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The Posterior Bed Nucleus of the Stria Terminalis Mediates Opposite-Sex Odor Preference in Male Syrian Hamsters (Mesocricetus Auratus)

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THE POSTERIOR BED NUCLEUS OF THE STRIA TERMINALIS MEDIATES OPPOSITE-SEX ODOR PREFERENCE IN MALE SYRIAN HAMSTERS (*MESOCRICETUS AURATUS*)

by

LAURA E. BEEN

Under the Direction of Aras Petrulis

ABSTRACT

In Syrian hamsters, social behavior is mediated exclusively by chemosensory cues and circulating gonadal steroid hormones. Where these two signals are processed in the brain is unknown, but the posterior bed nucleus of the stria terminalis (pBNST) has been suggested as a candidate site. Therefore, we tested male hamsters' preference for opposite-sex odors following excitotoxic lesions of the pBNST. Lesions of the pBNST (pBNST-X) eliminated male hamsters' preference for opposite-sex odors. Furthermore, pBNST-X males spent significantly less time investigating female odors than clean odors and significantly less time investigating female odors than control males did. Lesions of the pBNST did not change male hamsters' investigation of male odors. The deficits observed in pBNST-X males were not due to a failure to discriminate between odors, as pBNST-X males were able to distinguish between odors. Together, these data suggest the pBNST is critical for opposite-sex odor preference in male hamsters.

INDEX WORDS: Bed nucleus of the stria terminalis, Hamster, Odor, Hormones, Excitotoxic lesion, Social behavior, Odor preference, Reproduction, Extended amygdala

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LAURA E. BEEN

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Master of Arts

in the College of Arts and Sciences

Georgia State University

2008

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Introduction

To generate appropriate social behavior, most animals must integrate internal signals with the perception of external cues. In the Syrian hamster, for example, socio-sexual behavior requires both internal hormone signals and the perception of chemosensory cues from the environment (Johnston, 1990). These odor cues are processed by the main and/or accessory olfactory systems, which together compose the chemosensory system (Breer, 2003). In the main olfactory system, odors interact with sensory neurons in the main olfactory epithelium, which projects to the main olfactory bulbs, whereas in the accessory olfactory system, chemical signals bind to sensory neurons in the vomeronasal organ (VNO), which projects to the accessory olfactory bulbs (Meredith, 1991). The chemosensory system mediates appetitive aspects of reproductive behavior in the Syrian hamster, such as males' attraction to female vaginal secretion (Powers, Fields, & Winans, 1979). Moreover, inactivating the chemosensory system, either by olfactory bulbectomy (Murphy & Schneider, 1970) or simultaneous deafferentation of the main and accessory olfactory systems (Powers & Winans, 1975), eliminates copulation in the Syrian hamster. This complete elimination of copulation is unique to Syrian hamsters, as olfactory bulbectomy in the Norway rat (Edwards, Griffis, & Tardivel, 1990; Larsson, 1975) or mouse (Rowe & Edwards, 1972) only reduces copulatory behavior and is not mitigated by sexual experience.

In addition to chemosensory signals, Syrian hamster reproductive behavior is also tightly regulated by changes in gonadal steroid hormone levels. This internal signal regulates both consummatory and appetitive aspects of reproduction, as reducing systemic testosterone levels eliminates male Syrian hamster copulatory behavior (Morin & Zucker, 1978) and decreases males' attraction to female vaginal secretion (Petrulis & Johnston, 1995; Powers & Bergondy,

1983). These deficits in sexual behavior can be restored in castrated male hamsters by administration of testosterone, or its primary metabolites (Petrulis & Johnston, 1995; Powers, Bergondy, & Matochik, 1985).

Given the importance of their contributions to successful reproductive behavior, chemosensory and hormonal signals are likely integrated on the neural level. Where and how this integration takes place is relatively unknown, but several limbic nuclei known to mediate reproductive behavior have been suggested as candidate brain regions. The medial amygdala (ME), bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) all receive chemosensory information (Coolen & Wood, 1998; Kevetter & Winans, 1981a, 1981b) and contain steroid hormone receptors (Wood, Brabec, Swann, & Newman, 1992; Wood & Newman, 1993). In the Syrian hamster, unilateral testosterone implants into either the BNST/MPOA or the ME can partially restore copulatory behavior in castrated males, suggesting that steroid hormone action in these nuclei is important for the expression of sexual behavior (Wood & Newman, 1995b). However, unilateral olfactory bulbectomy on the side ipsilateral to steroid implants in the MPOA/BNST (Wood & Newman, 1995a) or ME (Wood & Coolen, 1997) prevents this restoration of copulatory behavior. Thus, the integration of both chemosensory and hormonal cues in these nuclei is required for male sexual behavior.

In particular, the BNST is a candidate site for the chemosensory and hormonal regulation of sexual behavior. However, the BNST is not a homogenous structure; only the posterior BNST (pBNST) has been implicated in the regulation of male sexual behavior, as it receives both chemosensory and hormonal signals. Although the pBNST receives a small, direct projection from the accessory olfactory bulbs (Davis, Macrides, Youngs, Schneider, & Rosene, 1978), most chemosensory information is conveyed to the pBNST via the corticomedial amygdala,

particularly from the ME (Gomez & Newman, 1992; Scalia & Winans, 1975). Specifically, the pBNST is densely interconnected with the ME (Wood & Swann, 2005), such that main and accessory olfactory information that converges on the ME (Newman, 1999) can terminate at the pBNST or continue to the MPOA via the BNST. In addition to receiving chemosensory information, the pBNST contains dense populations of steroid receptor-containing neurons (Li, Blaustein, De Vries, & Wade, 1993; Wood et al., 1992) that are activated during copulation (Wood & Newman, 1993). Furthermore, castration increases the absolute refractory period of neurons in the stria terminalis that project to the MPOA in the Norway rat (Kendrick & Drewett, 1979), suggesting that hormonal cues, potentially from the BNST, can directly affect neural transmission within the mating circuit. These data, when taken together, suggest the pBNST processes both chemosensory and hormonal cues.

Functionally, the pBNST may be involved in generating the motivation to investigate female odors. Large electrolytic lesions including the pBNST in male Syrian hamsters reduce investigation of female hamster vaginal secretion and reduce anogenital investigation during copulation, but only affect mating in a subset of subjects (Powers, Newman, & Bergondy, 1987). In the Norway rat, large lesions including the pBNST alter the copulatory sequence such that subjects display more intromissions before ejaculation, longer intervals between intromissions, and longer post-ejaculatory refractory periods (Valcourt & Sachs, 1979). Similarly, moderate-sized radiofrequency lesions including the pBNST in the Norway rat cause decreased penile erections in response to remote cues from estrous females (Liu, Salamone, & Sachs, 1997), suggesting that the pBNST may mediate male arousal to female cues. Additionally, the pBNST may function as a target for the hormonal modulation of reproduction, as unilateral testosterone implants into the Syrian hamster BNST/MPOA continuum facilitate mating behavior in castrated

males (Wood & Newman, 1995b). This evidence suggests that sexual motivation, mediated by the perception of female odor cues, may be generated in the pBNST.

