

cAMP-independent responses of olfactory neurons in *Xenopus laevis* tadpoles and their projection onto olfactory bulb neurons

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We report on responses of olfactory receptor neurons (ORNs) upon application of amino acids and forskolin using a novel slice preparation of the olfactory epithelium of *Xenopus laevis* tadpoles. Responses were measured using the patch-clamp technique. Both amino acids and forskolin proved to be potent stimuli. Interestingly, a number of ORNs that responded to amino acids did not respond to forskolin. This suggests that some amino acids activate transduction pathways other than the well-known cAMP-mediated one. The differential processing of cAMP-mediated stimuli on the one hand and amino acid stimuli on the other was further elucidated by calcium-imaging of olfactory bulb neurons using a novel nose–olfactory bulb preparation of *Xenopus laevis* tadpoles. The projection pattern of amino acid-sensitive ORNs to olfactory bulb neurons differed markedly from the projection pattern of forskolin-sensitive ORNs. Olfactory bulb neurons activated by amino acids were located laterally compared to those activated by forskolin, and only a small proportion responded to both stimuli. The ensemble of neurons activated by forskolin was also activated by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the membrane-permeant cAMP analogue 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (pCPT-cAMP). We therefore conclude that sensory transduction of a number of amino acids is cAMP independent, and amino acid- and cAMP-mediated responses are processed differentially at the level of the olfactory bulb.

(Resubmitted 3 September 2002; accepted after revision 19 September 2002; first published online 18 October 2002)

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Olfactory receptor neurons convey information about the chemical environment to the brain. Among the first steps in this signalling process are the binding of odorants to olfactory receptors (ORs) (Buck & Axel, 1991) and the transduction of this event into action potentials. The number of ORs in higher vertebrates is assumed to be about 1000. The question of whether all ORs couple to one or more transduction cascades has been debated over a number of years (Schild & Restrepo, 1998; Gold, 1999), and the simple view of cAMP as the only olfactory second messenger has had to be abandoned (Zufall & Munger, 2001). Rather there appears to be a diversity of transduction mechanisms in different kinds of ORNs (Michel & Ache, 1994; Zufall & Munger, 2001). Although a number of stimuli have been shown to be associated with either cAMP or IP₃ production (Sklar *et al.* 1986; Boekhoff *et al.* 1994, 1990), such relationships are unknown for most olfactory stimuli and the ORNs they act upon.

In aquatic species, amino acids are potent olfactory stimuli (Caprio & Byrd, 1984; Restrepo *et al.* 1990; Freitag *et al.* 1995; Kang & Caprio, 1995, 1997; Iida & Kashiwayanagi, 1999; Vogler & Schild 1999). Chemically they are a well-defined group of molecules, many of which act as

neurotransmitters or neurotransmitter precursors. In the CNS, amino acid neurotransmitter receptors couple to a variety of transduction mechanisms (Kandel *et al.* 2000). Their transduction pathways in ORNs are not known. Here we present evidence that the transduction pathways of many amino acids in ORNs are independent of cAMP.

METHODS

Slice preparation for patch-clamp recordings

Tadpoles of *Xenopus laevis* (stages 49–54) (Nieuwkoop & Faber, 1956) were chilled in a mixture of ice and water and decapitated, as approved by the Göttingen University Committee for Ethics in Animal Experimentation. A block of tissue containing the olfactory mucosae, the intact olfactory nerves and most 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, Bensheim, Germany) and cut horizontally into 120–125 μm -thick slices (Fig. 1A). The slices were placed under a grid in a recording chamber (Edwards *et al.* 1989) and viewed using Nomarski optics (Axioskop 2, Zeiss, Göttingen, Germany). Olfactory receptor neurons were easily recognized by their characteristic shape. Figure 1C and D shows a slice of the olfactory epithelium ('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 (see next section for staining procedures).

Staining of the olfactory mucosa and the olfactory bulb

Tadpoles were chilled and killed as described above. For staining the olfactory mucosa, a block of tissue containing the olfactory mucosa, the olfactory nerves and the anterior part of the brain, including the olfactory bulb, was cut out. The blocks of tissue were pinned to the bottom of a silicone-covered Petri dish, and the

tissue above the olfactory bulb was removed. Then, 5 μ l of DMSO was dropped onto the olfactory bulb and crystals of biocytin (Molecular Probes, Eugene, OR, USA) were pinched into the glomerular layer using a fine needle. After 40 min of nerve backfilling the blocks were put into bath solution (see below) for 1 h. The blocks were then fixed in 4% formaldehyde in

