Brain targeting and glomerulus formation of two olfactory neuron populations expressing related receptor types

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Abstract

Olfactory sensory neurons expressing different members of the mOR37 odourant receptor subfamily send their axons to distinct glomeruli located in the immediate vicinity in the olfactory bulb [Strotmann, J., Conzelmann, S., Beck, A., Feinstein, P., Breer, H. & Mombaerts, P. (2000) J. Neurosci., 20, 6927–6938]. In this study, the potential of transgenic mouse lines was used to explore the onset of receptor expression, the outgrowth of axons as well as the glomerulus formation for two neuron populations expressing different mOR37 subtypes. The data indicate a synchronous time course of these features for both neuron populations. From E15 until the day of birth, the axons of the two mOR37 populations terminate in a common, small area of the presumptive olfactory bulb. During a short postnatal phase, the two axon populations segregate into distinct, protoglomerular structures; some aberrant fibers can still be observed during this period.

Introduction

Olfactory sensory neurons (OSNs) located in the epithelium lining the nasal cavity project their axons to contact dendrites of target cells in the olfactory bulb, thereby forming specialized globose neuropil structures called glomeruli. The observation that odourants elicit spatially defined patterns of glomerular activity in the bulb (Stewart et al., 1979; Jourdan et al., 1980; Lancet et al., 1982; Guthrie et al., 1993; Xu et al., 2000) has led to the hypothesis that axons from neurons expressing the same odourant receptor may converge onto common target glomeruli in the bulb. This concept has recently been confirmed by a transgenic approach which allowed the projection of distinct neuron populations to be visualized (Mombaerts et al., 1996; Wang et al., 1998). These studies have demonstrated directly that all neurons expressing receptor type P2 send their axons onto two glomeruli in each bulb, one on the medial side and one on the lateral side. Based on the number of receptor genes in rodents (~1000) (Buck, 1996) and the number of glomeruli in each bulb (~1800) (Royet et al., 1988) it has been proposed that each glomerulus may receive input from only one population of OSNs. However, results of recent studies indicate that heterogeneity may exist in the innervation pattern of individual glomeruli. In a transgenic mouse line with one subset of OSNs labelled by X-gal staining and another population labelled with the lectin Dolichos biflorus agglutinin (DBA), the olfactory bulb comprised glomeruli with both markers, suggesting that these glomeruli receive input from at least two neuron populations (Treloar et al., 1996). Similar results came from studies on P2-ires-tauLacZ mice, demonstrating that, in addition to their regular targets, some P2 axons innervate extra-glomeruli which apparently contain fibers from neuron populations expressing other receptor subtypes (Royal & Key, 1999). Moreover, during development an intermixing of P2 axons with fibers not originating from P2 neurons was observed in the presumptive olfactory bulb; the nature of these non-P2 fibers is elusive. The appearance of more than one axon population within a glomerulus raises the question whether this may be due to an imperfect targeting of axons during glomerulus formation. It is conceivable that axon populations, which ultimately project into neighbouring glomeruli, are intermingled before the anatomical structures of distinct glomeruli are formed.

We have recently shown that axons from OSNs expressing different members of the closely related mOR37-odourant receptor subfamily converge onto glomeruli which are located in immediate vicinity in the anterior/ventral region of the olfactory bulb (Strotmann et al., 2000). The time course of the wiring processes and the interplay between axon populations in finding the common target area in the bulb but projecting onto distinct glomeruli is largely unknown. In this study, the potential of the mouse lines was employed to examine the trajectory of axons during target finding as well as the timing of glomerulus formation for neuron populations which express highly related receptor subtypes and innervate neighbouring glomeruli in the olfactory bulb.

Materials and methods

Tissue preparation

Homozygous mOR37A-ITLZ (A-lacZ) and mOR37C-ITGFP (C-GFP) mice (Strotmann et al., 2000) were mated for 2 h and subsequently examined for a vaginal plug; 24 h later was dated E1.0; the day of birth was postnatal day 0 (PN0). Pregnant mice were deeply anaesthetised by CO 2 asphyxiation and decapitated; embryos were harvested and then decapitated. The embryo head was fixed for 60 min in 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at room temperature and then incubated in 30% sucrose at 4 °C for 48 h. The tissue was embedded in TissueTek (Reichert-Jung, Nußdorf, Ger)
and frozen on dry ice. Coronal and sagittal sections (30, 50 and 100 μm thick) were cut on a Reichert-Jung Frigocut 3000 at −17 °C and adhered to Superfrost microslides (Fisher, Pittsburg, PA).