Although the pBNST has been shown to process both chemosensory and hormonal information, detailed anatomical evidence suggests that these two signals may be processed differentially by two subnuclei within the BNST, the posterointermediate BNST (BNSTpi) and the posteromedial BNST (BNSTpm). First, the BNSTpm and BNSTpi contain different concentrations of steroid receptors; the BNSTpm contains dense populations of neurons expressing androgen and estrogen receptors, whereas the BNSTpi contains relatively fewer of these steroid-sensitive neurons (Doherty & Sheridan, 1981; Wood et al., 1992; Wood & Swann, 2005). Furthermore, there is a sex difference in androgen receptor concentrations within the BNSTpm, such that females have significantly less androgen receptor-containing neurons than males in several species, including the Syrian hamster (Wood & Newman, 1999) and the Norway rat (Roselli, 1991). Finally, the BNSTpm and BNSTpi are strongly interconnected via reciprocal fibers (Wood & Swann, 2005). These pathways may serve to regulate appetitive aspects of reproductive behavior by integrating chemosensory signals with hormonal cues processed in the pBNST.

In addition to intra-BNST connectivity, the BNSTpi and BNSTpm are differentially connected with the neural circuitry that processes chemosensory information in the Syrian hamster. The BNSTpm receives both a small, direct projection from the accessory olfactory bulbs (Scalia & Winans, 1975) and indirect chemosensory information via the ME (Gomez & Newman, 1992), whereas the BNSTpi is limited to indirect odor stimuli via the corticomedial amygdala (Dong, Petrovich, & Swanson, 2001). Projections from the ME to the pBNST also show topographical differences such that the BNSTpi is preferentially connected with the

anterior medial amygdala (MeA), an area with extensive chemosensory input but fewer steroid-sensitive cells. In contrast, the steroid-responsive posterior medial amygdala (MePD), an area with fewer chemosensory inputs but greater numbers of steroid-sensitive cells, preferentially communicates with BNSTpm (Gomez & Newman, 1992). Both the BNSTpm and BNSTpi receive substantial projections from the corticomedial amygdala, including the anterior corticomedial amygdaloid nucleus (ACo), posterior lateral cortical amygdaloid nucleus (PLCo), posterior medial cortical amygdaloid nucleus (PMCo), and amygdalo-hippocampal area (AHA) in the Syrian hamster (Wood & Swann, 2005) as well as the Norway rat (Dong & Swanson, 2004).

Mirroring their differences in connectivity to the ME, the BNSTpi and BNSTpm also differ in their connectivity to diencephalic structures. The BNSTpm sends projections to the medial aspects of the preoptic area, including the medial preoptic nucleus (MPN), and maintains reciprocal connections with periventricular hypothalamic regions including the anteroventral periventricular nucleus (AVPe), ventromedial hypothalamus (VMH), ventral premammillary nucleus (PMV), and arcuate hypothalamic nucleus (ARC). In contrast, the BNSTpi is reciprocally connected to the lateral aspects of the hypothalamus, including the medial preoptic area (MPOA), anterior hypothalamus (AH), and lateral aspects of the VMH (Wood & Swann, 2005). Thus, the BNSTpi has broad connections throughout the limbic system and related areas, whereas the connections of the BNSTpm are restricted principally to other steroid-responsive regions within the limbic system. As such, the separation of chemosensory and hormonal information seems to be maintained in areas that regulate male reproductive behavior.

Ultimately, this evidence indicates that male Syrian hamster reproductive behavior requires chemosensory and hormonal cues, and that pBNST subnuclei may play a role in processing these

cues to produce appetitive sexual behavior. However, no studies have directly tested the functional significance of the pBNST alone in regulating appetitive aspects of reproductive behavior. Thus, the following experiments used site-specific, excitotoxic lesions of the pBNST to test role of this region in regulating male hamsters' preference for, and attraction to, social odors. We hypothesized that pBNST is necessary for generating the appropriate investigation of sexual odors. If so, then lesions of the pBNST should eliminate opposite-sex odor preference, suggesting this nucleus is required for the expression of appropriate appetitive reproductive behavior.

Experiment 1

The goal of Experiment 1 was to test the role of the pBNST in generating a preference to investigate opposite-sex odors in male hamsters. Subjects were tested for their preference for female odors over male odors when presented simultaneously in a Y-maze apparatus. Furthermore, because an observed preference for one odor may reflect an avoidance of the other odor, subjects were tested for their attraction to both male and female odors when presented opposite a clean odor.

Methods

Subjects

Male Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratories (Wilmington, MA, USA) at 3 weeks of age and singly-housed. All experimental subjects were sexually naïve and unrelated to each other (non-siblings). Between 2 and 3 months of age, experimental subjects were gonadectomized and immediately implanted with subcutaneous testosterone Silastic capsules. At least one week after gonadectomy, subjects were

randomly assigned to one of two experimental conditions: a posterior BNST lesion (pBNST-X; *n* = 45) or a sham posterior BNST lesion (SHAM; *n* = 15).

In addition to experimental subjects, a separate group of adult (3-8 months old), gonadally-intact male and female Syrian hamsters served as odor donors (*n* = 80). These odor donors were purchased from Charles River Laboratories at 3 months of age and group housed (3-4 same-sex animals/cage). All odor donors were unrelated to, and had no contact with, experimental subjects. Both experimental subjects and odor donors were housed in solid-bottom Plexiglas cages (36 cm x 30 cm x 60 cm) and maintained on a reversed 14 hr. light / 10 hr. dark photoperiod. Food and water were available *ad libitum*.

Surgery

Gonadectomy and Testosterone Implant. Experimental subjects were anesthetized with 1-2% isoflurane and a midline abdominal incision was made, exposing the testicles. Bilateral removal of the testicles was accomplished via cauterization of the ductus deferens and blood vessels. Vicryl suture (Ethicon, Somerville, NJ) was used to close the smooth muscle, and wound clips were used to close the skin. Immediately after gonadectomy, Silastic capsules (1.57 mm internal diameter, 2.41 mm external diameter, Dow Corning, Midland, MI) packed with 20 mm length of crystalline testosterone (Sigma, St. Louis, MO) were implanted subcutaneously via a small dorsal incision between the scapulae. Wound clips were used to close the incision.

Excitotoxic Lesions. At least one week following gonadectomy, experimental subjects were anesthetized with 1-2% isoflurane (7:3 oxygen: nitrous oxide mix) and positioned in a stereotaxic apparatus such that ear bars immobilized the head and the skull was level. After retracting the temporal muscles, small holes were drilled into the skull, exposing the dura.

Bilateral excitotoxic lesions were made by lowering a microinjection syringe (701R 10µl syringe, Hamilton, Reno, NV) under stereotaxic control into the BNST and injecting N-methyl-D-aspartic acid (NMDA; 20mg/ml) (Sigma, St. Louis, MO) into two bilateral sites targeting the BNSTpi and BNSTpm, respectively. Table 1 summarizes the stereotaxic coordinates and excitotoxin volume for all injection sites. Sham surgeries were identical to lesion surgeries with two exceptions: 1) the microinjection syringe was lowered to 1mm above the target injection site and 2) no excitotoxin was infused into the target injection sites. After all injections, skull holes were sealed using bone wax and incisions were closed with wound clips. Behavioral testing did not commence until at least two weeks after the lesion surgery.

Table 1: Stereotaxic coordinates for pBNST lesions. Bilateral injections of NMDA were directed at the BNSTpi and BNSTpm, respectively, resulting in a total injection volume of 120 nanoliters per hemisphere. Anterior-Posterior (A-P), Medial-Lateral (M-L), and Dorsal-Ventral (D-V) coordinates are all relative to bregma.