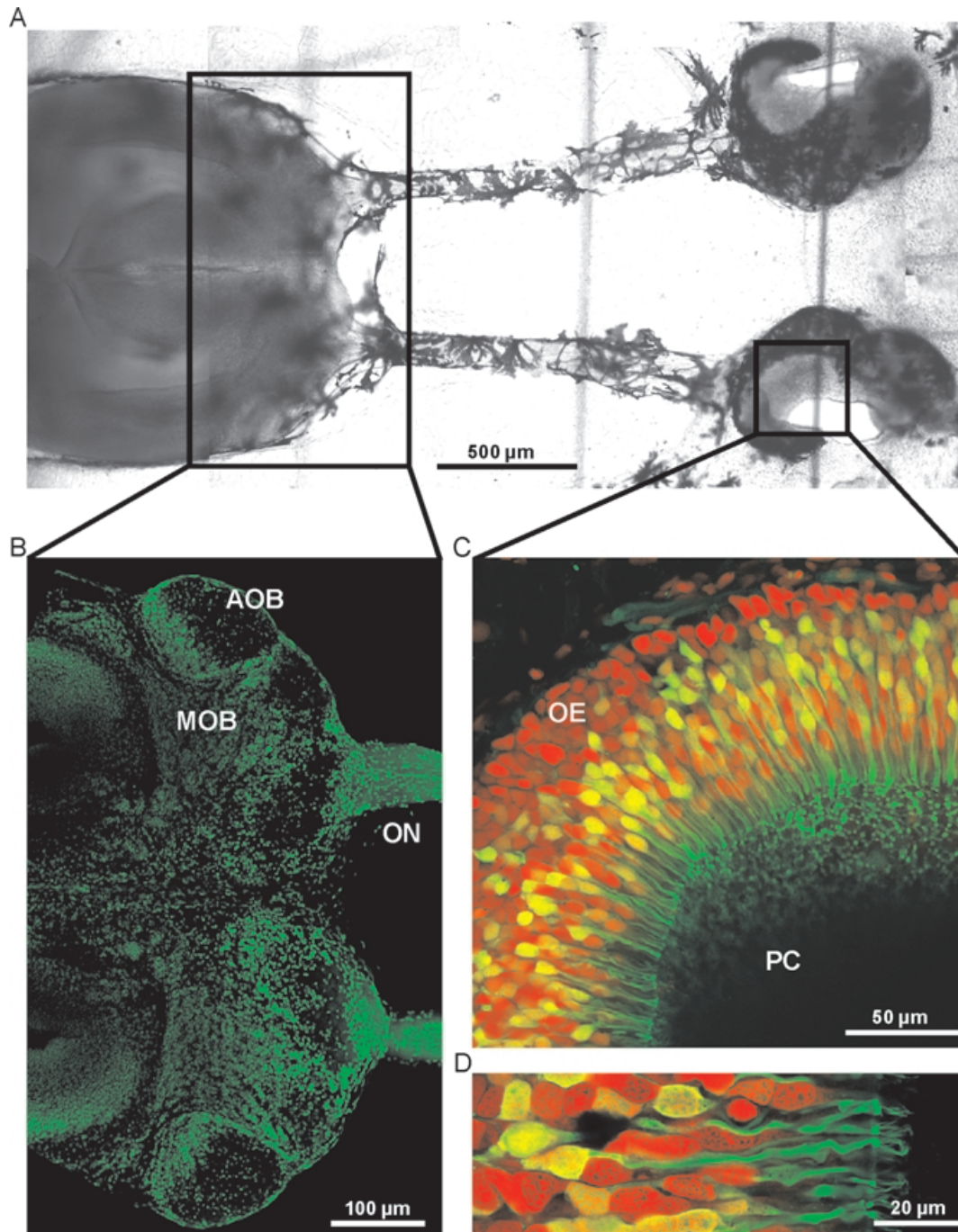


Figure 1. Slice of the olfactory mucosa and the olfactory bulb

A, image of the slice of the olfactory mucosa and the olfactory bulb. *B*, slice of the anterior part of the brain including the olfactory nerve (ON), the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB) stained with propidium iodide. *C*, horizontal overview of the olfactory epithelium (PC, principal cavity and OE, olfactory epithelium). The neurons were backfilled through the nerve using biocytin–avidin staining (green fluorescence), and the slice was counterstained with propidium iodide (red fluorescence). *D*, higher magnification of *C*, showing ORNs with dendrites, dendritic knobs and cilia.

phosphate-buffered saline (PBS, pH 7.4) overnight, washed in PBS, embedded in 5% low melting point agarose (Sigma, Deisenhofen, Germany), and sectioned at 70 μm thickness on a vibroslicer. Sections were washed in PBS containing 0.2% Triton X-100 (PBST), and the tissue was incubated in avidin, ALEXA 488 conjugated (Molecular Probes, 1:200 in PBS with 0.2% Triton X-100) for 2 h at room temperature. After washing off the avidin in PBS, sections were incubated for 15 min in 25 $\mu\text{g ml}^{-1}$ propidium iodide (Molecular Probes) in PBS to stain cell nuclei. Sections were washed in at least five changes of PBS and transferred into 60% glycerol–PBS for at least 1 h and finally mounted on slides in 80% glycerol–PBS. For staining cell nuclei of olfactory bulb neurons, slices of the anterior part of the brain were fixed in formaldehyde, incubated in propidium iodide, washed and mounted on slides as described above. Preparations were viewed and imaged using a laser-scanning confocal microscope (Zeiss LSM 510/Axiovert 100, Jena, Germany).

Patch-clamp recordings

Patch electrodes with a tip diameter of 1–2 μm and approximately 7–10 M Ω resistance were fabricated from borosilicate glass with 1.8 mm outer diameter (Hilgenberg, Malsfeld, Germany) using a two-stage electrode puller (Narishige, Tokyo, Japan) and fire-polished. Pulse protocols, data acquisition and evaluation programs were written in 'C'. Voltage pulses were delivered from a microcontroller (Schild *et al.* 1996) to a D/A converter and then to the patch-clamp amplifier (EPC7; List, Darmstadt, Germany) in order to assess the impedance in the on-cell and whole-cell configurations. Holding voltage in the on-cell configuration was 0 mV, while in whole-cell recordings, an average voltage of –75 mV was set by current injection in the current-clamp mode. Currents and voltages were recorded on video tape using a PCM unit (Instrutech, Elmont, NY, USA). The data were digitized off-line using an 8-pole Bessel filter, an A/D converter and a PC. Further data analysis was performed on a PC under the LINUX operating system.

Nose–olfactory bulb preparation and calcium imaging

Tadpoles (stages 51–53) were chilled in a mixture of ice and water and after subsequent decapitation a block of tissue containing the olfactory mucosae, olfactory nerves and the brain was cut out. The tissue was glued on a stage of a vibroslicer, and only the dorsal surface of the olfactory bulb was sliced off. The olfactory mucosae and olfactory nerves were left intact. The nose–olfactory bulb preparation was transferred to a recording chamber, and 200 μl of bath solution (see below) containing 50 μM Fura-2 AM (Molecular Probes) was added. After incubation for 1 h at room temperature on a shaker, the tissue block was rinsed with bath solution, glued into the recording chamber using 5% low melting point agarose, covered with bath solution and viewed under an upright microscope using a $\times 10$ objective (Axioskop 2, Zeiss, Göttingen, Germany). Preparations were rinsed with bath solution for at least 20 min under a bath flow at a rate of 550 $\mu\text{l min}^{-1}$ in the recording set-up. To estimate intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$), pairs of fluorescence images (F_{340} and F_{380} , alternating excitation at 340 and 380 nm; emission > 505 nm) of the olfactory bulb were taken using a frame transfer, back-illuminated CCD camera (Micromax, Visitron, München, Germany) and a custom-built monochromator with a xenon light source. Image pairs were acquired at 0.96 Hz and 500 ms exposure time per image. The $[\text{Ca}^{2+}]_i$ responses of mitral and granule cells were represented pixelwise as the ratio F_{340}/F_{380} , whereby the mean values of the autofluorescence of unstained slices were taken as background values. The background-corrected ratio R of the

Table 1. Water-soluble mixtures of L-amino acids

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

Mixtures of L-amino acids following groups established by Caprio & Byrd (1984). LCN, long chain neutral amino acids. SCN, short chain neutral amino acids. BAS, alkaline amino acids. ACID, acidic amino acids. AROM, aromatic amino acids.

fluorescence images excited at 340 and 380 nm (F_{340} and F_{380}) was taken as an estimate of $[\text{Ca}^{2+}]_i$, and correspondingly, the changes $\Delta R(t) = R(t) - R(t=0)$ were taken as estimates for $\Delta[\text{Ca}^{2+}]_i(t)$. Anatomical identification of mitral and granule cells was based on morphological criteria according to previous investigations by Nezlin & Schild (2000).

