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A direct main olfactory bulb projection to the ‘vomeronasal’ amygdala in female mice selectively responds to volatile pheromones from males

Ningdong Kang¹, Michael J. Baum¹, and James A. Cherry²

¹ Department of Biology, Boston University, Boston, MA, USA

² Department of Psychology, Boston University, Boston, MA 02215, USA

Abstract

The main olfactory system, like the accessory olfactory system, responds to pheromones involved in social communication. Whereas pheromones detected by the accessory system are transmitted to the hypothalamus via the medial (‘vomeronasal’) amygdala, the pathway by which pheromones are detected and transmitted by the main system is not well understood. We examined in female mice whether a direct projection from mitral/tufted (M/T) cells in the main olfactory bulb (MOB) to the medial amygdala exists, and whether medial amygdala-projecting M/T cells are activated by volatile urinary odors from conspecifics or a predator (cat). Simultaneous anterograde tracing using Phaseolus vulgaris leucoagglutinin and Fluoro-Ruby placed in the MOB and accessory olfactory bulb (AOB), respectively, revealed that axons of MOB M/T cells projected to superficial laminae of layer Ia in anterior and posterodorsal subdivisions of the medial amygdala, whereas projection neurons from the AOB sent axons to non-overlapping, deeper layer Ia laminae of the same subdivisions. Placement of the retrograde tracer cholera toxin B into the medial amygdala labeled M/T cells that were concentrated in the ventral MOB. Urinary volatiles from male mice, but not from female conspecifics or cat, induced Fos in medial amygdala-projecting MOB M/T cells of female subjects, suggesting that information about male odors is transmitted directly from the MOB to the ‘vomeronasal’ amygdala. The presence of a direct MOB-to-medial amygdala pathway in mice and other mammals could enable volatile, opposite-sex pheromones to gain privileged access to diencephalic structures that control mate recognition and reproduction.

Keywords

anterograde; mating; pheromone; retrograde; sex; vomeronasal organ

Introduction

Chemosignals are detected in mammals primarily by two distinct sensory systems. Whereas the conventional view has been that the main olfactory system is primarily responsible for detection of common volatile odors, and the accessory olfactory system responds to non-volatile pheromones involved in social communication, a number of recent papers have emphasized that both olfactory systems play significant roles in the processing of species-

Correspondence: Dr J. A. Cherry, as above. E-mail: jcherry@bu.edu.

Supporting information

Additional Supporting Information may be found in the online version of this article:

specific chemosensory signals important for reproduction (Pankevich *et al.*, 2004; Restrepo *et al.*, 2004; Brennan & Zufall, 2006; Shepherd, 2006).

The traditional view that the main olfactory system plays a subordinate role in the processing of reproductively relevant olfactory cues is based on anatomical distinctions between the main and accessory olfactory systems. The accessory system is comprised of receptor neurons in the vomeronasal organ (VNO) that innervate glomeruli in the accessory olfactory bulb (AOB). Efferents from AOB mitral cells project to the medial amygdala (Me), referred to as the 'vomeronasal amygdala' (Kevetter & Winans, 1981a), which in turn projects to the bed nucleus of the stria terminalis (BNST), ventromedial hypothalamus and medial preoptic area – structures that control neuroendocrine and behavioral aspects of reproduction. Conversely, receptor neurons in the main olfactory epithelium synapse with mitral cell dendrites in glomeruli of the main olfactory bulb (MOB). Although MOB mitral cells project heavily to sites throughout the olfactory cortex, it has long been recognized that amygdaloid nuclei, primarily the anterior and posterolateral cortical amygdala (ACo, PLCo), receive direct MOB input (Kevetter & Winans, 1981b; Licht & Meredith, 1987). In addition, there is now evidence in the sheep and rat (Jansen *et al.*, 1998; Pro-Sistiaga *et al.*, 2007) for direct connections from MOB mitral cells to the Me, suggesting that odors processed by the main olfactory system, via the Me, may have greater access to the hypothalamus than previously realized.

There is strong evidence in mice that volatile urinary odors from conspecifics activate populations of MOB glomeruli, as measured by induction of Fos in periglomerular cells, and that this glomerular response is similar in male and female subjects (Schaefer *et al.*, 2001, 2002; Martel *et al.*, 2007). What has not been addressed, however, is if detection of these urinary volatiles results in selective activation of MOB projection neurons that directly target the Me. Therefore, in an initial study using anterograde and retrograde tracing in female mice, we show that projection neurons (mitral and/or tufted cells, or M/T) in the MOB send axons directly to the Me. Then, using a combination of Fos and cholera toxin B (CTb) immunohistochemistry, we demonstrate that these Me-projecting M/T cells in the MOB of female mice selectively co-expressed Fos after exposure to volatile urinary odors from male conspecifics. These results reveal a projection pathway from the MOB to the Me that is selectively responsive to opposite-sex urinary volatiles, and raise the possibility that a direct route from the MOB to amygdalo-hypothalamic structures may exist in mammals – including humans – that is activated by volatile pheromones.

Materials and methods

Subjects and urine collection

Adult Swiss Webster mice (8–12 weeks) were purchased (Taconic Farms, Germantown, NY, USA) and maintained on a 12:12 h light:dark cycle with food and water available at all times. Mice were group housed (four per cage) in Experiments 1 and 2, and singly housed in Experiment 3. Urine was obtained from mice after restraining and gently applying abdominal pressure. Urine donor mice were 12 gonadally intact adult males and 15 ovariectomized females treated with 20 µg estradiol benzoate 48 and 24 h, and 500 µg progesterone 4 h prior to urine collection. Cat urine was obtained from three domestic female cats. Regular litter was replaced with Nosorb urine-collecting litter (Catco Vet Products, Cape Coral, FL, USA) overnight. Urine was then collected into clean containers. Urine of each type was pooled, then aliquoted and frozen at –80°C until use. All procedures involving animals followed NIH guidelines and were approved by the Boston University Charles River Campus Institutional Animal Care and Use Committee.

