

Classes and Narrowing Selectivity of Olfactory Receptor Neurons of *Xenopus laevis* Tadpoles

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ABSTRACT In olfactory receptor neurons (ORNs) of aquatic animals amino acids have been shown to be potent stimuli. Here we report on calcium imaging experiments in slices of the olfactory mucosa of *Xenopus laevis* tadpoles. We were able to determine the response profiles of 283 ORNs to 19 amino acids, where one profile comprises the responses of one ORN to 19 amino acids. 204 out of the 283 response profiles differed from each other. 36 response spectra occurred more than once, i.e., there were 36 classes of ORNs identically responding to the 19 amino acids. The number of ORNs that formed a class ranged from 2 to 13. Shape and duration of amino acid-elicited $[Ca^{2+}]_i$ transients showed a high degree of similarity upon repeated stimulation with the same amino acid. Different amino acids, however, in some cases led to clearly distinguishable calcium responses in individual ORNs. Furthermore, ORNs clearly appeared to gain selectivity over time, i.e., ORNs of later developmental stages responded to less amino acids than ORNs of earlier stages. We discuss the narrowing of ORN selectivity over stages in the context of expression of olfactory receptors.

KEY WORDS: mucosa slice • calcium imaging • amino acids • odorants

INTRODUCTION

Olfactory receptor neurons (ORNs) in fish and amphibia have been shown to respond to various groups of odorants, e.g., prostaglandins (Sorensen et al., 1988; Kitamura et al., 1994), nucleotides (Kang and Caprio, 1995), bile acids (Kang and Caprio, 1995; Sato and Suzuki, 2001), and amino acids (Caprio and Byrd, 1984; Kang and Caprio, 1995; Vogler and Schild, 1999; Sato and Suzuki, 2001; Manzini et al., 2002a,b; Manzini and Schild, 2003b). The olfactory receptors (ORs) to which these stimuli bind, their number, and their ontogenetic appearance are largely unknown. The way these stimuli are mapped onto the olfactory bulb depends primarily on the precise projections of the respective ORNs to the olfactory bulb. These are unknown, too. In our animal model, the tadpole of *Xenopus laevis*, four ORs of class I (fish-like receptors) have been characterized (Freitag et al., 1995, 1998; Mezler et al., 1999, 2001). Cross adaptation of responses to amino acids showed that these stimuli fall into several classes (Caprio and Byrd, 1984; Caprio et al., 1989), and recent evidence suggests (a) that amino acids are mapped to a lateral area in the olfactory bulb (Friedrich and Korsching, 1997; Manzini et al., 2002b) and (b) that they are not transduced by the well-known cAMP-dependent olfac-

tory transduction pathway (Manzini et al., 2002b; Manzini and Schild, 2003b).

According to the generally accepted scheme of olfactory coding every ORN expresses one 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 ORNs expressing the same receptor form a class of ORNs. One might expect a rather limited number of ORs sensitive to amino acids. However, amino acids may also unspecifically bind to putative olfactory peptide receptors, or to any other receptors, in which cases the total number of receptors sensitive to amino acids could be much larger.

Here we measured the responses of ORNs to amino acids in *Xenopus laevis* tadpoles. As the amount of odorant profile data that could result from patch-clamp experiments has proved limited in our hands we resorted to calcium imaging experiments of the olfactory mucosa (Manzini et al., 2002a,b). We imaged calcium responses upon application of amino acids in the main cavity of the olfactory epithelium of this species. We set out to determine the characteristics of the tadpoles' ORN responses and, in particular, whether there were classes of identically responding ORNs and, if so, how many.

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Abbreviations used in this paper: ACID, acidic amino acids; AROM, aromatic amino acids; BAS, basic amino acids; LCN, long chain neutral amino acids; OR, olfactory receptor; ORN, olfactory receptor neuron; SCN, short chain neutral amino acids.

Slice Preparation for Calcium Imaging Experiments

Tadpoles of *Xenopus laevis* (stages 51–56) (Nieuwkoop and Faber, 1994) were chilled in a mixture of ice and water and decapitated, in accordance with the Göttingen University Committee for Ethics in Animal Experimentation. A block of tissue containing the olfactory mucosae, the olfactory nerves, and the anterior two thirds of the brain was cut out and kept in bath solution (see below). The tissue was glued onto the stage of a vibroslicer (VT 1000S; Leica) and cut horizontally into 120–130- μm thick slices. Fig. 1, A and B, show a mucosa slice stained with biocytin/avidin by backfilling the receptor axons from the glomerular layer of the olfactory bulb. The slice was counterstained with propidium iodide (for staining procedures see Manzini et al., 2002a). For the imaging experiments the tissue slices were incubated in bath solution (see below) containing Fluo-4/AM (50 μM ; Molecular Probes) for 1 h, and then transferred to a recording chamber. The fluorescence of Fluo-4 increases with increasing intracellular calcium concentration. Fluo-4/AM was dissolved in DMSO (Sigma-Aldrich) and Pluronic F-127 (Molecular Probes). ORNs of *Xenopus laevis* tadpoles express multidrug resistance transporters (Manzini and Schild, 2003a) with a wide substrate spectrum, including calcium-indicator dyes. To avoid transporter-mediated destaining of the slices, MK571 (50 μM ; Alexis Biochemicals), a specific inhibitor of the multidrug resistance-associated proteins (MRP; Gekeler et al., 1995; Abrahamse and Rechkemmer, 2001), was added to the incubation solution. After incubation, the tissue slices were placed between two grids in a recording chamber to allow diffusion from both sides and placed on the microscope stage of an Axiovert 100M (ZEISS) to which a laser scanning unit (LSM 510; ZEISS) was attached. Before starting the calcium imaging experiments, the slices were rinsed with bath solution for at least 20 min.

