

# Development and Further Characterization of a Small Subclass of Rat Olfactory Receptor Neurons That Shows Immunoreactivity for the HSP70 Heat Shock Protein

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## ABSTRACT

We previously described a rat olfactory receptor neuron (ORN) subpopulation [the 2A4(+) ORNs] that shows uniquely strong reactivity with antibodies to the 70-kD heat shock protein (HSP70) family of molecular chaperones (Carr et al. [1994] *J. Comp. Neurol.* 348:150-160). The 2A4(+)ORNs are dispersed through zones II-IV of the olfactory epithelium (OE), and their axons project to only two or three glomeruli that are located consistently in each olfactory bulb (OB). To date, the 2A4(+)ORN subpopulation is the only cell population to show such distinct HSP70 immunoreactivity as well as the most discrete ORN subpopulation to be so labeled. The present report shows that 2A4(+)ORN neurons first appear between postnatal days 7 (P7) and P10. Initially, low cell numbers rise to a density of 0.1 2A4(+)ORNs/mm OE length by P14, plateau at 0.9 2A4(+)ORNs/mm by P49, then fall to adult values of 0.4 cells/mm. Autoradiographic birthdating indicates that almost all of these early appearing 2A4(+)ORNs are generated postnatally, in contrast to the prenatal generation of all ORN subpopulations characterized to date by their expression of olfactory receptor protein mRNAs. A developmentally related increase in the mean depth of 2A4(+)ORNs within the OE also occurs. In the OB, initial 2A4(+)axonal projections are to only two or three glomeruli, as in adults. Slight but significant rostral shifts in (+)glomerular location occur with development. The 2A4(+)ORN immunoreactivity was found to be due to expression of HSP70, the dominant stress-inducible member of the HSP70 family, rather than constitutively expressed HSC70. In addition, despite their presence in rat OE, no 2A4(+)ORNs were found in mice, gerbils, guinea pigs, or hamsters. *J. Comp. Neurol.* 404:375-386, 1999. © 1999 Wiley-Liss, Inc.

**Indexing terms:** stress proteins; olfactory epithelium; olfactory bulb; olfactory neurogenesis; immunohistochemistry

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We have previously described a unique subpopulation of olfactory receptor neurons (ORNs) in rat that is reactive with several antibodies directed to the human 70-kD heat shock protein (HSP) family (Carr et al., 1994). For one of these antibodies, monoclonal antibody 2A4 (Mab 2A4), immunoreactivity (IR) was shown to occur throughout the ORN cytoplasm, from the dendritic knob to the axonal termination in glomeruli in the olfactory bulb (OB). The 2A4 IR appears to be unique: To date, we are unaware of reports of any other neuronal subpopulation showing a similar HSP70-based identity. Moreover, within the olfactory system itself, only the ORN subpopulations character-

ized on the basis of their expression of mRNAs for the putative olfactory receptor proteins (Strotmann et al., 1992, 1994a,b; Ressler et al., 1993, 1994; Vassar et al., 1993, 1994) show similarly discrete labeling characteris-

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tics as the 2A4(+)ORNs. No other immunologically identified ORNs have been found to do so.

The 2A4(+)ORNs are scattered randomly throughout the olfactory epithelium (OE) that lines the nasal cavity except for that lining the dorsal half of the septum, the dorsal recess, and the medial aspect of the first endoturbinates (nomenclature of Liebich, 1975). Their location thus essentially corresponds with zones II–IV of the four-zone subdivision of the OE based on the distribution of putative olfactory receptor genes (Strotmann et al., 1992, 1994a,b; Ressler et al., 1993, 1994; Vassar et al., 1993, 1994). Centrally, 2A4(+)axons project to only two or three glomeruli, which are located at distinct locations, in each OB. Projection pathways from the OE to the OB are consistent and predictable from animal to animal.

Heat shock or stress proteins are cellular proteins the expression of which is up-regulated greatly following exposure of cells to a wide variety of physiological stresses, including heat shock, oxidative stress, and exposure to heavy metals; their expression is essential for survival of such conditions (see, e.g., Li and Werb, 1982; Li and Laszlo, 1985; Johnson and Kucey, 1988; Riabowol et al., 1988; Beckmann et al., 1992; Sato et al., 1996; for reviews, see Morimoto et al., 1990, 1994). However, stress proteins also play a major role as molecular chaperones in normal, ongoing cellular activities, such as protein folding and translocation and assembly of oligomeric complexes (see, e.g., Chirico et al., 1988; Deshaies et al., 1988; Beckmann et al., 1990; Black et al., 1991; Hightower, 1991; Agard, 1993; Craig, 1993; Welch, 1993; Rassow et al., 1995; Freeman and Morimoto, 1996; Hartl, 1996; Liang and MacRae, 1997).

The HSP70 family of heat shock proteins consists of multiple members that show various degrees of stress inducibility, ranging from strictly stress-inducible expression to largely constitutive expression. We demonstrated previously that, in the rat OE, two members of this HSP70 family show reactivity to monoclonal antibody (Mab) 2A4: HSP70, the dominant stress-inducible member of the HSP70 family, and the constitutively expressed HSC70 (Carr et al., 1994). Our observations also led us to conclude that the 2A4 IR we saw was not stress induced. Rather, it seemed most likely that 2A4 IR in the rat OE reflects the presence of some unique protein or situation in the 2A4(+)ORN subpopulation that requires the molecular chaperone function of either the HSP70, or HSC70, or both. Because of its constitutive expression, HSC70 was presumed the most likely candidate.

In the present study, we examined the development and maturation of the 2A4(+)ORN subpopulation and its bulbar innervation. This is the first such discrete ORN subpopulation to be followed through the entirety of its development. We also undertook initial efforts to characterize the nature of the 2A4 IR by using antibodies specific to either HSP70 or HSC70 or to HSP25 or HSP90 as well as by examining the species distribution of the 2A4(+)ORN subpopulation. Given the known role of the HSP70 family as molecular chaperones, our findings again point to the highly unusual nature of this ORN subpopulation. Portions of this work have appeared previously in abstract form (Carr and Farbman, 1995a,b; 1997) and in an extended abstract (Carr and Farbman, 1998).

## MATERIALS AND METHODS

### Histological preparation

Sprague Dawley rats (Harlan, Indianapolis, IN), from postnatal [postnatal day 1 (P1) = day of birth] to adult ages, were deeply anesthetized with sodium pentobarbital (50–100 mg/kg) and perfused transcardially with phosphate-buffered saline (PBS), pH 7.0, followed by Bouin's fixative. Embryonic rats were obtained from deeply anesthetized mothers. Embryonic day 14 (E14) and E18 rats (E1 = day when dam is sperm positive) were fixed by immersion in Bouin's; E21 pups were perfused by transcardial perfusion as described above. All procedures were approved by the Animal Care and Use Committee of Northwestern University.

Heads were trimmed of lower jaws and, with increasing animal age, of teeth, soft palates, skin, and muscle, as needed. Trimmed heads were stored overnight in Bouin's at 4°C. Heads were then rinsed extensively in 50% ethanol followed by running water, decalcified in RDO (Apex Engineering, Plainfield, IL), rinsed again in running water, dehydrated in increasing concentrations of ethanol, cleared in Histosol (National Diagnostics, Atlanta, GA), embedded in Paraplast, and sectioned coronally through the OE and the OB at 10 µm. Sections were mounted on silanated (for OE) or albumen-coated (for OB) slides.

In addition, three to four adults from each of several other species were examined to determine whether the 2A4(+)ORN subpopulation might occur in species other than rat. The additional species were Mongolian gerbils, POC-DH guinea pigs, and Swiss Webster mice. All animals were obtained from Harlan. All were anesthetized, perfused, and processed for immunohistology in the same manner as the adult rats. The nasal region of one Bouin's-perfused Lak:LVG Golden hamster (Charles River Lakeview, Wilmington, MA; generously provided by Dr. R. Costanzo, Virginia Commonwealth University) was processed similarly.

