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Chemosensory and hormone information are relayed directly between the medial amygdala, posterior bed nucleus of the stria terminalis, and medial preoptic area in male Syrian hamsters

Laura E. Been and Aras Petrulis

Georgia State University Neuroscience Institute 100 Piedmont Avenue Atlanta, GA, 30303, USA

Abstract

In many rodent species, including Syrian hamsters, the expression of appropriate social behavior depends critically on the perception and identification of conspecific odors. The behavioral response to these odors is mediated by a network of steroid-sensitive ventral forebrain nuclei including the medial amygdala (Me), posterior bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA). Although it is well-known that Me, BNST, and MPOA are densely interconnected and each uniquely modulates odor-guided social behaviors, the degree to which conspecific odor information and steroid hormone cues are directly relayed between these nuclei is unknown. To answer this question, we injected the retrograde tracer, cholera toxin B (CTB), into the BNST or MPOA of male subjects and identified whether retrogradely-labeled cells in Me and BNST 1) expressed immediate early genes (IEGs) following exposure to male and/or female odors or 2) expressed androgen receptor (AR). Although few retrogradely-labeled cells co-localized with IEGs, a higher percentage of BNST- and MPOA-projecting cells in the posterior Me (MeP) expressed IEGs in response to female odors than to male odors. The percentage of retrogradely-labeled cells that expressed IEGs did not, however, differ between female- and male- odor-exposed groups in the anterior Me (MeA), posterointermediate BNST (BNSTpi), or posteromedial BNST (BNSTpm). Many retrogradely-labeled cells co-localized with AR, and a higher percentage of retrogradely-labeled MeP and BNSTpm cells expressed AR than retrogradely-labeled MeA and BNSTpi cells, respectively. Together, these data demonstrate that Me, BNST, and MPOA interact as a functional circuit to process sex-specific odor cues and hormone information in male Syrian hamsters.

Keywords

Olfaction; Pheromone; Androgen Receptor; Fos; Retrograde Tracer; Odor Preference; Reproduction

Introduction

In many rodent species, including Syrian hamsters (*Mesocricetus auratus*), appropriate behavior towards conspecifics depends critically on the perception of odor cues from the environment (Baum and Kelliher, 2009; Johnston, 1990). Indeed, the detection and correct

Corresponding Author: Laura E. Been lbeen1@student.gsu.edu Phone: 404-413-5468 Fax: 404-413-5471.

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identification of sex-specific chemosignals is required for both appetitive and consummatory aspects of reproductive behavior in Syrian hamsters, including males' attraction to female vaginal secretion (Powers et al., 1979) and copulation (Murphy and Schneider, 1970; Powers and Winans, 1975). These chemosignals are processed by a network of ventral forebrain nuclei including the medial amygdala (Me), posterior bed nucleus of the stria terminalis (BNST), and medial preoptic area (MPOA) (Wood, 1997). Specifically, Me and BNST both receive chemosensory input (Scalia and Winans, 1975) and are densely interconnected to each other and to MPOA (Coolen and Wood, 1998; Gomez and Newman, 1992; Scalia and Winans, 1975; Wood and Swann, 2005). As such, main and accessory olfactory information that converges on Me can terminate at BNST or continue directly to MPOA (Coolen and Wood, 1998; Gomez and Newman, 1992), whereas accessory olfactory information received by BNST can be relayed to MPOA (Gomez and Newman, 1992; Wood and Swann, 2005).

In addition to these anatomical relationships, functional data suggest that Me, BNST, and MPOA all process the chemosignals required for the appropriate behavioral response to conspecifics. Exposure to female odors or copulation increases the expression of immediate early genes (IEGs) in the Me, BNST, and MPOA of male hamsters (Fernandez-Fewell and Meredith, 1994; Fiber et al., 1993; Kollack-Walker and Newman, 1997) and other species (Baum and Everitt, 1992; Heeb and Yahr, 1996; Robertson et al., 1991; Wang et al., 1997), suggesting that neurons in these nuclei are activated by sex-specific odor stimuli. Furthermore, lesions of Me (Maras and Petrulis, 2006) and MPOA (Been and Petrulis, 2010b) eliminate preference for volatile opposite-sex odors in male hamsters and severely impair copulatory behavior in hamsters (Been and Petrulis, 2010b; Lehman et al., 1980) and other species (De Jonge et al., 1989; Heeb and Yahr, 2000; Kondo, 1992). Similarly, lesions of BNST eliminate volatile opposite-sex odor preference in male hamsters (Been and Petrulis, 2010a), but cause more subtle, temporal deficits in copulatory behavior in male hamsters and other species (Been and Petrulis, 2010a; Claro et al., 1995; Liu et al., 1997). Together, these data suggest Me, BNST, and MPOA each play a unique and critical role in regulating chemosensory-dependent behaviors.

Conspecific chemosignals must also be integrated with internal indicators of an animal's own endocrine status in order to generate the appropriate behavioral response. Several lines of evidence suggest that this integration of chemosensory and hormone cues takes place within Me, BNST, and MPOA. Each of these three nuclei is sensitive to steroid hormones (Wood et al., 1992; Wood and Newman, 1995a) and site-specific implants of testosterone into either Me or BNST/MPOA restore reproductive behavior in castrated male hamsters (Wood and Newman, 1995c). Furthermore, removal of the olfactory bulb ipsilateral to the testosterone implant prevents this restoration of copulatory behavior, suggesting that chemosensory and hormonal cues are integrated within Me (Wood and Coolen, 1997) and BNST/MPOA (Wood and Newman, 1995b), and that this integration is required for the expression of reproductive behavior.

Despite anatomical and functional data suggesting that Me, BNST, and MPOA are individually required for chemosensory and hormonal modulation of behavior, how these three nuclei interact as a functional circuit to process these cues remains largely unknown. We hypothesized that sex-specific odor information and hormone information are directly relayed between Me, BNST, and MPOA. To test this hypothesis, we injected a retrograde tracer into either MPOA or BNST of male hamsters and subsequently identified whether retrogradely-labeled cells within Me and/or BNST (1) were activated following exposure to sex-specific odors, using IEG expression as an indirect marker of neuronal activation (Pfaus and Heeb, 1997), or (2) expressed androgen receptor (AR). These anatomical data add to the growing body of literature that suggests that Me, BNST, and MPOA interact as a functional

circuit to process both sex-specific odor cues and hormone information in male Syrian hamsters.

Materials and Methods

Animals

Adult male Syrian hamsters (*Mesocricetus auratus*) were purchased from Harlan Laboratories (Prattville, AL, USA) at eight weeks of age. Upon arrival, subjects were singly-housed and remained so for the duration of the study. All subjects ($n = 64$) were gonadally-intact and sexually-naïve. A separate group of group-housed (3–4 same-sex animals per cage), gonadally-intact, adult male and female hamsters ($n = 80$) were used to provide odor stimuli. Subjects were unrelated to, and had no previous contact with, these odor donor animals.

