Individual Olfactory Sensory Neurons Project into More Than One Glomerulus in *Xenopus laevis* Tadpole Olfactory Bulb

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ABSTRACT

An essential step in the coding of odorants is the way olfactory sensory neurons (OSNs) convey their information to the olfactory bulb. This projection determines how the specificities of OSNs are mapped onto the spatial activity patterns of the olfactory bulb (OB). Despite the fact that virtually nothing is known about how individual OSN axons project to glomeruli, it is generally believed that OSNs always project to one glomerulus each. Our recent findings in tadpoles of *Xenopus laevis* challenge this view. By injection of a tracer into individual OSNs, we show for the first time that axons typically project into more than one glomerulus and that they do so in a characteristic way. Upon entering the olfactory bulb, an axon bifurcates to give two primary branches. Each of these branches bifurcates again to give two subbranches, thus resulting in four subbranches per OSN. The two subbranches of each primary branch project into two different glomeruli. Variations of this characteristic innervation pattern include the innervation of three and, occasionally, one glomerulus. In any case, and independent of the number of glomeruli innervated by an OSN, each glomerulus receives at least two axonal branches of the same OSN. *J. Comp. Neurol.* 481:233–239, 2005.

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Following the characterization of a multitude of olfactory receptor proteins (ORs) (Buck and Axel, 1991), a number of studies have been carried out to understand how olfactory sensory neurons (OSNs) expressing specific ORs are connected to the olfactory bulb (OB) (e.g., Ressler et al., 1994; Vassar et al., 1994; Treloar et al., 1996). So far, two basic principles of olfactory coding have emerged from these studies. In mammals, each OSN appears to express one particular OR (Nef et al., 1992; Strotmann et al., 1992; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994; Malnic et al., 1999), and all OSNs that express the same OR form a class of sensory neurons project to one or a few glomeruli within the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts, 1996; Mombaerts et al., 1996). Thereby, it is explicitly or implicitly assumed that the individual axons of OSNs project to one glomerulus each. These features are considered the morphological basis of chemosensory maps connecting receptor specificities to the neuronal network of the OB.

However, for the latter feature there is no direct experimental evidence so far. Although axon intraglomerular arborizations within individual glomeruli have been stud-

ied (Hálasz and Greer, 1993), no data on the projections of individual OSNs into glomeruli exist. This is because the cribriform plate separates the olfactory mucosa from the olfactory bulb in all popular model vertebrates including mammals, reptiles, amphibians, and fish, making it currently impossible to inject a tracer into a single OSN and trace its fibers in the bulb. However, tadpoles of the clawed frog *Xenopus laevis* provide a favorable model sys-

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tem to address this question. They have a well-developed olfactory system (Byrd and Burd, 1991) and, due to the lack of the cribriform plate, allow making a preparation that contains both the sensory epithelium and the OB and, by using the patch-clamp technique, to record odorant responses in individual OSNs while injecting a tracer into them (Nezlin et al., 2003).

Therefore, we studied projections of individual OSNs in the OB of *Xenopus* tadpoles. Visualizing the projections in the whole-mount preparations reveals that OSNs regularly project to more than one glomerulus, and they do so in a stereotypical way. Basic mechanisms of olfactory transduction and neural processing in the peripheral olfactory pathway are considered to be universal across most species and phyla (Hildebrand and Shepherd, 1997; Eisthen, 2002). It is a future challenge to see whether this also applies to the findings reported here.

