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Odor-induced increases in c-*fos* mRNA expression reveal an anatomical "unit" for odor processing in olfactory bulb

(olfactory system/sensory representation/immediate-early gene/*in situ* hybridization/protooncogene)

KATHLEEN M. GUTHRIE*, AILEEN J. ANDERSON†, MICHAEL LEON†, AND CHRISTINE GALL*

Departments of *Anatomy and Neurobiology and †Psychobiology, University of California, Irvine, CA 92717

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ABSTRACT **Expression of the immediate-early gene c-*fos* was used to evaluate the coordinate activation of olfactory bulb neurons by brief exposure to specific odors in the alert rat. *In situ* hybridization to c-*fos* mRNA was compared to regional increases in 2-deoxy-D-[^{14}C]glucose incorporation in an adjacent section analysis. Levels of c-*fos* mRNA in olfactory bulb were high in rats recently removed from their home cage but were low in animals placed in a relatively odor-free chamber for 30 min. Presentation of specific odors to alert rats for as little as 5 min increased c-*fos* mRNA in radially distributed neuronal ensembles that spanned the lamina of the main olfactory bulb. The complementary RNA (cRNA)-labeled neuronal collectives consisted of cells in the glomerular layer that precisely defined the borders of individual glomeruli and underlying tufted, mitral, and granule cells. The activated fields were much broader in the granule cell layer than in the overlying glomerular layer and thus exhibited a flask-like, as opposed to a columnar, contour. The bulbar distribution of cRNA-labeled cell arrays differed with different odors and, in the glomerular layer, corresponded to focal regions of high 2-deoxy-D-[^{14}C]glucose uptake. Administration of the noncompetitive *N*-methyl-D-aspartate receptor antagonist MK801 did not attenuate the odor induction of c-*fos* but, instead, increased c-*fos* mRNA levels throughout the bulb. We propose that the neuronal ensembles expressing increased c-*fos* mRNA with odor stimulation represent principal functional units of sensory processing in the main olfactory bulb of the behaving rat.**

How the activities of the disparate elements that comprise the mammalian olfactory bulb are integrated into a functional representation of odors in behaving animals constitutes a major question in olfactory research. The olfactory bulb is a laminated cortical structure that receives diffusely distributed innervation from odor receptor neurons in the nasal epithelium and gives rise to axonal projections to olfactory cortex (1). The bulb is also massively innervated by pathways originating from other brain structures (e.g., olfactory cortex, anterior olfactory nucleus), suggesting that the processing of odor cues in the behaving animal reflects an interaction between primary sensory input and highly processed centrifugal feedback (1, 2). Multielectrode electroencephalogram recording studies have provided valuable information about patterns of activity in the olfactory bulb of alert animals during odor stimulation (3) but do not allow resolution of individual active elements. Use of the metabolic marker 2-deoxy-D-[^{14}C]glucose ([^{14}C]dGlc) in behaving rats has revealed that odors stimulate regional increases in [^{14}C]dGlc incorporation in the glomerular layer, where the olfactory nerve axons terminate, with different odors producing distinct patterns of focal uptake (4–6). This observation has led to the suggestion that individual glomeruli serve as functional units in odor processing (7). However, limitations of the [^{14}C]dGlc technique (e.g., [^{14}C]dGlc diffusion, potential axonal and glial uptake) (8) have hampered efforts to identify the types and spatial relationships of individual neurons activated by odor. In particular, the functional topography of activation within the mitral, tufted, and granule cell fields remains unknown, although anatomical and physiological data (1, 9) and the recent utilization of voltage-sensitive dye techniques (10) predict topographic activation of these neurons with odor stimulation.