Subjects and odor donors were housed in solid-bottom, polycarbonate cages (325 mm $\times$ 470 mm $\times$ 205 mm) with corncob litter (The Andersons, Maumee, OH, USA) and cotton bedding material (Ancare, Bellmore, NY, USA). Cages were self-contained in an exhaust-vented carousel caging system (OptiRat System, Animal Care Systems, Centennial, CO, USA). All animals were maintained on a reversed 16-h light/8-h dark photoperiod (lights off/on at 10 AM/6 PM). Food and water were available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and were approved by the Georgia State University Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Retrograde Tracer Injections

To visualize the cell bodies that project to MPOA and BNST, the retrograde tracer cholera toxin B (CTB) was deposited unilaterally into either MPOA ($n = 24$) or BNST ($n = 20$). CTB (List Biological Laboratories, Campbell, CA, USA) was prepared as a 0.5% solution in 0.1 M phosphate-buffered saline (PBS; pH 7.4) according to the manufacturer's instructions. Subjects were anesthetized with 2% isoflurane (Butler Schein Animal Health, Dublin, OH, USA) vaporized in a 70:30% oxygen/nitrous oxide mixture and were secured in the stereotaxic apparatus such that their skull was level in the anterior-posterior (A-P) and medial-lateral (M-L) planes. Following a midline scalp incision, the skin and temporal muscles were retracted to expose the skull and a hand-operated drill was used to expose dura. All A-P and M-L measurements were taken in mm relative to bregma and all dorsal-ventral (D-V) measurements were taken in mm relative to dura.

30 nl of CTB was deposited over a 1 minute period by lowering a microinjection syringe (701R 10 μ l syringe, Hamilton, Reno, NV, USA) under stereotaxic control (Microinjection Unit, Model 5002, David Kopf Instruments, Tujunga, CA, USA) into either MPOA (A-P: + 2.0 mm; M-L: $\pm$ 0.6 mm; D-V: -7.1 mm) or BNST (A-P: + 1.85 mm; M-L: $\pm$ 1.35 mm; D-V: -5.9 mm). Injections were alternated between the right and left hemispheres between subjects. To minimize the flow of CTB up the needle tract, the syringe was left in place for 15 minutes after each injection. After injections, skull holes were sealed using bone wax and incisions were closed with wound clips. To minimize post-operative pain, ketoprofen (5 mg/kg subcutaneously, Henry Schein, Melville, NY, USA) was administered intra-operatively.

IEG Induction

All males were sacrificed seven days following tracer injection to allow for sufficient transport of CTB (Vercelli et al., 2000). During this time, males were handled extensively and habituated to the testing room. On the day of sacrifice, males were brought into the

testing room and allowed to sit undisturbed for at least 1 hour prior to stimulus exposure. To determine if BNST- and/or MPOA-projecting neurons respond to same-sex and/or opposite-sex odors, a subset of injected males was exposed to either female (BNST $n = 8$; MPOA $n = 10$) or male (BNST $n = 7$; MPOA $n = 8$) odors. Sex-specific odor exposure was accomplished by placing subjects into a vacated odor-donor cage that had housed 3–4 same-sex animals and had not been changed for 4 days prior to use. In this way, subjects were exposed to a heterogeneous odor stimulus that contained volatile and non-volatile chemosignals (including those from the soiled litter, bedding, and cage walls) and reflected the composite sexual identity of the odor, rather than the individual identity of a single animal. Furthermore, as these cages had not been changed for four days, female stimulus cages included odors from across the entire estrous cycle, including behavioral estrus. To provide a measure of baseline activation, a third group of injected males was transferred to a clean cage (BNST $n = 5$; MPOA $n = 6$). Males were left undisturbed in the stimulus cage for 70 minutes, at which time they were injected with an overdose of sodium pentobarbital (100 mg/kg; Sleep Away, Fort Dodge, IA, USA) and allowed to reach a deep level of anesthesia (additional 10 minutes) prior to perfusion. This survival time is within the time range for peak IEG induction (60–90 min; Herdegen and Leah, 1998) and has been previously used for odor stimulus-induced expression of Fos (Delville et al., 2000; Fiber and Swann, 1996; Swann et al., 2001) and EGR-1 (Lai et al., 2004; Lai et al., 2005) in Syrian hamsters.

Histology and Immunohistochemistry

Males were transcardially perfused with 200 ml of 0.1 M PBS (pH 7.4), followed by 200 ml of 4% paraformaldehyde in phosphate buffer (pH 7.2). Brains were immediately removed and post-fixed in 4% paraformaldehyde overnight (4° C) and then cryoprotected for 48 hours in 30% sucrose in PBS. In order to distinguish between left and right brain hemispheres, an angle was cut into the right dorsal cortex of each brain. Coronal sections (30- μ m) of brain tissue were sectioned on a cryostat (–20°C), collected in a 1:6 series, and stored in cryoprotectant until immunohistochemical processing.

To determine whether BNST and/or MPOA afferents respond to male and/or female odor stimuli, one series of tissue was processed for immunohistochemical localization of Fos and CTB. To determine whether BNST and/or MPOA afferents are androgen-sensitive, a separate series of tissue was stained for AR and CTB. Finally, because we observed low levels of co-localization of Fos and CTB (see Results), a subset of female odor-exposed subjects' tissue (MPOA-injected $n = 3$; BNST-injected $n = 3$) was also processed for immunohistochemical localization of a different IEG, EGR-1, and CTB, in order to confirm that Fos is an accurate marker of neuronal activation in MPOA- and BNST-projecting cells.

Procedures for double-labeling Fos/CTB, AR/CTB, and EGR-1/CTB were identical except for the specific primary antibodies used. Sections were removed from cryoprotectant and rinsed thoroughly in PBS. To reduce endogenous peroxidase activity, tissue sections were incubated in 0.5% hydrogen peroxide in PBS for 15 minutes. After several rinses, sections were then incubated in the appropriate primary antibody to Fos, AR, or EGR-1 in 0.4% Triton-X 100 for 24 hours at room temperature. The primary antibody for Fos was a rabbit polyclonal antibody (1: 20,000, sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) generated against a peptide mapping the N-terminus of the human c-Fos. The primary antibody for AR was a rabbit polyclonal antibody (1: 3,000, sc-816, Santa Cruz Biotechnology) generated against a peptide mapping the N-terminus of the human AR. The primary antibody for EGR-1 was a rabbit polyclonal antibody (1: 20,000, sc-110, Santa Cruz Biotechnology) generated against a peptide mapping the C-terminus of the human EGR-1. The specificity of these primary antibodies has been previously confirmed (Creutz and Kritzer, 2004; Kollack-Walker and Newman, 1997; Lai et al., 2004)

After incubation in primary antibody, sections were rinsed in PBS and then incubated for 1 hour in anti-rabbit biotinylated secondary antibody (1:600 Jackson ImmunoResearch, West Grove, PA, USA) in PBS with 0.4% Triton-X 100. Sections were rinsed again in PBS and then incubated for 1 hour in avidin-biotin complex (4.5 μ l each of A and B reagents/ml PBS with 0.4% Triton-X 100, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). After rinsing in PBS and then in 0.175 M sodium acetate, sections were incubated in 3,3'-diaminobenzidine HCl (0.2 mg/ml, Sigma) and hydrogen peroxide (0.83 μ l/ml, Sigma) in a nickel-sulfate solution (25 mg/ml, Sigma) for 20 minutes, yielding a blue-black product. The reaction was stopped by rinsing sections in sodium acetate. Immediately following visualization of Fos, AR, and/or EGR-1, tissue sections were incubated in the primary antibody to CTB in PBS with 0.4% Triton-X 100 for 24 hours at room temperature. The primary antibody to CTB was a goat polyclonal antibody (1: 80,000, List Biological Laboratories, Campbell, CA, USA) generated against the CTB subunit. Procedures for labeling CTB were identical to those described above except that the biotinylated secondary antibody anti-goat (Vector Laboratories, Burlingame, CA) and CTB was visualized using 3,3'-diaminobenzidine HCl (0.2 mg/ml, Sigma) and hydrogen peroxide (0.83 μ l/ml, Sigma) in Tris Buffer (pH 7.2), yielding a brown reaction product. Stained tissue sections were mounted onto subbed glass slides and allowed to air-dry overnight. Slides were then dehydrated in alcohols, cleared in xylenes, and coverslipped using Permount (Fisher Scientific).

