 20 nl of CTB was injected over a two-minute period, and the needle was left in place for twenty minutes prior to removal from the brain. Bone wax was used to seal the hole in the skull, and the incision was closed with wound clips.

Stimulus exposure and tissue collection

All males were sacrificed eight to nine days following tracer injection to allow for sufficient transport of CTB (Vercelli *et al.*, 2000). During this time, males were extensively handled 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 one hour prior to any manipulation. To determine if MeA-MePD projecting neurons respond to opposite-sex or same-sex odors, a subset of injected males were exposed to female (MeA $n = 11$; MePD $n = 9$) or male (MeA $n = 9$; MePD $n = 11$) sexual odors, respectively. Exposure to sexual odors consisted of placing a subject into a vacated odor donor cage; subjects were therefore exposed to both the volatile and non-volatile components of sexual odors, including those from the soiled litter, bedding, and walls of the cage. In order to minimize differences in odor quality across individual odor donors and to provide a composite source of sexual (rather than individual) odors, all stimulus cages housed 3 – 4 female or male hamsters (Maras & Petrulis, in press). Furthermore, to ensure equivalent levels of odor stimuli, all stimulus cages had not been changed for 4 days prior to use; thus, all female stimulus cages included sexual odors across the entire estrous cycle, including behavioral estrus. Finally, to provide a measure of the baseline activation of MeA-MePD projecting neurons, a third group of injected males was handled and returned to their home cage (MeA $n = 3$; MePD $n = 2$).

Males were left undisturbed in the stimulus (or home) cage for 60 minutes, upon which time they were injected with an overdose of sodium pentobarbital (100 mg/kg; Sleep Away, Ft. Dodge, IA, USA) and allowed to reach a deep level of anesthesia prior to perfusion (an additional 10 – 15 minutes). This survival time is within the time range for peak Fos protein expression (60 – 90 minutes; Herdegen & Leah, 1998) and has previously been used for the

induction of Fos by odor stimuli in Syrian hamsters (Fiber & Swann, 1996; Delville *et al.*, 2000; Swann *et al.*, 2001; Lai *et al.*, 2004). Males were transcardially perfused with 200 ml of 0.1M phosphate-buffered saline (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) prior to being placed in 30% sucrose in PBS solution for 48 – 72 hours (4°C). In order to distinguish between left and right brain hemispheres, an angle was cut into the right dorsal cortex of each brain. Brains were sectioned coronally (35- μ m thickness) using a cryostat set at -20°C. Brain sections throughout the rostrocaudal extent of the MA (Morin & Wood, 2001) were collected in a 1:4 series and stored in cryoprotectant-antifreeze solution (Watson *et al.*, 1986) until processing.

Immunohistochemistry

To determine whether MeA-MePD projecting neurons (a) respond to sexual odors or (b) are androgen-sensitive, separate series of tissue sections were processed for Fos and CTB or AR and CTB, respectively. Procedures for double-labeling Fos/CTB and AR/CTB were identical except for the specific antibodies used, and for both procedures, sections were stained first for Fos or AR, followed by CTB. Briefly, sections were removed from cryoprotectant and rinsed thoroughly in PBS (pH 7.4). To reduce endogenous peroxidase activity, tissue sections were incubated in 0.5% H₂O₂ in PBS for 15 minutes. After several rinses, sections were then incubated in the primary antibody for Fos or AR in PBS with 0.4% Triton-X100 for 24 hours at room temperature. The primary antibody for Fos was a rabbit polyclonal antibody (Santa Cruz Biotechnology, sc(4)-52) generated against a peptide mapping the N-terminus of the human c-Fos and was used at a concentration of 1:20,000. The primary antibody for AR was a rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-816) generated against a peptide mapping the

N-terminus of the human AR and was used at a concentration of 1:3,000. The specificity of these Fos and AR primary antibodies has previously been confirmed by preabsorption with epitope peptide (Kollack-Walker & Newman, 1997; Creutz & Kritzer, 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-X100. Sections were rinsed again in PBS and then incubated for 1 hour in avidin-biotin complex (4.5 μ l each of A and B reagents/ml of PBS with 0.4% Triton-X100, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). After rinsing in PBS and then in 0.175M sodium acetate, Fos or AR was visualized using 3,3'-diaminobenzidine HCl (0.2mg/ml, Sigma) and H₂O₂ (0.83 μ l/ml, Sigma) in a nickel-sulfate solution (25mg/ml, Sigma) for 15 minutes. This staining reaction yields a blue-black product and was stopped by rinsing sections in sodium acetate.

Immediately following Fos or AR staining, tissue sections were incubated in the primary antibody for CTB in PBS with 0.4% Triton-X100 for 24 hours at room temperature. The primary antibody for CTB was a goat polyclonal antibody (List Biological Laboratories, Campbell, CA, USA) generated against cholera toxin B subunit and was used at a concentration of 1:80,000. Procedures for labeling CTB were identical to those described above except that (1) the biotinylated secondary antibody was anti-goat and (2) CTB was visualized using 3,3'-diaminobenzidine HCl (0.2mg/ml, Sigma) and H₂O₂ (0.83 μ l/ml, Sigma) in Tris buffer (pH 7.2), yielding a brown reaction product. In addition to double-labeling, a separate series of tissue sections was single-labeled for CTB alone using identical procedures to those described above. 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 ©.

Data analysis

A single researcher that was blind to the stimulus condition of the animal conducted all analyses. Tissue that was single-labeled for CTB was used to examine the placement and spread of CTB injections. Only those subjects with CTB deposition restricted to the target sub-region (see RESULTS) were analyzed. As projections between MeA and MePD are primarily ipsilateral (Coolen & Wood, 1998), CTB, Fos and AR labeling was analyzed within nuclei ipsilateral to the injection. In addition, to determine whether the accumulation of CTB decreases Fos expression within MA, we also quantified Fos immunoreactivity within nuclei contralateral to the injection.

Sections were examined using a Nikon Eclipse E800 microscope with a Synsys digital camera attached. Counting domains were generated by projecting the microscopic field (10X) onto a computer screen using iVision software (Atlanta, GA, USA). In MeA-injected males, cells were counted within three separate sections of MePD (Figure 5.1B, D, F). In MePD-injected males, cells were counted within three separate sections of MeA, including both dorsal and ventral regions (Figure 5.1A, C, E). The minimum distance between any two sections within a single analysis (Fos/CTB or AR/CTB) was 140 μm . The diameter of cells within MA ranges from 10 – 20 μm (McDonald, 1992), thereby precluding duplicate counting of cells within any two sections.

Fos-positive (Fos+) or AR-positive (AR+) cells were identified as having dark, blue-black nuclear staining. As CTB has slight anterograde properties (Kobbert *et al.*, 2000; Vercelli *et al.*, 2000), CTB-positive (CTB+) cells were counted only if they had brown cytoplasmic staining that filled the shape of the cell. Fos/CTB-positive (Fos/CTB+) or AR/CTB-positive (AR/CTB+) cells were identified as having a dark, blue-black nucleus surrounded by brown

cytoplasmic staining. The total numbers of Fos⁺, AR⁺, Fos/CTB⁺, and AR/CTB⁺ cells were calculated separately for MeA and MePD (total of three sections per area) and were divided by the total area analyzed for each region (MeA: 1.947 mm², MePD: 1.178 mm²) to reflect the density of immunoreactive cells per mm². These densities were then used to generate the percent of total Fos⁺ cells that are also Fos/CTB⁺, the percent of total CTB⁺ cells that are also Fos/CTB⁺, the percent of total AR⁺ cells that are also AR/CTB⁺, and the percent of total CTB⁺ cells that are also AR/CTB⁺ for each sub-region.

SPSS 16.0 (SPSS Inc, Chicago, IL, USA) was used for all data analyses, and significance was determined as $p < .05$. All data are reported as mean \pm SEM. To identify differences in the total numbers of Fos⁺ and Fos/CTB⁺ cells across stimulus conditions (Handled, Female, Male), separate one-way ANOVAs were used for each brain area (MeA, MePD), followed by Tukey's-B post-hoc tests. To compare the total numbers of CTB⁺, AR⁺, and AR/CTB⁺ cells between brain areas, data were combined across stimulus conditions and compared using separate independent t -tests. Finally, Mann-Whitney U tests were used to identify differences in the percentages of Fos/CTB⁺ or AR/CTB⁺ cells between brain areas.

Results

CTB deposition and retrograde labeling

Males were included in the MeA- or MePD-injection group only if dark CTB staining was observed around the deposition site within at least two atlas plates of section for MeA or MePD, respectively (Morin & Wood 2001; Figure 5.2). Males were excluded from the analyses if the injection was misplaced (MeA, $n = 3$; MePD, $n = 3$). Within the MeA-injection group, misplaced injections resulted in CTB deposition lateral to the MeA, including the nuclei of the

lateral and accessory olfactory tract. Within the MePD-injection group, misplaced injections resulted in CTB deposition either medial to the MePD into the optic tract ($n = 2$) or lateral to the MePD into the intercalated and central nuclei of the amygdala ($n = 1$). Males were also excluded from the analyses if the size of the injection was too small (ie. observed within only one atlas plate; MeA, $n = 5$; MePD, $n = 4$) or too large (ie. spread into the other sub-region of the MA; MeA, $n = 2$; MePD, $n = 3$). These inclusion and exclusion criteria resulted in a final total of 13 MeA-injected males (Female odor $n = 6$, Male odor $n = 5$, Handled $n = 2$) and 12 MePD-injected males (Female odor $n = 5$, Male odor $n = 5$, Handled $n = 2$). For every male included in the analyses, the placement and spread of CTB injections were traced by hand onto published hamster atlas plates (Morin & Wood, 2001), and the largest and smallest extents of CTB deposition are shown in Figure 5.2.

Within the MeA-injection group, CTB spread into both the dorsal and ventral sub-regions of the MeA in seven males, whereas injections were restricted to the dorsal MeA in six males. In nine MeA-injected males, injections were centered within the rostral section of the MeA (plate 24, Morin & Wood, 2001), whereas in four of these males, injections were centered within the middle section of the MeA (plate 25, Morin & Wood, 2001). Within the MePD-injection group, eight males had injections centered within the middle section of the MePD (plate 28, Morin & Wood, 2001), whereas four males had injections centered within the most caudal section of the MePD (plate 29, Morin & Wood, 2001). As shown in Figure 5.2, CTB deposition was primarily restricted to MeA or MePD, although a sub-set of MeA- and MePD-injected males had minor spread into adjacent nuclei, which included the anterior cortical amygdala (MeA $n = 3$), intercalated nucleus of the amygdala (MeA $n = 2$; MePD $n = 3$), and posteroventral MA (MePV $n = 4$). Importantly, CTB deposition into these adjacent nuclei was only partial, spreading into

less than 20% of the area of each nucleus at any one plate of section. Furthermore, within each injection group, the placement and spread of CTB deposition were comparable across the different stimulus conditions (Female odor, Male odor, Handled).

CTB injections into the MeA or MePD retrogradely labeled cell bodies within the MePD or MeA, respectively (Figure 5.3A-D, 5.5A-D), as well as within several previously identified afferents of MA (Coolen & Wood 1998), including the amygdalohippocampal area, anterior amygdaloid area, anterior cortical amygdala, bed nucleus of the stria terminalis, intercalated nucleus of the amygdala, posteromedial cortical amygdala, and ventromedial hypothalamus. Retrogradely labeled cells were also observed within the posterolateral cortical amygdala, endopiriform nucleus, and piriform cortex following injections into MeA (Coolen & Wood 1998). For both MeA- and MePD-injected males, there were no differences in the overall pattern of retrograde labeling between males with the smallest and largest spread of CTB deposition, although larger injections were generally associated with higher densities of retrograde labeling.

Co-localization of CTB and Fos

The density of CTB+, Fos+, and Fos/CTB+ cells within MeA and MePD are shown in Table 5.1. MeA and MePD had comparable levels of retrograde labeling, as indicated by the similar numbers of CTB+ cells ($p > .05$). The numbers of Fos+ and Fos/CTB+ cells following exposure to handling alone were very low, indicating low levels of constitutive Fos expression. Exposure to either female odors or male odors significantly increased the numbers of Fos+ and Fos/CTB+ cells relative to handling within both the MeA (Fos+: $F(2, 10) = 7.862, p < .01$; Fos/CTB+: $F(2, 10) = 20.912, p < .001$) and MePD (Fos+: $F(2, 9) = 5.706, p < .05$; Fos/CTB+:

$F(2, 9) = 7.793, p < .05$). Within both MeA and MePD, there were no differences in the numbers of Fos+ cells or Fos/CTB+ cells between males exposed to female odors or male odors ($p > .05$).

Many retrogradely labeled cells within MeA and MePD expressed Fos in response to sexual odors (Table 5.1; Figure 5.3A-D). To identify the primary direction in which odor information is conveyed between MeA and MePD, we first compared the percentages of CTB+ cells within MeA and MePD that were double-labeled for Fos/CTB following exposure to any sexual odor (Female and Male odor groups combined; MeA $n = 11$; MePD $n = 10$). Thirty-six percent of CTB+ cells within MeA (that project to MePD) were double-labeled for Fos/CTB, which was significantly higher than the 13% of CTB+ cells within MePD (that project to MeA) that were double-labeled for Fos/CTB, $z = 3.873, p < .001$ (Figure 5.3E). Similarly, 21% of Fos+ cells within MeA were double-labeled for Fos/CTB, compared to the only 12% of Fos+ cells within MePD that were double-labeled for Fos/CTB, $z = 3.450, p < .001$ (Figure 5.3F).