Calcium Imaging of Odor Responses

Intracellular calcium was monitored using a laser-scanning confocal microscope (ZEISS LSM 510/Axiovert 100M). The confocal pinhole was ~ 80 μm and excluded fluorescence detection from more than one cell layer. Fluorescence images (excitation at 488 nm; emission > 505 nm) of the olfactory mucosa were acquired at 0.25–1.27 Hz and 786.4 ms exposure time per image with 3–5 images taken as control images before the onset of odor delivery. The fluorescence changes $\Delta F/F$ were calculated for individual ORNs as $\Delta F/F = (F_1 - F_2)/F_2$, where F_1 was the fluorescence averaged over the pixels of an ORN, while F_2 was the average fluorescence of that ORN before stimulus application, averaged over three images. Background intensity was zero.

Data Evaluation

The fluorescence changes $\Delta F/F$ were exported from the ZEISS 510 image acquisition program to ASCII files containing the time courses of fluorescence changes for each responsive ORN. These data were used (a) for plotting (Figs. 2 and 3) and (b) for generating a binary response vector for each ORN indicating to which out of the 19 stimuli applied the respective ORN was sensitive to. The responses of the 283 ORNs tested were thus represented as a 283×19 matrix (see Fig. 4), the elements of which are 1 (response) or 0 (no response). Calculations on this matrix were done using a custom-written program in C++.

Solutions and Stimulus Application

The composition of the bath solution was (in mM): 98 NaCl, 2 KCl, 1 CaCl_2 , 2 MgCl_2 , 5 glucose, 5 Na-pyruvate, 10 HEPES. The pH of the bath solution was adjusted to 7.8. This is the physiolog-

ical pH in this poikilothermal species (Howell et al., 1970). The osmolarity of the bath solution was 230 mOsmol. As odorants, we used the amino acids (Sigma-Aldrich; listed in Table I) applied either as a mixture of 19 amino acids (AA), as submixtures, or as single amino acids. The amino acids were dissolved in bath solution (10-mM stock, each) and used at a final concentration of 200 μM in all of the experiments. Stimulus solutions were prepared immediately before use by dissolving the respective stock solution in bath solution. The bath solution was applied by gravity feed from a storage syringe through a funnel drug applicator (Schild, 1985) to the recording chamber. The flow was 350 $\mu\text{l}/\text{min}$. Odorants were pipetted directly into the funnel without stopping the flow. Outflow was through a syringe needle placed close to the mucosa to ensure that odorant molecules were removed rapidly. The minimum interstimulus interval between odorant applications was at least 2 min.

The dilution of the stimulus within the funnel was $< 1\%$. The dilution of the stimulus in the mucosa was determined by first putting a confocal volume ($\sim 1 \text{ fl} = 10^{-15} \text{ l}$) of a laser-scanning confocal microscope (ZEISS LSM 510/Axiovert 100) in front of the funnel outlet and, second, in front of the epithelial surface and measuring the respective fluorescences. For this control measurement we used the fluorescent probe tetramethylrhodamine (TMR, 500 nM; Sigma-Aldrich) as a “dummy stimulus”. The dilution factor was 0.91 ± 0.02 (mean \pm SD, $n = 7$). The delay between TMR leaving the funnel outlet and reaching the steady-state concentration at the mucosal surface was < 1 s, and after the end of stimulation, TMR was completely rinsed from the mucosa within 15 s.

RESULTS

For this study we analyzed changes in the intracellular calcium concentration $[\text{Ca}^{2+}]_i$ in 283 ORNs ($n = 49$ slices) of the olfactory epithelium of *Xenopus laevis* tadpoles (Fig. 1, A and B) using Fluo-4 as calcium-indicator dye and amino acids as stimuli. Fig. 1 C shows a mucosa slice stained with the calcium-indicator dye Fluo-4. The encircled ORNs were responsive to a mixture of 19 amino acids (AA) as seen from the increase of the $[\text{Ca}^{2+}]_i$ in Fig. 1, D–F. At interstimulus intervals of 2 min we applied the five submixtures of amino acids (LCN, SCN, BAS, ACID, and AROM, see Table I) and subsequently the 19 single amino acids, one after another.

The responses of four of these ORNs are shown in more detail in Fig. 2. ORN #1 was responsive to L-histidine and, accordingly, to the mixture of basic amino acids. ORN #2 reacted upon application of L-leucine, L-methionine, L-cysteine, L-glutamine, L-asparagine,

TABLE I
Water-soluble Mixtures of L-amino Acids

Mixture	Amino acids
LCN	Proline, valine, leucine, isoleucine, methionine
SCN	Glycine, alanine, serine, threonine, cysteine, asparagine, glutamine
BAS	Arginine, lysine, histidine
ACID	Glutamate, aspartate
AROM	Tryptophane, phenylalanine
AA	LCN, SCN, BAS, ACID, and AROM

Mixtures of L-amino acids following Caprio and Byrd (1984).

