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Blocking oxytocin receptors inhibits vaginal marking to male odors in female Syrian hamsters

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Abstract

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

Keywords

Medial preoptic area; Anterior hypothalamus; Bed nucleus of the stria terminalis; Olfaction; Preference; Reproduction; Precopulatory; Scent marking

1. Introduction

In female mammals, successful reproduction depends upon a suite of precopulatory behaviors that enhance the probability of locating and attracting a suitable mate [1,2].

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Precopulatory behaviors may be particularly important for reproductive success in species where adult members of the opposite sex live in isolation from each other, such as the Syrian hamster [3,4]. Vaginal marking is one form of precopulatory behavior in this species that serves to solicit potential mates [5,6]. This highly stereotyped behavior involves the female moving forward while maintaining contact between the perineum and the underlying substrate, thereby depositing vaginal secretion [4]. In agreement with its role as a precopulatory behavior, vaginal marks are highly attractive to male hamsters [7–9], and males may use vaginal marks to locate females for mating [6]. Furthermore, the expression of vaginal marking is critically dependent on internal hormonal state and external signals important for synchronizing mating activity [4]. Vaginal marking levels are highest on the reproductive cycle day of proestrus, and are completely absent on the following cycle day (estrus) when females are sexually receptive and will engage in mating [4,10]. Vaginal marking is also preferentially directed towards males, as females will vaginal mark at higher levels in response to male odors than in response to female odors or clean bedding [4,11–14].

Although the sensory stimuli and hormonal signals important for vaginal marking have been examined to a considerable extent [15], less is known about the brain areas and specific neurochemicals that may be important for regulating this behavior. One likely candidate is the neuropeptide oxytocin (OT) acting within the medial preoptic/anterior hypothalamus (MPOA-AH). OT is expressed in cell bodies and fibers within MPOA-AH [16], and large, bilateral electrolytic lesions of MPOA-AH inhibit vaginal marking [17]. Injection of OT into MPOA-AH of female hamsters [18] or rats [19] enhances the expression of lordosis, a reflexive copulatory posture assumed by females [20]. In contrast, blockade of oxytocin receptors (OTRs) in MPOA-AH via injection of a specific OTR antagonist (OTA) inhibits lordosis in both these species [18,21,22]. In addition to effects on lordosis, OT in MPOA-AH also increases the production of ultrasonic vocalizations [23], a precopulatory behavior that induces approach in males [24].

Taken together, the existing data suggest that OT in MPOA-AH may normally facilitate vaginal marking. To test this hypothesis, we injected proestrous female hamsters with OTA or vehicle into MPOA-AH and measured vaginal marking in response to stimuli present in male, female, and clean cages (Experiment 1). Given that vaginal marking is preferentially directed towards opposite-sex, rather than same-sex, stimuli [1], we predicted that OTA injections into MPOA-AH would decrease vaginal marking to male, but not to female or clean, cage odors. Following the results of Experiment 1, two subsequent experiments were conducted to assess the anatomical and functional specificity of OTA on vaginal marking. In Experiment 2, injections were made into either MPOA-AH or the bed nucleus of the stria terminalis (BNST), a brain area located immediately dorsal to MPOA-AH that contains both OT immunoreactivity and OTR binding in hamsters [16,25], and vaginal marking was measured in response to male odors only. Finally, we measured the effects of OTA on social odor investigation to determine if alterations in odor investigation might underlie the effects of OTA on male odor-induced vaginal marking (Experiment 3).