To determine whether the percentages of Fos/CTB+ cells varied according to the sexual identity of the odor stimuli, we next compared these percentages between males exposed to female odors or male odors for each brain area (Figure 5.4). Within both MeA and MePD, the percentages of CTB+ cells that were double-labeled for Fos/CTB were identical following exposure to either female odors or male odors (Figure 5.4A, B), as were the percentages of Fos+ cells that were double-labeled for Fos/CTB (Figure 5.4C, D) (all $p > .05$).

To determine whether accumulation of CTB altered levels of Fos expression, as has been observed with the use of other retrograde tracers (Greco *et al.*, 1998a), we compared the total numbers of Fos+ cells following exposure to either sexual odor (MeA $n = 11$; MePD $n = 10$) between nuclei ipsilateral and contralateral to the CTB injection (Table 5.2). Within both MeA

and MePD, the total numbers of Fos+ cells ipsilateral to the injection were significantly lower than the total numbers of Fos+ cells contralateral to the injection (MeA: $t(10) = 7.732$, $p < .001$, MePD: $t(9) = 10.516$, $p < .001$), resulting in a 28% decrease within MeA and a 26% decrease within MePD compared to contralateral levels (Table 5.2).

Co-localization of CTB and AR

The density of CTB+, AR+, and AR/CTB+ cells within MeA and MePD are shown in Table 5.3. MeA and MePD again had comparable levels of total retrograde labeling ($p > .05$), although the MePD had significantly more AR+ cells, $t(23) = 10.686$ $p < .001$, as well as AR/CTB+ cells, $t(23) = 11.826$ $p < .001$, than MeA. Within the MeA, the populations of AR+ and CTB+ occupied distinct, non-overlapping zones (Figure 5.5A, C), whereas the distribution of AR+ and CTB+ cells within MePD overlapped substantially (Figure 5.5B, D). This qualitative difference is supported by the statistical analysis of AR/CTB+ cells; 57% of CTB+ cells within MePD were double-labeled for AR/CTB, which was significantly higher than the 29% of CTB+ cells within MeA that were double-labeled for AR/CTB, $z = 3.521$, $p < .001$ (Figure 5.5E). Similarly, 17% of AR+ cells within MePD were double-labeled for AR/CTB, compared to the only 11% of AR+ cells within MeA that were double-labeled for AR/CTB, $z = 2.535$, $p < .05$ (Figure 5.5F).

Discussion

The MA has been identified as a possible site for the neural integration of chemosensory and steroid hormone input, as distinct sub-regions within the MA process each of these cues. Specifically, the MeA receives substantial chemosensory input (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Lehman & Winans, 1982), whereas the MePD is highly sensitive to steroid

hormones (Doherty & Sheridan, 1981; Wood *et al.*, 1992; Wood & Newman, 1993). These sub-regions share substantial reciprocal connections (Coolen & Wood, 1998), suggesting that the neural integration of chemosensory and hormone cues involves interactions directly between MeA and MePD. By combining the use of retrograde tracers with markers of odor-responsive neurons (Fos) or hormone-sensitive neurons (AR), the current experiments provide the first anatomical evidence that chemosensory and hormone cues are conveyed *directly* between MeA and MePD. Indeed, approximately 40% of MeA cells that project to MePD responded to sexual odors, and 60% of MePD cells that project to MeA expressed AR. These data suggest that the anatomical connections between MeA and MePD function to integrate chemosensory and hormone signals. Furthermore, by comparing the percentage of double-labeled cells between MeA and MePD, our results suggest that chemosensory information is conveyed primarily from MeA to MePD, whereas hormone information is conveyed primarily from MePD to MeA.

Methodological considerations

Previous studies have used CTB to label afferents of MA neurons in hamsters (Coolen *et al.*, 1998; Coolen & Wood, 1998), and in the current study, we observed a pattern of retrograde labeling similar to those reported previously. By combining the use of CTB with immunohistochemical markers for Fos and AR, we extended these previous findings by characterizing the odor-responsiveness and hormone sensitivity of MeA-MePD projecting neurons. Fos, an immediate early gene product (Sheng & Greenberg, 1990; Sheng *et al.*, 1990), has been used extensively as an indirect marker of neuronal excitation in response to stimulus exposure (Pfaus & Heeb, 1997), and we observed significant activation of MeA and MePD neurons following exposure to either opposite-sex or same-sex odors, confirming previous studies (Fiber *et al.*, 1993; Heeb & Yahr, 1996; Coolen *et al.*, 1997; Kelliher *et al.*, 1998; Halem

et al., 1999; Sheehan *et al.*, 2000; Meredith & Westberry, 2004; Samuelsen & Meredith, 2009). Constitutive Fos expression observed in the handled control group was very low in this experiment, as were the levels of cells double-labeled for Fos/CTB in this condition. Thus, the co-localization of CTB and Fos observed in males exposed to female or male odors likely reflects specific activation of MeA-MePD projecting neurons in response to sexual odor stimuli, rather than constitutively activated cells.

In the current study, we observed a significant decrease in the expression of Fos protein ipsilateral to the side of the CTB injection, indicating that accumulation of CTB may decrease the ability of neurons to express Fos. A decrease in Fos expression may be a general problem with the use of retrograde toxins, as similar decreases in Fos expression within MA have been reported with the use of FluoroGold in the MA of rats (Greco *et al.*, 1998a). The current results may therefore underestimate the absolute levels of MeA-MePD projecting neurons that show activation. Importantly, CTB deposition decreased Fos expression within both MeA and MePD, and the relative magnitude of these decreases was very similar between the two areas (28% within MeA and 26% within MePD). Thus, any underestimation of Fos expression due to uptake of CTB would be equivalent between MeA and MePD and would not bias the direct comparison of double-labeled cells between the two sub-regions.

Chemosensory information is conveyed from MeA to MePD

Our results suggest that many of the cells that project between MeA and MePD respond to sexual odors, although sexual odor information appears to be conveyed predominantly from MeA to MePD, rather than from MePD to MeA. Indeed, relatively more MeA cells that project to MePD responded to sexual odors than compared to MePD cells that project to MeA.

Similarly, a higher percentage of odor-responsive neurons within MeA projected directly to MePD compared to those within MePD that projected to MeA. The fact that sexual odor information is conveyed primarily from MeA to MePD is consistent with the fact that MeA receives relatively more chemosensory input compared to MePD (Scalia & Winans, 1975; Kevetter & Winans, 1981b; Lehman & Winans, 1982; Coolen & Wood, 1998). Functionally, MeA also appears to play a greater role in regulating odor-guided reproductive behaviors in male hamsters than MePD; MeA lesions completely eliminate the expression of copulatory behavior (Lehman *et al.*, 1980), similar to what is observed following destruction of the olfactory bulbs (Murphy & Schneider, 1970), whereas lesions of MePD only alter the temporal pattern of the copulatory sequence (Lehman *et al.*, 1983). The current results also confirm recent studies in our laboratory showing that MeA lesions substantially reduce odor-induced Fos expression within MePD, whereas lesions of MePD have no effect on Fos expression within MePD (Maras and Petrulis, under review). Interestingly, MeA lesions reduce Fos expression within MePD to an equal degree following exposure to either opposite-sex or same-sex odors (Maras and Petrulis, under review), and our current results indicate that both types of odor information may be conveyed directly from MeA to MePD.

To our knowledge, no previous studies have examined the activation of MeA-MePD projecting cells in response to odor presentation alone. However, the percentages of MeA-MePD projecting cells that were activated in response to sexual odors (MeA 36%, MePD 13%) are lower than the percentage of MA cells projecting to the medial preoptic area that are activated during sexual behavior (60 – 65%) (Coolen *et al.*, 1998; Greco *et al.*, 1998b). These lower levels of activation may reflect the fact that presentation of odors alone provides relatively less total sensory input to the MA than does engaging in sexual behavior (Kollack-Walker & Newman,

1997). In support of this idea, we observed similar levels of activation of MeA-MePD projecting cells compared to the percentage of MeA cells projecting to the AOB that are also activated in response to opposite-sex volatile odors in mice (Martel & Baum, 2009). Importantly, although MeA appears to transmit sexual odor information to MePD, our results indicate that many of the odor-responsive cells within MeA do not project to MePD. These odor-responsive cells may terminate locally on other MeA neurons, although the MA is characterized by few, if any, locally projecting cells, (McDonald, 1992), making this possibility unlikely. Instead, the odor-responsive cells not labeled by CTB likely represent cells that project to other ventral forebrain nuclei, such as the bed nucleus of the stria terminalis, medial preoptic area, and posteromedial cortical amygdala (Gomez & Newman, 1992; Canteras *et al.*, 1995; Coolen & Wood, 1998). In fact, lesions of MeA decrease odor-induced Fos expression within all of these regions (Maras and Petrulis, under review).

Hormone information is conveyed from MePD to MeA

Although neurons within both MeA and MePD expressed AR, there were substantially more AR+ cells within MePD compared to MeA. These results confirm previous reports of AR density within MA using immunohistochemistry (Wood & Newman, 1993; 1999), or autoradiography (Wood *et al.*, 1992). Consistent with these differences in the overall density of hormone-sensitive neurons, testosterone or estradiol implants facilitate the expression of copulatory behavior in castrated males when directed at MePD, but not MeA (Wood & Newman, 1995c; Huddleston *et al.*, 2003), suggesting a functional difference between MeA and MePD in their sensitivity to steroid hormones. Given these differences in hormone sensitivity, it is perhaps not surprising that the current study found relatively more double-labeled AR/CTB cells within MePD compared to MeA. Whereas approximately 57% of MePD cells that project to MeA

expressed AR, a relatively low percentage of MeA cells that project to MePD also contained AR (29%). These results suggest that, in contrast to the flow of chemosensory information, hormonal signals within MA are conveyed predominantly from MePD to MeA.

As in many rodent species (Moffatt, 2003), sufficient levels of gonadal hormones are critical for male hamsters' attraction to investigate female odors (Steel, 1982; Powers & Bergondy, 1983; Powers *et al.*, 1985; Petrulis & Johnston, 1995), suggesting that hormones modulate neural processing of sexual odors. As has been proposed by Cottingham and Pfaff (Cottingham & Pfaff, 1986) and later by Wood (Wood, 1998), gonadal steroids may act as a gating mechanism to enhance transmission of sexually relevant odors, and this mechanism likely involves reciprocal connections between hormone-sensitive and chemosensory-responsive subnuclei. Thus, the hormone-sensitive projections from MePD to MeA may function to increase transmission of opposite-sex odors by MeA. In hamsters, exposure to female vaginal secretions (FVS) causes a significant increase in Fos expression within MePD of males, but not females (Fiber & Swann, 1996). Although this FVS-induced Fos expression in males does not require circulating testosterone, testosterone treatment in females masculinizes the response of MePD neurons to FVS (Fiber & Swann, 1996). In male rats, castration decreases, whereas testosterone administration increases, Fos expression within both the MeA and MePD in response to female odors (Paredes *et al.*, 1998a). Given that MeA itself is not highly sensitive to steroid hormones (Wood, 1998), this effect may be mediated in part by projections from MePD. The current results provide anatomical support for an indirect model of chemosensory-hormone interaction within the MA, although further studies are needed to determine the functional significance of these hormone-sensitive projections.

Although the current study only examined the expression of AR within the MA, this nucleus also contains abundant estrogen receptor (ER)-containing cells (Doherty & Sheridan, 1981; Wood *et al.*, 1992; Wood & Newman, 1995a), and these ERs mediate many of the effects of steroid hormones on male reproductive behavior (Wood, 1996). Unfortunately, the only antibody that consistently labels ER in hamster brain is the H222 antibody (Li *et al.*, 1993; Wood & Newman, 1995a; Mangels *et al.*, 1998; Boers *et al.*, 1999; De La Iglesia *et al.*, 1999), and this antibody labels specifically unbound ER, precluding its use in any studies that require subjects to maintain normal levels of gonadal steroid hormones (Blaustein, 1993). In hamsters, individual MA cells often co-express AR and ER, such that cells expressing only ER constitute a small fraction (13%) of the total population of AR- and ER-expressing cells within this nucleus (Wood & Newman, 1995a). These data suggest that the current findings 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.

Concluding remarks

The interactions between chemosensory and hormone-sensitive sub-regions of the MA may represent a basic mechanism by which the brain integrates information about sexually relevant stimuli in the environment with hormonal indices of reproductive state (Wood, 1998; Newman, 1999). The connections between MeA and MePD may therefore provide a neural substrate for the refinement of odor responses and regulation of reproductive behaviors within a dynamic internal and external environment. The current findings provide the first anatomical evidence that chemosensory and steroid hormone cues are conveyed *directly* between MeA and MePD. Although we focus on chemosensory-hormone integration for the purposes of reproduction, other rodent social behaviors, such as aggressive or maternal behaviors, also rely

heavily on chemosensory and hormone signals processed by the MA (Luiten *et al.*, 1985; Numan & Sheehan, 1997; Blanchard *et al.*, 2003). Thus, the MeA-MePD interactions described here may be critical for regulating the appropriate behavioral responses to odor stimuli in a variety of social contexts.