Target	A-P	M-L	D-V	Volume
BNSTpi	- 1.9	± 1.65	- 5.7	60 nl
BNSTpm	- 1.9	± 1.05	- 5.8	60 nl
Total				120 nl

Odor Stimuli

Subjects were tested using two different types of odor stimuli: social odor stimuli (male and female) and clean odor stimuli. All social odor stimuli were collected weekly from group-housed animals (3-4 same-sex animals/cage), with two complete odor stimuli collected from a single cage. Each social odor stimulus consisted of soiled cotton bedding (4 Nestlets, 12 g, ANCARE, Bellmore, NY), soiled corncob litter (50ml) and one damp cotton gauze pad that was

used to wipe the inner walls of the cage. In addition, a damp gauze pad was used to wipe two of the four cage residents' anogenital region and bilateral flank glands ten times each. For female odor stimuli, vaginal secretion was also collected onto a gauze pad by gently palpating the vaginal area of an estrous female with a disposable probe, and was added to each odor stimulus. Clean odor stimuli consisted of clean cotton bedding (4 Nestlets), clean corncob litter (50 ml), and clean cotton gauze pads (2). For all odor stimuli collections, latex gloves were worn to prevent odor transfer during collection. All odor stimuli were stored in plastic bags at 4°C until twenty minutes before use. Odor stimuli older than one month were discarded, and care was taken to ensure that no subject was tested with the same social odor stimulus more than once.

Behavioral Testing

Each subject was tested in four Y-maze tests: Clean, Preference, Attraction to female, and Attraction to male. First, to habituate subjects to the Y-maze and also obtain baseline levels of activity, subjects were exposed to clean odor stimuli in each stimulus chamber (Clean Test). Next, to test for opposite-sex odor preferences, subjects were simultaneously exposed to male and female odors in opposite stimulus chambers (Preference Test). Last, to test for levels of attraction to female and male odors separately, subjects were exposed to each sex odor stimulus (female or male) against a clean odor stimulus (Attraction Tests) (Figure 1).

Preference for, and attraction to, odor stimuli were tested in an enclosed, Plexiglas Y-maze. The Y-maze consists of a stem arm (61 cm long) and two side arms (68 cm long). All arms of the maze are 10 cm wide and have walls 10 cm high. The side arms each angle away from the stem at 120° and, at half of its length, angle back inward 120°, forming a Y shape. A start chamber (20 cm long) with a removable perforated door lies at the distal end of the stem. Each side arm has an odor stimulus chamber (20 cm long) at its distal end, separated by

removable perforated doors that prevent contact with the odor stimulus but allow airflow. An electric fan behind the start chamber pulls air from the distal stimulus chambers through the entire length of the Y-maze.

Each test lasted ten minutes; subjects were placed in the start chamber for one minute, then the start chamber door was removed and subjects were allowed to explore the maze for nine minutes. All testing was done in the first six hours of the dark phase and under dim illumination. Surfaces of the Y-maze were cleaned with 70% alcohol and allowed to dry between subjects. The top of the maze was secured with a clear Plexiglas top to allow for overhead video recording of animals' behavior. To conserve stimulus odors, odor stimuli were re-used once. All Y-maze tests were conducted on separate days, with 1-3 days separating each test. The order of stimulus presentation and stimulus side was counterbalanced across lesion groups. For all tests, latex gloves were worn while handling odors to prevent odor transfer.

Video recordings of the tests were digitized onto a computer and scored using the Observer for Windows, version 5.0 (Noldus Information Technology B.V., Wageningen, Netherlands). The number of entries into each arm was used to assess general levels of motor activity, and the duration of time spent investigating the stimulus chamber was used to measure the subject's interest for that odor. Investigation of the stimulus chamber was scored when the subject contacted or directed its nose within 1 cm from the stimulus chamber door. All observers were blind to the condition of the subject and different observers reached at least an 85% inter-observer reliability score prior to scoring tests.

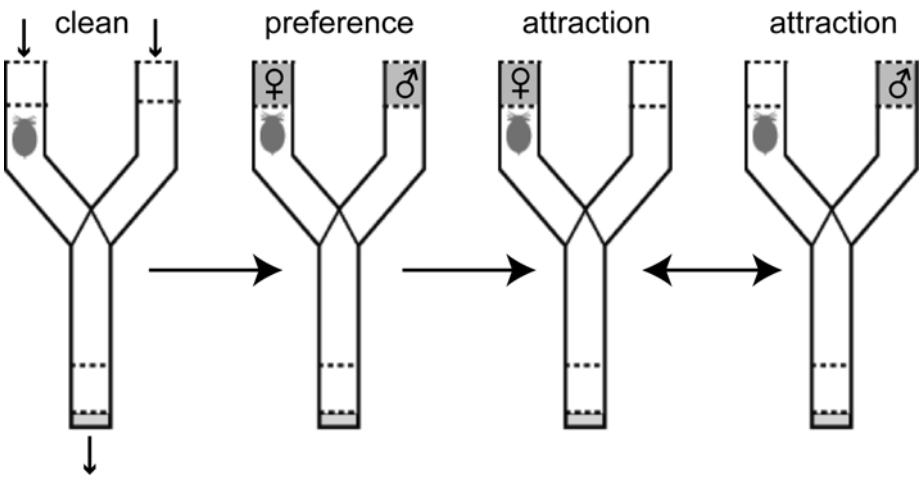

Figure 1: Illustration of Y-maze behavioral testing sequence. Subjects were tested in 4 Y-maze tests. Odorized air was pulled towards subjects from stimulus chambers containing clean vs. clean odors (Clean test), male vs. female odors (Preference test), and female or male vs. clean odors (Attraction tests). The order of Attraction tests was counterbalanced across lesion groups.

Histology and Lesion Verification

Following the last behavioral test, subjects were injected with an overdose (.15-.20 ml) of Sleepaway Euthanasia Solution (sodium pentobarbital with 7.8% isopropyl alcohol, Fort Dodge Animal Health, Fort Dodge, IA) and transcardially perfused with 200 ml of 0.1M phosphate-buffered saline (PBS, pH 7.4) followed by 200 ml of 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde at least overnight and then cryoprotected in 30% sucrose in PBS solution. Subsequently, brains were sectioned in the coronal plane at 30 μm on a cryostat and stored in cryoprotectant until staining.

In order to determine the location and extent of lesion damage, every third section of tissue was processed for immunohistochemical localization of neuronal nuclei protein (NeuN), a neuron-specific protein (Mullen, Buck, & Smith, 1992). Using NeuN to visualize excitotoxic lesions is advantageous because areas damaged by excitotoxin do not label with NeuN, thus

simplifying lesion analysis. Additionally, adjacent sections were Nissl-stained using cresyl violet (Sigma, St. Louis, MO), further aiding lesion analysis by allowing for the differentiation of fiber tracts from lesioned areas.

Immunohistochemistry

One in every three sections of tissue collected was processed for immunohistochemical localization of NeuN. Free-floating sections were washed ten times with PBS before incubation in a blocking solution for 30 minutes at room temperature. Following ten washes, sections were incubated overnight in a primary antibody to NeuN (1:100K, mouse anti-NeuN, Chemicon, Temecula, CA). The following day, sections were rinsed ten times and incubated sequentially in biotinylated secondary antibody (1:600, rabbit anti-mouse, Jackson Immunoresearch Laboratories, West Grove, PA), followed by avidin-biotinylated enzyme complex (Vectastain Elite ABC Kit, Vector, Burlingame, CA), with ten washes between each sequential step. The complex was visualized with diaminobenzidine with 30% hydrogen peroxide (Sigma, St. Louis, MO). Sections were mounted onto gelatin-coated slides, dehydrated, and coverslipped.