Figure 1B shows a slice of the anterior part of the brain including the olfactory nerves (ON), the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB) stained with propidium iodide (see above).

Solutions and stimulus application

The composition of the bath solution was (mM): 98 NaCl, 2 KCl, 1 CaCl_2 , 2 MgCl_2 , 5 glucose, 5 sodium pyruvate, 10 Hepes. The pipette solution used for whole-cell and on-cell recordings contained (mM): 2 NaCl, 47 KCl, 2 MgCl_2 , 43 potassium gluconate, 10 Hepes, 0.2 EGTA, 2 $\text{Na}_2\text{-ATP}$, 0.1 $\text{Na}_2\text{-GTP}$. All solutions were adjusted to pH 7.8. Osmolarities of the bath and pipette solutions were 230 mosmol l^{-1} and 190 mosmol l^{-1} , respectively. The amino acids listed in Table 1 were used as odorants, applied either as a mixture of 19 amino acids (AA), or as submixtures (LCN, SCN, BAS, ACID and AROM), or single amino acids. The amino acids, as well as the membrane-permeant cAMP analogue 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (pCPT-cAMP), were dissolved in bath solution (10 mM stock, each), while forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) were dissolved in DMSO (10 mM and 50 mM stock, respectively). All of these chemicals were purchased from Sigma. Stimulus solutions were prepared immediately before use by dissolving the respective stock solution in bath solution (for final concentrations see text and figure legends).

In patch-clamp experiments, the bath solution was applied to the recording chamber by gravity feed from a storage syringe through a funnel drug applicator (Schild, 1985). The tip of the applicator was placed as close as possible to the olfactory epithelium. The flow was 250 $\mu\text{l min}^{-1}$. Odorants were pipetted directly into the funnel without stopping the flow. The minimum interstimulus interval was 1 min. The dilution of the stimulus within the funnel was less than 1%. The dilution of the stimulus in the mucosa was determined by putting a confocal volume (~1 fl) of a laser-scanning confocal microscope (Zeiss LSM 510/Axiovert 100, Jena, Germany), firstly, in front of the funnel outlet and, secondly, in front of the epithelial surface and measuring the respective fluorescences. For this control measurement the fluorescent probe tetramethylrhodamine (TMR, 500 nm) was used as a 'dummy stimulus'. The dilution factor was 0.91 ± 0.02 (mean \pm S.D., $n = 7$). The delay between TMR leaving the funnel outlet and reaching the mucosal surface was less than 1 s, and after the end of stimulation, TMR was completely rinsed from the mucosa within 15 s.

In the calcium-imaging experiments, odorants were applied directly into the funnel as described above. The tip of the applicator was placed directly above the ipsilateral mucosa. Odorants were pipetted into the funnel without stopping the flow. An additional bath applicator was positioned close to the olfactory bulb using a higher flow rate of $550 \mu\text{l min}^{-1}$. Outflow was through a syringe needle placed close to the mucosa and the stimulus applicator to ensure that odorant molecules were removed rapidly with the fast bath stream. Suction was adjusted to balance the slower bath flow through the funnel applicator and the fast bath flow. The minimum interstimulus interval in calcium-imaging experiments was 5 min. To exclude any direct effects of odorant stimuli on olfactory bulb neurons, a series of control experiments were performed. After stimulation with the mix of amino acids and forskolin, the olfactory nerves were cut and the stimulation repeated. We did not observe any responses to either stimuli after transection of the olfactory nerves, and we found no differences from control conditions. However, to further exclude any direct effects on olfactory bulb neurons, we removed critical amino acids (L-glutamate, L-aspartate, L-glutamine, L-asparagine). Therefore, the amino acid mix we used in calcium-imaging experiments contained only 15 L-amino acids compared to the mix of 19 amino acids used for patch-clamp measurements in ORNs.

RESULTS

Responses to amino acids

We recorded the response behaviour of ORNs (stages 49–54) upon application of 19 amino acids using the patch-clamp technique. Two hundred and twenty-seven cells were tested,

111 in the on-cell configuration and 116 in the whole-cell configuration. The amino acids were applied either as a mixture of 19 amino acids (AA), each at a concentration of $200 \mu\text{M}$, or as amino acid submixtures (Fig. 2), or as single amino acids, each at $200 \mu\text{M}$. Twenty-seven ORNs out of 227 responded to at least one amino acid. In 11 cells, all 19 amino acids were tested (Table 2). In all cases the overall effect on spiking rate was excitatory, whereby the spontaneous spiking activity of the ORNs was always sufficiently high (Fig. 3) to detect a decrease if it should have occurred.

Figure 4 shows an example of a cell (Table 2, cell No. 3) which responded to the amino acid mixture, to the mixtures of the long chain neutral amino acids (LCN), the short chain neutral amino acids (SCN), and the alkaline amino acids (BAS), as well as to L-methionine, L-glutamine and L-histidine. It responded neither to the mixtures of aromatic amino acids (AROM) or acidic amino acids (ACID) nor to other long chain neutral, short chain neutral or alkaline amino acids. Stimulus responses came back to spontaneous activity within a variable time that depended on the specific stimulus and never exceeded 60 s.

Lack of correlation between responses to amino acids and to activators of the cAMP-mediated transduction pathway

Ninety ORNs were tested with forskolin ($10\text{--}100 \mu\text{M}$) and amino acids ($200 \mu\text{M}$) using the patch-clamp technique,