Data Analysis

A single researcher that was blind to the stimulus condition of the animal conducted all analyses. Tissue was first examined for the placement and spread of CTB injections. Only those subjects with CTB deposition restricted to the target region were analyzed. As projections between Me, BNST, and MPOA are primarily unilateral (Coolen and Wood, 1998; Wood and Swann, 2005), Fos/CTB, AR/CTB, and EGR-1/CTB was counted in the hemisphere ipsilateral to the injection. Furthermore, as sexual odor information flows primarily from Me and/or BNST to MPOA (Wood, 1997), cells were counted in both Me and BNST in males with MPOA injections, whereas in males with BNST injections, cells were only counted in Me. Sections were examined using a Nikon Eclipse E800 Microscope with a QImaging digital camera attached. Counting domains were generated by projecting the microscopic field (10 $\times$) onto a computer screen using iVision software (Atlanta, GA, USA). For data presentation, photomicrographs were taken in iVision and processed using Adobe Photoshop CS2 Version 9.0 (San Jose, CA, USA) only to enhance tone, brightness, and contrast.

Within Me, two sections of the anterior medial amygdala (MeA, Figure 1A and 1B) and two sections of the posterior medial amygdala (MeP, Figure 1C and 1D) were counted. Within BNST, two sections of the posterior intermediate BNST (BNSTpi) and posterointermediate BNST (BNSTpm) were counted (Figure 1E and 1F). Fos-positive (Fos+), AR-positive (AR+), and EGR-1-positive (EGR-1+) cells were identified as having dark, blue-black nuclear staining, whereas CTB-positive (CTB+) cells were identified as having brown, cytoplasmic staining that filled the shape of the cell. Fos/CTB-positive (Fos/CTB+), AR/CTB-positive (AR/CTB+), and EGR-1/CTB-positive (EGR-1/CTB+) cells were identified as having a dark, blue-black nucleus surrounded by brown cytoplasmic staining. The total numbers of Fos+, AR+, EGR-1+, Fos/CTB+, AR/CTB+, and EGR-1/CTB+ cells (two sections per domain) were calculated separately and were divided by the total area analyzed for each region (MeA and MeP = 1.298 mm², BNSTpi and BNSTpm = 0.649 mm²) to reflect the density of immunoreactive cells in cells/mm². The sum of the densities of double-labeled cells (Fos/CTB+ or AR/CTB+) was then divided by the sum of the densities of single-

labeled cells (CTB+, Fos+, or AR+) in order to generate the percentages of single-labeled cells that were double-labeled for each brain region.

SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for all data analyses and significance was determined as $P < 0.05$. Unless otherwise noted, all data are reported as mean $\pm$ SEM. To identify differences in the densities of single- or double-labeled cells across stimulus conditions (male, female, clean), separate one-way ANOVAs were used for each brain area (MeA, MeP, BNSTpi, BNSTpm) followed by Tukey's-B post hoc tests. To compare the densities of single- and double-labeled cells between brain areas (MeA vs. MeP and BNSTpi vs. BNSTpm), data were combined across odor stimulus conditions and compared using separate independent *t*-tests. Finally, *z*-tests for independent proportions with Bonferroni corrections for multiple comparisons were used to identify differences in the percentages of double-labeled cells between odor conditions (Fos co-localization experiment) and brain areas (AR co-localization experiment).

Results

CTB deposition and retrograde labeling

Males were included in the MPOA or BNST injection groups only if dark CTB staining was observed around the deposition site within at least two atlas plates of section for MPOA (Figure 2A) and BNST (Figure 2B), respectively (Morin and Wood, 2001). Males were excluded from analysis if the injection was misplaced (MPOA $n = 4$; BNST $n = 5$). Within the MPOA-injected group, misplaced injections resulted in CTB deposition rostral to MPOA, into BNST ($n = 2$), or caudal to MPOA, into the anterior hypothalamus ($n = 2$). Within the BNST-injected group, misplaced injections resulted in CTB deposition dorsal to BNST, into the lateral ventricles ($n = 4$) or the lateral septum ($n = 1$). These inclusion and exclusion criteria resulted in a final total of 16 MPOA-injected males (Female odor condition $n = 6$; Male odor condition $n = 6$; Clean odor condition $n = 4$) and 15 BNST-injected males (Female odor condition $n = 6$; Male odor condition $n = 5$; Clean odor condition $n = 4$).

The largest and smallest extent of CTB deposition in MPOA and BNST are shown in Figure 2C and 2D, respectively. In all subjects included in the MPOA-injected group, CTB was deposited into the mid-rostral MPOA, including the medial preoptic nucleus (MPN), below the most caudal extent of the anterior commissure (Bregma 0.5 mm). In most subjects, CTB was also deposited into MPOA at the level where the lateral and third ventricles fuse ($n = 14$; Bregma 0.2 mm) as well as the most caudal level of MPOA, including MPN and the magnocellular medial preoptic nucleus ($n = 12$; Bregma -0.1 mm). In fewer subjects, CTB deposition spread into the more rostral aspects of MPOA, including MPN, ($n = 8$; Bregma 0.8 mm). In all subjects in the BNST-injected group, CTB was deposited into the mid-rostral BNST (Bregma 0.2 mm), including BNSTpi and BNSTpm, as well as the posterolateral BNST. In most subjects ($n = 13$), CTB was also deposited into more rostral and anterior (Bregma 0.5 mm) parts of BNSTpi and BNSTpm and into the more caudal and ventral level of BNSTpi and BNSTpm ($n = 12$; Bregma -0.1 to -0.3 mm). There was no difference in the overall pattern of retrograde labeling between males with the smallest and largest injections, although larger injections were associated with higher densities of retrograde labeling. Furthermore, within each injection group, the placement and spread of CTB deposition were comparable across the different odor stimulus conditions.

In the current experiments, we only quantified retrograde labeling in Me (MPOA- and BNST-injected) and BNST (MPOA-injected only). However, CTB+ cells were also observed within several previously identified afferents of MPOA, including the anterior cortical amygdala, amygdalohippocampal area, lateral septum, and ventromedial

hypothalamus (Simerly and Swanson, 1986; Wang and Swann, 2006), and within several previously identified afferents of BNST, including the basolateral amygdala, posterior medial cortical amygdala, lateral septum, and ventromedial hypothalamus (Wood and Swann, 2005).

