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The medial preoptic area is necessary for sexual odor preference, but not sexual solicitation, in female Syrian hamsters

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Abstract

Precopulatory behaviors that are preferentially directed towards opposite-sex conspecifics are critical for successful reproduction, particularly in species wherein the sexes live in isolation, such as Syrian hamsters (*Mesocricetus auratus*). In females, these behaviors include sexual odor preference and vaginal scent marking. The neural regulation of precopulatory behaviors is thought to involve a network of forebrain areas that includes the medial amygdala (MA), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA). Although MA and BNST are necessary for sexual odor preference and preferential vaginal marking to male odors, respectively, the role of MPOA in odor-guided female precopulatory behaviors is not well understood. To address this issue, female Syrian hamsters with bilateral, excitotoxic lesions of MPOA (MPOA-X) or sham lesions (SHAM) were tested for sexual odor investigation, scent marking, and lordosis. MPOA-X females did not investigate male odors more than female odors in an odor preference test, indicating that MPOA may be necessary for normal sexual odor preference in female hamsters. This loss of preference cannot be attributed to a sensory deficit, since MPOA-X females successfully discriminated male odors from female odors during an odor discrimination test. Surprisingly, no deficits in vaginal scent marking were observed in MPOA-X females, although these females did exhibit decreased overall levels of flank marking compared to SHAM females. Finally, all MPOA-X females exhibited lordosis appropriately. These results suggest that MPOA plays a critical role in the neural regulation of certain aspects of odor-guided precopulatory behaviors in female Syrian hamsters.

Keywords

Olfaction; Chemosensory; Proceptive; Sexual motivation; Appetitive

Introduction

Precopulatory behaviors that aid in the identification and localization of potential mating partners are an important component of reproductive behavior for most mammals (Beach, 1976). For species typified by non-cohabitating sexes such as Syrian hamsters (*Mesocricetus auratus*), these behaviors are essential for successful mating (Gattermann et al., 2001; Pfaff et al., 2008). Female Syrian hamsters engage in a number of different precopulatory or

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solicitational behaviors, including vaginal marking (a stereotyped scent marking behavior resulting in deposition of vaginal secretion) and preferential approach towards, and investigation of, opposite-sex odors (Petrulis, 2009). Although both vaginal marking and opposite-sex odor preference are behavioral responses that are preferentially directed towards male compared to female odors (Johnston, 1977; Martinez and Petrulis, 2011; Petrulis and Johnston, 1999; Petrulis et al., 1999), odor preference is more clearly linked to the identification and localization of potential mating partners, whereas vaginal marking plays a key role in attracting mates. Indeed, the deposited secretion is highly attractive to male hamsters (Johnston and Schmidt, 1979; Johnston, 1974; Kwan and Johnston, 1980), and females deposit these marks in such a way as to direct the male to her nesting area (Lisk et al., 1983).

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

Although there is substantial evidence suggesting MPOA is broadly involved in regulating sexual behavior in rodents (Hull and Dominguez, 2007; Sakuma, 2008), its specific role in odor-guided female precopulatory behaviors is not clear. In rats, radiofrequency lesions of MPOA decrease the amount of solicitational behaviors towards, and time spent with, a sexually-experienced male, and disrupt females' preference for intact compared to castrated male rat odors (Xiao et al., 2005). Furthermore, excitotoxic lesions of MPOA decrease the preference of female rats to spend time with intact males compared to estrous females (Guarraci and Clark, 2006). However, it should be noted that other researchers have found no effects of electrolytic lesions of MPOA on sexual odor/partner preference in female rats (Paredes et al., 1998) or ferrets (Robarts and Baum, 2007). Although comparable data for the role of MPOA in sexual odor preference in female hamsters is not available, this area does appear to be involved in other precopulatory behaviors that can be induced by opposite-sex odors. Indeed, large electrolytic lesions of MPOA that also damaged BNST decrease vaginal marking during interactions with males (Malsbury et al., 1977), and radiofrequency lesions of MPOA decrease ultrasonic vocalizations by females following exposure to male hamsters (Floody, 1989).