### Immunohistochemical procedures

Immunoperoxidase procedures were carried out by using the avidin-biotin complex (ABC) Elite kit (Vector Laboratories, Burlingame, CA) as directed, with diaminobenzidine serving as the chromophore. Mab 2A4 was diluted from 1:25 to 1:100 with the blocking solution provided in the kit. Fast Green FCF served as a counterstain. Substitution of PBS for the primary antibody in this and all immunohistochemical procedures served as a control for specific immunoreactivity.

To further characterize the nature of the 2A4 IR, sections through rat nasal cavities were probed with several additional antibodies directed specifically to either the strictly inducible HSP70 or the constitutive HSC70 members of the HSP70 family (Table 1). The specificity of all antibodies in rat OE was confirmed by two-dimensional gel electrophoresis and subsequent Western blotting. Similar confirmatory results have been reported recently for rat brain by Manzerra et al. (1997). Immunoperoxidase procedures were carried out with the appropriate species ABC Elite kit as described above. Sections through rat eyes served as positive controls for these antibodies (Tytell, 1994).

Some sections also were processed by double-labeling immunofluorescent procedures for Mab 2A4 and some of the additional  $\alpha$ -HSP70 antibodies to verify that all re-

TABLE 1. Summary of Antibodies Tested

Antibody	Source species and type	Immunogen species	Dilution
$\alpha$ -HSP70 family 2A4	Mouse Mab	Human	1:25–1:100
Specifically $\alpha$ -HSP70			
Santa Cruz <sup>2</sup>	Goat Pab <sup>1</sup>	Human	1:200
StressGen <sup>3</sup>	Mouse Mab	Human	1:50–1:200
Tanguay 799 <sup>4</sup>	Rabbit Pab	Human	1:1,000–1:2,000
Tanguay 971	Rabbit Pab	Human	1:1,000–1:2,000
Specifically $\alpha$ -HSC70			
Santa Cruz	Goat Pab	Human/mouse	1:25–1:100
StressGen	Rat Mab	Hamster	1:50–1:200
Tanguay 1477	Rabbit Pab	Human	1:500–1:2,000

<sup>1</sup>HSP, heat shock protein; Pab, polyclonal antibody; Mab, monoclonal antibody.

<sup>2</sup>Obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

<sup>3</sup>Obtained from StressGen (Victoria, British Columbia, Canada).

<sup>4</sup>Antibodies 799, 971, and 1477 generously provided by Dr. R. Tanguay (Université Laval; Ste. Foy, Quebec, Canada).

acted with the same ORN subpopulation. For such studies, incubation with the first primary antibody was followed by incubation with the appropriate secondary antibody coupled to one fluorophore. This antibody pair was then followed sequentially by exposure to the second primary antibody and then to its appropriate secondary antibody coupled to a different fluorophore. Because of the lower sensitivity of the immunofluorescence procedures, all primary antibodies were used at four times the concentration required for the immunoperoxidase procedures. Dilutions were in PBS containing 1.5% bovine serum albumen. Donkey- $\alpha$ -mouse indocarbocyanine [Cy3; immunoglobulin G (IgG), 1:200–1:800; Jackson Immunoresearch Laboratories, West Grove, PA] served as the secondary antibody for Mab 2A4, rabbit  $\alpha$ -goat-fluorescein isothiocyanate (FITC; IgG, 1:50; Cappel Laboratories, Durham, NC) served as the secondary antibody for the Santa Cruz  $\alpha$ -HSP70, and goat  $\alpha$ -rabbit (IgG, 1:30; Cappel Laboratories) served as the secondary antibody for polyclonal antibodies (Pabs) 791 and 977.

In addition, a few sections were also examined for the presence of IR for other heat shock proteins. Antibodies used included rabbit Pabs to HSP25 (StressGen, Vancouver, British Columbia, Canada) and to HSP90 (generously provided by Dr. B. Freeman, University of California, San Francisco). These were used at dilutions of 1:25 and 1:500, respectively.

### Determination of 2A4(+)ORN density within the OE

The density of 2A4(+)ORNs within the OE was determined at weekly intervals from P14 through P63 as well as in adult rats. For these studies, the length of OE around the nasal cavity was measured in each of ten coronal sections at five locations distributed through the anterior-posterior extent of the OE by using the ONCOR V150 image-analysis system (Gaithersburg, MD). The selected locations corresponded with regions 2–6 of Weiler and Farbman (1997), extending from the area where the first and second ectoturbinates and the second endoturbinates are fully visible to the area where the fourth endoturbinates fuse with the dorsal portion of the nasal cavity. The two sections at each location were separated by at least 20  $\mu$ m and served as mutual controls. The number of 2A4(+)ORNs was determined for each section, and the overall density of 2A4(+)ORNs was determined by dividing the total number of 2A4(+)ORNs in all ten sections by the total length of OE summed from these sections. Six to nine animals were examined at each age.

### Basal-apical location of 2A4(+)ORNs within the OE

To determine the distribution of depths within the OE at which 2A4(+)ORNs cell bodies are located at different postnatal ages, the distances of the centers of 2A4(+)ORN nuclei from the OE basement membrane were measured and expressed as percentages of the total OE width at the location where each measurement was made. Measurements were made with a calibrated eyepiece micrometer at a total magnification of  $\times 400$ . The total OE width was taken to be the distance between the basement membrane and the top of the dendritic knob layer. For all 2A4(+)ORNs measured, the nucleus was clearly visible, and the ORNs themselves were situated in intact OE. Measurements were made in three animals each at P21, P28, P35, P42, and P63 as well as in adults. From 10 to 30 cells were examined in each of five sections distributed through the nasal cavity from each animal, as described above, giving a total of 200–350 ORNs measured per age. At P14, five pups were examined for a total of 76 cells.

### Autoradiographic procedures

For autoradiographic birthdating studies of 2A4(+)ORNs, rats were given three doses of tritiated thymidine ( $^3\text{H}$ -TdR; 20–40 Ci/mM; ICN Radiochemicals, Irvine, CA), 2  $\mu$ Ci/g body weight, i.p., at 8-hour intervals. For prenatal delivery, the isotope was given to the dams in the same per weight dosage. Pups were then perfused at P21, and tissues were sectioned and processed for 2A4 immunoperoxidase reactivity as described above. Slides bearing 2A4-treated sections were coated with NTB2 emulsion (Eastman Kodak, Rochester, NY), diluted 1:3 with distilled water, and air-dried. Autoradiographs were developed after a 3-week exposure, then counterstained, and cover-slipped as usual. Six pups were examined for each isotope delivery age. Ten sections distributed through the rostral-caudal extent of the nasal cavity, as described above, were examined per animal. A minimum of four silver grains was required to overlay the nucleus of any ORN for it to be counted as autoradiographically labeled.

### Location of 2A4(+)glomeruli within the OB

To determine the locations of glomeruli containing 2A4(+)ORN axons at different developmental ages, every third coronal section through the entire rostral-caudal extent of each OB was mounted on albumen-coated slides and processed for 2A4(+) immunoperoxidase histochemistry. Albumen-coated rather than silanated slides were used because, in our experience, brain tissue adheres noticeably better to the albumen-coated slides and because, given the relatively small extent of each glomerulus, it was essential that no sections be lost. The locations of 2A4-reactive glomeruli within an OB were noted and expressed as a percentage of the rostral-caudal extent of the OB.

