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The bed nucleus of the stria terminalis is critical for sexual solicitation, but not for opposite-sex odor preference, in female Syrian hamsters

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Abstract

Successful reproduction in vertebrates depends critically upon a suite of precopulatory behaviors that occur prior to mating. In Syrian hamsters (*Mesocricetus auratus*), these behaviors include vaginal scent marking and preferential investigation of male odors. The neural regulation of vaginal marking and opposite-sex odor preference likely involves an interconnected set of steroid-sensitive nuclei that includes the medial amygdala (MA), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA). For example, lesions of MA eliminate opposite-sex odor preference and reduce overall levels of vaginal marking, whereas lesions of MPOA decrease vaginal marking in response to male odors. Although BNST is densely interconnected with both MA and MPOA, little is known about the role of BNST in female precopulatory behaviors. To address this question, females received either bilateral, excitotoxic lesions of BNST (BNST-X) or sham lesions (SHAM), and were tested for scent marking and for investigatory responses to male and female odors. Whereas SHAM females vaginal marked more to male odors than female odors on two days of the estrous cycle, BNST-X females marked at equivalent levels to both odors. This deficit is not due to alterations in social odor investigation, as both BNST-X and SHAM females investigated male odors more than female odors. Finally, BNST lesions did not generally disrupt the cyclic changes in reproductive behaviors that occur across the estrous cycle. Taken together, these results demonstrate that BNST is critical for the normal expression of solicitational behaviors by females in response to male odor stimuli.

Keywords

Olfaction; Reproduction; Chemosensory; Scent marking; Appetitive

Introduction

Successful reproduction in most vertebrates depends critically on precopulatory or solicitation behaviors that occur prior to mating (Beach, 1976). Although these behaviors can take many species-specific forms, they all serve to facilitate or coordinate mating behavior. Precopulatory behaviors are therefore particularly important for mating coordination in species where the sexes live in isolation from each other, such as Syrian hamsters (Gattermann et al., 2001; Lisk et al., 1983; Pfaff et al., 2008). In this species,

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females engage in several forms of precopulatory behaviors that include vaginal scent marking and the preference to investigate opposite-sex odors. Vaginal marking is a highly stereotyped behavior typified by the female lowering her perineum to the substrate and depositing a small amount of vaginal secretion as she moves forward (Johnston, 1977). Vaginal marks likely serve to advertise a female's location to males, as females vaginal mark to form a path linking the male and female's nesting areas when tested in a large, semi-natural environment (Lisk et al., 1983), and males vigorously investigate and follow these marks (Johnston, 1974; Johnston and Schmidt, 1979; Kwan and Johnston, 1980; Lisk et al., 1983).

The expression of vaginal marking relies on the perception of odor cues from conspecifics, as females vaginal mark at much higher levels in response to males or their odors compared to odors from other females (Johnston, 1977; Maras and Petrulis, 2008; Martinez et al., 2010; Petrulis and Johnston, 1999; Petrulis et al., 1999). In hamsters as well as other vertebrates, social odors are processed by an interconnected set of forebrain neural structures that includes the olfactory bulbs, the medial amygdala (MA), and the bed nucleus of the stria terminalis (BNST) (Wood, 1998). Some of these structures have been implicated in the regulation of female precopulatory behavior. For example, disruption of the main olfactory system decreases overall levels of vaginal marking and ultrasonic vocalizations (Johnston, 1992), whereas disruption of the accessory olfactory system eliminates preferential vaginal marking to male odors (Petrulis et al., 1999). Input from the main and accessory olfactory systems converge on MA (Coolen and Wood, 1998), and bilateral electrolytic lesions centered on MA eliminate opposite-sex odor preference and decrease vaginal marking (Petrulis and Johnston, 1999; Takahashi and Gladstone, 1988). Finally, electrolytic lesions that target the entire corticomедial amygdala decrease ultrasonic vocalizations by females in response to males (Kirn and Floody, 1985).

