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Spatial distribution of neural activity in the anterior olfactory nucleus evoked by odor and electrical stimulation

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Abstract

Several lines of evidence indicate that complex odorant stimuli are parsed into separate data streams in the glomeruli of the olfactory bulb, yielding a combinatorial “odotopic map.” However, this pattern does not appear to be maintained in the piriform cortex, where stimuli appear to be coded in a distributed fashion. The anterior olfactory nucleus (AON) is intermediate and reciprocally interconnected between these two structures, and also provides a route for the interhemispheric transfer of olfactory information. The present study examined potential coding strategies used by the AON. Rats were exposed to either caproic acid, butyric acid, limonene, or purified air and the spatial distribution of Fos-immunolabeled cells was quantified. The two major subregions of the AON exhibited different results. Distinct odor-specific spatial patterns of activity were observed in pars externa, suggesting that it employs a topographic strategy for odor representation similar to the olfactory bulb. A spatially distributed pattern that did not appear to depend on odor identity was observed in pars principalis, suggesting that it employs a distributed representation of odors more similar to that seen in the piriform cortex.

Indexing terms

olfactory cortex; olfactory system organization; olfactory peduncle

Odors entering the nasal cavity are detected by olfactory sensory neurons (OSNs) residing in the olfactory mucosa. In rodents, with few exceptions, each of these neurons expresses one (or perhaps a very small number) of the nearly 1,000 varieties of olfactory receptor proteins (Serizawa et al., 2003). Most odors are complex mixtures of chemical compounds that activate a large number of different sensory neurons, and each sensory neuron is capable of responding to a large number of chemically diverse odorants (Zhao et al., 1998; Rubin and Katz, 1999; Araneda et al., 2000; Bozza et al., 2002; Araneda et al., 2004).

Axons from OSNs expressing the same receptor protein converge in a small number of defined glomeruli in the olfactory bulb (OB), thereby parsing odor information into receptor-specific channels. The nature of these inputs suggests that odor quality may be

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encoded in part by the location of activity in a spatial map within the OB, and studies employing several techniques have been used to support this claim (e.g., 2-DG autoradiography: Sharp et al., 1975; Johnson and Leon, 2007; immunostaining for MAPK: Mirich et al., 2004; the visualization of immediate early genes [IEGs]: Morgan et al., 1987; Sallaz and Jourdan 1993, 1996; Guthrie and Gall, 1995, 2003; Zou and Buck, 2006; high-resolution functional magnetic resonance imaging [fMRI]: Yang et al., 1998; Schafer et al., 2006; and electrophysiology: Mori et al., 1992, 2006).

The axons of the bulb's mitral and tufted cells project through the lateral olfactory tract to synapse in a number of third-order structures, including the anterior olfactory nucleus (AON; also called the "anterior olfactory cortex"; Haberly, 2001; Brunjes et al., 2005) and piriform cortex (Price and Sprich, 1975). Relatively few studies have examined whether the receptor-based organization seen in the OB is conserved in these regions. Examinations of IEG expression in the rodent piriform cortex indicate that odor exposure leads to widespread rather than patchy postsynaptic cellular activation (Illig and Haberly, 2003; Zou and Buck, 2006; Illig, 2007), and single-unit and whole-cell voltage clamp recordings suggest that cells with similar odor response profiles do not cluster together (Rennaker et al., 2007; Poo and Isaacson, 2009). Live calcium imaging of piriform cortex also indicates that odors activate unique but dispersed ensembles of neurons (Stettler and Axel, 2009). Thus, functional evidence suggests that rather than utilizing a spatially ordered "map" of odor quality, the piriform cortex employs a spatially distributed, ensemble code for odors.

The AON's cytoarchitectural features and substantial connections with other olfactory areas indicate that it plays a significant role in olfactory processing (Haberly and Price, 1978b; Luskin and Price, 1983; Haberly, 2001; Brunjes et al., 2005; Illig, 2005; Meyer et al., 2006; Illig and Eudy, 2009), but little is known about its functional organization. The AON is typically divided into two major subregions: pars externa and pars principalis. Pars externa, unique in its location, structure, and projections, is a thin sheet of cells encircling pars principalis in the rostral end of the olfactory peduncle. Pars principalis is a two-layered cortical structure: an outer plexiform layer containing projections from the OB (layer Ia) and other inputs (layer Ib), and an inner cellular layer (layer II) containing the cell bodies of pyramidal and other cells. It is often divided into four subregions based on relative location within the structure: pars medialis, pars lateralis, pars dorsalis, and pars ventroposterior. Although cell morphology, neurochemical phenotypes, and projections have been shown to vary from region to region (Meyer et al., 2006), there is little experimental foundation for defining subregional boundaries (see Brunjes et al., 2005).

