Coordinated shift of olfactory amino acid responses and V2R expression to an amphibian water nose during metamorphosis

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Short title: Olfaction during metamorphosis

Abstract

All olfactory receptors identified in teleost fish are expressed in a single sensory surface, whereas mammalian olfactory receptor gene families segregate into different olfactory organs, chief among them the main olfactory epithelium expressing ORs and TAARs, and the vomeronasal organ expressing V1Rs and V2Rs. A transitional stage is embodied by amphibians, with their vomeronasal organ expressing more 'modern', later diverging V2Rs, whereas more 'ancient', earlier diverging V2Rs are expressed in the main olfactory epithelium. During metamorphosis the main olfactory epithelium of *Xenopus* tadpoles transforms into an air-filled cavity (principal cavity, air nose), whereas a newly formed cavity (middle cavity) takes over the function of a water nose. We report here that larval expression of ancient V2Rs is gradually lost from the main olfactory epithelium as it transforms into the air nose. Concomitantly, ancient *v2r* gene expression begins to appear in the basal layers of the newly forming water nose. We observe the same transition for responses to amino acid odorants, consistent with the hypothesis that amino acid responses may be carried by V2R receptors.

Introduction

Amphibians occupy an intermediate stage in the water-to-land transition that occurred during vertebrate evolution. Thus they have to cope with the drastically different demands placed by these two environments on their olfactory systems (see [1]). The olfactory organ of an aquatic species needs to sense water-borne, hydrophilic odorants, whereas a terrestrial species has to detect a mostly non-overlapping set of hydrophobic, airborne odorants. Amphibians solve this problem by adapting the larval olfactory organ during metamorphosis to meet the requirements of the adult lifestyle [2]. Anurans reconstruct the main olfactory epithelium (MOE) of their aquatic tadpoles into a so-called air nose (principal cavity) during metamorphosis [2]. Secondarily aquatic pipid frogs such as *Xenopus laevis* have an additional olfactory epithelium, the so-called water nose (middle cavity), that develops newly during metamorphosis [3–5], and is responsible for detecting water-borne odors (in terrestrial anurans the middle cavity is non-sensory [2]).

During this metamorphotic reorganization massive apoptotic cell death occurs, former larval olfactory receptor neurons (ORNs) are replaced and newly generated neurons form the middle cavity [4]. Thus one expects massive changes in the molecular and functional response characteristics of olfactory sensory neurons during metamorphosis. However, so far, these changes have not been investigated, neither at the molecular nor the functional level.

We have recently identified an early diverging subclade of vomeronasal type 2 receptors (V2Rs) that surprisingly was expressed in the MOE of tadpoles [6], together with transient receptor potential cation channel (TRPC2*)*, an element of the signal transduction cascade for V2Rs [7]. Remarkably, ORNs activated by amino acids, a major odor group for fish and frogs [8–11], show a similar spatial distribution as the V2R and TRPC2 expression [6]. It may be expected that amino acid responses and possibly V2R expression do not remain unaltered during metamorphosis, as there appears to be no use for receptors to detect water-borne odorants such as amino acids in the air nose of an adult frog.

We have therefore analysed the amino acid responses of both water and air nose during metamorphosis and in post-metamorphotic frogs and report a gradual loss of response in what used to be larval MOE, and a concomitant increase in amino acid responses in the newly formed water nose. Exactly the same transition is seen for the expression pattern of larval MOE-specific *v2r* genes, with a gradual loss of expression in the larval MOE, and a concomitant increase of expression in the middle cavity. Moreover, spatial segregation *within* the middle cavity is very similar for amino acid responses and V2R expression. These results strengthen the hypothesis of V2R receptors carrying the olfactory response to amino acids in the

3

amphibian sense of smell.