In contrast to MA, less is known about the role of BNST in the regulation of precopulatory behaviors. BNST receives input from the olfactory system both directly (Davis et al., 1978), as well as indirectly via MA and other amygdaloid nuclei (Coolen and Wood, 1998; Wood and Swann, 2005) and in male hamsters, lesions of BNST eliminate opposite-sex odor preference (Been and Petrulis, 2010). In female hamsters, large bilateral electrolytic lesions of the medial preoptic area (MPOA) that also damage BNST decrease vaginal marking in response to males (Malsbury et al., 1977). Furthermore, injection of a selective oxytocin receptor antagonist into BNST of female hamsters decreases vaginal marking to male odors, without affecting marking to female odors or the preference to investigate opposite-sex odors (Martinez et al., 2010). This effect, however, cannot be specifically attributed to BNST, as injections into the underlying MPOA also were effective in decreasing marking to male odors. In order to gain a more complete understanding of the role of BNST in precopulatory behaviors, we examined the effects of site-specific, excitotoxic lesions of BNST on vaginal marking, as well as investigatory responses to male and female odors. Based on previous data, we predicted that BNST lesions would disrupt both preferential vaginal marking and odor investigation. As BNST is a steroid-responsive brain area (Krieger et al., 1976; Li et al., 1993) and vaginal marking varies with changes in circulating gonadal steroids (Takahashi and Lisk, 1983) we tested females for scent marking on multiple days of the estrous cycle (diestrous day 2 and proestrus), to determine if BNST mediates the effects of circulating gonadal steroids on vaginal marking.

Materials and Methods

Experimental design

Subjects were first assessed for levels of vaginal marking to male odors in order to screen out animals that failed to mark at sufficiently high levels (see below) (Figure 1A). Subjects

then received either bilateral, excitotoxic lesions of BNST 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). After an initial habituation test (Clean test), subjects were tested both when direct contact with the odor stimuli was prevented (Non-contact test) and when it was allowed (Contact test). Second, subjects were 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 BNST 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 7–8 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 (16:10 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. All efforts were made to minimize the number of animals used and their suffering.

Estrous cycle monitoring

Prior to behavioral testing, 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 using a disposable probe, and the consistency of the secretion was examined for stringy consistency indicative of behavioral estrus (Orsini, 1961). Once 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. 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 BNST lesion group (BNST-X; $n = 28$) or a sham lesion group (SHAM; $n = 14$). A matched random assignment procedure was used, such that initial levels of vaginal marking in response to male odors on proestrus were equivalent across both experimental conditions (see below). Subjects were first deeply 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 BNST-X subjects, the excitotoxin *N*-methyl-D-aspartic acid (20 mg/ml, 20 nl per injection site; Sigma, St. Louis, MO) was injected bilaterally via a Hamilton microinjection syringe (701R 10 μ l syringe; Hamilton, Reno, NV) under stereotaxic control. Subjects in the BNST-X group received two injections/side, one targeting the posterointermediate BNST (anterior-posterior, +1.65; medial-lateral, ± 1.65 ; dorsal-ventral, -5.80) and the other the posteromedial BNST (anterior-posterior, +1.65;

medial-lateral, ± 1.05 ; dorsal-ventral, -5.90). The anterior-posterior and medial-lateral measurements were made relative to bregma, whereas dorsal-ventral measurements were made relative to dura, according to 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 excitotoxin 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 stimulus and apparatus—Stimuli for scent marking tests consisted of vacated cages ($43 \times 22 \times 20$ cm) that had previously been occupied by male or female stimulus animals for four days (Figure 1B). 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 cage stimuli were representative 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 $40 \text{ cm} \times 19 \text{ cm} \times 0.4 \text{ cm}$ perforated Plexiglas plate was placed over the bedding just prior to testing. The surface of the plate was painted with black non-toxic chalkboard paint (Rust-oleum, Vernon Hills, IL) such that the plate was divided into four painted quadrants separated by an unpainted area in the shape of a cross. This plate facilitated the 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 throughout the course of 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. Similar to vaginal marking, flank marking is a discrete, stereotyped behavior that is 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. 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.

Prior to surgical procedures, subjects were initially screened for vaginal marking responses to male odors (Figure 1A). Subjects that failed to mark at least 5 times during a ten-minute test ($n = 2$) were removed from the study. After recovering from surgery, subjects were tested for scent marking responses to male or female odors, on both diestrous day 2 and proestrus. The order of testing was counterbalanced across subjects.

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.