**X-Gal staining**

The sections were air-dried and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as follows. They were washed with buffer A (100 mM phosphate buffer, pH 7.4, 2 mM MgCl₂ and 5 mM EGTA) once for 5 min and once for 25 min at room temperature, followed by two incubations of 5 min at room temperature in buffer B (100 mM phosphate buffer, pH 7.4, 2 mM MgCl₂, 0.01% sodium deoxycholate and 0.02% Nonidet P-40). The blue precipitate was generated by exposure at 37 °C to buffer C (buffer B with 5 mM potassium-ferricyanide, 5 mM potassium-ferrocyanide and 1 mg/mL X-gal).

**In situ hybridization**

Digoxigenin labelled mOR37A sense and antisense riboprobes were generated using the SP6/T7 in vitro transcription system (Roche Diagnostic, Mannheim, Ger). In brief, 2 μg of linearized vector was transcribed in the presence of 70 nmol digoxigenin-11-uridine-5′-triphosphate. RNA was precipitated with ethanol and resuspended in 20 μL of ‘in situ grade’ hybridization buffer (Amersham Pharmacia Biotech, Freiburg, Ger) containing 50% deionized formamide. For in situ hybridization, tissue sections were covered with 10 μL of hybridization solution containing approximately 3–5 ng digoxigenin-labelled RNA and coveredslipped. Hybridization and posthybridization washes were performed as described earlier (Strotmann et al., 1994)

**Immunohistochemistry**

For double immunohistochemistry, X-Gal stained sections of heterozygous double mutants (mOR37A-ITLZ/mOR37C-GFP) were incubated in 1% H₂O₂ in 0.1 M phosphate buffer for 15 min at room temperature. Tissue-sections were incubated with the primary rabbit anti-GFP antibody (Molecular Probes, Eugene, OR) diluted 1 : 500 at 4 °C overnight. The immunoperoxidase system ‘Vectastain-Elite’ (Vector Laboratories, Burlingame, CA) was used to localize the unlabelled primary antibody; horse radish peroxidase (HRP) activity was detected using the chromogen diaminobenzidine 1% in phosphate buffer. Alternately, double fluorescence immunohistochemistry was performed using a monoclonal anti-α-galactosidase antibody (Clontech, Palo Alto, CA) 1 : 500 at 4 °C overnight, followed by a secondary antibody conjugated to Alexa568 (Molecular Probes) 1 : 500 for 2 h at room temperature. The GFP-signal was enhanced using a polyclonal rabbit anti-GFP antibody (Molecular Probes) 1 : 500 at 4 °C overnight followed by an anti-rabbit Alexa488 (Molecular Probes) 1 : 500 secondary antibody incubated for 2 h at room temperature. Sections were mounted in Vectashield (Vector Laboratories).

**Microscopy and photography**

Sections were photographed using a Zeiss Axiophot connected to a CCD camera (SensiCam; PCO Computer Optics, Kelheim, Ger) and the Zeiss Axiovision imaging system. Fluorescence was examined using the appropriate filter sets for Alexa488 and Alexa568. Photomicrographs were transferred to Corel Photopaint (Corel Corporation, Ottawa, Ontario, Can) for further editing.

**Confocal laser scanning microscopy**

The preparations were viewed with a Leica TCS-4D confocal laser scanning microscope (Wetzlar, Ger) equipped with a Leitz DM RBE microscope and a krypton/argon laser light source. For preparations containing Alexa568-labelled axons the rhodamine filter (excitation wavelength 568 nm) was used. For preparations containing dual staining of olfactory nerve axons (Alexa568 and Alexa488) the rhodamine and the fluorescein filter (excitation wavelength 488 nm) were used. Serial optical sections were imaged at intervals of 0.5 μm through the depth of the cryostat sections and subsequently compiled in two-dimensional reconstructions; two-dimensional projections of series or parts of series (23–35 sections) were generated with Imaris software (Bitplane, Zurich, Switzerland). In preparations with dual staining, projections were created for each channel by using different pseudocolors for each channel (red for Alexa568 and green for Alexa488), and the images were subsequently merged. The digitized images were processed and modified to enhance contrast with Corel Photopaint.