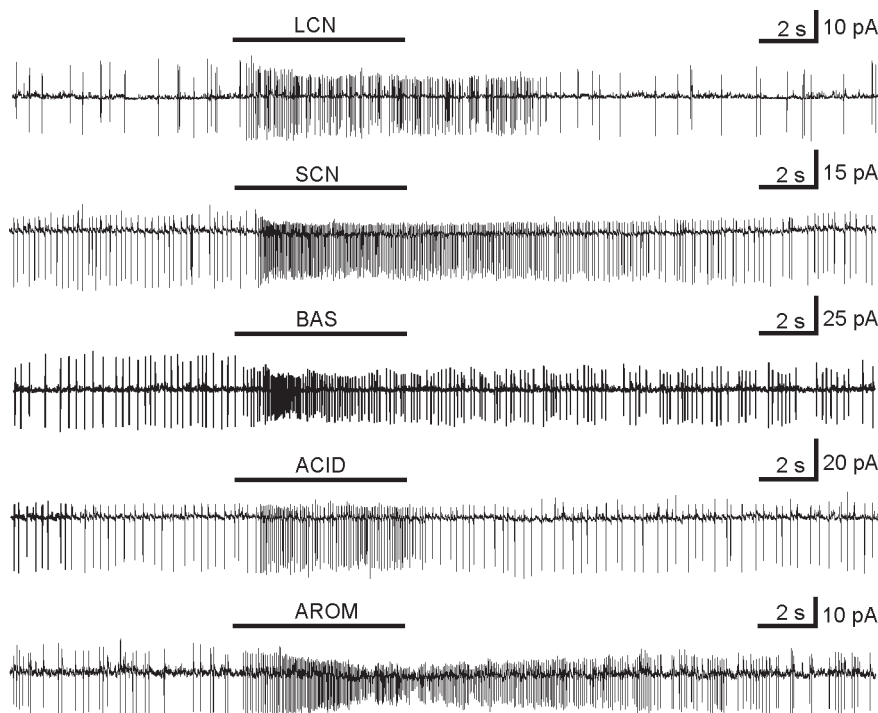


Figure 2. Odorant responses of different ORNs (stages 53 and 54) in the mucosa slice recorded in the on-cell configuration

The current traces from top to bottom show excitatory responses to the mixtures of long chain neutral (LCN), short chain neutral (SCN), alkaline (BAS), acidic (ACID) and aromatic (AROM) amino acids. In all of the submixtures the concentration of the individual amino acids was $200 \mu\text{M}$.

Table 2. Responses of 11 ORNs to single amino acids

Cell No.		1	2	3	4	5	6	7	8	9	10	11
SCN	Gly											
	Ala											●
	Ser											
	Thr											
	Cys											
	Asn											
	Gln			●								●
LCN	Val	●							●			
	Leu								●	●		
	Ile								●			
	Me	●		●	●		●			●		
	Pro											
ACID	Asp		●									
	Glu											
BAS	Arg					●		●		●		
	Lys					●		●				
	His			●								
AROM	Phe							●				
	Trp							●				

Of the 27 ORNs that responded to amino acids 11 were tested with all 19 amino acids. The dot size in the table represents the relative increase of spiking rate with respect to spontaneous activity: ● : > 350 %, ● : 250–350 %, ● : 130–250 %, no dot: no significant change from spontaneous activity.

51 in the on-cell configuration and 39 in the whole-cell configuration. Twenty-one out of these ORNs exhibited a response. Nine of these 21 ORNs responded to forskolin, but not to amino acids (Fig. 5B and D). The remaining 12 ORNs responded to amino acids but, interestingly, only 5 of these 12 ORNs also responded to forskolin (Fig. 5A and D), while 7 responded to amino acids only (Fig. 5C and D). This result, though based on small numbers, clearly indicates that some amino acids are transduced in a cAMP-independent way.

Considering the general view that different odorant-receptor specificities are mapped onto different areas of the olfactory bulb (Ressler *et al.* 1994; Mombaerts *et al.* 1996; Fuss & Korsching, 2001; Nikonov & Caprio, 2001), it should be expected that the projection area of amino acid-sensitive ORNs is not congruent with the projection area of forskolin-sensitive ORNs. We tested this hypothesis using calcium-imaging of the postsynaptic population of olfactory bulb neurons, while applying forskolin (100 μM) or amino acids (100 μM) to the olfactory mucosa. Using this method we were able to monitor changes in the somatic [Ca²⁺]_i in olfactory bulb neurons. We used a mix of 15 amino acids (see Methods). Figure 6A–F shows representative examples of calcium responses in mitral and granule cells in response to stimulation with amino acids (shown in red) and forskolin (shown in green). The spatial distributions of olfactory bulb neurons that responded to amino acids and forskolin were clearly different and showed very little overlap (Fig. 6C–E). The

majority of olfactory bulb neurons responding to stimulation of the olfactory mucosa with the mixture of amino acids were always located in the lateral half of the olfactory bulb (n = 33 slices), whereas the population of neurons responding to forskolin was located in the medial half of the olfactory bulb (n = 8 slices). In cases where both stimuli were used in the same preparation, less than 5 % of the neurons responded to both stimuli (n = 8 slices). These neurons were located at an intermediate position

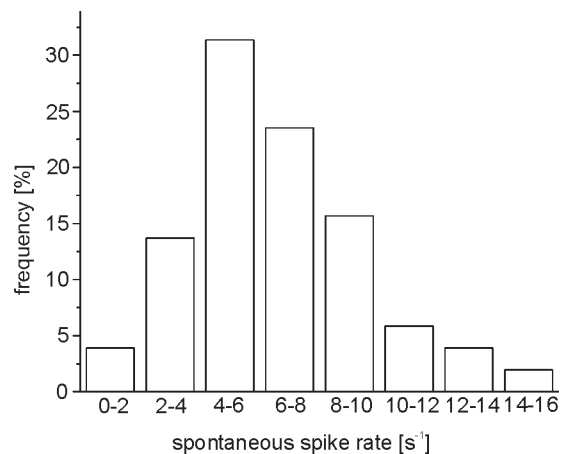


Figure 3. Frequency histogram of spontaneous spiking activities of ORNs in the mucosa slice

Fifty-one ORNs (stages 49–54), recorded in the on-cell mode and tested for both forskolin and amino acids, were evaluated for this histogram.

(Fig. 6F). The time courses of calcium responses to amino acids and forskolin were very similar with a steep increase in intracellular calcium after stimulus onset followed by a plateau and a slow decrease outlasting the stimulus application. Figure 6G and H shows three representative time courses of $[Ca^{2+}]_i$ in neurons within the mitral cell layer upon stimulation with amino acids and forskolin.

Though it was clear at this point of our study that the map of amino acid-sensitive ORNs upon the olfactory bulb differed from that of forskolin-sensitive ORNs, there was

still an uncertainty as to whether forskolin, particularly at the high concentrations used, acted by activating adenylate cyclase. In addition to or instead of its effect upon adenylate cyclase, forskolin might have acted as an odorant. To remove this ambiguity we tested, in addition to forskolin (50 or 100 μM), the effects of IBMX (500 μM), and pCPT-cAMP (2.5 mM). The neurons activated by forskolin were also activated by IBMX and pCPT-cAMP (Fig. 7). The overlap with the neuron ensemble activated by amino acids was as small as that described above, though slightly higher for pCPT-cAMP. In the 12 slices