A significant limitation of previous studies examining the role of MPOA in odor-guided precopulatory behaviors is the lack of specificity in disrupting MPOA vs. nearby areas such as BNST. This is a critical issue given that these areas are highly interconnected and share

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

Materials and Methods

Overview of design

Subjects were initially screened for adequate levels of vaginal marking to male odors (> 5 marks/10 min), and then received either bilateral, excitotoxic lesions of MPOA or sham surgeries. Following recovery, subjects underwent a series of behavioral tests. First, subjects were tested for their investigatory responses to male and female odors (Odor investigation tests). This consisted of an initial test to familiarize subjects with the testing apparatus (Clean test), followed by a volatile odor preference test utilizing conspecific odor stimuli (Preference test). Second, subjects were tested for their ability to discriminate the sexual identity of odor stimuli using a habituation-discrimination task (Odor discrimination test). Subjects were then tested for scent-marking responses to male or female stimuli on two days of the estrous cycle, diestrous day 2 and proestrus. Finally, to verify that MPOA lesions had not disrupted the ability of females to display copulatory behavior, subjects were tested during behavioral estrus for receptive sexual responses to a sexually experienced male.

Subjects

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

Estrous cycle monitoring

Prior to screening for sufficient vaginal marking levels, subjects were examined daily for eight consecutive days in order to determine their stage of the estrous cycle. Subjects were

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

Surgery

At two to three months of age, subjects were assigned to either a MPOA lesion group (MPOA-X) or a sham lesion group (SHAM). A matched random assignment procedure was used, such that initial levels of vaginal marking in response to male odors on proestrus were equivalent across subjects assigned to the MPOA-X and SHAM groups (see below). Subjects were first anesthetized with 2–3% isoflurane gas (Baxter, Deerfield, IL) in an oxygen (70%) and nitrous oxide (30%) mixture, and then placed within a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with ear- and incisor-bars positioned such that the top of the skull was level. Following a midline scalp incision, the skin and underlying temporal muscles were retracted to expose the skull. A hand-operated drill was then used to make holes in the skull in order to expose dura. For MPOA-X subjects, the excitotoxin *N*-methyl-D-aspartic acid (20 mg/ml, 25 nl per injection site; Sigma, St. Louis, MO) was injected bilaterally via a Hamilton microinjection syringe (701R 10 µl syringe; Hamilton, Reno, NV) under stereotaxic control. A single injection of excitotoxin was made per hemisphere, using the following coordinates: Anterior-posterior, +1.7 mm (relative to bregma); medial-lateral, ±0.6 mm (relative to bregma); dorsal-ventral, −7.4 mm (relative to dura) using published anatomical plates of the Syrian hamster brain (Morin and Wood, 2001). The excitotoxin was expelled over the course of one minute, and the syringe needle was then left in place for an additional nine minutes to allow sufficient time for the injection volume to disperse from the syringe tip. Sham surgeries were conducted in a similar manner to lesion surgeries except that no liquid was infused into the target sites.

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

Scent-marking tests

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

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

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

Odor investigation tests

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

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

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

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

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

Odor discrimination test

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

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

Lordosis test

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

Histology and lesion verification

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

Immunohistochemistry

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

Data analysis

All data were analyzed using SPSS for Windows, version 18.0 (SPSS Inc, Chicago, IL). Data were first examined to determine if the assumptions of parametric statistical tests were met. When assumptions were violated, attempts were first made to normalize the distributions using applicable data transformations (Osborne and Overbay, 2004; Sheskin,

2000); however, for the duration of investigation of male/female odors in the odor preference test, these transformations were unsuccessful. In this case the deletion of an extreme outlier (> 3 SD above the mean) was necessary. For all statistical tests, results were considered to be statistically significant if $p < .05$.

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

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

Results

Lesion reconstruction

Subjects were included in the MPOA-X group ($n = 9$) if bilateral damage to approximately 60% of MPOA was observed on two consecutive plates of the Syrian hamster atlas (Figure 1) (Morin and Wood, 2001). Although some MPOA-X females sustained bilateral damage at more anterior levels of MPOA (0.5 mm anterior to bregma, $n = 4$), every subject in this group sustained substantial bilateral damage to MPOA at the level of the juncture of the lateral and third ventricles (0.2 mm anterior to bregma, $n = 9$). Furthermore, most MPOA-X females sustained bilateral damage at more posterior levels of MPOA (-0.1 mm posterior to bregma, $n = 7$). Finally, one MPOA-X subject had significant bilateral damage to the anterior hypothalamus (AH; bregma -0.3 mm, $n = 1$). It is important to note that subjects included in the MPOA-X group also sustained minor damage to BNST (less than 25% bilaterally on any single atlas plate).