**Results**

In order to determine the onset of mOR37 receptor expression, transgenic mouse lines were employed in which a mOR37 gene was modified in a way that neurons which activate it coexpress tau-lacZ (henceforth referred to as lacZ) or tau-GFP (referred to as GFP) (Strotmann et al., 2000). On serial sections through the nasal cavity of mOR37-lacZ transgenic mice, X-gal stained cells were first detected on embryonic day 11.25; at this age, blue cells were seen in some, but not in all embryos (Table 1). Labelled cells were visible only after substrate incubation for approximately 30 h at 37 °C, which is approximately ten times longer than in adult animals; this weak staining indicates a very low level of expression. At E11.5, i.e. 6 h later in development, at least one reactive cell was visible in each of the mOR37-lacZ mice. In the majority of animals up to three X-gal positive cells were found (Table 1). In mice from different mOR37-lacZ lines, similar numbers of cells were labelled, suggesting that the onset of expression for these receptor subtypes occurs within the same narrow time window between E11.25 and E11.5. To confirm this observation, expression of two receptor types, mOR37A and mOR37C, was analyzed within the same individual. For this purpose double heterozygous animals were generated that carry the two mOR37 genes in the developing olfactory epithelium within the same series of sections. At E11.5, labelled cells were present 3, 1, 2 3, 2, 3 2, 1, 0 6, 2, 3

**Table 1. Number of neurons expressing mOR37 genes in the developing olfactory epithelium**

<table>
<thead>
<tr>
<th>Embryonic age</th>
<th>A-lacZ</th>
<th>B-lacZ</th>
<th>C-lacZ</th>
<th>mOR37A antisense</th>
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</thead>
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<tr>
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<td>2, 1, 0</td>
<td>6, 7, 5</td>
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<td>E11.5</td>
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<td>0, 0, 0</td>
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<tr>
<td></td>
<td>0, 0, 2</td>
<td>1, 0, 0</td>
<td>6, 4, 8</td>
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Quantification of neurons expressing mOR37 genes in the olfactory epithelium of homozygous A-lacZ, B-lacZ and C-lacZ mice, respectively, by X-gal staining. Nine animals were analyzed at each developmental stage originating from three different litters. The number of cells in the olfactory epithelium hybridizing to an mOR37A antisense riboprobe was determined in wild-type mice (C57Bl6) (n = 6 at each developmental stage; from two different litters).
present in the olfactory epithelium either for both receptor types (Fig. 1a) \((n = 5)\) or for none of the two \((n = 3)\); in no cases were cells of only one subtype found. These results thus confirm the simultaneous onset of expression for both receptor types. A characteristic feature of all labelled cells at E11.5 was the staining of only the cell body (Fig. 1a and b); neither a dendritic process extending to the luminal surface nor an axonal process was visible; also on adjacent sections no staining was detectable at the corresponding positions (data not shown). Due to the rather weak staining it seemed possible that the appearance of the marker in these cells is delayed from the expression of the corresponding mRNA; therefore, control \(\textit{in situ}\) hybridization experiments were performed using a digoxigenin-labelled antisense riboprobe corresponding to the \(mOR37A\) coding sequence. On serial sections through the olfactory epithelium of wild-type mice at E11.25, a few hybridization-positive cells in each individual were detectable (Table 1). The number of cells detected by \(\textit{in situ}\) hybridization was higher than that labelled by X-gal staining in the transgenic animals; this is probably due to a cross-hybridization of the probe to different subfamily members, which share a sequence identity between 87.1% and 93.1%. Control experiments using a sense probe resulted in no hybridization signals (data not shown). At E11, only a single hybridization signal was detected in a total number of six animals; these results thus demonstrate that visualization of the histological markers faithfully reflects the onset of \(mOR37\) receptor expression in olfactory sensory neurons.