Reagents

Phaseolus vulgaris leucoagglutinin (PHA-L, L-1110), goat anti-PHA-L AS-2224, biotinylated rabbit anti-goat, ABC *Elite* reagent and diaminobenzidine (DAB) with nickel enhancement were obtained from Vector Laboratories, Burlingame, CA, USA.

Dextran-tetramethylrhodamine conjugate (Fluoro-Ruby, D-1820), TO-PRO-3 iodide and Alexa Fluor 488-Streptavidin were purchased from Molecular Probes, Eugene, OR, USA; CTb (#104) and goat anti-CTb antibody, #703 were from List Biological Laboratories, Campbell, CA, USA; rabbit anti-cFos antibody, SC-52, was from Santa Cruz Biotechnology, Santa Cruz, CA, USA; and biotinylated donkey anti-rabbit antibody was from Jackson ImmunoResearch Laboratories, West Grove, PA, USA. Anti-gonadotropin-releasing hormone (GnRH) antibody (LR-1, 1:100 000 dilution) was obtained from Dr R. Benoit (Montreal, Quebec, Canada). The sensitivity and specificity of this antibody in mouse brain was previously evaluated (Gill *et al.*, 2008).

Stereotaxic injections of tracers

All stereotaxic injections (Table 1) were made with pulled glass micropipettes (tip diameter = 10–15 μm) and a constant current source (Stoelting, Wood Dale, IL, USA). Female mice were anesthetized using 1% isoflurane vapor, and the head was fixed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) using ear bars. The skull was exposed, whereupon lambda and bregma were placed in the same horizontal plane by adjusting the incisor bar. A small hole was drilled above the injection target, and the pipette was carefully lowered. In Experiment 1, PHA-L (2.5% in 0.01 M phosphate buffer, pH 8.0; PB) was injected iontophoretically into the ventral and anterior MOB using coordinates rostral to the inferior cerebral vein, lateral to the midline, and below the dura as follows: 1.0, 0.8, 2.2 mm (ventral); and 1.2, 0.7, 2.0 mm (anterior). For mice that received anterograde tracers into both the MOB and the AOB, Fluoro-Ruby (5% in 0.01 M PB, pH 8.0) was injected iontophoretically into the AOB of the same hemisphere of the PHA-L MOB injection at a 50° angle off the horizontal plane. The coordinates for the AOB were 1 mm anterior to the inferior cerebral vein, 0.8 mm lateral to the midline and 2.0 mm below the dura. Subsequent analysis revealed that these injection sites were biased towards the caudal AOB (see Results). A +5 μA continuous current was applied for 10 min to deliver the tracers into the MOB/AOB. In Experiments 2 and 3 the retrograde tracer CTb (0.5% in 0.1 M PB, pH 6.0) was placed iontophoretically into the Me, ACo or piriform cortex (Pir) using coordinates posterior to bregma, lateral to the midline, and below the dura as follows: 1.25, 2.0, 4.6 mm (Me); −1.0, 2.4, 5.3 mm (ACo); and −1.35, 3.75, 4.7 mm (Pir). A +2 μA alternating (7 s on/7 s off) current was given for 10 min to deliver each tracer. The pipette was left in place for 5 min after termination of each injection and was withdrawn from the brain under a −2 μA current. A piece of sterile aluminum foil was glued to the skull using a cyanoacryl ester adhesive to seal the hole, and the skin was sutured to close the wound. Seven (Experiments 1 and 2) or 10–14 (Experiment 3) days after surgery, animals were killed and the brain was removed for processing by immunocytochemistry.

Immunocytochemistry

Mice were deeply anesthetized with sodium pentobarbital (150 mg/kg) and perfused transcardially with 100 mL 0.1 M phosphate-buffered saline (PBS) followed by 150 mL 4% paraformaldehyde. Brains were removed and post-fixed in the same fixative for 2 h before soaking in 30% sucrose overnight at 4°C. Free-floating sagittal (olfactory bulb) and coronal (forebrain) sections (40 μm) were cut on a freezing microtome and washed in 0.1% Triton X-100 in PBS (PBST). For single-labeling, peroxidases were first inactivated by incubating sections in 40% methanol and 1% H_2O_2 in PBS for 10 min. Sections were then incubated in blocking solution (PBST containing 5% rabbit or goat serum) for 1 h at RT, followed by an overnight incubation at 4°C in one of the following primary antibodies in blocking solution:

goat anti-PHA-L (1:4000), goat anti-CTb (1:40 000), or rabbit anti-cFos (1:1000). After washes in PBST, sections were transferred to biotinylated secondary antibodies – either biotinylated rabbit anti-goat (1:400) or biotinylated donkey anti-rabbit (1:600) – in blocking solution for 2 h at RT. After PBST washes, sections were incubated with ABC (1:200), rinsed in PBST and 0.05 M Tris–Cl buffer (pH 7.6), and visualized in DAB with nickel enhancement. Double-labeling for Fos and CTb was accomplished by first immunostaining for Fos, then re-fixing sections in 4% paraformaldehyde for 10 min and washing in PBST before a second immunostaining for CTb. Rabbit serum (5%) and 2% goat serum were added to the secondary antibody solution. Two different chromogens (DAB-nickel, black; and DAB-only, brown) were used for Fos and CTb staining, respectively. After labeling, sections were rinsed in water, mounted and dried in air overnight. Some sections were counterstained with 1% neutral red before coverslipping.

Fluorescent immunocytochemistry was used for PHA-L. After incubating with blocking solution for 1 h, free-floating sections were incubated with primary (goat anti-PHA-L, 1:4000, overnight at 4°C) and secondary antibodies (biotinylated rabbit anti-goat, 1:400, 1.5 h at RT) in blocking solution before visualizing with Alexa Fluor 488-Streptavidin (1:800).