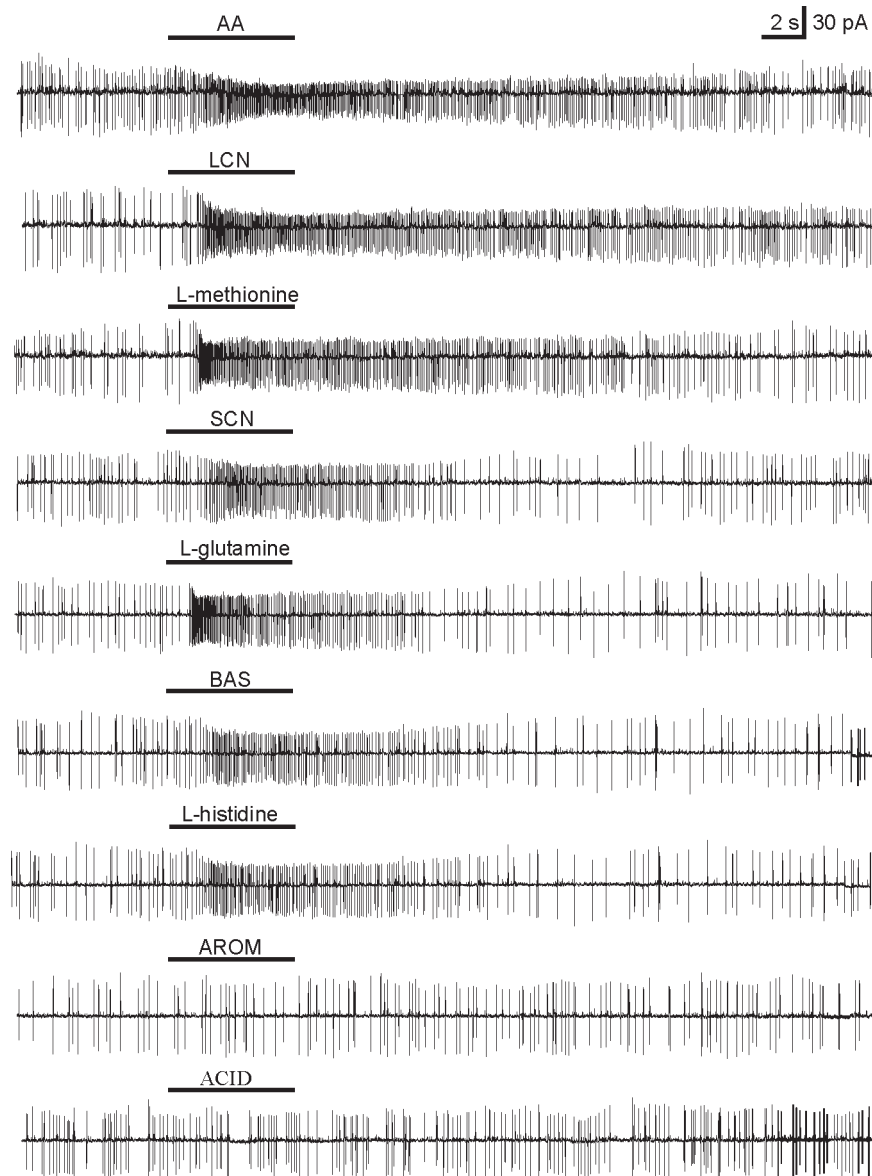


Figure 4. Odorant responses of a single ORN in the mucosa slice from a tadpole (stage 53) recorded in on-cell configuration

The current traces from top to bottom show responses to: the mixture of amino acids (AA), the mixture of long chain neutral amino acids (LCN), L-methionine, the mixture of short chain neutral amino acids (SCN), L-glutamine, the mixture of alkaline amino acids (BAS) and to L-histidine. There was no response to the mixtures of aromatic (AROM) and acidic (ACID) amino acids. All amino acids were applied at a concentration of 200 μM . The traces are arranged according to the intensities of the responses to the different stimuli.

tested the overlap was less than 5 % for forskolin or IBMX and less than 9 % for pCPT-cAMP.

DISCUSSION

Amino acid responses

Amino acids are known to be olfactory stimuli in *Xenopus laevis* (Vogler & Schild, 1999; Iida & Kashiwayanagi, 2000). Regarding the responses to single amino acids, Table 2 shows that some ORNs (5 out of 11) responded to one amino acid, while others responded to amino acids of one

or more groups. This nicely confirms the classification of ORNs in fish by Caprio & Byrd (1984), which was set up by cross-adaptation experiments. In their study, responses to L-histidine were correlated with responses to the LCN group of amino acids, a correlation also seen in Table 2 (cell No. 3).

It was not the aim of this study to analyse specificity profiles of tadpole ORNs in more detail, though extending Table 2 to a complete set of response patterns is a tempting idea. However, using the patch-clamp technique this