In the present study, Fos protein expression was used as a marker for activity in AON cells to examine whether the spatially segregated processing of different stimuli in the OB is maintained in patterns of activation in the AON, and whether subregions differentially participate in olfactory information processing. Three odorants were chosen on the basis of their molecular structure, perceived odor, and representation within the OB. Caproic (CAS 142-62-1) and butyric (CAS 67-43-6) acids have a similar molecular structure (six and five carbon aliphatic acids, respectively), perceived odor (rancid cheese/butter), and are represented in the dorsomedial portion of the OB. Limonene (CAS 5989-27-5) is quite different in every way: it is a cyclic terpene hydrocarbon with a perceived odor of citrus, and activates large regions in the lateral and medial parts of the OB (Johnson et al., 1999). If topographic coding occurs in the AON as it does in the OB, one would predict that Fos patterns elicited by caproic and butyric acid would be similar, but both would be very different than that seen after exposure to limonene. In addition, we examined Fos expression following focal electrical stimulation of the dorsolateral or dorsomedial OB.

MATERIALS AND METHODS

Animals

Forty-one male Long-Evans rats (250–350 g; Harlan, Houston, TX) were used. All procedures were performed according to National Institutes of Health (NIH) guidelines and protocols approved by the University of Virginia Institutional Animal Care and Use Committee (IACUC). Animals were housed in standard polypropylene cages with food (Harlan Rat Chow 8604) and water ad libitum. The colony was maintained on a 12:12 light:dark cycle in a temperature- and humidity-controlled room (22°C; 40% humidity).

Odor exposure and tissue preparation

Animals were placed in individual chambers of an odor delivery setup (Illig and Haberly, 2003) and exposed to purified air for ≈ 18 hours (overnight). Subsequently, stimuli (or purified air for control subjects) were delivered for 1 hour with 30-second odor presentations followed by clean air for 90 seconds to reduce habituation (see Illig and Haberly, 2003, for details). High and low concentrations of three odors were used. For limonene, the high concentration was produced by bubbling nitrogen (N₂) through undiluted reagent and delivering it at a final concentration of 1:200 in air. The low concentration was produced by passing N₂ through the reagent diluted 1:10 in mineral oil and then diluting the stream to a final concentration of 1:2,000. The high and low concentrations of both caproic and butyric acid were produced in a similar fashion, diluted to achieve similar vapor pressures for each concentration as for limonene (high concentration: N₂ bubbled through caproic or butyric acid diluted 1:50 in mineral oil and diluted to a final concentration of 1:10,000 in air; low concentration: N₂ bubbled through caproic or butyric acid diluted 1:100 in mineral oil and diluted to a final concentration of 1:200,000 in air; data used for vapor pressure calculation were obtained from the National Institute of Standards and Technology). The odors were chosen for differences in molecular structure, perceived odor, and spatial patterns expressed in the OB following odor stimulation (see Johnson et al., 1998, 1999; Illig and Haberly, 2003). One hour after initial odor exposure, animals were deeply anesthetized with sodium pentobarbital (150 mg/kg) and perfused transcardially with 0.01M phosphate-buffered saline (PBS; pH 7.4) containing heparin followed by 4% buffered formaldehyde freshly depolymerized from paraformaldehyde. Brains were removed, postfixed for ≈ 4 hours, then cryoprotected in 30% sucrose/PBS. The following day, brains were sectioned on a cryostat at 50 μ m. Every second section through the entire extent of the bulb and AON was taken for immunohistochemical processing and analysis. Five animals were examined in each of the odor-exposed conditions and in the controls that received only purified air, for a total of 35 subjects.

Focal electrical stimulation

Six additional rats were anesthetized (pentobarbital sodium, 40 mg/kg), placed on a heating pad to maintain body temperature, and tracheotomized. The external nares were occluded with petroleum jelly to prevent odor-evoked activation of cells in the OB. The head was secured in a stereotaxic frame, a midline incision was made, and a craniotomy drilled in the skull above the intended stimulation site in the OB. The dura was removed and a bipolar stimulating electrode consisting of Teflon-coated silver wire (A-M Systems, Carlsborg, WA) exposed at the end to achieve a final size of ≈ 100 μ m was lowered into the glomerular layer. The exposed area was covered with 4% agarose and the preparation was left to stabilize for at least 60 minutes. Approximately 2 hours after initial nose plug, 0.1 ms, 1 μ A, 0.1 Hz stimulation was delivered through the stimulating electrode for 1 minute. These parameters were chosen based on pilot studies showing that this low stimulus level resulted in highly restricted focal activity within the OB. One hour following stimulus delivery animals were perfused and tissue was prepared in same manner as for odor-exposed rats.