Acknowledgments

We would like to thank Anaam Mohammed and Nina King for experimental assistance. 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 5 Tables

Table 5.1 Total numbers of CTB+, Fos+, and Fos/CTB+ cells (per mm²) within MeA and MePD. Within both the MeA and MePD, exposure to either female odors or male odors increased the numbers of Fos+ cells and Fos/CTB+ cells. Identical letters represent homologous means within each brain area (Tukey's-B post-hoc comparisons, $p < .05$).

Total/mm ²		MeA	MePD
CTB+		110.86 ± 6.84	108.09 ± 5.17
Fos+	<i>Handled</i>	56.25 ± 4.37 ^a	52.19 ± 1.80 ^a
	<i>Female</i>	189.11 ± 22.80 ^b	147.33 ± 21.07 ^b
	<i>Male</i>	207.96 ± 16.90 ^b	113.54 ± 10.49 ^b
Fos/CTB+	<i>Handled</i>	4.37 ± 0.26 ^a	6.15 ± 0.21 ^a
	<i>Female</i>	36.22 ± 3.90 ^b	15.28 ± 1.44 ^b
	<i>Male</i>	43.05 ± 1.84 ^b	13.94 ± 1.27 ^b

Table 5.2 Total numbers of Fos+ cells (per mm²) within MeA and MePD observed ipsilateral or contralateral to CTB injection. Within both MeA and MePD, fewer Fos+ cells were observed ipsilateral compared contralateral to the CTB injection. * $p < .05$ compared to ipsilateral totals within each brain area.

	Total numbers of Fos+ cells/mm ²	
	Ipsilateral	Contralateral
MeA	198.54 ± 13.24	275.28 ± 11.18 *
MePD	129.38 ± 15.75	175.79 ± 14.14 *

Table 5.3 Total numbers of CTB+, AR+, and AR/CTB+ cells (per mm²) within MeA and MePD. More AR+ and AR/CTB+ cells were observed within MePD compared to MeA (* $p < .05$).

Total/mm²	MeA	MePD
CTB+	108.54 ± 5.29	120.26 ± 3.82
AR+	237.20 ± 10.58	463.47 ± 21.30*
AR/CTB+	27.41 ± 1.98	77.30 ± 3.55*

Chapter 5 Figures

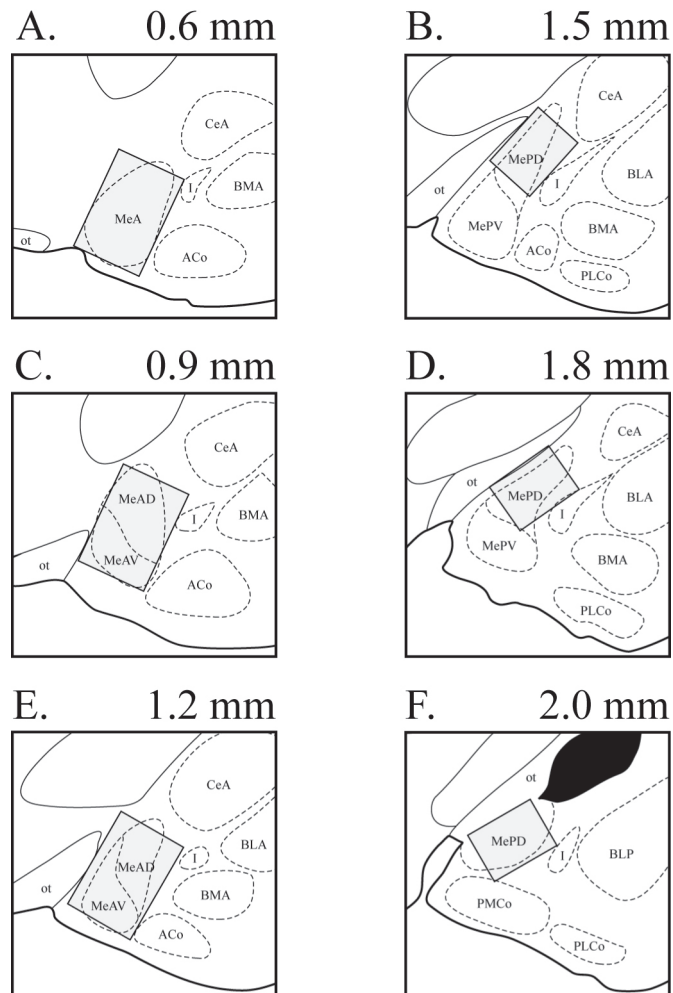


Figure 5.1 Counting domains for analysis of CTB, Fos, and AR. Illustration adapted and modified from hamster brain atlas of Morin and Wood (2001) showing placement of counting domains (gray rectangles) for MeA (A, C, E) and MePD (B, D, F). Total numbers of immunoreactive cells therefore represent the sum of three counting domains for each region. Values given in millimeters indicate distance posterior to bregma. ACo, anterior cortical amygdala; BLA, anterior basolateral amygdala; BLP, posterior basolateral amygdala; BMA, basomedial amygdala; CeA, central amygdala; I, intercalated amygdala; MeA, anterior medial amygdala; MeAD, anterodorsal medial amygdala; MeAV, anteroventral medial amygdala; MePD, posterodorsal medial amygdala; MePV, posteroventral medial amygdala; ot, optic tract; PLCo, posterolateral cortical amygdala; PMCo, posteromedial cortical amygdala.

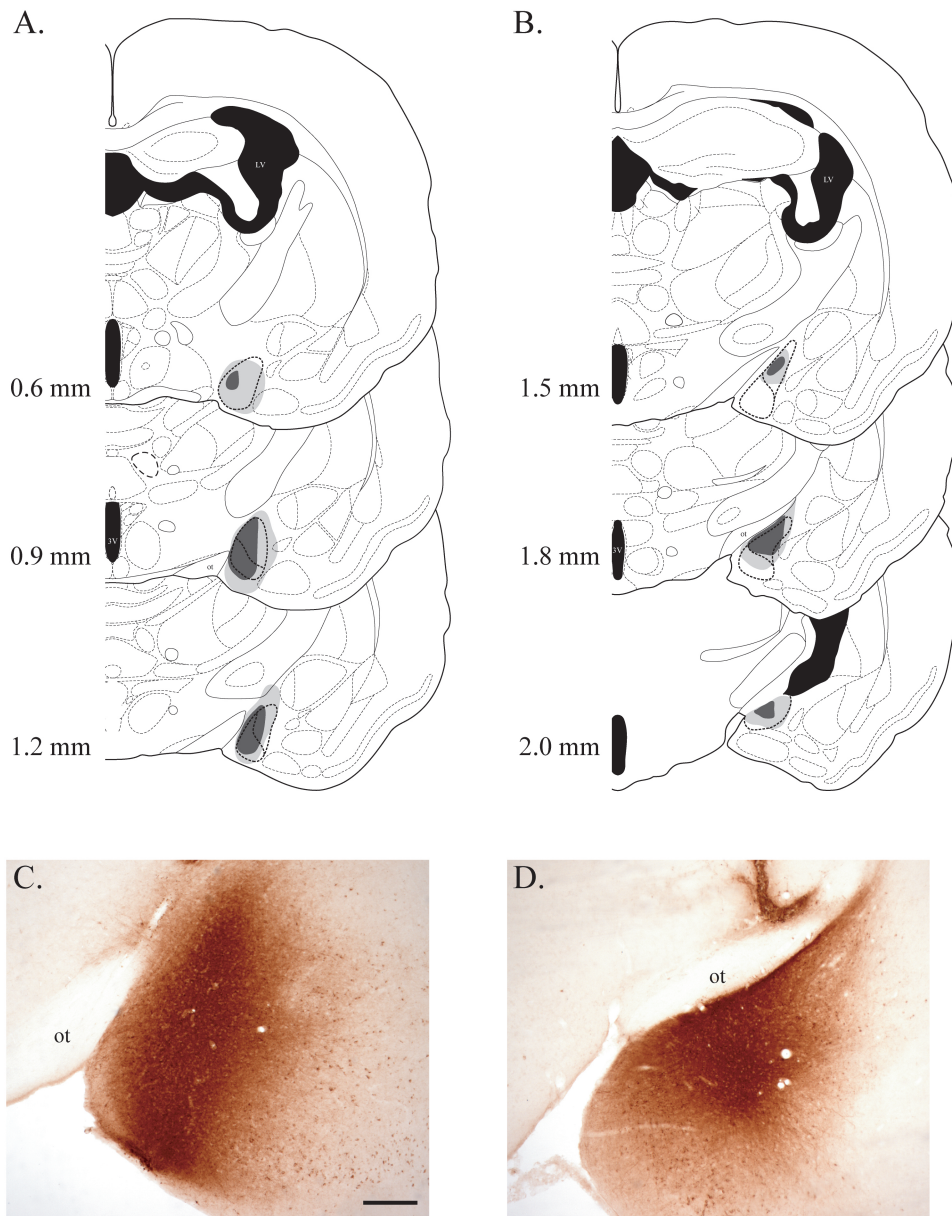


Figure 5.2 Verification of CTB injections. Placement and spread of CTB deposition in MeA-injected males (A, C) and MePD-injected males (B, D). Reconstruction of coronal sections of the largest (light gray) and smallest (dark gray) extents of CTB deposition within the MeA (A) or MePD (B). Numbers represent the distance posterior to bregma in millimeters. Photomicrographs depict typical CTB injection into the MeA (C) or MePD (D). Scale bar = 200 μ m applies to both C and D. ot, optic tract; 3V, third ventricle.

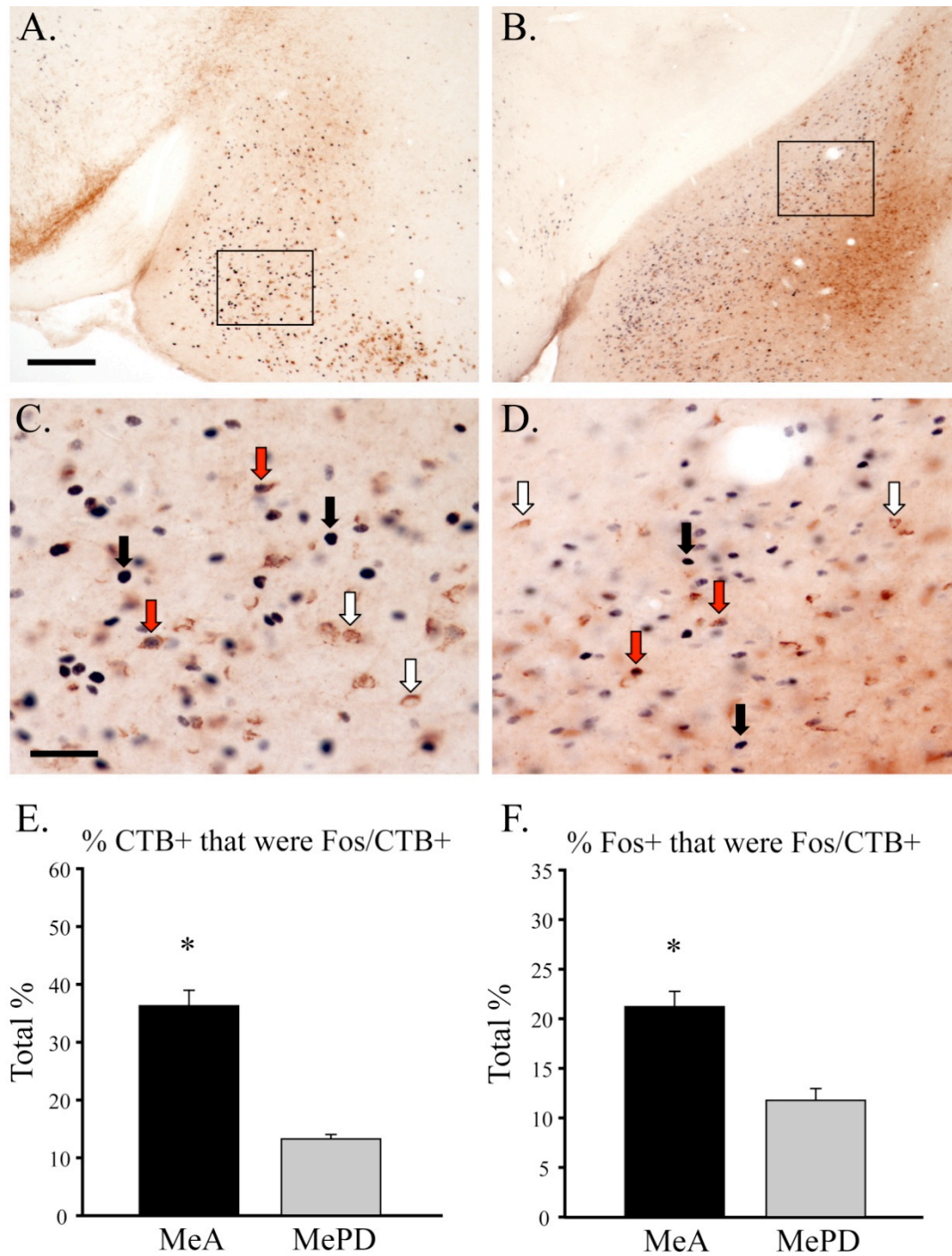
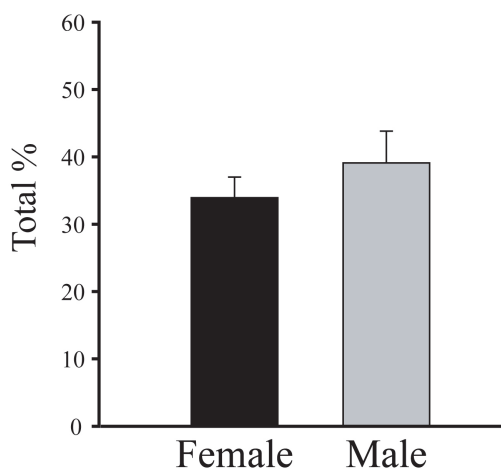