MATERIALS AND METHODS

Animals and tissue preparation

Tadpoles of laboratory-bred *Xenopus laevis* (stages 51–56; staged after Nieuwkoop and Faber, 1994) were used. Late premetamorphic stages were chosen because the OB is morphologically fully developed by this time (Byrd and Burd, 1991; Nezlin and Schild, 2000). The animals were anesthetized and immobilized in a mixture of ice and water for at least 5 minutes and subsequently decapitated, as approved by the Göttingen University Committee for Ethics in Animal Experimentation. To stain individual OSNs and their axons we used the *X. laevis* tadpole nose-brain preparation as indicated in Figure 1. A block of tissue containing the olfactory mucosae, the olfactory nerves, and the anterior two-thirds of the brain was cut out (Fig. 1A), transferred into frog physiological saline (FS) containing (in mM): 101 NaCl, 2 KCl, 1 CaCl₂, 2 Mg Cl₂, 5 glucose, 5 Na-pyruvate, 10 HEPES (Geiling and Schild, 1996) and mounted on the stage of a vibratome (Leica VT1000S) as shown in Figure 1B. The upper part of the mucosae was cut off to have free access to the olfactory epithelium while the nerves and the telencephalon were left intact. The preparation was then placed in a chamber under a harp-like grid for patch-clamp experiments (Fig. 1C). All chemicals were from Sigma (St. Louis, MO) unless otherwise specified.

Tracing of neuronal processes

Axons of individual OSNs were traced by injecting biocytin through a patch pipette into the soma as described elsewhere (Czesnik et al., 2001). Briefly, the “nose-brain” preparations were used in a setup equipped with an upright microscope with differential interference contrast (Axioskop 2, Zeiss, Göttingen, Germany). Patch electrodes with a tip diameter of approximately 1 μ m and a resistance of 6–8 M Ω were fabricated from borosilicate glass (1.8 mm outer diameter; Hilgenberg, Malsfeld, Germany) using a two-stage electrode puller (PP-83, Narishige, Tokyo, Japan). The pipettes were fire-polished. The pipette solution contained (in mM): 80 K-gluconate, 11 KCl, 2 NaCl, 2 MgSO₄, 10 HEPES, 0.2 EGTA, 1 Na₂-ATP, 0.1 Na₂-GTP, and 0.5% biocytin (Molecular Probes, Eugene, OR). The neurons to be investigated were selected randomly from various regions of the mucosa. The seal between pipette and OSN was established quickly to limit

Fig. 1. “Nose-brain” preparation of a *Xenopus laevis* tadpole, stage 54. **A:** Dorsal view of the tadpole head, the olfactory mucosa, the olfactory nerves, and the telencephalon being situated within the rectangular block indicated. **B:** The extirpated block is mounted on the stage of a vibratome and sliced (dotted line) in a way that allows patch clamping of neurons in the olfactory mucosa while leaving the olfactory nerves and the telencephalon intact (side view). **C:** Preparation in the experimental chamber under a harp-like grid. Scale bars = 1 mm.

spill-over of the dye into the bath to a minimum. Spilled dye was rapidly removed by a continuously flowing bath (bath volume 500 μ l; flux was $\sim$ 2.5 ml/min). After establishing the whole-cell configuration a mixture of amino acids was applied and stimulus responses were recorded in the current clamp mode as described in Manzini et al. (2002a). Then biocytin was injected using depolarizing current pulses (200 ms pulses at 0.5 Hz for 20 minutes). In each olfactory mucosa one cell was injected. Preparations were left in fresh FS for 1 hour and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 8–12 hours at 4°C. Then the preparations were washed in PBS (3 $\times$ 30 minutes), the brain together with the olfactory nerves and mucosae were dissected from the connective tissue and processed for biocytin visualization and immunochemistry.

Immunocytochemistry

Glomeruli were visualized using a polyclonal anti-synaptophysin antibody (rabbit, G113/p38 frog, kind do-

nation of Dr. Reinhard Jahn, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) (Jahn et al., 1985). Nonspecific binding was blocked using 2% normal goat serum (ICN Biomedicals, Germany) and 0.2% Triton X-100 in PBS for 2 hours at room temperature. The tissue was then incubated overnight at 4°C with the antibody diluted 1:1,000 in blocking solution. Primary antibodies were washed off with PBS (3 × 30 minutes), and Alexa546-conjugated goat anti-rabbit IgG and Alexa488-conjugated avidin (A 11003 and A-21370, Molecular Probes) were applied at a dilution of 1:250 in 1% normal goat serum in PBS for 8–12 hours at 4°C. The preparations were finally washed in at least five changes of PBS, transferred to 60% glycerol in PBS for at least 1 hour, and finally mounted on slides for microscopy in 80% glycerol in PBS.