2. Materials and Methods

2.1. Subjects

Female Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratories (Wilmington, MA, USA) at approximately 22 days of age. Subjects were individually housed upon arrival within the animal facility in an all-female room. In addition to experimental subjects, a separate group of male and female Syrian hamsters served as odor donors. Odor donors were also purchased from Charles River Laboratories as sexually mature adults (approximately 60 days of age). Odor donors were either individually housed

or group housed (3–4 same sex animals per cage); in all cases, odor donors were unrelated to, and had no previous contact with, subjects prior to use in experiments. All animals were housed in solid-bottom Plexiglas cages (43 cm × 22 cm × 20 cm) containing corncob bedding and cotton nesting material (Nestlets; Ancare, Bellmore, NY) and maintained on a reversed light cycle (14:10 light:dark; lights out at 9 am). Behavior testing occurred during the first four hours of the dark portion of the light cycle. Food and water were available *ad libitum*. Animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23; revised 1996) and approved by the Georgia State University Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

2.2. Estrous Cycle Monitoring

Adult subjects were examined daily for eight consecutive days to determine stage of the estrous cycle. Subjects were gently restrained while vaginal secretion was manually extruded and the consistency of the secretion was examined [26]. Estrous cycles were also monitored for eight days following surgery to ensure that the procedure had not disrupted cyclicity. For all experiments, the cycle day immediately prior to behavioral estrus was defined as proestrus, whereas the two cycle days immediately following behavioral estrus were defined as diestrus day 1, and diestrus day 2, respectively. In all cases, day refers to the dark phase of the light cycle.

2.3. Surgery

At two to three months of age, subjects were unilaterally implanted with guide cannulae. Subjects were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p.; Nembutal, Ovation Pharmaceuticals, Deerfield, IL) and placed within a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with ear- and incisor-bars positioned such that the top of the skull was level. Each subject was then fitted with a single 4-mm 26-gauge guide cannula (Plastics One, Roanoke, VA), implanted at a 10° angle and directed at the following coordinates: 1.1 mm anterior to bregma, 1.7 mm lateral to the midline suture, and 4.0 mm ventral to dura. Injection cannulae were cut to extend the additional distance necessary to reach from the tip of the guide cannula to the target brain region. For Experiments 1 and 3, injection cannulae extended a total distance of 7.3 mm ventral to dura, effectively targeting the juncture between MPOA-AH and BNST. For experiment 2, injection cannulae extended either 6.8 mm or 7.8 mm ventral to dura, in order to more specifically target BNST or MPOA-AH, respectively. In all cases, stereotaxic coordinates were derived from published neuroanatomical plates for the Syrian hamster brain [27]. Guide cannulae were secured to the skull with dental cement and skull screws, and immediately prior to the completion of the surgical procedure, subjects were injected subcutaneously with an analgesic agent (Ketofen; Fort Dodge Animal Health, Fort Dodge, IA). All subjects were allowed to recover for at least 10 days prior to behavioral testing.

2.4. Drug Injections

OTA ([d(CH₂)₅¹, Tyr(Me)², Thr⁴, Orn⁸, des-Gly-NH₂⁹]-vasotocin; Bachem, Torrance, CA) was diluted in sterile saline to a final concentration of 900 μM and stored in aliquots of 10 μl at –20°C until use. OTA is a highly selective OTR antagonist that demonstrates a 17-fold greater potency for antagonizing OTRs compared to vasopressin 1a receptors (V1aRs) [28]. Thirty minutes prior to behavioral testing, subjects were injected via guide cannulae with either 200 nl of OTA (900 μM) or vehicle (saline). This concentration and volume of OTA is effective in eliciting behavioral effects in female hamsters when administered 30 minutes prior to testing [29]. OTA or vehicle was delivered to the target brain area through an injection cannula connected to a 1 μl Hamilton syringe. Subjects were lightly restrained for

the 20 seconds while the drug was injected, and for an additional 20 seconds post-injection to allow the drug to diffuse away from the tip of the injection cannula.