Blood Collection and Radioimmunoassay

To confirm that Silastic testosterone capsule implants maintained physiologic levels of hormone, serum testosterone levels were measured by radioimmunoassay (RIA). Blood samples were collected from the inferior vena cava immediately prior to perfusion and stored in vacutainer collection tubes (VWR, West Chester, PA, 4 ml draw) on ice until centrifuging. Samples were centrifuged at 3200 rpms, at 4°C for 20 minutes. Serum was stored in 200 μl aliquots at -20°C until the testosterone assay. Testosterone levels were measured by RIA kits from Diagnostics System Laboratories (DSL 4000 Testosterone) with an inter-assay reliability of

3%, as previously validated for Syrian hamster serum (Cooper, Clancy, Karom, Moore, & Albers, 2000). Subjects with systemic testosterone levels that failed to reach physiologic levels (2-7 ng/ml) were excluded from analysis.

Statistical Analysis

Separate repeated-measures ANOVAs for each test type (Clean test, Preference test, Attraction tests) were used to compare the durations of investigation of each odor stimulus. Each of these analyses had experimental group (pBNST-X and SHAM) as the between-subjects factor and stimulus odor (male, female, or clean) as the repeated measure. Because SHAM males failed to show a significant attraction to female odors, paired *t*-tests (2-tailed) were used to test within-group effects in the female Attraction test. A one-way ANOVA was used to compare testosterone levels across lesion groups. To compare overall levels of activity across experimental groups, the total number of arm entries during the clean tests was analyzed using an ANOVA with experimental group as the between-subjects factor.

To eliminate extreme cases of side bias during Y-maze testing, a side preference ratio (the amount of time spent investigating the right side of the maze divided by the time spent investigating both sides of the maze during the clean test) was calculated for each subject. Subjects that obtained a side preference ratio > 0.8 (lesion $n = 3$, sham $n = 2$) or < 0.2 (lesion $n = 2$, sham $n = 1$) were considered to have an extreme side bias and were removed from the analysis. In order to control for side bias in the remaining subjects, the difference between the amount of time spent on the right and left sides of the maze during the clean test was entered as a covariate in all remaining analyses. Additionally, a variable coding for which social odor was on the right side of the maze was included as an additional between-subjects factor in all remaining analyses.

Results

Lesion Reconstruction

Based on analysis of both NeuN and Nissl stains, subjects with lesions that failed to damage a significant portion of the pBNST (*n* = 21), or extended significantly outside of the pBNST (*n* = 10), were excluded from analysis. Subjects were included in the pBNST-X lesion group (*n* = 14) only if they had extensive bilateral damage to the pBNST. Specifically, subjects were included only if they had at least 60% bilateral pBNST damage in at least two planes of section (Morin & Wood, 2001).

All fourteen subjects included in the pBNST-X lesion group had damage primarily restricted to the pBNST. At the largest part of the pBNST (Bregma 0.2mm), twelve males had bilateral lesion damage; eleven of these males had bilateral damage to the BNSTpi, BNSTpm, and BNSTpl. More rostral and anterior (Bregma 0.5mm), eight males had bilateral lesion damage; four of these males had damage to both the BNSTpi and BNSTpm, whereas two males had damage only to the BNSTpi or BNSTpm, respectively. Three males also had bilateral damage of the BNSTpl at this level. More caudal and posterior (Bregma -0.1mm), ten males had bilateral lesion damage; of these males, five had bilateral damage of both the BNSTpi and BNSTpm, and five had bilateral damage only to the BNSTpm. Two males also had bilateral damage of the BNSTpl at this level. At the most caudal and posterior level of the pBNST (Bregma -0.1mm), six males had bilateral lesion damage; four of these males had damage to the BNSTpi and BNSTpm, while two of these males had bilateral damage to only to the BNSTpm.

Subjects in the pBNST-X group sustained partial or unilateral damage to other adjacent nuclei. This included nuclei within the anterior BNST, such as the anterointermediate BNST (BNSTai; *n* = 4), anteromedial BNST (BNSTam; *n* = 3), and anteroventral BNST (BNSTav; *n* =

5). Outside of the BNST, partial or unilateral damage was sustained to sub-cortical nuclei including the ventral lateral septum (LSV; *n* = 3), paraventricular hypothalamic nucleus (Pa; *n* = 4), and globus pallidus (GP; *n* = 2). Finally, partial or unilateral damage was also incurred in thalamic nuclei, such as the anterodorsal thalamic nucleus (AD; *n* = 2), anteroventral thalamic nucleus (AV; *n* = 2), ventrolateral anteroventral thalamic nucleus (AVVL; *n* = 1), reuniens thalamic nucleus (Re; *n* = 5), and reticular thalamic nucleus (Rt; *n* = 3). Two males also sustained unilateral damage to overlying cortex. These males did not differ in behavior from males without damage to adjacent nuclei and were kept in the analysis.

In most SHAM lesion males (*n* = 14), only injection needle tracts were visible. One SHAM male sustained significant lesion damage to the pBNST and was excluded from the analysis. One SHAM male also had unilateral damage to overlying cortex, but did not differ behaviorally from other SHAM males so was kept in the analysis.

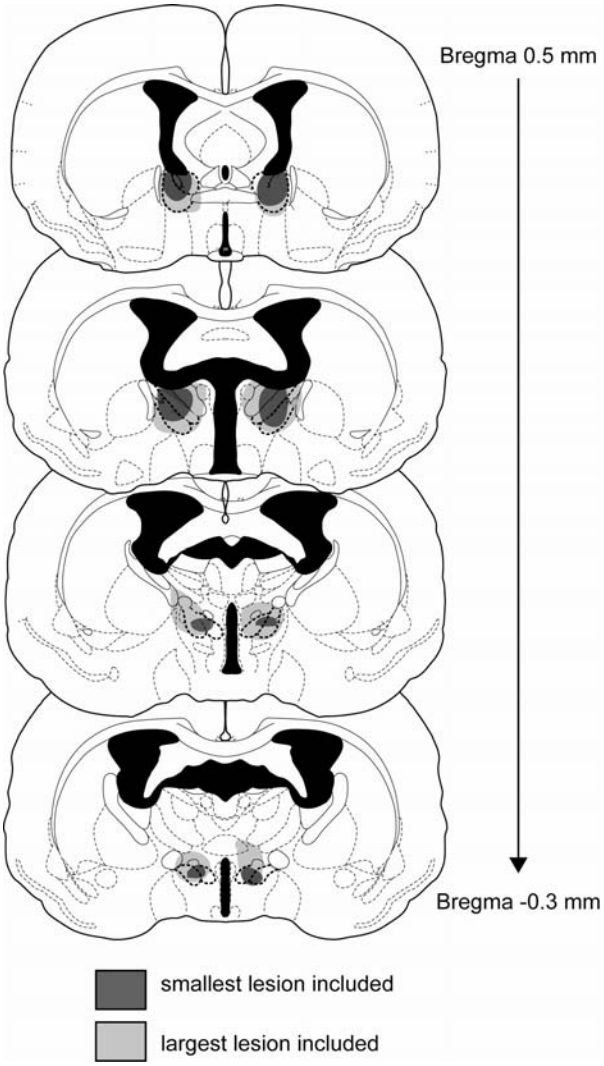

Figure 2: Reconstruction of the smallest (dark grey) and largest (light grey) lesions in pBNST-X males. The pBNST is indicated with bold dashed lines; sections proceed from the most rostral to the most caudal level.