Immunostaining: antibody characterization

Table 1 provides a list of the primary antibodies used in the present study. The rabbit polyclonal anti-c-fos antibody employed recognized a ≈ 55 -kDa protein on Western blots of stimulated rat brain tissue corresponding to the molecular weight of Fos (manufacturer's technical information). This antibody has been screened for positive reactivity with floating rat brain sections induced for c-Fos expression and it does not react with the highly similar 39-kDa c-Jun protein (manufacturer's technical information). Our tissue from odor-exposed rats yielded staining patterns comparable to those seen in a previous study (Illig and Haberly, 2003) and similar to patterns observed with 2DG uptake following odor exposure in the OB (Johnson et al., 1998, 1999).

The mouse monoclonal anti-GAD 67 antibody employed recognizes a single band of 67 kDa molecular weight on Western blot from rat brain lysate (manufacturer's technical information; Fong et al., 2005). This antibody was raised against a recombinant fusion protein containing the N-terminal region of human GAD 67 that is not shared by GAD 65 (manufacturer's technical information). We used olfactory bulb sections to confirm that nearly all granule cells bodies were positive for GAD staining with this antibody, whereas mitral/tufted cells were negative.

Immunostaining: procedures

Fos—Sections were rinsed twice in 0.01M PBS, incubated in 0.03% H₂O₂ for 30 minutes at room temperature, then rinsed in a wash buffer containing 0.01M PBS, 2% bovine serum albumin (BSA), and 0.3% Triton-X 100 (TX). Next, sections were incubated in a blocking buffer containing 0.01M PBS, 2% BSA, and 0.3% TX and 20% normal goat serum (NGS) for 1 hour at room temperature. Subsequently, tissue was labeled with rabbit polyclonal anti-Fos antibody in buffer containing 0.01M PBS, 0.5% TX, 2% NGS via incubation of sections on a shaker overnight at room temperature. The following morning, sections were rinsed in wash buffer twice for 5 minutes then twice for 15 minutes. Next, sections were incubated in secondary antibody at room temperature for 3 hours (1:1,000, biotinylated goat-antirabbit IgG (H+L), Vector, Burlingame, CA). Following secondary incubation, sections were rinsed in wash buffer again (twice for 5 minutes then two times for 15 minutes) and incubated in avidin-biotin complex (ABC elite standard kit, Vector) for 1 hour at room temperature. Finally, sections were rinsed and stained with DAB. Omission of the primary antibody during processing eliminated all tissue staining.

Fos/GAD-67 double label—Gad-67 and Fos were double-labeled using fluorescence immunohistochemistry as described previously (Illig and Haberly, 2003). A small amount of TX was added to the appropriate steps in order to facilitate Fos antibody absorption without significantly compromising the numbers of GAD-positive cell bodies. Briefly, sections were rinsed in 0.01M PBS twice followed by incubation in 0.03% H₂O₂ for 30 minutes at room temperature then rinsed in a wash buffer containing 0.01M PBS, 2% BSA, and 0.05% TX. Next, sections were incubated in a blocking buffer containing 0.1M PBS, 2% BSA, 0.05% TX, and 20% normal goat serum (NGS) for 1 hour at room temperature. Tissue subsequently was labeled with same primary antibody employed above and mouse anti-GAD-67 monoclonal antibody (see Table 1) in buffer containing 0.01M PBS, 2% NGS, and 0.05% TX via incubation of sections on shaker overnight at room temperature. The following morning, sections were rinsed 6×10 minutes in wash buffer and incubated in fluorescence-tagged secondary antibody (1:500 goat-antimouse; AlexaFluor; Molecular Probes, Eugene, OR) at room temperature in the dark for 3 hours. Finally, sections were rinsed 3 times with PBS and mounted on slides. Omission of the primary antibody during processing eliminated all tissue staining.

Image acquisition and analysis

Tissue selection—Separate analyses were made for the OB, pars externa, and pars principalis. For each OB specimen the section that represented the caudalmost extent of the tissue not containing a portion of the AON was located. This reference point became the first 50- μ m section to be scored. Every other section anterior to the standardized location was measured until a population of 21 sections had been accumulated. Labeled cells found within the glomerular layer of the OB were examined.

For each AON specimen the first section chosen for analysis contained the rostralmost extent of pars externa (Fig. 1A). {FIG 1} Thereafter, every other 50- μ m section was chosen. The pars externa samples included the first nine of these sections while the pars principalis sample included 13 sections, ending with the caudal transitional areas of the AON where pars medialis and pars ventroposterior disappear and the piriform cortex begins (Fig. 1E; see Brunjes et al., 2005).

All analyses were made by observers blind to the experimental group of the subjects. Composite images of each section were made using a microscope with a 20 $\times$ objective and the tiled-field mapping routine of an image analysis software package (MCID 7.0 Elite; Interfocus Imaging, Linton, UK). Fiduciary points on each map were used to sequentially rotate and align sections in the coronal plane. In order to assure that the measurement of each section was calibrated with respect to the intensity of staining, “Fos-immunoreactive cells” were defined using an optical density threshold defined as a percentage (50%) of the difference between the optical density of the darkest stained cell nucleus and that of the noncellular background in layer IA (after Illig and Haberly, 2003). The software package also plotted the X-Y coordinates of each target cell.