2.5. Scent-marking Tests

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

2.5.2. Testing protocol—For each 15-minute scent-marking test, females were placed within a soiled stimulus cage and the number of vaginal and flank marks was scored live using a hand counter. Vaginal and flank marking are discrete, stereotyped events that are easily recognized [4]. A vaginal mark was scored each time the female moved forward with tail deflected upwards while maintaining contact between the perineum and the underlying substrate. A flank mark was scored each time the female moved forward while maintaining contact between the flank region and the side of the stimulus cage. Tests were recorded using an overhead camera connected to a VHS video recorder. Videos were scored for the number of quadrant entries by researchers blind to the experimental conditions of the subjects, with inter-rater reliability of 90% or greater. Entry into a quadrant was scored whenever greater than 50% of the body mass of the subject crossed from one quadrant into another.

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

2.6. Odor Investigation Tests

2.6.1. Apparatus—For odor investigation tests (Experiment 3), the 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 one short wall of the apparatus. Each odor container had a perforated front door to allow subjects to investigate the volatile components of the stimuli without allowing direct access to the contents of the container. The top of the apparatus was secured with a clear Plexiglas lid, allowing for overhead video recording of the subject throughout the behavioral test.

2.6.2. Stimuli—Odor stimuli were collected from group-housed odor donor hamsters (3–4 same-sex animals per cage, bi-weekly cage changes). During collection, approximately 50 ml of soiled corncob bedding and 12 g of soiled cotton nesting material were placed in a one-quart re-sealable plastic collection bag. In addition, separate damp gauze pads were used to wipe down the walls of the cage, the anogenital region, and the bilateral flank glands of odor donors, and these pads were included in the collection bag. Vaginal secretion from odor donor females was collected onto an additional gauze pad by gently palpating the vaginal area with a disposable probe, and included in female odor stimuli collection bags. Hamsters investigate female odors collected on different days of the estrous cycle at relatively equivalent levels [30]; therefore, female stimuli (vaginal secretion, cage stimuli, and body odors) were collected irrespective of cycle day of odor donors and were likely representative of multiple cycle days. Clean odor stimuli consisted of 10 ml of clean corncob bedding, four grams of clean cotton nesting material, and one clean cotton gauze pad. Once collected, all odor stimuli were stored at 4°C until 30 minutes prior to use. Male and female odor stimuli were stored for up to one month and discarded if not used within that time.

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

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

All behavior tests were recorded using an overhead video camera connected to a VHS video recorder. Videos from each subject were then digitized and scored using the Observer for windows, version 5.0 (Noldus Information Technology B.V., Wageningen, the Netherlands). Researchers blind to the experimental conditions of the subjects scored the videos, with an inter-rater reliability of 90% or greater. Within each test, the number of entries into each of the two compartments and the duration of investigation of each of the three odor containers were scored. Investigation was scored whenever the snout of a subject came within one cm of the perforated front panel of an odor box. Entry into a compartment was scored whenever greater than 50% of the body mass of the subject crossed from one compartment into another.

2.7. Histology and Brain Injection Site Verification

Upon completion of behavioral testing, subjects were administered a lethal dose of sodium pentobarbital (0.2 ml, i.p.; SleepAway, Fort Dodge Animal Health, Fort Dodge, IA), and 200 nl india ink was infused via cannulae. Brains were removed, postfixed in 10% neutral buffered formalin and 50 μ M sections were taken using a vibrating microtome (Vibratome, Richmond, IL). Sections were examined under light microscopy for ink penetration and compared against published neuroanatomical plates for the Syrian hamster brain [27]. The microinjection site was considered to be within the section of tissue containing the most ventral location of deposited ink.