Figure 2 shows a representative sagittal section through the head of an A-lacZ mouse at E13. A-lacZ expressing cells are clustered within a distinct region of the developing olfactory epithelium. Their axons grow parallel to the border of the telencephalon in anterior-dorsal direction. oe, olfactory epithelium; tel, telencephalon. The dotted line indicates the border between mesenchyme and telencephalon. The arrow indicates the position of axon endings within the mesenchyme. Scale bar, 40 \(\mu\)m anterior to the left; dorsal to the top.
border of the telencephalon. In a total number of eight A-lacZ animals analyzed at E13, no blue colored structures were visible within the developing brain, indicating that the A-lacZ axons have not yet entered their target tissue. On serial sections through the head of B-lacZ- (n = 7) and C-lacZ-mice (n = 6), respectively, the same picture emerged (data not shown), indicating that at E13 axons from the different mOR37 populations have reached approximately the same position.

At E15 axons from mOR37 expressing neurons were first detectable beyond the developing cribriform plate within the telencephalon (n = 9). At this age, 153 ± 34 (n = 3) A-lacZ expressing neurons and 133 ± 23 (n = 3) C-lacZ expressing neurons were detectable on serial sections through the olfactory epithelium of homozygous animals. As shown by histochemical staining of a representative cross section through the head of an A-lacZ×C-GFP double heterozygous animal (Fig. 3a), axons from both neuron populations accumulated in a small area within the ventro-medial region of the telencephalic vesicle. The two adjacent sections showed almost no staining (data not shown), indicating that these axons apparently have reached their final destination within this particular region of the presumptive olfactory bulb. A separation of the telencephalon into a nerve fiber layer and an underlying glomerular layer was not yet visible. Higher magnification (Fig. 3b) reveals that the two axon populations were rather diffusely arranged in this area; in each bulb examined at this age (n = 14) the axons were intermingled such that a distinct target area for each population was not noticeable.

Two days later in development, at E17, the characteristic layering of the developing bulb became visible when cross sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole.2HCl) (Fig. 3c). As seen on this representative section through the head of an A-lacZ×C-GFP double heterozygous mouse, the green fluorescent C-GFP axons accumulate in the ventral part of the bulb (Fig. 3c). Higher magnification (Fig. 3d) reveals that, in contrast to the diffuse arrangements of axons at E15 (see Fig. 3b) now at E17 condensations of axons are forming at discrete spots. Analyzing the distribution of A-lacZ axons revealed a similar condensation for this population. An overlay of the two markers (Fig. 3d) shows that axons from both neuron populations still share a common target field. In nine bulbs examined at this age very similar pictures emerged; a separation of axons into distinct target areas had not yet occurred.

At the day of birth, when olfactory sensory neurons first encounter airborne odours, a large number of stained axons were visible projecting from the left nasal cavity exclusively into the left bulb and from the right cavity into the right bulb. In each of the seven homozygous A-lacZ animals examined, axons were seen converging at very similar positions in the ventral region of each bulb (Fig. 4a). The developing glomerular structure appeared not as compact as in adult animals, in some bulbs (4 out of 14) condensation spots were visible at several sites (arrows in Fig. 4a); in these cases they appeared to be interconnected by thick bundles of axons. Similar results were obtained when A-lacZ axons were examined in A-lacZ×C-GFP double heterozygous animals by immunofluorescence (Fig. 4b–h). A confocal micrograph at high resolution of A-lacZ axons converging at a single site (Fig. 4c) demonstrates that even within the immediate vicinity of this protoglomerular structure very thin fibers are detectable, suggesting that individual axons which are apparently not fasciculated with their sister axons approach this target.