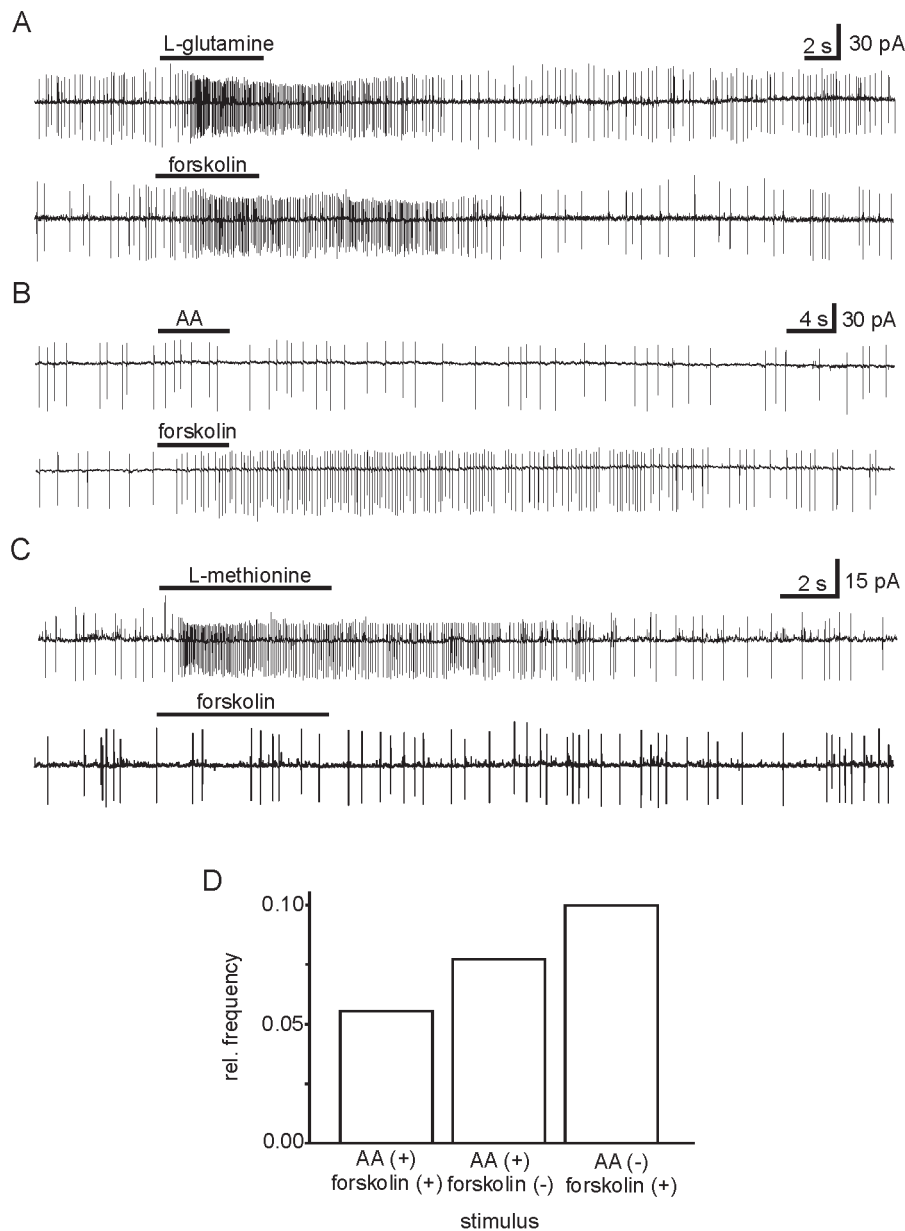


Figure 5. Correlation of odorant and forskolin responses

A, ORN (stage 53) excited by both L-glutamine (200 μM) and forskolin (50 μM). B, ORN (stage 51) excited by forskolin (50 μM) but not by the mixture of amino acids (AA, 200 μM). C, ORN (stage 54) excited by an amino acid (L-methionine, 200 μM) but not by forskolin (50 μM). D, occurrences of correlated and uncorrelated responses to forskolin and amino acids. Sixty-nine out of the 90 ORNs tested responded neither to amino acids nor to forskolin.

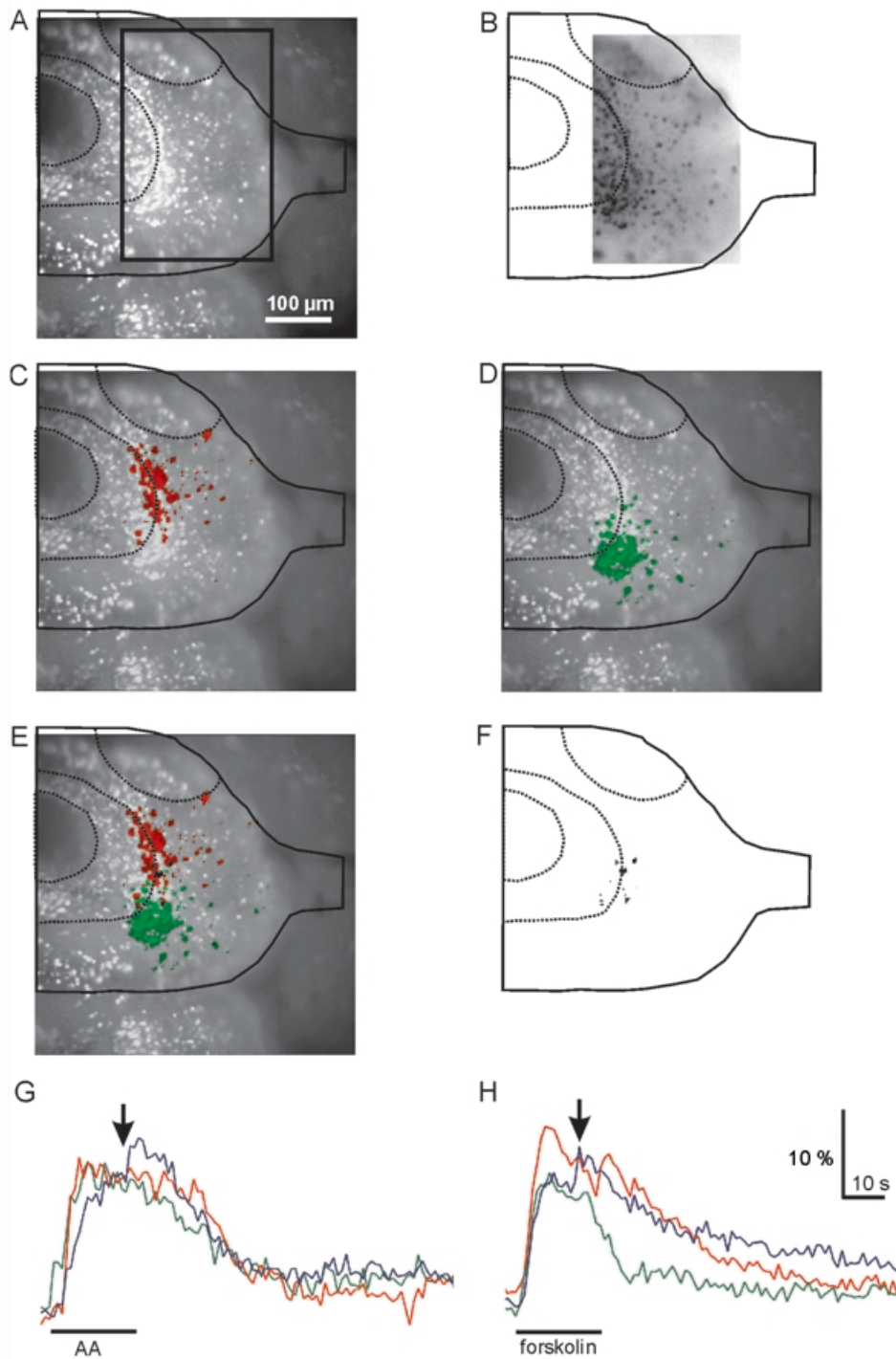


Figure 6. Comparison of changes of $[Ca^{2+}]_i$ in olfactory bulb neurons in response to stimulation of the olfactory mucosa with amino acids and forskolin

A, fluorescence image (excitation at 380 nm) of the olfactory bulb of a tadpole (stage 51) with outlines indicating the borders of the olfactory bulb, olfactory nerve, ventricle and the approximate borderlines between the mitral and granule cell layers and of the accessory olfactory bulb. Cell bodies of mitral and granule cells exhibit bright fluorescence. The box indicates the region of interest used for calcium imaging. B, ratio image at the time $t = 0$ s. C and D, spatial response patterns to stimulation with amino acids ($100 \mu\text{M}$, C, in red) and forskolin ($100 \mu\text{M}$, D, in green) expressed as stimulus-induced ratio changes. For identification of the location of olfactory bulb neurons, the ratio changes were superimposed on the fluorescence image shown in A. E, superimposed images of the responses to amino acids and forskolin. F, areas of overlap between the responses to stimulation with amino acids and forskolin. G and H, time courses of calcium responses (expressed as ratio changes) in olfactory bulb neurons in the mitral cell layer to stimulation with amino acids (G) and forskolin (H). Three representative examples are shown in each case. The arrows indicate the time chosen for the spatial response patterns shown in the images in panels C–F.

would presumably require thousands of experiments given the huge number of parameters (i.e. number of possible stimuli, different concentrations, number of receptors proteins, possibility of more than one receptor per cell...). Other methods, in particular optical imaging techniques, allowing the recording of many cells simultaneously, are more promising in this respect.