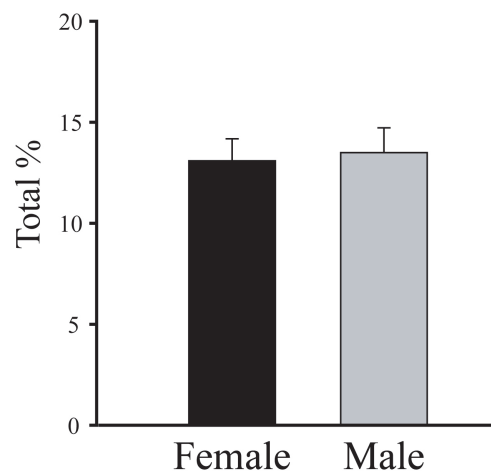
Figure 5.3 Co-localization of CTB and Fos. Photomicrographs of representative sections of the MeA (A, C) and MePD (B, D) that were double-labeled for Fos/CTB. C and D provide higher magnifications of the areas indicated by the boxes in A and B, respectively. Cells stained brown are CTB+ (white arrows), nuclei stained black are Fos+ (black arrows), and brown cells with black nuclei are Fos/CTB+ (red arrows). Scale bar = 100 μ m (A, B) and 50 μ m (C, D). A higher percentage of CTB+ cells within MeA were double-labeled for Fos/CTB compared to MePD (E), and a higher percentage Fos+ cells within MeA were double-labeled for Fos/CTB compared to MePD (F). * $p < .05$, Mann-Whitney U tests ($\alpha_{fw} = .05$).

% CTB+ cells that were Fos/CTB+

A. MeA

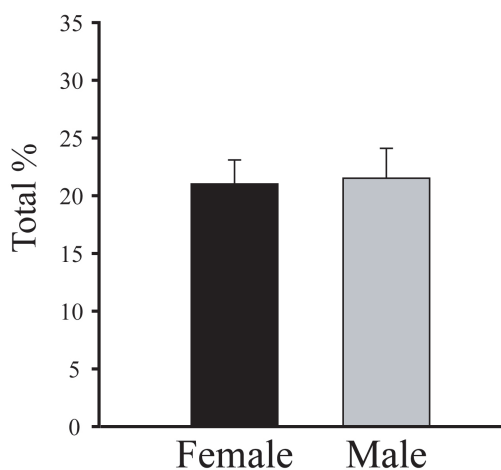


B. MePD



% Fos+ cells that were Fos/CTB+

C. MeA



D. MePD

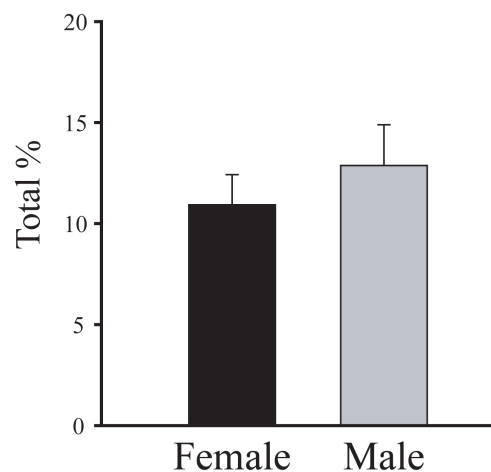


Figure 5.4 Fos/CTB double-labeling observed in males exposed to female odors or male odors. For both MeA (A, C) and MePD (B, D), there were no significant differences in either the percentages of CTB+ cells that were double-labeled for Fos/CTB (top) or the percentages of Fos+ cells that were double-labeled for Fos/CTB (bottom).

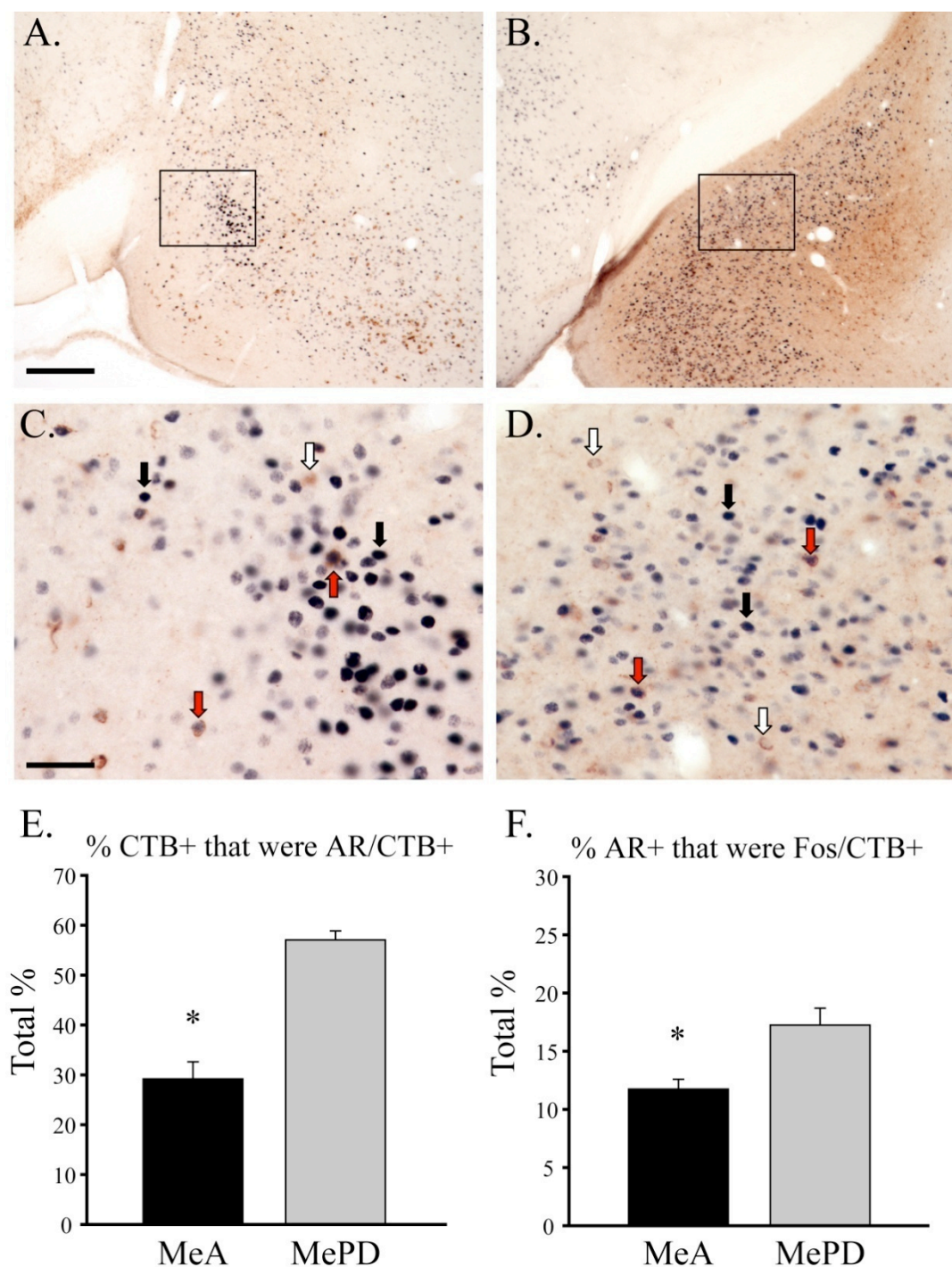


Figure 5.5 Co-localization of CTB and AR. Photomicrographs of representative sections of the MeA (A, C) and MePD (B, D) that were double-labeled for AR/CTB. C and D provide higher magnifications of the areas indicated by the boxes in A and B, respectively. Cells stained brown are CTB+ (white arrows), nuclei stained black are AR+ (black arrows), and brown cells with black nuclei are AR/CTB+ (red arrows). Scale bar = 100 μ m (A, B) and 50 μ m (C, D). A higher percentage of CTB+ cells within MePD were double-labeled for AR/CTB compared to MeA (E),

and a higher percentage AR⁺ cells within MePD were double-labeled for AR/CTB compared to MeA (F). * $p < .05$, Mann-Whitney U tests ($\alpha_{fw} = .05$).

CHAPTER 6: General Discussion

Overview

Male reproductive behavior in rodents requires neural integration of chemosensory and steroid hormone cues (Wood, 1998; Hull *et al.*, 2002). Within the corticomedial amygdala, the posteromedial cortical (PMCo) and medial (MA) amygdala nuclei process odor and hormone signals (Scalia & Winans, 1975; Wood *et al.*, 1992; Wood, 1997), suggesting that one or both of these nuclei mediate odor-guided aspects of male reproductive behavior. The experiments presented in this dissertation therefore examined the roles of PMCo and MA in processing sexual odors and generating appropriate behavioral responses to these stimuli. Using site-specific lesions of the PMCo, we demonstrated that this nucleus is not required for the preference to investigate opposite-sex odors, although it does regulate two critical aspects of male copulatory behavior, inhibiting inappropriate olfactory investigation of the female and timing the onset of sexual satiety (Chapter 2). These results suggest that PMCo has a minimal role in generating attraction to sexual odors. The remainder of the experiments therefore addressed the role of the MA in odor processing, and in particular, how the anterior (MeA) and posterodorsal (MePD) sub-regions within MA interact to generate attraction to sexual odors. First, using an asymmetrical lesion technique, we demonstrated that functional interactions between MeA and MePD are required for the preference to investigate the volatile components of opposite-sex odors, although these interactions are not required when additional non-volatile odors are available (Chapter 3). Next, to identify the nature of this interaction during odor processing, we examined odor-induced Fos expression in males with lesions of either MeA or MePD (Chapter 4). These results indicate that MeA normally enhances excitation of MePD neurons in response to sexual odors, but that MePD does not regulate sexual odor responses within MeA. Finally, using retrograde tracers to identify neurons that project directly between MeA and MePD, we

asked whether these neurons either express Fos following odor exposure or contain androgen receptors (Chapter 5). We found that many MeA cells that project to MePD also respond to sexual odors, whereas many MePD cells that project to MeA are also hormone-sensitive. These anatomical data provide the first evidence that chemosensory and hormone cues are integrated directly between MeA and MePD, and together, these studies identify possible mechanisms for the neural integration of sensory and hormone cues.

Differential roles for PMCo and MA

Combined with previous data from our laboratory and others, our results indicate that the PMCo and MA mediate related, but distinct aspects of odor processing and olfactory investigation. Although both PMCo and MA receive substantial chemosensory input (Scalia & Winans, 1975; Pro-Sistiaga *et al.*, 2007), only the MA is critical for generating the preference for, and attraction to, opposite-sex odors. Indeed, lesions of the MA completely eliminate opposite-sex odor preferences in male (Maras & Petrulis, 2006) and female (Petrulis & Johnston, 1999) hamsters, whereas lesions of the PMCo have no effect on these preferences in male hamsters (Maras & Petrulis, 2008b). Both MA and PMCo regulate olfactory investigation during copulatory behavior, although these nuclei mediate qualitatively distinct aspects of investigation. Whereas the MA is critical for generating attraction to investigate the female's anogenital area (Lehman *et al.*, 1980; Lehman & Winans, 1982), which contains the most potent female odors (Johnston, 1975; 1986), the PMCo is critical for inhibiting inappropriate investigation of the female's non-anogenital area (Maras & Petrulis, 2008b). The PMCo and MA may therefore work together during copulation to direct olfactory investigation toward the most salient of the female odors, which are critical for stimulating and maintaining other aspects of the copulatory sequence (Johnston, 1975; Bunnell *et al.*, 1977; Johnston, 1986).

Furthermore, the MA, but not the PMCo, is required for the expression of male copulatory behavior. In fact, males with lesions of the PMCo display all elements of the male copulatory sequence, including ejaculations (Maras & Petrulis, 2008b). This pattern of results is in striking contrast with those observed following lesions of MA in hamsters (Lehman *et al.*, 1980), rats (Kondo, 1992), or gerbils (Heeb & Yahr, 2000), in which the expression of male copulatory behavior is severely impaired. These data suggest that within the corticomedial amygdala, the MA is the primary regulator of male copulatory behaviors. The differential deficits in copulation following MA or PMCo lesions are consistent with the fact that MA, but not PMCo, projects to downstream nuclei that are known to control the output of mating behavior, such as the medial preoptic area (MPOA) (Kevetter & Winans, 1981a; Canteras *et al.*, 1992; 1995; Hull *et al.*, 2002).