When the same range of optical sections as shown in Fig. 4c was analyzed for the presence of C-GFP axons it turned out that many C-GFP axons were apparently approaching this protoglomerular structure (Fig. 4d). Merging the two images indeed results in yellow spots at sites in the outer nerve layer and within the protoglomerulus (Fig. 4e); these results thus indicate that fibers from both neuron populations comigrate to roughly the same target area. Figure 4f–h shows another representative example from the bulb of an A-lacZ×C-GFP double heterozygous animal. Confocal scans through this developing glomerulus demonstrate that A-lacZ fibers (Fig. 4f) and C-GFP fibers (Fig. 4g) are found within the same area in the bulb also in this case; merging of these pictures confirms this notion (Fig. 4h) as yellow spots appear at discrete positions. In ten bulbs examined, similar pictures were seen, thus indicating an intermixing of axons from these two populations. It could, however, be possible that axons only appear intermingled because of overlaying multiple scans from different levels. Therefore individual optical sections were analyzed to examine the arrangement of the two axon populations. Figure 5 shows the organization of the two axon populations within the glomerulus from Fig. 4(c–e) in more detail. The analysis of individual optical sections at 3 μm equidistant intervals through the entire structure revealed that on each level both types of fibers were detectable; this was particularly pronounced in the centre (Fig. 5c) of the structure, whereas the outer margins were dominated by one population, A-lacZ in upper levels (Fig. 5a) and C-GFP in the lower levels (Fig. 5e). In all glomeruli analyzed by confocal laser scanning microscopy it became evident that they actually consisted of subcompartments which were occupied by the different axon populations. In two bulbs from the A-lacZ×C-GFP animals that were examined, two distinct loci were visible with A-lacZ axons converging, very similar to the situation shown in Fig. 4a. The analysis of these spots revealed that also C-GFP axons were present at both sites (data not shown). Altogether, these results demonstrate that the segregation of axons is not complete on the day of birth.

To monitor the progress in glomerular formation during postnatal development, cross sections through olfactory bulbs of A-lacZ×C-GFP mice at postnatal day 3 (PN3) were examined by confocal laser scanning microscopy. Two largely distinct glomerular structures were observed (Fig. 6a and b); an overlap of the two structures is hardly noticeable. Thus, separation appears to be completed to a large extent at PN3. The targeting, however, is still slightly imperfect at this age, as a few fibers or fiber bundles could be observed within the ‘inappropriate’ glomerulus in 17 of 21 glomeruli examined (data not shown). These misprojections still occurred several days later in development, at PN7. Figure 6c shows a representative thin optical section through a C-GFP glomerulus at PN7 containing an A-lacZ fiber. The same phenomenon was observed vice versa, individual C-GFP fibers were present within A-lacZ glomeruli (Fig. 6d). From 19 glomeruli examined at this stage, 13 contained fibers from the other axon population; in all these cases they could be followed from the point of entry into the glomerulus until their exit, indicating that they apparently did not terminate within the ‘inappropriate’ target region but penetrated the complex neuropil structure on the way towards the correct target glomerulus.

Discussion

In the present study critical events in the development of OSNs, including the onset of receptor expression and the precise axon projection were investigated for neurons that express the highly related mOR37 receptor genes. For all three genes that were examined, expression was first detectable around E11.5; thus the onset of receptor expression in neurons that are clustered in a small area of the epithelium is similar to those neurons that are widely

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distributed in broad zones (Sullivan et al., 1995). The molecular mechanisms underlying the control of OR gene expression are still elusive. The genomic organization of OR genes may provide a basis for regulatory principles, as shown for other multigene families (Krumlauf, 1992). Mapping studies have shown that the mOR37 genes are organized in a distinct gene cluster on chromosome 4.

Fig. 3. (a) Cross section through the head of an A-lacZ×C-GFP double heterozygous mouse at E15; axons from A-lacZ expressing cells are blue, those from C-GFP expressing cells are brown. Axons from both populations have entered the presumptive olfactory bulb. Lateral to the left, dorsal to the top. (b) Higher magnification of the area indicated in (a); a diffuse arrangement of axons from both populations within roughly the same area of the bulb is visible. (c) Cross section through the olfactory bulb of an A-lacZ×C-GFP double heterozygous mouse at E17 counterstained with DAPI; axons from C-GFP expressing cells are green fluorescent. Lateral to the left, dorsal to the top. (d) Higher magnification of the area indicated in (c) with an overlay of axons from red fluorescent A-lacZ expressing cells. cp, cribriform plate; ns, nasal septum; tel., telencephalon. Scale bars, 50 μm in (a); 30 μm in (b) and (d); 200 μm in (c).
comprising receptor genes that all share a unique clustered expression pattern within the olfactory epithelium (Strotmann et al., 1999). Detailed sequence analyzes have revealed the presence of common sequence motifs in the 5' region of these genes which may be involved in the regulation of their expression (Hoppe et al., 2000). Based on these data it is conceivable that genes from this cluster are