Image processing

Fluorescent images were captured on an Olympus Fluoview confocal microscope. Each fluorophor was captured separately in stacked slices. Individual color channels were collapsed in the Z dimension and combined to yield a color image using Image J software (NIH, v. 3.2). Light images were captured with an Olympus (BH2) microscope equipped with a Nikon DXM1200 digital camera. Images were cropped, arranged and labeled in Adobe Photoshop (v. 5.5). Drawings were made using a Neurolucida System (Micro BrightField, Williston, VT, USA). Structures were delineated based on neutral red or TO-PRO-3 staining. In referring to labeled brain regions we adhere to the designations given in the mouse brain atlas of Paxinos & Franklin (2001). In particular, this atlas describes five subnuclei of the medial amygdala: anteroventral (MeAV), anterodorsal (MeAD), posteroventral (MePV), posterodorsal (MePD) and anterior (MeA) medial amygdala, with the MeA shown as a region separating the anterior and posterior subnuclei.

Specific experiments

Ovary-intact female mice were used at unspecified stages of the estrous cycle in Experiments 1 and 2. In Experiment 1, females received injections of anterograde tracer into the MOB alone (PHA-L), or into both the MOB (PHA-L) and AOB (Fluoro-Ruby). After 1 week, mice were killed and brains were processed for immunohistochemistry. In Experiment 2, female mice were given bilateral injections of the retrograde tracer, CTb, aimed at layer Ia of the Me. To visualize the distribution of CTb-labeled cells in the MOB, cell positions were traced from sagittal sections using a Neurolucida system. Olfactory bulb sections were categorized as lateral (> 0.92 mm), central (between 0.92 and 0.64 mm) or medial (< 0.64 mm), respectively, based on their estimated distance from the midline (see Fig. S1). Cells traced from sections in the same category were collapsed to generate maps representing lateral, central and medial views of the olfactory bulb. Sections assigned to the central segment were further divided into dorsal and ventral parts by a virtual line drawn parallel to the ventral surface of the olfactory bulb and across the most rostral point of the arch of the MOB mitral cell layer (see Fig. 4).

Only tissue from hemispheres that received successful injections of tracer were analysed. For PHA-L and Fluoro-Ruby injections, a hemisphere was excluded from the study if we could not see an obvious injection site in the MOB and/or AOB in addition to anterogradely labeled axons in the lateral olfactory tract. For CTb injections, exclusion criteria included: an injection outside of the Me or deeper than layer Ia of the Me, or no apparent AOB mitral cell labeling

(see Fig. S2). The overall success rate for Me injections of CTb delivered to subjects in Experiments 2 and 3 was 38% (see Table 1 for a summary of all injections). There were few subjects that received successful Me injections bilaterally; in these animals, single- and double-labeled cell counts and ratios (see Experiment 3) were averaged to achieve a single measure for each subject.

In Experiment 3, female mice that had been given bilateral CTb injections into the Me 10–14 days earlier (the longer interval between CTb injection and odor exposure compared with Experiments 1 and 2 was to reduce surgery-related Fos expression in MOB and forebrain) were placed individually into a narrow plastic box (5 × 10 × 25 cm) equipped with a suspended metal floor with rows of holes so that subjects' urine and feces fell below them. These female subjects had been ovariectomized under 1% isoflurane anesthesia and later treated sequentially with 20 µg estradiol benzoate 3 and 1 days prior to the administration of 500 µg progesterone, which was followed 4 h later by odor presentation. Previous work (Halem *et al.*, 1999) suggested that maximal forebrain Fos responses to non-volatile pheromonal cues in female mice occurred after treatment with ovarian hormones. Therefore in the present study we wanted to assess females' MOB and forebrain Fos responses to volatile urinary odors when they were in estrus. The use of ovariectomy and exogenous ovarian hormones avoided any potentially disruptive, stress-induced effects of brain surgery on the occurrence of estrus in ovary-intact females. Forced air (1.5 L/min) was blown into the chamber from one end and exhausted into a fume hood via an opening on the opposite end. Clean air filtered with activated charcoal was passed through the chamber in the first hour. In the following 30 min, animals either continued to receive clean air ($n = 7$) or were exposed to air blown over one of three experimental stimuli: male urine ($n = 8$); estrous female urine ($n = 8$); or cat urine ($n = 8$). Urine vapors were generated by passing clean air over 5 mL of 20% urine diluted in water, as in previous studies (Schaefer *et al.*, 2001; Martel & Baum, 2007). Odor delivery consisted of six 3-min exposures, each separated by 2 min of clean air. Clean air was also pulsed off and then on again at these same intervals. One hour after receiving the final stimulus, animals were killed, and olfactory bulbs were isolated and sectioned for double-immunolabeling using CTb and Fos. In order to assess the effects of different urinary volatiles on Fos expression in different forebrain regions, forebrain tissue was also obtained. Alternate forebrain sections were either single-labeled with CTb antibody to confirm the location of Me injection sites or single-labeled with Fos antibody to examine the effects of volatile odor exposure on forebrain Fos expression. Some subjects in which CTb injections missed the Me were included in this latter analysis.