Results

Metamorphotic transition of amino acid odor sensitivity from the larval principal cavity to the emerging middle cavity

Amino acids constitute an important odor class in aquatic organisms, signalling the presence of food [8, 11]. To examine the fate of olfactory responses during metamorphosis of *Xenopus laevis* we monitored responses to amino acids at three ontogenetic stages, 57/58, 61/62 and 66+ (beginning metamorphosis with functional ORNs already present in the MC, mid-metamorphosis, and post-metamorphosis, respectively; see Fig. 1 and [12, 13]). At the same stages we concomitantly measured forskolin responses to visualize neurons that use a cAMP-mediated signal transduction cascade and are presumably ciliated [10]. Amino acid odor- and forskolin-induced responses of ORNs were measured as somatic Ca^{2+} transients in acute slice preparations of the epithelia of the MC and PC. Representative traces from individual ORNs are shown for all stages (Fig. 1).

In all stages the two stimuli activated mutually exclusive subsets of ORNs (Fig. 1, Table 1), with the sole exception of two neurons found at stage 66+, which reacted to both stimuli (Table 1). The MC contained amino acid-responsive as well as forskolin-responsive cells at all stages examined, similar to the larval MOE [10]. In contrast, the developing PC retains forskolin responses, but gradually loses all amino acid responses (Fig. 1).

Forskolin-responsive ORN were found in roughly similar frequency in the middle and principal cavity during all metamorphosis stages analysed (Fig. 1). Likewise, in early metamorphosis (stage 57/58), the responses to amino acids were nearly equally distributed across the middle and principal cavity (Fig. 1). However, during mid-metamorphosis the balance shifted massively, with only 5% of amino-acid-responsive neurons located in the principal cavity (Fig. 1). Finally, post-metamorphosis (stage 66+) not a single amino acid-responsive neuron was found in the principal cavity, whereas many such cells were present in the newly formed middle cavity (Fig. 1).

During metamorphosis, expression of the broadly expressed olfactory receptor v2r-C ceases in

the principal cavity, and concomitantly appears in the newly forming middle cavity

We have previously suggested that the amphibian olfactory response to amino acids is carried by V2R receptors, based on spatial co-segregation within the tadpole olfactory organ [6]. Here we show that olfactory amino acid responses migrate to the newly forming middle cavity during metamorphosis. If V2R receptors do mediate these responses, we expect their expression pattern to switch in parallel with the amino acid responses during metamorphosis.

Amphibian *v2r* genes are subdivided in 3 subfamilies (*v2r-A1, A2*, and *A3*), plus *v2r-C* as the most ancestral gene of the family. This gene is broadly expressed in possibly all microvillous neurons of the tadpole MOE, whereas other *v2r* genes are expressed in sparse subsets of neurons [6, 7]. Thus, *V2R-C* can serve as a general indicator for V2R expression. We performed *in situ* hybridization on tissue sections of the olfactory organ to follow expression of *V2R-C* over the course of metamorphosis up to the young post-metamorphotic frog stage.

We report that during metamorphosis expression of *V2R-C* becomes gradually less frequent in the MOE (Fig. 2). At stage 57/58, *V2R-C*-expressing neurons are found in high frequency in both the MOE and the newly forming middle cavity (water nose). At stage 61/62, i.e. during mid-metamorphosis of the former MOE into the adult principal cavity (air nose), very little expression remains in the MOE. Post metamorphosis, at stage 66+, not a single *V2R-C*-expressing cell could be found in the principal cavity among several hundred labeled neurons examined (Fig. 2). For TRPC2, a component of the signal transduction pathway for V2Rs, a very similar gradual shift in expression from the MOE to the middle cavity was observed (data not shown). Incidentally, *V2R-C* is also completely absent from the VNO (Fig. 2).

Taken together, *V2R-C*-expressing cells show a very similar transition from larval MOE to middle cavity, as observed for the amino acid responses (Fig. 1), supporting the hypothesis that V2R-expressing neurons mediate amino acid responses.

Post-metamorphosis ancient v2r genes and a component of their signal transduction cascade are completely absent from the principal cavity

Next we evaluated the spatial expression pattern of two ancient *v2r* genes, *v2r-A1a*, and *v2r-A1b*, after metamorphosis was completed. In tadpoles ORNs expressing these genes appear to constitute a subpopulation of *V2R-C*-expressing cells [6]. Both genes were found exclusively expressed in the middle cavity, and absent from principal cavity and VNO (Figs. 3, 4). Thus expression of these genes co-migrates both with the *V2R-C* expression and the amino acid responses. In contrast, a late-diverging *v2r* gene known to be expressed exclusively in the VNO

of tadpoles (*v2r-A3 E-1*), is still exclusively found in VNO after metamorphosis (Fig. 3). The probe for *v2r-A3 E-1* crossreacts with 95 other late-diverging *v2r* genes [6], in other words reflects the expression pattern of up to 95 *v2r* genes. This suggests that the larval split in expression pattern between early and late-diverging *v2r* genes is faithfully reproduced during metamorphosis, even though the site of expression changes for the early-diverging genes.

