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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Highly specific responses to amine odorants of individual olfactory receptor neurons *in situ*

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Abstract

The main olfactory system of larval *Xenopus laevis* is made up of at least two subsystems consisting of subsets of olfactory receptor neurons (ORNs) with different transduction mechanisms. One ORN subset lacks the canonical cAMP transduction pathway and responds to amino acid odorants. The second subset has the cAMP transduction pathway but as yet suitable odorants are unknown. Here we report the identification of amines as proper olfactory stimuli for larval *X. laevis* using functional Ca^{2+} imaging and slice preparations of the olfactory system. The response profiles of individual ORNs to a number of amines were extremely complex and mostly highly specific. The great majority of amine-sensitive ORNs responded also to forskolin, an activator of the olfactory cAMP transduction pathway. Most amine-induced responses could be attenuated by the cyclic nucleotide-gated channel inhibitor LY83583. This confirms that most amine-responsive olfactory receptors (ORs) are coupled to the cAMP-dependent transduction pathway. Furthermore, we show that trace amine-associated receptors (TAARs), which have been shown to act as specific ORs for amines in mammals, are expressed in the olfactory organ of *X. laevis*. The TAARs expressed in *Xenopus* cannot, however, explain the complex responses of individual ORNs to amines because there are too few of them. This indicates that, in addition to TAARs, there must be other receptor families involved in the detection of amines.

Introduction

In vertebrates, the olfactory sensory organs consist of specialized neuroepithelia containing olfactory receptor neurons (ORNs) that are able to sense a myriad of different odor molecules, sustentacular cells, which share common properties with glial and epithelial cells, and basal cells including olfactory stem cells, which maintain the regenerative capacity of the olfactory epithelium (OE; Graziadei & Metcalf, 1971; Graziadei, 1971). One of the first steps in the detection of odorous compounds is their binding to G protein-coupled receptors expressed in ORNs (Buck & Axel, 1991; Dulac & Axel, 1995; Buck, 2000). The nature of the detected odorants depends on the animal's habitat. While terrestrial species are able to smell a myriad of volatile compounds, aquatic species can smell a relatively small group of chemically well-defined water-soluble molecules such as amino acids (Caprio & Byrd, 1984; Kang & Caprio, 1995; Manzini *et al.*, 2002; Manzini & Schild, 2003a, 2004; Schild & Manzini, 2004), bile salts (Kang & Caprio, 1995; Sato & Suzuki, 2001), amines (Carr & Derby, 1986; Carr *et al.*, 1990; Rolen *et al.*, 2003), gonadal steroids (Sorensen & Caprio, 1998), prostaglandins (Sorensen *et al.*, 1988; Kitamura *et al.*, 1994) and nucleotides (Derby *et al.*, 1984; Carr *et al.*, 1986; Kang & Caprio, 1995; Rolen *et al.*, 2003). There are also considerable differences in the olfactory transduction pathways employed by ORNs of terrestrial and aquatic species. In terrestrial vertebrates the vast majority of canonical ORNs possess the cAMP-mediated transduction pathway, but some ORN subgroups have been shown to possess other transduction cascades (Ma, 2007; Breer *et al.*, 2006). In aquatic vertebrates, cAMP-independent transduction mechanisms appear to be much more widespread (Ma & Michel, 1998; Delay & Dionne, 2002; Manzini *et al.*, 2002; Hansen *et al.*, 2003; Manzini & Schild, 2003a).

The OE of the principal cavity (PC) of larval *Xenopus laevis* is the main olfactory system (Hansen *et al.*, 1998). It consists of diverse subsystems comprising at least two subsets of ORNs with different transduction mechanisms, varying projection patterns to the olfactory bulb (OB) and an unequal composition of presynaptic proteins (Manzini *et al.*, 2002, 2007a; Manzini & Schild, 2003a). One subset of ORNs, lacking the canonical cAMP transduction pathway, responds to amino acid odorants (Manzini & Schild, 2003a); ORNs of the second subset possess the cAMP transduction pathway but suitable odorants are as yet unknown (Manzini *et al.*, 2002, 2007a; Manzini & Schild, 2003a). During metamorphosis a complete rearrangement of the main olfactory system takes place; finally, in the adult animal, the PC is filled with air serving as an 'air-nose' whereas the newly formed middle cavity (MC) is filled with water serving as a 'water-nose' (Altner, 1962; Hansen *et al.*, 1998). In addition, both larval and adult *X. laevis* possess a vomeronasal organ (VNO; Hansen *et al.*, 1998).

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Here we describe specific odorants for the larval ORN subset possessing the cAMP transduction pathway, and in preliminary experiments we identified amines as promising candidates. Therefore, here we focus on amine odorants and carefully examine the amine responsiveness of the olfactory system in larval *X. laevis*. In nature amines are mainly produced by bacterial decarboxylation of free amino acids (Silla Santos, 1996) and they have been reported to act as odorants for terrestrial as well as for aquatic vertebrates (Michel *et al.*, 2003; Rolen *et al.*, 2003; Takahashi *et al.*, 2004; Mori *et al.*, 2006). Amines have been used as odorants in electrophysiological (Michel *et al.*, 2003; Rolen *et al.*, 2003) and imaging (Takahashi *et al.*, 2004; Mori *et al.*, 2006) experiments. However, odorant effects mediated by amines in individual ORNs *in situ* have not yet been reported.

Recently, trace amine-associated receptors (TAARs), a class of G protein-coupled receptors with a broad tissue distribution (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001), have been shown to be expressed in ORNs of mouse OE in a similar way to olfactory receptors (ORs; Liberles & Buck, 2006). These ORNs coexpress $G\alpha_{olf}$, indicating that TAARs transduce signals via the classical cAMP transduction pathway (Liberles & Buck, 2006). Furthermore, Liberles & Buck (2006) showed that mouse and human TAARs specifically detect amines. TAARs have also been shown to be present in cells of the murine Grueneberg ganglion, a putative chemosensory system (Fleischer *et al.*, 2007) and in the olfactory organs of two fish species (Hashiguchi & Nishida, 2007). Together, these results strongly suggest that TAARs function as chemosensory receptors for amines. As the TAAR gene family has been shown to be present also in amphibians (Hashiguchi & Nishida, 2007), here we investigate whether TAARs could be the ORs for amine odorants also in larval *X. laevis*.

Materials and methods

Slices of the olfactory epithelium and nose–brain preparations

Tadpoles of *Xenopus laevis* (stages 50–52; staged after Nieuwkoop & Faber, 1994; see Fig. 1) were cooled to produce complete immobility, and then killed by transection of the brain at its transition to the spinal cord. All procedures for animal handling and tissue dissections were carried out according to the guidelines of the Göttingen University Committee for Ethics in Animal Experimentation. A block of tissue containing the olfactory epithelia, the olfactory nerves and the brain was cut out and kept in bath solution (see below). For the preparation of slices of the OE the tissue was glued onto the stage of a vibroslicer (VT 1000S, Leica, Bensheim, Germany) and cut horizontally into 140–150-μm-thick slices. For nose–brain preparations only the dorsal surface of the olfactory bulbs (OBs) was sliced off. Thereby the cutting angle was chosen in a way to enter the OBs straight above the olfactory nerve entrance. The olfactory epithelia were left intact. For a more detailed description of these preparations see earlier studies from our laboratory (Manzini *et al.*, 2002; Manzini & Schild, 2003a; Czesnik *et al.*, 2003). The slices of the OE and the nose–brain preparations (see Fig. 1) were then transferred to a recording chamber, and 200 μl of bath solution (see later) containing 50 μM Fluo-4/AM (Molecular Probes, Leiden, The Netherlands) was added. Fluo-4/AM was dissolved in DMSO (Sigma, Deisenhofen, Germany) and Pluronic F-127 (Molecular Probes). ORNs of larval *X. laevis* express multidrug resistance transporters (Manzini & Schild, 2003b; Manzini *et al.*, 2008) with a wide substrate spectrum, including Ca^{2+}-indicator dyes. To avoid transporter-mediated destaining of the slices, 50 μM MK571 (Alexis Biochemicals, Grünberg, Germany), an inhibitor of multidrug transporters, was added to the incubation solution. After incubation on a shaker at room temperature for 35 mins, the tissue slices were fixed with a grid in a recording chamber and placed on the microscope stage of an Axiovert 100M (Zeiss, Jena, Germany) to which a laser scanning unit (LSM 510, Zeiss) was attached. Before starting the Ca^{2+} imaging experiments, the slices were rinsed with bath solution for at least 10 mins.