### Image processing

Image processing was carried out with Adobe Photoshop 4.0 software (Adobe Systems, Mountain View, CA). For composite figures (Figs. 1, 5), which show developmentally related changes in immunoreactivity levels, all necessary contrast and brightness adjustments were made on the composite image rather than selectively on individual component images to maintain the relative degrees of staining intensities for the different ages depicted.

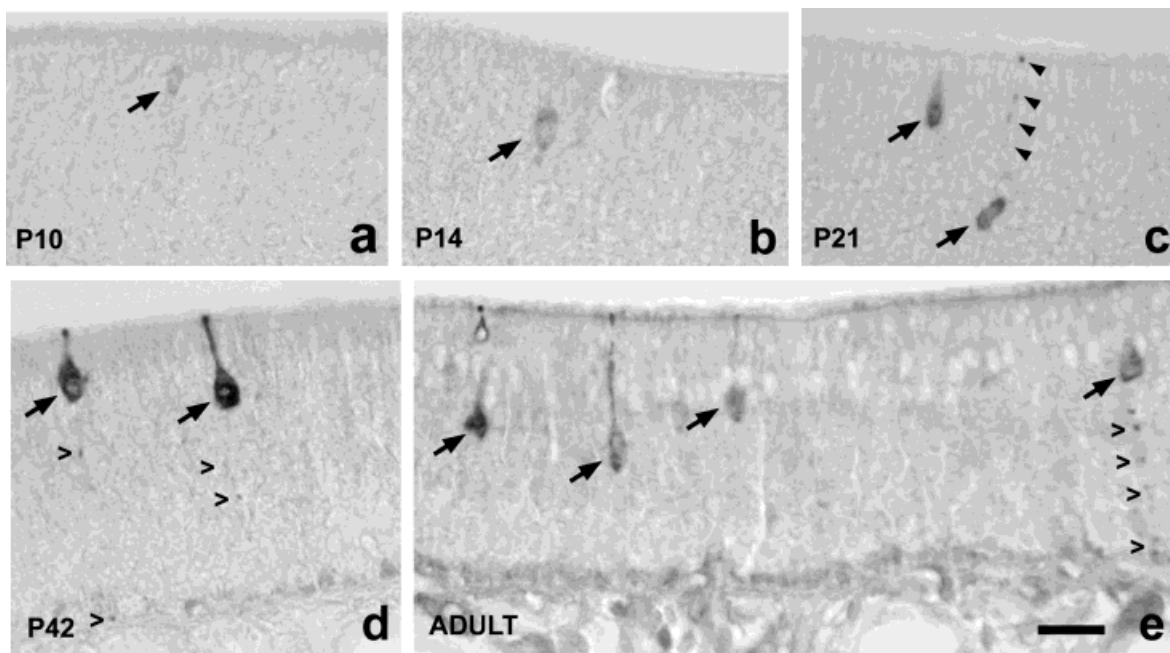


Fig. 1. Immunoperoxidase staining of monoclonal antibody (Mab) 2A4(+) olfactory receptor neurons (ORNs) in frontal sections through the olfactory epithelium (OE) of rats at various stages of development from postnatal day 10 (P10) to adulthood. Note the increasing intensity of immunoreactivity through P42. The 2A4(+) cell bodies are

indicated by arrows in a–e. The lower arrow in c indicates two juxtaposed positive cell bodies. Dendrites (indicated in c by arrowheads) can be seen projecting to the apical surface in c–e. Basally directed axons are indicated in d and e by open arrowheads. Scale bar = 10  $\mu$ m.

## RESULTS

### Development of the 2A4(+)ORN subpopulation

**Immunohistochemical studies.** 2A4(+)ORNs first appear in the rat OE postnatally, between P7 and P10 (Fig. 1). Immunoreactivity is relatively faint, and the reactive ORNs are scattered and are very few in number (e.g., five cells in a total of 20 sections from one P10 pup). This postnatal initial appearance of the 2A4(+)ORN subpopulation is relatively late compared with the midembryonic appearance of the putative olfactory receptor-based ORN subpopulations studies to date (Strotmann et al., 1995; Sullivan et al., 1995). By P14, the 2A4(+)ORN density within the OE begins to rise (Fig. 2). The average 2A4(ORN) density at P14 is 0.11 2A4(+)ORNs/mm, with values in individual rats ranging from 0.02 to 0.39 2A4(+)ORNs/mm [representing counts of 5–95 2A4(+)ORNs in 10 sections]. Densities continue to increase through P49, when the average value reaches 0.86 2A4(+)ORNs/mm [range, 423–928 2A4(+)ORNs in 10 sections]. Immunoreactivities increase during this time as well (Fig. 1). Densities then decline to an average adult density of 0.42 2A4(+)ORNs/mm [range, 177–452 2A4(+)ORNs in 10 sections].

At each age examined, reactive ORNs were found throughout the OE except in the dorsal half of the septum, the dorsal recess, and the medial aspect of the first ectoturbinate (Fig. 3). They also occurred only rarely on the medial aspect of the second endoturbinate. Thus, similar to the expression of the putative olfactory receptor messages in situ (Strotmann et al., 1995; Sullivan et al., 1995), initial 2A4(+)ORN distribution in the OE is the same as that in adults (Carr et al., 1994), i.e., in zones

II–IV (Ressler et al., 1993, 1994; Vassar et al., 1993, 1994) but not in region I.

**Basal-apical location of 2A4(+)ORN nuclei within the OE.** A developmentally associated change also occurs in the laminar distribution of 2A4(+)ORN cell bodies within the OE. At the earliest times of their presence in the OE, a striking proportion of the 2A4(+)ORN cell bodies is located quite apically and shows fairly short dendrites (Fig. 1a). However, with continued development, some 2A4(+)ORN cell bodies become situated more basally as well (Fig. 1c,e). Quantitative determination of 2A4(+)ORN nuclear depth within the OE (Table 2) corroborates these visual observations. Mean adult 2A4(+)ORN nuclear location was found to be 63.9% of the basal-to-apical OE width, confirming our previous visual observations that 2A4(+)ORNs tend to be situated in the apical half of the OE (Carr et al., 1994). In contrast, postnatal and juvenile rats show mean nuclear 2A4(+)ORN locations of 73–77% of this width, significantly more apical than that of adults (Mann-Whitney U test; one-tailed). This location appears to begin to shift basally to 68% of the basal-to-apical width between P42 and P63. Further examination was not carried out after P63.

### Autoradiographic birthdating studies

Because the postnatal initial appearance of the 2A4(+)ORN subpopulation is so late compared with the appearance of the putative olfactory receptor-based subpopulations (Strotmann et al., 1995; Sullivan et al., 1995), the question arose as to whether the 2A4(+)ORNs indeed were indeed generated postnatally and expressed the 2A4 epitope concomitantly with their neuronal development or whether these neurons were “born” prenatally but did not