Cell counts—The Abercrombie (1946; Guillery, 2002) correction, based on mean profile diameter, was employed to make unbiased estimates of the number of Fos-labeled cells. Tissue sections from four animals (one randomly selected from animals exposed to limonene, caproic, and butyric acids, and purified air) were selected to establish the calibration parameters. In each section the diameters of 50 randomly selected Fos-positive cell nuclei were measured with a 40 $\times$ objective. No differences were observed in the size of the nuclei among subjects, so the data were pooled to determine the mean profile diameter, and that number used in the correction factor employed to estimate cell number.

Density measurements—The following methods were employed to test the hypothesis that odor exposure resulted in spatially discrete concentrations of activated cells. The XY position data for each labeled nucleus was transformed so that the data could be expressed in polar coordinates (Fig. 1F,G). Polar plots of the points for each section were then divided into 5° bins, the number of labeled nuclei in each bin determined, and the counts corrected as above. In order to address the wide intersubject variations in the total numbers of immunopositive cells (Figs. 2, 3), {FIG 2–3} the data were normalized to percentages by dividing the counts in each bin by the total number of Fos-labeled cells (corrected as above) found in that subject. These density estimates were then plotted as color-coded contour graphs, with oranges and reds representing the areas with highest densities of Fos-expressing cells and blue representing the areas with the lowest densities (Figs. 1J,K, 2A, 3A, 4A). {FIG 4} Pairwise comparisons of the densities in the 5° bins were calculated between all subjects with the Pearson correlation coefficient, r . These correlation values were then averaged for within- and between-condition comparisons.

Boundary lines representing the internal organization of the OB, pars externa, and pars principalis were overlaid on the contour plots. The coronal sections of the OB were divided into dorsal, ventral, medial, and lateral regions. Due to pars externa's unique shape it was

simply divided into dorsal and ventral regions. For pars principalis, boundary lines between pars medialis, pars ventroposterior, pars lateralis, and pars dorsalis were defined by the criteria of Haberly and Price (1978). The distribution of Fos-positive cells found in each subdivision in each anterior/posterior section was calculated (number of labeled cells in region/total population of immunoreactive cells) for each subject, and mean percentages for each odor condition calculated and plotted.

Identifying clusters of Fos-positive cells—Using a 20× objective, areas containing Fos-positive cells were quantified by tracing around clusters of labeled cells in every second 50-μm section throughout the OB and in the 13 sections of the AON used prior for contour plots. A “cluster” of labeled cells was only defined if it was separated from another by a zone of at least 500 μm that contained no labeled cells. For both odor-exposed and electrically stimulated subjects, the volume of OB Fos-positive cells was plotted against the volume of AON Fos-positive cells and a three-parameter sigmoidal function of best fit for these data calculated (SigmaPlot 10.0; Systat Software, Point Richmond, CA; Fig. 4D).

Fos/GAD-67 double stain—An Olympus Fluoview confocal microscope was used to obtain stacks of images of the fluorescently labeled tissue which were analyzed as described previously (Meyer et al., 2006). Briefly, five standardized areas were chosen for analysis (one each in pars dorsalis, medialis, and ventroposterior, and two in pars lateralis), and the images for each were acquired in a random order. This method allowed an overview of several regions of pars principalis while avoiding photobleaching. Ten images were obtained at each location, each 1 μm thick, and separated by 1 μm. Each focal plane was scanned four times and the results averaged for both Fos and GAD-67 labeling. Subsequently the images were combined and counts were made for the number of Fos, GAD-67, and double-labeled cells. Images were acquired and minimally adjusted for brightness and contrast with Adobe Photoshop CS2; plates were constructed with Adobe Illustrator (San Jose, CA).

RESULTS

Odor stimulation

Olfactory bulb—As expected, Fos-immunoreactive cells were sparse in the OBs of control animals but plentiful in odor-exposed subjects. As described in previous studies using a variety of methods, exposure to caproic acid or butyric acid led to staining in the dorsomedial portion of the anterior OB, while exposure to limonene resulted in dense labeling along the lateral side. To confirm that our quantification method detects spatial patterns, Fos-positive cells in the glomerular layer of five animals exposed to butyric acid were examined using the sampling procedures described above. As expected, a large average correlation was found among the five animals exposed to the same odor ($r(1512) = 0.41$, $P < 0.001$, $SEM = 0.03$).

Pars Externa—A similar pattern was observed in pars externa: animals exposed to purified air displayed very few Fos-labeled cells (fewer than 6 cells/animal), while odor exposure resulted in substantial numbers of darkly immunoreactive cells. Odor exposed animals had significantly more labeled cells than controls ($t(33) = 4.33$, $P < 0.001$; Fig. 2D). There were also significant differences in the number of labeled cells elicited by high and low concentrations of limonene ($t(8) = 5.39$, $P < 0.001$), and butyric acid ($t(8) = 2.76$, $P < 0.05$). The difference between high and low concentrations of caproic acid was not significant ($t(8) = 1.99$, $P = 0.081$).