Laser scanning confocal microscopy and image processing

Preparations were selected for further analysis only if 1) one cell body was stained in the olfactory epithelium, and 2) if one axon could be traced along the whole olfactory nerve, and 3) if the axon terminated in the OB forming at least one characteristic glomerular tuft. Neurons that did not reach the glomerular layer were considered immature and discarded. The preparations were viewed using inverted confocal optics (LSM 510 attached to an Axiovert 100M, Zeiss). Serial optical sections were taken and saved as 3D stacks. Three-dimensional, rotatable reconstructions were generated from an image stack and analyzed using the LSM-510 software. For illustrations, series of optical sections were projected into one image with greater focal depth and imported into an image processing program. Where needed, image contrast was enhanced, only to merge images from consecutive sections of the same preparation, or to form montages of images from adjacent regions. Image processing and labeling of figures were performed with one or more of the following programs: LSM 510 (Zeiss, Jena, Germany), Adobe PhotoShop (San Jose, CA), and Corel Draw (Corel, Canada). The number and step size of optical sections are given for each image in the captions.

RESULTS

To stain axons of individual OSNs and trace them to the olfactory bulb's glomeruli we used the *Xenopus* tadpole nose-brain preparation (Fig. 1). We checked OSNs for stimulus responses and injected biocytin through a patch pipette. In each mucosa only one OSN was injected (Fig. 2). Twelve out of 96 OSNs responded with a series of action potentials to the application of a mixture of amino acid (Fig. 2A), confirming previous evidence of amino acid sensitivity of some *X. laevis* tadpole OSNs (Manzini and Schild, 2002b, 2004) and proving that the injected neurons were functional and responsive. No differences in axonal projection patterns between amino acid responsive and nonresponsive OSNs were found. Immature neurons never responded to amino acids.

In all successfully filled preparations ($n = 65$), one OSN could clearly be seen in the epithelium of the mucosa (Fig. 2B). The characteristic cellular compartments of OSNs, i.e., cilia, dendrite, soma, and axon were present (Fig. 2C). The axon could be traced along the olfactory nerve (Fig.

2D). Once the axon arrived in the olfactory bulb, it bifurcated into two axonal branches, *a* and *b*. Downstream, each of these branches bifurcated again giving rise to subbranches a_1 and a_2 , as well as b_1 and b_2 . The subbranches a_1 and b_1 innervated one glomerulus, and subbranches a_2 and b_2 ran into another glomerulus (Fig. 2E, schematically depicted in Fig. 2G; see Supplementary Material for a 3D rotatable reconstruction). Branch a_2 bifurcated again and the subbranches a_{2-1} and a_{2-2} entered glomerulus G2 from opposite sides. The two glomeruli involved were neighboring but not adjacent, as shown by the anti-synaptophysin staining of glomeruli (Fig. 2F).

The specific paths of the branches and subbranches varied from preparation to preparation. In the example given in Figure 3 the axon bifurcated into two branches *a* and *b* that projected into the glomeruli G1 and G2 (Fig. 3A,B). Branch *a* bifurcated well before entering G1, sending both a_1 and a_2 towards G1. Note, however, that branch a_2 shortly before entering glomerulus G1 bifurcated again (arrowhead in the figure) sending one axonal sister process a_{2-1} into glomerulus G1, and a second one, a_{2-2} , to glomerulus G2. Similarly, branch *b* innervated glomerulus G2 but, before entering into it, issues an additional branch, b_1 , to glomerulus G1 (Fig. 3B, schematically depicted in Fig. 3D). The branches entered the glomerulus from opposite sides and appeared to innervate opposite parts of it (Fig. 3C).