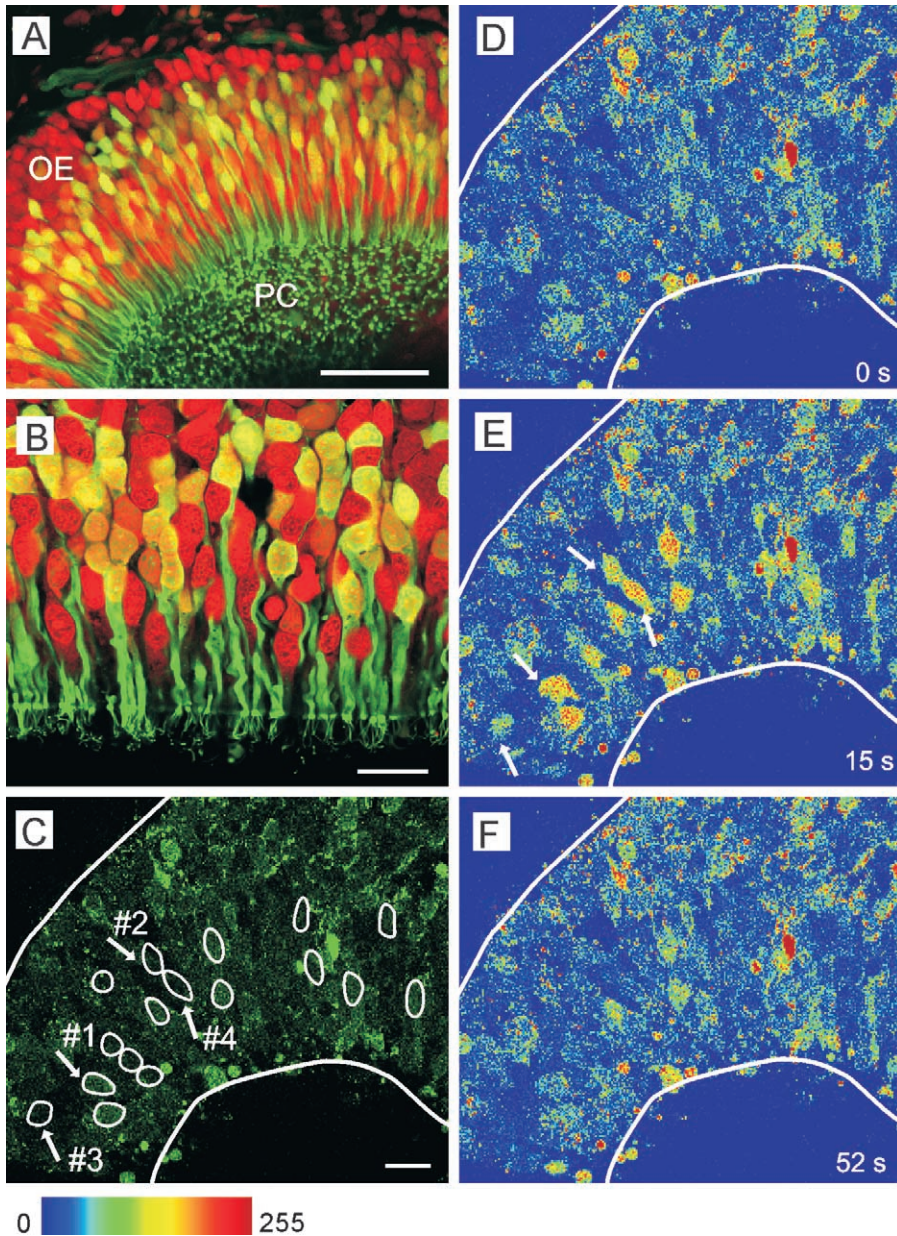


FIGURE 1. Slice of the olfactory epithelium of a *Xenopus laevis* tadpole and amino acid-induced $[Ca^{2+}]_i$ increases in individual ORNs in a mucosa slice. (A) Overview of a horizontal slice of the olfactory epithelium of a *Xenopus laevis* tadpole (stage 54, PC, principal cavity and OE, olfactory epithelium). The neurons were backfilled through the nerve using biocytin/avidin staining (green fluorescence), and then the slice was counterstained with propidium iodide (red fluorescence). Cell bodies of ORNs thus appear yellow. (B) Higher magnification of a part of the olfactory epithelium shown in A. Olfactory receptor neurons with dendrites, dendritic knobs, and cilia are easily recognizable. (C) Fluorescence image of a mucosa slice (stage 55, image acquired at rest) stained with Fluo-4. Cell bodies of amino acid-sensitive ORNs are encircled. The responses to amino acids of the ORNs indicated by arrows are shown in Fig. 2. (D–F) Sequence of three pseudocolored images of the slice showing that stimulation with a mixture of amino acids (200 μ M each) transiently increases calcium-dependent fluorescence in the ORNs encircled in C. (D) Image taken before the application of the amino acid mixture ($t = 0$ s). (E) Image taken at the peak of the response ($t = 15$ s). (F) Image taken after return to the base line fluorescence ($t = 52$ s). Bars: (A) 50 μ m; (B–F) 20 μ m.

and L-arginine and to the respective submixtures, whereas ORN #3 was sensitive just to L-asparagine and the short chain neutral submixture. The fourth ORN shown in this figure responded to L-isoleucine, L-methionine, L-glutamine, and the submixtures of long chain neutral and short chain neutral amino acids. The number of ORNs in a slice responding to amino acids varied from slice to slice, the average being 5.8 ORNs/slice ($n = 49$ slices). When ORNs were tested several times to the same stimulus, the responses exhibited a high degree of similarity (Fig. 3). In some cases different stimuli induced different response time courses (Fig. 3 A, L-cysteine vs. L-methionine; Fig. 3 B, L-methionine vs. L-leucine), while in other cases the response to one stimulus appeared just

down- or upscaled with respect to another one (Fig. 3 C, L-glutamine vs. L-isoleucine).