Expression of the immediate-early gene (IEG) c-*fos* can be rapidly induced by a variety of extracellular stimuli, including depolarization in the presence of calcium (11–13). Activity-dependent changes in IEG expression have proven useful in mapping neuronal responses in several brain regions (14–16) and, in some instances, have been shown to involve *N*-methyl-D-aspartate (NMDA)-mediated synaptic activity (17, 18). In the present studies, *in situ* hybridization analysis of odor-induced increases in c-*fos* mRNA expression was used to identify the types and spatial distributions of bulb neurons activated by brief odor stimulation in the behaving rat. Our results indicate that odors stimulate c-*fos* expression in multiple, topographically distributed neuronal ensembles, each of which includes neurons surrounding individual glomeruli and cells distributed within spatially limited regions of the underlying external plexiform, mitral, and granule cell layers. We suggest that the odor-activated neuronal array visualized with c-*fos* complementary RNA (cRNA) hybridization constitutes a primary functional unit of odor processing in the olfactory bulb. Portions of these results have been published in abstract form (19).

MATERIALS AND METHODS

Animal Treatments. Young male Wistar rats (21–22 days of age) were used in all experiments. In rats sacrificed immediately after removal from the home cage, basal levels of c-*fos* mRNA were highest in olfactory areas. To determine if bulb c-*fos* mRNA levels could be reduced by maintaining animals in a relatively odor-free environment, rats were placed individually in a clean, open glass jar inside a laboratory fume hood (air flow = 24.4–30.5 linear m/min) for 30 min prior to sacrifice. The fume hood door was lowered to within 5 cm of the base and care was taken to eliminate odor sources from the dedicated test room. For controlled odor presentation, alert rats were first placed in the clean air environment for 30 min, injected i.p. with [^{14}C]dGlc (20 μCi/100 g; 1 Ci = 37 GBq), and then transferred to a closed jar for exposure to either peppermint or isoamyl acetate odor (n = 7 per odor). Odors were delivered as a 1:10 dilution of saturated vapor in air at 2 liters/min for 1 min at 4-min intervals over a 30-min period (20). Air-control littermates (n = 7) were placed in the clean air chamber (30 min), injected with [^{14}C]dGlc, and

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Abbreviations: dGlc, 2-deoxy-D-glucose; cRNA, complementary RNA; NMDA, *N*-methyl-D-aspartate.

returned to the clean air chamber. All rats in this series were killed 30 min after [^{14}C]dGlc injection. To determine if odor induction of c-*fos* requires NMDA receptor activation, additional rats received the noncompetitive NMDA antagonist MK801 (i.p., 1 mg/kg, $n = 4$) or isotonic saline (equivalent volume, $n = 4$) 30 min prior to placement in the clean air chamber for 30 min. They were then exposed to peppermint odor (30 min, as above) and sacrificed. For analysis of the time course of c-*fos* mRNA induction, a final series of rats were placed individually in the clean air chamber (30 min), exposed continuously to peppermint odor for 5 min, and then returned to the clean chamber until sacrifice 15, 30, or 60 min ($n = 3$ each) after odor onset.

In all cases rats were decapitated, brains were frozen in 2-methylbutane (−50°C), and adjacent series of cryostat sections (coronal, 20 μm) were processed for [^{14}C]dGlc autoradiography (20) or *in situ* hybridization with ^{35}S-labeled c-*fos* cRNA (21). For [^{14}C]dGlc autoradiograms, tissue was exposed to Amersham β-max Hyperfilm for 7–10 days.

***In Situ* Hybridization and Autoradiographic Analysis.** Levels of c-*fos* mRNA were examined in tissue sections through the olfactory bulbs using *in situ* hybridization of an ^{35}S-labeled RNA probe complementary to rat c-*fos* mRNA. The antisense cRNA was transcribed from *Pst* I-linearized recombinant clone pBS/rfos with T7 RNA polymerase in the presence of uridine 5′-[α-[^{35}S]thio]triphosphate. The resulting cRNA is complementary to positions 583–1250 of clone pc-fos(rat)-1 by Curran *et al.* (22). The sense RNA sequence was generated from the same template using T3 RNA polymerase after linearization with *Pvu* II.