Behavioral Measures

Clean Test. Levels of activity, as measured by the total number of arm entries made in the clean test, were not different across experimental groups $F(1, 24) = 0.389$, $p > .05$. Furthermore, when the investigation times were summed for the left and right arms, experimental groups did

not differ in their total levels of investigation $F(1, 24) = .001$, $p > .05$. However, both experimental groups showed a significant bias towards the right side of the Y-maze in the clean test. Specifically, pBNST-X males spent significantly more time investigating the right stimulus chamber than the left stimulus chamber $F(1, 11) = 9.276$, $p < .05$, and SHAM males entered the right arm significantly more than the left arm $F(1, 11) = 4.054$, $p < .05$. In subsequent tests, both pBNST-X and SHAM males showed evidence of a right-side bias. Table 2 summarizes the measures of investigation of the right and left sides of the Y-maze for all tests across experimental groups.

Table 2: Mean investigation times (seconds) for the right and left stimulus chambers and mean times (seconds) spent in the right and left arms in the Y-maze. pBNST-X males spent more time investigating the right stimulus chamber during the clean test, whereas SHAM males spent more time investigating the right stimulus chamber and more time in the right arm of the Y-maze during the Preference and Attraction to female tests, * $p < .05$.

	Mean stimulus investigation time				Mean time spent in arm			
	pBNST-X		SHAM		pBNST-X		SHAM	
Test	Left	Right	Left	Right	Left	Right	Left	Right
Clean	43.85	79.21*	71.59	51.46	175.98	212.31	184.32	166.99
Pref	72.71	82.28	62.68	92.08*	189.15	225.61	183.79	220.03*
Attract-F	52.70	76.60	63.51	83.73*	189.67	194.73	163.76	192.85*
Attract-M	59.45	62.68	63.19	66.58	192.02	186.41	172.95	189.41

Preference Test. The difference between the amount of time spent on the right and left sides of the maze during the clean test was entered as a covariate, and a variable coding for which social odor was on the right side of the maze was included as an additional between-subjects factor to control for side bias in the analysis of the Preference test. There was a significant interaction between experimental group and preference for investigating female over male odors, $F(2, 15) = 5.261$, $p < .05$. SHAM males preferred to investigate female odors, as indicated by their longer investigation of female odors compared to male odors $F(1, 9) = 5.586$, $p < .05$. In contrast, pBNST-X males showed no preference for either odor and investigated the two social odors equally $F(1, 7) = .312$, $p > .05$ (Figure 3a).

Attraction Tests. The same statistical controls used in the Preference test were applied to the analysis of the Attraction tests. No interaction between experimental group and attraction to female odors was observed $F(2, 15) = 1.070$, $p > .05$. However, when analyzed within-group, pBNST-X males investigated female odors significantly less than clean odors $t(7) = 2.230$, $p < .05$. Moreover, pBNST-X males spent less time investigating female odors than SHAM males during the attraction test $t(17) = 1.586$, $p < .05$ (Figure 3b). No interaction between experimental group and attraction to male odors was observed $F(2, 15) = .204$, $p > .05$, suggesting that both SHAM and pBNST-X males spent equal amounts of time investigating male and clean odors (Figure 3c).

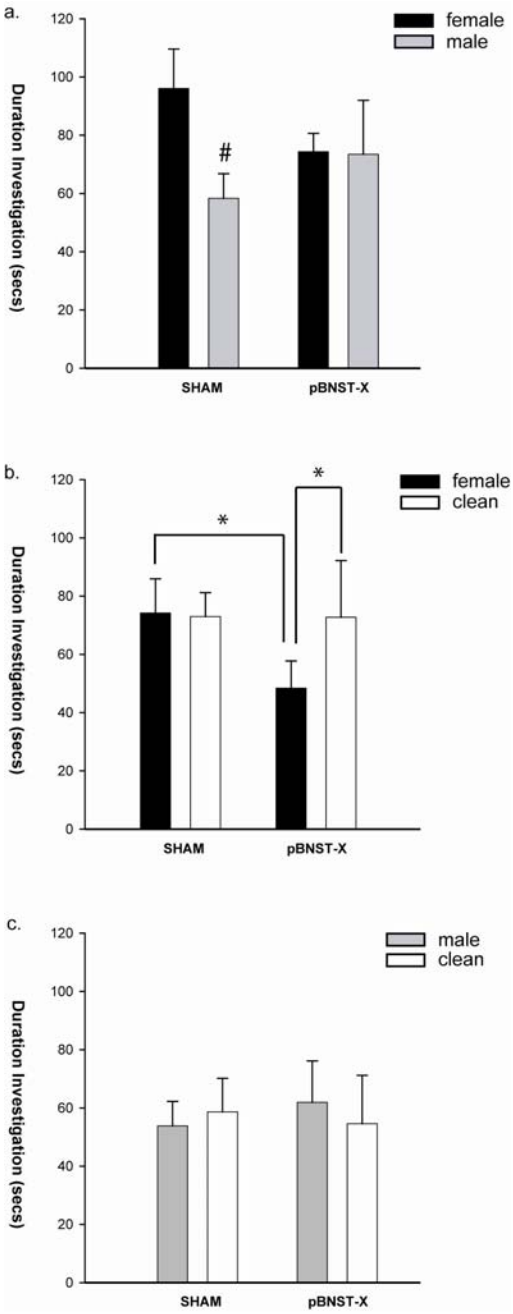

Figure 3: Mean investigation times (± S.E.M.) from each Y-maze test. (a) SHAM males, but not pBNST-X males, preferred to investigate female over male odors, # indicates significant interaction between experimental group and preference to investigate female odor, $p < .05$. (b) pBNST-X males spent less time investigating female odors than clean odors and spent less time investigating female odors than SHAM males did, * indicates significant difference between investigation times, $p < .05$. (c) Both pBNST-X and SHAM males spent equal amounts of time investigating male and clean odors.

Testosterone Assay

All subjects' testosterone levels were within the physiologically-relevant (2 -7 ng/nl) range. There was no difference in mean testosterone levels between experimental groups; pBNST-X = 2.77 ± 0.511 ng/nl; SHAM = 2.56 ± 0.349 ng/nl, $F(1, 16) = .114$, $p > .05$.

Summary

The results of Experiment 1 demonstrate that the pBNST-X males show no preference to investigate male over female odors, suggesting the pBNST mediates the preference to investigate opposite-sex odors in male hamsters. Additionally, the pBNST appears to play a role in the attraction to female odors in male Syrian hamsters. Although SHAM males did not show an attraction to female odors, making it difficult to interpret the behavior of pBNST-X males, pBNST-X males spent significantly less time investigating female odors when they were presented opposite clean odors. Moreover, pBNST-X males spent significantly less time investigating female odors than SHAM males did. In contrast, the pBNST does not appear to affect male hamsters' investigation of male odors, as both pBNST-X and SHAM males spent equal amounts of time investigating male and clean odors.

