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Anatomical connections between the anterior and posterodorsal sub-regions of the medial amygdala: Integration of odor and hormone signals

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Abstract

In many rodent species, such as Syrian hamsters, reproductive behavior requires neural integration of chemosensory information and steroid hormone cues. The medial amygdala processes both of these signals through anatomically distinct sub-regions; the anterior region (MeA) receives substantial chemosensory input, but contains few steroid receptor-labeled neurons, whereas the posterodorsal region (MePD) receives less chemosensory input, but contains dense populations of androgen and estrogen receptors. Importantly, these sub-regions have considerable reciprocal connections, and previous studies in our lab have shown that functional interactions between MeA and MePD are required for the preference to investigate opposite-sex odors in male hamsters. We therefore hypothesized that chemosensory and hormone signals are conveyed *directly* between MeA and MePD. To test this hypothesis, we injected the retrograde tracer, cholera toxin B (CTB), into either MeA or MePD of male subjects and identified whether retrogradely labeled cells within MePD or MeA, respectively, expressed (1) Fos protein following exposure to female or male odors or (2) androgen receptors (AR). Approximately 36% of CTB-labeled cells within MeA (that project to MePD) also expressed Fos following exposure to either social odor, compared to the only 13% of CTB-labeled cells within MePD (that project to MeA) that also expressed odor-induced Fos. In contrast, 57% of CTB-labeled cells within MePD also contained AR, compared to the 28% of CTB-labeled cells within MeA that were double-labeled for AR/CTB. These results provide the first anatomical evidence that chemosensory and hormone cues are conveyed directly between MeA and MePD. Furthermore, these data suggest that chemosensory information is conveyed primarily from MeA to MePD, whereas hormone information is conveyed primarily from MePD to MeA. More broadly, the interactions between MeA and MePD may represent a basic mechanism by which the brain integrates information about social cues in the environment with hormonal indices of reproductive state.

Keywords

Reproductive behavior; Hamsters; Olfaction; Fos; Androgen receptor; Retrograde tracer

Adaptive reproductive behavior requires integrating external signals about possible mates in the environment with internal signals of reproductive state (Wingfield et al., 1997, Gowaty and Hubbell, 2009). In rodent species, including Syrian hamsters, sex-specific odors provide

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the primary signal for mate recognition (Johnston, 1983, 1990, Keverne, 2004); male hamsters are highly motivated to investigate female odors (Murphy, 1973, Johnston, 1974), and these chemosignals stimulate the expression of male copulatory behavior (Murphy, 1973, Johnston, 1975). As in other vertebrates, internal signals of reproductive state in hamsters are conveyed through changes in circulating levels of gonadal steroid hormones, such as testosterone (Beyer et al., 1976, Morin and Zucker, 1978, Hull and Dominguez, 2007). Testosterone and its primary metabolites, estradiol and dihydrotestosterone, are critical not only for the expression of male hamster copulatory behavior (Morin and Zucker, 1978, Powers et al., 1985), but also for male's attraction to investigate female odors (Steel, 1982, Powers and Bergondy, 1983, Powers et al., 1985, Petrulis and Johnston, 1995). Consequently, the expression of reproductive behavior in male hamsters involves the neural integration of chemosensory and hormonal cues (Wood, 1998).

The medial amygdala (MA) has been suggested as one candidate site for the integration of chemosensory and hormonal cues, as it receives both types of information (Wood, 1998). Lesions of MA disrupt the odor-guided aspects of reproductive behavior in many rodent species, including hamsters (Lehman et al., 1980, Petrulis and Johnston, 1999, Maras and Petrulis, 2006), rats (Kondo, 1992, Kondo et al., 1997, Stark et al., 1998), and gerbils (Heeb and Yahr, 2000). The MA also mediates hormonal control of reproductive behavior, as unilateral testosterone implants directly into MA facilitate the expression of copulatory behavior in castrated male hamsters (Wood and Newman, 1995c) and rats (Huddleston et al., 2003, Huddleston et al., 2006). Unilateral olfactory bulbectomy ipsilateral to the steroid implant, however, prevents this facilitation in male hamsters (Wood and Newman, 1995b, Wood and Coolen, 1997), suggesting that processing of both chemosensory and steroid cues within MA is critical for the expression of reproductive behavior in male hamsters.

Detailed analysis of the MA suggests that chemosensory and hormone cues are processed within separate, but interconnected sub-regions of MA. In particular, the anterior MA (MeA) receives the majority of chemosensory input, as it has extensive connections with both the main and accessory olfactory systems (Scalia and Winans, 1975, Kevetter and Winans, 1981, Lehman and Winans, 1982, Coolen and Wood, 1998, Kang et al., 2009). Although the posterodorsal MA (MePD) receives input from the olfactory bulbs, these projections are less substantial than compared to MeA, and the MePD also has much more limited connections with secondary nuclei of the main olfactory system compared to MeA (Scalia and Winans, 1975, Kevetter and Winans, 1981, Lehman and Winans, 1982, Coolen and Wood, 1998, Kang et al., 2009). In contrast, the processing of steroid hormone information appears to be processed specifically within MePD, as the majority of steroid receptor-containing MA neurons are localized specifically within MePD rather than the MeA (Doherty and Sheridan, 1981, Wood et al., 1992, Wood and Newman, 1993). This anatomical separation of chemosensory and hormone processing is maintained throughout much of the extended forebrain circuit; MeA and MePD preferentially project to chemosensory and steroid-sensitive sub-regions, respectively, of the bed nucleus of the stria terminalis and medial preoptic area (Gomez and Newman, 1992, Canteras et al., 1995, Coolen and Wood, 1998). Importantly, MeA and MePD share dense reciprocal connections (Coolen and Wood, 1998), and functional interactions between these sub-regions are critical for the preference to investigate opposite-sex odors in male hamsters (Maras and Petrulis, 2010).