Lack of correlation between responses to amino acids and to pharmacological agents activating the cAMP transduction pathway

The cAMP-mediated olfactory pathway can be activated in various odorant-independent ways, e.g. by dialysis of cAMP through a patch pipette into the cytosol (e.g. Iida & Kashiwayanagi, 2000), or by application of forskolin (e.g. Frings & Lindemann, 1991), IBMX (e.g. Kashiwayanagi & Kurihara, 1995; Zufall *et al.* 2000) or pCPT-cAMP (e.g. Kashiwayanagi & Kurihara, 1995; Reisert & Matthews, 2001) to the bath solution.

In our patch-clamp experiments about 40 % of the ORNs that responded to amino acids were also excited by forskolin, which is consistent with cAMP-mediated olfactory transduction. Further, it was not unexpected that forskolin excited some ORNs that were not excited by amino acids. In these cases amino acids were not the appropriate stimuli. Interestingly, however, 7 out of 12 ORNs (about 60 %) that responded to amino acids were not sensitive to forskolin (Fig. 5C). In these cases, the odorants activated a cAMP-independent olfactory pathway. Thus, amino acid transduction is probably not a subset of cAMP-dependent transduction.

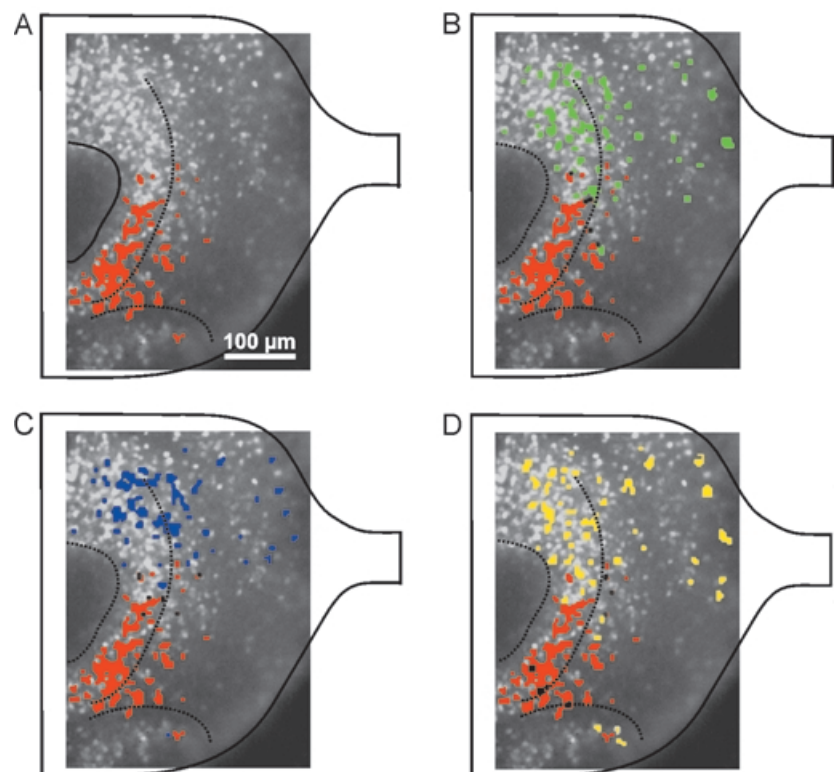
To support this evidence we investigated the projection of olfactory stimuli to the olfactory bulb and analysed the $[Ca^{2+}]_i$ response patterns in mitral and granule cells upon stimulation with amino acids, on the one hand, and with forskolin, IBMX or pCPT-cAMP, on the other. Olfactory bulb neurons responding to amino acids preferentially were located in the lateral half of the olfactory bulb, indicating that glomeruli in the lateral olfactory bulb process information about amino acids. Interestingly, a projection of amino acid-sensitive ORNs to a cluster of glomeruli in the lateral olfactory bulb has also been observed in imaging studies in the zebra fish olfactory bulb (Friedrich & Korsching, 1997).

Few olfactory bulb neurons in *Xenopus laevis* showed a response to stimulation with both amino acids and pharmacological agents that activate the cAMP-mediated transduction pathway. On the other hand, the relatively large number of neurons responding to stimulation with forskolin, IBMX or pCPT-cAMP suggests that there is a significant number of ORNs responding to as yet unknown odorants mediated by the cAMP transduction pathway. Forskolin activated virtually the same ensemble of olfactory bulb neurons as IBMX and pCPT-cAMP did. Forskolin, therefore, did not appear to act as an odorant. We can instead conclude that axons of ORNs responsive to cAMP-mediated stimuli project to glomeruli in the medial region of the olfactory bulb.

The pCPT-cAMP-sensitive neuron ensemble and the amino acid-sensitive ensemble showed a larger overlap than the forskolin- or IBMX-sensitive ensemble with the

Figure 7. Comparison of $[Ca^{2+}]_i$ responses in olfactory bulb neurons upon stimulation of the olfactory mucosa with amino acids, forskolin, IBMX and pCPT-cAMP

Background in A–D is as in the preceding figure. A, $[Ca^{2+}]_i$ activation pattern in response to the mixture of amino acids (100 μ M, shown in red) superimposed on the background image. B–D, $[Ca^{2+}]_i$ activation patterns in response to forskolin (50 μ M, B, green), IBMX (500 μ M, C, blue) and pCPT-cAMP (2.5 mM, D, yellow) were superimposed on the image shown in A. The areas of overlap are shown in black.



amino acid-sensitive ensemble. This is possibly due to pCPT-cAMP acting, in addition to the effect upon the adenylate cyclase, as an odorant in *Xenopus laevis* tadpoles. Nucleotides are known to be important olfactory stimuli in fish (Kang & Caprio, 1995). We did not analyse this possibility here.