The identification of retrogradely labeled MOB M/T cells that either were or were not co-expressing Fos protein was based on differences in color and cellular localization of staining (Fos: black, nucleus; CTb: brown, cytoplasm) under a 40× objective. Alternate sagittal sections of the olfactory bulb were included in this analysis. Cells in the following categories were counted without the investigator having any knowledge of treatment group: (1) Me-projecting but not activated by odor (CTb-labeled only); and (2) both Me-projecting and activated by odor (CTb/Fos double-labeled). The percentage of CTb-immunoreactive (IR) cells that co-expressed Fos was calculated for three different segments of the MOB that contain large numbers of these Me-projecting M/T cells using a Neurolucida system. Within each MOB segment, a parametric ANOVA was used to compare group differences in both the number of Me-projecting M/T cells and the percentage of these cells that were co-labeled with Fos in response to different odors. Following a significant overall group effect, *post hoc* comparisons of pairs of means were carried out using Student's Newman–Keuls tests. We did not count MOB M/T cells that were only labeled with Fos after odor exposure because, unlike the activation of MOB glomeruli by urinary volatiles (Schaefer *et al.*, 2002), Fos-positive M/T cells were numerous and ubiquitous throughout the MOB after odor exposure. Moreover, previous attempts in mice to identify sex differences in MOB M/T cell activation by odors were not successful (H. Halem, M.J. Baum & J.A. Cherry, unpublished observations). Fos

counts in forebrain structures were averaged for each subject from two brain sections containing each structure of interest, and comparisons between groups were carried out by *post hoc* Student's Newman–Keuls tests following a significant one-way ANOVA.

After completing this functional anatomical study, we asked whether the selective ability of male urinary volatiles to stimulate Fos expression in Me-projecting MOB M/T cells and in several forebrain nuclei (details in Results) may have been associated with subjects' exposure to heightened concentrations of male, as opposed to female or cat, urinary volatiles, due to subjects' increased nasal contact with the odor input port during the pulsed presentation of odors to different groups. We had not monitored subjects' behavior during the anatomical study. Therefore, in order to address this question eight additional ovariectomized female mice were given estradiol benzoate and progesterone to induce behavioral estrus. These animals were exposed to clean air or to urinary volatiles from either male mice, estrous female mice or cat in the same apparatus described above. Subjects were exposed to each of the four odor conditions on separate days, with 4 days separating test sessions. During each test session an odor was presented six times (3 min on and 2 min off over 30 min), and the time subjects put their nose against the odor input port was recorded. The order in which odors was presented was counter-balanced across the eight subjects. The investigation times of estrous female mice for different odors were analysed using a two-way repeated measures ANOVA, with odor condition and time of repeated presentation during each test session serving as factors.

Results

Experiment 1: anterograde tracer injections into the olfactory bulb

Iontophoretic injections of PHA-L placed into the ventral MOB mitral cell layer produced successful labeling in six mice (Fig. 1A). Consistent with previous studies in the rat (Scalia & Winans, 1975; Shipley & Adamek, 1984; Pro-Sistiaga *et al.*, 2007), anterogradely labeled axons were found in a number of targets, including the anterior olfactory nuclei, olfactory tubercle, Pir, lateral entorhinal cortex, nucleus of lateral olfactory tract, bed nucleus of accessory olfactory tract, ACo, PLCo and ventral anterior amygdaloid area. However, unexpectedly, labeled axons were also found in several subnuclei of the Me: the MeAV, the MeA and the MePD (Fig. 1C–H). Notably, fibers in the MePD and MeA appeared to be completely isolated from fibers in the ACo by the MePV, in which no labeled fibers were detected (Fig. 1E, G and H). Similar to the MOB projection to the Pir (Cajal, 1995), processes in the MeA and MePD were concentrated in layer Ia of these nuclei and extended varicose collaterals into layer II (Fig. 1F and Fig. S3).

Two additional mice received successful injections of PHA-L into the anterior MOB, which resulted in anterograde labeling of the Me similar to what was seen with ventral MOB injections (not shown). Several attempts to place PHA-L injections restricted to the dorsal MOB either failed to produce anterograde labeling anywhere in the brain and/or resulted in spread of tracer to the medial or lateral walls of the MOB.

As medial amygdaloid nuclei are known targets of the AOB (Scalia & Winans, 1975), the spatial relationship between MeA/MePD-projecting fibers from the MOB and AOB was next examined by placing iontophoretic injections of PHA-L and Fluoro-Ruby into the ventral MOB and AOB, respectively, of the same hemisphere of three adult female mice (Fig. 2A). Double-immunolabeling revealed that anterogradely labeled PHA-L fibers from the MOB were concentrated in superficial laminae of layer Ia of the Me (both MeA and MePD), whereas Fluoro-Ruby-labeled fibers from the AOB were found in deeper layer Ia laminae (Fig. 2B and C). Thus, direct projections from the MOB and AOB converged on the Me such that labeled fibers from each site terminated in abutting laminae of the MeA and MePD.

We next considered the possibility that anterograde tracer placed into the MOB may have inadvertently migrated to the terminal nerve. We stained the olfactory bulb and forebrain tissue from three female mice with anti-GnRH antibody to visualize the course of the terminal nerve that runs above the nerve layer of the olfactory bulb and innervates the ventral forebrain (Schwanzel-Fukuda *et al.*, 1992). No GnRH-IR fibers were seen in the MeA or MePD. In the olfactory bulb, although GnRH-IR cell bodies and bouton-like structures were found in close proximity to the olfactory nerve, no cells in the mitral layer were GnRH-IR (data not shown). Thus, Me afferents labeled by ventral MOB injections of anterograde tracer are unlikely to arise from the terminal nerve.

Experiment 2: retrograde tracer injections into the medial amygdala

Results from Experiment 1 suggested that projection neurons located in ventral and anterior segments of the MOB directly target the MeA and MePD. To determine both the precise location of these cells and whether M/T cells in other parts of the MOB also project to the Me, we injected CTb into the Me (including the MeA and MePD) of female mice to localize retrogradely labeled, Me-projecting cells in the MOB (Fig. 3A). As expected, retrogradely labeled mitral cells were found throughout the AOB (Fig. 3B). In addition, Me injections resulted in strong labeling of projection neurons that were concentrated in three abutting regions of the MOB, which included a region found at the caudal edge of the olfactory bulb in lateral and medial segments (Fig. 4, area 1); a central ventral region, which is the most ventral part of the MOB (Figs 3D and 4, area 2); and the anterior medial region, which includes the most anterior part of the medial wall (Fig. 4, area 3). For ease of reference, we refer to this labeling as 'ventral MOB'. Most of the labeled cells were located in the mitral cell layer, but occasionally back-filled cells were found in the adjacent external plexiform layer. These were usually smaller cells that fit the general description of an internal tufted cell or a displaced mitral cell (Shepherd, 1990). Labeled cells are thus referred to as M/T cells.