Taken together, these data indicate that although MeA and MePD process chemosensory and hormone signals differently, these signals are likely integrated at the level of the MA. We hypothesized that chemosensory and hormone signals are conveyed directly between MeA and MePD, such that odor-responsive neurons within MeA project to MePD, and hormone-sensitive neurons within MePD project to MeA. To test this hypothesis, we injected a retrograde tracer into either MeA or MePD of male hamsters and identified whether labeled

cells within MePD or MeA, respectively, expressed (1) Fos protein (an indirect marker of neuronal activation) following exposure to sex-specific odors or (2) androgen receptors (AR). These anatomical data provide the first evidence that chemosensory and hormone cues are conveyed directly between MeA and MePD and identify a possible substrate for the neural integration of these signals within MA.

Experimental Procedures

Animals

Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratory (Wilmington, MA) at eight weeks of age. All subjects (total $n = 45$) were gonadally intact, sexually naïve males (3 – 5 months old, 120 – 150g) that were singly housed upon arrival and remained singly housed for the duration of the study. A separate group of gonadally intact male and female hamsters (3 – 8 months old, $n = 80$) were used to provide odor stimuli. Subjects were unrelated to, and had no previous contact with, these odor donor animals. All animals were housed in solid-bottom Plexiglas cages (36 cm $\times$ 30 cm $\times$ 16 cm) with corn cob litter (the Andersons, Maumee, OH, USA) and cotton bedding material (Ancare, Bellmore, NY, USA) and were maintained on a reversed 14-h light/ 10-h dark photoperiod (lights off/on at 9 am/7 pm). Food and water were available *ad libitum*. All animal 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 injection

To visualize afferents of MeA or MePD (hereafter referred to as MeA-MePD projecting neurons), the retrograde tracer, cholera toxin B (CTB), was deposited into each sub-region (MeA $n = 23$; MePD $n = 22$). CTB (List Biological Laboratories, Campbell, CA, USA) was prepared as a 0.5% solution in 0.1M PBS (pH 7.5) according to manufacturer's instructions. For deposition of the tracer, males were anesthetized with 2% isoflurane anesthesia and placed into a stereotaxic apparatus so that the skull was flat. The temporal muscles were retracted from the skull, and small holes were drilled to expose the surface of the brain. CTB was loaded into a 10- μ l Hamilton microsyringe, which was lowered under stereotaxic control into either MeA (AP + 0.75 mm, ML $\pm$ 2.60 mm, DV – 7.95 mm) or MePD (AP – 0.30 mm, ML $\pm$ 2.80 mm, DV – 7.80 mm). For each sub-region, 20 nl of CTB was injected over a two-minute period, and the needle was left in place for twenty minutes prior to removal from the brain. Bone wax was used to seal the hole in the skull, and the incision was closed with wound clips.

Stimulus exposure and tissue collection

All males were sacrificed eight to nine days following tracer injection to allow for sufficient transport of CTB (Vercelli *et al.*, 2000). During this time, males were extensively handled 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 one hour prior to any manipulation. MA neurons have been shown to display increases in Fos expression in response to either opposite-sex or same-sex odor stimuli (Fiber *et al.*, 1993, Halem *et al.*, 1999, Meredith and Westberry, 2004, Samuelsen and Meredith, 2009), and both types of odor information may be conveyed between the MeA and MePD (Maras and Petrulis, *in press*). Thus, to determine if MeA-MePD projecting neurons respond to opposite-sex or same-sex odors, a sub-set of injected males were exposed to either female (MeA $n = 11$; MePD $n = 9$) or male (MeA $n = 9$; MePD $n = 11$) odors, respectively. Exposure to sex-specific odors consisted of placing a

subject into a vacated odor donor cage; subjects were therefore exposed to both the volatile and non-volatile components of odor stimuli, including those from the soiled litter, bedding, and walls of the cage. In order to minimize differences in odor quality across individual odor donors and to provide a composite source of sex-specific (rather than individual-specific) odors, all stimulus cages housed 3 – 4 female or male hamsters (Maras and Petrulis, in press). Furthermore, to ensure equivalent levels of odor stimuli, all stimulus cages had not been changed for 4 days prior to use; thus, all female stimulus cages included odors from across the entire estrous cycle, including behavioral estrus. Finally, to provide a measure of the baseline activation of MeA-MePD projecting neurons, a third group of injected males was handled and returned to their home cage (MeA $n = 3$; MePD $n = 2$).

Males were left undisturbed in the stimulus (or home) cage for 60 minutes, upon which time they were injected with an overdose of sodium pentobarbital (100 mg/kg; Sleep Away, Ft. Dodge, IA, USA) and allowed to reach a deep level of anesthesia prior to perfusion (an additional 10 – 15 minutes). This survival time is within the time range for peak Fos protein expression (60 – 90 minutes; Herdegen and Leah, 1998) and has previously been used for the induction of Fos by odor stimuli in Syrian hamsters (Fiber and Swann, 1996, Delville et al., 2000, Swann et al., 2001, Lai et al., 2004). Males were transcardially perfused with 200 ml of 0.1M phosphate-buffered saline (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) prior to being placed in 30% sucrose in PBS solution for 48 – 72 hours (4°C). In order to distinguish between left and right brain hemispheres, an angle was cut into the right dorsal cortex of each brain. Brains were sectioned coronally (35- μ m thickness) using a cryostat set at -20°C. Brain sections throughout the rostrocaudal extent of the MA (Morin and Wood, 2001) were collected in a 1:4 series and stored in cryoprotectant-antifreeze solution (Watson *et al.*, 1986) until processing.