All 283 ORNs recorded were tested for their responses to 19 amino acids (see Table I), resulting in the 283×19 response matrix shown in Fig. 4. For the purpose of this paper we indicated a response of an ORN to a particular stimulus by a “1” in the response matrix, while a “0” in the matrix means “no response”. Time courses and response amplitudes were thus neglected.

The frequency by which the 19 amino acids used elicited a response varied markedly from amino acid to amino acid (Fig. 5). L-methionine and L-proline were the most and the least effective stimuli, leading to responses in 64% and 3.5% of all responsive ORNs, respectively (Fig. 5).

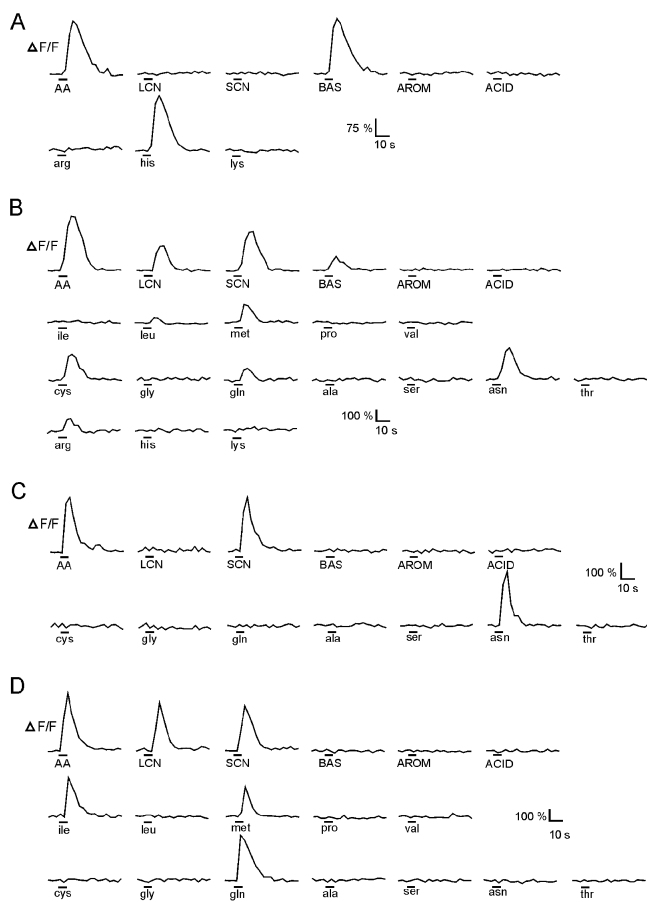


FIGURE 2. Amino acid-induced changes in calcium-dependent fluorescence of four individual ORNs in a mucosa slice. (A) Time course of $[Ca^{2+}]_i$ transients of an ORN (ORN #1 in Fig. 1 C) evoked by the application of amino acids. The traces show the responses to the mixture of 19 amino acids (AA), to the mixture of basic amino acids (BAS) and to L-histidine. No response to the mixtures of the long chain neutral (LCN), the short chain neutral (SCN), the aromatic (AROM), and the acidic (ACID) amino acids. No response to the remaining single amino acids of the BAS mixture. (B) ORN #2 (Fig. 1 C) responding to the mixture of AA, LCN, L-methionine, L-leucine (though slightly weaker), SCN, L-cysteine, L-glutamine, L-asparagine, BAS, and to L-arginine. No response to the mixtures AROM or ACID, nor to the remaining single amino acids of the responsive groups. (C) ORN #3 (Fig. 1 C) responding to the mixtures of AA, SCN, and to L-asparagine. No response to the other mixtures, nor to the remaining single amino acids of the SCN mixture. (D) ORN #4 (Fig. 1 C) responding to the mixture of AA and LCN, to L-isoleucine and to L-methionine, the mixture of SCN, and to L-glutamine. No response to the mixtures BAS, AROM, or ACID, nor to the remaining single amino acids of the responsive groups. All amino acids were applied at a concentration of 200 μ M.

As most ORNs (239 of 283) responded to more than one stimulus (Fig. 4), the frequencies shown in Fig. 5 sum up to a value $>100\%$.

Of the 283 responses recorded 204 patterns were unique in the sense that they differed from all other patterns. While 168 of these occurred just once, 36 pat-