2.8. Data Analysis

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

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

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

3. Results

3.1. Histology

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

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

Microinjections of OTA into MPOA-AH decreased vaginal marking in response to male, but not in response to female or clean, odors (Figure 2). The interaction of drug by odor stimulus on the number of vaginal marks was statistically significant, $F(2,27) = 5.485, p = .010$. In the male odor condition ($n = 11$), OTA injections into MPOA-AH significantly decreased the number of vaginal marks compared to vehicle injections, $t(10) = -3.420, p = .007$. In contrast, OTA did not impair vaginal marking in either the female odor condition ($n = 10$), $t(9) = .805, p = .442$, or the clean odor condition ($n = 9$), $t(8) = -1.348, p = .214$. Although there was a statistically significant effect of odor condition on the number of vaginal marks for both OTA- and vehicle-injected subjects (Vehicle: $F(2,27) = 15.334, p < .001$; OTA: $F(2,27) = 5.391, p = .011$), post-hoc comparisons revealed that marking was significantly higher in the male odor condition compared to the female odor condition for OTA-injected subjects, whereas for vehicle-injected subjects, marking was higher in the male odor condition compared to either the clean or female odor conditions. No significant effects of drug, odor condition, or drug by odor condition interaction, were observed for the number of flank marks or the number of quadrant entries (Table 1).

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

OTA injections into MPOA-AH ($n = 10$) or BNST ($n = 10$) significantly decreased the number of vaginal marks to male odors compared to vehicle injections, $F(1, 18) = 6.172, p = .023$ (Table 2). No significant effect of brain area or interaction of drug by brain area on the number of vaginal marks was observed, however. Similar to results from Experiment 1, there were no significant effects of brain area, drug, or brain area by drug interaction on the number of flank marks or quadrant entries (Table 2).

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

3.4.1. Clean odor tests—When tested prior to group assignment, subjects differentially investigated the three odor boxes when the same stimuli (clean cage materials) were presented in each box, $\chi^2(2) = 20.222, p < .001$. Specifically, subjects investigated the center box less than either the left box, $z = -4.060, p < .05$, or the right box, $z = -3.820, p < .05$. The investigation times for the two outer boxes, however, were not significantly different, $z = -.793, p > .05$. In addition, there were no pre-existing differences between the OTA ($n = 16$) and vehicle ($n = 11$) groups for odor box investigation or number of midline crosses (Table 3).

3.4.2. Odor preference tests—Irrespective of OTA ($n = 16$) or vehicle ($n = 11$) injection, subjects differentially investigated male, clean and female cage stimuli, $\chi^2(2) = 21.308, p < .001$ (Figure 3). Subjects investigated male odors more than either clean odors, $z = -4.178, p < .05$, or female odors, $z = -3.695, p < .05$. The main effect of drug and the drug by odor stimulus interaction on the duration of odor investigation were not significant. Furthermore, the effect of drug on the number of midline crosses was not significant (Table 3).

4. Discussion

We show here, for the first time, that disrupting OT signaling via injection of OTA decreases vaginal marking induced by male, but not female or clean, odors. This decrease in vaginal marking cannot be attributed as a general effect of OTA on scent marking behavior or as an indirect effect of OTA on general locomotor activity, as OTA injections did not significantly alter the number of quadrant entries or flank marks. Injections of OTA into

MPOA-AH or BNST were equally effective in decreasing marking, suggesting that either brain area may mediate the effects of OTA. Finally, OTA does not alter the normal pattern of investigation of social odors by female hamsters, as OTA-injected subjects investigated male, female, and clean cage odors at levels equivalent to vehicle-injected subjects. Taken together, these results support our hypothesis that OT normally facilitates the elevated levels of vaginal marking induced by male odors, through its actions within MPOA-AH and/or BNST.

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

4.1.1. Vaginal marking behavior—The results of the present study indicate that the decrease in vaginal marking observed following injections of OTA on male odor-induced vaginal marking may be mediated by either MPOA-AH or BNST. This is consistent with previous studies examining the effects of either OT or OTA in MPOA-AH on other reproductive behaviors, as these studies had injection sites nearby, and likely including, BNST [18,23,31]. Prior lesion studies examining the role of hypothalamic areas in vaginal marking also did not distinguish between MPOA-AH and BNST [17]. It is possible that these brain areas work together as a functional unit to regulate vaginal marking, as MPOA-AH and BNST are densely interconnected [32,33]. Both areas receive chemosensory input relevant for vaginal marking behavior via connections with the medial amygdala (ME) [33–35], and project to midbrain areas important for motoric aspects of vaginal marking, including the periaqueductal gray [15]. Finally, both MPOA-AH and BNST bind estradiol and express OT [16,36]. Alternatively, the results of the present experiments are consistent with the conclusion that BNST exclusively mediates the observed effects of OTA. BNST overlays much of the rostral-caudal extent of MPOA-AH; thus, OTA injected in MPOA-AH may have spread back up to BNST, and any effects attributed to MPOA-AH may instead represent effects of OTA in BNST. This conclusion is further supported by the presence of OTR in BNST, but not MPOA-AH, of Syrian hamsters [25].