Immunohistochemistry

To determine whether MeA-MePD projecting neurons (a) respond to odor stimuli or (b) are androgen-sensitive, separate series of tissue sections were processed for Fos and CTB or AR and CTB, respectively. Procedures for double-labeling Fos/CTB and AR/CTB were identical except for the specific antibodies used, and for both procedures, sections were stained first for Fos or AR, followed by CTB. Briefly, sections were removed from cryoprotectant and rinsed thoroughly in PBS (pH 7.4). To reduce endogenous peroxidase activity, tissue sections were incubated in 0.5% H₂O₂ in PBS for 15 minutes. After several rinses, sections were then incubated in the primary antibody for Fos or AR in PBS with 0.4% Triton-X100 for 24 hours at room temperature. The primary antibody for Fos was a rabbit polyclonal antibody (Santa Cruz Biotechnology, sc(4)-52) generated against a peptide mapping the N-terminus of the human c-Fos and was used at a concentration of 1:20,000. The primary antibody for AR was a rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-816) generated against a peptide mapping the N-terminus of the human AR and was used at a concentration of 1:3,000. The specificity of these Fos and AR primary antibodies has previously been confirmed by preabsorption with epitope peptide (Kollack-Walker and Newman, 1997, Creutz and Kritzer, 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-X100. 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 of PBS with 0.4% Triton-X100, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). After rinsing in PBS and then in 0.175M sodium acetate, Fos or AR was visualized using 3,3'-diaminobenzidine HCl (0.2mg/ml, Sigma) and H₂O₂ (0.83 μ l/ml, Sigma) in a nickel-

sulfate solution (25mg/ml, Sigma) for 15 minutes. This staining reaction yields a blue-black product and was stopped by rinsing sections in sodium acetate.

Immediately following Fos or AR staining, tissue sections were incubated in the primary antibody for CTB in PBS with 0.4% Triton-X100 for 24 hours at room temperature. The primary antibody for CTB was a goat polyclonal antibody (List Biological Laboratories, Campbell, CA, USA) generated against cholera toxin B subunit and was used at a concentration of 1:80,000. Procedures for labeling CTB were identical to those described above except that (1) the biotinylated secondary antibody was anti-goat and (2) CTB was visualized using 3,3'-diaminobenzidine HCl (0.2mg/ml, Sigma) and H₂O₂ (0.83 µl/ml, Sigma) in Tris buffer (pH 7.2), yielding a brown reaction product. In addition to double-labeling, a separate series of tissue sections was single-labeled for CTB alone using identical procedures to those described above. 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 ©.

Data analysis

A single researcher that was blind to the stimulus condition of the animal conducted all analyses. Tissue that was single-labeled for CTB was used to examine the placement and spread of CTB injections. Only those subjects with CTB deposition restricted to the target sub-region (see RESULTS) were analyzed. As projections between MeA and MePD are primarily ipsilateral (Coolen and Wood, 1998), CTB, Fos and AR labeling was analyzed within nuclei ipsilateral to the injection. In addition, to determine whether the accumulation of CTB decreases Fos expression within MA, we also quantified Fos immunoreactivity within nuclei contralateral to the injection.

Sections were examined using a Nikon Eclipse E800 microscope with a Synsys digital camera attached. Counting domains were generated by projecting the microscopic field (10×) onto a computer screen using iVision software (Atlanta, GA, USA). In MeA-injected males, cells were counted within three separate sections of MePD (Figure 1B, D, F). In MePD-injected males, cells were counted within three separate sections of MeA, including both dorsal and ventral regions (Figure 1A, C, E). The minimum distance between any two sections within a single analysis (Fos/CTB or AR/CTB) was 140 µm. The diameter of cells within MA ranges from 10 – 20 µm (McDonald, 1992), thereby precluding duplicate counting of cells within any two sections.

Fos-positive (Fos+) or AR-positive (AR+) cells were identified as having dark, blue-black nuclear staining. As CTB has slight anterograde properties (Kobbert et al., 2000, Vercelli et al., 2000), CTB-positive (CTB+) cells were counted only if they had brown cytoplasmic staining that filled the shape of the cell. Fos/CTB-positive (Fos/CTB+) or AR/CTB-positive (AR/CTB+) cells were identified as having a dark, blue-black nucleus surrounded by brown cytoplasmic staining. The total numbers of Fos+, AR+, Fos/CTB+, and AR/CTB+ cells were calculated separately for MeA and MePD (total of three sections per area) and were divided by the total area analyzed for each region (MeA: 1.947 mm², MePD: 1.178 mm²) to reflect the density of immunoreactive cells per mm². These densities were then used to generate the percent of total Fos+ cells that are also Fos/CTB+, the percent of total CTB+ cells that are also Fos/CTB+, the percent of total AR+ cells that are also AR/CTB+, and the percent of total CTB+ cells that are also AR/CTB+ for each region.

SPSS 16.0 (SPSS Inc, Chicago, IL, USA) was used for all data analyses, and significance was determined as $p < .05$. All data are reported as mean $\pm$ SEM. To identify differences in the densities of Fos+ and Fos/CTB+ cells across stimulus conditions (Handled, Female, Male), separate one-way ANOVAs were used for each brain area (MeA, MePD), followed

by Tukey's-B post-hoc tests. To compare the densities of CTB+, AR+, and AR/CTB+ cells between brain areas, data were combined across stimulus conditions and compared using separate independent *t*-tests. Finally, Mann-Whitney U tests were used to identify differences in the percentages of Fos/CTB+ or AR/CTB+ cells between brain MeA and MePD.