Finally we examined the expression pattern of two marker genes for ciliated and microvillous receptor neurons (OMP and TRPC2, respectively; [6, 7]). Strong OMP expression was seen in both middle and principal cavity post metamorphosis (Figs. 3, 4). This result is consistent with the presence of forskolin-responsive neurons in both middle and principal cavity (Fig. 1). As expected, the VNO did not exhibit OMP expression (Fig. 3). On the other hand, TRPC2, a signal transduction component of microvillous neurons [7, 14], was found expressed in the middle cavity and the VNO, but absent from the principal cavity (Figs. 3, 4). Thus, the middle cavity mimicks the mixed ORN population of the larval main olfactory epithelium, with both ciliated and microvillous receptor neurons, and responses to forskolin as well as amino acids, even though this organ is built *de novo* during metamorphosis [4].

Ancient V2Rs form a basal expression domain in the middle cavity very similar to that observed for expression in tadpole main olfactory epithelium

In the tadpole main olfactory epithelium, V2R-, TRPC2-, and OMP-expressing receptor neurons largely segregate according to height, with V2R- and TRPC2-expressing neurons found in a basal layer, whereas OMP-positive neurons occupy more apical positions [6, 7]. Presumably these populations resemble microvillous and ciliated ORNs, respectively.

We were interested to see, whether this spatial expression pattern would re-form in the *de novo* generated middle cavity after metamorphosis. Relative height within organ was measured for post-metamorphotic stage 66+. V2R-*C*-expressing cells shows a basal peak at 0.4 relative height and the same peak was observed for V2R-*A1a* and V2R-*A1b* expression as well as for TRPC2. In contrast, OMP expression showed an apical peak at 0.7 relative height (Fig. 4). These values are very similar to those observed in the tadpole main olfactory epithelium (0.3-0.4 for *v2r-C*, *v2r-A1a*, and *v2r-A1b* compared to 0.8 for *omp2*, [6]). Thus, the segregation in a basal V2R-expressing domain and an apical domain for *OMP*-expressing neurons is conserved in the *de novo* formed post-metamorphotic middle cavity.

Discussion

During metamorphosis an extensive remodelling of the amphibian bauplan takes place, including a reconstruction of the olfactory system reflecting the transition from an aquatic to a terrestrial habitat. The larval main olfactory epithelium of anurans transforms into the principal cavity, the so-called air nose, dedicated to the detection of airborne smells. In secondarily aquatic anurans such as *Xenopus* a newly formed middle cavity, the so-called water nose, takes over the detection of water-borne odors.

Here we report that olfactory neuronal responses to a behaviorally important class of aquatic odors - amino acids, which serve as food odor - migrate during metamorphosis from the larval main olfactory epithelium to the middle cavity. After metamorphosis, the principal cavity retains not a single amino acid-responsive neuron. In parallel, the expression of early-derived V2Rs disappears from the larval MOE, as it transforms into the adult principal cavity, and concomitantly appears in the newly generated middle cavity. This is in line with our previous findings that neuronal populations of the olfactory organ change substantially during metamorphosis: larval ORNs undergo apoptotic cell death, do not persist in the long run and are replaced by newly generated neurons [4]. The basal enrichment of V2R-expressing cells within the middle cavity is indistinguishable from that of TRPC2-expressing neurons, very similar to the situation observed in the tadpole MOE [7]. Thus the correlation between V2R and TRPC2 expression is retained in a newly formed organ [4], the middle cavity. In the same vein, the distribution of amino acid-responsive cells between the middle cavity and the principal cavity closely parallels the distribution of *V2R-C*-expressing cells, consistent with the hypothesis that V2Rs expressed in microvillous neurons mediate the amino acid-responses in amphibians, similar to what has been shown for teleost fish [15].