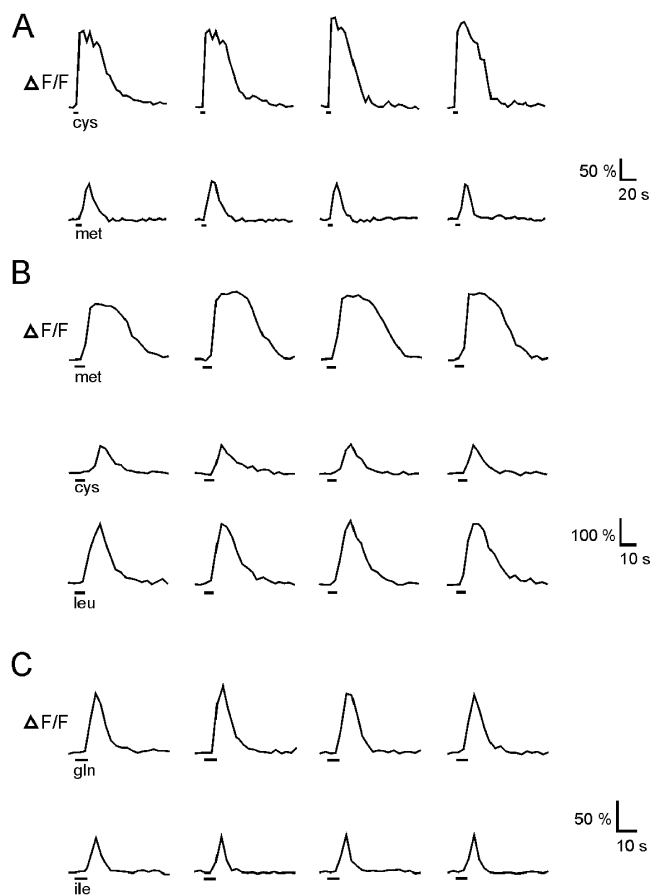


FIGURE 3. Different amino acids elicit distinguishable and highly reproducible $[Ca^{2+}]_i$ transients in individual ORNs in a mucosa slice. Time courses of amino acid-induced $[Ca^{2+}]_i$ transients of three individual ORNs (A–C). The traces show responses to successive applications of the amino acids L-cysteine and L-methionine (A); L-methionine, L-cysteine, and L-leucine (B); and L-glutamine and L-isoleucine (C). Shape and duration of the $[Ca^{2+}]_i$ transients stayed approximately constant if the same amino acid was applied several times.

terns occurred more than once. All ORNs that showed the same response pattern to the sequence of 19 amino acids formed a class of ORNs, respectively. Among the 283 ORNs recorded there were 36 classes, each of which contained between 2 and 13 identically responding ORNs (Table II).

The ORNs of six classes (class #2, 3, 10, 15, 18, and 32) responded to one stimulus each and these stimuli were L-histidine, L-arginine, L-lysine, L-asparagine, L-glutamine, and L-methionine. Among the individual ORNs that responded differently from all others, there were four ORNs responding to one amino acid, i.e., to L-glycine, L-cysteine, L-tryptophane, and L-leucine. Together there were 44 ORNs that responded to one stimulus, 40 of them being members of cell classes and 4 of them being individual ORNs.