Co-localization of CTB and Fos

MPOA-projecting cells—Overall, we observed low levels of co-localization of CTB and Fos in Me (Figure 3A) following injection of CTB into MPOA. The densities of CTB+, Fos +, and Fos/CTB+ cells for MPOA-projecting cells are shown in Table 1. MeP had significantly more retrogradely-labeled cells than MeA ($t(10) = 2.78$, $P = 0.02$), whereas the density of retrogradely-labeled cells did not differ between BNSTpm and BNSTpi ($t(10) = 1.94$, $P = 0.08$). In MeA and MeP, the density of Fos+ cells did not differ between female odor-exposed and male odor-exposed subjects (MeA: $F(2,13) = 13.78$, $P = 0.001$; MeP: $F(2,13) = 15.45$, $P < 0.001$), although exposure to either female (MeA: $P = 0.002$, MeP: $P = 0.001$) or male (MeA: $P = 0.001$, MeP: $P = 0.001$) odors significantly increased the densities of Fos+ cells above clean odor-exposed (i.e. baseline) densities. Similarly, the density of Fos + cells did not differ between female odor-exposed and male odor-exposed subjects in BNSTpi ($F(2,13) = 5.56$, $P = 0.02$) or BNSTpm ($F(2,13) = 5.78$, $P = 0.02$). In both BNSTpi and BNSTpm, exposure to both female (BNSTpi: $P = 0.014$; BNSTpm: $P = 0.03$) and male (BNSTpi: $P = 0.02$; BNSTpm: $P = 0.01$) odors increased the density of Fos+ cells above baseline levels. The densities of Fos/CTB+ cells did not differ between subjects exposed to female, male, or clean odors in MeA ($F(2,13) = 0.21$, $P = 0.81$), MeP ($F(2,13) = 1.08$, $P = 0.37$), BNSTpi ($F(2,13) = 1.08$, $P = 0.37$), and BNSTpm ($F(2,13) = 1.42$, $P = 0.28$).

Figure 4A depicts the percentage of CTB+ cells that were also Fos+ in response to each odor exposure condition for MPOA-projecting cells. In MeA, the percentage of CTB+ cells that were double-labeled with Fos following exposure to female odors (18.45%) and male odors (14.56%) did not differ from each other ($z = 1.62$, $P = 0.05$), but both were significantly higher than the percentage of CTB+ cells that were also Fos+ following exposure to clean odors (8.67%; female $z = 3.10$, male $z = 4.37$, both $P < 0.001$). In contrast, the percentage of CTB+ cells in MeP that were also Fos+ following exposure to female odors (15.84%) was significantly higher than the percentage of CTB+ cells that also expressed Fos following exposure to male odors (11.58%; $z = 2.36$, $P = 0.009$). Furthermore, the percentage of CTB+ cells in MeP that were Fos+ following exposure to either female ($z = 5.05$, $P < 0.001$) or male ($z = 3.17$, $P < 0.001$) odors was significantly higher than the percentage of CTB+ cells that were Fos+ following clean odor exposure (7.10%).

As in Me, we observed low levels of co-localization of CTB and Fos in BNST (Figure 3B) following injection of CTB into MPOA. In BNSTpi and BNSTpm, the percentages of CTB+ cells that were Fos + following female (BNSTpi: 12.73%; BNSTpm: 11.09%) and male (BNSTpi: 11.69%; BNSTpm: 8.19%) odor exposure did not differ from each other (BNSTpi: $z = 0.36$, $P = 0.36$; BNSTpm: $z = 1.44$, $P = 0.07$). The percentages of CTB+ cells that were Fos+ following exposure to male (BNSTpi: $z = 4.40$; BNSTpm: $z = 4.13$, both $P < 0.001$) and female (BNSTpi: $z = 4.09$; BNSTpm: $z = 3.15$, both $P < 0.001$) odors, however, were higher than the percentages of CTB+ cells that were also Fos+ following exposure to clean odors in BNSTpi (1.53%) and BNSTpm (1.85%) (Figure 4A).

Figure 4B depicts the percentage of Fos+ cells responding to each odor exposure condition that were also CTB+ in MPOA-injected males. In MeA, the percentage of Fos+ cells following exposure to female odors that were also CTB+ (4.76%) was significantly greater than the percentage of Fos+ cells following exposure to male odors that were also CTB+ (3.40%; $z = -2.15$, $P = 0.01$). The percentages of Fos+ cells following exposure to either female or male odors that were CTB+, however, were significantly lower than the

percentage of Fos+ following exposure to clean odors that were CTB+ (10.38%; $z = -6.35$ and 4.57 , both $P < 0.001$). Similarly, in MeP, the percentage of Fos+ cells following exposure to female odors that were CTB+ (7.49%) was significantly higher than the percentage of Fos+ cells following exposure to male odors that were CTB+ (5.52%; $z = -2.23$, $P = 0.01$) and the percentages of Fos+ cells following exposure to either female or male odors that were CTB+ were significantly lower than the percentage of Fos+ following exposure to clean odors that were CTB+ (21.07%; $z = -8.70$ and -6.92 , both $P < 0.001$).

In contrast, in BNSTpi and BNSTpm, there was no difference in the percentage of Fos+ cells that were CTB+ between female odor-exposed (BNSTpi: 8.21%; BNSTpm: 10.56%) and male odor-exposed (BNSTpi: 7.76%; BNSTpm: 10.69%) groups (BNSTpi: $z = -0.22$, $P = 0.41$; BNSTpm: $z = 0.05$, $P = 0.47$), nor did the percentage of Fos+ cells that were CTB+ differ between the clean odor-exposed group (BNSTpi: 18.75%; BNSTpm: 18.18%) and either the female odor-exposed group (BNSTpi: $z = -1.46$, $P = 0.07$; BNSTpm: $z = -1.09$, $P = 0.14$) or the male odor-exposed group (BNSTpi: $z = -1.58$, $P = 0.06$; BNSTpm: $z = -1.10$, $P = 0.13$) (Figure 4B).

BNST-projecting cells—The densities of CTB+, Fos+, and Fos/CTB+ cells for BNST-projecting cells are shown in Table 2. There was no difference in the density of retrogradely-labeled cells between MeA and MeP ($t(9) = 1.71$, $P = 0.21$). In MeA, the densities of Fos+ cells were greater following exposure to either female odors ($P = 0.001$) or male odors ($P = 0.003$) compared to the density of Fos+ cells following exposure to clean odors ($F(2,12) = 14.14$, $P = 0.001$), although there was no difference between the female odor-exposed and male odor-exposed groups ($P = 0.72$). In MeP, however, the densities of Fos+ cells differed significantly between all three odor-exposure conditions ($F(2,12) = 94.37$, $P < 0.001$). Exposure to either female ($P < 0.001$) or male ($P = 0.003$) odors increased the density of Fos+ cells over exposure to clean odors, and the density of Fos+ cells was significantly greater following exposure to female odors than it was following exposure to male odors ($P < 0.001$). The densities of Fos/CTB+ did not differ following exposure to any of the three odor conditions in MeA ($F(2,12) = 0.57$, $P = 0.58$) or MeP ($F(2,12) = 0.03$, $P = 0.97$).

In MeA, the percentage of CTB+ cells that were double-labeled with Fos following exposure to female odors (15.70%) and male odors (16.69%) did not differ from each other ($z = 0.46$, $P = 0.32$), although exposure to both female ($z = 3.61$, $P < 0.001$) and male ($z = 4.10$, $P < 0.001$) odors increased the percentage of CTB+ cells that were Fos+ over the percentage of CTB+ that were Fos+ following exposure to clean odors (9.34%) (Figure 5A). In contrast, the percentage of CTB+ cells that were Fos+ in MeP following exposure to female odors (15.95%) was significantly higher than the percentage of CTB+ cells that expressed Fos+ following exposure to male odors (12.33%; $z = -2.14$, $P = 0.016$). Furthermore, the percentages of CTB+ cells that were Fos+ in MeP following exposure to either female ($z = 4.82$, $P < 0.001$) or male ($z = 2.70$, $P = 0.003$) odors were significantly higher than the percentage of CTB+ cells that were Fos+ following clean odor exposure (8.59%) (Figure 5A).