Subjects that received excitotoxin injections resulting in less than 60% bilateral damage to MPOA on at least two consecutive atlas plates were excluded from the MPOA-X group. In these females, MPOA damage was restricted to the same rostral-caudal extent as in MPOA-X females. Females with partial MPOA lesions were further divided into two groups based on the extent of concurrent BNST damage: Partial MPOA-X (less than 25% bilateral damage to BNST on any atlas plate; $n = 19$), and Partial BNST/MPOA-X (greater than 25% bilateral damage to BNST on any atlas plate; $n = 18$). In order to determine if this concurrent damage to BNST (in the absence of more substantial MPOA damage) was sufficient to drive behavioral effects, both partial lesion groups were directly compared to shams for all measures of odor investigation, scent marking and copulatory behavior. Neither Partial MPOA-X nor Partial BNST/MPOA-X females differed from SHAMs on any measure (all $p > .05$); therefore, data from partial lesion groups were not included in any comparisons of

MPOA-X vs. SHAM females (Table 1). Although needle tracts were typically visible in the brains of SHAMs, no significant damage was observed to any brain areas in these females.

Odor investigation tests

Clean test—Females did not differentially investigate the left and right boxes when both boxes contained clean cage stimuli, $F(1,23) = 2.00$, $p = .17$, $\eta_p^2 = .08$, indicating that females were not biased in their investigation across the two outer boxes. Furthermore, there was no evidence to suggest that there were any differences in odor box investigation between the two lesion groups, as neither the main effect of lesion ($F(1,23) = 0.22$, $p = .64$, $\eta_p^2 = .01$) nor the odor box by lesion interaction ($F(1,23) = 0.59$, $p = .45$, $\eta_p^2 = .03$) were statistically significant. Finally, locomotor activity was equivalent across both lesion groups, $t(23) = 1.51$, $p = .14$, $d = .65$.

Odor preference test

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

Odor discrimination test

Females with lesions of MPOA were able to discriminate male odors from female odors in the odor discrimination test (Figure 3). Irrespective of lesion group, females differentially investigated the presented odors, $F(2,44) = 21.40$, $p < .001$, $\eta_p^2 = .49$. Specifically, females successfully habituated to repeated presentation of female odors, $t(23) = 3.72$, $p < .05$, $d = 1.01$, and discriminated female odors from male odors, $t(23) = -5.71$, $p < .05$, $d = 1.64$. The interaction of odor by lesion group was not significant, $F(2,44) = 1.24$, $p = .30$, $\eta_p^2 = .05$, indicating that the effect of odor was consistent across the two lesion groups.

Scent-marking tests

Vaginal marking—There were no significant effects of MPOA lesions on vaginal marking responses to male or female odors (Figure 4). Overall, females vaginal marked more on proestrus compared to diestrous day 2, $F(1,23) = 79.79$, $p < .001$, $\eta_p^2 = .78$, and more in response to male odors compared to female odors, $F(1,23) = 17.97$, $p < .001$, $\eta_p^2 = .44$. This effect of odor was not consistent across cycle days, $F(1,23) = 6.81$, $p = .02$, $\eta_p^2 = .23$. Specifically, females vaginal marked more to male odors compared to female odors only on proestrus, $t(24) = 4.73$, $p < .001$, $d = .95$, not diestrous day 2, $t(24) = 1.46$, $p = .15$, $d = .30$. Overall levels of vaginal marking did not differ across lesion groups, $F(1,23) = 1.77$, $p = .20$, $\eta_p^2 = .07$, and the effects of cycle day and odor condition on vaginal marking (as described above) did not differ across lesion groups (cycle day $\times$ lesion: $F(1,23) = .03$, $p = .87$, $\eta_p^2 = .001$; odor $\times$ lesion: $F(1,23) = 2.84$, $p = .11$, $\eta_p^2 = .11$).