Interestingly, odor-exposed animals displayed consistent patterns of activation across all subjects (Fig. 2A,B); subjects presented with limonene displayed the highest proportion of

immunolabeled cells in the dorsal-posterior area of pars externa, while tissue from animals exposed to caproic acid or butyric acid had Fos-labeled cells found primarily in the ventral-anterior portion of the region. Contour plot patterns for the low-concentration groups were similar to their high-concentration counterparts. No significant differences were found in either the average pairwise correlations between animals exposed to either the high or low concentrations of the same odor ($t(133) = 0.72$, $P = \text{ns}$) or analysis of variance by region (ANOVA, $F_{1,72} = 2.08$, $P = \text{ns}$) and therefore high- and low-concentration subjects were pooled within an odor group. The average correlation coefficient for comparisons of animals exposed to the same odor ($r(646) = 0.34$, $P < 0.001$, $\text{SEM} = 0.02$) was significantly higher than comparisons of animals exposed to different odors ($r(646) = 0.24$, $P < 0.001$, $\text{SEM} = 0.02$; $t(433) = 6.13$, $P < 0.001$). Detailed quantitative analyses of subregions revealed a significant difference in the distribution of Fos-labeled cells between limonene and the two other stimuli [caproic acid: dorsal (ANOVA, $F_{1,162} = 45.09$, $P < 0.01$), ventral: (ANOVA, $F_{1,162} = 69.66$, $P < 0.01$); butyric acid: dorsal (ANOVA, $F_{1,162} = 93.51$, $P < 0.01$), ventral (ANOVA, $F_{1,162} = 77.14$, $P < 0.01$)]. No significant differences in Fos distribution were found between caproic and butyric acids in either the dorsal (ANOVA, $F_{1,162} = 0.16$, $P = \text{ns}$) or ventral region (ANOVA, $F_{1,162} = 0.41$, $P = \text{ns}$). Thus, the pattern of Fos activation elicited in pars externa by limonene was significantly different than those elicited by butyric or caproic acids, which did not differ from each other.

Pars principalis—While considerable numbers of labeled cells were found throughout pars principalis in all subjects, no significant differences were observed in the number of Fos-immunoreactive cells between odor exposed and control animals ($t(33) = 0.275$, $P = \text{ns}$) or between high and low concentrations of the same odor ($t(8) = 0.252$, $P = \text{ns}$; Fig. 3c). Patterns of staining were highly variable in control animals and specific, fine-scale foci of activity were not consistent between subjects (Fig. 3). The average intersubject correlation among control animals ($r(934) = 0.20$, $P < 0.001$, $\text{SEM} = 0.03$) was not significantly different from correlations between control and odor-exposed animals ($r(934) = 0.16$, $P < 0.001$, $\text{SEM} = 0.02$; $t(158) = 1.56$, $P = \text{ns}$).

Animals exposed to high concentrations of the odorants had darkly labeled cells that were distributed throughout all subregions. While areas dense with labeled cells were seen in each subject (Fig. 3A), pairwise correlations within the five animals receiving the same odor stimulus revealed mean correlations ($r(934) = 0.13$, $P < 0.001$, $\text{SEM} = 0.02$) that were not different from average correlations obtained between animals exposed to different odors ($r(934) = 0.14$, $P < 0.01$, $\text{SEM} = 0.01$; $t(103) = 0.14$, $P = \text{ns}$).

To ensure that high odor concentrations were not obscuring patterns by saturating neural responses, a second group of animals was exposed to reduced levels of the same odors (see Materials and Methods). Whereas patterns elicited in pars externa were similar across odor concentration, contour plots in pars principalis failed to display any stereotyped patterns (Fig. 3A–E). Areas of relatively dense Fos immunoreactivity were observed in individual animals, but comparisons among animals indicated that these foci did not appear consistently in the same regions. Comparisons revealed no similarities within odor groups; correlations between animals within low concentration odor groups ($r(934) = 0.18$, $P < 0.001$, $\text{SEM} = 0.02$) were no stronger than those observed between low concentration odor groups ($r(934) = 0.16$, $P < 0.001$, $\text{SEM} = 0.01$: comparisons between the average correlation; $t(178) = 1.53$, $P = \text{ns}$). Similarly, the correlations observed among animals exposed to low and high odor concentration of the same odor ($r(934) = 0.14$, $P < 0.001$, $\text{SEM} = 0.01$) were not significantly different than correlations between odors ($r(934) = 0.14$, $P < 0.001$, $\text{SEM} = 0.01$; $t(448) = 0.01$, $P = \text{ns}$). Additionally, when the average percentages of labeled cells found in each subregion of pars principalis were calculated for each anterior/posterior section (Fig. 3B–E), this broader measure of odor-evoked activation revealed no

significant differences among any of the odor or control groups (ANOVA, $F_{1,118} = 0.36$, $P = \text{ns}$). Thus, patterns of Fos activation in pars principalis elicited by odor exposure could not reliably be attributed to odor quality or odor concentration.