In MeA, the percentage of Fos+ cells following exposure to female odors that were also CTB+ (7.50%) was significantly greater than the percentage of Fos+ cells following exposure to male odors that were CTB+ (5.3%; $z = 2.38$, $P = 0.008$), although the percentage of Fos+ cells following exposure to either female odors ($z = -11.16$, $P < 0.001$) or male odors ($z = -8.57$, $P < 0.001$) that were also CTB+ was significantly lower than the percentage of double-labeled cells following exposure to clean odors (24.28%) (Figure 5B). Similarly, in MeP, the percentage of Fos+ cells following exposure to female odors that were CTB+ (13.44%) was significantly higher than the percentage of Fos+ cells following exposure to male odors that were CTB+ (6.52%; $z = 5.95$, $P < 0.001$) and the percentages of

Fos+ cells following exposure to either female odors ($z = -10.93$, $P < 0.001$) or male odors ($z = -4.86$, $P < 0.001$) that were CTB+ were significantly lower than the percentage of Fos+ following exposure to clean odors that were CTB+ (25%) (Figure 5B).

Co-localization of CTB and EGR-1

The overall pattern of labeling observed in tissue double-labeled for EGR-1 and CTB was very similar to that of tissue double-labeled for Fos and CTB, suggesting that the low levels of CTB/Fos co-localization were not specific to Fos, but rather, may be a veridical feature of the IEG response (Figure 3C). The densities of CTB+, EGR-1+ and EGR-1/CTB+ cells following exposure to female odors are shown in Table 3. In MPOA-projecting cells (Table 3A), the densities of EGR-1+ cells did not differ from the densities of Fos+ cells in MeA ($t(7) = 0.76$, $P = 0.47$), MeP ($t(7) = 0.53$, $P = 0.61$), BNSTpi ($t(7) = 0.09$, $P = 0.93$), or BNSTpm ($t(7) = 0.67$, $P = 0.53$). Furthermore, the densities of EGR-1/CTB+ cells did not differ from the densities of Fos/EGR-1+ cells in MeA ($t(7) = 0.79$, $P = 0.45$), MeP ($t(7) = 1.74$, $P = 0.12$), BNSTpi ($t(7) = 0.87$, $P = 0.41$), or BNSTpm ($t(7) = 0.09$, $P = 0.92$). Similarly, in BNST-projecting cells (Table 3B), the densities of EGR-1+ and EGR-1/CTB+ did not differ from the densities of Fos+ or Fos/EGR-1+ cells in MeA (single-labeled: $t(7) = 0.09$, $P = 0.93$; double-labeled: $t(7) = 0.24$, $P = 0.82$) or MeP (single-labeled: $t(7) = 2.46$, $P = 0.04$; double-labeled: $t(7) = 0.06$, $P = 0.57$).

Co-localization of CTB and AR

MPOA-projecting cells—The densities of CTB+, AR+, and AR/CTB+ cells for MPOA-projecting cells are shown in Table 4A. There were no differences in the densities of cells single- or double-labeled with AR across odor exposure conditions so data was collapsed across odor conditions. MeP had significantly more retrogradely-labeled cells than MeA ($t(20) = 5.09$, $P < 0.001$) and BNSTpm had significantly more retrogradely-labeled cells than BNSTpi ($t(20) = 8.28$, $P < 0.001$). Similarly, MeP had more AR+ ($t(20) = 2.35$, $P = 0.03$) and AR/CTB+ ($t(20) = 3.50$, $P = 0.002$) cells than MeA, and BNSTpm had more AR+ ($t(20) = 13.21$, $P < 0.001$) and AR/CTB+ ($t(20) = 9.32$, $P < 0.001$) cells than BNSTpi.

We observed high levels of co-localization of CTB and AR within both Me (Figure 6A) and BNST (Figure 6B). The percentage of CTB+ cells that were AR+ was significantly higher in MeP (90.63%) than in MeA (75%; $z = -11.67$, $P < 0.001$). Similarly, the percentage of CTB+ cells that were AR+ was significantly higher in BNSTpm (88.01%) than in BNSTpi (67.71%; $z = -8.32$, $P < 0.001$) (Figure 7A). The percentage of AR+ cells that were CTB+ was significantly higher in MeP (38.60%) than in MeA (29.71%; $z = -7.57$, $P < 0.001$) as well as significantly higher in BNSTpm (45.89%) than in BNSTpi (23.92%; $z = -3.03$, $P = 0.001$) (Figure 7B).

BNST-projecting cells—The densities of CTB+, AR+, and AR/CTB+ cells for BNST-projecting cells are shown in Table 4B. Again, there were no differences in the densities of cells single- or double-labeled with AR across odor exposure conditions so data was collapsed across odor conditions. MeP had significantly more retrogradely labeled cells than MeA ($t(22) = 2.92$, $P = 0.007$). Furthermore, the densities of AR+ ($t(22) = 5.21$, $P < 0.001$) and AR/CTB+ ($t(22) = 5.21$, $P < 0.001$) cells were significantly higher in MeP than in MeA. The percentage of CTB+ cells that were AR+ was significantly higher in MeP (87.38%) than in MeA (68.37%; $z = -15.35$, $P < 0.001$) (Figure 8A). There was no difference, however, in the percentage of AR+ cells that were CTB+ between MeA (45.89%) and MeP (45.48%; $z = -0.33$, $P = 0.37$) (Figure 8B).

Discussion

Methodological Considerations

Previous studies have used CTB to label afferents of Me (Coolen and Wood, 1998; Maras and Petrulis, 2010a) and BNST (Wood and Swann, 2005) in hamsters, and we observed a pattern of retrograde labeling similar to previous reports. By combining CTB retrograde tracing with immunohistochemical markers for IEGs and AR, we extended these previous findings by characterizing the nature of odor-responsive and hormone-sensitive Me and BNST projection neurons.

IEGs have been widely used as indirect markers of neuronal activation in response to discrete stimulus presentations, including odor stimuli (Pfaus and Heeb, 1997). Although we observed low levels of co-localization of Fos and CTB within cells of Me and BNST, the total number of cells expressing Fos were similar to previous reports of Fos expression following odor stimulus exposure (Fernandez-Fewell and Meredith, 1994; Fiber et al., 1993; Kollack-Walker and Newman, 1997). Furthermore, we observed low levels of Fos expression in the clean control group, suggesting that the co-localization of Fos and CTB observed in males exposed to sexual odors likely reflects specific activation of Me and BNST afferents in response to sexual odor stimuli, rather than constitutive expression. The overall numbers of cells participating in coding social odors, however, are likely to be underestimated by IEG expression, as it primarily reflects the cumulative number of neurons activated via excitatory, NMDA-receptor mediated inputs and is therefore not a sensitive measure of inhibitory processing or rapid, non-NMDA receptor-mediated excitation (Flavell and Greenberg, 2008).

It is also possible that we would observe a different pattern of IEG activity and/or co-localization with CTB in sexually-experienced males than we do in the current, sexually-naïve subjects. Indeed, previous research has demonstrated that sexual experience can alter male hamsters' neural (Fewell and Meredith, 2002; Kollack-Walker and Newman, 1997) and behavioral (Been and Petrulis, 2010a; Been and Petrulis, 2010b) responses to females and/or their odors. Unlike other rodent species, however, male hamsters do not require sexual experience to show a preference for (or discriminate between) female vs. male odors (Ballard and Wood, 2007; Been and Petrulis, 2010a; Been and Petrulis, 2010b; Maras and Petrulis, 2006). If the pattern of neural activation observed in the current study mediates this opposite-sex odor preference, then we might expect to see a similar, or more pronounced, difference between female and male odor-responsive projection cells in sexually-experienced males.