Cryostat sections were collected onto Vectabond-treated slides (Vector Laboratories), fixed in 4% paraformaldehyde, and processed for *in situ* hybridization as described (21, 23). Hybridization was conducted at 60°C for 16–18 hr with the cRNA at a concentration of 1×10^6 cpm/100 μl. The distribution of hybridization was evaluated using Amersham β-max Hyperfilm and Kodak NTB2 emulsion autoradiography with exposure intervals of 4–7 days and 2–3 weeks, respectively. Soluble dGlc is washed out during *in situ* hybridization procedures and does not interfere with autoradiographic localization of cRNA hybridization, as verified by the absence of regional labeling in tissue processed with the ^{35}S-labeled sense RNA sequence. No cellular labeling was seen in tissue hybridized to the sense probe or treated with ribonuclease A before hybridization. For densitometric analysis, film autoradiograms were calibrated and measured relative to ^{14}C-labeled brain paste standards using the MicroComputer Imaging Device (Imaging Research, St. Catherine's, ON, Canada).

RESULTS

In rats removed from their home cage immediately prior to sacrifice, hybridization to c-*fos* mRNA was dense in olfactory structures, including the glomerular layer of the main olfactory bulb, the granule cell layer of the main and accessory bulbs, and the cellular layers of the anterior olfactory nucleus and piriform cortex. The possibility that high basal levels of c-*fos* mRNA in olfactory bulb reflect continuous stimulation by ambient odors in the home cage was tested by maintaining rats in comparatively clean air for 30 min prior to sacrifice. This period was chosen because c-*fos* mRNA has a half-life of about 20 min (24). Even though the environment was not specifically deodorized, hybridization density in the olfactory bulbs of these animals was substantially less than in bulbs from paired littermates recently removed from the home cage. This result suggests that in alert rats, odors normally encountered in the environment stimulate c-*fos* expression by olfactory bulb neurons and, moreover, that 30 min in a relatively odor-free environment is sufficient for levels of mRNA induced by prior odor exposure to decline. In subsequent experiments, animals were sacrificed a total of 1 hr after removal from the home cage, allowing additional time for the effects of prior odor exposure to subside.

In animals placed in the clean air chamber for 30 min and then exposed to either isoamyl acetate or peppermint odor for 30 min, regional elevations in hybridization to c-*fos* mRNA in the main olfactory bulb were observed and the distributions of these regions were different, though overlapping, for the two odor groups. Results from a typical experiment are shown in Fig. 1. Fig. 1 *A* and *B* show that intense hybridization is associated with (*i*) individual, spatially segregated glomeruli (arrows) and (*ii*) underlying portions of the granule cell layer that extend well beyond the vertical zone defined by a single glomerulus (arrowheads). As shown in Fig. 2*A*, autoradiographic grains were concentrated over small neurons surrounding individual glomeruli. Cell labeling in the vicinity of neighboring glomeruli was conspicuously absent. The size and location of labeled cells in the glomerular layer suggest the majority are periglomerular neurons. Underlying the activated glomeruli, grain density was also high over scattered neurons in the external plexiform layer (EPL) (arrow) and fewer neurons in the mitral cell layer (arrowhead). Based on size and distribution, these neurons are probable external and middle tufted, and mitral/deep tufted cells, respectively. Dense hybridization was also associated with large numbers of granule cells underlying activated glomeruli (Fig. 2*A*). In the dorsoventral and rostocaudal axes, granule cell activation generally extended well beyond the width of an activated glomerulus but, nonetheless, retained well-defined boundaries. In some instances, where labeled glomerular foci were located close together, areas of dense hybridization in the underlying granule cell layer appeared continuous, lacking an intervening zone of unlabeled cells (Fig. 1*A*). Although labeled neurons were present throughout the depth of the granule cell layer, densely labeled cells were more prevalent in superficial aspects. It is noteworthy that superficial granule cells receive excitatory input (via reciprocal dendrodendritic synapses) from the secondary dendrites of tufted cells in the EPL, many of which were labeled after odor presentation, whereas deeper granule cells are contacted primarily by mitral cells, fewer of which were labeled after odor exposure (25, 26). Physiological studies have also noted a higher incidence of odor-responsive granule cells in the superficial aspects of this layer (27).