Because labeled M/T cells appeared to be most abundant in the ventral MOB, the number of labeled cells in the ventral vs. the dorsal region was compared using the ratio of ventral to dorsal cell labeling. Although the overall number of labeled MOB M/T cells varied from about 300 to 1000 across animals (see Fig. S4 for individual examples), the mean ($\pm$ SEM) ratio of the number of cells in the ventral to dorsal MOB in 16 bulbs examined (from 15 subjects, including some animals from Experiment 3) was $8.8 (\pm 2.2)$, significantly different from a ratio of 1, $t_{14} = 4.95$, $P < 0.001$, indicating that relatively more M/T cells in the ventral as opposed to the dorsal MOB project to the Me. In agreement with a previous study that described interconnectivity among medial amygdaloid nuclei (Coolen & Wood, 1998), we also noted a moderate number of CTb-labeled cells after Me injection of CTb in the anterior amygdaloid area, bed nucleus of the accessory olfactory tract, ACo, PLCo, posteromedial cortical amygdala, the endopiriform nucleus and posterior Pir. Sparse labeling of cells was also seen in the anterior Pir (data not shown).

To assess whether CTb-labeling in the MOB of Me-injected mice may have been due to spread of CTb along the injection needle into adjacent sites known to receive MOB projections, and to ensure that our procedures were capable of producing back-filled cells throughout the entire MOB mitral cell layer, additional subjects received CTb injections into the ACo (Fig. 3E) and posterior Pir (Fig. S5A). In three subjects that received injections restricted to the ACo, a site known to receive MOB projections (Scalia & Winans, 1975), retrogradely labeled cells were found all throughout the MOB mitral cell layer (Fig. 3F–H). Similarly, in four subjects that received a CTb injection restricted to the posterior Pir, a primary target of the MOB (Shipley & Adamek, 1984), labeled M/T cells could be seen throughout the MOB (Fig. S5). The observation that CTb injected into the ACo or posterior Pir retrogradely labeled M/T cells in all segments of the MOB, whereas Me injections labeled a more restricted (generally ventrally

located) set of MOB M/T cells, provides critical support for our claim that only a fraction of MOB output conveys information to the ‘vomeronasal’ amygdala.

Experiment 3: identification of MOB mitral cells that project to the Me and express Fos in response to volatile urinary odors

To determine whether the direct MOB-to-Me pathway responds to social and/or predatory odors, estrous female subjects that previously received CTb tracer in the Me were exposed to volatile urinary odors from adult male or estrous female mice or from a cat (predator), and the olfactory bulbs were collected for immunolabeling of Fos and CTb (Fig. 5A). Our results (Fig. 5B) suggest that Me-projecting MOB M/T cells were activated by opposite-sex urinary volatiles but not by urinary volatiles from either same-sex (female) conspecifics or from a predator (cat). Thus, after exposure to male mouse urinary odors, a significantly higher percentage of M/T cells in the medial segment of the MOB co-expressed Fos than after exposure to urinary volatiles from either female mice or cats or after clean air ($F_{3,30} = 8.54$, $P < 0.001$). The central-ventral segment of the MOB had the same profile of group differences in CTb/Fos double-labeling ($F_{3,30} = 4.38$, $P < 0.05$). In the lateral segment of the MOB, male mouse urinary volatiles induced Fos expression in a significantly greater percentage of CTb-labeled M/T cells than did female mouse urine or clean air ($F_{3,30} = 5.80$, $P < 0.01$). In this lateral MOB segment the value for animals exposed to cat urinary volatiles did not differ significantly from the values of mice exposed either to clean air or mouse urinary odors. In no MOB segments were significant differences found in the percentage of CTb-IR M/T cells that also co-expressed Fos between groups of mice exposed to either clean air or volatiles from estrous female urine or cat urine. The total number of CTb-labeled cells in each MOB segment across different odor exposure groups did not differ significantly (values are shown above each bar in Fig. 5B). Thus, observed group differences in Fos expression in Me-projecting MOB M/T cells did not reflect group differences in the number of these cells that were successfully retrogradely labeled by CTb injections into the Me.

We reasoned that an increased intrinsic interest in male mouse urinary volatiles might have caused female subjects to spend more time in close proximity to the odor source, resulting in greater relative exposure to these volatiles than to other odor stimuli and a consequent greater increase in the expression of Fos in MOB M/T cells projecting to the Me. Therefore, in a separate group of estrous female mice we observed investigatory responses to the same odors used to induce Fos in our functional neuroanatomical experiment. We found no significant group differences in time spent investigating each odor ($F_{3,191} = 1.10$, $P = 0.37$; Fig. S6). Investigation times decreased significantly across consecutive presentations of each odor during the 30-min tests ($F_{5,239} = 50.1$, $P < 0.001$). We conclude that it is unlikely that the significant group differences in the number of CTb/Fos double-labeled MOB M/T cells following exposure to different urinary volatiles can be attributed to group differences in the exposure concentrations of the odors presented.

Consistent with the Fos expression profile seen in MOB M/T cells that project to the Me, significantly more Fos-positive cells were found in subnuclei of the medial amygdala (including the MeA, MePD, MePV) after exposure to urinary volatiles from male mice as opposed to urinary volatiles from female mice or cat or to clean air (Table 2). Sites that receive inputs from the ‘vomeronasal’ amygdala were similarly selectively activated by male mouse odor. These areas included the BNST, the medial preoptic area and the ventrolateral portion of the ventromedial hypothalamus. Finally, exposure to male mouse urinary volatiles significantly augmented Fos expression in two previously known amygdaloid targets of main olfactory input, the ACo and the PLCo, as well as the nucleus accumbens shell.