Me afferents relay sex-specific odor information to BNST and MPOA

The current results provide the first direct evidence that opposite-sex odors activate a higher percentage of Me afferents than do same-sex odors. Specifically, within MeP, female odors activated a higher percentage of neurons that project to either MPOA or BNST than did male odors. Furthermore, within both MeA and MeP, a higher percentage of female odor-responsive neurons than male odor-responsive neurons projected to BNST and/or MPOA. Together, these data suggest that male hamsters' preference for opposite-sex odors may rely on this differential projection of female- versus male-responsive neurons, particularly from MeP to MPOA and/or BNST. This finding is consistent with previous studies demonstrating that lesions of Me eliminate opposite-sex odor preference in hamsters (Maras and Petrulis, 2006), and cause deficits in other odor-guided reproductive behaviors in hamsters (Lehman et al., 1980; Petrulis and Johnston, 1999), rats (Kondo, 1992; Kondo et al., 1997) and gerbils (Heeb and Yahr, 2000). Furthermore, functionally disconnecting Me from MPOA using asymmetrical pathway lesions disrupts copulation in male rats (Kondo and Arai, 1995) and

gerbils (Heeb and Yahr, 2000), suggesting Me projections to MPOA may be particularly critical for reproductive behavior. Future studies will determine whether connections between Me and MPOA/BNST are similarly required for the appropriate behavioral response to sex-specific chemosignals in hamsters.

Although exposure to either female or male odors consistently activated more cells than exposure to clean control odors, the mean densities of Me neurons expressing Fos did not consistently differ between female and male odor-exposed groups. Exposure to female and male odors activated equivalent numbers of neurons in MeA, as is typical in studies that directly compare Fos induction following exposure to different categories of social odors (Maras and Petrulis, 2010a; Maras and Petrulis, 2010b; Meredith and Westberry, 2004; Samuelsen and Meredith, 2009). In MeP, however, female odors did not consistently activate more cells than male odors, unlike previous reports (Maras and Petrulis, 2010b Meredith and Westberry, 2004; Samuelsen and Meredith, 2009). This difference may be explained by the fact that MeP is a heterogeneous nucleus (Canteras et al., 1992) and subtle differences in the placement of counting domains within and between studies may therefore contribute to variability in the total number of Fos-immunoreactive cells counted in this area.

The percentage of Me cells that were activated by female or male odors and projected to MPOA and/or BNST was also lower than the percentage of cells that were activated by clean odors and projected to MPOA and/or BNST. One possible explanation for this finding is that the cells activated by clean odors comprise a portion of the cells activated by female and/or male odors. That is, it is possible that some cells may express Fos in response to female, male, and/or clean odors. If this is case, then it is likely that the small numbers of cells in Me and BNST that respond to clean odors also respond to female and/or male odors, and therefore encode chemoinvestigatory behavior rather than stimulus identity. Unfortunately, conventional IEG methods cannot distinguish whether the same cells are activated by different categories of sexual odor stimuli.

The percentage of Me cells that project to MPOA and are also activated by female odor stimuli in the current study (16–18%) is markedly lower than the percentage of Me-MPOA projecting cells activated by copulation (60–65%) in rats (Coolen et al., 1998; Greco et al., 1998). These lower levels of activation likely reflect the fact that presentation of female odors alone provides only part of the total sensory input to Me during copulatory behavior (Kollack-Walker and Newman, 1997). As such, the immediate early gene response in the current study likely corresponds to the perception and identification of female odors. The large number of retrogradely-labeled cells that did not express Fos in response to female odor stimuli may therefore represent neurons that require the additional sensory input received during copulatory behavior to be activated. Conversely, the large number of odor-responsive cells that were not retrogradely-labeled may represent Me cells that project to other brain areas important for odor-guided reproductive behaviors, such as the ventromedial hypothalamus or nucleus accumbens (Heeb and Yahr, 1996; Kippin et al., 2003; Robarts and Baum, 2007), or to brain areas important for other, non-reproductive, odor-guided behaviors (Blanchard et al., 2005; Choi et al., 2005).

BNST afferents relay nonspecific sexual odor information to MPOA

As in Me, exposure to either female or male odors consistently activated more BNST cells than exposure to clean control odors, although the mean densities of Me neurons expressing Fos did not differ between female and male odor-exposed groups. In contrast to Me afferents, however, the sex-specificity of odor information was lost at the population level of BNST projections to MPOA. The percentage of BNST cells that project to MPOA and were also activated in response to female odor stimuli (11–13%) is lower than the percentage Me

afferents to MPOA that were also activated by female odors in the current study, and far lower than the percentage of BNST cells that project to MPOA and are also activated by copulation (55–60%) in rats (Coolen et al., 1998; Greco et al., 1998). These results are consistent with previous studies that suggest BNST may play a less critical role than Me in generating the behavioral response to sex-specific odors. Whereas lesions of Me decrease female odor-induced Fos expression in BNST and MPOA (Maras and Petrulis, 2010b), lesions of BNST do not reduce Fos expression in MPOA or other downstream brain areas (Been and Petrulis, 2008). Similarly, lesions of the vomeronasal organ in male hamsters do not decrease female odor-induced Fos expression in BNST, suggesting that BNST may only be involved in the response to volatile female odors, or may mediate integrative or motoric components of chemoinvestigation, rather than investigation of directly-contacted female odors specifically (Fernandez-Fewell and Meredith, 1994). Finally, whereas Me lesions cause severe deficits in male copulatory behavior (Kondo, 1992; Lehman et al., 1980), lesions of BNST alter the temporal sequence of copulation but do not severely impair mating (Been and Petrulis, 2010a; Claro et al., 1995; Liu et al., 1997; Valcourt and Sachs, 1979).

Hormone Information is directly conveyed between Me, BNST, and MPOA

As in many rodent species (Moffatt, 2003), appropriate levels of gonadal hormones are critical for male hamsters' attraction to female odors (Petrulis and Johnston, 1995; Powers and Bergondy, 1983; Powers et al., 1985; Steel, 1982), suggesting hormones modulate neural processing of conspecific odors (Fiber and Swann, 1996; Kelliher et al., 1998). In agreement with previous reports, we observed higher densities of cells expressing AR in MeP and BNSTpm than in MeA and BNSTpi, respectively (Wood et al., 1992; Wood and Newman, 1999), and we have extended these findings by demonstrating that this pattern is preserved with regard to the number of AR-sensitive neurons that project within this circuit. Interestingly, both the percentage of AR-containing cells that project to MPOA and the percentage of MPOA-projecting cells that contain AR are greater in MeP and BNSTpm than in MeA and BNSTpi, respectively. In contrast, only the percentage of BNST-projecting cells that contain AR, but not the percentage of AR-containing neurons that project to BNST, is greater MeP than in MeA. These data suggest that MPOA may be the primary target of hormonal information from MeP and BNSTpm, whereas MeP projections to BNST may play a secondary role in the hormonal modulation of neural processing.