The pattern of hybridization described above was obtained in each of the 14 rats exposed to odors and was not seen in rats maintained in clean air (Figs. 1*C* and 2*B*). Densitometric analysis of film autoradiograms revealed that hybridization densities in odor-stimulated regions of the glomerular and granule cell layers were 4.4- and 1.9-fold higher, respectively, than in nonactivated portions of these same laminae. In quiescent regions, hybridization was 2.3-fold higher in the granule cell layer than in the overlying glomerular layer, whereas in the odor-activated zones, hybridization densities were essentially equivalent in radially aligned portions of these two laminae.

Studies using [^{14}C]dGlc incorporation have established that individual odors activate topographically discrete regions of the glomerular layer (4, 5). To determine if c-*fos* cRNA hybridization reliably detects these same regions of activation, patterns of cRNA hybridization and [^{14}C]dGlc incorporation were compared in adjacent sections through the olfactory bulbs of odor-exposed rats. As illustrated by autoradiograms from a rat exposed to peppermint odor (Fig. 3 *A* and *B*), very good correspondence between regions of labeling within the glomerular layer was observed. Areas of focal [^{14}C]dGlc uptake coincided with glomerular cell clusters labeled with cRNA in at least one adjacent section. However, serial reconstructions revealed additional areas of increased

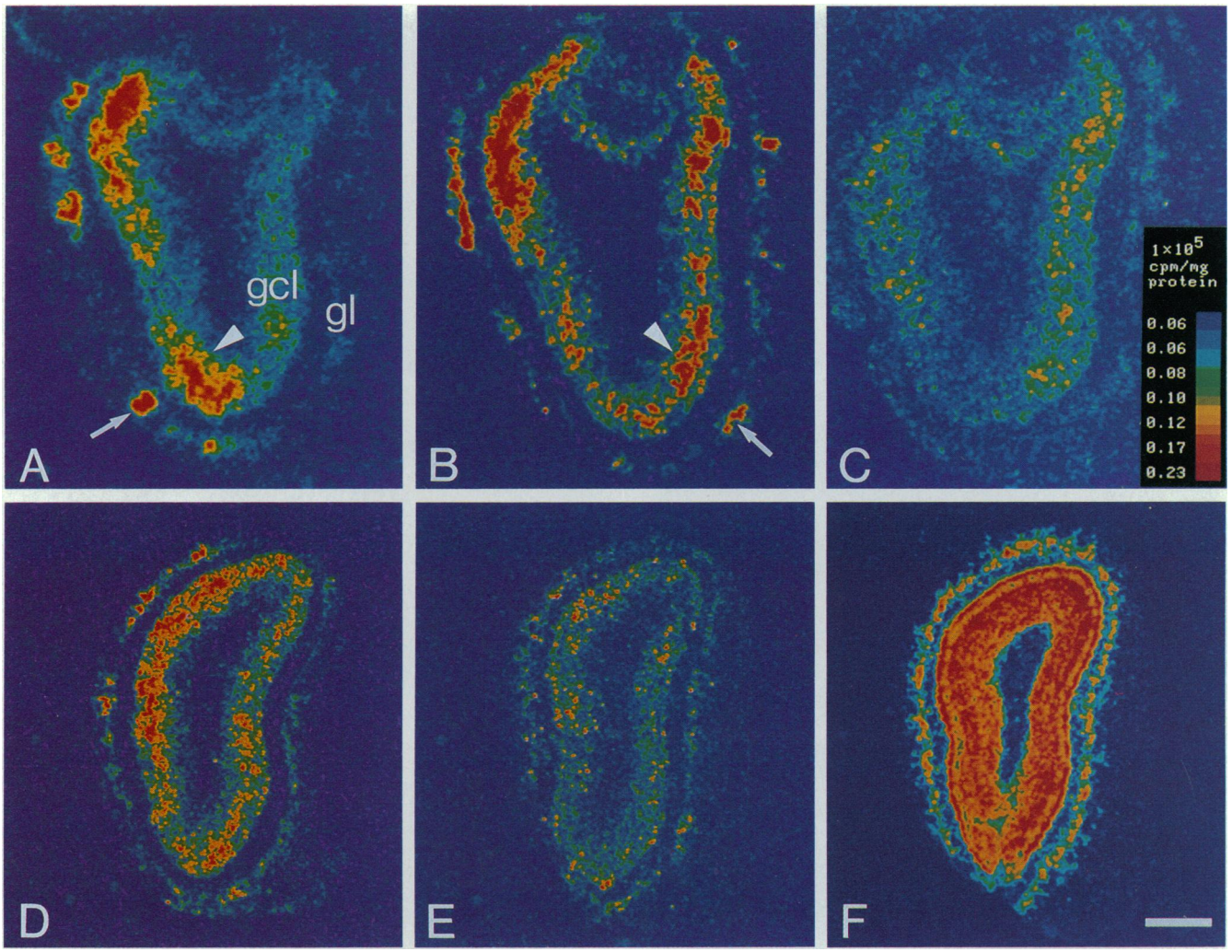

FIG. 1. (*A–C*) Pseudocolor images of film autoradiograms showing the density of ^{35}S-labeled c-*fos* cRNA hybridization in coronal sections through caudal olfactory bulb of rats exposed to peppermint odor (*A*), isoamyl acetate odor (*B*), or clean air (*C*) for 30 min. With odor stimulation (*A* and *B*), dense hybridization occurs in focal regions of the glomerular layer (gl, solid arrows) and the underlying granule cell layer (gcl, arrowheads). Note that the hybridization