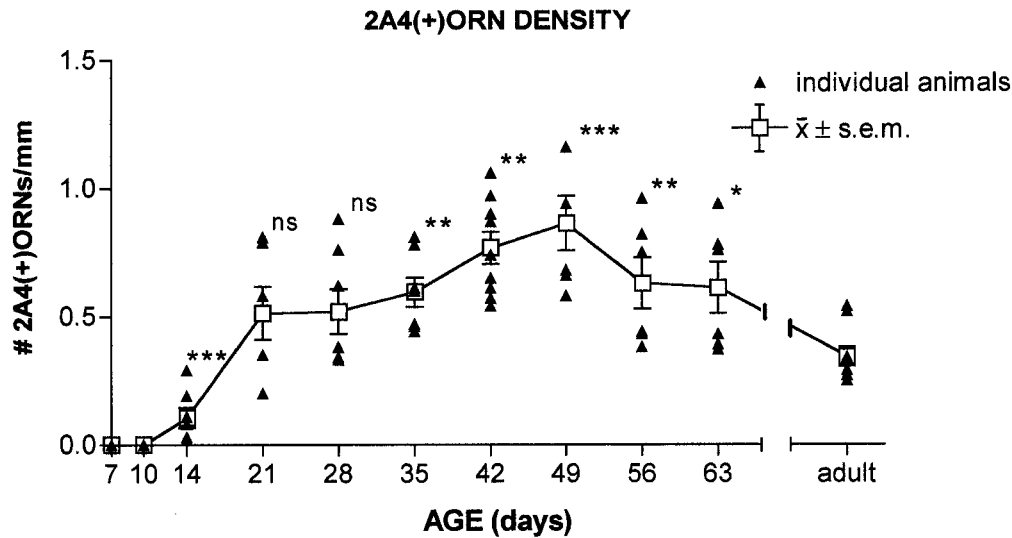


Fig. 2. Density of 2A4(+)ORNs in the OE at different days of postnatal development. Single asterisk,  $0.05 > P > 0.01$ ; double asterisks,  $0.01 > P > 0.001$ ; triple asterisks,  $P < 0.001$ ; ns, no significant difference compared with adults (unpaired t test).

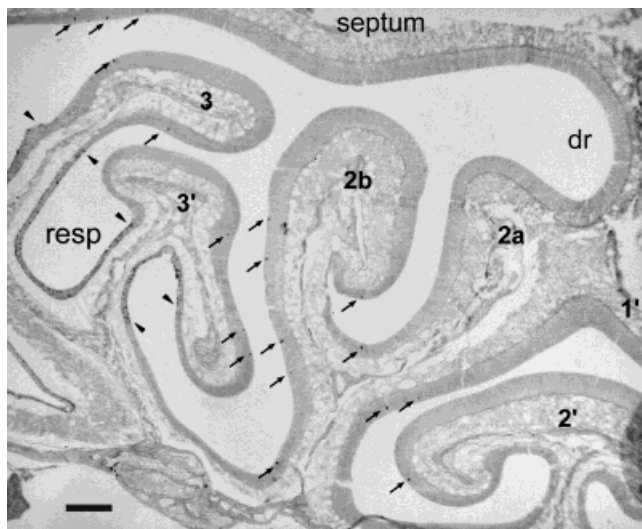


Fig. 3. Low-magnification photomicrograph of a frontal section through the nasal cavity of a P21 rat pup showing the distribution of all 2A4(+)ORNs (arrows) within the OE (dorsal is to the right, ventral is to the left). Higher density of reactive neurons in the ventral septum and more ventral and lateral aspects of turbinates is obvious. dr, dorsal recess; 1'-3', first through third ectoturbinates, respectively; 2a, 2b, dorsal and ventral arms, respectively, of the second endoturbinates; 3, third endoturbinates. The contrast has been digitally enhanced in an effort to show more clearly the 2A4(+)ORNs. Consequently, characteristic immunoreactivity in the respiratory epithelium (resp) also is apparent; its extent is delimited by arrowheads. Scale bar = 200  $\mu$ m.

express the 2A4 epitope until postnatal life. To address this question, [ $H^3$ ]-TdR autoradiographic birthdating studies were carried out in P21 pups. P21 is the first age at which a sufficient number of 2A4(+)ORNs are present to provide meaningful data. Isotope injections were given at E17, E21, P3, P7, P10, and P14; the pups were killed at P21. Results show (Fig. 4) that relatively few of the P21 2A4(+)ORNs become labeled with prenatal isotope deliv-

TABLE 2. Mean  $\pm$  S.E.M. and Median Basal-Apical Locations of 2A4(+) Olfactory Receptor Neuron Cell Bodies Within Olfactory Epithelia During Postnatal Development<sup>1</sup>

Age	Percent ( $\pm$ S.E.M.)	Median (%)	P*
P14	73.0 $\pm$ 1.7	76.5	0.039
P21	72.4 $\pm$ 0.8	73.0	<0.0001
P28	75.6 $\pm$ 0.5	77.0	<0.0001
P35	76.6 $\pm$ 0.5	79.0	<0.0001
P42	72.1 $\pm$ 0.7	74.0	<0.0001
P63	68.2 $\pm$ 0.7	70.0	0.002
Adult	63.9 $\pm$ 0.9	66.0	—

<sup>1</sup>Basal-apical locations are expressed as the percent of distance from the basal lamina to the top of dendritic knobs. Means are included to provide S.E.M. Medians are provided because they are the basis for the Mann-Whitney test. P, postnatal day.

\*One-tailed Mann-Whitney U test (based on median values) compared with adult. This test was used because the standard deviations for the mean values at the different ages were not all equal, an assumption of the Student's t test.

ery but that a large increase in labeling occurs postnatally, such that approximately 60% of the P21 2A4(+)ORNs become labeled between P3 and P10. Thus, the vast majority of the 2A4(+)ORNs present at P21 are indeed "born" postnatally and express the 2A4 epitope as they mature.

### Developmental appearance of 2A4(+)glomeruli within the OB during development

To determine when 2A4-reactive glomeruli first appear within the OB and whether the pattern and location of innervated glomeruli might change during development, every third coronal section through the entire rostral-caudal extent of each OB was examined for 2A4(+)glomeruli and axons. From 6 to 18 OBs from three to nine animals at each of the weekly intervals from P14 to P42 and from adults were examined. Results show (Fig. 5, Table 3) that the appearance of reactive glomeruli parallels the emergence of the 2A4(+)ORNs in the OE as axons from the increasing numbers of 2A4(+)ORNs converge on the OBs. Glomeruli-bearing 2A4(+)axons can be seen first at P14 but, at this age, show only faint IR and occur in only about half of the animals examined. Their size does not

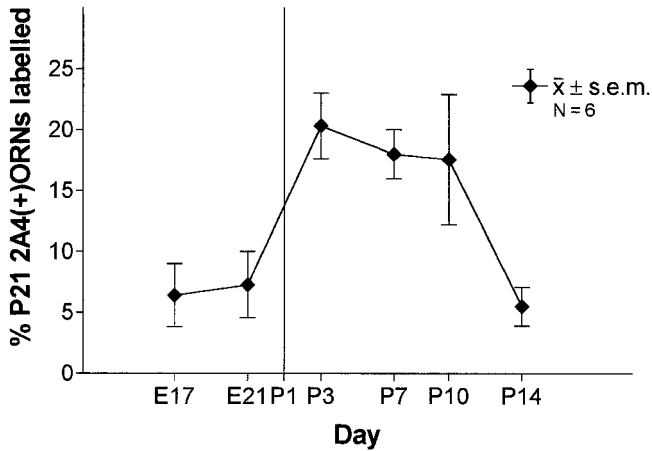


Fig. 4. The percentage of 2A4(+) ORNs present at P21 that became labeled autoradiographically by tritiated thymidine administered at the indicated times.

appear smaller than that of neighboring glomeruli. With further development, the proportion of pups showing reactive glomeruli increases; after P21 they occur in all animals examined. Intensity of glomerular 2A4 IR increases as well. There is also a shift from the situation at P14, in which innervating 2A4(+) axons generally appear dispersed throughout a target glomerulus, to the adult situation, in which innervating 2A4(+) axons often appear compartmentalized within smaller regions of target glomeruli. However, the degree of such compartmentalization varies between animals (compare Fig. 5g with Fig. 5h).