For data presentation, photomicrographs were taken using a Synsys digital camera attached to a Nikon Eclipse E800 microscope (4× or 20×). Images were captured using iVision (Atlanta, GA, USA) and processed using Adobe Photoshop CS2 Version 9.0 (San Jose, CA, USA) only to enhance brightness and contrast. In addition, to illustrate the spatial patterns of AR and CTB labeling observed within MeA and MePD, a representative male from each injection group was selected and the distributions of AR+, CTB+, and AR/CTB+ cells were plotted directly from a photomicrograph (4×) onto published hamster atlas plates (Morin and Wood, 2001) using Adobe Illustrator CS2 Version 9.0.

Results

CTB deposition and retrograde labeling

Males were included in the MeA- or MePD-injection group only if dark CTB staining was observed around the deposition site within at least two atlas plates of section for MeA or MePD, respectively (Morin and Wood, 2001; Figure 2). Males were excluded from the analyses if the injection was misplaced (MeA, *n* = 3; MePD, *n* = 3). Within the MeA-injection group, misplaced injections resulted in CTB deposition lateral to the MeA, including the nuclei of the lateral and accessory olfactory tract. Within the MePD-injection group, misplaced injections resulted in CTB deposition either medial to the MePD into the optic tract (*n* = 2) or lateral to the MePD into the intercalated and central nuclei of the amygdala (*n* = 1). Males were also excluded from the analyses if the size of the injection was too small (i.e. observed within only one atlas plate; MeA, *n* = 5; MePD, *n* = 4) or too large (i.e. spread into the other sub-region of the MA; MeA, *n* = 2; MePD, *n* = 3). These inclusion and exclusion criteria resulted in a final total of 13 MeA-injected males (Female odor *n* = 6, Male odor *n* = 5, Handled *n* = 2) and 12 MePD-injected males (Female odor *n* = 5, Male odor *n* = 5, Handled *n* = 2). For every male included in the analyses, the placement and spread of CTB injections were traced by hand onto published hamster atlas plates (Morin and Wood, 2001), and the largest and smallest extents of CTB deposition are shown in Figure 2.

Within the MeA-injection group, CTB spread into both the dorsal and ventral sub-regions of the MeA in seven males, whereas injections were restricted to the dorsal MeA in six males. In nine MeA-injected males, injections were centered within the rostral section of the MeA (plate 24; Morin and Wood, 2001), whereas in four of these males, injections were centered within the middle section of the MeA (plate 25; Morin and Wood, 2001). Within the MePD-injection group, eight males had injections centered within the middle section of the MePD (plate 28; Morin and Wood, 2001), whereas four males had injections centered within the most caudal section of the MePD (plate 29; Morin and Wood, 2001). As shown in Figure 2, CTB deposition was primarily restricted to MeA or MePD, although a sub-set of MeA- and MePD-injected males had minor spread into adjacent nuclei, which included the anterior cortical amygdala (MeA *n* = 3), intercalated nucleus of the amygdala (MeA *n* = 2; MePD *n* = 3), and posteroventral MA (MePD *n* = 4). Importantly, CTB deposition into these adjacent nuclei was only partial, spreading into less than 20% of the area of each nucleus at any one plate of section. Furthermore, within each injection group, the placement and spread of CTB deposition were comparable across the different stimulus conditions (Female odor, Male odor, Handled).

CTB injections into the MeA or MePD retrogradely labeled cell bodies within the MePD or MeA, respectively (Figure 3A-D, 5A-D), as well as within several previously identified afferents of MA (Coolen and Wood, 1998), including the amygdalohippocampal area, anterior amygdaloid area, anterior cortical amygdala, bed nucleus of the stria terminalis, intercalated nucleus of the amygdala, posteromedial cortical amygdala, and ventromedial hypothalamus. Retrogradely labeled cells were also observed within the posterolateral cortical amygdala, endopiriform nucleus, and piriform cortex following injections into MeA (Coolen and Wood, 1998). For both MeA- and MePD-injected males, there were no differences in the overall pattern of retrograde labeling between males with the smallest and largest spread of CTB deposition, although larger injections were generally associated with higher densities of retrograde labeling.

Co-localization of CTB and Fos

The densities of CTB+, Fos+, and Fos/CTB+ cells within MeA and MePD are shown in Table 1. MeA and MePD had comparable levels of retrograde labeling, as indicated by the similar densities of CTB+ cells ($p > .05$). The densities of Fos+ and Fos/CTB+ cells following exposure to handling alone were very low, indicating low levels of constitutive Fos expression. Exposure to either female odors or male odors significantly increased the densities of Fos+ and Fos/CTB+ cells relative to handling within both the MeA (Fos+: $F(2, 10) = 7.862, p < .01$; Fos/CTB+: $F(2, 10) = 20.912, p < .001$) and MePD (Fos+: $F(2, 9) = 5.706, p < .05$; Fos/CTB+: $F(2, 9) = 7.793, p < .05$). Within both MeA and MePD, there were no differences in the densities of Fos+ cells or Fos/CTB+ cells between males exposed to female odors or male odors ($p > .05$).