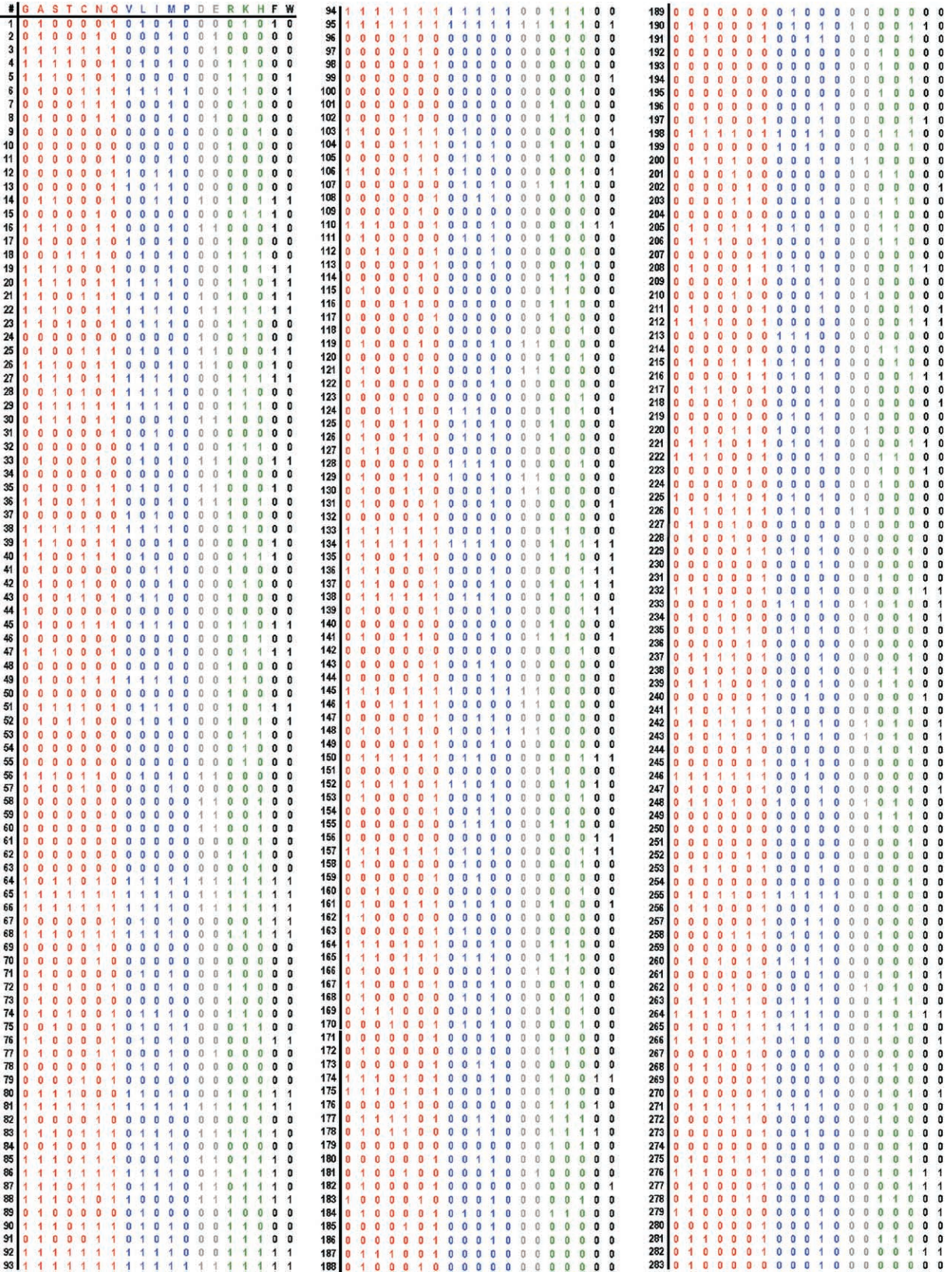


FIGURE 4. Response profiles of 283 olfactory receptor neurones to amino acids. 283×19 matrix representing the responses of the 283 ORNs each tested for 19 amino acids indicated by the common one-letter code for amino acids (first line). A 1 or a 0 in the matrix indicates whether or not a particular ORN responded to a particular amino acid (1 = response; 0 = no response). A response was assumed if the following two criteria were met: (a) the first two intensity values after stimulus arrival at the mucosa, $I(t_1)$ and $I(t_2)$, had to be larger than the maximum of the prestimulus intensities; (b) $I(t_2) > I(t_1)$ with $t_2 > t_1$.

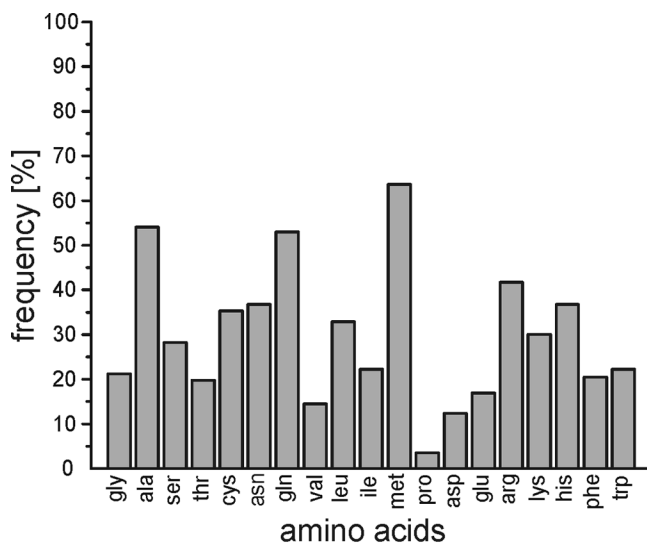


FIGURE 5. Histogram of response frequencies to amino acids. The histogram shows for each of the 19 amino acids used in how many ORNs (out of 283) a response was observed. Results are plotted as relative numbers, i.e., normalized to 283. As most of the ORNs ($n = 283$) responded to more than one amino acid, the sum of the response frequencies is much higher than 100%.

As a next step of our evaluation we asked how many of the 283 ORNs responded to two stimuli, how many to three stimuli, and so forth. The resulting frequency distribution (including the 44 ORNs responding to one stimulus) is shown in Fig. 6. It indicates a clear tendency of ORNs to respond to few rather than to many stimuli.

The responses of an ORN to multiple stimuli could be explained either by the expression of a relatively nonspecific receptor or by the expression of more than one receptor per ORN. Though the expression of more than one OR in one ORN contradicts all evidence in adult ORNs of higher vertebrates, it is a priori not excluded in larval stages of lower vertebrates. In this case, however, the sensitivity spectra of tadpole ORNs might be expected to change over time and stages.

We therefore split the dataset of Fig. 4 in two subsets, i.e., ORNs recorded at stage 51, 52, 53, and ORNs recorded at stage 54, 55, 56. Fig. 4 is arranged in a way that the first subset includes ORNs #0–143 (first set of stages) and the second subset includes ORNs #144–283 (second set of stages). Frequency histograms of the number of effective stimuli in each subset of ORNs are plotted in Fig. 7, A and B. It is obvious that ORNs at later developmental stages respond to less stimuli, indicating that *Xenopus laevis* tadpole ORNs indeed gain specificity over stages.

DISCUSSION

Amino acids are chemically well-known and biologically relevant stimuli in *Xenopus laevis* (Iida and Kashiwayanagi, 1999; Vogler and Schild, 1999; Manzini et al.,

2002a,b; Manzini and Schild, 2003b). From an experimental point of view they are convenient stimuli because they form a finite and small group of stimuli and dissolve well in water.

To obtain specificity profiles of ORNs to many odorants using the patch-clamp technique is inherently difficult due to two major problems. First, it is rather troublesome to find individual ORNs that respond to a particular known group of odorants and, second, in the rare case where such an ORN is found, the ORN is notoriously lost before it has been tested for all of the odorants of that set (see Manzini et al., 2002a,b). A way out of this intrinsic problem is using calcium imaging in the slice of the olfactory epithelium. This way it is feasible to monitor responses of many ORNs in parallel, thereby increasing the detection probability of responses considerably. The specificity profiles to amino acids of a number of ORNs can thus be determined simultaneously. Another important advantage of this method is that calcium responses can be observed over a long period of time, markedly longer than ORNs can usually be held on a patch pipette, thereby allowing to test all stimuli of a test set. To our knowledge the data presented here is the largest olfactory response matrix (283 ORNs, each tested for 19 odorants) reported so far.

In our study we set out to determine the specificity profiles of a large number of ORNs to the 19 amino acids listed in Table I, using Fluo-4 as calcium-indicator dye. Using this approach we determined the specificity profiles to amino acids of 283 ORNs of *Xenopus laevis* tadpoles (stages 51–56). As to the evaluation of the resulting data, we here concentrated on the three most prominent issues, i.e., (a) reproducibility and variability, (b) response classes, and (c) changing selectivities over developmental stages.