Although MA and PMCo have different roles in mediating the expression of male copulatory behavior, both of these nuclei appear to be involved in regulating the timing of sexual satiety during the copulatory sequence. In hamsters, lesions of either PMCo (Maras & Petrulis, 2008b) or the caudal region of the MePD (Parfitt & Newman, 1998) delay the expression of long intromissions, an indication of the onset of sexual satiety in this species (Bunnell *et al.*, 1977; Parfitt & Newman, 1998). Furthermore, Fos expression within MePD correlates specifically with the expression of long intromissions (Parfitt & Newman, 1998), although the PMCo has not been analyzed using this experimental design. The reciprocal connections between the PMCo and MePD (Kevetter & Winans, 1981a; Coolen & Wood, 1998) suggest a possible functional relationship between these nuclei in regulating the temporal aspects of copulatory behavior. Given that the PMCo does not directly project to hypothalamic or brainstem nuclei known to regulate ejaculatory responses (Kevetter & Winans, 1981a; Canteras *et al.*, 1992; Coolen *et al.*,

2004; Normandin & Murphy, 2008), one possibility is that PMCo indirectly modulates ejaculation centers via interactions with MePD (Kevetter & Winans, 1981a; Canteras *et al.*, 1995; Coolen & Wood, 1998; Coolen *et al.*, 2004).

Taken together, these data indicate that the MA and PMCo regulate overlapping, but distinct aspects of male reproductive behavior. Although PMCo mediates specific aspects of male copulatory behavior, this nucleus appears to have a minimal role in generating attraction to sexual odors. In contrast, the MA is critical not only for the expression of male copulatory behaviors, but also for regulating behavioral responses to sexual odors outside of the mating context.

Integration of chemosensory and hormone cues within MA

The MA is a heterogeneous nucleus, containing both chemosensory and steroid-sensitive sub-regions (MeA and MePD, respectively). Although most research has focused on the role of the MA as a whole in regulating reproductive behavior, previous lesion studies in our laboratory provide direct evidence for functional differences between MeA and MePD in regulating attraction to sexual odors (Maras & Petrulis, 2006). Lesions of either MeA or MePD eliminate the preference to investigate opposite-sex over same-sex odors, but these lesions are associated with distinct changes in odor investigation; males with MePD lesions display decreased attraction to female odors, whereas males with MeA lesions are highly attracted to both female and male odors (Maras & Petrulis, 2006). This pattern of results suggests that MePD generates motivation to investigate opposite-sex odors, whereas MeA evaluates the sexual relevance of different social odors and directs investigation specifically toward opposite-sex stimuli.

The anatomical and functional differences between MeA and MePD suggest parallel processing of chemosensory and hormone cues (Wood, 1997; 1998; Newman, 1999), both of which are critical for generating the final behavioral response to sexual odors (Wood & Coolen, 1997; Wood, 1998). This parallel processing is maintained throughout much of the ventral forebrain circuit, including the bed nucleus of the stria terminalis (BNST), MPOA, and ventromedial hypothalamus (VMH) (Wood, 1997; Newman, 1999), and the integration of chemosensory and hormone cues may therefore occur at any or all of these levels. Our data suggest, however, that at least some of this integration occurs within the MA itself. In fact, functional interactions between MeA and MePD are required for the preference to investigate the volatile components of opposite-sex odors (Maras & Petrulis, 2010). Given our results, we propose an updated model of chemosensory-hormone integration within MA (Figure 6.1).

Specifically, we propose that sexual odor information is conveyed primarily from MeA to MePD. Indeed, lesions of MeA substantially reduce Fos expression within MePD in response to sexual odors, but this interaction is not reciprocal (Chapter 4). In contrast to our original model (see Chapter 1, Figure 1.3), however, our results suggest that MeA does not function as a “chemosensory filter”, enhancing or suppressing odor responses within MePD depending on the sexual relevance of the odor stimuli. Instead, MeA appears to provide an important source of chemosensory input to MePD, enhancing responses to *either* opposite-sex or same-sex odors. MeA likely transmits sexual odor information to MePD directly, as many of the MeA cells that project to MePD also respond to opposite-sex or same-sex odors (Chapter 5).

One interpretation of these data is that MeA functions as a chemosensory relay, passively transmitting chemosensory information to MePD and other forebrain nuclei. This interpretation is consistent with the fact that MeA lesions in hamsters completely eliminate male copulatory

behavior (Lehman *et al.*, 1980), similar to deficits observed following removal of the olfactory bulbs themselves (Murphy & Schneider, 1970). Outside of the mating context, however, MeA lesions actually *increase* attraction to investigate same-sex odors (Maras & Petrulis, 2006), suggesting that MeA itself provides an important evaluative function during odor processing. Although the mechanisms of this evaluative function remain unclear, our results suggest that it does not involve suppression of odor responses within MePD.

Although MePD does not appear to regulate odor responses within MeA (Chapter 4), this nucleus may mediate the permissive effects of steroid hormones on attraction to sexual odors. As has been proposed by Cottingham and Pfaff (1986) and later by Wood (1998), hormones may act as a gating mechanism to enhance transmission of sexually relevant cues, and there are many examples of hormonal modulation of sensory processing in vertebrates (Remage-Healey *et al.*; Moffatt, 2003; Zakon, 2004; Doty & Cameron, 2009). Given that MePD processes both hormone signals (directly via hormone receptors) as well as chemosensory information (indirectly via projections from MeA), the MePD provides a likely target for the permissive effects of steroid hormones on odor processing (Kevetter & Winans, 1981a; Wood & Newman, 1995a; Wood, 1998). Indeed, either testosterone or estradiol implants directly into MePD are sufficient to generate attraction to investigate female odors in castrated male hamsters (Wood & Newman, 1995b) and rats (Baum *et al.*, 1982; Bialy & Sachs, 2002; Huddleston *et al.*, 2003), suggesting that hormones acting specifically within MePD are sufficient to modify behavioral responses to odor stimuli.

Gonadal hormones may act directly to shape odor responses of MePD neurons themselves. There is in fact substantial evidence that gonadal hormones alter the morphology of MePD neurons; gonad status and/or hormone treatment has been shown to affect somal area,

dendritic branching, and/or spine density of MePD neurons (Gomez & Newman, 1991; Rasia-Filho *et al.*, 1999; Cooke *et al.*, 2003; Cooke, 2006; de Castilhos *et al.*, 2008). Importantly, these morphological changes may translate into physiological changes in the response properties of MePD neurons (Cooke & Woolley, 2009), providing a mechanism for hormonal modulation of odor processing within MePD. In hamsters, exposure to female vaginal secretions (FVS) causes a significant increase in Fos expression within MePD of males, but not females (Fiber & Swann, 1996). Although this FVS-induced Fos expression in males does not require circulating testosterone, testosterone treatment in females masculinizes the response of MePD neurons to FVS (Fiber & Swann, 1996). Furthermore, in both male and female rats, testosterone treatment enhances Fos expression within MePD in response to female odors (Paredes *et al.*, 1998a). These studies provide initial evidence that gonadal hormones modulate odor responses within MePD, although an examination of the electrophysiological responses of MePD neurons under different hormonal conditions is needed to fully address this question.

In addition to acting directly within MePD, hormones may indirectly gate chemosensory processing via feedback projections from MePD to MeA. As originally proposed by Wood (1998), this model recognizes that the primary neural sites for processing chemosensory and hormone cues are in fact anatomically separate, and the integration of these signals likely involves reciprocal connections between hormone-sensitive and chemosensory-responsive subnuclei. Our work has identified direct projections from hormone-sensitive neurons within MePD to MeA (Chapter 5), providing anatomical support for this indirect action of steroid hormones. Although the functional significance of these projections remains to be tested, one possible experiment would be to determine if blocking androgen and/or estrogen receptors within MePD alters odor responses within MeA. Importantly, these direct and indirect models of hormone-

sensory interaction are not mutually exclusive, but may instead provide mechanisms for the recurrent modification of social odor information within MA (Wood, 1997).

An extended circuit for generating attraction to sexual odors

As mentioned above, the anatomical separation of chemosensory and hormone cues observed within MA is maintained throughout much of the extended forebrain circuit (Wood, 1997; Coolen & Wood, 1998). The posterior medial zone of the BNST (BNSTpm), medial subdivisions of MPOA (MPOAm), and ventrolateral regions of VMH (VMHvl) are all characterized by dense concentrations of steroid receptor-containing neurons (Simerly *et al.*, 1990; Li *et al.*, 1993; Wood & Newman, 1993) and are preferentially connected to MePD (Gomez & Newman, 1992; Canteras *et al.*, 1995; Coolen & Wood, 1998). In contrast, the posterior intermediate zone of the BNST (BNSTpi), lateral subdivisions of the MPOA (MPOAl), and dorsomedial VMH (VMHdm) express only modest concentrations of steroid receptors (Simerly *et al.*, 1990; Li *et al.*, 1993; Wood & Newman, 1993) and are preferentially connected with the MeA (Gomez & Newman, 1992; Canteras *et al.*, 1995; Coolen & Wood, 1998). Although the functional significance of this separation outside of the MA remains unclear, lesion studies suggest that the expression of sexual preferences requires intact processing at all levels of this circuit; male rats (Paredes *et al.*, 1998b) and ferrets (Paredes & Baum, 1995) within lesions of the MPOA, including both chemosensory and steroid-sensitive regions, display reversed partner preferences.

Given the limited existing knowledge regarding the nature of the connections between MA and downstream nuclei, we can only speculate on how this circuit generates the final behavioral response to sexual odors. Although the MA contains many GABAergic neurons

(Mugnaini & Oertel, 1985; McDonald & Augustine, 1993; Stefanova, 1998), populations of glutamatergic neurons have also been identified within this nucleus (Simmons & Yahr, 2003; Choi *et al.*, 2005; Bian *et al.*, 2008), and both inhibitory and excitatory systems may be critical for transmitting odor information (Choi *et al.*, 2005; Donato *et al.*, 2010). Furthermore, there are significant differences between MeA and MePD in their projections to downstream nuclei; although both MeA and MePD project to nuclei that promote reproductive behavior, MeA also projects to nuclei that generally promote aggressive or defensive behaviors (Figure 6.3) (Gomez & Newman, 1992; Canteras *et al.*, 1995; Swanson, 2000).

One simple model for generating appropriate mating behavior would be that the “reproductive” and “non-reproductive” downstream nuclei are reciprocally inhibitory, and the final behavioral response to different social odors depends on the relative activity of these nuclei (Figure 6.3). The MeA may differentially regulate the activity of reproductive and non-reproductive nuclei depending on the category of the social odor, whereas the MePD may provide an additional gain on the system via its projections to hormone-sensitive sub-regions of the reproductive circuit. Thus, when sexually relevant (opposite-sex conspecific) odors are detected (Figure 6.3A), MeA excites reproductive nuclei, but inhibits non-reproductive nuclei. Sexually relevant odors are also processed by MePD, which may subsequently enhance responses within reproductive nuclei. The reciprocal excitatory connections between chemosensory and hormone-sensitive sub-regions within the reproductive circuit may further enhance processing of sexually relevant odors. This network would result in relatively more activation of the reproductive compared to non-reproductive nuclei when sexually relevant odors are processed, thereby increasing the bias toward the expression of mating behavior.

Sexually irrelevant (same-sex or heterospecific) odors also activate MeA, but in response to these stimuli, MeA would primarily excite non-reproductive nuclei (Figure 6.3B). The MeA may also inhibit reproductive nuclei in response to sexually irrelevant odors, although the fact that lesions of the MeA do not increase Fos expression within BNST/MPOA in response to same-sex odors (Chapter 4) suggests that MeA does not provide a substantial source of inhibition to these reproductive nuclei. The MePD displays low levels of activation in response to sexually irrelevant odors, although MePD projections may suppress reproductive nuclei in response to these odors. Thus, when sexually irrelevant odors are perceived, the relative activity of reproductive and non-reproductive nuclei would be shifted, decreasing the probability that mating behaviors would be displayed. Although this model is likely an oversimplified view of the circuits mediating reproductive behavior, it provides an initial framework to understand how the brain generates opposing behavioral responses to different categories of social stimuli in the environment and can be used to generate future experimental questions.

The role of the corticomedial amygdala in regulating other social behaviors

In addition to regulating male reproductive behavior, there is substantial evidence that the corticomedial amygdala, and in particular the MA, mediate many aspects of social behavior. Although few studies have directly tested the role of the PMCo in regulating non-reproductive social behaviors, the MA has been implicated in the regulation of female reproductive behaviors (Coopersmith *et al.*, 1996; Erskine & Hanrahan, 1997; Polston & Erskine, 2001), parental behaviors (Numan *et al.*, 1993; Keller *et al.*, 2004), aggressive behaviors (Koolhaas *et al.*, 1980; Luiten *et al.*, 1985; Potegal *et al.*, 1996a) defensive behaviors (Dielenberg & McGregor, 2001; Li *et al.*, 2004), and scent-marking behaviors (Takahashi & Gladstone, 1988; Petrulis & Johnston, 1999).