Experiment 2

Experiment 1 demonstrated that males with pBNST lesions do not show a preference to investigate female odors over male odors. This lack of preference, however, may reflect either a decrease in the motivation to investigate female odors or a sensory deficit in the ability to discriminate between the two odors. Thus, in Experiment 2, a habituation-dishabituation model was used to test the ability of the subjects in Experiment 1 to discriminate between male and female odors. In addition, to demonstrate that any deficits observed in this test were specific to

social odors, a subset of subjects (pBNST-X n = 5, SHAM n = 9) was also tested for their ability to discriminate between two complex, non-social odor stimuli.

Methods

Behavioral Testing

A habituation-dishabituation test was used to determine whether subjects were able discriminate between odor stimuli. This test involves repeated presentations of the same odor source followed by a test presentation of a novel odor source. A decrease in investigation time (habituation) during the repeated presentations indicates a perception of the odors as being the same or familiar, whereas an increase in investigation time (dishabituation) during presentation of the novel odor indicates an ability to discriminate between the two odors.

The testing sequence consisted of four, 3-minute presentations of repeated odors (habituation), followed by a fifth, 3-minute presentation of a novel odor (dishabituation test). Five-minute inter-trial intervals separated each odor presentation trial. Odor stimuli were presented in modified 50 ml polypropylene collection tubes with 1 cm holes drilled 1 cm apart along the surface of the tube. Wire mesh lined the inner surface of the odor container to prevent contact with the odor stimulus.

The odor container was placed in the center of the subject's cage, and investigation time was measured with a stopwatch. Investigation of the odor was scored when the subject's nose contacted, or came within 1 cm, of the odor container. Subjects were tested in pairs, such that one subject was tested with an individual odor stimulus first, and that odor stimulus was then transferred to a clean odor container to be used as the stimulus for the other subject in the pair. In this way, each odor was used two times, but was always presented in a clean odor container to avoid transfer of subject odor across trials. The pair order was reversed across test days and

counterbalanced across lesion groups. Odor containers were cleaned with 70% alcohol and allowed to dry for at least 12 hours prior to re-use. Each habituation-dishabituation test was conducted on separate days, with 1-3 days separating each test. The order of testing sequence was counterbalanced across lesion groups.

Odor stimuli

Social odor stimuli were collected as in Experiment 1, but the volumes were reduced to fit the odor presentation containers. Each odor stimulus consisted of samples of the flank and anogenital region (two gauze pads), odors from the inside walls of a soiled cage (one gauze pad), soiled cotton bedding (1/2 Nestlet, 2g) and soiled corncob litter (10 ml). The female odor containers also included vaginal secretions (one gauze pad). In non-social odor tests, odor stimuli consisted of two clean gauze pads, clean cotton bedding (1/2 Nestlet), clean corncob litter (10 ml), and 3 synthetic strawberry- or baby powder-scented beads (International Flavors and Fragrances, Inc., New York, NY). As in Experiment 1, latex gloves were worn during odor collection and behavior testing to prevent odor transfer. All odor stimuli were stored in plastic bags at 4°C until twenty minutes before use. Odor stimuli older than one month were discarded, and care was taken to ensure that no subject was tested with the same social odor stimulus more than once.

Discrimination of male and female odors. To determine if pBNST-X males could discriminate between social odors, subjects were tested with a habituation-dishabituation model using male and female odors as the odor stimuli. The testing sequence consisted of four, 3-minute presentations of different male odor stimuli followed by a fifth, 3-minute presentation of a female odor stimulus (Figure 4a). Which social odor was used for the habituation stimulus

could not be counterbalanced, as male hamsters do not habituate to repeated presentations of female odors (Maras & Petrulis, 2006).

Discrimination of non-social odors. In addition to being tested for the ability to discriminate between social odors, a sub-set of subjects was tested for the ability to discriminate between two complex, non-social odors. The testing sequence consisted of four, 3-minute presentations of either strawberry or baby powder odor stimuli, followed by a fifth, 3-minute presentation of the opposite non-social odor stimulus. Which odor was used for the habituation stimulus was counterbalanced across lesion groups (Figure 4b).

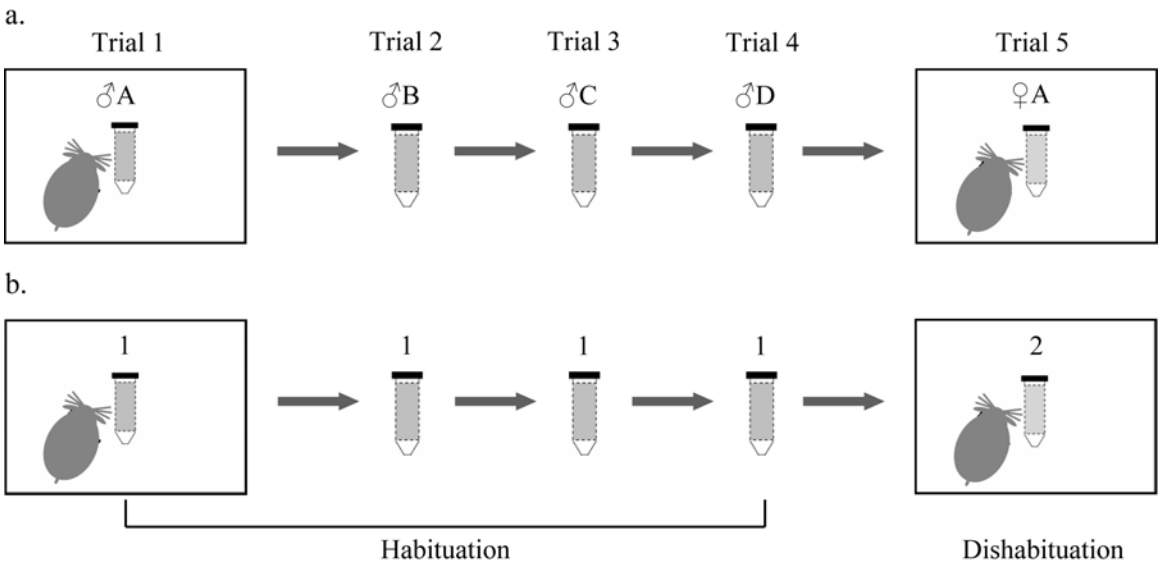

Figure 4: Illustration of Habituation-Dishabituation testing sequence. All tests consisted of four habituation trials followed by a dishabituation test. (a) Social discrimination tests involved repeated presentations of individual male odors followed by a test presentation of a female odor. (b) Non-social discrimination tests involved repeated presentations of a complex, non-social odor (either strawberry or baby powder) followed by a test presentation of the opposite non-social odor.

Statistical Analysis

To determine whether subjects habituated to the repeated odor stimulus in the habituation-dishabituation tests, paired *t*-tests (1-tailed) were used to compare the time spent investigating the odor on the first and fourth trials. A significant decrease in investigation between the first and fourth trial reflected a habituation to the repeated odor stimulus. To assess whether subjects could discriminate between the habituated odor and the novel odor, paired *t*-tests (2-tailed) were used to compare the investigation times during the fourth trial of the repeated odor and the test trial with the novel odor. A significant increase in investigation of the novel odor reflected a discrimination between the repeated and test odors.