Discussion

Although it has previously been recognized that amygdaloid subnuclei receive input from the MOB, the Me has traditionally been thought to receive only indirect MOB signals via afferents from the ACo (Scalia & Winans, 1975; Licht & Meredith, 1987). In principle, signals carried by a monosynaptic pathway may be less subject to modification and would enable MOB M/T cells to gain unconditioned access to the Me. Such a pathway may be more suitable for the regulation of innate, stereotyped behaviors evoked by pheromones or other biologically relevant chemosensory cues such as predator odors. We provide evidence using anatomical tracing that MOB M/T cells project directly to the Me in mice. Moreover, we corroborate previous reports (Schaefer *et al.*, 2001; Ma *et al.*, 2003; Xu *et al.*, 2005; Martel *et al.*, 2007) that the mouse MOB responds to volatile, biologically significant pheromones. Finally, by combining Fos immunohisto-chemistry with retrograde labeling of Me-projecting M/T cells, we provide evidence in estrous female mice that volatiles from male conspecifics, but not from other female mice or from a predator (cat), can stimulate these cells.

In male and female mice, urinary volatiles from the opposite sex induce Fos in the AOB via a pathway that requires a functional main olfactory system (Martel & Baum, 2007). The Me-projecting MOB M/T cells identified in the present study may convey information about opposite-sex odors to the MeA and then back to the AOB via a centrifugal pathway (Barber, 1982; Martel & Baum, 2009). The present observation of a selective ability of male urinary volatiles to stimulate Fos expression in both the classic vomeronasal and olfactory subnuclei of the amygdala as well as in other segments of the vomeronasal projection pathway (e.g. BNST, ventrolateral portion of the ventromedial hypothalamus and medial preoptic area) and the mesolimbic dopamine system (e.g. nucleus accumbens shell) of estrous female subjects replicates and extends the results of a similar study (Martel & Baum, 2007) carried out in ovariectomized female mice given no hormone priming. In that previous study, odor-induced stimulation of forebrain Fos expression was eliminated after chemical lesions of the main olfactory epithelium, further emphasizing the critical role of main olfactory epithelium receptors in mediating these effects of opposite-sex urinary volatiles. A recent study shows that female-typical central processing of sexually relevant olfactory cues was shaped by early estrogens (Pierman *et al.*, 2008). Presumably, the forebrain targets of this main olfactory input control essential aspects of courtship behavior and reproduction.

Double-labeling with anterograde tracers revealed that AOB and MOB M/T cell axonal projections converged in the Me in adjacent laminae of layer 1, a relatively cell-free zone rich in synapses (Ichikawa, 1987). Although ventral MOB injections labeled the outermost layer, axonal branches also penetrated into deeper layers (e.g. Fig. 1F and Fig. S3), suggesting that synaptic interactions between MOB and AOB afferents to the Me, as well as with intrinsic Me neurons, are likely. This interaction may be the means by which the accessory olfactory system, through a process of associative conditioning, transfers the saliency of social odor cues to the main olfactory system. For example, removal of the VNO in male mice prior to receiving heterosexual mating experience prevented subjects from emitting ultrasonic vocalizations (a component of mating behavior) in response to anesthetized females or female urine (Wysocki *et al.*, 1982). In chemically naïve female mice, lesions of the AOB disrupted the development of a preference for volatile male odors (Martinez-Ricos *et al.*, 2008). In male hamsters, VNO removal before, but not after, acquisition of sexual experience impaired mating behavior (Meredith, 1986) and, in male guinea pigs, initial interest in female urinary odors declined in the weeks following VNO removal (but not in control subjects), suggesting an extinction in the salience of cues processed by the main olfactory system in the absence of reinforcement provided by the accessory system (Beauchamp *et al.*, 1982). Our evidence that MOB and AOB projections converge on the MeA and MePD now implicate these sites in such odor conditioning.

A recent report in the rat (Pro-Sistiaga *et al.*, 2007) has also demonstrated convergence of main and accessory olfactory inputs to medial amygdaloid nuclei. Unfortunately, due to differences between the location of these structures described in the mouse (Paxinos & Franklin, 2001) and rat (Paxinos & Watson, 2005) atlases, direct comparisons with the present study are difficult. Whereas we found in the mouse that the AOB and MOB both send axons to the MeA and MePD, convergent AOB/MOB projections in the rat were seen in the anterior, rather than the posterior, medial amygdala (Pro-Sistiaga *et al.*, 2007). In addition, we saw no AOB afferents to the ACo, which Pro-Sistiaga *et al.* reported as a site of AOB/MOB convergence in the rat. Thus, despite general agreement that the Me receives projections from both the AOB and MOB, there may be species differences in the details of the specific locations involved.

Electrophysiological and anatomical evidence show that the main olfactory system and hypothalamus are linked (Pfaff & Pfaffmann, 1969; Price *et al.*, 1991; Boehm *et al.*, 2005; Yoon *et al.*, 2005). These data, together with our results and a growing number of reports (Petrulis *et al.*, 1999; Pankevich *et al.*, 2004; Baxi *et al.*, 2006; Shepherd, 2006), indicate that the accessory olfactory system is unlikely to be the sole access point for social odors that are involved in intraspecific communication. The renewed focus on the main olfactory system as an important transducer of social odors is particularly relevant for humans. Neither an AOB nor a projection from the VNO to the brain has been identified in humans (Knecht *et al.*, 2001, 2003; Meredith, 2001; Witt & Hummel, 2006). Nevertheless, exposure to extracts from human sweat or to volatile steroids such as androstadienone (a putative pheromone excreted in male sweat) can produce in human subjects: (1) sex- and sexual orientation-specific activation of the hypothalamus (Savic *et al.*, 2001, 2005; Berglund *et al.*, 2006); and (2) an increased incidence of luteinizing hormone pulses (Shinohara *et al.*, 2001; Preti *et al.*, 2003; Witt & Hummel, 2006). The lack of a functional accessory system in humans has raised the possibility that circuitry within the MOB is responsible for the initial processing and transmission of pheromones that result in these neurobehavioral responses. Our current findings in the mouse point to a MOB–Me pathway that may mediate such actions.