For tests involving direct contact with odors, additional stimuli were collected directly onto glass microscope slides ($25 \times 75 \times 1$ mm). Stimulus animals were gently restrained while clean slides were rubbed along the flank and anogenital regions. For male odor slides, one half of the slide contained flank gland secretions and the other half contained anogenital secretions. For female odor slides, the slide was divided into three sections rather than two, with the center section containing a sample of vaginal secretion collected from a stimulus female using a disposable probe. In all cases, individual slides contained stimuli from multiple animals, as each type of stimuli collected onto a slide was derived from a different stimulus animal. This was done to minimize the impact of individual differences in stimulus quality on subsequent investigatory behavior by experimental subjects during testing. Following collection, slides containing same-sex stimuli were stored together in airtight containers at 4°C until 30 minutes prior to use. All collected odor stimuli were stored for up to one month and discarded if not used within that time.

Apparatus & testing protocol—Odor investigation tests were conducted in a three-choice odor investigation apparatus (Figure 1C) by a researcher blind to the experimental condition of the subjects. This apparatus consisted of a modified $51 \text{ cm} \times 25.5 \text{ cm} \times 30.5 \text{ cm}$ 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 three separate occasions in the three-choice odor investigation apparatus (Clean test, Non-contact test, and Contact test) (Figure 1A). Testing occurred across three 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 Non-contact test followed by the Contact test. During these tests, 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. For the Contact test, a single slide containing stimuli sex-matched to the contents of the odor container (male, female, or clean) was affixed to the center of the exposed, perforated side of the odor container, such that the long axis of the slide was parallel to the floor of the apparatus.

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. Entry into a compartment was scored whenever greater than 50% of the body mass of the subject crossed from one compartment into another.

Lordosis tests—Subjects were tested for the latency and expression of sexual receptivity (lordosis) on the predicted day of behavioral estrus (Figure 1A) 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), and so were not allowed to mount the female. The latency to assume lordosis (measured from the time the subject was introduced into the male's cage) and duration of lordosis 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 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde (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, 1% Triton-X (Sigma, St. Louis, MO), and 1:30,000 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 rabbit anti-mouse secondary antibody (315-065-003, Jackson ImmunoResearch Laboratories, West Grove, PA) 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, Burlingame, CA) 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% H₂O₂ 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 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 $p < .05$.

Behavioral measures from scent marking tests (number of vaginal and flank marks and number of quadrant entries) were first examined using mixed-design 2 X 2 X 2 ANOVAs with experimental group (BNST-X or SHAM) as a between-subjects factor, and odor stimulus (male or female) and cycle day (diestrous day 2, proestrus) as within-subjects factors. Statistically significant two-way interactions were further examined for the effect of odor within each lesion group using paired-samples *t*-tests. The number of quadrant entries was compared across experimental group using independent-samples *t*-tests.

For the Clean odor test, investigation durations were examined using mixed-design 2 X 3 ANOVAs with experimental group (BNST-X or SHAM) as a between-subjects factor and odor box position (left, center, right) as a within-subjects factor. Significant main effects of odor box position were further examined using Friedman tests followed by Wilcoxon sign-ranks tests with Bonferroni correction. For the Non-contact and Contact odor tests, investigation durations were examined using mixed-design 2 X 2 ANOVAs with experimental group (BNST-X or SHAM) as a between-subjects factor and odor stimulus (male or female) as a within-subjects factor. Significant main effects of odor were further examined using Wilcoxon sign-ranks tests. Within each the odor investigation tests, odor box investigation times and the number of compartment entries were compared across experimental group using Mann-Whitney *U* tests. Lastly, behaviors measured during copulatory tests (latency to assume lordosis, duration of lordosis) were compared across experimental group using Mann-Whitney *U* tests.

Results

Lesion reconstruction

Subjects were included in the BNST-X group if bilateral damage comprised at least 50% damage to BNST on at least two consecutive stereotaxic planes (Figure 2A) (Morin and Wood, 2001). All subjects included in the BNST-X group sustained substantial bilateral damage to BNST at the level of the most caudal pole of the anterior commissure (0.5 mm anterior to Bregma, $n = 17$) (Figure 2C). Furthermore, most subjects in this group also sustained bilateral damage to more posterior levels of BNST (Bregma +0.2 mm, $n = 14$;

Bregma -0.1 mm, $n = 1$). A few BNST-X subjects also had bilateral damage at more rostral levels of BNST (Bregma $+0.8$ mm, $n = 3$).