It is unknown whether MPOA- and BNST-projecting neurons that express Fos in response to male and female odors are a subset of the MPOA- and BNST-projecting neurons that express AR. In male hamsters, approximately 40% of Me neurons and 47% of BNST neurons that express Fos following copulation also express AR (Wood and Newman, 1993). Similarly, in rats, 89% of Me neurons that express Fos in response to copulation also express AR, and of those neurons 82% also project to MPOA (Greco et al., 1998). Although we observed lower percentages of cells double-labeled with Fos/CTB following exposure to sexual odors than others have observed following copulation, these previous studies suggest that we might expect to see a substantial amount of overlap between Fos/CTB+ and AR/CTB+ cells in Me and BNST, likely to a proportionally smaller degree.

Although in the current study we only examined the expression of AR in Me, BNST, and MPOA, these nuclei also contain abundant estrogen receptor (ER)-containing cells (Wood et al., 1992; Wood and Newman, 1995a). Unfortunately, the only antibody that consistently labels ER in hamster brain tissue is the H222 antibody (Boers et al., 1999; Li et al., 1993; Mangels et al., 1998; Wood and Newman, 1995a), and this antibody labels unbound ER, precluding its use in any studies that require subjects to maintain normal levels of steroid hormones (Blaustein, 1993). It is worth noting, however, that ERs may mediate of the effects of steroid hormones on males' behavioral response to conspecifics odors (Wood,

1996). Although systemic estradiol administration, either alone or in combination with dihydrotestosterone, does not restore levels of female odor investigation in castrated males as effectively as systemic testosterone administration (Powers and Bergondy, 1983), the aromatization of testosterone into estradiol may play a role in the identification and investigation of female odors in male hamsters. Indeed, administration of testosterone propionate, which can be aromatized, restores males' preference for female odors in castrated males (Gregory et al., 1975). Furthermore, inhibiting aromatase activity decreases olfactory investigation of a female and, at high doses, eliminates males' ability to discriminate between individual females' vaginal secretions, whereas subsequent treatment with estradiol can reverse these deficits (Steel and Hutchison, 1987; Steel and Hutchison, 1988). In hamsters, cells in MA and BNST often co-express AR and ER, such that cells expressing only ER constitute a small proportion (12–18%) of the total population of AR- and ER-expressing cells within these nuclei (Wood and Newman, 1995a). These data would suggest that the current findings about AR-containing cells may generalize to ER-containing cells, and we would therefore expect to observe similar proportions of ER/CTB double-labeling as were observed for AR/CTB double-labeling.

Summary and Conclusions

This study demonstrates that sex-specific odor information and steroid hormone cues are directly relayed between Me, BNST, and MPOA in male Syrian hamsters. As few MPOA- or BNST-projecting cells expressed IEGs in response to female or male odors, the present results show that sparse and discrete populations of cells send male and female chemosignals to BNST and MPOA. Sex-specific odor information may be carried primarily by MeP, as IEG expression in this region is greater to female odors than to male odors in MPOA- and BNST-projecting cells, whereas female and male odors induced equivalent levels of IEGs within retrogradely-labeled MeA, BNSTpi, and BNSTpm cells (Figure 9A). We observed high levels of co-localization of AR with cells that project to MPOA or BNST, and these hormone-sensitive cells also followed a distinct pattern. Specifically, a higher percentage of retrogradely-labeled MeP and BNSTpm cells also expressed AR than retrogradely-labeled MeA and BNSTpi cells, respectively (Figure 9B). Together, these data demonstrate that Me, BNST, and MPOA interact to transmit chemosensory and hormonal information within this ventral forebrain circuit. These odor-responsive and steroid-sensitive projections may provide the neural basis for the appropriate behavioral response to conspecific odors.

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Abbreviations

A-P	anterior-posterior
AR	androgen receptors
BNST	posterior bed nucleus of the stria terminalis
BNSTpi	posterointermediate bed nucleus of the stria terminalis
BNSTpm	posteromedial bed nucleus of the stria terminalis
CTB	cholera toxin B

D-V	dorsal-ventral
ER	estrogen receptors
IEG	immediate early gene
Me	medial amygdala
MeA	anterior medial amygdala
MeP	posterior medial amygdala
M-L	medial-lateral
MPN	medial preoptic nucleus
MPOA	medial preoptic area
PBS	phosphate buffered saline

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Figure 1.

Counting domains for analysis of cholera toxin B (CTB), immediate early genes (Fos, EGR-1) and androgen receptor (AR). The total number of single- and double-labeled cells within the specified counting domains (grey rectangles) were counted and summed across two sections for the anterior medial amygdala (MeA; A and B), posterior medial amygdala (MeP; C and D), and posterointermediate and posteromedial bed nucleus of the stria terminalis (BNSTpi and BNSTpm; E and F). Illustration modified from hamster brain atlas (Morin and Wood, 2001). ACo, anterior cortical amygdala; BLA, basolateral amygdala; BLP, posterior basolateral amygdala; BMA, basomedial amygdala; CeA, central amygdala; f, fornix; GP, globus pallidus; I, intercalated amygdala; ic, internal capsule; LPO, lateral preoptic area; ot, optic tract; MPN, medial preoptic nucleus; MPO, medial preoptic area; PLCo posterolateral cortical amygdala; sm, stria medullaris; St, stria terminalis.

Figure 2. Verification of cholera toxin B (CTB) injections. Photomicrographs depict typical deposition of CTB in A) the medial preoptic area (MPOA) and B) the posterior bed nucleus of the stria terminalis (BNST). Reconstruction of largest (light grey) and smallest (dark grey) CTB injections (numbers represent distance posterior to bregma) through the rostral-caudal extent of (C) MPOA and (D) BNST. Scale bars = 100 μ m.

Figure 3.

Co-localization of cholera toxin B (CTB) and immediate early genes (Fos, EGR-1).

Photomicrographs from representative sections immunoreactive for CTB and Fos in A) the medial amygdala (Me) and B) the posterior bed nucleus of the stria terminalis (BNST), and C) from a representative section immunoreactive for CTB and EGR-1 in Me. Images to right provide higher magnifications of areas included in the boxes. Cells with brown cytoplasmic staining (white arrows) are CTB+, cells with black nuclear staining (black arrows) are Fos+ (A and B) or EGR-1+ (C), and cells with black nuclear staining surrounded by brown cytoplasmic staining (grey arrows) are Fos/CTB+ (A and B) or EGR-1/CTB+ (C). Scale bars = 100 μ m and 25 μ m for lower and higher magnifications, respectively.

Figure 4. Percentages of Fos/CTB double-labeled cells in males with medial preoptic area (MPOA) injections. A) Total percentage of CTB+ cells that were also Fos+ in the anterior medial amygdala (MeA), posterior medial amygdala (MeP), posterointermediate bed nucleus of the stria terminalis (BNSTpi), and posteromedial bed nucleus of the stria terminalis (BNSTpm). B) Total percentage of Fos+ that were also CTB+ in MeA, MeP, BNSTpi, and BNSTpm. Error bars represent standard errors of mean group percentages, * reflects significant differences between brain areas, $P < 0.05$ (z-test for independent proportions, $\alpha_{FW} = 0.05$).

Figure 5.

Percentages of Fos/CTB double-labeled cells in males with posterior bed nucleus of the stria terminalis (BNST) injections. A) Total percentage of CTB+ cells that were also Fos+ in the anterior medial amygdala (MeA) and posterior medial amygdala (MeP). B) Total percentage of Fos+ that were also CTB+ in MeA and MeP. Error bars represent standard errors of mean group percentages, * reflects significant differences between brain areas, $P < 0.05$ (z -test for independent proportions, $\alpha_{fw} = 0.05$).

Figure 6.