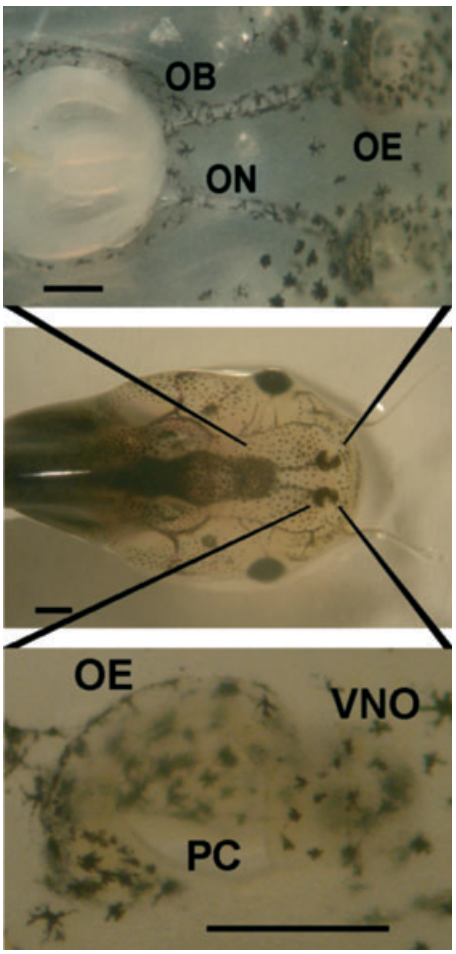


FIG. 1. Acute slice preparation of the olfactory epithelium and nose–brain preparation of larval *Xenopus laevis*. Larval *X. laevis* (middle image). The upper image shows the nose–brain preparation. Note that the OE and the olfactory nerve are left intact in this preparation; only the upper part of the brain, i.e. the OB, has been cut to gain access to the OB cells. The lower image shows an acute slice of the OE. OE, olfactory epithelium; ON, olfactory nerve; OB, olfactory bulb; VNO, vomeronasal organ; PC, principal cavity. Scale bars, 500 μm (upper and lower image); 1 mm (middle image).

Ca^{2+} imaging and data evaluation

Intracellular Ca^{2+} was monitored using a laser-scanning confocal microscope (LSM 510/Axiovert 100M, Zeiss). The thickness of the optical slice excluded fluorescence from more than one cell layer. Fluorescence images (excitation at 488 nm; emission > 505 nm) of the OE or the OB were acquired at 1.27 Hz and 786.4 ms exposure time per image with about 10 images taken as control images before the onset of stimulus delivery. Fluorescence changes $\Delta F/F$ were calculated for individual cells as $\Delta F/F = (F_1 - F_2)/F_2$, where F_1 was the fluorescence averaged over the pixels of a cell, while F_2 was the average fluorescence of that cell prior to stimulus application, averaged over five images. A response was assumed if the following two criteria were met: (i) the first two fluorescence intensity values

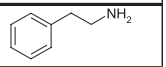
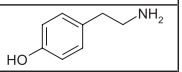
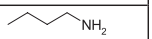
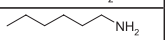
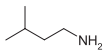
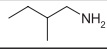
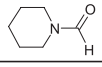
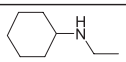
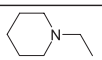
after stimulus arrival at the OE, $I(t_1)$ and $I(t_2)$, had to be larger than the maximum of the prestimulus fluorescence intensities; and (ii) $I(t_2) > I(t_1)$ with $t_2 > t_1$.

The data were analyzed using custom written programs in MATLAB (Mathworks, Natick, MA, USA). To facilitate the selection of regions of interest, a 'pixel correlation map' was obtained by calculating the cross-correlation between the fluorescence signals of a pixel to that of its immediate neighbors and then displaying the resulting value as a grayscale map. As physiological responses often give similar signals in adjacent pixels, this method specifically highlights those pixels. In contrast, pixels that contain only noise show uncorrelated traces and thus appear dark in the cross-correlation map.

Solutions and stimulus application

The composition of the standard bath solution was (in mM): 98 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 glucose, 5 Na-pyruvate, 10 HEPES, 230 mOsmol/l, pH 7.8. All of the chemicals used for the preparation of the bath solution were purchased from Merck (Darmstadt, Germany) or Sigma (St Louis, MO, USA) and were of the highest purity available. As odorants, we used 13 amines (Sigma, listed in Table 1) applied either as a mixture or as single amines. In every experiment the individual amines were applied in random order. Amines were dissolved in bath solution (10 mM stock, each) and used at a final concentration of 200 μM in all of the experiments. Forskolin (Sigma) was dissolved in DMSO (stock of 10 mM) and used at a final concentration of 50 μM. LY83583 (Sigma) was dissolved in DMSO (stock of 2 mM) and used at a final concentration of 200 μM. Stimulus solutions were prepared immediately before use by dissolving the respective stock in bath solution. The bath solution was applied by gravity feed from a storage syringe through a funnel drug applicator to the recording chamber. The tip of the applicator was placed directly above the OE. The odorants were pipetted directly into the funnel without stopping the flow. Outflow was through a syringe needle placed close to the OE.

TABLE 1. List of amines (all used at a final concentration of 200 μM)

Amine	Structure
2-Phenylethylamine (1)	
Tyramine (2)	
Butylamine (3)	
Cyclohexylamine (4)	
Hexylamine (5)	
3-Methylbutylamine (6)	
N,N-Dimethylethylamine (7)	
2-Methylbutylamine (8)	
1-Formylpiperidine (9)	
2-Methylpiperidine (10)	
N-Ethylcyclohexylamine (11)	
1-Ethylpiperidine (12)	
Piperidine (13)	

Identification, isolation, sequencing and expression of TAAR genes in X. laevis

The *Xenopus tropicalis* TAAR gene family (Ensembl Family ID: fam50v00000000600) includes three genes. Two of them (TAAR 1, Ensembl Gene ID ENSXETG00000000106; TAAR 2, Ensembl Gene ID ENSXETG00000000103) are clearly annotated as TAARs. The third gene of this family (Ensembl Gene ID ENSXETG00000010365) is not clearly annotated as a TAAR. For this reason we focused our analysis only on the two clearly annotated TAAR genes. To verify the existence of these TAARs in *X. laevis*, we prepared genomic DNA from the tail of a larval *X. laevis* using a DNA Isolation Kit (Hain Lifescience GmbH, Nehren, Germany). The homologous *X. laevis* TAARs were isolated via PCR using primers designed for the two *X. tropicalis* TAAR sequences mentioned above. The primers were: TAAR 1 fw (5′-GCCTTCACAATGGTATTTCTGG-3′), TAAR 1 rev (5′-CCTATCTCTGCTTCGGGACAC- 3′), TAAR 2 fw (5′-ACT-TGGTCTGTTTCCTGTGTGTTT T-3′), TAAR 2 rev (5′-TGGAAAC-TATGGTGGTTATGTACAAG-3′). The following conditions were used: 94 °C denaturation (2 min), 47 °C annealing (2 min), 72 °C extension (4 min) followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 47 °C (1 min) and 72 °C (1.5 min) and a final extension at 72 °C (7 min). PCR products were sequenced by Seqlab-Sequence Laboratories (Göttingen, Germany). To test TAAR expression in the olfactory organ and the brain of *X. laevis*, we isolated total RNA from the olfactory organ as well as from the brain of *Xenopus* larvae (stage 50–52; Nieuwkoop & Faber, 1994), while in adult *X. laevis* we isolated the RNA from the PC, MC and the brain. Standard protocols including DNase treatment were used. cDNA was generated with $(dT)_{15}$ and random hexamer primers. PCR amplifications were performed using TAAR primers and conditions as above. GAPDH (*X. laevis*; GenBank accession number U41753) with the following specific intron-spanning primer pair: fw, 5′-TTCACAAC-CACAGAGAAGGC-3′, rev 5′-TGGACTGTTGTCATGAGTCC-3′ or beta-actin (*X. laevis*; GenBank accession number: AF079161) with the following specific intron-spanning primer pair: fw 5′-AC-CAGAAGAACACCCAGTGCT-3′, rev 5′-TGGGTTACACCATCA-CCTGAG-3′ were chosen as reference genes. PCR conditions were the same as for TAAR primers except for the annealing temperature, which was set to 51 °C for GAPDH and 58 °C for beta-actin.