Many retrogradely labeled cells within MeA and MePD expressed Fos in response to social odors (Table 1; Figure 3A-D). To identify the primary direction in which odor information is conveyed between MeA and MePD, we first compared the percentages of CTB+ cells within MeA and MePD that were double-labeled for Fos/CTB following exposure to either social odor (Female and Male odor groups combined; MeA $n = 11$; MePD $n = 10$). Thirty-six percent of CTB+ cells within MeA (that project to MePD) were double-labeled for Fos/CTB, which was significantly higher than the 13% of CTB+ cells within MePD (that project to MeA) that were double-labeled for Fos/CTB, $z = 3.873, p < .001$ (Figure 3E). Similarly, 21% of Fos+ cells within MeA were double-labeled for Fos/CTB, compared to the only 12% of Fos+ cells within MePD that were double-labeled for Fos/CTB, $z = 3.450, p < .001$ (Figure 3F).

To determine whether the percentages of Fos/CTB+ cells varied according to the sexual identity of the odor stimuli, we next compared these percentages between males exposed to female odors (opposite-sex) and males exposed to male odors (same-sex) for each brain area (Figure 4). Within both MeA and MePD, the percentages of CTB+ cells that were double-labeled for Fos/CTB were identical following exposure to either female odors or male odors (Figure 4A, B), as were the percentages of Fos+ cells that were double-labeled for Fos/CTB (Figure 4C, D) (all $p > .05$).

To determine whether accumulation of CTB altered levels of Fos expression, as has been observed with the use of other retrograde tracers (Greco *et al.*, 1998a), we compared the densities of Fos+ cells following exposure to either social odor (Female and Male odor groups combined; MeA $n = 11$; MePD $n = 10$) between nuclei ipsilateral and contralateral to the CTB injection (Table 2). Within both MeA and MePD, the densities of Fos+ cells ipsilateral to the injection were significantly lower than the densities of Fos+ cells contralateral to the injection (MeA: $t(10) = 7.732, p < .001$, MePD: $t(9) = 10.516, p < .001$), resulting in a 28% decrease within MeA and a 26% decrease within MePD compared to contralateral levels (Table 2).

Co-localization of CTB and AR

The densities of CTB+, AR+, and AR/CTB+ cells within MeA and MePD are shown in Table 3. MeA and MePD again had comparable levels of total retrograde labeling ($p > .05$), although the MePD had a significantly higher density of AR+ cells, $t(23) = 10.686$ $p < .001$, as well as AR/CTB+ cells, $t(23) = 11.826$ $p < .001$, than MeA. Furthermore, we noticed a consistent difference in the spatial distributions of AR+ and CTB+ cells between MeA and MePD; within the MeA, the populations of AR+ and CTB+ cells appeared to occupy distinct, relatively non-overlapping zones (Figure 5A), whereas these populations overlapped substantially within MePD (Figure 5B). As is further illustrated in Figure 6, the majority of AR+ cells within the MeA were located within the dorsal region, whereas the majority of CTB+ cells were located within the ventral region (Figure 6A). Although this was not an exclusive separation (i.e. some AR+ cells were located within the ventral MeA and some CTB+ cells were located within the dorsal MeA), this general distinction was observed in almost every subject (11/12). The MeA did contain some AR/CTB+ cells (Table 3, Figure 5), and these were often observed along the border of the AR+ or CTB+ zones (Figure 6A). Importantly, both the dorsal and ventral regions of the MeA were included in the data analysis (see Figure 1), and we were therefore able to capture each population of cells, as well as any double-labeled cells. In contrast to the MeA, both AR+ and CTB+ cells were scattered across the entire MePD, resulting in a large overlap of AR+ and CTB+ cells, and AR/CTB+ cells were observed throughout the nucleus (Figure 6B).

Finally, when we compared the percentage of AR/CTB+ cells between the two areas, the MePD had significantly higher percentages of double-labeled cells than MeA; 57% of CTB+ cells within MePD were double-labeled for AR/CTB, compared to the 29% of CTB+ cells within MeA that were double-labeled for AR/CTB, $z = 3.521$, $p < .001$ (Figure 5E). Similarly, 17% of AR+ cells within MePD were double-labeled for AR/CTB, compared to the 11% of AR+ cells within MeA that were double-labeled for AR/CTB, $z = 2.535$, $p < .05$ (Figure 5F).

Discussion

The MA has been identified as a possible site for the neural integration of chemosensory and steroid hormone input, as distinct sub-regions within the MA process each of these cues. Specifically, the MeA receives substantial chemosensory input (Scalia and Winans, 1975, Kevetter and Winans, 1981, Lehman and Winans, 1982), whereas the MePD is highly sensitive to steroid hormones (Doherty and Sheridan, 1981, Wood et al., 1992, Wood and Newman, 1993). These sub-regions share substantial reciprocal connections (Coolen and Wood, 1998), suggesting that the neural integration of chemosensory and hormone cues involves interactions directly between MeA and MePD. By combining the use of retrograde tracers with markers of odor-responsive neurons (Fos) or hormone-sensitive neurons (AR), the current experiments provide the first anatomical evidence that chemosensory and hormone cues are conveyed *directly* between MeA and MePD. Indeed, approximately 40% of MeA cells that project to MePD responded to social odors, and 60% of MePD cells that project to MeA expressed AR. These data suggest that the anatomical connections between MeA and MePD function to integrate chemosensory and hormone signals. Furthermore, by comparing the percentage of double-labeled cells between MeA and MePD, our results suggest that chemosensory information is conveyed primarily from MeA to MePD, whereas hormone information is conveyed primarily from MePD to MeA.