There is a common feature in Figures 2 and 3. The axon bifurcated and each of the branches bifurcated again, giving a total of four subbranches, whereby the subbranches of each primary branch projected to different glomeruli. We observed this innervation pattern in 37 preparations (57%). In 19 cases (29%), an OSN projected into three glomeruli, while in nine preparations (14%), only one glomerulus appeared to be innervated. But even in these cases the axon split into two branches, which innervated a glomerulus from opposite sides. The innervation pattern did not appear to depend on whether an OSN responded to the mixture of amino acids or not. Thus, among 12 responsive to amino acids OSNs, eight were successfully injected; one of them innervated three glomeruli, six innervated two, and one appeared to innervate one glomerulus.

Figure 4 shows an example where three glomeruli were innervated. After entering the olfactory bulb (arrow), the axon took a U-turn, and after about 150 μm bifurcated multiply to innervate two glomeruli, G1 and G2 (upper inset). In addition, one of the subbranches left the region of these glomeruli, crossed the midline of the brain, entered the contralateral olfactory bulb, and bifurcated there to innervate a glomerulus close to the midline (lower inset).

In all cases, irrespectively of the number of glomeruli innervated by one primary axon, not less than two axonal branches entered each glomerulus, usually from opposite sides, as shown in Figure 3C.

In Amphibia, the olfactory bulb's mitral cells receive afferent input from two or more glomeruli (Ramon y Cajal, 1896). This has also been described in the olfactory bulb of *X. laevis* tadpoles (Nezlin and Schild, 2000). Although the detailed cytoarchitecture of mitral cells is beyond the scope of the present article, we would like to note a particularity. Tracer injection into individual mitral cells showed that their dendrites projected in a characteristic way, mainly into two or a few glomeruli (Fig. 5; see Supplementary Material for a 3D rotatable reconstruction). In

Fig. 2. Projection of an amino acid-responsive OSN to two glomeruli. **A:** Application of a mixture of amino acids (solid line) evoked a burst of action potentials (scale bars: 1 s; 50 mV). **B:** Same preparation after visualization of biocytin. Fluorescence of this OSN was clearly recognized in the epithelium. **C:** High magnification image of the OSN showing the dendrite with apical cilia and the axon, which disappears into a deeper layer of the slice. **D:** In the olfactory nerve of the same preparation the axon could be traced along the whole length of the nerve. **E:** Representative innervation pattern of two glomeruli by the

same OSN. The axon bifurcates into two branches *a* and *b*, each of them bifurcating again into *a*₁ and *a*₂, and *b*₁ and *b*₂, respectively. The fibers *a*₁ and *b*₁ innervate glomerulus *G1*, and the branches *a*₂ and *b*₂ run into glomerulus *G2*. Before entering the glomerulus, the fiber *a*₂ divides into *a*₂₋₁ and *a*₂₋₂. **F:** Double-staining with anti-synaptophysin antibody shows two glomeruli interposed between the innervated ones. **G:** Schematic drawing of *E* showing branch *a* in green, and *b* in red. Scale bars = 50 μ m in *B*; 10 μ m in *C–F*.

the mitral cell shown in Figure 5A,B, one dendrite issued from the soma and projected into two different glomeruli in a way similar to the OSN projections described above. The dendrite bifurcated (into *a* and *b*) and both dendritic branches bifurcated again and entered two glomeruli; *a*₁ and *b*₁ ran into the small glomerulus *G1*; *a*₂ and *b*₂ ran into the big glomerulus *G2*. In another mitral cell (Fig. 5C), two primary dendrites *a* and *b* issued from the soma and projected into two glomeruli in the same criss-cross way. The branch *a* divided into *a*₁ and *a*₂, then *a*₁ divided again and both branches entered glomerulus *G1*, whereas *a*₂ ran into glomerulus *G2*. The branch *b* divided into *b*₁ and *b*₂, and *b*₂ ran into the glomerulus *G2*, whereas *b*₁ divided again and one its branches (*b*₁₋₁) innervated *G1*, while the other one (*b*₁₋₂) ran into *G2*. We have observed the same criss-cross dendritic glomerular innervations in 14 (out of

20) mitral cells. Six mitral cells showed even more complex projections into three different glomeruli. None of the mitral cells filled through the patch pipette projected into a single glomerulus.