Electrical stimulation

Since exposure to even simple, monomolecular odors leads to activation of large parts of the OB (e.g., Johnson and Leon, 2007), electrical stimulation of the OB was employed to determine whether small focal activation would lead to discrete Fos labeling in the AON. Preliminary studies indicated that the spatial extent of Fos-immunoreactivity correlated with the intensity of the current and with the duration of the stimulation. Thus, a very low level of electrical stimulation (0.1 ms, 1 μA , 0.1 Hz for 1 minute) was delivered to the bulb to elicit activity in only a very small number of cells (Fig. 4A,D). Even with this very low stimulation, considerable variation was seen in the number of Fos-positive cells in the OB. Nevertheless, a significant correlation between the extent of immunolabeled cells found in the OB and in pars principalis was observed ($R^2 = 0.527$, $P < 0.001$, Fig. 4D).

Using the same quantification measures described above, contour plots for three of the subjects with the smallest zone of activation in the OB were prepared (Fig. 4A). In these subjects, only two to three Fos-positive cells were observed in the dorsal-anterior region of pars externa. In pars principalis, a more robust response was observed, and regions with dense Fos immunoreactivity were found in posterior pars medialis and anterior pars lateralis. Fos-labeled cells were relatively absent from posterior pars lateralis, anterior pars dorsalis, and pars ventralis. Nevertheless, despite the focal nature of the bulb stimulation, no patterns of Fos-labeling were observed that were consistent across animals.

Fos expression in GABAergic cells—Previous studies have shown that odor stimulation evokes Fos expression in different cell populations in the OB and piriform cortex; GABAergic cells in the OB show induction of c-fos (Guthrie et al., 1993), but GABAergic cells in the piriform cortex are rarely Fos-positive (Illig and Haberly, 2003). It is possible, therefore, that Fos labeling in a large number of GABAergic neurons might obscure a spatial code for odor quality in the principal neurons in the AON. To examine the neurophenotype of the Fos-positive AON cells, tissue from odor-exposed animals was double-labeled for Fos protein and the enzyme GAD-67 (a marker of GABAergic cells; Kaufman et al., 1986). While many cells were double-labeled in the OB (Fig. 4E,G,I), no double-labeled nuclei were found in a random sample of 177 Fos-positive cells in pars principalis from nine animals (Fig. 4F,H,J).

DISCUSSION

Spatial organization of olfactory information

The present study was designed to investigate whether olfactory stimulation results in patterns of odor-evoked Fos activation in the AON similar to that found in the OB, or in diffuse, structure-wide activation similar to that found in piriform cortex. Because Fos staining allows for the visualization of activity within a large population of cells, it has been widely employed in assessing patterns of evoked activity in the olfactory system and other sensory modalities (e.g., Morgan et al., 1987; Filipkowski, 2000; Caputto, 2000; Kovacs, 2008).

The results presented above indicate that odor exposure evoked spatial patterns of Fos expression in the OB that are consistent with those reported by other studies employing several markers of cellular activation. Our quantification methods detected the correlation between these OB patterns across animals. Odor-evoked activity in pars externa also

followed a spatially discrete pattern, with aliphatic acids and limonene eliciting significantly different patterns of Fos expression (Fig. 2). Furthermore, responses were graded in pars externa; few labeled cells were observed in control animals exposed to purified air, high odor concentrations induced substantial Fos expression, and lower concentrations resulted in intermediate results. These results suggest that activity within pars externa is organized according to location and intensity of bulbar activation, in agreement with previous reports (Lei et al., 2006; Yan et al., 2008; Kikuta et al., 2010).

In contrast, odor-evoked Fos expression in pars principalis was widespread and without any consistent spatial patterns (Fig. 3). While discrete accumulations (clusters) of Fos-positive cells could be found throughout pars principalis in individual subjects, particularly in pars dorsalis and pars medialis, both the number and spatial locations of labeled cells were not consistent among subjects exposed to the same odor, and therefore could not be attributed to odor quality, odor concentration, or position of OB activation. These results suggest that odor-evoked activity in pars principalis is not organized into a spatially discrete “spot code” as is seen in the OB, but rather takes on a distributed organization similar to that found in the piriform cortex (Illig and Haberly, 2003; Poo and Isaacson, 2009; Stettler and Axel, 2009).