Flank marking—Lesions of MPOA disrupted the expression of flank marking by females (Figure 5). Although females in both groups flank marked more in response to female odors compared to male odors, $F(1,23) = 22.90$, $p < .001$, $\eta_p^2 = .50$, the overall levels of flank

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

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

Discussion

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

Role of MPOA in sexual odor investigation

Our finding that females with MPOA lesions fail to investigate male odors more than female odors is in agreement with previous studies that have identified MPOA as a critical area in regulating appetitive sexual behavior in females. For example, lesions of MPOA in female rats decrease the expression of solicitational movements to a stimulus male (Hoshina et al., 1994; Whitney, 1986) and increase the latency to return to the male during paced mating tests (Guarraci et al., 2004). Furthermore, lesions of MPOA essentially eliminate the preference for intact vs. castrated male odors in female rats (Xiao et al., 2005), as well as the preference to spend time with intact males vs. estrous females when contact with stimulus animals was allowed (Guarraci and Clark, 2006). These data are not consistent, however, with findings of other studies that did not find an effect of MPOA lesions on partner preference involving direct contact with stimulus animals in female rats (Paredes et al., 1998), or in preference tests in female ferrets that either prevented or allowed direct contact with stimulus animals (Robarts and Baum, 2007). Given that the differences in the results of these studies cannot be easily attributed to the manner of stimulus presentation, the species tested, or the size and placement of MPOA lesions, it seems likely that MPOA plays a highly nuanced role in sexual odor preference in females. This stands in contrast to males, wherein MPOA has been found to be essential for the normal expression of sexual odor preference in several different species across multiple testing protocols (Been and Petrulis, 2010; Hurtazo and Paredes, 2005; Paredes and Baum, 1995; Paredes et al., 1998). It may be other hypothalamic nuclei play a role in female sexual odor preference that is directly comparable to that of MPOA in males; however, with the exception of the established role of the ventromedial hypothalamus in female ferret odor/partner preference (Robarts and Baum, 2007), these data are presently lacking.

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

The underlying neurochemical mechanisms that mediate the effects of MPOA on investigatory responses to sexual odors in females are not well understood. Neurons in MPOA express receptors for estradiol and progesterone (Du et al., 1996; Li et al., 1993), and gonadal steroids strongly regulate other precopulatory behaviors that are disrupted following MPOA lesions, such as ultrasonic vocalizations (Floody, 1989; Floody et al., 1979). Although there is evidence in rats to suggest that estrogen alone or estrogen plus progesterone treatment may enhance the preference for male stimuli in females (Clark et al., 2004; Xiao et al., 2005), this does not appear to be the case in either mice (Moncho-Bogani et al., 2004) or hamsters (Eidson et al., 2007). Indeed, female hamsters display a robust preference for male odors across the estrous cycle (Eidson et al., 2007; Martinez and Petrulis, 2011; Martinez et al., 2010) and following ovariectomy (Eidson et al., 2007). Therefore, it seems unlikely that either estradiol or progesterone acts within MPOA to critically regulate the expression of sexual odor preference in female hamsters.

Role of MPOA in scent marking

It was surprising to observe no deficit in vaginal marking in MPOA-X females. Previously, Malsbury et al. (1977) reported that females with large, electrolytic lesions of MPOA vaginal mark less in response to a stimulus male compared to sham-operated controls. There are several important differences between the two studies that may explain the differences in findings. First, the lesions of MPOA reported by Malsbury and colleagues were fairly large and appear to have substantially damaged BNST at anterior as well as posterior levels. We have previously reported that fairly discrete, excitotoxic lesions of BNST decrease vaginal marking to male odors (Martinez and Petrulis, 2011); this raises the possibility that the effects of MPOA lesions on vaginal marking reported by Malsbury and colleagues were mediated by BNST, not MPOA. Furthermore, the method whereby the MPOA was lesioned differed across the two studies, with the present study employing excitotoxic lesions and the study by Malsbury and colleagues employing electrolytic or radiofrequency lesions. Although all of these lesion types destroy cell bodies within the targeted area, electrolytic and radiofrequency lesions also result in damage to fibers of passage (Jarrard, 2002). Given that projection fibers from other brain areas such as the lateral septum pass through MPOA, and that disruption of these fibers has behavioral consequences that are independent of direct damage to neuronal cell bodies within MPOA (Hoshina et al., 1994), it may be that differences in lesion effects between the two studies are due to differential damage to fibers of passage. Finally, the present study also differs from that of Malsbury and colleagues in the manner whereby females were exposed to male stimuli. In the present study, scent-marking tests were conducted in vacated cages that had previously contained males, as females that are allowed to directly interact with males can engage in behaviors that are incompatible with the expression of vaginal marking, such as lordosis. Indeed, MPOA-lesioned females in the study by Malsbury et al. (1977) did express