Methodological considerations

Previous studies have used CTB to label afferents of MA neurons in hamsters (Coolen et al., 1998, Coolen and Wood, 1998), and in the current study, we observed a pattern of

retrograde labeling similar to those reported previously. By combining the use of CTB with immunohistochemical markers for Fos and AR, we extended these previous findings by characterizing the odor-responsiveness and hormone sensitivity of MeA-MePD projecting neurons. Fos, an immediate early gene product (Sheng and Greenberg, 1990, Sheng et al., 1990), has been used extensively as an indirect marker of neuronal excitation in response to stimulus exposure (Pfaus and Heeb, 1997). Constitutive Fos expression observed in the handled control group was very low in this experiment, as were the levels of cells double-labeled for Fos/CTB in this condition. Thus, the co-localization of CTB and Fos observed in males exposed to female or male odors likely reflects specific activation of MeA-MePD projecting neurons in response to social odor stimuli, rather than constitutively activated cells.

In the current study, we observed a significant decrease in the expression of Fos protein ipsilateral to the side of the CTB injection, indicating that accumulation of CTB may decrease the ability of neurons to express Fos. A decrease in Fos expression may be a general problem with the use of retrograde toxins, as similar decreases in Fos expression within MA have been reported with the use of FluoroGold in the MA of rats (Greco *et al.*, 1998a). The current results may therefore underestimate the absolute levels of MeA-MePD projecting neurons that show activation. Importantly, CTB deposition decreased Fos expression within both MeA and MePD, and the relative magnitude of these decreases was very similar between the two areas (28% within MeA and 26% within MePD). Thus, any underestimation of Fos expression due to uptake of CTB would be equivalent between MeA and MePD and would not bias the direct comparison of double-labeled cells between the two sub-regions.

Odor-induced Fos expression

Previous IEG studies have shown that different types of social odors can activate MA neurons (Fiber et al., 1993, Heeb and Yahr, 1996, Coolen et al., 1997, Kelliher et al., 1998, Halem et al., 1999, Sheehan et al., 2000, Meredith and Westberry, 2004, Samuelsen and Meredith, 2009). In the current study, exposure to odors from either female or male hamster cages resulted in significant activation of MeA and MePD neurons, consistent with these previous findings. Furthermore, we found that opposite-sex and same-sex odor stimuli resulted in equivalent levels of Fos expression within both the MeA and MePD. Although few studies have directly compared levels of IEG expression within the MA following exposure to different categories of social odors, these studies have shown that MeA neurons display similar levels of activation in response to opposite-sex and same-sex odors (Maras and Petrulis, in press; Meredith and Westberry, 2004; Samuelsen and Meredith, 2009), similar to the results observed here. Neurons within the MePD can also be activated by either opposite-sex or same-sex odors, although the levels of IEG expression are typically higher following exposure to opposite-sex odors (Maras and Petrulis, in press; Meredith and Westberry, 2004; Samuelsen and Meredith, 2009). Differences in either the source of the odor stimuli (scent gland secretions or composite odors) and/or the exact placement of the counting domains within the MA may account for the different levels of IEG expression reported across studies. Nevertheless, the current results are consistent with the fact that MePD neurons are not exclusively activated by sexual stimuli (Kollack-Walker and Newman, 1995, Veening et al., 2005) and suggest that this nucleus may be involved in processing the non-sexual aspects of odor stimuli as well.

Chemosensory information is conveyed from MeA to MePD

Our results suggest that many of the cells that project between MeA and MePD respond to social odors, although this odor information appears to be conveyed predominantly from MeA to MePD, rather than from MePD to MeA. Indeed, relatively more MeA cells that

project to MePD responded to social odors than did MePD cells that project to MeA. Similarly, a higher percentage of odor-responsive neurons within MeA projected directly to MePD compared to those within MePD that projected to MeA. The fact that odor information is conveyed primarily from MeA to MePD is consistent with the fact that MeA receives relatively more chemosensory input compared to MePD (Scalia and Winans, 1975, Kevetter and Winans, 1981, Lehman and Winans, 1982, Coolen and Wood, 1998). Functionally, MeA also appears to play a greater role in regulating odor-guided reproductive behaviors in male hamsters than MePD; MeA lesions completely eliminate the expression of copulatory behavior (Lehman *et al.*, 1980), similar to what is observed following destruction of the olfactory bulbs (Murphy and Schneider, 1970), whereas lesions of MePD only alter the temporal pattern of the copulatory sequence (Lehman *et al.*, 1983). The current results also confirm recent studies in our laboratory showing that MeA lesions substantially reduce odor-induced Fos expression within MePD, whereas lesions of MePD have no effect on Fos expression within MePD (Maras and Petrulis, in press). Interestingly, MeA lesions reduce Fos expression within MePD to an equal degree following exposure to either opposite-sex or same-sex odors (Maras and Petrulis, in press), and our current results indicate that both types of odor information may be conveyed directly from MeA to MePD.

To our knowledge, no previous studies have examined the activation of MeA-MePD projecting cells in response to odor presentation alone. However, the percentages of MeA-MePD projecting cells that were activated in response to odor stimuli observed here (MeA 36%, MePD 13%) are lower than the percentage of MA cells projecting to the medial preoptic area that are activated during sexual behavior (60 – 65%) (Coolen *et al.*, 1998, Greco *et al.*, 1998b). These lower levels of activation may reflect the fact that presentation of odors alone provides relatively less total sensory input to the MA than does engaging in sexual behavior (Kollack-Walker and Newman, 1997). In support of this idea, we observed similar levels of activation of MeA-MePD projecting cells compared to the percentage of MeA cells projecting to the AOB that are also activated in response to opposite-sex volatile odors in mice (Martel and Baum, 2009). Importantly, although MeA appears to transmit information about social odors to MePD, our results indicate that many of the odor-responsive cells within MeA do not project to MePD. These odor-responsive cells may terminate locally on other MeA neurons, although the MA is characterized by few, if any, locally projecting cells, (McDonald, 1992), making this possibility unlikely. Instead, the odor-responsive cells not labeled by CTB likely represent cells that project to other ventral forebrain nuclei, such as the bed nucleus of the stria terminalis, medial preoptic area, and posteromedial cortical amygdala (Gomez and Newman, 1992, Canteras *et al.*, 1995, Coolen and Wood, 1998). In fact, lesions of MeA decrease odor-induced Fos expression within all of these regions (Maras and Petrulis, in press).

