Published in final edited form as:

Neuroscience. 2010 February 17; 165(4): 1052. doi:10.1016/j.neuroscience.2009.11.024.

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*)

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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.

Keywords

Reproductive behavior; Social behavior; Olfaction; Chemosensory; Sexual preference

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Section editor: Dr. Geoffrey Schoenbaum

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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 and 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 and Schneider, 1970; Rowe and 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 and 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 and Zucker, 1978; Powers et al., 1985), but also for male's attraction to investigate female odors (Steel, 1982; Powers and Bergondy, 1983; Powers et al., 1985; Petrulis and 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 and Johnston, 1999; Maras and Petrulis, 2006), rats (Kondo, 1992; Kondo et al., 1997; Stark et al., 1998), and gerbils (Heeb and 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 medial amygdala (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 and Winans, 1975; Kevetter and Winans, 1981b; Lehman and Winans, 1982; Coolen and Wood, 1998; Kang et al., 2009). Although the 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 and Winans, 1975; Kevetter and Winans, 1981b; Lehman and Winans, 1982; Coolen and 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 and Sheridan, 1981; Wood et al., 1992; Wood and 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 and 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 and 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 and 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 and Westberry, 2004; Kiyokawa et al., 2005; delBarco-Trillo et al., 2009; Samuelsen and 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 and Newman, 1992; Coolen and 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 and 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 and Westberry, 2004; Maras and Petrulis, 2006), (2) the connections between MeA and MePD are almost exclusively ipsilateral (Gomez and Newman, 1992; Coolen and Wood, 1998), and (3) unilateral lesions of MeA or MePD by themselves do not affect opposite-sex odor preferences in male hamsters (Maras and 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).

EXPERIMENTAL PROCEDURES

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 × 30 cm × 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 and Petrulis, 2006), while limiting collateral damage to nearby nuclei or major fiber tracts (Table 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 $\times$ 75 mm $\times$ 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 $\times$ 3 in $\times$ 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 × 10 in × 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 and 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 and Keverne, 2002).

The testing sequence consisted of four, 3-minute presentations of repeated odors (habituation) followed by a fifth, 3-minute presentation of a novel odor (dishabituation). Five-minute inter-trial intervals separated each odor presentation. Under these testing parameters, male hamsters consistently display a lack of habituation to repeated presentations of female odors (Maras and Petrulis, 2006), and so all subjects were tested using male odors as the habituation stimuli and female odors as the dishabituation stimuli. 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 and 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) $\times$ 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 and Wood, 2001); Figure 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 (see Figure 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 (ASYM $n = 4$; UNI $n = 2$). Importantly, damage to adjacent nuclei was minimal (Figure 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 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 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 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 Greenhouse-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 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 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 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 2B).

Odor discrimination

In the Non-Contact Discrimination test (Figure 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 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 and Winans, 1973) and eliminates the preference to investigate the volatile components of sexual odors in male and female mice (Keller et al., 2006b, 2006a). Compared to MeA, MePD receives relatively few projections from the MOS and therefore relies heavily on MeA to receive main olfactory input (Scalia and Winans, 1975; Kevetter and Winans, 1981b; Coolen and 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 and Winans, 1975; Kevetter and 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 and 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 and 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 and 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 and Winans, 1975; Kevetter and Winans, 1981a, 1981b; Lehman and Winans, 1982; Coolen and 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 and 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 and Newman, 1995), as well as exposure to many types of conspecific and heterospecific odors (Meredith and Westberry, 2004; Kiyokawa et al., 2005; delBarco-Trillo et al., 2009; Samuelsen and 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 and Winans, 1975; Kevetter and 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 and Sheridan, 1981; Wood et al., 1992; Wood and 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 and Bergondy, 1983; Bean et al., 1986; Ferkin and Gorman, 1992), and implants of testosterone or estradiol directly into MePD can facilitate many aspects of reproductive behavior in gonadectomized male hamsters (Wood and Newman, 1995; Wood, 1996) and rats (Bialy and 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 and 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 and 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 and Winans, 1975; Kevetter and Winans, 1981b; Coolen and 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 and Westberry, 2004; Kiyokawa et al., 2005; delBarco-Trillo et al., 2009; Samuelsen and 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 and 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 and 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 and Wood, 1998). This model suggests that MeA provides the primary output to BNST and/or MPOA (Gomez and 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 and 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 and Newman, 1992; Martel and 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 and 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 and Sheridan, 1981; Wood et al., 1992; Li et al., 1993) and are strongly linked to the MePD (Gomez and Newman, 1992; Coolen and 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 and Sheridan, 1981; Wood et al., 1992; Li et al., 1993) and are preferentially connected with the MeA (Gomez and Newman, 1992; Coolen and Wood, 1998). Given the reciprocal connections within MA, BNST, and MPOA (Simerly and Swanson, 1986, 1988; Coolen and Wood, 1998; Wood and 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 and Sheehan, 1997; Newman, 1999; Dielenberg and 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.

Glossary

List of Abbreviations

AOB	Accessory olfactory bulb
AOS	Accessory olfactory system
ASYM	Asymmetrical lesion group

BNST	Bed nucleus of the stria terminalis
MA	Medial amygdala
MeA	Medial amygdala, anterior region
MePD	Medial amygdala, posterodorsal region
MOS	Main olfactory system
MPOA	Medial preoptic area
SHAM	Sham surgery group
UNI	Unilateral lesion group

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Asymmetrical (ASYM)

Unilateral (UNI)

Figure 1.

Reconstruction of coronal sections 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 2.

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.

Figure 3.

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).

Table 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	6
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 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 $n = 15$).

	Total number midline crosses	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

* indicates significant differences between center container and both left and right containers using pairwise comparisons, $p < .05$, $\alpha_{FW} = .05$.