Fig. 4. (a) Cross section through the head of an A-lacZ animal at PN0 stained with X-Gal. Protoglomerular structures are visible in the ventro-medial region of the left and right olfactory bulb. (b) Cross section through the head of an A-lacZ×C-GFP double heterozygous animal at PN0. Red fluorescent axons from A-lacZ expressing neurons are visible symmetrically positioned in the ventral region of the left and right olfactory bulb. (c) Confocal micrograph showing a stack of 35 × 0.5 μm optical sections through the A-lacZ glomerulus shown in (b). (d) Confocal image of the same area shown in (c) visualizing axons from green fluorescent C-GFP axons. (e) Merged images of the two pictures in (c) and (d), resulting in yellow colour spots at discrete positions. (f) Confocal micrograph showing a stack of 24 × 0.5 μm optical sections through another representative A-lacZ glomerulus. (g) Confocal image of the same area shown in (f), visualizing axons from green fluorescent C-GFP axons. (h) Merged images of the two pictures shown in (f) and (g), showing the intermingling of the two axon populations. dr, dorsal recess; ns, nasal septum. Scale bars, 200 μm in (a) and (b); 20 μm in (c–h).
under common expression control that also involves their coordinated onset during development. The data available so far suggest that in mammals expression of all OR genes starts at approximately the same time; this is in contrast to the situation in zebrafish, where a different timing of expression onset for various olfactory receptor subtypes has been observed (Barth et al., 1996). Also in birds, a sequential onset of expression for distinct receptor types has been described (Nef et al., 1996).

At the time of expression onset (E11.5), only cell bodies of neurons expressing mOR37-lacZ or C-GFP were stained; this could be due to the very low level of marker protein which might not be sufficient to allow visualization of all cellular compartments. Alternatively, these neurons may not have elaborated dendritic and axonal processes at that time; this would mean that expression of the receptor begins before axons are extended towards the olfactory bulb and thus long before target selection. These data are consistent with the hypothesis that the receptor protein itself may be involved in the process of targeting axons to their correct position in the olfactory bulb (Singer et al., 1995; Mombaerts et al., 1996; Wang et al., 1998).

The synchrony of developmental processes in sensory cells expressing distinct mOR37 receptor genes also holds true for the outgrowth of axons which reach the developing telencephalic vesicle around E13 and then simultaneously invade it approximately one or two days later. Thus, axons of mOR37 expressing neurons seem to remain waiting for a certain period of time at the surface of the prospective olfactory bulb, before a synchronized invasion into the central nervous system occurs. These observations are in line with several reports (Valverde et al., 1992; Kobayashi et al., 1997) and it has been proposed that the waiting period prevents axons from entering their target prematurely (Shepherd et al., 1997). In fact, when the axons enter the brain early, they tend to overshoot the area that will be their prospective target region later in development (Renzi et al., 2000). The stop of axons may not be a general phenomenon true for all cell populations as it was not observed in rats by Treloar et al., (1999).

The simultaneous visualization of axons from two OSN populations in the same animal allowed us for the first time to demonstrate that axons of neurons expressing highly related olfactory receptor genes initially project to largely overlapping areas within the developing olfactory bulb; only during the perinatal and postnatal phase of development they segregate into distinct glomeruli. This is partly reminiscent of the situation in the visual system, where axons of retinal ganglion cells from both eyes initially invade overlapping areas in the lateral geniculate nucleus; when development proceeds, axons are gradually retracted from inappropriate regions and eventually form eye-specific domains (Katz & Shatz, 1996). Interestingly, this domain-specific segregation does not take place if electrical activity of retinal ganglion cells is blocked (Katz & Shatz, 1996). This observation raises the question concerning a putative role of electrical activity in the wiring refinement of the olfactory system.