Subjects that received NMDA injections but did not exhibit significant bilateral damage to BNST on two consecutive stereotaxic planes were not included in the BNST-X group. Specifically, these subjects all had partial (less than 50%), bilateral damage to BNST (Partial Lesion, $n = 15$) restricted within the same rostral-caudal extent of BNST as BNST-X subjects. Importantly, these subjects did not differ significantly from SHAM subjects on any measure of vaginal marking or odor investigation ($p > .05$ for all comparisons).

In both BNST-X and Partial Lesion subjects, minimal and predominantly unilateral damage to surrounding nuclei was occasionally observed (defined as less than 20% of the nuclei on any stereotaxic plane) (Morin and Wood, 2001). This damage was seen in the ventrolateral septum ($n = 9$), the medial preoptic area ($n = 3$), the medial preoptic nucleus ($n = 1$), the ventral pallidum ($n = 3$), the globus pallidus ($n = 1$), and the caudate putamen ($n = 1$). No differences were observed in any behaviors between subjects with damage to any of these nuclei compared to BNST-X and Partial Lesion subjects. Although needle tracts were typically visible in the brains of SHAM subjects (Figure 2B), no significant damage to any brain structure was observed in these subjects.

Scent marking tests

Preferential vaginal marking to male vs. female odors was eliminated in females with bilateral lesions of BNST (Figure 3). As expected (see Introduction), females vaginal marked more on proestrus compared to diestrous day 2, $F(1,39) = 52.925$, $p < .001$, and more to male odors than to female odors, $F(1,39) = 38.516$, $p < .001$. The interaction of lesion group by odor condition, however, was statistically significant, $F(1,25) = 10.160$, $p < .01$, indicating that the effect of odor condition was driven predominantly by SHAM females marking more to male odors on both diestrous day 2, $t(9) = 4.80$, $p < .01$, and proestrus, $t(9) = 3.94$, $p < .01$. In contrast, BNST-X females vaginal marked at equivalent levels to male and female odors on both cycle days (diestrous day 2: $t(16) = 1.11$, $p = .28$; proestrus: $t(16) = .518$, $p = .61$). This loss of preferential vaginal marking to male odors did not reflect an overall decrease in vaginal marking levels in BNST-X females, as the total number of vaginal marks (summed across cycle days and odor conditions) did not differ between BNST-X and SHAM females, $F(1,25) = .132$, $p = .72$. BNST-X and SHAM females also did not differ in the number of line crosses during scent marking tests, in response to either male, $t(23) = -.88$, $p = .39$, or female, $t(21) = -1.28$, $p = .22$, odors. Similar to SHAM females, vaginal marking in the Partial Lesion group was significantly higher to male compared to female odors on both diestrous day 2 and proestrus (Table 1).

There were no significant differences in flank marking behavior between BNST-X and SHAM females (Table 2). Although females flank marked more in response to female odors compared to male odors, $F(1,39) = 8.74$, $p < .01$, the interaction of odor condition by lesion group was not significant, $F(2,39) = 1.11$, $p = .34$. BNST-X and SHAM females also did not differ in the overall number of flank marks, $F(1,25) = .71$, $p = .41$.

Odor investigation tests

Clean test—Subjects differentially investigated the three odor boxes when clean cage materials were presented in each box, $\chi^2(2) = 30.25$, $p < .001$ (data not shown). Specifically, females investigated the center box less than either the left box, $z = -4.26$, $p < .001$, or the right box, $z = -4.23$, $p < .001$. Importantly, the investigation times of the two outer boxes were not significantly different, $z = -.49$, $p = .63$. Furthermore, there were no significant differences in odor box investigation (left box: $z = -.82$, $p = .412$; center box: $z = -.59$, $p = .55$).

56; right box: $z = -.176$, $p = .86$) or midline crosses, $z = -.53$, $p = .60$, between BNST-X and SHAM females.

Non-contact test—Irrespective of lesion condition, females differed in their investigation of male and female cage stimuli when direct contact with the odor source was prevented, $z = -3.87$, $p < .001$ (Figure 4A). Both SHAM ($z = -2.70$, $p < .01$) and BNST-X ($z = -2.79$, $p < .01$) females investigated male odors more than female odors. The main effect of lesion, $z = -.264$, $p = .79$, and the lesion by odor stimulus interaction, $F(1,24) = .64$, $p = .43$, were not significant. Similar to SHAM and BNST-X females, Partial Lesion subjects also investigated male odors more than female odors (Table 1).