The MA can either facilitate or inhibit social behaviors

Depending on the particular type of social behavior, MA can either facilitate or inhibit behavioral output. For example, lesions of MA reduce or eliminate the expression of territorial flank marks and proceptive vaginal marks (Takahashi & Gladstone, 1988; Petrulis & Johnston, 1999), suggesting that MA generally facilitates scent-marking behaviors. In contrast, the MA has been shown to inhibit the expression of maternal behavior in nulliparous female rats; female rats that have not given birth do not display maternal behavior when presented with foster pups, but lesions of MA increase maternal responses in these females (Numan *et al.*, 1993; Sheehan *et al.*, 2001). Nulliparous females treat pups (and their odors) as aversive, and MA appears to be involved in processing the aversive quality of these stimuli (Sheehan *et al.*, 2000; Sheehan *et al.*, 2001).

For the regulation of aggressive behaviors, the stimulatory or inhibitory role of MA varies according to the type of aggression. Specifically, in cats, MA suppresses the expression of predatory attack, but stimulates the expression of defensive rage (Shaikh *et al.*, 1993; Han *et al.*, 1996b; a). These different behaviors represent mutually exclusive forms of feline aggression, and the MA differentially regulates the expression of both behaviors through the release of substance P into the VMH (Shaikh *et al.*, 1993; Han *et al.*, 1996b; a). The MA facilitates territorial aggression, as lesions of MA reduce (Koolhaas *et al.*, 1980; Potegal *et al.*, 1996a), and electrical stimulation of MA increases (Potegal *et al.*, 1996b), aggressive responses to intruders. The fact that MA either facilitates or inhibits social behavior depending on the category of odor stimuli received suggests that MA is critical for identifying the relevance and context of different social odors.

The MA is involved in neural plasticity

In addition to simply facilitating or inhibiting the expression of these behaviors, there is growing evidence that MA mediates experience-dependent changes in many social behaviors. Indeed, some of the earliest studies of the role of MA in regulating territorial aggression noted that the decreases in attack and chase behaviors following MA lesions are greater in males that have previous aggressive experience (Vochteloo & Koolhaas, 1987). Furthermore, lesions or inactivation of MA prevent the normal increases in submissive behaviors following social defeat (Bolhuis *et al.*, 1984; Markham & Huhman, 2008). A single social defeat encounter causes robust increases in Fos expression within MA, and many of these activated cells also express corticotropin releasing factor type 2 receptors (Fekete *et al.*, 2009), indicating a role for MA in the transduction of social stressors. Moreover, exposure to repeated social defeats have recently been shown to increase the expression of brain-derived neurotrophic factor (BDNF) within the MA (Fanous *et al.*), suggesting that MA may itself be a critical site for the neuroplasticity underlying experience-dependent changes in agonistic behavior.

Using various models of social recognition, several studies have also identified a critical role for MA in the formation of social memories. For example, the MA is required for the formation of maternal olfactory memories in ewes; ewes that had lidocaine infused into MA during the period when maternal memories are formed subsequently fail to recognize the odor of their own lambs (Keller *et al.*, 2004). Using a similar model of maternal memory in rats, work by Nephew and Bridges (2008) suggests that the formation of maternal memories requires activation of vasopressin receptors specifically within MA. The MA also appears to be critical for the ability to recognize familiar vs. unfamiliar individuals in rats and mice, and this form of

social recognition has been shown to involve both oxytocin and estrogen receptors within MA (Spiteri *et al.*; Ferguson *et al.*, 2001; Choleris *et al.*, 2007).

Finally, several studies by Erskine and colleagues demonstrate that plasticity within MA is critical for encoding a short-term memory for the pattern of vaginocervical stimulation (VCS) that a female rat receives during mating. The total amount and temporal pattern of VCS received by females during mating is critical for initiating the prolactin surges associated with early pregnancy or pseudopregnancy (PSP) (Erskine *et al.*, 2004). Female rats control this temporal pattern by actively pacing the reception of intromissions from the male, and pacing behavior therefore involves generating a short-term memory for the pattern and accumulation of VCS received (Paredes & Vazquez, 1999; Erskine *et al.*, 2004). The MA, in particular the MePD, seems to encode the quality of VCS; higher levels of Fos expression are observed within the MePD of female rats following paced compared to non-paced mating encounters (Erskine & Hanrahan, 1997), and the levels of Fos expression within MePD correlate with the total number of intromissions received by the female during mating (Polston & Erskine, 1995). The MePD is in fact critical for the initiation of PSP, as lidocaine infusions into MePD completely block the induction of PSP following mating (Coopersmith *et al.*, 1996). Furthermore, the activation of NMDA receptors within MePD is both necessary and sufficient for the induction of PSP following artificial VCS (Polston *et al.*, 2001), suggesting that NMDA-dependent mechanisms of neuronal plasticity mediate the transduction of VCS within MePD. Importantly, protein synthesis inhibitors within MePD do not block PSP induction (Polston *et al.*, 2001), indicating that the long-term storage of the VCS memory does not occur within MePD, but may instead occur within downstream targets of MePD (Polston *et al.*, 2001; Lehmann & Erskine, 2005). Together, these studies demonstrate that MePD is critical for the transduction of VCS during mating, and

more broadly support the role of the MA in processing salient aspects of sensory information associated with neural plasticity.

The MA may recognize salience in the environment

Given that MA regulates so many aspects of social behavior, what can we infer about the underlying function of this nucleus? The substantial chemosensory inputs to MA indicate a primary role in processing and transmitting social odor information, and a wealth of immediate early gene studies clearly supports this concept (Fiber *et al.*, 1993; Coolen *et al.*, 1997; Dielenberg *et al.*, 2001; Day *et al.*, 2004; Meredith & Westberry, 2004; Choi *et al.*, 2005; Kiyokawa *et al.*, 2005). Beyond relaying chemosensory information, however, the MA appears to have a more complicated role in directing social behavior and mediating behavioral plasticity. Furthermore, the fact that MA neurons can be activated by stimuli or behaviors that are neither olfactory nor social indicates that MA may play a much broader role in regulating behavior than originally thought. In fact, MA neurons display increases in immediate early gene expression following fear conditioning (Pezzone *et al.*, 1992; Knapska *et al.*, 2006), avoidance training (Duncan *et al.*, 1996; Savonenko *et al.*, 1999), or even exposure to elevated plus maze (Silveira *et al.*, 1993; Savonenko *et al.*, 1999).

The extensive anatomical connections of MA are consistent with a broad function for this nucleus (Figure 6.3). In addition to the well-known chemosensory inputs to MA (Scalia & Winans, 1975; Pro-Sistiaga *et al.*, 2007), MA also receives multimodal sensory information from thalamic and lower brainstem relays (Ottersen & Ben-Ari, 1979; Ottersen, 1981; Coolen & Wood, 1998). The MA is also characterized by its extensive reciprocal connections with several basal forebrain and hypothalamic nuclei (Simerly & Swanson, 1986; Canteras *et al.*, 1994;

Risold *et al.*, 1994; Canteras *et al.*, 1995; Dong *et al.*, 2001; Dong & Swanson, 2004). Thus, the MA is anatomically situated to integrate diverse exteroceptive and interoceptive sensory inputs with autonomic and endocrine mediators of a variety of behavioral systems. The MA also has reciprocal connections with brain areas known to function in learning and memory, including the ventral subiculum of the hippocampus and basolateral and lateral nuclei of the amygdala (Canteras & Swanson, 1992; Canteras *et al.*, 1995; Coolen & Wood, 1998; Swanson & Petrovich, 1998), and these connections provide a substrate by which MA can mediate experience-dependent changes in social behavior. Finally, the MA sends modest projections to several nuclei within the ventral striatopallidal complex, including the ventral pallidum, nucleus accumbens (shell), and olfactory tubercle (Canteras *et al.*, 1995; Coolen & Wood, 1998), indicating that MA may interact with reward circuits of the brain to direct motivated behaviors (Smith *et al.*, 2009).

Given that such a variety of stimuli can activate MA, one possible interpretation of these data is that MA processes any novel stimulus in the environment and increases general arousal toward these stimuli. There is in fact evidence that MeA neurons specifically respond to novelty (Day *et al.*, 2001; Knapska *et al.*, 2007) and Fos expression within MA has been shown to habituate to repeated stimulus presentations (Juhila *et al.*, 2003). Other studies, however, report little to no activation of MA when novel stimuli are clearly presented (Milanovic *et al.*, 1998; Radulovic *et al.*, 1998; Rosen *et al.*, 1998; Delville *et al.*, 2000; Silveira *et al.*, 2001; Knapska *et al.*, 2007), rejecting the idea that novelty alone drives activation of MA neurons.

An alternative interpretation of these data is that MA functions to recognize highly salient sensory stimuli in the environment and subsequently recruit the activation of other brain areas (Figure 6.3) to direct appropriate behavioral responses to these stimuli. Depending on the context

in which the stimuli are presented (social or non-social, reproductive, aggressive, etc.), MA likely recruits distinct, but overlapping networks to regulate the final output of behavior. Indeed, Sarah Newman (1999) proposed that the MA functions as a critical node for integrating sensory inputs with an extended forebrain circuit for the control of rodent social behavior. The final output of behavior reflects the relative activity within, and strength of connections between, the different nuclei in this circuit. The current review of the literature supports this concept and further indicates that the function of MA extends beyond purely social behaviors to regulate the expression of a variety of motivated behaviors.

What is the role of the PMCo in regulating social behaviors?

Few studies have directly tested the role of the PMCo in mediating non-reproductive behaviors, making it difficult to define a role for this nucleus in regulating other social behaviors. The substantial chemosensory inputs to PMCo (Scalia & Winans, 1975; Pro-Sistiaga *et al.*, 2007) suggest that, similar to MA, this nucleus functions primarily to process social odors. Consistent with this role, the PMCo is required for the formation of olfactory memories in ewes (Keller *et al.*, 2004). Given the reciprocal connections between the PMCo and ventral subiculum (Kevetter & Winans, 1981a; Canteras *et al.*, 1992; Petrovich *et al.*, 2001), these data suggest that the PMCo may provide critical chemosensory input to the hippocampus during olfactory learning processes. Importantly, the PMCo has relatively limited connections with other brain areas and, in particular, has few direct connections with hypothalamic nuclei (Ottersen, 1980; Kevetter & Winans, 1981a; Ottersen, 1981; Canteras *et al.*, 1992). Thus, rather than driving the output of specific social behaviors, the PMCo may provide additional modification of chemosensory processing and modulate behavioral output via interactions with the BNST and MA (Kevetter & Winans, 1981a; Canteras *et al.*, 1992).

Structure and function of the amygdala in other species

The amygdala complex is highly conserved among tetrapods, sharing several critical features relating to embryological origin, anatomical connections, and apparent function of the different amygdala nuclei (Moreno & Gonzalez, 2006; 2007b). During development, the mammalian amygdala originates from both pallial and subpallial components, and this developmental organization is also observed in non-mammalian tetrapods, including reptiles, anurans and birds (Puelles *et al.*, 2000; Bachy *et al.*, 2002; Martinez-Garcia *et al.*, 2002; Moreno & Gonzalez, 2003; 2006). Furthermore, the main components of the amygdala have been identified in all tetrapods, although the mammalian amygdala contains additional “newer” nuclei not observed in non-mammalian species (Zeier & Karten, 1971; Price, 2003; Moreno & Gonzalez, 2007b). Although the classification of the various components of the amygdala complex remain under debate (Swanson & Petrovich, 1998; Swanson, 2003), the available evidence is consistent with a highly conserved organization and suggests that the precursors to the mammalian amygdala are present in ancestral anamniotes (Moreno & Gonzalez, 2007b).

Conserved function of the amygdala

Several characteristics of the amygdala are common among tetrapods and therefore suggest a conserved function for this structure in regulating social and/or reproductive behaviors. First, substantial olfactory inputs are a core feature of the amygdala in most tetrapods (Martinez-Garcia *et al.*, 1991; Lanuza & Halpern, 1998; Moreno *et al.*, 2005; Moreno & Gonzalez, 2007b; a), highlighting the role of the amygdala in mediating chemosensory communication. Although a vomeronasal system has not been identified in birds (Balthazart & Taziaux, 2009), the putative avian homolog to the mammalian amygdala complex, the archistriatum, does receive projections

from the main olfactory bulbs (Zeier & Karten, 1971; Cheng *et al.*, 1999). Second, the amygdala of all tetrapods is characterized by strong connections with endocrine and autonomic nuclei of the hypothalamus (Zeier & Karten, 1971; Bruce & Neary, 1995; Lanuza *et al.*, 1997; Cheng *et al.*, 1999; Martinez-Marcos *et al.*, 1999; Price, 2003; Moreno & Gonzalez, 2005). These connections represent a highly conserved fiber pathway (Price, 2003; Moreno & Gonzalez, 2007b) and reflect the importance of amygdalo-hypothalamic connections in regulating the output of various motivated behaviors. Finally, steroid hormone receptors are expressed within specific sub-regions of the amygdala in all tetrapods studied to date, including reptiles, birds, and anurans (Balthazart *et al.*, 1989; Balthazart *et al.*, 1998; Rosen *et al.*, 2002; Guerriero *et al.*, 2005). The hormonal sensitivity of the amygdala across tetrapods indicates that this structure mediates hormone-dependent behaviors in species with divergent mechanisms of sexual determination and differentiation.