Given the spatial coding of odorants in the OB and the broadly topographical arrangement of axons in the lateral olfactory tract, it may be surprising that no evidence was observed for spatial patterns of odor-evoked activity in pars principalis. There are at least four alternative views that can explain these results. First, it is possible that the analyses employed had insufficient spatial resolution. Given the large number of odor stimuli rats can discriminate and the small size of pars principalis, any spatially discrete organization of odor-specific clusters of cells might have to be very small to avoid overlapping representations. Several sources of measurement variation could obscure such areas of activation, including individual differences in the plane of section between animals, the 50- μ m “gap” between sections used for analysis, and the difficulty in precisely mapping radial coordinates between animals. Nevertheless, this alternative does not seem probable: Fos-labeled cells were found throughout the AON following odor exposure, suggesting that odor-evoked activation is widespread, rather than confined to small, discrete clusters of cells. If odor identity is coded by small clusters of cells in pars principalis, then widespread activation as found in our study would not be expected. Indeed, such activation would be counterproductive to a rigid, spatially discrete coding scheme.

A second alternative is that immunostaining for Fos protein is not the proper tool to detect a spatially discrete organization. All IEG imaging methods have shortcomings limiting interpretation (Kovacs, 2008). For Fos immunostaining, these restrictions include low temporal resolution, the fact that Fos protein may be activated by means other than odor stimulation, and the fact that there are no assurances that cells stimulated by the test odorants will express the protein (see also Illig and Haberly, 2003). Thus, even though Fos immunostaining successfully detected spatially discrete activation in the OB and pars externa, the relationship between odor-evoked activity and Fos expression in pars principalis may be more complex, and therefore may not accurately reflect odor-evoked activity there.

Third, it is possible that spatial patterns of odor-evoked activity evolve with time. Indeed, examinations of activity in the OB at short time scales has suggested complicated and evolving coding schemes (e.g., Spors et al., 2006; Liu and Shipley, 2008; Rabinovich et al., 2008; Bathellier et al., 2008; Soucy et al., 2009). Perhaps in the early moments following odor exposure, odor-associated activity is spatially discrete within pars principalis in order to serve initial sensory processing and reflect the driving input from the OB. As the odor identity is incorporated with other information, however (e.g., contextual or mnemonic cues available through the rich interconnections among the AON, PC, orbitofrontal cortex, and

other regions; Haberly and Price, 1978a,b; Luskin and Price, 1983; Illig, 2005), this activity may become more spatially diffuse. Thus, initial Fos protein expression in small, spatially discrete patches of cells would be obscured by Fos protein expressed by cells activated during later processing.

Our results may suggest that pars principalis uses a spatially distributed ensemble code similar to that postulated for piriform cortex (Illig and Haberly, 2003; Poo and Isaacson, 2009; Stettler and Axel, 2009). A distributed approach to sensory information processing can be found elsewhere in the brain, particularly in higher-order sensory cortices. For example, reports suggest that higher-order visual cortex in primates contain cells responsive to complex objects, but the location of cells responsive to a particular object is distributed and varies among animals. Further, it appears that coding for objects is based on the responses of a large ensemble of neurons, each of which is involved in coding many different objects (e.g., Kiani et al., 2007). Therefore, it may be unnecessary to duplicate a spatial code established at early sensory processing levels for higher-order information processing, particularly when heavy reciprocal projections exist among processing regions (as among the OB, AON, piriform cortex, and other olfactory regions).

Several computational advantages exist for a spatially distributed architecture. The number and relationship of elements within the set of odors and odor mixtures that can be represented, for example, would not be limited by the amount of cortical space available. Further, encoding associations among odors would not depend on the a priori juxtaposition of certain “odor-processing modules”; the representational power of the system is improved by utilizing a highly associative architecture where responses are distributed across the surface of the cortex, and where the elements of the neural network participate in the coding of a wide variety of odor stimuli. Our results indicate that while pars externa appears to utilize a spatially discrete code to maintain the localized processing observed in the olfactory bulb, pars principalis employs a distributed code similar to that seen in the piriform cortex. Taken together with previous research indicating its central position in the olfactory circuit, our results suggest that pars principalis likely plays a key role in integrating and processing olfactory information.

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Fig. 1.

Method for quantification of Fos-positive cells in pars externa (left column) and pars principalis (right column). See text for details. A–E: Nissl-stained coronal sections show the location of pars externa (white arrows) and pars principalis. Nine and 13 sections containing the AON were measured for pars externa and pars principalis, respectively. F,G: For each section the cellular region was divided radially, with the medial (M), dorsal (D), lateral (L), and ventral (V) regions represented by 0°, 90°, 180°, and 270°, respectively. The radial positions of the Fos-labeled cells were transferred onto a linear coordinate system (for example, the asterisks shown become tick marks on the vertical linear plot to the left of each section). H,I: Linearized data for all Fos-labeled cells in every second 50- μ m section were graphed from anterior to posterior. Each horizontal line represents a single Fos-labeled cell,

and each column is the data from a single section. J,K: For each section, data from radial position plots were pooled into 72 5° bins and the number of cells in each bin determined. The counts were then normalized by dividing the number of cells in each bin by the total number of labeled cells in the animal. The data were then plotted using color-coded contour plots with areas of relatively high density cells shown in red and orange and low density in blues. Areas outside the region of interest were shaded gray. For pars principalis, boundary lines were overlaid on the plots between the subdivisions of the AON as defined by Haberly and Price (1978b). The white number in the bottom left of each panel is the total number of Fos-positive cells counted in that animal.