Initial relative locations of both the lateroventral (LV) and the medioventral (MV) 2A4(+) glomeruli were found to lie significantly caudal to their relative adult locations (Fig. 6, Table 3). This is especially obvious for the LV glomeruli, which showed a rostral shift from a mean location at 24% of the anterior-posterior length of the OB at P14 to the mean adult location at approximately 16% of anterior-posterior OB length. The MV (+) glomeruli showed only a shift from 40% to 35% of the OB length. However, there appears to be more variability in the location of individual MV (+) than LV (+) glomeruli during the course of development. This may be reflected in the patterns of significance shown in Figure 6.

Table 3 also indicates that in pups, as in adults, the 2A4(+) axons occasionally project to two, rather than one, LV or MV glomeruli in a bulb. In such cases, the two glomeruli often appear to be slightly smaller than a normal, single 2A4(+) glomerulus. Similar observations of occasional multiple glomeruli have also been made for using putative receptor-based ORN subpopulations (Ressler et al., 1994; Mombaerts et al., 1996).

### Further characterization of the 2A4(+) ORNs

**Reactivity with HSP70- and HSC70-specific antibodies.** We showed previously that, in the rat OE, Mab 2A4 reacts with both the inducible HSP70 and the constitutively expressed HSC70 members of the HSP70 family (Carr et al., 1994). To start to analyze the nature of the 2A4 IR in the reactive ORNs, sections of adult OE were reacted with antibodies specific to either HSP70 or HSC70. Immunoperoxidase techniques revealed numerous ORNs reactive with the  $\alpha$ -HSP70 antibodies obtained from all sources.

Reactive ORNs were scattered through the OE in a pattern similar to that seen with Mab 2A4. Double-labeling immunofluorescence studies with all  $\alpha$ -HSP70 antibodies showed that every 2A4(+) ORN was also HSP70(+) and that every HSP70(+) ORN was also 2A4(+) (Fig. 7). In contrast, no intensely labeled ORN subpopulation was observed with any of the HSC70 antibodies despite the presence of strong  $\alpha$ -HSC70 IR in the retinal ganglion cells, the inner nuclear layer, and the inner segments of the rat eyes that were used as positive control tissue. Thus, the IR of the 2A4(+) ORN subpopulation is due specifically to HSP70 alone.

**Reactivity with HSP25- and HSP90-specific antibodies.** Neither HSP25-specific nor HSP90-specific antibodies showed IR in association with the 2A4(+) ORN subpopulation, again supporting the absence of general stressing conditions that could be responsible for the 2A4 IR in these ORNs. The  $\alpha$ -HSP25 IR in particular was examined, because constitutive expression of HSP25 has been seen recently in both sensory and motor neuron subpopulations in rat (Plumier et al., 1997) and because HSP25 has been associated with regulation of cellular levels of glutathione (Mehlen et al., 1995, 1996, 1997), a well-recognized participant in xenobiotic biotransformation and processing active in the OE (see, e.g., Kirstein et al., 1991; Rama Krishna et al., 1992; Starcevic and Zielinski, 1997). Although a few ORNs did show HSP25 IR similar to that seen with 2A4 (not shown), these were very few in number and were expressed inconsistently from animal to animal. HSP90, another cytosolic stress protein, which is recognized especially as a molecular chaperone of steroid aporeceptors and tubulin (see, e.g., Freeman and Morimoto, 1996; Williams and Nelson, 1997), showed no apparent constitutive neuronal expression within the OE.

### Species-dependent nature of 2A4(+) ORNs

Examination of sections through the nasal cavities of animals from species other than rat showed that none of the other species tested (mice, gerbil, guinea pig, or hamster) contained an ORN subpopulation with the labeling characteristics of the rat 2A4(+) ORN subpopulation. At most, only very occasional intensely 2A4-reactive ORNs, microvillar cells, or axons were observed in the gerbil and guinea pig sections. Mice and the single hamster tested showed no intensely 2A4-reactive ORNs whatsoever. Significantly, in each species, this apparent absence of a 2A4(+) ORN subpopulation occurred despite 2A4 labeling of a variety of other cell types in and around the nasal cavity that normally show 2A4 IR in rat (e.g. basal cells, respiratory epithelial cells; see Carr et al., 1994).

Fig. 5. a-h: Immunoperoxidase staining of glomeruli showing 2A4(+) axonal innervation in frontal sections through the olfactory bulb (OB) at various stages of development. The contrast has been enhanced to facilitate visualization of the 2A4 immunoreactivity (IR) at P14 and P21. Large arrows indicate 2A4(+) glomeruli. Small arrows indicate nonreactive glomeruli. e-g: Higher magnifications of the glomeruli shown in a, b, and d, respectively. The increasing IR intensity of 2A4(+) glomeruli with development is obvious with regard both to neighboring, nonreactive glomeruli and to 2A4(+) glomeruli in younger animals. Note the extreme degree of spatial compartmentalization of 2A4(+) axons in the adult glomerulus shown in h: Open arrowheads indicate several 2A4(+) axon fascicles within the reactive glomerulus. Such compartmentalization is not as obvious in the glomerulus shown in g. Arrowhead in g indicates aggregate of 2A4(+) axons in the OB olfactory nerve layer. Scale bar in h = 100  $\mu$ m in a-d, 25  $\mu$ m in e-h.