Results

Identification of amines as olfactory stimuli in larval X. laevis

We tested whether amines are suitable olfactory stimuli in larval *X. laevis* with the aid of functional Ca^{2+} imaging in acute slice preparations of the OE. Figure 2(A) shows an OE slice stained with the calcium-indicator dye Fluo-4. The encircled ORNs were responsive to a mixture of 13 amines (see Table 1; 200 μM each) as seen from the increase of the intracellular calcium concentration ($[Ca^{2+}]_i$) in Fig. 2B–D. Figure 2(E) shows amine-induced time courses of $[Ca^{2+}]_i$ transients of the four amine-responsive ORNs of this OE slice. Shape and duration of the $[Ca^{2+}]_i$ transients were highly reproducible when the mixture of amines was applied repeatedly. Application of bath

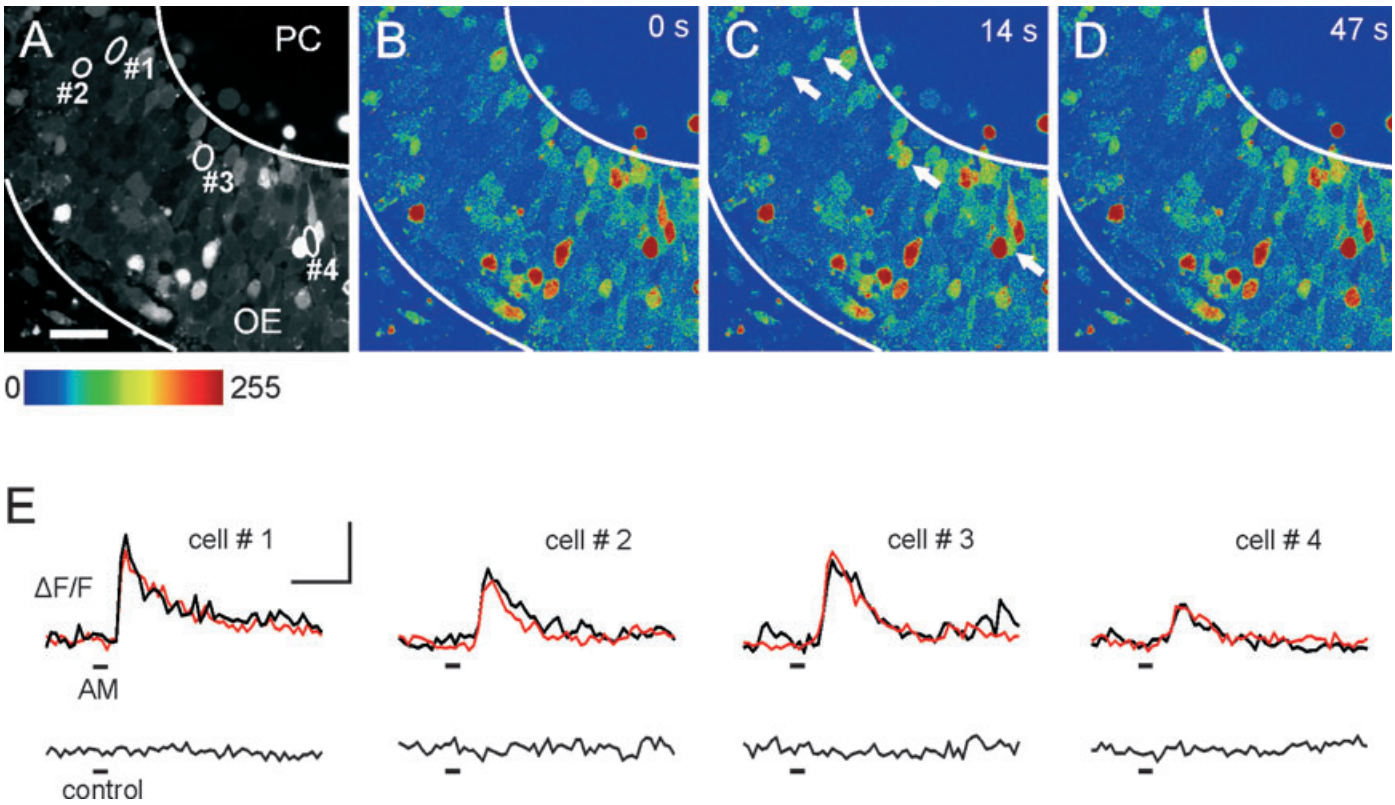


FIG. 2. Acute slice of the olfactory epithelium and amine-induced changes in calcium-dependent fluorescence in individual olfactory receptor neurons. (A) Fluorescence image of an acute slice of the OE (image acquired at rest; OE, olfactory epithelium; PC, principal cavity) stained with Fluo-4/AM. Amine-sensitive ORNs are encircled and marked with #1 to #4. (B–D) Sequence of pseudocolored images of the OE slice showing that stimulation with the mixture of 13 amines (AM; 200 μM each) transiently increases the calcium-dependent fluorescence in the ORNs encircled in A (see arrows in C). (B) ORNs before stimulus application [time (t), 0 s]. (C) At the peak of the response (t, 14 s) and (D) after return to the basal fluorescence level (t, 47 s). (E) Time courses of $[Ca^{2+}]_i$ transients of the responsive ORNs labeled in A. The time course and amplitude of amine-induced $[Ca^{2+}]_i$ increases in individual ORNs were highly reproducible if the mixture of amines was applied repeatedly (black trace, 1st application; red trace, 2nd application). Application of bath solution as a control stimulus showed no comparable response (lower traces). Scale bar in A, 30 μM. Scale bars in E, 10 s and $\Delta F/F$ 100%.

solution as a negative control stimulus did not evoke any comparable response in these (Fig. 2E, lower traces) nor in any of the other 60 ORNs (n = 14 slices) that responded upon application of the mixture of amines.

The observed odorant-induced $[Ca^{2+}]_i$ changes in ORNs allow only indirect conclusions regarding the information conveyed to higher olfactory centers. We therefore used a nose–brain preparation (see Methods) and tested whether application of amines onto the intact OE induces responses in OB neurons. Figure 3(A) shows the OB of a nose–brain preparation stained with Fluo-4. Application of the mixture of amines to the intact ipsilateral OE induced changes of the Ca^{2+}-dependent fluorescence in cells of the mitral and the granule cell layer of the corresponding OB (Fig. 3B). In all tested nose–brain preparations mucosal amine application led to activation of OB cells (76 cells, n = 9 slices). The reproducibility of this and other responses shown throughout this paper was verified by regularly repeating the application of most of the odorants at least twice. The corresponding time courses of the $[Ca^{2+}]_i$ transient of the three OB cells marked in Fig. 3B are plotted in Fig. 3C. Application of bath solution as a negative control stimulus did not evoke any comparable response (Fig. 3D). After transection of the ipsilateral olfactory nerve these OB cells no longer responded (Fig. 3E). To verify the viability of the nose–brain preparation following nerve transection, we applied the mixture of amines to the contralateral OE and recorded responses of cells of the contralateral OB. Figure 3(F) shows time courses of $[Ca^{2+}]_i$ transients of three randomly chosen cells of the contralateral OB. Taken together, these experiments leave no doubt that amines are suitable olfactory stimuli in larval *X. laevis*.

Transduction mechanisms of amine-sensitive ORNs

To gain information about the transduction mechanism of amine-responsive ORNs we additionally applied forskolin as a pseudostimulus to a number of OE slices. In this way it was possible to stimulate all ORNs in a slice that would, under appropriate natural conditions, respond to a cAMP-mediated odorant. Figure 4(A) and B display the ORNs of the same OE slice activated upon mucosal application of the mixture of amines (200 μM) and forskolin (50 μM), respectively. Figure 4(C) summarizes these results by showing the forskolin-sensitive ORNs in blue, the amine-sensitive ORNs in red and those sensitive to both, forskolin and amines, in green. In this slice, three of the four amine-sensitive ORNs were sensitive also to forskolin, showing that these three ORNs possess the cAMP-mediated transduction pathway. Figure 4(D) shows time courses of the $[Ca^{2+}]_i$ transients of the ORN marked with an asterisk in Fig. 4C. This ORN responded to both the mixture of amines and forskolin, while