DISCUSSION

The main result of this study is that the axons of individual OSNs regularly project to more than one glomerulus, mostly to two glomerula, one of them being in the vicinity of but not necessarily adjacent to the other. This evidence must not be confused with previous findings showing that OSNs expressing the same OR project to one or a few glomeruli (e.g., Mombaerts, 2004). While the latter evidence does not imply anything regarding the projections of individual nerve fibers, the evidence shown

Fig. 3. Projection of an OSN axon into two glomeruli. **A:** In the olfactory bulb, an axon bifurcates into two branches *a* and *b* innervating glomeruli *G1* and *G2*. **B:** The branch *a* runs to the glomerulus *G1*, bifurcates in its vicinity (arrowhead) and sends a short fiber (*a*₂₋₁) into glomerulus *G1*, and a long fiber (*a*₂₋₂) to *G2*. Similarly, branch *b* runs to the glomerulus *G2* but, before entering into it, sends an additional branch (*b*₁) to the glomerulus *G1*. **C:** Higher magnification; the two branches innervate two different halves of the glomerulus labeled with anti-synaptophysin antibody. **D:** Schematic drawing of **B** showing branch *a* in green and *b* in red. Scale bars = 20 μ m in **A**, **B**; 10 μ m in **C**.

in the present study does not allow any conclusion as to how many glomeruli are innervated by OSNs expressing the same OR.

On the basis of the light microscopic observation of an avidin-stained fiber in the olfactory nerve layer, it is often impossible to distinguish whether this fiber consists of one, two, or more axons. It was therefore crucial to make sure individual axons were stained. We did so by exploiting patch-clamp technique, and by dialyzing biocytin from the patch pipette into individual OSNs in the olfactory

Fig. 4. Projection of an OSN axon into three glomeruli. The axon enters the bulb (arrow) and innervates two glomeruli in the ipsilateral bulb (*G1* and *G2*; upper inset) and one in the contralateral bulb (*G3*, lower inset). Scale bar = 50 μ m.

mucosa, one OSN per preparation. The whole-cell configuration was kept intact for up to 1 hour to allow sufficient diffusion of biocytin. The tight seal between pipette and cell prevented biocytin from leaking out of the pipette into the bath or the tissue. There was only a short period of time (less than 60 seconds) when biocytin could leak into the bath, i.e., when the patch pipette approached the neuron. However, the continuous bath perfusion system and the dilution in the bath were presumably the main reasons why there was no detectable biocytin-avidin staining in epithelial cells other than the patch-clamped OSN. Accordingly, one fiber could be traced from the mucosa into the nerve, confirming that one OSN had been injected.

Axons of OSNs in some species are known to be coupled by gap junctions, potentially allowing the spread of biocytin between axons. If so, more than one axon would be inevitably stained in the olfactory nerve. However, we never used for analysis preparations with more than one stained OSN soma, and never observed one OSN stained in the epithelium with more than one axon stained in the nerve. Therefore, we discount such a possibility.

In conclusion, 1) one individual OSN and its axon were stained per experiment, and 2) the primary and secondary bifurcations of the fiber observed in the olfactory bulb

Fig. 5. Projections of mitral cells into glomeruli. **A:** A primary dendrite bifurcates into *a* and *b*. **B:** Both branches split again, and innervate two glomeruli (G1 and G2). The branches *a₁* and *b₁* run into the small glomerulus G1, while the branches *a₂* and *b₂* run into the big glomerulus G2. **C:** Two primary dendrites (*a* and *b*) issue from the soma, bifurcate several times, and terminate in two glomeruli (G1 and G2). Secondary dendrites *a₁* and *b₁* run into the G1, while *a₂* and *b₂* run into the G2. Scale bars = 10 μ m.

were no doubt the bifurcations of one individual axon. As a consequence, in *X. laevis* tadpoles individual OSNs innervate more than one glomerulus. Individual glomeruli in the OB could be clearly recognized as distinct spherical or oval clusters of synaptophysin immunoreactivity colocalized with glomerular arborizations of OSN axons (Nezlin et al., 2003). Even in the few cases where we detected just one glomerulus innervated, we occasionally observed thin branchings of the innervating fiber that were too faint to be traced. Whether these entered the same or another glomerulus could not be established.