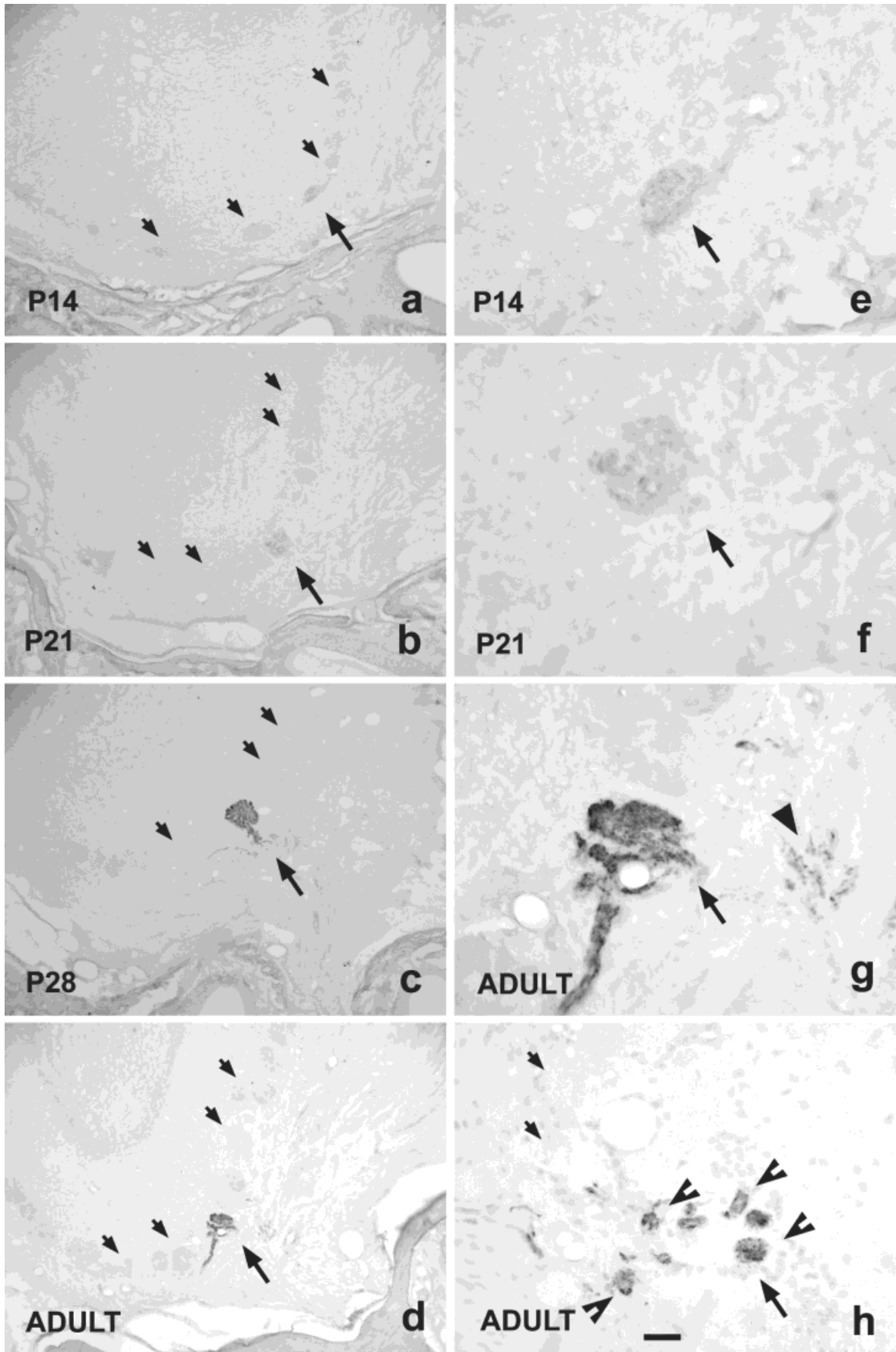


Figure 5

TABLE 3. Location of 2A4(+)Olfactor Receptor Neuron Target Glomeruli as a Percentage of Olfactory Bulb Anterior-Posterior Length

Age	Lateroventral			Medioventral		
	Left	Right	Mean	Left	Right	Mean
<i>P14</i>						
1	26	(Missing)	24	None	None	40
2	20	27		43	41	
3	24	24		36	38	
4	24	None		41	43	
<i>P21</i>						
1	20	21	21	45	41	42
2	21	35		52	52	
3	24	25		35	49	
4	14	14		32	34	
5	None	None		None	None	
6	22	19		29, 38 <sup>1</sup>	49	
<i>P28</i>						
1	21	19	20	42	40	38
2	18	22		37, 42 <sup>1</sup>	41	
3	19	18		37	37	
4	17	14		35	29, 32 <sup>1</sup>	
5	22	25		38	42	
6	19	24		41	41	
<i>P35</i>						
1	16	14	19	36	34	40
2	25	22		44	45	
3	15	16		36	36	
4	22	21		43	42	
<i>P42</i>						
1	24	17	18	41	39	39
2	22	15		41	42	
3	16	14		34	38	
<i>Adult</i>						
1	16	18	16	33	35	35
2	16	21		35	38	
3	17, 19 <sup>1</sup>	14		37	36	
4	15	14		34	35	
5	19	19		33	37	
6	13	13		38	34	

<sup>1</sup>Two small glomeruli were present. P, postnatal day.

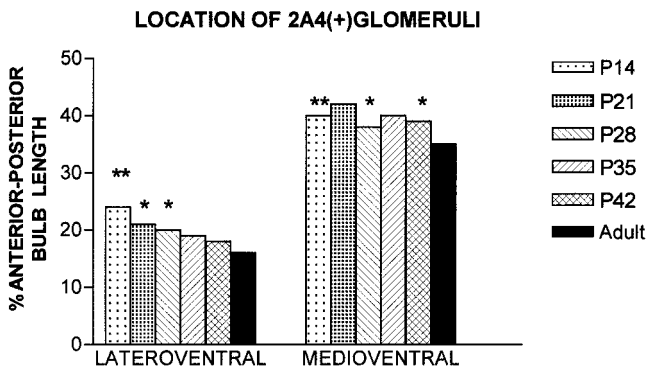


Fig. 6. The location of lateroventral and medioventral 2A4(+)glomeruli as a function of total anterior-posterior OB length at different postnatal ages. Single asterisks,  $0.05 > P > 0.01$ ; double asterisks,  $0.01 > P > 0.001$  (compared with adult values; Mann-Whitney U test).

## DISCUSSION

The observations reported above describe the initial appearance and development of the 2A4(+)ORN subpopulation of the rat OE. This is the only mammalian ORN subpopulation to date for which such a temporally extensive developmental study has been carried out. Of even greater significance, however, is the fact that the distinguishing characteristic of this subpopulation is its unique high level of constitutive expression of HSP70 IR. Given the extensively documented role of HSP70 as a molecular chaperone both in preventing stress-related protein misfolding and in normal protein biosynthesis, transport, and turnover, the presence of this degree of IR points to an

unusual nature of at least some aspect of the metabolism of this ORN subpopulation. Although the emphasis of the present paper is on the insights into ORN development provided by this uniquely reactive subpopulation, the ultimate goal for the studies must lie in understanding the basis for this uniqueness.

## Postnatal appearance of 2A4(+)ORNs

Our results show that the 2A4(+)ORNs are a postnatally appearing ORN subpopulation. This is in contrast to the embryonic initial appearance of those rodent ORN subpopulations characterized by their expression of putative olfactory receptor mRNAs that have been studied to date (Vassar et al., 1994; Strotmann et al., 1995; Sullivan et al., 1995). Those subpopulations first appear concomitantly with the earliest ORN neurogenesis. In addition, from the earliest time of expression, the proportion of the total ORN population comprised by each of the receptor-based subpopulations examined is about the same as in adults, 0.1–0.2% (Strotmann et al., 1994, 1995; Sullivan et al., 1995). Such consistent maintenance of this proportionality clearly is not the case for the 2A4(+)ORN subpopulation, in which neuronal numbers and density are exceedingly low at the time of first appearance but then rise rapidly.

Despite these differences, striking similarities do exist between the 2A4(+) and putative receptor-based ORN subpopulations. For both types of subpopulations, constituent ORNs are scattered through zonally limited regions of the OE. Both also project to only two or three glomeruli in each OB, and the locations of target glomeruli are relatively consistent from animal to animal. Moreover, odorant receptors have been shown to play an instructive role in specific axonal targeting to particular glomeruli (Mombaerts et al., 1996; Wang et al., 1998). Thus, it is highly likely that the 2A4(+)ORN subpopulation itself is associated with a unique, as yet unidentified, receptor protein.

Given this likely association plus the embryonic appearance of all rodent receptor-based subpopulations studied to date, it was necessary to determine whether the postnatally appearing 2A4(+)ORN subpopulation itself is actually generated embryonically but only expresses the 2A4 epitope postnatally. The autoradiographic birthdating studies clearly show that is not the case. Rather, of the 2A4(+)ORNs present at P21, the earliest age showing a sufficient density of these ORNs for such analysis, the vast majority are "born" postnatally. Only a small proportion arises embryonically. Obviously, animals younger than P21 would show a greater proportion of their extant 2A4(+)ORNs to have arisen before birth. However, so few 2A4(+)ORNs are present earlier than P21 that any prenatally generated 2A4(+)ORNs must quickly, and overwhelmingly, be eclipsed by the vastly more numerous, postnatally arising 2A4(+)ORNs. Finally, if the 2A4(+)ORN subpopulation were born embryonically, in the same short time frame as the receptor-based subpopulations, but with the 2A4 epitope appearing only later, in response to a developmentally related signal, then the initial appearance of 2A4 IR would seem likely to occur relatively synchronously throughout the entire subpopulation. This, again, is not the case. Rather, 2A4(+)ORN density increases over a fairly extended period (Fig. 2).