![Image](image_url)
Recent observations on CNG-channel knock-out mice seem to argue against a role in axonal convergence; despite the lack of detectable odourant-evoked electrical activity in these cells, P2-receptor expressing OSNs converge on their appropriate glomerular targets (Lin et al., 2000). This result, however, cannot be generalized, as neurons expressing M72 are affected in their projection by the mutation (Zheng et al., 2000). Evidence that odourant-evoked activity may contribute to the organization and maintenance of axonal projections in the olfactory system was recently obtained by a genetic approach in which two different populations of olfactory neurons were generated by disruption of the OCNC-1 channel (Zhao & Reed, 2001). OCNC-1 deficient OSNs were specifically depleted from the olfactory epithelium when faced with their functional counterparts. It has been argued that they may not be able to compete for target sites in the bulb due to the lack of electrical activity. A similar mechanism may also be involved in the segregation process of mOR37 axons during development. Because of the high sequence identity between the different mOR37 subtypes, it is likely that OSNs expressing these subtypes respond to very similar compounds, however, with slightly different thresholds. This variation could result in a different electrical activity pattern and thus a different ability to compete for the mitral cells in the same glomerulus they initially project to. It is conceivable that this different level of activity may ultimately lead to a segregation of the axons into distinct glomeruli.

Prior to electrical activity, molecular cues are considered as major parameters for guiding axons to their appropriate position in the telencephalic vesicle. Based on our data it seems possible that the different mOR37 populations interact with similar, eventually even

Fig. 6. (a and b) Representative confocal micrographs of glomeruli formed by A-lacZ (red) and C-GFP (green) axons in a double heterozygous animal at PN3. Axons target largely distinct glomeruli. (c and d) Representative confocal micrographs of glomeruli formed by A-lacZ (red) and C-GFP (green) axons in a double heterozygous animal at PN7. Individual fibers or fiber bundles are visible passing through the ‘inappropriate’ glomeruli. Scale bars, 20 μm.
the same, guidance cues, which are present in a defined domain of the bulb. The fact that axons were sometimes found at multiple loci and both populations examined were present at these spots can be viewed in favour of this idea.

A still open question is, who provides these guidance cues in the developing bulb. The ultimate cellular interaction partners are of course the target cells, the mitral and tufted cells. In early development, mitral cells form numerous, widespread dendritic processes which contact the area of several glomeruli; these exuberant arbors are retracted over time (Malun & Brunjes, 1996). This process coincides with the separation of the intermingled olfactory axons while forming the appropriate glomeruli, suggesting an influence on the refinement process. Recent observations seem to argue against such a view; in mutant mice which lack mitral and tufted cells, olfactory axons still converge onto a common target region in the bulb (Bulfone et al., 1998). However, before generalizing one has to keep in mind that only a single neuron population has been analyzed so far, and it is unclear whether neurons expressing highly related receptors would segregate their axons appropriately under these circumstances.

In other regions of the CNS, glial cells play a crucial role in creating compartmented neuropils, e.g. the somatosensory barrel fields (Crossin et al., 1989; Steinholder, 1993) and there are suggestions that glia also play a critical role in the formation of glomeruli (Valverde et al., 1992; Bailey et al., 1999; Treloar et al., 1999). Radial glia are present in the developing bulb at the time when axon terminals invade; their end-feet reach into the outer layers of the telencephalic vesicle where the olfactory neurons make their first contact with the brain. It is conceivable that these cells may present targeting cues for ingrowing axons, and in fact, radial glia in the chick visual system has been shown to express Ephrin ligands that play a critical role in establishing the topographic map (Drescher et al., 1995). In addition, another glial cell type appears to play an important role; the olfactory ensheathing cells which comigrate with the outgrowing axons and separate developing glomeruli by forming ‘walls’ around them. Also ensheathing cells do express molecules which are supposed to be involved in axon guidance, like, e.g. semaphorin3A (Kobayashi et al., 1997; Renzi et al., 2000) and thus could help directing axons towards their specific destinations.

The precise connection of neurons with their distinct glomeruli is considered as an essential prerequisite for discriminating odours; in particular, information processing in focal areas of the bulb depends largely on a correct connectivity. This study as well our previous analyses of the olfactory receptor gene cluster mOR37 on mouse chromosome 4. Genomics, 66, 284–295.