Results

Behavioral Measures

Discrimination of social odors. Both experimental groups habituated to the repeated presentations of different male odors, as indicated by decreased investigation of the male odor on the fourth trial compared to the first trial (pBNST-X, $t(13) = 2.998$, $p < .01$; SHAM $t(13) = 3.612$, $p < .01$). Importantly, all lesion groups also dishabituated to the novel female odor, as indicated by an increased investigation of the test female odor compared to the last presentation of the habituated male odor (pBNST-X, $t(13) = 4.616$, $p < .01$; SHAM $t(13) = 5.213$, $p < .01$). These results suggest that lesions of the pBNST do not disrupt male hamsters' ability to discriminate between male and female odors (Figure 5a).

Discrimination of non-social odors. Both experimental groups habituated to repeated presentations of the non-social odors, as indicated by a decreased investigation on the fourth trial compared to the first trial (pBNST-X $t(4)= 4.121$, $p < .01$; SHAM $t(8) = 3.164$, $p < .01$). Importantly, both experimental groups also discriminated between the habituated odor and the

other non-social odor, as indicated by an increased investigation of the test odor compared to the last presentation of the habituated odor (pBNST-X $t(4) = 3.053$, $p < .01$; SHAM $t(8) = 3.028$, $p < .01$). These results suggest that lesions of the pBNST do not impair male hamsters' ability to discriminate between non-social odors (Figure 5b).

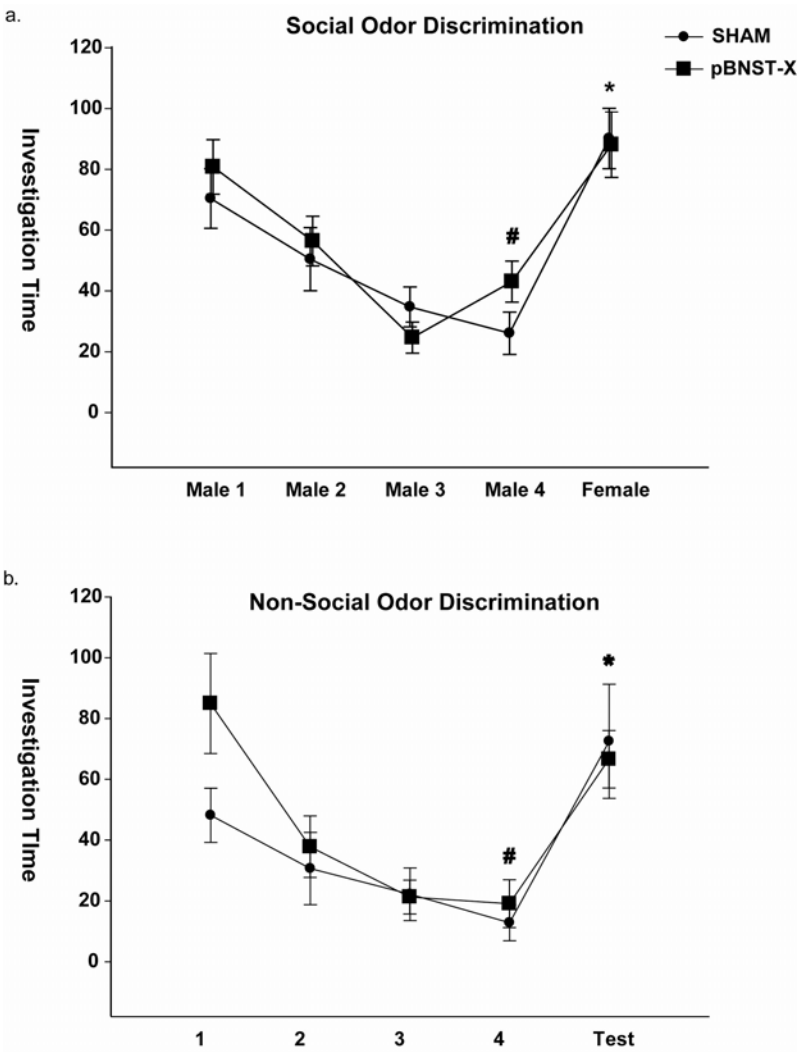

Figure 5: Mean investigation times (seconds ± S.E.M.) for Habituation-Dishabituation tests. (a) In the social odor discrimination tests, both pBNST-X and SHAM males habituated to repeated presentations of individual male odors, # indicates $p < .05$ for Male 1 vs. Male 4, and dishabituated to the female test presentation, * indicates $p < .05$ for Male 4 vs. Female. (b) In the non-social odor discrimination tests, both pBNST-X and SHAM males habituated to repeated presentations of a non-social odor, # indicates $p < .05$ for 1 vs. 4, and dishabituated to the non-social odor test presentation, * indicates $p < .05$ for 4 vs. Test.

Summary

The results of Experiment 2 demonstrate that pBNST-X and SHAM males are able to discriminate between social odors when presented sequentially. This suggests that the deficit in opposite-sex odor preference observed in pBNST-X males in Experiment 1 was not due to an inability to discriminate between the male and female odors. Moreover, pBNST-X and SHAM males were able to discriminate between non-social odors, further confirming that lesions of the pBNST do not cause general chemosensory deficits. Thus, lesions of the pBNST do not interfere with the ability to discriminate between social or non-social odors.

General Discussion

Taken together, the results of Experiment 1 and Experiment 2 demonstrate that the pBNST is critical for opposite-sex odor preference in male Syrian hamsters. Lesions of the pBNST eliminated male hamsters' preference for female odors over male odors when presented simultaneously. Furthermore, pBNST-X males spent significantly less time investigating female odors than clean odors and significantly less time investigating female odors than SHAM males did. Although SHAM males did not show a significant attraction to female odors, making it difficult to interpret the behavior of pBNST-X males, these results suggest that lesions of the pBNST decrease male hamsters' attraction to female odors. Interestingly, this effect seems to be specific to female odors, as lesions of the pBNST had no effect on male hamsters' investigation of male odors. Finally, the deficits observed in pBNST-X males are not due to a failure to discriminate between odors, as both pBNST-X and SHAM males were able to distinguish between both social and non-social odors in habituation-dishabituation tests.

Data from previous lesion studies support our finding that the pBNST mediates the appetitive or motivational aspects of male sexual behavior. In the rat, radiofrequency lesions

including the pBNST delay the copulatory sequence (Valcourt & Sachs, 1979) and decrease non-contact erections to female stimuli (Liu et al., 1997). In male hamsters, large, electrolytic lesions including the pBNST reduce investigation of female hamster vaginal secretion and anogenital investigation during copulation (Powers et al., 1987). These studies are in agreement with our present findings, as pBNST-X males spent less time investigating female odors than clean odors, suggesting a decreased motivation to investigate opposite-sex odors. Moreover, the current results suggest that the deficits observed in previous studies may be attributed to the pBNST specifically, rather than the BNST as a whole.