patterns for peppermint and isoamyl acetate odor differ. (*D*) After 5 min of exposure to peppermint odor, hybridization levels are elevated at 30 min but have declined by 60 min (*E*). (*F*) Following MK801 treatment of a peppermint-exposed rat, hybridization is increased throughout the bulb. (Bar = 500 μm for *A–C* and 760 μm for *D–F*; color calibration applies to *A–E*.)

c-*fos* cRNA hybridization in the glomerular layer that were not evident in adjacent [^{14}C]dGlc autoradiograms (Fig. 3*C*). Moreover, consistent increases in [^{14}C]dGlc uptake in the granule cell layer were not observed in areas that exhibited elevated cRNA hybridization.

In the above experiments, rats were exposed to odors for 30 min prior to sacrifice. Similar topographic and quantitative results were obtained with shorter periods of stimulation. As illustrated in Fig. 1*D*, exposure of rats to odor for as little as 5 min increased c-*fos* mRNA expression >3-fold in discrete glomerular clusters and underlying fields of the granule cell layer as measured 30 min after stimulus onset. By 60 min, this expression had declined to control, clean air, levels (Fig. 1*E*). Thus, stimulation approximating the duration of odor exposure likely to be encountered by the behaving rat is sufficient to increase c-*fos* mRNA content in olfactory bulb.

The noncompetitive NMDA antagonist MK801 dramatically increased c-*fos* mRNA levels throughout the external plexiform, mitral, and granule cell layers (Fig. 1*F*). Labeled mitral cells formed a band around the circumference of the bulb and labeled tufted cells defined a continuous scalloped border beneath the glomerular layer (Fig. 2*C*). Hybridization was not substantially increased in the superficial aspect of the glomerular layer, suggesting that periglomerular cells were largely unaffected by this treatment. The spatially restricted nature of the odor response, seen clearly in saline-treated controls, could not be detected in MK801-treated rats.