The underlying neural mechanisms whereby OTA inhibits male-odor induced vaginal marking are not clear. Failure of antibodies generated against OTR to consistently label neurons in rodent brain tissue has limited the characterization of OTR-bearing neurons in MPOA-AH or BNST (unpublished observations). Although the neural mechanisms whereby OT regulates the processing of vaginal marking-relevant signals within these brain areas has not been examined, evidence from behavioral studies suggests that OT may interact with the progesterone system to regulate reproductive behaviors. In hamsters and rats, OT can substitute for progesterone treatment in inducing lordosis in ovariectomized, estradiol-primed females, and OTA can block the facilitation of lordosis by progesterone treatment [18,19,31]. These results raise the possibility that in the current study, OTA may have inhibited vaginal marking by blocking the downstream effects of progesterone. This seems unlikely, as proestrous female hamsters have relatively low levels of serum progesterone [37] and the surge in progesterone levels that occurs just prior to the onset of behavioral receptivity appears to inhibit vaginal marking [4,38,39]. In contrast, estradiol treatment facilitates vaginal marking in response to males, either when administered systemically [17,40,41] or when implanted directly into MPOA-AH [39,42]. Given that OT and OTR expression in MPOA-AH and BNST are enhanced by estradiol in rats [43,44], it seems likely that OT may mediate the effects of estradiol on vaginal marking. More research is required, however, to determine the mechanisms whereby OT acting downstream of estradiol specifically enhances the vaginal marking response to male odors.

Although the decrease in vaginal marking following OTA injection was statistically significant in both Experiment 1 and Experiment 2, the magnitude of the decrease was not large (Experiment 1: 31%; Experiment 2: 22%). This may be due to the specific procedures

employed in this study. For all experiments, subjects were injected unilaterally with 200 nl of OTA (900 μ M) and tested 30 minutes following injection. When administered unilaterally, this volume and concentration of OTA effectively stimulates aggression in female hamsters 30 minutes, but not immediately, following injection [29]. OTA may be working through different mechanisms to modulate aggression and vaginal marking; therefore, bilateral injections or other alterations to the timing, dose, or volume of injections may be necessary for OTA in MPOA-AH or BNST to maximally inhibit vaginal marking behavior.

4.1.2. Flank marking behavior—In contrast to vaginal marking, injections of OTA in MPOA or BNST had no effect on the number of flank marks in response to male, clean, or female odors. Although it has been reported that OT injections in MPOA-AH can induce flank marking in male hamsters [45–47], in females this effect is dependent on the presence of a familiar, subordinate partner [46]. In the current study, however, subjects were only tested with odors from unfamiliar stimulus animals. Interestingly, there were also no differences in flank marking to male, female, or clean odors, irrespective of drug condition. Female hamsters flank mark at higher levels in response to same-, rather than opposite-sex stimuli [4]; however, this effect is not consistently observed in proestrous female hamsters [12,13]. Therefore, it is likely that a significant effect of odor condition on flank marking was not observed in the current study due to testing females only on the cycle day of proestrus.