There is compelling evidence that the innate avoidance of aversive odors is regulated in mice by dorsal MOB glomeruli (Kobayakawa *et al.*, 2007). Our results suggest that different categories of behaviors, such as those involved in mating, are regulated by odors processed via a different set of MOB glomeruli. Thus, we observed that Me-projecting M/T cells that co-expressed Fos in response to male urinary odors were disproportionately located in the ventral MOB, which is consistent with other evidence (Schaefer *et al.*, 2001, 2002; Martel *et al.*, 2007) in mice, suggesting that volatiles in urine activate glomeruli located primarily in the ventral MOB. In another recent study in mice, it was reported that mature olfactory sensory neurons that express the transient receptor potential channel M5 (TRPM5) send axons to a small number of glomeruli in the ventral MOB (Lin *et al.*, 2007). These TRPM5-expressing olfactory sensory neurons were stimulated by a variety of semiochemicals, including the putative male urinary pheromone, 2,5-dimethylpyrazine, (methylthio) methanethiol, as measured by increased Fos expression in periglomerular cells of the MOB glomeruli that receive input from these neurons. This small population of TRPM5-expressing sensory neurons, which is concentrated in the ventrolateral olfactory epithelium, may represent the sensory segment of a pathway that signals information regarding volatile pheromones from the MOB to the Me and subsequently to hypothalamic targets that control heterosexual mate recognition and sexual arousal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACo	anterior cortical amygdala
AOB	accessory olfactory bulb
BNST	bed nucleus of the stria terminalis
CTb	cholera toxin B
DAB	diaminobenzidine
GnRH	gonadotropin-releasing hormone
IR	immunoreactive
M/T	mitral/tufted
Me	medial amygdala
MeA	anterior medial amygdala
MeAD	anterodorsal medial amygdala
MeAV	anteroventral medial amygdala
MePD	posterodorsal medial amygdala
MePV	posteroventral medial amygdala
MOB	main olfactory bulb
PB	phosphate buffer
PBS	phosphate-buffered saline
PBST	

Triton X-100 in phosphate-buffered saline

PHA-L

Phaseolus vulgaris leucoagglutinin

Pir

piriform cortex

PLCo

posterolateral cortical amygdala

TRPM5

transient receptor potential channel M5

VNO

vomerolateral organ

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Fig. 1.

Neurolucida drawings (2.25 mm² brain space) show labeled axons 7 days following a PHA-L injection into the ventral part of the MOB (A) in a female mouse. The photos shown in (A) and (F) were taken from the same animal. Medial amygdala-projecting fibers can be seen running along the ventral surface of the brain and innervating the MeAV (B and C). At the level of the MeA (D), labeled fibers were concentrated on the medial edge of the MeA (solid arrow), with a noticeable thinning of fibers (open arrow) present between the MeA and the ACo. MePD-projecting fibers (solid arrow in E) were separated from those projecting to the ACo by the unlabeled MePV. A photo taken from the boxed region in (E) shows labeled fibers with varicosities (two arrows in F). A thin layer of PHA-L-positive fibers was seen on the medial edge of the MePD (solid arrow in G). In the most caudal MePD, layer Ia was sparsely labeled (solid arrow in H). Numbers at the right bottom of each drawing indicate distances (in mm) behind bregma. Open and closed arrowheads in (A) point to the anterior olfactory nucleus and lateral olfactory tract, respectively. ACo, anterior cortical amygdala; AOB, accessory olfactory bulb; BAOT, bed nucleus of the accessory olfactory tract; MeA, anterior medial amygdala; MeAD, anterodorsal medial amygdala; MeAV, anteroventral medial amygdala; MePD, posterodorsal medial amygdala; MePV, posteroventral medial amygdala; MOB, main olfactory bulb; opt, optic tract; PLCo, posterolateral cortical amygdala.

Fig. 2.

Fluorescently labeled MOB and AOB projection cell axons travel to adjacent laminae in subdivisions of the medial amygdala of a female mouse. (A) A PHA-L injection site in the MOB (solid arrow) and a Fluoro-Ruby injection site in the AOB (open arrow) are shown in a sagittal section of the olfactory bulb 7 days after injection of anterograde tracers into these respective sites (counterstained with TO-PRO-3 – see blue pseudo color in on-line graphic). Open and closed arrowheads indicate the anterior olfactory nucleus and lateral olfactory tract, respectively. (B and C) PHA-L-labeled MOB M/T cell axons (solid arrows) and Fluoro-Ruby-labeled AOB mitral cell axons (open arrows) can be seen abutting each other in the superficial and deeper regions of layer Ia in coronal sections taken through the MeA (B; 1.2 mm behind bregma) and the MePD (C; 1.46 mm behind bregma), respectively.

Fig. 3.

Retrograde labeling of the AOB and MOB after injection of CTb into the Me or ACo. (A) 7 days after tracer injection, CTb (dark staining) was centered in layer I of the Me. (B) 7 days after injection of CTb into the Me, CTb-labeled cells were seen in the AOB and the ventral portion of the MOB. (C) A higher magnification image shows that the dorsal MOB contained no CTb-labeled cells. (D) A higher magnification image shows that CTb-labeled cells (arrows) were located in the mitral cell layer of the ventral MOB. Following an injection of CTb into the ACo (E), M/T cells were labeled throughout the MOB (F). (G and H) Higher magnification images show CTb-labeled M/T cells (arrows) in the dorsal and ventral segments of the MOB, respectively, after an ACo injection of CTb. Tissue was counterstained with neutral red.