Contact test—Both SHAM and BNST-X females investigated the odor stimuli for differing amounts of time when contact with the odor stimuli was allowed, $z = -4.18$, $p < .001$ (Figure 4B). Females in both conditions investigated male odors more female odors (SHAM: $z = -2.80$, $p < .01$; BNST-X: $z = -3.15$, $p < .01$). There was no significant main effect of lesion group, $z = -.30$, $p = .76$, or significant lesion by odor stimulus interaction, $F(1,24) = 1.18$, $p = .29$. This pattern of results was also seen in females in the Partial Lesion group (Table 1).

Lordosis tests—All females exhibited lordosis on the predicted day of behavioral estrus in response to a stimulus male. BNST-X and SHAM females did not differ in either the latency, $z = -.40$, $p = .69$, or duration, $z = -.18$, $p = .86$, of their lordotic response to males (Table 2).

Discussion

We show here, for the first time, that discrete, bilateral lesions of BNST specifically disrupt the expression of female sexual solicitation to conspecific stimuli, as evidenced by the elimination of preferential vaginal marking to male odors compared to female odors in BNST-X females. This loss of preferential vaginal marking cannot be attributed to a deficit in social odor investigation, as BNST-X females investigated male odors more than female odors both when direct contact with the odor source was allowed and when it was prevented. BNST-X females also displayed the normal pattern of elevated flank marking to female odors, and had no deficits in the expression of sexual receptivity. Together, these results support our hypothesis that BNST is required for the normal expression of odor-guided precopulatory behaviors in female Syrian hamsters.

Role of BNST in sexual solicitation

The finding that lesions of BNST eliminate preferential vaginal marking to male odors is consistent with a previous study demonstrating that bilateral electrolytic lesions targeted at MPOA, that also damaged BNST, decrease vaginal marking to males (Malsbury et al., 1977). BNST receives substantial input from the olfactory bulbs, predominantly via connections with corticomedial amygdaloid nuclei such as MA (Wood and Swann, 2005), and the normal pattern of vaginal marking responses to conspecifics is severely disrupted following damage to these olfactory systems (Johnston, 1992; Petrulis et al., 1999). Lesions of MA, however, do not eliminate preferential vaginal marking to male odors, but rather result in an overall decrease in vaginal marking regardless of odor condition (Petrulis and Johnston, 1999). These data suggest, therefore, that MA may process chemosensory information important for supporting overall levels of vaginal marking to conspecific odors, whereas information critical for preferential vaginal marking may be processed by BNST independent of MA, perhaps involving the direct connections between AOB and BNST (Davis et al., 1978; Fan and Luo, 2009).

Although the neurochemical mechanisms underlying the regulation of preferential vaginal marking by BNST have not been extensively examined, there is some evidence to suggest that the neuropeptide oxytocin may be involved. BNST appears to be sensitive to oxytocin, given that it contains both oxytocin-immunoreactive fibers (Whitman and Albers, 1998) and binding sites for oxytocin (Dubois-Dauphin et al., 1992), and neurons in BNST exhibit excitatory responses following application of oxytocin (Ingram et al., 1990). In a recent study we found that unilateral injections of a specific oxytocin receptor antagonist into BNST decrease vaginal marking to male odors, without affecting marking to female odors or preferential investigation of male odors (Martinez et al., 2010). Although these data suggest that oxytocin normally acts in BNST to support male odor-guided vaginal marking without more generally affecting investigatory responses to male odors, it is important to note that BNST is also important for other precopulatory behaviors in females such as ultrasonic vocalizations (Kirn and Floody, 1985). Indeed, injections of oxytocin into MPOA increase ultrasonic vocalizations to males (Floody et al., 1998). Further research is needed, therefore, in order to clarify the role of oxytocin in BNST across the full range of female precopulatory behaviors.