Co-localization of cholera toxin B (CTB) and androgen receptor (AR). Photomicrographs from representative sections immunoreactive for CTB and AR in A) the medial amygdala (Me) and B) the posterior bed nucleus of the stria terminalis (BNST). Images to right provide higher magnifications of areas included in the boxes. Cells with brown cytoplasmic staining (white arrows) are CTB+, cells with black nuclear staining (black arrows) are AR+, and cells with black nuclear staining surrounded by brown cytoplasmic staining (grey arrows) are AR/CTB+. Scale bars = 100 μ m and 25 μ m for lower and higher magnifications, respectively.

Figure 7. Percentages of AR/CTB double-labeled cells in males with medial preoptic area (MPOA) injections. A) Total percentage of CTB+ cells that were also AR+ in the anterior medial amygdala (MeA), posterior medial amygdala (MeP), posterointermediate bed nucleus of the stria terminalis (BNSTpi) and posteromedial bed nucleus of the stria terminalis (BNSTpm). B) Total percentage of AR+ cells that were also CTB+ in MeA, MeP, BNSTpi, and BNSTpm. Error bars represent standard errors of mean group percentages, * reflects significant differences between brain areas, $P < 0.05$ (z -test for independent proportions, $\alpha_{fw} = 0.05$).

Figure 8. Percentages of AR/CTB double-labeled cells in males with posterior bed nucleus of the stria terminalis (BNST) injections. A) Total percentage of CTB+ cells that were also AR+ in the anterior medial amygdala (MeA) and posterior medial amygdala (MeP). B) Total percentage of AR+ cells that were also CTB+ in MeA and MeP. Error bars represent standard errors of mean group percentages, * reflects significant differences between brain areas, $P < .05$ (z -test for independent proportions, $\alpha_{fw} = 0.05$).

Figure 9.

Summary of chemosensory and steroid-sensitive projections to medial preoptic area (MPOA) and posterior bed nucleus of the stria terminalis (BNST). A) A low percentage of cells that respond to female odors (filled circles) or male odors (open circles) project to MPOA (left) or BNST (right). In the posterior medial amygdala (MeP), however, a higher percentage of female-odor responsive cells project to BNST and/or MPOA than do male odor-responsive cells. B) A high percentage of androgen receptor (AR)-bearing cells (squares) project to MPOA (left) and BNST (right). Specifically, more AR+ cells in MeP than in the anterior medial amygdala (MeA) project to MPOA and/or BNST; similarly, more AR+ cells in the posteromedial BNST (BNSTpm) than in the posterointermediate BNST (BNSTpi) project to MPOA. Solid arrows indicate afferent projections; dashed arrows represent direct olfactory projections.

Table 1

Densities of CTB+, Fos+, and Fos/CTB+ cells in cells that project to the medial preoptic area (MPOA). Data reported as means $\pm$ standard errors of the means.

Total/mm ²	MeA	MeP	BNSTpi	BNSTpm
CTB+	73.86 $\pm$ 11.83	144.44 $\pm$ 22.46*	36.06 $\pm$ 3.20	95.37 $\pm$ 37.43
Fos+				
Female	238.06 $\pm$ 37.12 ^a	196.97 $\pm$ 6.46 ^a	101.39 $\pm$ 19.67 ^a	72.93 $\pm$ 5.39 ^a
Male	260.14 $\pm$ 17.75 ^a	200.18 $\pm$ 24.53 ^a	115.82 $\pm$ 18.06 ^a	111.26 $\pm$ 43.21 ^a
Clean	87.25 $\pm$ 21.40 ^b	50.27 $\pm$ 14.27 ^b	6.16 $\pm$ 1.54 ^b	2.15 $\pm$ 0.77 ^b
Fos/CTB+				
Female	13.10 $\pm$ 3.93 ^a	14.77 $\pm$ 1.13 ^a	8.32 $\pm$ 3.92 ^a	16.17 $\pm$ 8.26 ^a
Male	8.86 $\pm$ 2.79 ^a	11.04 $\pm$ 1.48 ^a	8.99 $\pm$ 2.88 ^a	7.70 $\pm$ 3.75 ^a
Clean	9.05 $\pm$ 2.44 ^a	10.59 $\pm$ 4.31 ^a	1.15 $\pm$ 0.77 ^a	0.77 $\pm$ 0.77 ^a

* reflects significant differences between brain areas, dissimilar letters reflect significant differences between odor stimulus conditions (Tukey's-B post-hoc comparisons, $P < .05$). MeA, anterior medial amygdala; MeP, posterior medial amygdala; BNSTpi, posterointermediate bed nucleus of the stria terminalis; BNSTpm, posteromedial bed nucleus of the stria terminalis.

Table 2

Densities of CTB+, Fos+, and Fos/CTB+ cells that project to the posterior bed nucleus of the stria terminalis (BNST). Data reported as means±standard errors of the means. Dissimilar letters reflect significant differences between odor stimulus conditions (Tukey's-B post-hoc comparisons, $P < .05$). MeA, anterior medial amygdala; MeP, posterior medial amygdala.

Total/mm ²	MeA	MeP
CTB+	101.44±12.89	140.22±16.12
Fos+		
Female	220.21±15.5 ^a	249.56±12.56 ^a
Male	197.22±28.42 ^a	138.29±11.43 ^b
Clean	60.29±15.49 ^b	68.27±6.39 ^c
Fos/CTB+		
Female	11.81±1.60 ^a	16.29±5.25 ^a
Male	14.79±3.35 ^a	19.97±3.16 ^a
Clean	14.64±7.07 ^a	17.07±5.27 ^a

Table 3

Densities of CTB+, ERG-1+, and CTB/ERG-1+ cells following Female odor exposure in cells the project to (A) the medial preoptic area (MPOA) and (B) the posterior bed nucleus of the stria terminalis (BNST). MeA, anterior medial amygdala; MeP, posterior medial amygdala; BNSTpi, posterointermediate bed nucleus of the stria terminalis; BNSTpm, posteromedial bed nucleus of the stria terminalis.

Total/mm ²	MeA	MeP	BNSTpi	BNSTpm
A) MPOA-projecting				
EGR-1+	195.61±21.55	208.63±30.53	98.46±24.87	88.06±33.84
EGR-1/CTB+	17.70±1.18	11.16±1.89	13.29±4.72	12.37±2.65
B) BNST-projecting				
EGR-1+	223.01±30.17	197.58±16.00		
EGR-1/CTB+	11.04±3.34	11.56±2.67		

Table 4

Densities of CTB+, AR+, and CTB/AR+ cells in cells that project to A) the medial preoptic area (MPOA) and B) the posterior bed nucleus of the stria terminalis (BNST).

Total/mm ²	MeA	MeP	BNSTpi	BNSTpm
A) MPOA-projecting				
CTB+	78.58±1.63	192.60±22.33*	9.75±2.02	171.54±19.43*
AR+	185.93±39.82	306.63±32.37*	93.47±12.93	362.60±15.74*
AR/CTB+	68.05±3.33	167.69±24.04*	2.56±0.68	156.65±16.52*
B) BNST-projecting				
CTB+	105.03±5.54	197.57±31.12*		
AR+	171.54±25.44	376.24±29.94*		
AR/CTB+	68.82±6.42	172.64±28.94*		

* reflects significant differences between brain areas (Independent *t*-tests, *P* < .05). MeA, anterior medial amygdala; MeP, posterior medial amygdala; BNSTpi, posterointermediate bed nucleus of the stria terminalis; BNSTpm, posteromedial bed nucleus of the stria terminalis.