Although previous studies have addressed the role of the BNST in other appetitive and consummatory aspects of male sexual behavior (Liu et al., 1997; Powers et al., 1987; Valcourt & Sachs, 1979), the lesions in those studies were either electrolytic or radiofrequency, raising the question of whether the effects could be attributed to cell damage within the BNST or disruption of fibers passing through or nearby. This issue is of particular relevance for the BNST, as the fibers of the stria terminalis (St) and stria medullaris (Sm) pass through and adjacent to the BNST, respectively. Most notably, fibers within the St convey information from the ME to the BNST and MPOA (Wood & Swann, 2005). Given the importance of these nuclei in male sexual behavior (Hull & Dominguez, 2007), it is possible that previous reports of lesion-induced deficits were due to disruption of information transfer between these nuclei, rather than damage to the BNST itself. This disambiguation may be particularly pertinent when distinguishing between deficits mediated by the BNST and MPOA, as studies that directly compare these two nuclei find that they regulate different aspects of male sexual behavior (Liu et al., 1997; Powers et al., 1987). Additionally, the St and Sm also contain projections to and from other nuclei important for sexual behavior, such as the VMH, PMV, and PMCo (Saper, Swanson, & Cowan,

1976). The use of the excitotoxins, as in the current study, prevents interpretational difficulties that result from damage to these fiber tracts.

Within the pBNST, anatomically separate and functionally dissociated subnuclei may be responsible for the deficits in attraction and preference observed in the current study. Specifically, several lines of evidence suggest that the BNSTpm may mediate appetitive sexual behavior in males. First, electrolytic lesions limited to the BNSTpm increase latencies to first mount and first intromission, and cause inappropriate anogenital investigation during the mating sequence in sexually-naïve Norway rats (Claro, Segovia, Guilamon, & Del Abril, 1995). Second, the BNSTpm contains dense populations of steroid receptor-containing neurons that are activated during copulatory behavior in male hamsters (Kollack & Newman, 1992). Notably, neither zinc sulfate lesions of the main olfactory epithelium (Fernandez-Fewell & Meredith, 1998) nor VNO removal (Fernandez-Fewell & Meredith, 1994) prevent this mating-induced c-fos induction, suggesting that the main or accessory olfactory systems are individually capable of producing activation in the BNSTpm. Alternatively, this activation may be a result of non-sensory aspects of chemoinvestigation, such as approach behavior. Together, this evidence suggests that the BNSTpm might mediate the deficits in female odor investigation observed in the current study.

Although the BNSTpm receives a small, direct projection from the accessory olfactory bulb, most of the chemosensory information the pBNST receives is transmitted indirectly from other corticomedial amygdala structures to the BNSTpi (Scalia & Winans, 1975). Less is known about the BNSTpi than the BNSTpm, but data from c-fos studies suggest it may be involved generating the appropriate response to social odors. For example, in addition to responding to reproductive odors, the BNSTpi shows increased c-fos expression in response to agonistic

encounters in male hamsters (Kollack-Walker & Newman, 1995) and exposure to predator odors in rats (Dielenberg, Hunt, & McGregor, 2001). This suggests that the BNSTpi may play a role in categorizing all social odors, not just sexual odors, and generating the appropriate response. Although there is less evidence to support this claim, damage to the BNSTpi may be responsible for the preference and attraction deficits observed in the current study.

In addition to the BNSTpi and BNSTpm, the posterolateral subnucleus of the BNST (BNSTpl) sustained significant damage in most pBNST-X males, raising the possibility that the post-lesion deficits in preference and attraction in the current study are due to BNSTpl damage. The BNSTpl is a small, but distinct group of cells that lies lateral to the BNSTpi in the more rostral portions of the pBNST. However, unlike the BNSTpi and BNSTpm, the BNSTpl is connected primarily with neural areas that are involved in motivated behaviors other than reproduction (i.e. basolateral and central amygdala, lateral preoptic area and lateral hypothalamus, striatum and pallidum) (Wood & Swann, 2005). In the absence of any direct evidence, the anatomy suggests that it is unlikely that BNSTpl damage is the primary mediator of preference for, and attraction to, opposite-sex odors within the pBNST.

The mechanism by which pBNST lesions produce deficits in preference and attraction is unknown, but these deficits may be due in part to alterations in the perceived rewarding properties of female odors. Sexual pheromones are regarded as natural reinforcers in many species, including the hamster (Lanuza et al., 2008). Indeed, male hamsters are highly motivated to approach and investigate female odors (Johnston, 1974). Anatomical evidence suggests the pBNST may play a role in evaluating the reinforcing properties of social odors, as it sends and receives direct projections to the mesolimbic dopamine system in the hamster. Specifically, the BNSTpm and BNSTpi both send moderate projections to the ventral tegmental area (VTA) and

the nucleus accumbens (NAc) shell. The BNSTpl also sends projections to the VTA and has bidirectional connections with both the shell and core of the NAc (Wood & Swann, 2005). Given this connectivity, the pBNST may provide social odor information to the dopaminergic circuitry of the mesolimbic reward system, allowing for the assignment of reward value to odor stimuli.

A constraint of the current study is the finding that males from both groups spent, on average, more time on the right side of the Y-maze. There are several possible reasons for this side bias. First, it is possible that the bias is due to unintended lesion damage to basal ganglia structures adjacent to the pBNST; unilateral damage to these structures is known to cause contralateral turning behavior (Papadopoulos & Huston, 1979). This possibility is unlikely, however, as a right-side bias was observed in both pBNST-X and SHAM males. Another explanation for the observed side bias is that, during testing, subjects developed a preference for extra-maze cues on the right side of the room. This possibility also is not likely, as the sides of the maze are opaque, restricting subjects' use of visual cues outside the maze. Furthermore, testing was conducted in two separate rooms that had different objects around the maze, making it improbable that subjects using different sets of extra-maze cues would all develop a preference for the objects on the right.

There are, however, several reports of a right-side bias in rodents, which indicate that our results may reflect an intrinsic directional bias in hamsters. For example, in an odor preference test, female mice show an unbalanced exploration of a rectangular testing arena, such that they spend more time on the right side of the arena (Martinez-Ricos, Agustin-Pavon, Lanuza, & Martinez-Garcia, 2008). Similarly, rats make more right turns both within and between arms of an elevated plus-maze and show a bias to turn into the right arm of the T-maze across repeated

trials (Alonso, Castellano, & Rodriguez, 1991; Schwarting & Borta, 2005). Finally, when started in the center of an elevated platform, almost 80% of Syrian hamsters choose to descend on the right side (Giehrl & Distel, 1980), suggesting that male hamsters also have a spontaneous directional preference for the right. Although it is possible that the side bias may have interfered with the results of the Y-maze tests in the current study, appropriate measures were taken to statistically control for this possibility. To circumvent this bias in the future, a testing apparatus with less pronounced directional architecture will be used.

Ultimately, the results of the current study add support to the growing body of evidence that suggests the pBNST plays an important role in the regulation of male reproductive behavior. However, it remains unknown how the chemosensory and steroid signals crucial for male reproductive behavior are processed within the pBNST. To this end, we plan to directly test the roles of the BNSTpi and BNSTpm in opposite-sex odor preference using site-specific, excitotoxic lesions of these individual subnuclei. Recent work in our laboratory has shown that the MeA and MePD, which preferentially project to the BNSTpi and BNSTpm, respectively, regulate distinct aspects of opposite-sex odor preference in male Syrian hamsters. Specifically, MePD lesions eliminate preference for female odors and decrease attraction to female odors, suggesting decreased sexual motivation. In contrast, MeA lesions also eliminate preference for female odors, but drastically increase levels of investigation to both male and female odors, suggesting an inability to categorize the relevance of the odor stimuli (Maras & Petrulis, 2006). Given the conservation of dissociated chemosensory and steroid-sensitive pathways throughout the circuit, we might expect a similar behavioral dissociation of the pBNST sub-nuclei.

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