In summary, forskolin, IBMX and pCPT-cAMP, on the one hand, and amino acids, on the other, induce very distinct and almost exclusive spatial patterns of neuronal activity in the olfactory bulb. This clearly shows that cAMP-mediated responses and responses to amino acids are transduced, processed and mapped differentially.

REFERENCES

- BOEKHOFF, I., MICHEL, W. C., BREER, H. & ACHE, B. W. (1994). Single odors differentially stimulate dual second messenger pathways in lobster olfactory receptor cells. *Journal of Neuroscience* **14**, 3304–3309.
- BOEKHOFF, I., TAREILUS, E., STROTMANN, J. & BREER, H. (1990). Rapid activation of alternative second messenger pathways in olfactory cilia from rats by different odorants. *EMBO Journal* **9**, 2453–2458.
- BUCK, L. & AXEL, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* **65**, 175–187.
- CAPRIO, J. & BYRD, R. P. (1984). Electrophysiological evidence for acidic, basic, and neutral amino acid olfactory sites in the catfish. *Journal of General Physiology* **84**, 403–422.
- EDWARDS, F. A., KONNERTH, A., SAKMANN, B. & TAKAHASHI, T. (1989). A thin slice preparation for patch-clamp recordings from neurones of the mammalian central nervous system. *Pflügers Archiv* **414**, 600–612.
- FREITAG, J., KRIEGER, J., STROTMANN, J. & BREER, H. (1995). Two classes of olfactory receptors in *Xenopus laevis*. *Neuron* **15**, 1383–1392.
- FRIEDRICH, R. W. & KORSCHING, S. I. (1997). Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. *Neuron* **18**, 737–752.
- FRINGS, S. & LINDEMANN, B. (1991). Current recording from sensory cilia of olfactory receptor cells *in situ*. I. The neuronal response to cyclic nucleotides. *Journal of General Physiology* **97**, 1–16.
- FUSS, S. H. & KORSCHING, S. I. (2001). Odorant feature detection: activity mapping of structure response relationships in the zebrafish olfactory bulb. *Journal of Neuroscience* **21**, 8396–8407.
- GOLD, G. H. (1999). Controversial issues in vertebrate olfactory transduction. *Annual Review of Physiology* **61**, 857–871.
- IIDA, A. & KASHIWAYANAGI, M. (1999). Responses of *Xenopus laevis* water nose to water-soluble and volatile odorants. *Journal of General Physiology* **114**, 85–92.
- IIDA, A. & KASHIWAYANAGI, M. (2000). Responses to putative second messengers and odorants in water nose olfactory neurons of *Xenopus laevis*. *Chemical Senses* **25**, 55–59.
- KANDEL, E. R., SCHWARTZ, J. H. & JESSELL, T. M. (2000). In *Principles of Neural Sciences*, 4th edn, chap. 15. McGraw-Hill, New York.
- KANG, J. & CAPRIO, J. (1995). *In vivo* responses of single olfactory receptor neurons in the channel catfish, *Ictalurus punctatus*. *Journal of Neurophysiology* **73**, 172–177.
- KANG, J. & CAPRIO, J. (1997). *In vivo* responses of single olfactory receptor neurons of channel catfish to binary mixtures of amino acids. *Journal of Neurophysiology* **77**, 1–8.
- KASHIWAYANAGI, M. & KURIHARA, K. (1995). Odor responses after complete desensitization of the cAMP-dependent pathway in turtle olfactory cells. *Neuroscience Letters* **193**, 61–64.
- MICHEL, W. C. & ACHE, B. W. (1994). Odor-evoked inhibition in primary olfactory receptor neurons. *Chemical Senses* **19**, 11–24.
- MOMBAERTS, P., WANG, F., DULAC, C., CHAO, S. K., NEMES, A., MENDELSON, M., EDMONDSON, J. & AXEL, R. (1996). Visualizing an olfactory sensory map. *Cell* **87**, 675–686.
- NEZLIN, L. P. & SCHILD, D. (2000). Structure of the olfactory bulb in tadpoles of *Xenopus laevis*. *Cell and Tissue Research* **302**, 21–29.
- NIEUWKOOP, P. D. & FABER, J. (1956). In *Normal Table of Xenopus laevis* (Daudin). North Holland Company, Amsterdam.
- NIKONOV, A. A. & CAPRIO, J. (2001). Electrophysiological evidence for a chemotopy of biologically relevant odors in the olfactory bulb of the channel catfish. *Journal of Neurophysiology* **86**, 1869–1876.
- REISERT, J. & MATTHEWS, H. R. (2001). Responses to prolonged odour stimulation in frog olfactory receptor cells. *Journal of Physiology* **534**, 179–191.
- RESSLER, K. J., SULLIVAN, S. L. & BUCK, L. B. (1994). Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. *Cell* **79**, 1245–1255.
- RESTREPO, D., MIYAMOTO, T., BRYANT, B. P. & TEETER, J. H. (1990). Odor stimuli trigger influx of calcium into olfactory neurons of the channel catfish. *Science* **249**, 1166–1168.
- SCHILD, D. (1985). A computer-controlled device for the application of odours to aquatic animals. *Journal of Electrophysiological Techniques* **12**, 71–79.
- SCHILD, D., GENNERICH, A. & SCHULTENS, H. A. (1996). Microcontrollers as inexpensive pulse generators and parallel processors in electrophysiological experiments. *Medical and Biological Engineering and Computing* **34**, 305–307.
- SCHILD, D. & RESTREPO, D. (1998). Transduction mechanisms in vertebrate olfactory receptor cells. *Physiological Reviews* **78**, 429–466.
- SKLAR, P. B., ANHOLT, R. R. & SNYDER, S. H. (1986). The odorant-sensitive adenylate cyclase of olfactory receptor cells. Differential stimulation by distinct classes of odorants. *Journal of Biological Chemistry* **261**, 15538–15543.
- VOGLER, C. & SCHILD, D. (1999). Inhibitory and excitatory responses of olfactory receptor neurons of *Xenopus laevis* tadpoles to stimulation with amino acids. *Journal of Experimental Biology* **202**, 997–1003.
- ZUFALL, F., LEIDNERS-ZUFALL, T. & GREER, C. A. (2000). Amplification of odor-induced Ca²⁺ transients by store-operated Ca²⁺ release and its role in olfactory signal transduction. *Journal of Neurophysiology* **83**, 501–512.
- ZUFALL, F. & MUNGER, S. D. (2001). From odor and pheromone transduction to the organization of the sense of smell. *Trends in Neurosciences* **24**, 191–193.

Acknowledgements

We thank Arne Gennerich for assembling the fluorescence excitatory pathway and the monochromator.