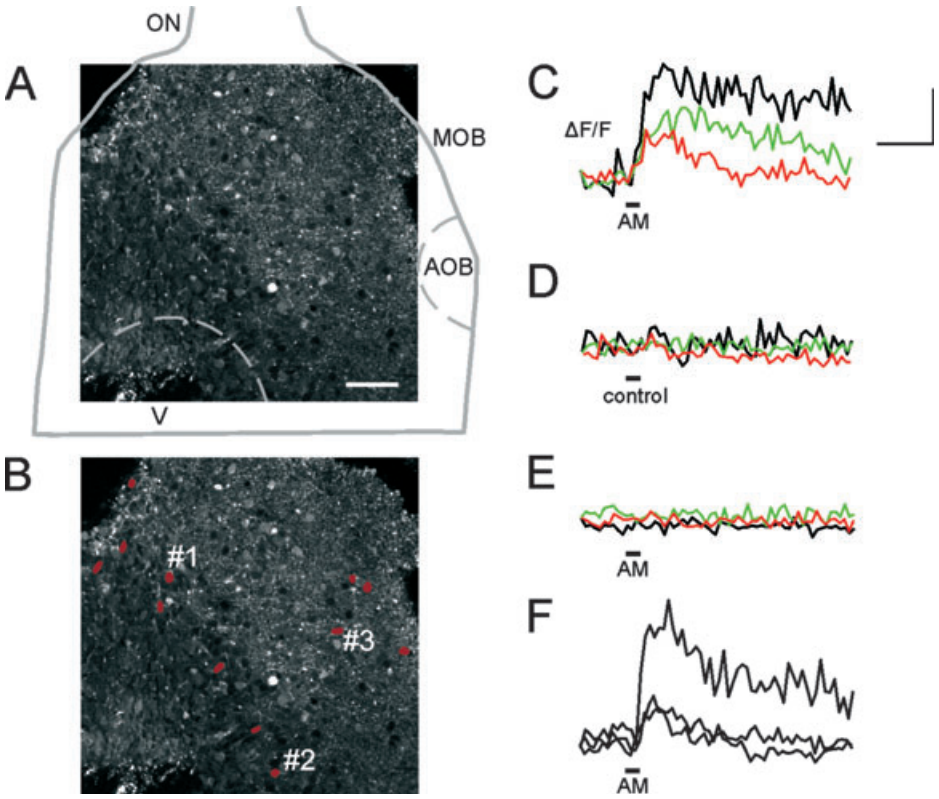


FIG. 3. Nose-brain preparation and amine-induced changes in calcium-dependent fluorescence in individual olfactory bulb neurons. (A) The imaged region of a Fluo-4/AM-stained nose–brain preparation has been superimposed to a sketch of an OB (image acquired at rest; ON, olfactory nerve; AOB, accessory olfactory bulb; MOB, main olfactory bulb; V, ventricle). (B) Cells activated by mucosal application of a mixture of 13 amines (AM; 200 μM each) are marked in red. (C) Time courses of $[Ca^{2+}]_i$ transients of the three OB cells marked in B evoked by mucosal application of the mixture of amines (cell #1, red trace; cell #2, green trace and cell #3, black trace). (D) Mucosal application of bath solution as a control stimulus showed no comparable response. (E) After transection of the ipsilateral ON these OB cells no longer respond to mucosal amine application. (F) Cells of the contralateral OB of the same nose–brain preparation still respond to mucosal application of the amine mixture. Scale bar in A, 50 μm. Scale bars for C–F, 10 s and $\Delta F/F$ 100%.

application of bath solution as negative control stimulus elicited no response. Thereby, the two classes of stimuli used, i.e. amines on the one hand and forskolin on the other, differed in the time course of their respective responses. The responses to amines always had a fast time course and the responses to forskolin typically showed a much slower time course (Fig. 4D). In all the slices tested (n = 27), 693 ORNs responded either to the mixture of amines or to forskolin. Thirty-six ORNs responded solely to the mixture of amines, 580 ORN solely to forskolin and 77 ORNs responded to both stimuli (Fig. 4E). Averaged over all slices the frequencies for an uncorrelated and correlated response were 33.4 ± 5.7 and 66.6 ± 5.7% (mean ± SEM; Fig. 4F), respectively. To substantiate the above results we performed another set of experiments and employed LY83583, which inhibits soluble guanylyl cyclase and cyclic nucleotide-gated (CNG) channels. Fig. 4 (G_{1-3}) shows that the $[Ca^{2+}]_i$ increase of an individual ORN induced by application of the mixture of amines is almost completely blocked by 200 μM LY83583 and recovers after drug wash-out. The amine responses of 24 of 36 ORNs (n = 12 OE slices) were affected by the inhibitor. On average 35.3 ± 11.2% of the amine-sensitive ORNs were not affected by LY83583, whereas 64.7 ± 11.2% were affected by LY83583 (Fig. 4H). These results confirm the above forskolin experiments. Together the two sets of experiments show that the great majority of amine-responsive ORNs possess the cAMP-mediated transduction pathway and suggest that the transduction cascade of the remaining amine-responsive ORNs does not involve cyclic nucleotides.

Response profiles of ORNs to amines

We determined the exact response profile of 102 ORNs (n = 13 OE slices) to the 13 amines included in the amine mixture (see Table 1; 200 μM each). The response profiles of all 102 ORNs are shown in Fig. 5A. The time courses of the $[Ca^{2+}]_i$ transients in response to application of the 13 single amines of three of these 102 ORNs are given in Fig. 5B. The first ORN (blue traces; ORN 18 in Fig. 5A) responded to hexylamine, *N*,*N*-dimethylethylamine, *N*-ethyl-cyclohexylamine, 1-ethylpiperidine and piperidine. The second ORN (red traces; ORN 70 in Fig. 5B) reacted upon application of 2-phenylethylamine and tyramine. The third ORN (green traces; ORN 10 in Fig. 5B) responded only to cyclohexylamine. Within the 102 response profiles, 56 differed from each other and thus formed ORN classes. While 35 of these response profiles were found just once, 21 response profiles occurred more than once (see Table 2). The 21 repetitive response profiles occurred between two and eight times (Table 2). The frequency by which each amine induced a response did not vary markedly between the amines used in this study. 1-formylpiperidine and 1-ethylpiperidine were the least and most effective stimuli, respectively (for more details see Fig. 5C). As more than half of the ORNs responded to more than one amine, the frequencies shown in Fig. 5C total more than 100%. Further evaluation of all response profiles produced a frequency distribution, which displays the proportion of ORNs responding to a definite number of effective stimuli (Fig. 5D). Notably, almost half of the 102