Only a limited number of studies have tested the effects of amygdala lesions in non-mammalian species. In reptiles, the dorsal ventricular ridge (DVR) corresponds to the mammalian amygdala complex (Striedter, 1997), and lesions of this structure decrease aggressive behavior in crocodiles (Keating *et al.*, 1970) and iguanid lizards (Tarr, 1977). As mentioned above, the archistriatum represents the avian homolog to the mammalian amygdala, and the nucleus taeniae (TnA) has been identified as a possible homolog specifically to the MA (Thompson *et al.*, 1998; Cheng *et al.*, 1999). Similar to the mammalian MA, the TnA expresses high levels of androgen and estrogen receptors (Balthazart *et al.*, 1989; Balthazart *et al.*, 1998) and projects to the preoptic area and hypothalamus (Thompson *et al.*, 1998; Cheng *et al.*, 1999). Furthermore, lesion studies in quail provide evidence for a functional homology between the TnA and MA; male quail with lesions of the caudal TnA spend less time in the proximity of a

female and are slower to initiate mating compared to control males, indicating a decrease in sexual motivation or arousal (Thompson *et al.*, 1998). When lesions are restricted to the rostral portion of the TnA, however, males show normal levels of sexual motivation but display increased copulatory behaviors compared to control males (Absil *et al.*, 2002). The authors interpret these increases in copulatory behavior to reflect a failure to reach sexual satiety in lesioned males, a pattern that is similar to the deficits following lesions of the caudal MePD in male hamsters (Parfitt *et al.*, 1996). Although the exact homology between the distinct subregions of the TnA and MA remain unclear, these studies suggest that the functional heterogeneity observed within the mammalian MA (Newman, 1999) may reflect a conserved property of this nucleus.

Amygdala function in primates

There is substantial evidence that the amygdala mediates social and emotional processing in primates (Kling, 1992). Indeed, early lesion studies in nonhuman primates report decreases in aggression and emotionality as well as increases in sexual behaviors following large lesions of the temporal lobes (Kling, 1992). Results from subsequent lesion studies indicate that many of the changes in social behavior associated with these lesions were due to damage of the amygdala, although the exact pattern of the behavioral deficits often depends on the sex and age of the subjects, as well as the size and complexity of the social group in which the subjects are tested (Emery *et al.*, 2001; Machado & Bachevalier, 2007; Machado *et al.*, 2008). One consistent finding from these studies is that lesions of the amygdala are associated with a reduction in the responses to threatening or fearful stimuli. Thus, compared to controls, male rhesus monkeys with bilateral neurotoxic lesions of the amygdala behave less anxiously in response to novel social interactions, are overly (and inappropriately) affiliative toward novel animals, and respond

less to threats and challenge displays by other males (Emery *et al.*, 2001; Machado & Bachevalier, 2007; Machado *et al.*, 2008). These studies indicate that, similar to the function of the amygdala in other mammalian species, the amygdala in primates is critical for recognizing social signals in the environment and generating the appropriate behavioral response to these stimuli.

The available evidence in humans supports a role for the amygdala in processing salient stimuli. In fact, a wealth of functional imaging studies report increases in amygdala activation in response to a variety of emotional or arousing stimuli, and amygdala activation is particularly sensitive to stimuli with an aversive or negative quality (Zald, 2003). The amygdala also responds to viewing pictures of human faces, and in general, presentations of fearful faces activate the amygdala more reliably than do presentations of happy, sad or neutral faces (Adolphs, 2003; 2008; Fusar-Poli *et al.*, 2009). The role of the amygdala in processing facial stimuli is supported by the fact that patients with bilateral amygdala damage show deficits in their ability to identify and match emotions from static pictures of faces (Adolphs *et al.*, 1994; Young *et al.*, 1995; Adolphs, 2003). Amygdala damage appears to particularly affect recognition of fearful emotions in faces, although there is considerable variability across subjects (Adolphs *et al.*, 1995; Adolphs *et al.*, 2005; Adolphs, 2008). Furthermore, amygdala damage also impairs the ability to recognize complex mental states (guilt, admiration, flirtatiousness) or make accurate character judgments (trustworthiness) from pictures of faces (Adolphs *et al.*, 1998; Adolphs *et al.*, 2002), indicating a more complicated function than just recognizing basic emotions. Interestingly, individuals with amygdala damage rate even highly negative faces as approachable and trustworthy, suggesting that amygdala damage has disrupted their ability to recognize salient features of social signals within the face. Moreover, these specific impairments are similar to the

deficits observed in monkeys with bilateral lesions of the amygdala (Emery *et al.*, 2001; Machado & Bachevalier, 2007; Machado *et al.*, 2008) and thus provide further support for the role of the amygdala in processing salient social signals.

The amygdala theory of autism

Autism spectrum disorders (ASD) are characterized in part by severe impairments in social interactions (Reading, 2008; Gepner & Feron, 2009). Although many brain areas have been implicated in the development of ASD (Amaral *et al.*, 2008), a growing body of evidence suggests that dysfunction of the amygdala may underlie the deficits in social processing associated with the disorder (Baron-Cohen *et al.*, 2000). Much of this evidence comes from the fact that damage to the amygdala is associated with changes in social function in human and nonhuman primates (described above). In fact, individuals with autism often display deficits in recognizing emotions from facial expressions (Ashwin *et al.*, 2006; Jemel *et al.*, 2006; Hadjikhani *et al.*, 2007; Kleinhans *et al.*, 2009) that are similar to the deficits observed in individuals with overt amygdala damage (Adolphs, 2003). The mechanisms of these deficits remain unclear; different studies report increases, decreases, or no changes in amygdala activation in response to viewing faces (Jemel *et al.*, 2006). One possible mechanism is suggested by a recent study that looked at activation of the amygdala over repeated presentations of facial stimuli (Kleinhans *et al.*, 2009). Specifically, the authors found that in addition to increased amygdala activation in response to the initial presentations of facial stimuli, the amygdala of autistic individuals fails to habituate to repeated facial presentations. Furthermore, the degree of hyperactivity of the amygdala is correlated with behavioral deficits, such that individuals with the least habituation of the amygdala have the worst social deficits. These

results suggest that dysfunction in a basic sensory learning process may underlie some of the deficits associated with ASD, although more research is needed to confirm these findings.

These data indicate that the amygdala may be a primary site for the neuropathology of ASD, and several studies have found structural differences in the amygdala between autistic and non-autistic individuals. Early in childhood (2 – 5 years of age), the amygdala of autistic children is enlarged 13 – 16 % compared to age-matched controls, and amygdala volume is negatively correlated with verbal and social function in autistic children (Sparks *et al.*, 2002; Schumann *et al.*, 2009). This increase in amygdala volume is observed late into childhood (8 – 12 years), but is not apparent in adolescents (13 – 18 years) with autism (Schumann *et al.*, 2004). Thus, the increase in amygdala volume that normally occurs around puberty is absent in individuals with autism (Amaral *et al.*, 2008). Furthermore, data from postmortem analysis of the amygdala suggest that autism is associated with fewer amygdala neurons, and this difference is primarily due to decreases in the number of neurons specifically within the lateral nuclei (Schumann & Amaral, 2006). Together, these studies indicate that the neuropathology of autism is associated with a premature overgrowth of the amygdala early in development, but a subsequent loss of neurons later in life.

Summary

Several lines of evidence suggest that the amygdala has a conserved function for recognizing socially relevant stimuli in the environment and generating appropriate behavioral responses to these stimuli. In rodents, the corticomedial amygdala has a particularly critical role for processing sexual odors and generating reproductive behaviors. The results presented in this dissertation identify the critical substrates for odor processing within the corticomedial amygdala

and suggest that the medial amygdala in particular functions to integrate sensory and hormone information.

Chapter 6 Figures

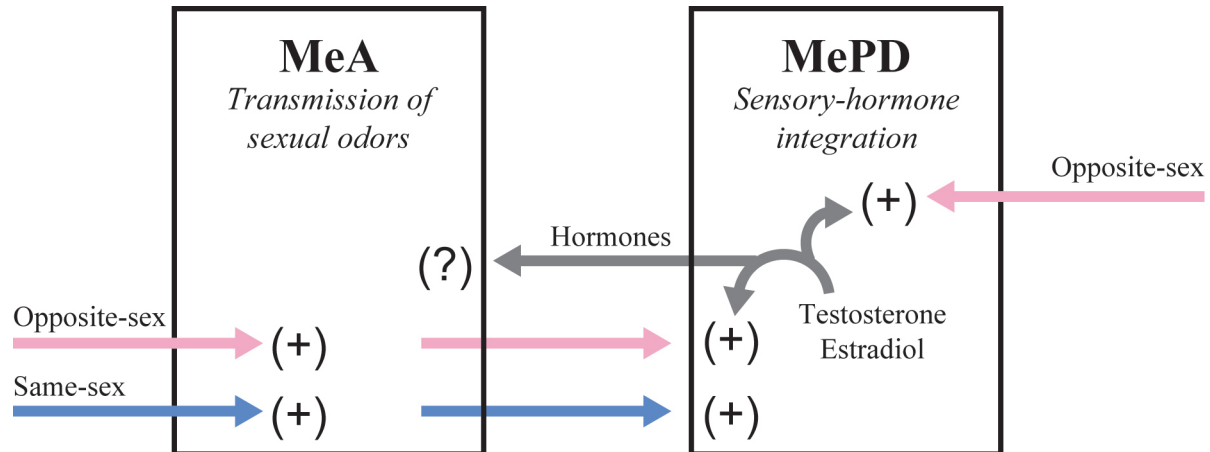


Figure 6.1 Updated model of MeA-MePD interaction during social odor processing. Our results suggest that social odors are transmitted primarily from MeA to MePD, and not vice versa. Furthermore, MeA provides an important source of chemosensory input to MePD, normally increasing responses to either opposite-sex or same-sex odors within MePD. Hormone-sensitive cells within MePD project directly to MeA, although the functional significance of these projections is unclear. Pink and blue lines represent opposite-sex and same-sex odors, respectively. (+) enhancement or (-) suppression of odor responses.

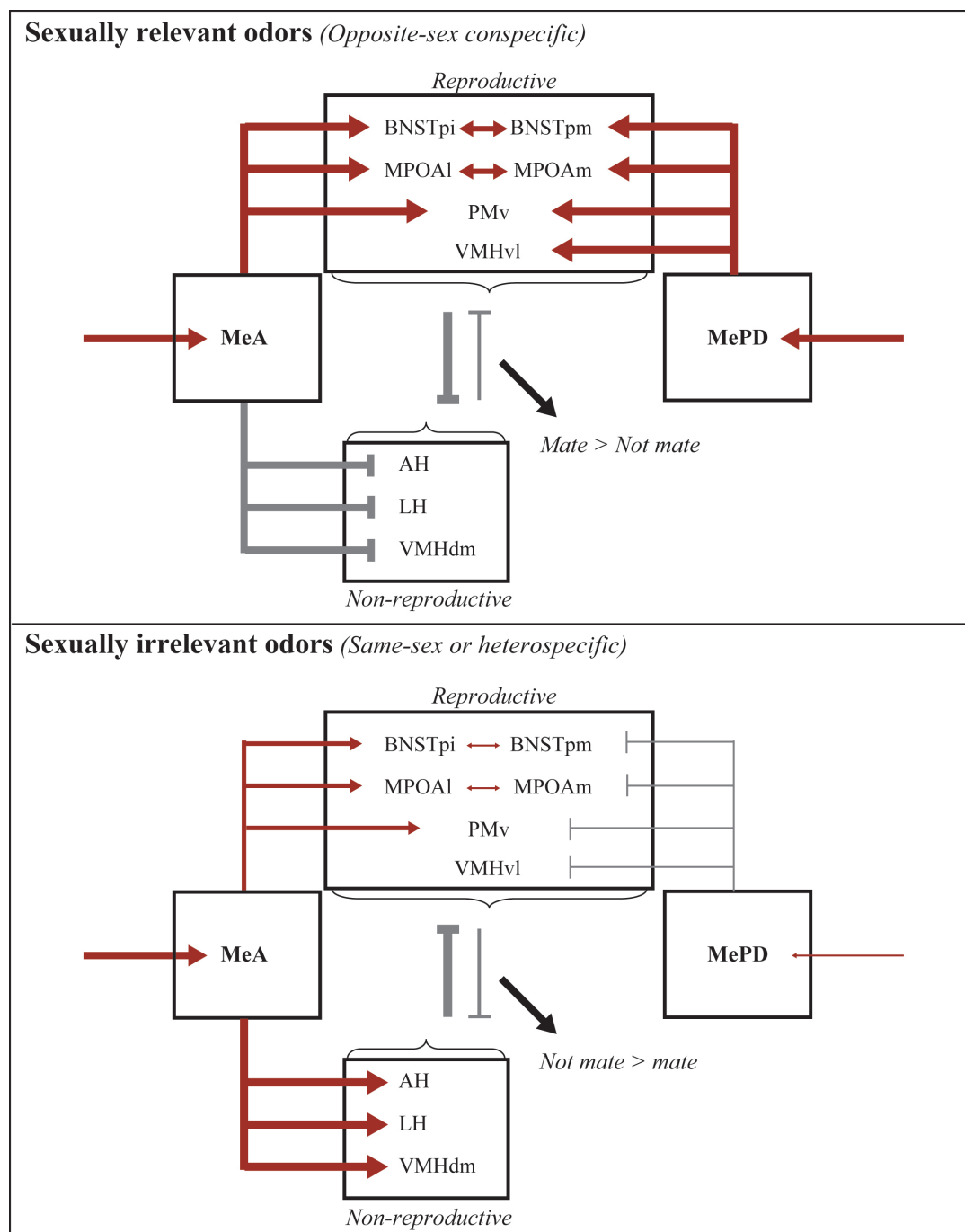


Figure 6.2 Proposed model for the regulation of reproductive behaviors in response to different categories of social odors. Both MeA and MePD project to downstream nuclei that promote reproductive behaviors, although MeA also projects to nuclei that promote non-reproductive (aggression, defensive) behaviors. We propose a simple model in which different categories of social odors processed via the MeA and MePD differentially excite (red lines) or inhibit (gray lines) these downstream nuclei. The final behavioral response to a social odor would depend on the relative activity of reproductive and non-reproductive nuclei. The thickness of the lines represents the relative strength of excitatory or inhibitory input.