The postnatal generation of the 2A4(+)ORN subpopulation raises the possibilities that the birth of these ORNs is related to the demands of postnatal life in the rat and, consequently, that other discrete ORN subpopulations

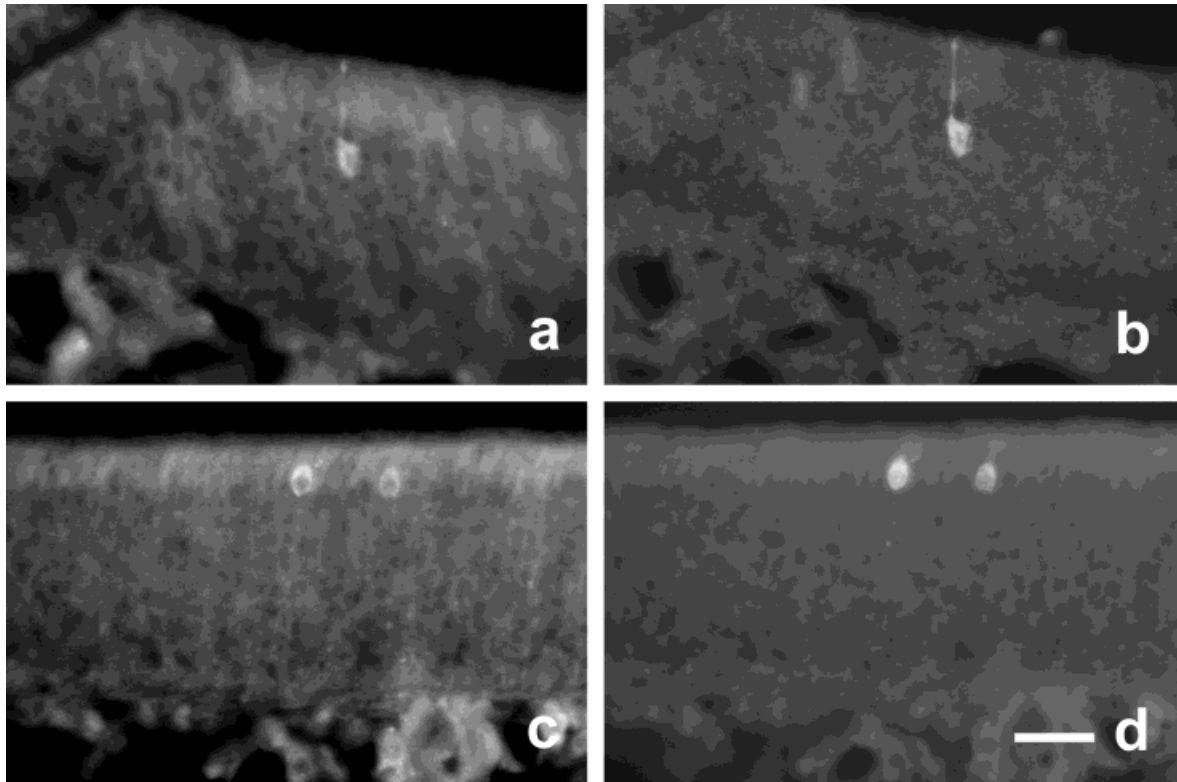


Fig. 7. Double-immunofluorescent preparations showing reactive ORNs. **a,b:** ORN incubated first with 2A4 and an indocarbocyanine (Cy3)-conjugated secondary antibody (a) and then with the Santa Cruz  $\alpha$ -heat shock protein 70 (HSP70) and a fluorescein isothiocyanate

(FITC)-conjugated secondary antibody (b). **c,d:** Pair of ORNs incubated first with Mab 2A4 and a Cy3-conjugated secondary antibody (c) and then with antibody  $\alpha$ -HSP70 polyclonal antibody 799 and an FITC-conjugated secondary antibody (d). Scale bar = 20  $\mu$ m.

may arise at distinct developmental stages as well. Although such possibilities appear to be at odds with the findings for the rodent receptor-based subpopulations, developmentally related changes in OR gene expression have been found for both chick (Nef et al., 1996) and zebra fish (Byrd et al., 1996). In the rodent studies, probably because early expression indeed was observed for all putative olfactory receptor genes examined, investigation of possible onset of receptor gene expression at later times, quite reasonably, was not undertaken. However, it is possible that, if development of a larger number of rodent receptor-based ORN subpopulations were to be examined, then a diversity in times of initial appearance, would, in fact, become manifest. Moreover, physiological studies indeed have demonstrated a developmentally associated increase in the specificity of ORN responses to odorants in rat (Gesteland et al., 1982), and such findings have yet to be correlated with a totally embryonic appearance of putative receptor messages. The existence of later appearing ORN subpopulations would resolve this discrepancy.

#### 2A4(+)ORN birthdating kinetics

A final point to be made from the birthdating studies comes from comparison of the labeling kinetics of 2A4(+)ORNs in P21 versus adult animals. Such a comparison shows that, at each postexposure period examined, the P21 values are sixfold higher than those of the comparable postexposure period in adults (cf. Carr et al., 1994). Thus, in the present study, the percentages of P21 2A4(+)ORNs labeled 1 week and 4 weeks after isotope delivery are

approximately 6%; the comparable values in adults are approximately 1%. Similarly, the labeling peak 18 days after isotope delivery is 20% at P21 but only 3.4% 21 days after isotope delivery in adults. These differences correspond exactly to those found by Weiler and Farbman (1997) for the ORN population as a whole.

#### 2A4(+)ORN density

The change that occurs in 2A4(+)ORN cell density, with values rising through P49 and then falling slowly into adulthood, is striking (Fig. 2). This pattern likely reflects two factors: first, the full complementation of the 2A4(+)ORN subpopulation and, second, the subsequent decline in overall ORN density after P40 described by Weiler and Farbman (1997). Thus, just as the number of 2A4(+)ORNs within the OE reaches its maximum, the overall ORN density begins to decline, and, with it, the 2A4(+)ORN density as well. Our findings, therefore, again not only corroborate those of Weiler and Farbman (1997) but extend them to a discrete ORN subpopulation as well.

#### 2A4(+)ORN location within the OE

The developmental shift we observe in the basal-apical location of 2A4(+)ORN cell bodies within the OE is interesting in light of the findings of Strotmann et al. (1996) of a laminar segregation of different receptor-based ORN subpopulations within the adult OE. Those investigators did not examine any developmental aspects of their observations. However, given our findings for the

2A4(+)ORN subpopulation, it would be of interest to determine whether the receptor-based subpopulations undergo similar shifts and, if they do, what factors might be responsible. It is also not known whether the shift we observe simply reflects a change in cell body location for individual ORNs or whether it reflects a later appearance either of greater ORN numbers more basally or of greater amounts of ORN death more apically. Whatever the cause, all of these findings, as Strotmann et al. (1996) point out, must be related to the well-known basal-to-apical migration of maturing ORNs (Moulton et al., 1970; Graziadei and Monti Graziadei, 1978; Caggiano et al., 1994).

A second observation by Strotmann et al. (1996) is that, at any particular OE depth, several ORNs expressing the same putative olfactory receptor gene often appear to occur in small linear arrays and that, within such arrays, neighboring ORNs appear to be separated by well-defined intervals. For the vast majority of 2A4(+)ORNs, however, such periodicity appears not to be the case. Rather, 2A4(+)ORNs usually occur individually, situated apparently randomly and often remotely from each other, as can be seen from Figure 1 in our previous report (Carr et al., 1994) and in Figure 1 in the present study. This apparently more random distribution of 2A4(+)ORNs within the OE likely reflects an apparently lower density of these ORNs than of the receptor-based subpopulations within their respective zones. This can be seen by comparing Figures 2 and 4 of Vassar et al. (1993) with ours cited above. More quantitatively, the 2A4(+)subpopulation is dispersed through three of the four zones of the OE rather than in just one, as is the case for most of the receptor-based subpopulations examined to date. Furthermore, each of the receptor-based subpopulations has been shown to comprise a similar proportion of the total ORN population, 0.1–0.2%, regardless of whether they are confined to a single zone, as is the majority, or confined to a more restricted region, as is the case for the OR37 subfamily (Strotmann et al., 1994, 1995; Sullivan et al., 1995; Kubick et al., 1997). If the 2A4(+)ORN subpopulation can be presumed to also contain this same proportion of the total ORNs, then distribution over three zones would indeed lead to a relatively lower 2A4(+)ORN density. Such decreased density could readily obscure any regular 2A4(+)ORN arrangement. The facts that the 2A4(+)ORNs project to the same proportion of the 2,000–3,000 glomeruli counted in each OB (Meisami, 1979; Meisami and Safari, 1981) as each receptor-based subpopulation studied to date and that this proportion is 0.1–0.15%, a value similar to that of the total ORN population comprised by each ORN subpopulation, may serve as circumstantial support for the above presumption of equivalent ORN subpopulation sizes.