In addition to regulation by odor signals from conspecifics, the expression of vaginal marking also varies in response to changes in circulating gonadal steroids. Vaginal marking levels increase across the estrous cycle and peak on proestrus (Johnston, 1977; Takahashi and Lisk, 1983). In the present study, BNST-X females vaginal marked more on proestrus compared to diestrous day 2, indicating that BNST lesions did not disrupt the normal cyclic pattern of vaginal marking. This was surprising, given that estradiol receptors are expressed in many neurons throughout BNST (Li et al., 1993), and that that overall levels of vaginal marking appear to be mediated by estradiol acting within several hypothalamic structures that are interconnected with BNST (Takahashi and Lisk, 1985, 1987; Takahashi et al., 1985). Taken together, the evidence suggests that the neural circuit mediating the effects of cyclic changes in estradiol on precopulatory and other reproductive behaviors does not include BNST. This conclusion is consistent with our observation that all BNST-X females, like SHAM females, were sexually receptive on the predicted day of behavioral estrous.

Role of BNST in social odor investigation

Females with BNST lesions showed the normal preference to investigate male odors more than female odors, indicating that BNST is not critical for the normal expression of opposite-sex odor preference in females. These results were unexpected, given that in male hamsters, similar lesions of BNST completely eliminate opposite-sex odor preference (Been and Petrulis, 2010), and larger, electrolytic lesions of BNST decrease investigation of female hamster vaginal secretion (Powers et al., 1987). Moreover, both males and females require a functional MA, the major input structure for BNST, for odor preference (Maras and Petrulis, 2006; Petrulis and Johnston, 1999), and both sexes show similar neural responses to opposite-sex odors in MA (DelBarco-Trillo et al., 2009; Maras and Petrulis, 2010; Meredith and Westberry, 2004) and BNST (Hosokawa and Chiba, 2007; Maras and Petrulis, 2010). This suggests that with the exception of MA, a fundamental sex difference may exist in the neural architecture regulating opposite-sex odor preference. This is congruent with evidence that the sexes differ in their requirement for the vomeronasal organ (VNO), the primary sensory organ of the accessory olfactory system, in processing of opposite-sex odors (Keller et al., 2006; Pankevich et al., 2004; Pankevich et al., 2006).

The intact opposite-sex odor preference in females with BNST lesions suggests that their deficits in preferential vaginal marking cannot be attributed to an inability to discriminate between male and female odors. It also argues for the existence of partially separate and independent neural circuits regulating different forms of precopulatory behaviors. Sexual identity information critical for preferential vaginal marking may be processed by a neural

circuit that includes VNO/AOB and BNST, as BNST receives direct input from AOB (Davis et al., 1978) and lesions of either VNO (Petrulis et al., 1999) or BNST (present study) eliminate the normal pattern of elevated marking to males without eliminating social odor preference. Despite its essential role in supporting overall levels of vaginal marking, MA is not critical for preferential vaginal marking (Petrulis and Johnston, 1999) and may be more important for regulating opposite-sex odor preference in females via a BNST-independent pathway.

Role of BNST in flank marking

In contrast to vaginal marking, the normal pattern of elevated flank marking to same-sex odors (Johnston, 1977; Petrulis, 2009) was not disrupted in females with BNST lesions. Although the role of BNST in flank marking has not been extensively examined, lesions of the lateral septum (LS) that also result in BNST damage decrease flank marking by males in response to male odors (Ferris et al., 1990). Furthermore, injections of arginine vasopressin (AVP), a compound known to induce high levels of flank marking when injected into specific brain areas (Ferris et al., 1984), also induce flank marking when injected into the LS/BNST of males (Irvin et al., 1990). Given the present data, it is likely that LS, rather than BNST, is the critical site for regulating flank marking via its connections with MPOA and MA (Coolen and Wood, 1998; Ferris et al., 1990).

Anatomical considerations

Lesions of animals included in the BNST-X group included damage to the anterior and/or posterior divisions of BNST. Therefore, it is difficult to assign the observed behavioral deficits to particular subdivisions of BNST. There are known anatomical and functional differences between the anterior and posterior divisions (Dong et al., 2001). For example, the posterior division of BNST is more heavily interconnected with MA, and has been implicated in the control of reproductive behaviors (Been and Petrulis, 2010; Wood, 1998). Although it also receives some input from MA, the anterior division is more strongly interconnected with the central nucleus of the amygdala and appears to be involved in anxiety and stress responses (Davis and Shi, 1999). Given this evidence, it seems likely that deficits in vaginal marking observed in the present study were the result of posterior, rather than anterior, BNST damage. No correlation was observed, however, between the anterior-posterior positioning of lesions and loss of preferential marking, indicating that damage throughout this axis may be sufficient to disrupt preferential marking to males.