The time course of the calcium responses of the ORNs showed a high degree of similarity (Fig. 3) when an amino acid was tested repeatedly on a mucosal slice. Interestingly, the time courses of the $[Ca^{2+}]_i$ transients elicited by some amino acids on an individual ORN differed from each other, i.e., shape and duration clearly depended on the stimulus applied (Fig. 3). This could be explained by individual ORNs each expressing one unspecific receptor with different ligand affinities. Obviously, this interpretation would imply the existence of a large number of amino acid-sensitive ORs. For instance in the case of L-histidine, there would be one OR sensitive to L-histidine (ORNs of class 2, Table II), but in addition many more ORs (in the case of L-histidine 79 ORs) would be sensitive to a combination of amino acids that includes L-histidine (Fig. 4). Alternatively, the differing $[Ca^{2+}]_i$ transients in an individual ORN elicited by different stimuli could be explained by the expression of more than one OR per ORN. While this hypothesis is not consistent with other findings in adult higher vertebrates (Nef et al., 1992; Strotmann et

TABLE II
Classes of ORNs

Class	Response pattern to amino acids	ORN#
	GASTCNQVLIMPDERKHFV	
1	0100011000100100000	8; 77
2	0000000000000000100	9; 46; 61; 63; 100; 118; 123; 142; 194; 251; 254; 274
3	0000001000100000000	10; 34; 48; 50; 101; 140; 151; 192; 193; 195; 204; 224; 250
4	0000001000100000000	11; 149; 171; 186; 280
5	0000001101100000000	12; 13
6	1110011000101100010	16; 26
7	1110001000100010111	19; 276
8	0000001001000000000	31; 159; 245; 273
9	0100111111100011000	49; 265
10	0000000000000001000	54; 55
11	0100100000100000000	57; 228
12	0000000000001100100	58; 59; 60
13	0000000000000011100	62; 249
14	1111111111101111111	65; 66
15	0000010000000000000	69; 70; 109; 132; 236; 252; 267
16	0100000010100010000	71; 111
17	1111111111111111111	81; 93
18	0000001000000000000	98; 207; 269
19	0000100000000011000	102; 116; 201
20	1100111010000000101	103; 106
21	0000001001100000000	108; 143; 147; 154; 173; 180; 257
22	1110001000100000111	110; 136; 232
23	0100000000000011000	115; 172
24	0000001000000010000	117; 231
25	0000000000000010100	120; 179
26	0100110000101100000	121; 130
27	0100000000100000000	122; 283
28	0100110010100010000	125; 126
29	0100001000100000111	139; 277; 282
30	0000010000100000000	144; 209
31	0100100000100101000	166; 262
32	0000000000100000000	196; 230; 259
33	0111001000100011000	206; 239; 268
34	0100001000100000101	211; 247
35	0111001000100000000	217; 253
36	0101101010100101001	242; 243

Classes of ORNs extracted from Fig. 4. Classes of identically responding ORNs are represented by their class index, the response pattern, and the member ORNs, respectively.

al., 1992; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994; Malnic et al., 1999), it would certainly be an explanation for the above results. A decision of whether the first interpretation, the second one, or a combination of both is true will presumably come from a more detailed characterization of *Xenopus laevis* ORNs and single-cell PCR experiments in ORNs of this species.

The concentration of the amino acids used is important. Throughout the experiments reported herein we used an amino acid concentration of 200 μ M. Though *Xenopus laevis* ORNs respond to lower concentrations as well, the used concentration is sufficiently high to acti-

vate ORs with high as well as with low affinity for amino acids, but not as high as in many other studies (e.g., Michel et al., 1991; Iida and Kashiwayanagi, 1999, 2000; Sato and Suzuki, 2000, 2001).

Of the 283 response profiles observed 36 patterns occurred more than once. These were seen in a total of 115 ORNs (see Table II), while 168 response profiles were recorded just once. There were thus 36 classes of ORNs, each comprising a number of ORNs (from 2 to 13). Among these 115 ORNs 40 responded to one amino acid, while the others responded to more than one amino acid. What was surprising was the large num-

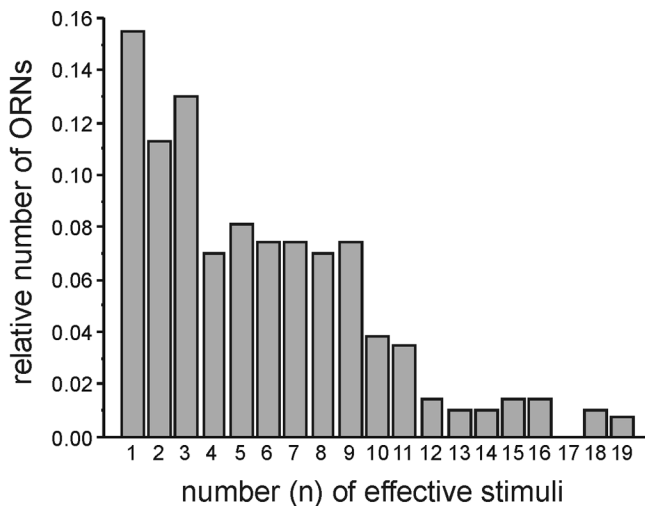


FIGURE 6. Histogram of the number of effective amino acids per ORN. Frequencies of ORNs ($n = 283$, 49 slices) that responded to a certain number n of amino acids (n out of 19 amino acids, Table I). Every possible number of effective stimuli, except 17, occurred. The distribution was, however, rather skewed, with small numbers of effective stimuli occurring much more often than larger numbers.

ber of different response patterns. At the beginning of our experiments we started out with the hypothesis that, assuming one OR per ORN, there would be a limited number between 10 and 30 of amino acid receptors and a correspondingly limited number of ORN classes. In contrast, our data showed 36 classes and, in addition, 168 individual response patterns that were different from all others. Assuming we had measured k more ORNs, what would have been the number of classes and the number of individual response patterns? $36 + k$ classes and 168 individual patterns, or 36 classes and $168 + k$ individual patterns? Presumably the result would have been in between. In any case this thought experiment shows that 36 is a minimum estimate for the number of amino acid-sensitive ORN classes.