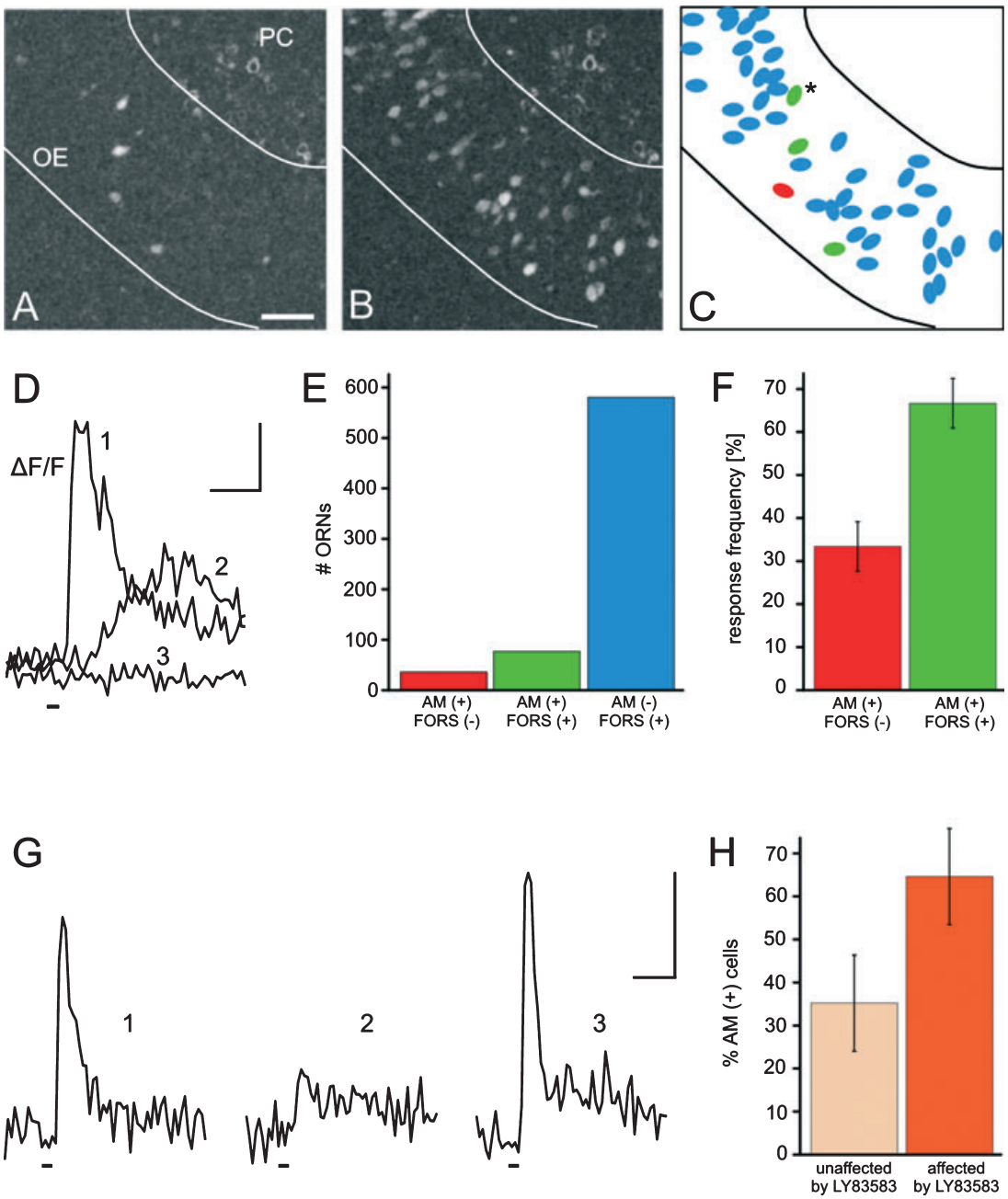
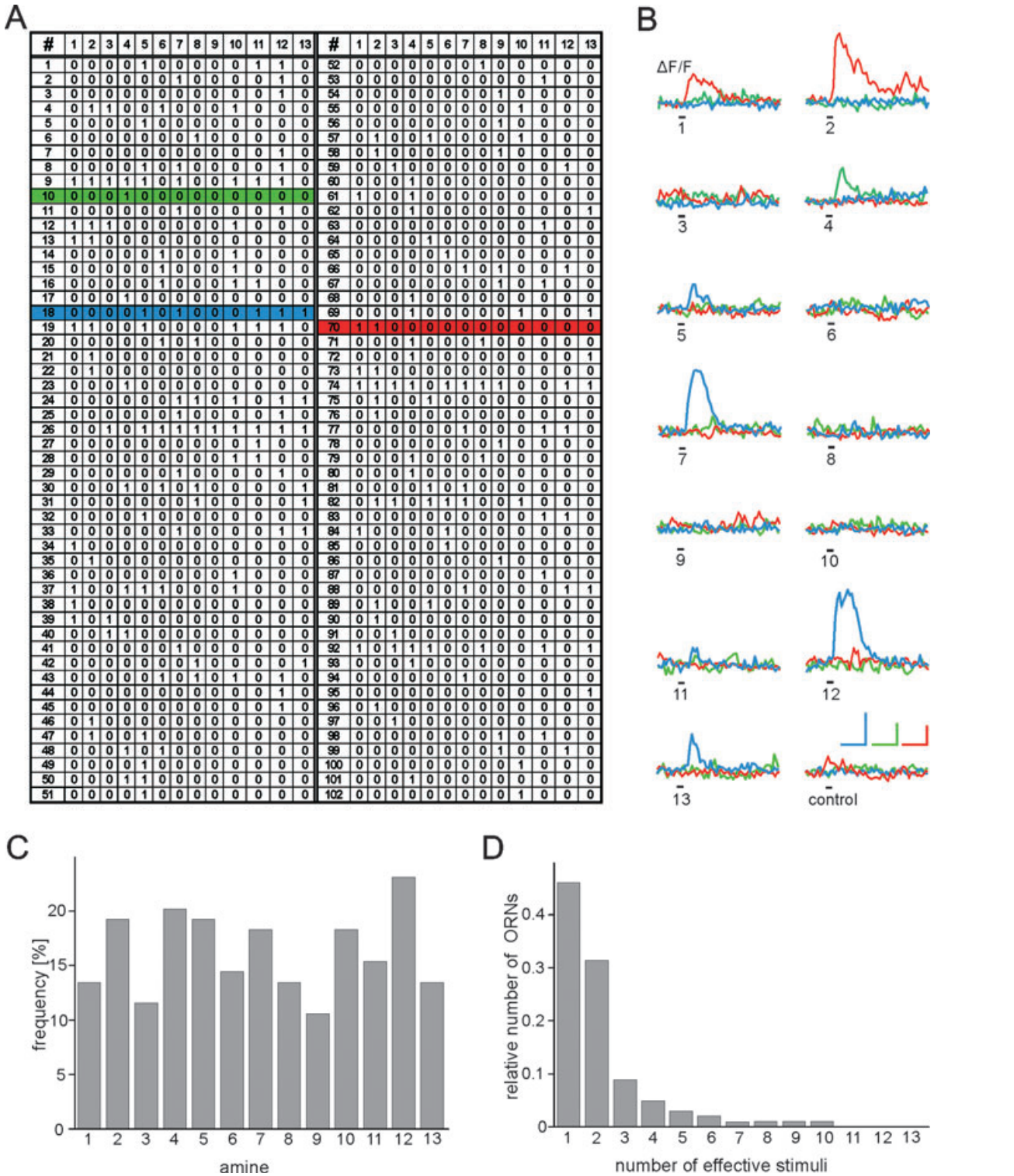


FIG. 4. Comparison of changes of calcium-dependent fluorescence in olfactory receptor neurons of an acute slice of the olfactory epithelium in response to stimulation with amines and forskolin and influence of LY83583 on amine-induced responses. (A) ORNs of an acute slice preparation of the OE (grayscale map, see Methods) activated by application of the mixture of 13 amines (AM; 200 μM each). (B) ORNs of the same acute slice preparation as in A (grayscale map) activated by application of forskolin (50 μM). (C) Schematic diagram showing superposition of amine- and forskolin-sensitive ORNs (visible in A and B). ORNs sensitive to amines (red), to forskolin (blue) and to both stimuli (green). (D) Time course of $[Ca^{2+}]_i$ transients of the ORN marked with an asterisk in C. Calcium-dependent fluorescence changes upon application of the mixture of amines (trace 1), forskolin (trace 2) and bath solution as a control stimulus (trace 3). (E) Occurrences of correlated and uncorrelated responses to amines and forskolin plotted as a histogram (n = 27 slices). (F) Frequency of correlated and uncorrelated responses to amines and forskolin in all amine-sensitive ORNs given as the mean ± SEM over slices (n = 27 slices, 113 cells). (G) Time course of $[Ca^{2+}]_i$ transients of an individual ORN sensitive to amines taken from a different OE slice. Calcium-dependent fluorescence changes upon application of the mixture of amines (200 μM; trace 1). After addition of LY83583 (200 μM) to the bath solution the amine-induced $[Ca^{2+}]_i$ transient was clearly smaller but still present (trace 2). After drug wash-out the amine-induced $[Ca^{2+}]_i$ transient recovered completely (trace 3). (H) Percentages of ORNs responsive to the mixture of amines, which were affected and unaffected by LY83583 given as the mean ± SEM over slices (n = 12 slices, 36 cells). Scale bar in A, 30 μm. Scale bars in D and G, 10 s and $\Delta F/F$ 50%.

ORNs responded only to one of the 13 amines. This unambiguous tendency of ORNs to respond to few rather than to many amines indicates that most of the amine-sensitive ORNs are highly specific for individual amines.

Expression of TAAR genes in the olfactory organ of X. laevis

Recently, it has been shown that in the mouse olfactory system TAARs may function as ORs for amines (Liberles & Buck, 2006). To check