Conclusion

The finding that lesions of BNST eliminate preferential vaginal marking to male odors is in general agreement with a role for BNST in the regulation of odor-guided precopulatory behaviors. Specifically, BNST may process odor signals derived from primary olfactory structures to ultimately generate a preferential behavioral response to opposite-sex odors. In female hamsters, this area appears to play a rather specific role in preferential vaginal marking, whereas in males it is critical for preferential odor investigation. These data suggest that the neural circuitry regulating precopulatory behaviors are not only differentiated between types of female precopulatory behaviors such as vaginal marking and opposite-sex odor preference, but that this circuit is also differentiated between the sexes. Further studies are needed to understand the underlying neural mechanisms whereby BNST regulates context-appropriate precopulatory responses in both males and females.

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Highlights

- Bilateral lesions of BNST eliminate preferential vaginal marking to male odors
- This loss of preferential vaginal marking is not due to odor preference disruption
- The hormonal regulation of reproductive behaviors is intact in BNST-lesioned females

Figure 1. Timeline and Testing Apparatus. A. Timeline of behavioral testing and surgeries in experiment. D2=diestrus day 2; PE=proestrus. B. Side view of testing apparatus for Scent marking tests. C. Top-down view of apparatus used for Odor investigation tests.

Figure 2.

Lesion Reconstruction. A. Coronal sections depicting largest (light gray) and smallest (dark gray) excitotoxic lesion damage in bed nucleus of the stria terminalis of female hamsters included in the BNST-X group. Sections are organized from anterior (top) to posterior (bottom), all relative to bregma. Neuronal damage was visualized following immunohistochemistry for NeuN for both (B) SHAM and (C) BNST-X subjects. In all cases, damage depicted on atlas plates that comprised less than 50% of BNST was derived from females with greater than 50% damage to BNST on two other consecutive plates (see Results section). Scale bar = 200 μ M. 3V=third ventricle; LV=lateral ventricle; ac=anterior commissure; sm=stria medullaris; f=fornix.

Figure 3. Mean ($\pm$ SEM) Number of Vaginal Marks to Male and Female Odors. SHAM, but not BNST-X, females preferentially marked at higher levels to male odors compared to female odors on both (A) diestrus day 2, and (B) proestrus. * $p < .01$, male vs. female odor condition.

Figure 4. Median ($\pm$ IQR) Duration of Odor Investigation. Subjects investigated male odors more than female odors, irrespective of lesion condition, in both the (A) Non-contact and (B) Contact tests. * $p < .01$, male vs. female within each test.

Table 1

Odor preference and vaginal marking of Partial Lesion subjects.

Vaginal marking Diestrous day 2	Proestrous		Non-contact odor preference		Contact odor preference	
	Male	Female	Male	Female	Male	Female
7.0 ± 1.9 [#]	3.6 ± 1.0	15.7 ± 2.1 [*]	8.1 ± 1.8	47.9 ± 3.7 [*]	25.2 ± 5.1	64.4 ± 6.2 [*]
						26.7 ± 2.7

Partial Lesion (see Introduction) females vaginal marked more in response to male compared to female odors on diestrous day 2 and proestrus. These females also investigated male odors more than the female in both the Non-contact and Contact tests.

[#] $p < .05$ and

^{*} $p < .01$ for male vs. female within each test. All data are mean ± SEM.

Table 2

Flank marking and lordosis of SHAM and BNST-X subjects.

	Number of flank marks		Lordosis duration	Lordosis latency
	Male	Female		
SHAM	13.4 ± 3.0	17.2 ± 4.6	49.2 ± 17.3	16.9 ± 3.1
BNST-X	7.8 ± 1.6	16.1 ± 3.2	45.6 ± 9.6	17.7 ± 2.0

During scent marking tests, females flank marked more to female odors than to male odors, $p < .01$. The interaction of odor by lesion group was not significant, $p = .34$. BNST-X females did not significantly differ from SHAM females in lordosis latency or duration, all $p > .05$. Data are presented as mean ± SEM.