Explaining the diversity of response patterns delineated above by assuming one OR per ORN would again imply the existence of a large number of amino acid-binding ORs. It has been shown that in higher vertebrates every ORN expresses one 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). Admittedly, however, little is known about OR expression in lower vertebrates, especially at larval stages. Therefore, it cannot be excluded that ORNs of *Xenopus laevis* tadpoles express a number of ORs at lower stages, lose most of them during development, and finally express one OR.

In this case the sensitivity spectra of tadpole ORNs must be expected to change over stages, i.e., under such a condition of narrowing selectivity over stages, ORNs would respond to less stimuli at higher stages

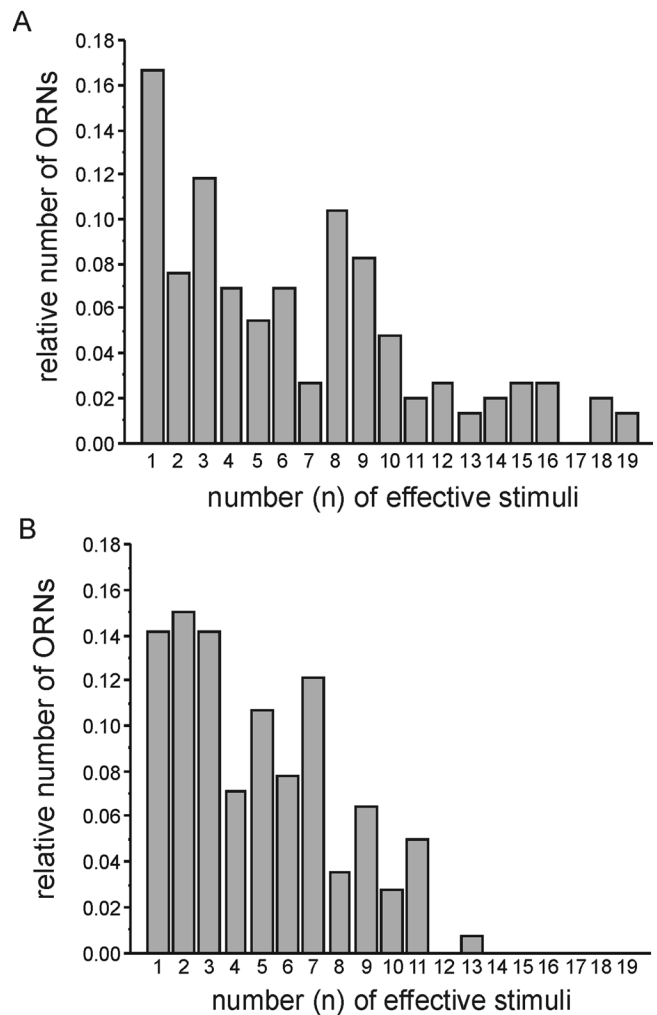


FIGURE 7. Histograms of the number of effective amino acids per ORN in two groups of ORNs of different stages. (A) Frequency distribution of ORNs ($n = 143$, 27 slices) responding to n stimuli, evaluated for earlier stages (stages 51, 52, and 53). (B) Frequency distribution of ORNs of later stages ($n = 140$, 22 slices, stages 54, 55, and 56). In the second subset (later stages) the individual ORNs clearly respond to less stimuli.

than at lower stages. To verify this hypothesis we split the dataset of Fig. 4 into two subsets, the first subset containing ORNs of earlier stages (51, 52, 53), the second subset containing ORNs of later stages (54, 55, 56). Tadpoles were staged after Nieuwkoop and Faber (1994), using the size of the tadpole's fore and hind limbs as a major classification criterion. However, as development is a continuous process, any categorization into discrete stages is necessarily error-prone. In addition, distinguishing certain stages from each other (e.g., stage 52 from 53) is more difficult than distinguishing others (e.g., stage 53 from 54). To minimize errors of stage classification, we therefore chose to split the ORNs in two subsets of stages. We then calculated histograms for either subset giving the frequencies by

which the respective ORNs responded to a certain number of stimuli. The earlier stage subset (Fig. 7 A) comprises ORNs responding to up to 19 amino acids, while the ORNs of the later stage subset (Fig. 7 B) never responded to >13 amino acids. While 58% of the early stage ORNs responded to seven or less amino acids, over 80% of the late stage ORNs responded to seven or less amino acids. These data unambiguously show a trend demonstrating that ORNs of *Xenopus laevis* gain specificity over stages. Thus, OR expression patterns do not appear to be constant over stages. To substantiate this assertion, the genes coding for amino acid ORs need to be characterized, a task certainly beyond the scope of this study. Furthermore, it would be interesting to study whether or not the specificity of ORNs of adult *Xenopus laevis* is consistently confined to one stimulus per ORN, as seen in a portion of tadpoles ORNs. We have as yet failed, however, to develop a slice preparation from adult *Xenopus*' water mucosa, which would allow us to answer this question. Hopefully the obstacles involved will be overcome in the future.

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