The experiments reported here have been done for one particular species and are currently not feasible in other preparations, including adult frogs and higher vertebrates. However, they are sufficient to falsify the general assumption that OSN axons project into one glomerulus each. At least, this is not the case in one species at one particular developmental stage.

Two tentative interpretations of the bifurcations observed are proposed here. The first pertains to the chemosensory map from the sensitivities of OSNs to the spatio-temporal activity pattern of OB neurons. This sensitivity-to-space map may not be as precise and focused in larval or embryonic stages as in adults. As a second or additional interpretation, one might assume that in the course of development more than one receptor is expressed in individual receptor cells. This would make it plausible that OSNs would project to more than one glomerulus. Although there is presently no proof for this assumption, the large number of receptor classes responsive to amino acids in *Xenopus* tadpole OSNs might hint in this direction (Manzini and Schild, 2004). Obviously, further studies are needed to address these hypotheses.

A collateral observation was the criss-cross bifurcation pattern of OSN axons prior to entering (mostly) two glomeruli. It is intriguing to speculate on the physiological implications of this particular branching pattern. As we have recorded stimulus responses to odorants, we can be sure action potentials were conveyed from the OSNs visualized to the OB. From what is known about axonal action potential propagation we have to assume that an action potential generated in a particular OSN is duplicated at every bifurcation of the axon, so that usually at least four action potentials enter two glomeruli almost simultaneously, not considering further intraglomerular bifurcations. Such projection patterns could have various electrophysiological implications. It could increase the fidelity of transmission to the dendrite of a projection neuron, and it would also allow excitation of different projection neurons. Perhaps more importantly, due to the length differences of the subbranches, which can amount to as much as 60 μ m, the correlated action potentials must be assumed to arrive at slightly different times at their synaptic sites. The action potential propagation speed in *Xenopus* olfactory nerves is not known. Given room temperature and very thin unmyelinated processes, 0.1 m/s is presumably realistic. This would result in a delay of 500 μ s per 50 μ m length difference. Interestingly, this is longer than AMPA channels need to activate (Grosskreutz et al., 2003). Intraglomerular synaptic transmission has been shown to depend on AMPA as well as on NMDA channels (e.g., Bardoni et al., 1996; Berkowicz et al., 1994; own observation in *X. laevis* tadpoles). The bifurcation mechanism would thus almost certainly allow some AMPA channels to be activated immediately prior to the activation of NMDA channels, which may be important for the plastic-

ity at glomerular synapses. This might be particularly important considering that OSN firing rates are low in *X. laevis* (up to 20 Hz; Manzini et al., 2002a,b). Consequently, epsps on mitral cell dendrites brought about by hypothetical nonbifurcated OSN axons may lack any effect on each other, whereas, on the other hand, bifurcated axons guarantee temporally correlated input to the postsynaptic sites within glomeruli.

Taken together, our results suggest that, contrary to the widespread belief that OSN axons have an unbranched phenotype across phyla, they in fact do branch in tadpoles of *X. laevis*, and moreover do so in a stereotyped way. *Xenopus* differs anatomically in the OB from mammals, as mitral cells innervate multiple glomeruli and this feature may underlie the OSN axonal branching pattern reported above. However, it raises the possibility that a similar branching pattern may exist in the olfactory systems of other species. Thus, the unbranched phenotype widely ascribed to OSN axons across species needs to be reexamined.

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