Fig. 2.

Distribution of Fos-labeled cells in pars externa. A: Contour plots show the density and location of Fos-labeled cells by their radial position (vertical axis), their anterior-posterior position (horizontal axis). See Figure 1 for details. Each panel represents the data collected from a single animal; the white number in the bottom left of each panel is the number of Fos-positive cells counted in that animal. Each column groups plots from animals exposed to the same odor and odor concentration. B,C: The average percentage of Fos-labeled cells in dorsal and ventral regions plotted separately and sorted by odor group. In dorsal pars externa, caproic and butyric acid-exposed subjects (red and green lines) have a higher proportion of Fos-labeled cells in the medial and posterior region compared to subjects exposed to limonene (blue line). In ventral pars externa, limonene-exposed subjects have a higher proportion of Fos-labeled cells in the posterior region compared to subjects exposed to caproic and butyric acids. An ANOVA revealed the significant differences among groups; asterisks indicate where comparisons between limonene and the aliphatic acids are significantly different using Tukey's HSD post-hoc analysis. D: Average cell counts for each

odor condition ($\pm$ SEM). Asterisks indicate statistically significant differences using a one-tailed t-test ($P < 0.05$). E,F: Photomicrographs showing anterior (E) and posterior (F) sections containing Fos-positive cells. Black arrows indicate the boundaries of pars externa. Scale bar = 250 μ m.

Fig. 3.

Distribution of Fos-labeled cells in pars principalis. A: Contour plots show the density of Fos-labeled cells by their radial position and anterior-posterior section (see Fig. 1). Each panel represents the data collected from a single animal; columns group plots from animals exposed to the same odor and odor concentration. White lines are overlaid to show the boundaries between pars medialis (M), pars dorsalis (D), pars lateralis (L), and pars ventroposterior (V) as defined by Haberly and Price (1978b). Note that patterns of Fos-labeled cells display as much between-subject variability within groups as they do across experimental condition. B–E: Line graphs plotting the mean percentage of cells in each subregion across the anterior/posterior sections (Note: pars medialis and ventroposterior do

not extend into the anterior AON). There were no significant differences between the odor or control groups in any of the measurements. F: Average cell counts for each odor condition ($\pm$ SEM). No significant differences were found.

Fig. 4.

A: Patterns of Fos activation in pars principalis induced by focal electrical stimulation of the olfactory bulb. Conventions as in Figure 1. Note restricted patterning compared to Figure 3, with most activation occurring in pars lateralis and medialis. B,C: Photomicrographs of matched sections through pars principalis following electrical bulb stimulation (B) and odor exposure (C) subject from high-concentration limonene group. Note that focal electrical stimulation of the bulb results in a more discrete cluster of Fos-labeled cells in pars principalis. D: Scatterplot showing the relationship between the volume of the olfactory bulb and pars principalis containing dense Fos-positive cells. Rats receiving electrical bulb stimulation are marked by circles, animals exposed to low concentrations of either limonene, caproic acid, or butyric acid marked by squares, and those exposed to high concentrations of odor are marked by triangles. The dotted line shows a three-parameter sigmoidal function of best fit for these data. E–J: Fos expression in GABAergic cells. Tissue from an odor-exposed animal was double-labeled for Fos protein (magenta) and GAD-67 (green). Solid arrows indicate Fos-positive cells in the OB (left) and AON (right). Open arrows indicate GABAergic cells in both regions. While some double-labeled cells were found in the OB, no cells in the AON were found to be labeled for both Fos protein and GAD-67. Scale bars = 500 μm in B,C for large photomicrographs and 250 μm for insets; 50 μm in F,H,J, AON; 25 μm in E,G,I, OB.

TABLE 1

Primary Antibodies Used

Antigen	Immunogen	Antibody type	Host	Manufacturer's details	Working dilution
c-Fos	Synthetic peptide, amino acids 4-17 of human c-Fos	Polyclonal	Rabbit	Cat. # PC38, Ab-5, Calbiochem, EMD Biosciences (San Diego, CA)	1:30,000
Glutamic acid decarboxylase 67	N-terminal amino acids 4-101 of human GAD67	Monoclonal	Mouse	Cat. # MAB5406, clone 1G10.2 Chemicon, Millipore (Temecula, CA)	1:10,000