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3	0	0	0	0	0	0	0	0	0	0	0	1	0
4	0	1	1	0	0	1	0	0	0	1	0	0	0
5	0	0	0	0	1	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	1	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	1	0
8	0	0	0	0	1	0	1	0	0	0	0	1	0
9	1	1	1	1	1	0	1	0	0	1	1	1	0
10	0	0	0	1	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	1	0	0	0	0	1	0
12	1	1	1	0	0	0	0	0	0	1	0	0	0
13	1	1	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	1	0	0	0	1	0	0	0
15	0	0	0	0	0	1	0	0	0	1	0	0	0
16	0	0	0	0	0	1	0	0	0	1	1	0	0
17	0	0	0	0	1	0	0	0	0	0	0	0	0
18	0	0	0	0	0	1	0	1	0	0	0	1	1
19	1	1	0	0	1	0	0	0	0	1	1	1	0
20	0	0	0	0	0	1	0	1	0	0	0	0	0
21	0	1	0	0	0	0	0	0	0	0	0	0	0
22	0	1	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	1	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	1	1	0	1	0	1	1
25	0	0	0	0	0	0	1	0	0	0	0	1	0
26	0	0	1	0	1	1	1	1	1	1	1	1	1
27	0	0	0	0	0	0	0	0	0	1	0	0	0
28	0	0	0	0	0	0	0	0	0	1	1	0	0
29	0	0	0	0	0	0	1	0	0	0	0	1	0
30	0	0	0	1	0	1	0	1	0	0	0	0	1
31	0	0	0	0	0	0	0	1	0	0	0	0	1
32	0	0	0	0	1	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	1	0	0	0	0	1	1
34	1	0	0	0	0	0	0	0	0	0	0	0	0
35	0	1	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	1	0	0	0
37	1	0	0	1	1	1	0	0	0	1	0	0	0
38	1	0	0	0	0	0	0	0	0	0	0	0	0
39	1	0	1	0	0	0	0	0	0	0	0	0	0
40	0	0	1	1	0	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	1	0	0	0	0	0	0
42	0	0	0	0	0	0	0	1	0	0	0	0	1
43	0	0	0	0	0	1	0	1	0	1	0	1	0
44	0	0	0	0	0	0	0	0	0	0	0	1	0
45	0	0	0	0	0	0	0	0	0	0	0	1	0
46	0	1	0	0	0	0	0	0	0	0	0	0	0
47	0	1	0	0	1	0	0	0	0	0	0	0	0
48	0	0	0	1	0	1	0	0	0	0	0	0	0
49	0	0	0	0	1	0	0	0	0	0	0	0	0
50	0	0	0	1	1	0	0	0	0	0	0	0	0
51	0	0	0	0	1	0	0	0	0	0	0	0	0
52	0	0	0	0	0	0	0	1	0	0	0	0	0
53	0	0	0	0	0	0	0	0	0	0	1	0	0
54	0	0	0	0	0	0	0	0	1	0	0	0	0
55	0	0	0	0	0	0	0	0	0	1	0	0	0
56	0	0	0	0	0	0	0	0	1	0	0	0	0
57	0	1	0	0	1	0	0	0	0	1	0	0	0
58	0	1	0	0	0	0	0	0	1	0	0	0	0
59	0	0	1	0	0	0	0	0	0	0	0	1	0
60	0	0	0	1	0	0	0	0	0	0	0	0	0
61	1	0	0	1	0	0	0	0	0	0	0	0	0
62	0	0	0	1	0	0	0	0	0	0	0	0	1
63	0	0	0	0	0	0	0	0	0	0	1	0	0
64	0	0	0	0	1	0	0	0	0	0	0	0	0
65	0	0	0	0	0	1	0	0	0	0	0	0	0
66	0	0	0	0	0	0	1	0	1	0	0	1	0
67	0	0	0	0	0	0	0	0	1	0	1	0	0
68	0	0	0	1	0	0	0	0	0	0	0	0	0
69	0	0	0	1	0	0	0	0	0	1	0	0	1
70	1	1	0	0	0	0	0	0	0	0	0	0	0
71	0	0	0	1	0	0	0	1	0	0	0	0	0
72	0	0	0	1	0	0	0	0	0	0	0	0	1
73	1	1	0	0	0	0	0	0	0	0	0	0	0
74	1	1	1	1	0	1	1	1	1	0	0	1	1
75	0	1	0	0	1	0	0	0	0	0	0	0	0
76	0	1	0	0	0	0	0	0	0	0	0	0	0
77	0	0	0	0	0	0	1	0	0	0	1	1	0
78	0	0	0	0	0	0	0	0	0	1	0	0	0
79	0	0	0	1	0	0	0	1	0	0	0	0	0
80	0	0	0	1	0	0	0	0	0	0	0	0	0
81	0	0	0	0	1	0	1	0	0	0	0	0	0
82	0	1	1	0	1	1	1	0	0	1	0	0	0
83	0	0	0	0	0	0	0	0	0	0	1	1	0
84	1	0	0	0	0	1	0	0	0	0	0	0	0
85	0	0	0	0	0	1	0	0	0	0	0	0	0
86	0	0	0	0	0	0	0	0	1	0	0	0	0
87	0	0	0	0	0	0	0	0	0	0	1	0	0
88	0	0	0	0	0	0	0	1	0	0	0	1	1
89	0	1	0	0	1	0	0	0	0	0	0	0	0
90	0	1	0	0	0	0	0	0	0	0	0	0	0
91	0	0	1	0	0	0	0	0	0	0	0	0	0
92	1	0	1	1	1	0	0	1	0	0	1	0	1
93	0	0	0	1	0	0	0	0	0	0	0	0	0
94	0	0	0	0	0	0	0	1	0	0	0	0	0
95	0	0	0	0	0	0	0	0	0	0	0	0	1
96	0	1	0	0	0	0	0	0	0	0	0	0	0
97	0	0	1	0	0	0	0	0	0	0	0	0	0
98	0	0	0	0	0	0	0	0	1	0	1	0	0
99	0	0	0	0	0	0	0	0	1	0	0	1	0
100	0	0	0	0	0	0	0	0	0	1	0	0	0
101	0	0	0	1	0	0	0	0	0	0	0	0	0
102	0	0	0	0	0	0	0	0	0	1	0	0	0

FIG. 5. Response profiles of olfactory receptor neurons to amines and evaluation histograms of response frequencies to amines and number of effective amines per olfactory receptor neuron. (A) 102 × 13 matrix representing the responses of the 102 ORNs each tested for 13 amines (1 = 2-phenylethylamine; 2 = tyramine; 3 = butylamine; 4 = cyclohexylamine; 5 = hexylamine; 6 = 3-methylbutylamine; 7 = *N*,*N*-dimethylethylamine; 8 = 2-methylbutylamine; 9 = 1-formylpiperidine; 10 = 2-methylpiperidine; 11 = *N*-ethylcyclohexylamine; 12 = 1-ethylpiperidine; 13 = piperidine). Each amine was applied at a concentration of 200 μM. A '1' or a '0' in the matrix indicates whether or not a particular ORN responded to a particular amine (1 = response; 0 = no response). Time courses and response amplitudes were neglected. (B) Time courses of $[Ca^{2+}]_i$ transients of three individual ORNs selected from the matrix (green trace: ORN #10; blue trace: ORN #18; red trace: ORN #70). (C) The histogram shows for each of the 13 amines used in how many ORNs (of 102) a response was observed. Results are plotted as relative numbers, i.e. normalized to 102. As various ORNs responded to more than one amine, the sum of the response frequencies is higher than 100%. (Response frequencies in detail: 1 = 13.5%; 2 = 19.2%; 3 = 11.5%; 4 = 20.2%; 5 = 19.2%; 6 = 14.4%; 7 = 18.3%; 8 = 13.5%; 9 = 10.6%; 10 = 18.3%; 11 = 15.4%; 12 = 23.1%; 13 = 13.5%.) (D) Frequencies of ORNs (n = 102) that responded to a certain number n of amines (n out of 13 amines). Scale bars: 10 s (green, blue and red traces); $\Delta F/F$ 50% (red and green traces); $\Delta F/F$ 100% (blue traces).

TABLE 2. Repetitive ORN classes extracted from Fig. 5

Class	Response profiles to amines													ORN numbers (see Fig. 5)
	1	2	3	4	5	6	7	8	9	10	11	12	13	
1	1	1	0	0	0	0	0	0	0	0	0	0	0	13; 70
2	1	0	0	0	0	0	0	0	0	0	0	0	0	34; 38
3	0	1	0	0	0	0	0	0	0	0	0	0	0	21; 22; 35; 46; 76; 90; 96
4	0	1	0	0	1	0	0	0	0	0	0	0	0	47; 75; 89
5	0	0	1	0	0	0	0	0	0	0	0	0	0	91; 97
6	0	0	0	1	0	0	0	0	0	0	0	0	0	10; 17; 23; 60; 68; 80; 93; 101
7	0	0	0	1	0	0	0	0	0	0	0	0	1	62; 72
8	0	0	0	1	0	0	0	1	0	0	0	0	0	71; 79
9	0	0	0	0	1	0	0	0	0	0	0	0	0	5; 32; 49; 51; 64
10	0	0	0	0	0	1	0	0	0	1	0	0	0	14; 15
11	0	0	0	0	0	1	0	0	0	0	0	0	0	65; 85
12	0	0	0	0	0	0	1	0	0	0	0	1	0	2; 11; 25; 29
13	0	0	0	0	0	0	1	0	0	0	0	1	1	33; 88
14	0	0	0	0	0	0	1	0	0	0	0	0	0	41; 94
15	0	0	0	0	0	0	0	1	0	0	0	0	1	31; 42
16	0	0	0	0	0	0	0	1	0	0	0	0	0	6; 52
17	0	0	0	0	0	0	0	0	1	0	1	0	0	67; 98
18	0	0	0	0	0	0	0	0	1	0	0	0	0	54; 56; 78; 86
19	0	0	0	0	0	0	0	0	0	1	0	0	0	36; 55; 100; 102
20	0	0	0	0	0	0	0	0	0	0	1	0	0	27; 53; 63; 87
21	0	0	0	0	0	0	0	0	0	0	0	1	0	3; 7; 44; 45