Hormone information is conveyed from MePD to MeA

Although neurons within both MeA and MePD expressed AR, there were substantially more AR+ cells within MePD compared to MeA. These results confirm previous reports of AR density within MA using immunohistochemistry (Wood and Newman, 1993, 1999) or autoradiography (Wood *et al.*, 1992). Consistent with these differences in the overall density of hormone-sensitive neurons, testosterone or estradiol implants facilitate the expression of copulatory behavior in castrated males when directed at MePD, but not MeA (Wood and Newman, 1995c, Huddleston *et al.*, 2003), suggesting a functional difference between MeA and MePD in their sensitivity to steroid hormones. Given these differences in hormone sensitivity, it is perhaps not surprising that the current study found relatively more double-labeled AR/CTB cells within MePD compared to MeA. Whereas approximately 57% of MePD cells that project to MeA expressed AR, a relatively low percentage of MeA cells that project to MePD also contained AR (29%). These results suggest that, in contrast to the flow

of chemosensory information, hormonal signals within MA are conveyed predominantly from MePD to MeA.

As in many rodent species (Moffatt, 2003), sufficient levels of gonadal hormones are critical for male hamsters' attraction to investigate female odors (Steel, 1982, Powers and Bergondy, 1983, Powers et al., 1985, Petrulis and Johnston, 1995), suggesting that hormones modulate neural processing of sexual odors. As has been proposed by Cottingham and Pfaff (Cottingham and Pfaff, 1986) and later by Wood (Wood, 1998), gonadal steroids may act as a gating mechanism to enhance transmission of sexually relevant odors, and this mechanism likely involves reciprocal connections between hormone-sensitive and chemosensory-responsive sub-nuclei. Thus, the hormone-sensitive projections from MePD to MeA may function to increase transmission of information about opposite-sex odors by MeA. In hamsters, exposure to female vaginal secretions (FVS) causes a significant increase in Fos expression within MePD of males, but not females (Fiber and Swann, 1996). Although this FVS-induced Fos expression in males does not require circulating testosterone, testosterone treatment in females masculinizes the response of MePD neurons to FVS (Fiber and Swann, 1996). In male rats, castration decreases, whereas testosterone administration increases, Fos expression within both the MeA and MePD in response to female odors (Paredes *et al.*, 1998). Given that MeA itself is not highly sensitive to steroid hormones (Wood, 1998), this effect may be mediated in part by projections from MePD. The current results provide anatomical support for an indirect model of chemosensory-hormone interaction within the MA, although further studies are needed to determine the functional significance of these hormone-sensitive projections.

Although the current study only examined the expression of AR within the MA, this nucleus also contains abundant estrogen receptor (ER)-containing cells (Doherty and Sheridan, 1981, Wood et al., 1992, Wood and Newman, 1995a), and these ERs mediate many of the effects of steroid hormones on male reproductive behavior (Wood, 1996). Unfortunately, the only antibody that consistently labels ER in hamster brain is the H222 antibody (Li et al., 1993, Wood and Newman, 1995a, Mangels et al., 1998, Boers et al., 1999, De La Iglesia et al., 1999), and this antibody labels specifically unbound ER, precluding its use in any studies that require subjects to maintain normal levels of gonadal steroid hormones (Blaustein, 1993). In hamsters, individual MA cells often co-express AR and ER, such that cells expressing only ER constitute a small fraction (13%) of the total population of AR- and ER-expressing cells within this nucleus (Wood and Newman, 1995a). These data suggest that the current findings 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.

Concluding remarks

The interactions between chemosensory and hormone-sensitive sub-regions of the MA may represent a basic mechanism by which the brain integrates information about social stimuli in the environment with hormonal indices of reproductive state (Wood, 1998, Newman, 1999). The connections between MeA and MePD may therefore provide a neural substrate for the refinement of odor responses and regulation of reproductive behaviors within a dynamic internal and external environment. The current findings provide the first anatomical evidence that chemosensory and steroid hormone cues are conveyed *directly* between MeA and MePD. Although we focus on chemosensory-hormone integration for the purposes of reproduction, other rodent social behaviors, such as aggressive or maternal behaviors, also rely heavily on chemosensory and hormone signals processed by the MA (Luiten et al., 1985, Numan and Sheehan, 1997, Blanchard et al., 2003). Thus, the MeA-MePD connections described here may be critical for regulating the appropriate behavioral responses to odor stimuli in a variety of social contexts.

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List of Abbreviations

AR	Androgen receptors
CTB	Cholera toxin B
ER	Estrogen receptors
MA	Medial amygdala
MeA	Medial amygdala, anterior region
MePD	Medial amygdala, posterodorsal region

Figure 1. Counting domains for analysis of CTB, Fos, and AR

Illustration adapted and modified from hamster brain atlas of Morin and Wood (2001) showing placement of counting domains (gray rectangles) for MeA (A, C, E) and MePD (B, D, F). Total numbers of immunoreactive cells represent the sum of three counting domains for each region. Values given in millimeters indicate distance posterior to bregma. ACo, anterior cortical amygdala; BLA, anterior basolateral amygdala; BLP, posterior basolateral amygdala; BMA, basomedial amygdala; CeA, central amygdala; I, intercalated amygdala; MeA, anterior medial amygdala; MeAD, anterodorsal medial amygdala; MeAV, anteroventral medial amygdala; MePD, posterodorsal medial amygdala; MePV, posteroventral medial amygdala; ot, optic tract; PLCo, posterolateral cortical amygdala; PMCo, posteromedial cortical amygdala.