In contrast to the present results suggesting that the normal expression of vaginal marking is reliant on OT signaling, flank marking appears to depend more critically on the related neuropeptide arginine vasopressin (AVP). For example, AVP injections into MPOA-AH strongly stimulate flank marking in both male and female hamsters [47], whereas administration of V1aR antagonists block flank marking induced by AVP, as well as by stimuli present in conspecific home cages [45,48]. In addition, these behaviors appear to be important for different aspects of intraspecific communication. Vaginal marking is important for mate solicitation, and therefore is most prevalent in response to males or their odors, whereas flank marking appears to function in the establishment and maintenance of social hierarchies and territorial advertisement, and is most strongly induced by same-sex odors [1]. There is a high degree of overlap, however, both in the brain areas that regulate vaginal and flank marking, as well as in the expression patterns of OT and AVP within these areas [1,49,50]. Given that OTA exhibits some level of V1aR antagonism [51], and V1aR expression has been confirmed in both MPOA-AH and BNST of Syrian hamsters [52], the possibility exists that the effects observed in the present study are the result of V1aR antagonism by OTA. This seems unlikely given that OTA had no effect on flank marking, a behavior that depends critically on V1aR signaling. Furthermore, preliminary studies examining the effects of a specific V1aR antagonist, Manning compound, in MPOA-AH or BNST found no effects of this drug on vaginal marking (unpublished observations), suggesting that the effects of OTA on vaginal marking are likely mediated by OTRs.

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

In the present study, OTA decreased vaginal marking in response to male odors without affecting social odor investigation levels. Subjects were prevented from directly contacting the odor source in all social odor investigation tests, whereas during scent-marking tests, subjects were allowed direct contact with odor stimuli. Therefore, OTA may decrease social odor investigation but only in situations when direct contact with the odor stimulus is allowed. This is in agreement with data from mice, wherein some deficits in odor preference following manipulation of chemosensory structures are only evident in tests wherein direct contact with the odor source is allowed [53,54]. Other studies examining the effects of OT in

MPOA-AH on socio-sexual behaviors with strong chemosensory components such as ultrasonic vocalizations in hamsters [23] and lamb acceptance in ewes [55] did not control contact with the odor source, so it is not known if direct contact is necessary for OT to modulate these behavioral responses. Alternatively, OTA in MPOA-AH or BNST may specifically target the male odor-induced component of vaginal marking without generally affecting male odor investigation, suggesting that separate neural pathways regulate these two behaviors. This is supported by previous work in hamsters, as females with lesions of the vomeronasal organ fail to vaginal mark more to male vs. female odors but show normal patterns of preferential investigation of male odors, whereas ME lesions eliminate opposite-sex odor preference without affecting preferential marking in response to male odors [12,13,56].

4.3. Conclusion

Together, these results suggest that OT within MPOA-AH and/or BNST facilitates vaginal marking in female Syrian hamsters. This is in agreement with the role of OT in other sociosexual behaviors, and suggests that the general role of OT is to facilitate sociosexual interactions [57]. Importantly, the present study found that the effects of OTA are specific to the male odor-induced component of vaginal marking, indicating that OT in these areas can modulate the behavioral response to chemosensory cues important for social behaviors.

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Figure 1. Histological Verification of Injection Sites

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

Figure 2. Number of Vaginal Marks During Scent-Marking Tests

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

Figure 3. Odor Box Investigation During Preference Tests

Subjects investigated male odors more than either female or clean odors, irrespective of drug (OTA or vehicle) injected into MPOA-AH ($p < .05$ with *Bonferroni* correction).

Investigation duration times were adjusted to account for pre-existing biases in investigation due to odor box positioning, as described in the *Data Analysis* section. Graphed data represent unadjusted investigation times.

Table 1

Summary of behavioral measures from Experiment 1.

	Number of flank marks		Number of quadrant entries	
	Male	Clean	Female	Clean
Drug				
Vehicle	3.2 ± 1.4	3.3 ± 1.5	4.6 ± 1.7	79.6 ± 9.9
OTA	2.5 ± 0.7	2.3 ± 0.7	8.0 ± 2.0	72.2 ± 5.8

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

Table 2

Summary of behavioral measures from Experiment 2.

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

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

Table 3

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

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

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