Classes of identically responding ORNs are represented by their class index, the response profile, and the member ORNs, respectively.

this hypothesis in *X. laevis*, we verified the expression of TAAR genes in its olfactory organ. Based on the respective *X. tropicalis* sequences found in the Ensembl genome database (see Methods), we were able to isolate the three genes included in the *X. tropicalis* TAAR gene family from *X. laevis* genomic DNA. Only two of these genes are clearly annotated as TAARs, namely as TAAR 1 and TAAR 2, in the Ensembl genome database (see Methods). For this reason we focused our analysis only on these two genes. Sequence analysis of the PCR products shows a high homology between the receptors of the two species, with over 94% sequence identity at DNA and protein level (see Supporting information, Figs S1 and S2). To determine whether the TAARs are expressed in cells of the olfactory organ of larval and adult *X. laevis*, we performed RT-PCR using cDNA preparations from their olfactory organ (Fig. 6). Additionally, we checked whether *Xenopus* TAARs were expressed in the brains of larvae and adults. In larvae, TAAR 2, but not TAAR 1, was expressed in the olfactory organ. Neither of the two genes were expresses in brain tissue. In adult *Xenopus*, expression of both TAAR 1 and TAAR 2 was found in the olfactory organ [‘air-nose’ (PC) and ‘water-nose’ (MC)] as well as in the brain.

Discussion

Amines are olfactory stimuli in larval X. laevis

Amines are known olfactory stimuli in terrestrial as well as in aquatic species (Carr & Derby, 1986; Carr *et al.*, 1990; Rolen *et al.*, 2003; Takahashi *et al.*, 2004; Mori *et al.*, 2006). In terrestrial species amine odorants signal the presence of spoiled food and elicit avoidance reactions (Takahashi *et al.*, 2004; Mori *et al.*, 2006). In contrast, for aquatic species there is strong evidence that amine odorants function as feeding cues (Carr & Derby, 1986; Rolen *et al.*, 2003). Application of amines to acute slice preparations of the OE of larval *X. laevis* clearly induced calcium-dependent fluorescence changes in a subset of ORNs. At this point it is certainly important to note that the concentration of the amines applied is important. Although *X. laevis* ORNs do respond to lower amine concentrations as well (data not shown), throughout the experiments reported herein we applied amines at a concentration of 200 μM. This concentration is sufficiently high to activate ORs with high as well as with low affinity for amines. The amines used in the present study were partly the same or structurally related to those used in the study of Liberles & Buck (2006). $[Ca^{2+}]_i$ imaging is a reliable tool to identify cellular sensitivity to the applied substances (Manzini & Schild, 2003a, 2004; Czesnik *et al.*, 2006), but it does not provide definitive proof that the activation occurred via ORs and that the signal is relayed to the OB, i.e. OR-independent calcium increases in OE cells could be erroneously interpreted as odorant responses. The fact that most of the known water-borne odorants, e.g. amino acids, bile acids, nucleotides or amines, are mainly endogenic molecules involved in many metabolic processes enhances the possibility of such a misinterpretation. This eventuality can be excluded by directly measuring the activation of second-order neurons in the OB upon mucosal odorant application. We therefore employed a nose–brain preparation of our experimental animal (Manzini *et al.*, 2002; Czesnik *et al.*, 2003) and showed that the signals generated by ORNs, when stimulated with amines, are relayed to the OB. In this way we could clearly show that in addition to amino acids (Manzini *et al.*, 2002; Manzini & Schild, 2003a), also amines are a class of molecules sensed by the *X. laevis* olfactory system.

Correlation between ORN subset and sensitivity to amines

We have previously shown that the main olfactory system of larval *X. laevis* is made up of at least two diverse subsystems consisting of ORNs with different transduction mechanisms (cAMP-independent and cAMP-dependent), different projection targets in the OB and an unequal expression of presynaptic proteins (Manzini *et al.*, 2002, 2007a; b; Manzini & Schild, 2003a). ORNs lacking the cAMP-dependent transduction mechanism project to glomeruli in the lateral

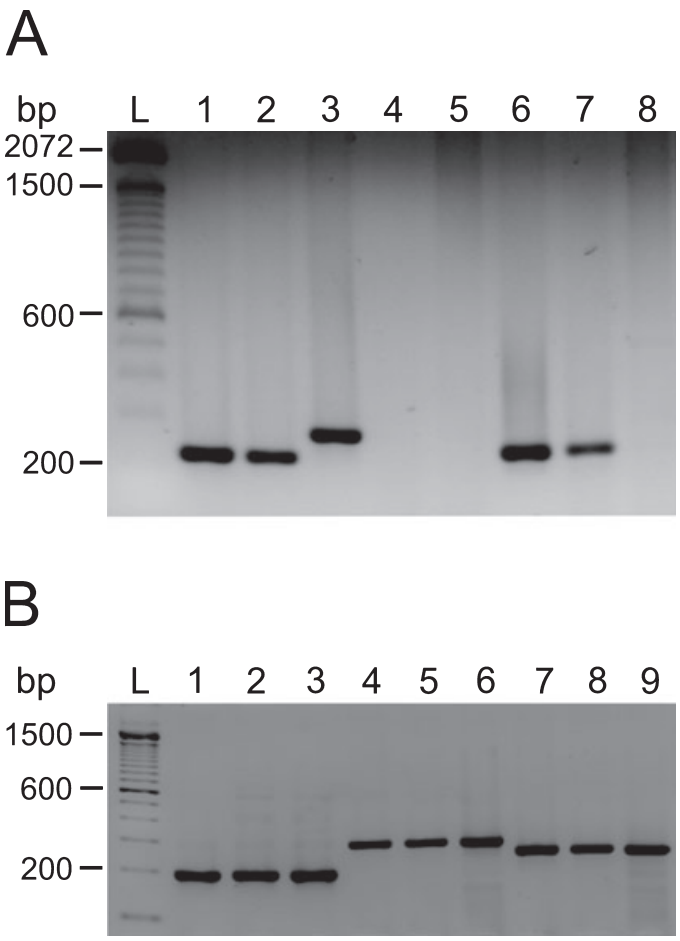

FIG. 6. Identification of TAAR gene expression in the olfactory organ and brain of larval and adult *X. laevis.* RT-PCR analysis of TAAR 1 and TAAR 2 (for detailed information see Methods) mRNA in the olfactory organ and brain of larval (A) and adult (B) *X. laevis*. The 285-bp and 251-bp products represent *X. laevis* TAAR 1 and TAAR 2, respectively. Control GAPDH (233 bp) and beta-actin (191 bp) RT-PCR reactions are shown. (A) L, DNA-Ladder; 1, GAPDH (olfactory organ); 2, GAPDH (brain); 3, TAAR 1 (genomic DNA); 4, TAAR 1 (olfactory organ); 5, TAAR 1 (brain); 6, TAAR 2 (genomic DNA); 7, TAAR 2 (olfactory organ); 8, TAAR 2 (brain). (B) L, DNA-Ladder; 1, beta-actin (middle cavity); 2, beta-actin (principal cavity); 3, beta-actin (brain); 4, TAAR 1 (middle cavity); 5, TAAR 1 (principal cavity); 6, TAAR 1 (brain); 7, TAAR 2 (middle cavity); 8, TAAR 2 (principal cavity); 9, TAAR 2 (brain).