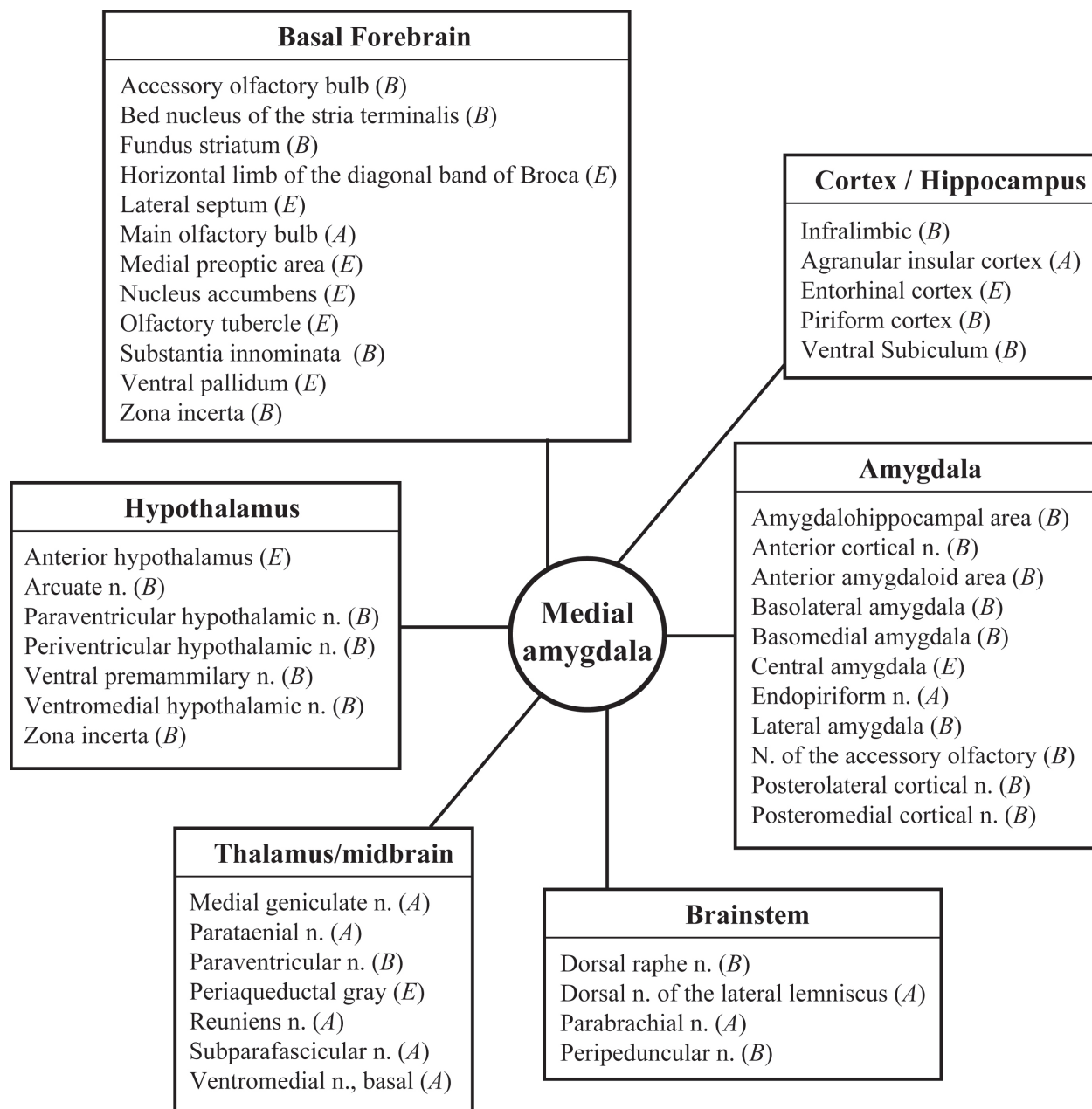


Figure 6.3 Summary of major afferent and efferent connections of the medial amygdala. The medial amygdala has extensive connections throughout the brain. (*A*) primarily afferent connections; (*E*) primarily efferent connections; (*B*) bilateral connections.

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APPENDIX: Curriculum Vitae

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MAJOR: Neuropsychology and Behavioral Neuroscience

INSTITUTION: Georgia State University, Atlanta, GA (2003-2005)

AWARDS:

Outstanding Young Investigator Award, Society for Behavioral Neuroendocrinology (2010)

Hewitt Foundation Postdoctoral Fellowship (2010)

Poster Presentation Award, Atlanta Chapter Society for Neuroscience (2009)

Dissertation Award, Georgia State University (2009)

Best Graduate Student, Center for Behavioral Neuroscience (2006)

Poster Presentation Award, Atlanta Chapter Society for Neuroscience (2005)

Magna Cum Laude graduate, Florida State University (2003)

Florida Bright Futures Academic Scholarship (1999-2003)

Dunedin Elks Club Academic Scholarship (1999-2000)

PROFESSIONAL ORGANIZATIONS:

Center for Behavioral Neuroscience, Atlanta, GA

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Society for Neuroscience

Golden Key National Honor Society

National Society Collegiate Scholars

PUBLICATIONS:

PEER-REVIEWED PRIMARY RESEARCH PAPERS

1. Maras, P.M. and Petrulis, A. Chemosensory and steroid-responsive regions of the medial amygdala regulate distinct aspects of opposite-sex odor preference in male Syrian hamsters. *European Journal of Neuroscience*, 24(12):3541-3552, 2006.
2. Eidson, L., Maras, P.M., Epperson, E. & Petrulis, A. Female hamster preference for odors is not regulated by circulating gonadal hormones. *Physiology and Behavior*, 91(1):134-141, 2007.
3. Maras, P.M. and Petrulis, A. Olfactory experience and the development of odor preference and vaginal marking in female Syrian hamsters. *Physiology and Behavior*, 94(4): 545-551, 2008.
4. Maras, P.M. and Petrulis, A. The posteromedial cortical amygdala regulates copulatory behavior, but not sexual odor preference, in the male Syrian hamster (*Mesocricetus auratus*). *Neuroscience*, 156: 425-435, 2008.
5. Maras, P.M. and Petrulis, A. Lesions that functionally disconnect the anterior and posterodorsal sub-regions of the medial amygdala eliminate opposite-sex odor preference in male Syrian hamsters (*Mesocricetus auratus*). *Neuroscience* 165: 1052–1062, 2010.
6. Maras, P.M. and Petrulis, A. The anterior medial amygdala transmits sexual odor information to the posterior medial amygdala and related forebrain nuclei. In press, *European Journal of Neuroscience*. April, 2010.

INVITED PEER-REVIEWED BOOK CHAPTERS

1. Maras, P.M. and Petrulis, A. The role of early olfactory experience in the development of adult odor preferences in rodents. In J.L. Hurst, R.J. Beynon, S.C. Roberts and T. Wyatt (Eds.), *Chemical Signals in Vertebrates 11*, Springer Press, 2007: 251-260.

PRESENTATIONS:

POSTERS

1. Maras, P.M. and Petrulis, A. The posterodorsal region of the medial amygdala regulates sex specific odor preferences in male hamsters. Society for Behavioral Neuroendocrinology, Austin, TX, 2005.
2. Maras, P.M. and Petrulis, A. Parallel processing of chemosensory and steroidal cues in the medial amygdala regulates opposite-sex odor preferences and attraction in male Syrian hamsters (*Mesocricetus auratus*). Society for Neuroscience, Washington D.C., 2005.
3. Maras, P.M. and Petrulis, A. Chemosensory experience and opposite-sex odor preference in female Syrian hamsters. Society for Behavioral Neuroendocrinology, Pittsburgh, PA, 2006.
4. Maras, P.M. and Petrulis, A. The role of early chemosensory experience in the expression of odor preference and vaginal marking in female Syrian hamsters. Chemical Signals in Vertebrates, Chester, England, 2006.
5. Maras, P.M. and Petrulis, A. The role of the posteromedial cortical nucleus of the amygdala in generating opposite-sex odor preference and copulatory behavior in male Syrian hamsters. Society for Neuroscience, Atlanta, GA, 2006.

6. Maras, P.M. and Petrulis, A. The role of the posteromedial cortical nucleus of the amygdala in generating opposite-sex odor preference and copulatory behavior in male Syrian hamsters. Society for Behavioral Neuroendocrinology, Asilomar, CA, 2007.
7. Maras, P.M. and Petrulis, A. Inhibitory neurons within the medial amygdala respond to same-sex odors in male Syrian hamsters. Society for Neuroscience, San Diego, CA, 2007.
8. Maras, P.M. and Petrulis, A. Functional interactions between sub-regions of the medial amygdala during the processing of sexual odors in male Syrian hamsters. Society for Behavioral Neuroendocrinology, Groningen, the Netherlands July 2008.
9. Maras, P.M. and Petrulis, A. Social odor processing within sub-regions of the medial amygdala: functional interactions and chemical phenotype. Society for Neuroscience, Washington D.C., 2008.
10. Maras, P.M. and Petrulis, A. Excitotoxic lesions of the anterior medial amygdala reduce Fos expression within the posterodorsal medial amygdala in response to female or male odors. Society for Behavioral Neuroendocrinology, East Lansing MI, 2009.
11. Maras, P.M. and Petrulis, A. Lesions that disconnect the anterior and posterodorsal regions of the medial amygdala eliminate opposite-sex odor preferences in male Syrian hamsters. Society for Neuroscience, Chicago, IL, 2009.

INVITED TALKS

1. Keynote Address, Center for Behavioral Neuroscience Annual Retreat: "Social odor processing within the medial amygdala: Integration of chemosensory and steroid hormone cues". September 2009.

2. Neuroscience Institute's Brown Bag Lunch: "Social odor processing within the medial amygdala: Integration of chemosensory and steroid hormone cues". December 2009.

DEPARTMENTAL/UNIVERSITY SERVICE:

Department of Psychology

2006-2007 Program Representative, Graduate Association of Student Psychologists

2007-2008 Executive Committee, Graduate Association of Student Psychologists

Neuroscience Institute

2008-2009 Student organizer, Neuroscience Institute Brown Bag Lunch

Center for Behavioral Neuroscience

2004 Research Mentor, ION program

2005 Organizing Committee Member, Spring Symposium "Stress in Early Development"

2005, 2006 Career Panel Member/Judge, Brain Bee

2006 Organizing Committee Member, Spring Symposium "Brain Mechanisms of Reward and Reinforcement"

2006 Volunteer, Brain Balloon Project

2007 Research Mentor, Brain Program

2005-2008 Volunteer, Annual Neuroscience Expo

2008 Volunteer, Brain awareness month, Redan Middle School

2009 Volunteer, Morningside Elementary Family Science Night

TEACHING EXPERIENCE:

Undergraduate Courses

Animal Behavior Laboratory, teaching assistant (2003)

Neuroanatomy Laboratory, teaching assistant (2003)

Introduction to Drugs and Behavior, teaching assistant (2003-2004)

Leadership and Group Dynamics, teaching assistant (2004)

RESEARCH EXPERIENCE:

POSITION: Undergraduate Research assistant

SPONSOR: Frank Johnson

INSTITUTION: Florida State University (2000-2002)

FIELD: Neuroendocrinology of song learning in Zebra Finches

POSITION: Undergraduate Research assistant

SPONSOR: James Smith

INSTITUTION: Florida State University (2002-2003)

FIELD: Taste and ingestive behavior in rodents

POSITION: Undergraduate Research assistant

SPONSOR: Richard Hyson

INSTITUTION: Florida State University (2003)

FIELD: Development of avian auditory system

POSITION: Graduate Research assistant

SPONSOR: Aras Petrulis

INSTITUTION: Georgia State University (2003-present)

FIELD: Neurobiology of sexual behavior and attraction in rodents