lordosis to the male stimulus animal, thereby reducing the total amount of time those females were free to engage in locomotor-dependent activities such as vaginal marking.

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

In contrast to MPOA, BNST appears to be a critical component of the neural circuit regulating the odor-guided aspects of vaginal marking (Martinez and Petrulis, 2011). Given the present findings that MPOA is required for the normal expression of sexual odor preference, it would appear that there is a clear disassociation between the neural circuitry that regulates these two forms of odor-guided precopulatory behaviors in females. This dissociation may allow for these two behaviors to be differentially regulated by internal or external factors. Indeed, vaginal marking is disrupted following either ovariectomy or blockade of oxytocin receptors, whereas sexual odor preference is normally expressed despite these manipulations (Eidson et al., 2007; Martinez et al., 2010; Takahashi and Lisk, 1983).

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

Anatomical considerations

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

analyzed as a separate group (Partial BNST/MPOA-X), and these females did not differ significantly from SHAM females on any behavioral measure.

Conclusion

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

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Highlights

- Bilateral lesions of MPOA disrupt preferential sexual odor preference
- This effect is not due to a deficit in discriminating sexual odors
- Lesions of MPOA do not disrupt vaginal marking to sexual odors

Figure 1.

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

Figure 2. Mean ($\pm$ SEM) Duration of Odor Investigation (Odor Preference Test). In contrast to SHAM females, MPOA-X females failed to investigate male odors significantly more than female odors. # $p < .01$, duration of male odor investigation vs. female odor investigation within the SHAM lesion group.

Figure 3. Mean ($\pm$ SEM) Duration of Odor Investigation (Odor Discrimination Test). Females successfully habituated to repeated presentations of female odors and discriminated male odors from female odors. This response to odors did not differ across lesion groups.

Figure 4. Mean ($\pm$ SEM) Number of Vaginal Marks. Females vaginal marked more on (B) proestrus compared to (A) diestrous day 2, and more in response to male odors compared to female odors (only on proestrus). These effects did not differ across lesion groups.

Figure 5. Mean ($\pm$ SEM) Number of Flank Marks. Although both MPOA-X and SHAM females flank marked at higher levels to female compared to male odors on (A) diestrous day 2 and (B) proestrus, overall levels of flank marking were lower in MPOA-X females compared to SHAM females.

Table 1

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

	Odor Preference		Vaginal Marking		Flank Marking	
	Male	Female	Male	Female	Male	Female
Partial MPOA-X	84 ± 11	46 ± 10 *	9 ± 2	6 ± 2	13 ± 3	22 ± 3
Partial BNST-X/MPOA-X	88 ± 10	41 ± 5 #	5 ± 1	5 ± 1	6 ± 1	19 ± 4 #

Females with partial lesions that predominantly damaged MPOA (Partial MPOA-X) or damaged both BNST and MPOA (Partial BNST-X/MPOA-X) investigated male odors more than female odors in the odor preference test. Females with partial lesions also vaginal marked more to male odors compared to female odors on proestrus, but not diestrus day 2, and flank marked more to female odors compared to male odor on proestrus (both groups) and diestrus day 2 (Partial BNST-X/MPOA-X only). Data represented as mean ± standard error of the mean.

* = $p < .05$;

= $p < .01$ (responses to male vs. female odors within each partial lesion group, for each behavioral test).