DISCUSSION

The pattern of increased c-*fos* mRNA expression reported here illustrates a coordinated response by individual anatomical elements of the mammalian olfactory bulb to odor stimulation. The response is topographic and odor-specific and incorporates a radial array of periglomerular, tufted/mitral, and granule neurons. Since the full array is activated with relatively brief odor presentation in freely moving rats, it is likely to constitute a functional anatomical unit activated by odors at behaviorally relevant concentrations (20).

An unexpected result was that the interneuronal populations (i.e., periglomerular and granule cells) dominated the response to odor. Labeled periglomerular cells defined complete borders around individual glomeruli, suggesting that a sizable proportion of the neurons in these fields had been stimulated by odor. Similarly, in underlying fields, a high proportion of the granule cells showed increased c-*fos* cRNA hybridization. In contrast, only a small proportion of the output neurons (tufted/mitral cells) was densely labeled, with

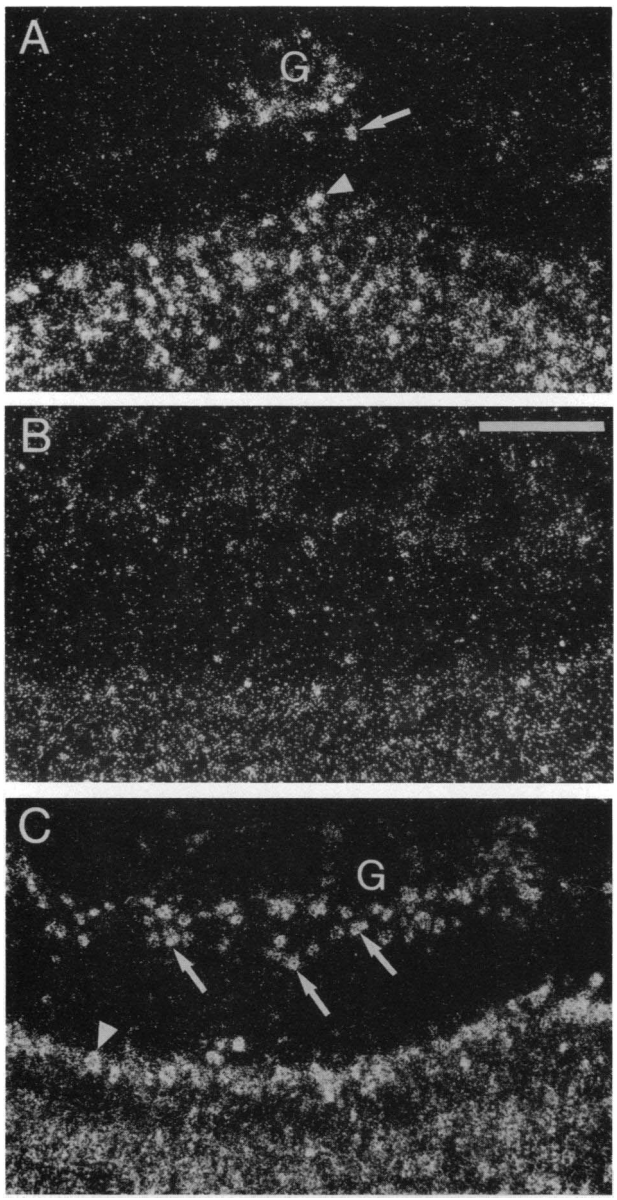

FIG. 2. High-magnification photomicrographs showing c-*fos* cRNA hybridization in olfactory bulbs of rats exposed to peppermint odor (*A*), clean air (*B*), or peppermint odor after MK801 treatment (*C*). In *A*, autoradiographic grains (white puncta) are concentrated over neurons surrounding a single glomerulus (G) and over underlying tufted (arrow), mitral (arrowhead), and granule cells (bottom). There is an absence of labeled cells around adjacent glomeruli. Little hybridization is seen in the air-control tissue (*B*), whereas there is dense labeling of mitral (arrowhead), tufted (arrows), and granule cells (bottom) after MK801 administration (*C*). (Bar = 100 μm.)