Figure 2. Verification of CTB injections

Placement and spread of CTB deposition in MeA-injected males (A, C) and MePD-injected males (B, D). Reconstruction of coronal sections of the largest (light gray) and smallest (dark gray) extents of CTB deposition within the MeA (A) or MePD (B). Numbers represent the distance posterior to bregma in millimeters. Photomicrographs depict typical CTB injection into the MeA (C) or MePD (D). Scale bar = 200 μm applies to both C and D. ot, optic tract; 3V, third ventricle.

Figure 3. Co-localization of CTB and Fos

Photomicrographs of representative sections of the MeA (A, C) and MePD (B, D) that were double-labeled for Fos/CTB. C and D provide higher magnifications of the areas indicated by the boxes in A and B, respectively. Cells stained brown are CTB+ (white arrows), nuclei stained black are Fos+ (black arrows), and brown cells with black nuclei are Fos/CTB+ (red arrows). Scale bar = 100 μ m (A, B) and 50 μ m (C, D). A higher percentage of CTB+ cells within MeA were double-labeled for Fos/CTB compared to MePD (E), and a higher percentage Fos+ cells within MeA were double-labeled for Fos/CTB compared to MePD (F). * $p < .05$, Mann-Whitney U tests ($\alpha_{fw} = .05$).

% CTB+ cells that were Fos/CTB+

% Fos+ cells that were Fos/CTB+

Figure 4. Fos/CTB double-labeling observed in males exposed to female odors or male odors
For both MeA (A, C) and MePD (B, D), there were no significant differences in either the percentages of CTB+ cells that were double-labeled for Fos/CTB (top) or the percentages of Fos+ cells that were double-labeled for Fos/CTB (bottom) between males exposed to female or male odors.

Figure 5. Co-localization of CTB and AR

Photomicrographs of representative sections of the MeA (A, C) and MePD (B, D) that were double-labeled for AR/CTB. C and D provide higher magnifications of the areas indicated by the boxes in A and B, respectively. Cells stained brown are CTB+ (white arrows), nuclei stained black are AR+ (black arrows), and brown cells with black nuclei are AR/CTB+ (red arrows). Scale bar = 100 μ m (A, B) and 50 μ m (C, D). A higher percentage of CTB+ cells within MePD were double-labeled for AR/CTB compared to MeA (E), and a higher percentage AR+ cells within MePD were double-labeled for AR/CTB compared to MeA (F).

* $p < .05$, Mann-Whitney U tests ($\alpha_{FW} = .05$).

Figure 6. Distribution of AR and CTB within the MeA and MePD

The spatial distribution of AR+, CTB+ and AR/CTB+ cells within the MeA (A; CTB injected into MePD) and MePD (B; CTB injected into MeA) were plotted from a single representative male onto published hamster atlas plates (Morin and Wood, 2001). Cells single-labeled for AR (black dots) or CTB (open circles) or double-labeled for AR and CTB (×) were identified. Within the MeA (A), AR+ and CTB+ cells occupied largely distinct, non-overlapping zones, whereas these populations of cells overlapped substantially within the MePD (B).

Table 1
Density of CTB+, Fos+, and Fos/CTB+ cells within MeA and MePD

Within both the MeA and MePD, exposure to either female odors or male odors increased the numbers of Fos+ cells and Fos/CTB+ cells compared to handling exposure. Identical letters represent homologous means within each brain area (Tukey's-B post-hoc comparisons, $p < .05$).

Total/mm ²		MeA	MePD
CTB+		110.86 ± 6.84	108.09 ± 5.17
Fos+	<i>Handled</i>	56.25 ± 4.37 ^a	52.19 ± 1.80 ^a
	<i>Female</i>	189.11 ± 22.80 ^b	147.33 ± 21.07 ^b
	<i>Male</i>	207.96 ± 16.90 ^b	113.54 ± 10.49 ^b
Fos/CTB+	<i>Handled</i>	4.37 ± 0.26 ^a	6.15 ± 0.21 ^a
	<i>Female</i>	36.22 ± 3.90 ^b	15.28 ± 1.44 ^b
	<i>Male</i>	43.05 ± 1.84 ^b	13.94 ± 1.27 ^b

Table 2
Density of Fos+ cells within MeA and MePD observed ipsilateral or contralateral to CTB injection

Within both MeA and MePD, fewer Fos+ cells were observed ipsilateral compared contralateral to the CTB injection. * $p < .05$ compared to ipsilateral totals within each brain area.

	Total Fos+ cells/mm ²	
	Ipsilateral	Contralateral
MeA	198.54 ± 13.24	275.28 ± 11.18 *
MePD	129.38 ± 15.75	175.79 ± 14.14 *

Table 3
Density of CTB+, AR+, and AR/CTB+ cells within MeA and MePD

More AR+ and AR/CTB+ cells were observed within MePD compared to MeA (* $p < .05$).

Total/mm ²	MeA	MePD
CTB+	108.54 ± 5.29	120.26 ± 3.82
AR+	237.20 ± 10.58	463.47 ± 21.30*
AR/CTB+	27.41 ± 1.98	77.30 ± 3.55*