Fig. 4.

Collapsed stacks of sagittal Neurolucida drawings from a representative female mouse show the distribution of CTb-labeled cells (black dots) in the lateral, central and medial segments of the MOB (shown in the sagittal plane; rostral surface on the left side) following an injection of CTb into the medial amygdala (Me; center image). The central segment of the MOB is further divided into dorsal and ventral regions by an imaginary line drawn parallel to the ventral surface of the olfactory bulb and across the arch of the mitral cell layer at the most rostral position. The drawing of a coronal section of the olfactory bulb (top left panel) is used to illustrate the four segments. CTb-IR cells were concentrated in three regions, including the caudal region that surrounds the stem of the olfactory bulb (area number 1 in the lateral and medial segments), the central-ventral region (area number 2 in the central segment), and the anterior medial region (area number 3 in the medial segment). Numerous CTb-labeled AOB mitral cells were not plotted.

Fig. 5.

Effect of volatile urinary odor exposure on the expression of Fos in MOB M/T cells of estrous female mice that were retrogradely labeled by a prior injection of cholera toxin B (CTb) into the Me. (A) Three possible types of labeling occurred in MOB M/T cells following Me CTb injections and urinary odor exposure: CTb/Fos double-labeled (CTb + Fos), CTb-single labeled (CTb) and Fos-single labeled (Fos) M/T cells. (B) The percentage of CTb-labeled (Me-projecting) M/T cells that co-expressed Fos is shown across three segments of the MOB following exposure to different volatile urinary odors or clean air. The number (mean $\pm$ SEM) of CTb-labeled M/T cells is shown above the error bar for each group. * P = 0.001; # P < 0.05, Student's Newman-Keuls *post hoc* comparisons between groups following a significant overall ANOVA.

Table 1
Summary of the number of injections of anterograde and retrograde tracers placed into the brains of 94 female mice in Experiments 1–3

First injection site	Second injection site	Hit	Miss
Experiment 1			
MOB mitral cell layer (ventral)/PHA-L	–	6	13
MOB mitral cell layer (anterior)/PHA-L	–	2	3
AOB mitral cell layer/PHA-L	–	1	3
MOB mitral cell layer (ventral)/PHA-L	AOB mitral cell layer (caudal)/ Fluoro-Ruby	3	5
Experiment 2			
Me layer Ia/CTb	–	9	17
ACo layer Ia/CTb	–	3	3
PPC layer Ia/CTb	–	4	5
Experiment 3			
Me layer Ia/CTb	–	42	68

The number of injected hemispheres of female mice is listed under the hit and miss categories for each single or combined injection. A hit was defined by labeling: (i) at the injection site; and (ii) in the lateral olfactory tract (for MOB injections) or the AOB (for Me injections). ACo, anterior cortical amygdala; AOB, accessory olfactory bulb; CTb, cholera toxin B; Fluoro-Ruby, dextran-tetramethylrhodamine conjugate; Me, medial amygdala; MOB, main olfactory bulb; PHA-L, *Phaseolus vulgaris* leucoagglutinin; PPC, posterior piriform cortex.

Table 2
Effect of different volatile odor stimuli on forebrain Fos expression in estrous female mice

Brain region	Volatile odor stimuli				$F_{3,28}$ (P -value)
	Male mouse urine ($n = 6$)	Female mouse urine ($n = 8$)	Cat urine ($n = 8$)	Clean air ($n = 7$)	
Classic vomeronasal projection pathway					
MeA	50 ± 5 ^b	10 ± 2 ^a	10 ± 3 ^a	8 ± 2 ^a	33.7 (< 0.001)
MePD	43 ± 4 ^b	11 ± 2 ^a	9 ± 2 ^a	12 ± 3 ^a	29.7 (< 0.001)
MePV	69 ± 18 ^b	11 ± 2 ^a	16 ± 3 ^a	12 ± 3 ^a	25.5 (< 0.001)
BNST	70 ± 9 ^b	32 ± 6 ^a	27 ± 7 ^a	23 ± 4 ^a	8.9 (< 0.001)
MPA	114 ± 20 ^b	89 ± 8 ^a	66 ± 9 ^a	73 ± 9 ^a	3.1 (0.045)
VMHVL	18 ± 5 ^b	8 ± 2 ^a	10 ± 3 ^a	6 ± 2 ^a	4.9 (0.001)
Classic main olfactory projection pathway					
ACo	88 ± 9 ^b	18 ± 2 ^a	18 ± 6 ^a	22 ± 5 ^a	33.5 (< 0.001)
PLCo	77 ± 4 ^b	20 ± 5 ^a	23 ± 6 ^a	35 ± 10 ^a	19.0 (< 0.001)
Mesolimbic dopamine system					
AcbC	11 ± 6 ^a	3 ± 1 ^a	1 ± 0.3 ^a	1 ± 1 ^a	2.9 (0.06)
AcbSh	86 ± 19 ^b	33 ± 7 ^a	28 ± 5 ^a	40 ± 7 ^a	6.2 (0.004)

Data are expressed as the mean (± SEM) number of Fos-IR cells per standard counting area. Different superscript letters (^a and ^b) indicate significant differences between groups, *P* < 0.05 (Student's Newman-Keuls *post hoc* tests following a significant overall anova). AcbC, nucleus accumbens core, shell; ACo, PLCo, anterior, posterolateral cortical amygdaloid nucleus; BNST, bed nucleus of the stria terminalis; MeA, MePD, MePV, anterior, posterodorsal and posteroventral medial amygdala; MPA, medial preoptic area; VMHVL, ventrolateral portion of the ventromedial hypothalamus.