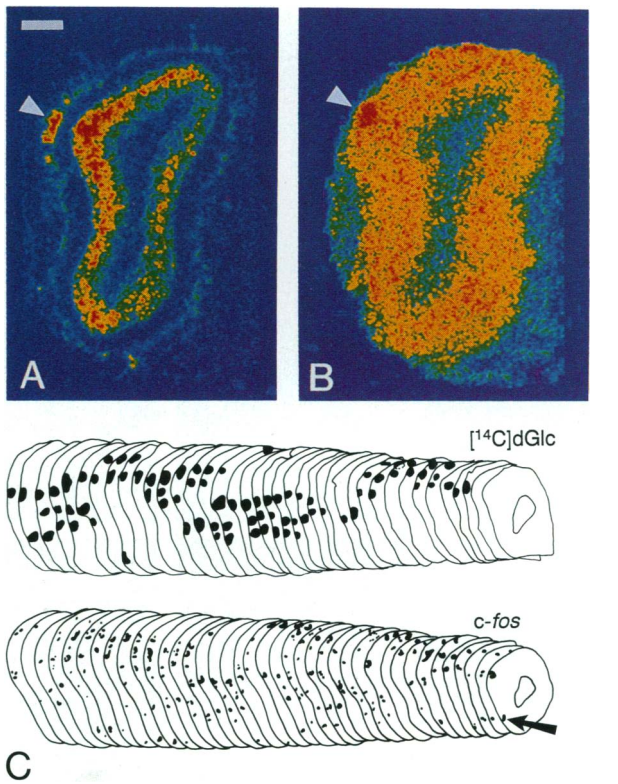

FIG. 3. (*A* and *B*) Adjacent sections through the olfactory bulb of a rat exposed to peppermint odor processed for hybridization with c-*fos* cRNA (*A*) or for [^{14}C]dGlc autoradiography (*B*). Glomerular layer regions of dense hybridization coincide with foci of elevated [^{14}C]dGlc uptake (arrowheads). (Bar = 500 μm.) (*C*) Adjacent serial reconstructions of the lateral aspect of olfactory bulb from a rat exposed to isoamyl acetate odor showing the distribution of focal [^{14}C]dGlc uptake (top) and dense c-*fos* cRNA hybridization (bottom) (olfactory nerve layer omitted; dorsal is up and rostral is to the right). dGlc foci correspond to regions of dense hybridization; however, additional areas of elevated c-*fos* expression are not evident in adjacent [^{14}C]dGlc autoradiograms (arrow). (Bar = 500 μm for *A* and *B*.)

tufted cells more frequently labeled than mitral cells. The sparse labeling of output neurons is clearly not due to phenotypic constraints on c-*fos* expression: the mitral cells exhibit robust c-*fos* expression after MK801 administration and in the neonatal rat following odor stimulation (K.M.G., unpublished observation). Although tufted and mitral cells are directly contacted by olfactory nerve afferents, physiological studies have shown that tufted cells have lower firing thresholds than mitral cells (28, 29), which might account for the differential response of these neurons to odor. Activation of the inhibitory interneuronal populations would also account for the comparatively sparse response of the output neurons. Lateral projections of odor-activated periglomerular cells, some of which are inhibitory, would inhibit mitral cells associated with neighboring glomeruli. Albeit low, the numbers of labeled mitral cells we observe are close to what one would expect if only those cells associated with maximally activated glomeruli exhibit increased expression. Mitral cells extend a primary dendrite into a single glomerulus and in the weanling rat, about 15 mitral cells are associated with each glomerulus (30). This estimate predicts that for each "activated" glomerulus, 1–3 mitral cells would exhibit elevated c-*fos* mRNA expression within a given histological section, which agrees with the pattern of labeling we observe.