A final point to be made concerning 2A4(+)ORN distribution within the OE concerns the association with nasal zones II–IV. These three zones have been correlated recently with the sites of expression of two other olfactory markers (Ring et al., 1997): One is recognized by Mab R4B12 of Mori et al. (1985), now shown to be a neural cell adhesion molecule-related homophilic adhesion glycoprotein (Yoshihara et al., 1997), and the other is the membrane-associated protein recognized by Mab RB-8 (Schwob and Gottlieb, 1986, 1988; for further discussion of other specific zonal features, see Menco and Jackson, 1997). This coincidence of expression raises the question of whether there might be a special function associated with these zones or,

conversely, with zone I alone. Again, this highlights the uniqueness of the 2A4(+)ORN subpopulation.

### Development of 2A4(+)ORN bulbar projections

The 2A4(+)axonal IR allows discernment of small reactive fascicles and even single axons, and we have used this IR to follow early innervation of the OB by 2A4(+)ORNs. Several points emerge. First, we find that even early appearing 2A4(+)ORN axons project directly to only two or three glomeruli and that these glomeruli are located consistently from animal to animal, as they are in adults. If 2A4(+)ORNs projected to larger numbers of glomeruli, then this would have been apparent in the numerous OB sections examined at P14 and P21. It was not. Only very rarely were even single reactive axons or axon bundles detected in glomeruli other than those receiving the major 2A4(+)axonal input. This developmental situation is in contrast to that seen after methyl bromide treatment and subsequent OE reconstitution (Carr et al., 1998). Under such reinnervation conditions, numerous 2A4(+)axons appear in glomeruli scattered throughout the ventral rostral bulb. Obviously, the developmental situation reflects at least partially the fact that, by the time of appearance of the 2A4(+)ORNs and their initial axonal outgrowth, the OE-OB projections already have been established for 2 weeks. Fasciculating 2A4(+)ORNs, thus, would grow to their correct targets along well-established routes. However, the same axonal targeting specificity to only two or three glomeruli is also seen for the receptor-based ORN subpopulations (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998), in which initial axonal outgrowth occurs early, when the OE-OB projection pathways themselves are just being established (Sullivan et al., 1995). Such observations argue for a certain degree of rigidity in this projection system (Sullivan et al., 1995; Mombaerts et al., 1996). Nevertheless, the not infrequent occurrence of three rather than two target glomeruli in both postnatal and adult OBs clearly indicates that a certain degree of flexibility is permitted in OE-OB innervation as well.

A second point concerns spatial compartmentalization of innervating ORN axons within a glomerulus. Recently, such apparent compartmentalization has been demonstrated in adult rat at the electron microscope level (Kasowski et al., 1996). The 2A4(+)axonal innervation patterns in at least some adults we have studied (Fig. 5h) show similar patterns. Other adults, however, show a more uniform filling of their 2A4(+)glomeruli (Fig. 5g). It is possible that this apparent uniformity would itself appear compartmentalized at the electron microscope level. In contrast, during the early period of 2A4(+)axonal bulb innervation, 2A4(+)axons routinely appear to fill an entire target glomerulus, albeit sparsely, rather than showing any spatial localization. Compartmentalization only becomes obvious later and, thus, may be a progressive process during OB development, most likely reflecting increased 2A4(+)axon numbers and increased axonal arborization (Klenoff and Greer, 1998) within a glomerulus. Although these observations need to be confirmed at the electron microscopic level, they could provide at least a partial basis for developmentally related refinement of olfactory function (Gesteland et al., 1982). Recent work by Klenoff and Greer (1998) has eliminated the likelihood that progressive ORN axonal pruning accounts for such functional refinement.

Third, it is not clear whether the earliest appearing 2A4(+)glomeruli are newly forming or not. Addition of glomeruli to the OB has been shown to continue through the first 3 weeks of the postnatal period in mice (LaMantia and Purves, 1989); presumably, the situation is similar in rats as well. However, if the 2A4(+)glomeruli are newly formed concomitantly with their innervation by 2A4(+)axons, then they might be expected to be smaller than previously established neighboring glomeruli. This was not observed. Rather, 2A4(+)glomeruli at P14 appear to be about the same size as their neighbors. Furthermore, any increases in 2A4(+)glomerular size during this postnatal period seem to parallel that of glomeruli in general.

It is noteworthy that the apparent existence of glomeruli to be innervated by 2A4(+)axons prior to this innervation and the compartmentalization of axons within a glomerulus both indicate that a given glomerulus may be innervated by more than one ORN subclass. This possibility would clearly have significant ramifications for olfactory functioning.

Finally, the rostral shift in the relative location of the (+)glomeruli during development almost certainly reflects OB growth during the postnatal period rather than an actual change in the specific glomeruli receiving 2A4(+)axons. The postnatal period is a time of much growth and addition of new glomeruli (Meisami, 1979; Brunjes and Frazier, 1986; LaMantia and Purves, 1989). The larger shift for the LV (+) than the more caudally situated MV (+)glomeruli would indicate that a significant portion of this bulbar growth occurs in the region between these two glomeruli. The greater degree of variability in MV than in LV glomerular location (Fig. 6, Table 3) could reflect such differential growth as well.

### Characterization of the 2A4(+)ORNs themselves

To begin to understand the nature of the 2A4(+)ORN subpopulation, we have examined the specificity of the 2A4 IR as well as the species distribution of this ORN subpopulation. Results show that the 2A4 ORN IR is due to the largely inducible HSP70 rather than to the constitutively expressed HSC70. Because the 2A4 IR is unlikely to be due to stress, per se, and because HSP70 is primarily a stress-inducible protein, this finding is the opposite of what was expected. Clearly, the 2A4(+)ORN subpopulation itself must have an unusual metabolic condition or express an unusual protein; the predominantly inducible HSP70 must be serving a chaperone function for such high levels to be expressed.

The surprising absence of 2A4(+)ORN subpopulations in the other rodent species examined further testifies to the unique nature of the 2A4(+)ORN subpopulation. It also provides additional circumstantial support for the HSP70 expression being unrelated to stress, per se, because all species examined were exposed to the same housing and laboratory conditions. Any condition responsible for stress-related induction of 2A4(+)ORN expression in rat would seem likely to have affected at least one of the other species examined as well.

In conclusion, our continued investigations of this rat ORN subpopulation with its constitutive expression of the largely inducible HSP70 protein have served to confirm the unique nature of this subpopulation. The questions raised by our observations are more than a little intriguing. We now need to determine how the HSP70 chaperone functions that are presumed to be responsible for the

uniquely high levels of  $\alpha$ -HSP70 IR in this population are related to its olfactory role and to whatever olfactory receptor protein it may express; why this subpopulation only appears postnatally and what postnatal conditions it addresses; whether the high HSP70 levels affect differentially the turnover of this subpopulation; and, ultimately, how all of these functions are related to the overall olfactory capabilities of the rat and the interaction of this species with its environment.

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