OB, and ORNs possessing the cAMP-dependent transduction mechanism project to glomeruli in the medial OB. A similar subdivision of the olfactory system has been reported also in other aquatic species (channel catfish: Hansen *et al.*, 2003; zebrafish: Sato *et al.*, 2005). In larval *X. laevis*, amino acid stimuli have been shown to be transduced in a cAMP-independent way (Manzini *et al.*, 2002, 2007a,b; Manzini & Schild, 2003a). To allocate amine stimuli to one of the above mentioned subsystems, in some experiments we applied forskolin, an activator of the cAMP-dependent transduction pathway, in addition to amines. Forskolin has been successfully used in studies with *X. laevis* ORNs (Manzini *et al.*, 2002; Manzini & Schild, 2003a). The fact that about two-thirds of the amine-sensitive ORNs could be activated also by forskolin shows that the great majority of amine-sensitive ORNs belong to the ORN subset possessing the cAMP transduction pathway. To confirm the above results, in another set of experiments we employed LY83583, an inhibitor of the soluble guanylyl cyclase and of CNG-gated channels (Leinders-Zufall & Zufall, 1995; Leinders-Zufall *et al.*, 1997). Fully in line with the forskolin experiments this inhibitor blocked the amine responses in about two-thirds of the ORNs. At present the transduction pathway of the remaining amine-sensitive ORNs is unknown. The results of the present work suggest that their transduction cascade does not involve cyclic nucleotides. The phospholipase C/IP_3-mediated transduction cascade is certainly a candidate (for a review see Ref. Schild & Restrepo, 1998), but this remains speculation. In addition, it cannot be ruled out that some of these ORNs have multiple transduction cascades. As we always applied a mixture of 13 amines, we do not know whether particular amines of the mixture are responsible for the activation of the cAMP-dependent and cAMP-independent ORN subgroups. At this point it is certainly interesting to note that the subset of amine-responsive ORNs lacking the cAMP-dependent transduction pathway never responded to amino acids (data not shown). This shows that in larval *X. laevis* there is a complete disjunction of amino acid- and amine-sensitive ORNs. This implies that the odorant-receptive sites of ORs specific for amino acids and for amines do not solely bind the amino group ($-NH_2$), present in both groups of molecules. Similarly, amino acids and amines have been shown to have independent receptor sites also in the olfactory organ of zebrafish and goldfish (Michel *et al.*, 2003; Rolen *et al.*, 2003).

Response profiles to amines of individual ORNs

We determined the specificity profiles of an extensive number of ORNs to the 13 amines listed in Table 1. The number of different amine response profiles recorded is fairly high. Fifty-six, i.e. 54%, of the 102 response profiles were different from each other. In comparison, 204, i.e. 72%, of the 283 response profiles for amino acids recorded in a previous study differed from each other (Manzini & Schild, 2004). Regarding the number of effective stimuli per ORN, amine-responsive ORNs show a clear tendency to respond to fewer stimuli than amino acid-responsive ORNs. While more than 75% of the amine-sensitive ORNs responded only to one or two amines and only 6% responded to more than five amines, only about 27% of the amino acid-sensitive ORNs responded to one or two amino acids and about 50% of them responded to more than five amino acids (Manzini & Schild, 2004). This shows that many of the ORNs responsive to amines were highly specific in the sense that they responded only to one of the 13 amines. This, in turn, suggests that in larval *X. laevis* there are at least 13 different ORs that respond to amines. The large number of 56 different response profiles to amines can, however, hardly be explained with 13 ORs responsive to amines, at least not if assuming that every ORN expresses only one type of OR, as is generally accepted for adult higher vertebrates (Nef *et al.*, 1992; Strotmann *et al.*, 1992; Ressler *et al.*, 1993; Vassar *et al.*, 1993; Chess *et al.*, 1994; Malnic *et al.*, 1999; Serizawa *et al.*, 2000). The possibility that ORNs of *X. laevis*, at least in its larval stages, express more than one OR per ORN has already been discussed for ORNs responsive to amino acids (Manzini & Schild, 2004; Schild & Manzini, 2004; Manzini *et al.*, 2007b). The 'one OR per ORN' rule has never been conclusively demonstrated (Mombaerts, 2004). One study even provided evidence for coexpression of two particular rat ORs (Rawson *et al.*, 2000), but it was not determined whether both are functional. A recent study in mouse has shown that ORNs in the septal organ are also able to express multiple ORs (Tian & Ma, 2008). In invertebrates, e.g. *Caenorhabditis elegans* (Troemel *et al.*, 1995; Robertson, 1998) or *Drosophila* (Goldman *et al.*, 2005; Ray *et al.*, 2007), multiple OR expression in single chemosensory cells/ORNs has already been documented.

TAAR expression in X. laevis

Recent studies in mice have shown that a number of TAARs are selectively expressed in ORNs of the main OE and in neurons of the Grueneberg ganglion in a similar way as ORs (Liberles & Buck, 2006; Fleischer *et al.*, 2007). TAAR genes have been shown to be expressed also in the olfactory organs of zebrafish and stickleback (Hashiguchi & Nishida, 2007), two aquatic vertebrates. Moreover, mouse and human TAARs heterologously expressed in HEK 293 cells specifically respond to amines (Liberles & Buck, 2006). These data strongly suggest that TAARs could function as specific ORs for amines. Motivated by the above evidence we verified whether TAAR genes are expressed also in the olfactory organ of *X. laevis*. It is reported that the genome of *X. tropicalis* includes 2–6 putatively functional TAAR genes (Hashiguchi & Nishida, 2007; Grus *et al.*, 2007; Grus & Zhang, 2008). In the Ensembl genome database (http://www.ensembl.org/) the *X. tropicalis* TAAR gene family contains three genes. Only two of them (TAAR 1 and TAAR 2) are clearly annotated as TAARs; the third gene of this family is not further described (see Methods for further details). For this reason we focused our analysis solely on TAAR 1 and TAAR 2. Note that the *Xenopus* TAAR 2 gene is an ortholog of mouse TAAR 4 (S. I. Korsching, personal communication). We identified the ortholog genes of these TAARs in the genome of *X. laevis* and found that only one of them, namely TAAR 2, is expressed in the olfactory organ of larval *X. laevis*. Interestingly, both TAARs are expressed in the adult olfactory organ [‘air-nose’ (PC) and ‘water-nose’ (MC)]. At this point it should be noted that also the third gene included in the TAAR gene family in the Ensembl genome database (see above and Methods) is not expressed in the olfactory organ of larval *X. laevis* but is expressed in the olfactory organ of the adult (data not shown). The fact that we did not find any evidence for TAAR 1 and TAAR 2 expression in the brain of *Xenopus* larvae and that both TAARs are expressed in the brain of adult *Xenopus* is also interesting. Several studies have reported that TAARs are expressed in various mouse and human brain regions (Borowsky *et al.*, 2001; Vanti *et al.*, 2003), whereas Liberles & Buck (2006) did not find any evidence for TAAR expression in the mouse brain.

The evidence that TAARs are expressed in the olfactory organs of larval and adult *X. laevis* is definitely in line with the results of other reports, showing that TAARs appear to be consistently present in olfactory organs of vertebrates (Liberles & Buck, 2006; Fleischer *et al.*, 2007; Hashiguchi & Nishida, 2007). The *Xenopus* genome includes only very few TAARs (Hashiguchi & Nishida, 2007; Grus *et al.*, 2007; Grus & Zhang, 2008), and only one TAAR is expressed in the larval olfactory organ. A single TAAR cannot account for the complex amine response profiles recorded in the present study. This shows that in larval *X. laevis* TAARs are certainly not the only receptor family responsible for the detection of amines. TAAR 2 alone could serve as an odorant receptor, but likewise it could simply play a modulatory role in which case amine odorants would not be detected by TAARs at all. In recent years, the importance of modulatory processes in the peripheral olfactory system has been becoming increasingly evident. The list of substances with modulatory potential in the peripheral olfactory system includes various neurotransmitters (Mousley *et al.*, 2006), purines (Hegg *et al.*, 2003; Hassenklöver *et al.*, 2008), endocannabinoids (Czesnik *et al.*, 2007) and hormones (Kawai *et al.*, 1999). At present a potential modulatory role of TAARs in olfactory organs cannot be completely ruled out, being consistent with TAARs involved in neuromodulatory events (Borowsky *et al.*, 2001; Lindemann & Hoener, 2005). The fact that in adult *X. laevis* TAARs are expressed also in the brain strongly suggests that these receptors do not exclusively function as ORs in this species. This finding rather supports a neuromodulatory function of TAARs in *Xenopus*.

In addition to TAARs, three olfactory receptor families are known, the OR, V1R/ORA and the V2R/olfC families (Korsching, 2008). It has been shown in larval *Xenopus* that ORs (*X. laevis*) and V1R/ORA (*X. tropicalis*) are expressed in the main olfactory system, while V2R/olfC (*X. laevis*) are almost exclusively expressed in the VNO (Mezler *et al.*, 1999; Hagino-Yamagishi *et al.*, 2004; Date-Ito *et al.*, 2008). This suggests that the receptor proteins of larval *X. laevis* which underlay the detection of amines are probably members of the OR and/or V1R/ORA receptor families.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Alignment of the partial DNA sequences encoding the two TAAR genes of *Xenopus laevis* and *Xenopus tropicalis*.

Fig. S2. Alignment of the deduced partial amino acid sequences encoded by the two TAAR genes of *Xenopus laevis* and *Xenopus tropicalis*.

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Abbreviations

MC, middle cavity; OB, olfactory bulb; OE, olfactory epithelium; ORNs, olfactory receptor neurons; ORs, olfactory receptors; PC, principal cavity; TAARs, trace amine-associated receptors; VNO, vomeronasal organ.

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