Periglomerular, tufted, and mitral cells receive direct olfactory nerve input, whereas granule cells do not. Therefore, odor-induced increases in c-*fos* mRNA in granule cells indicate transynaptic stimulation via the secondary dendrites of tufted/mitral cells. The dimensions of the labeled granule cell field likely reflect the area encompassed by these obliquely projecting basal dendrites, although centrifugal afferents may also contribute to the pattern of activity in this lamina. As the ratio of granule to mitral cells is about 50–100:1, the activation of even a small number of mitral cells would lead to the stimulation of large numbers of granule cells and consequent inhibition of neighboring output neurons (1). Physiological studies have directly demonstrated this spatially restricted inhibition of mitral cells with odor stimulation (31).

The rapid and transient increase in c-*fos* mRNA levels in olfactory bulb following odor exposure is similar to that reported for other brain regions following various forms of

electrophysiological activity (32, 33). Increases in bulb c-*fos* mRNA levels are detected as early as 15 min after odor stimulus onset and decay to near basal levels by 1 hr. Other investigators have reported that MK801 attenuates synaptic activation of immediate-early gene expression (17, 18), and there is evidence that the mitral to granule cell synapse may utilize glutamatergic neurotransmission with an NMDA component (34–36). We find that MK801 increases rather than decreases c-*fos* mRNA expression by mitral, tufted, and granule cells, suggesting that this drug increases the physiological activity of these neurons. There is precedence for this result; MK801 increases Fos immunoreactivity in dorsal thalamic nuclei (37). Whether the present MK801 response reflects a direct action on olfactory bulb cells or changes in the activity of centrifugal afferents cannot be determined from the present results. Given the large bulbar response to MK801, its use does not allow us to resolve the normal role of NMDA receptor activation in odor-regulated c-*fos* expression.

Further studies are needed to determine if odor-induced changes in c-*fos* mRNA in behaving rats are normally accompanied by changes in the levels of Fos protein. However, it has been recently reported that in anesthetized rats given 2 hr of odor stimulation after 30 hr of continuous anesthesia, regional increases in Fos immunoreactivity can be localized to cells of the mitral, granule, and, to a lesser extent, glomerular layers (38). The functional consequences of c-*fos* mRNA induction in olfactory bulb neurons are not known, but the role of Fos protein as a transcriptional regulatory factor suggests that odors may regulate other genes in these cells via a c-*fos* mechanism. In combination with other immediate-early gene products, Fos binds the DNA sequence known as the AP-1 consensus site and, thereby, can alter the transcription of other, phenotype-specific target genes (39). Genes that encode nerve growth factor (NGF) (40), proenkephalin (41), and tyrosine hydroxylase (TH) (42) are among those thought to be regulated, at least in part, by the Fos/AP-1 mechanism. Many glomerular layer neurons express TH and regulation of this expression by olfactory input has been well documented (43). Neurons in the glomerular and granule cell layers express preproenkephalin mRNA (44), and NGF mRNA has been localized to glomerular layer neurons (45). It will be interesting to determine if odor-stimulated increases in c-*fos* mRNA in the alert rat are linked similarly to topographic changes in the expression of these target genes.

The present findings demonstrate the utility of using c-*fos* mRNA expression for defining the composite odor response of the olfactory bulb in behaving rats in terms of the neurons involved and their regional distribution. The demonstration that odors activate radially aligned, heterogeneous neuronal arrays that appear similar in regard to constituent neuronal types and translaminar dimensions, regardless of the particular odor presented, illustrates the presence of a basic anatomical/functional unit of odor representation within the olfactory bulb. Using the techniques employed here it should be possible to study how this unit responds to changes in odor intensity and duration and to determine the effects that behavioral circumstances, such as olfactory learning, have on its operation.

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