Like the larval MOE, the middle cavity contains both amino acid-responsive and forskolin-responsive neurons. In larval MOE, amino acid responses have been shown to be mediated primarily by microvillous neurons, but to some extent also by forskolin-responsive (and forskolin-insensitive) ciliated neurons [7, 10, 16]. A small minority of both forskolin- and amino acid-responsive neurons is also observed in the middle cavity (this study), but not in the post-metamorphotic PC. Thus this subpopulation is dying out in the PC, but to some extent replaced during ontogenesis of the MC. Considering that forskolin-responsiveness is a hallmark of the adenylate cyclase signalling pathway characteristic for OR-expressing ciliated neurons and that OMP expression closely parallels forskolin-responsiveness ([6, 10], this study), it may be assumed that the forskolin-responsive neurons of the middle cavity represent ciliated neurons, whose ligands are to be found among water-borne odors, and may also include amino acids.

Such a dichotomic neuronal representation of amino acids appears to be an evolutionary ancient feature of neuronal representation of amino acids, since a contribution of both ciliated and microvillous ORNs has been suggested for several teleost fish species [17].

Previous work by the Breer group [18] has suggested that the adult middle cavity contains OR class I-expressing neurons (subgroup *alpha* in Niimura and Nei nomenclature, [19, 20]). For one of these OR class I receptors amino acid responses have been shown [21], so this class of receptors would be a plausible candidate for mediating amino acid responses in ciliated neurons.

Taken together, our results confirm and extend the molecular and functional segregation of olfaction in the middle *vs.* the principal cavity. Furthermore, they show that the unusual division of a large olfactory receptor gene family between two main olfactory organs is faithfully kept during metamorphosis: only those *v2r* genes expressed in tadpoles main olfactory epithelium later become expressed in the middle cavity (water nose). Thus the dichotomy of V2R expression in two different organs is not an immature feature of the *Xenopus* olfactory system, but stably maintained in adulthood. As both V2R subclades are expressed in microvillous ORNs, it will be informative to identify the regulatory elements which direct microvillous neurons of the main olfactory epithelium towards expression of early diverging V2Rs (subfamilies C and A1), and to compare them with those resulting in late diverging V2Rs (subfamilies A2 and A3) being expressed in the vomeronasal microvillous neurons.

Another feature of V2R expression stable during metamorphosis is the basal expression domain, suggesting the same segregation of basal microvillous and apical ciliated ORNs in larval MOE and post-metamorphotic middle cavity. Interestingly, a similar segregation between ciliated and microvillous neurons is found in the single olfactory surface of teleost fish, although the relative positions are inversed, with microvillous neurons occupying apical positions [15].

Finally it is worth noting that in adult secondarily aquatic amphibians detection of non-volatile odors is performed by two olfactory organs, the middle cavity/water nose, and the vomeronasal organ. We describe here post-metamorphosis the persistent expression of ancient V2Rs in the water nose, and of 'modern' V2Rs in the VNO, suggesting a corresponding segregation of functional properties between these two organs. It is tempting to speculate that the odors detected by the water nose are no longer relevant for strictly terrestrial species such as reptiles and mammals, whereas the VNO with few exceptions has kept its relevance throughout vertebrate evolution as detector of non-volatile pheromones.

Materials and Methods

Animal handling and preparation of acute slices

Xenopus laevis (of either sex, larval stages 57-58; 61-62 and post-metamorphotic froglets stage 66+; see [22]) were cooled in iced water to produce complete immobility and killed by transection of the brain at its transition to the spinal cord, as approved by the Göttingen University Committee for Ethics in Animal Experimentation. A block of tissue containing the olfactory organ, the olfactory nerves and the forebrain was cut out, and in post-metamorphotic animals parts of the skull were removed.

For acute slices the tissue block was glued onto the stage of a vibroslicer (VT 1200s, Leica, Bensheim, Germany), covered with amphibian Ringer's solution (see below) and sliced horizontally into 130-150 µm thick slices. Slices included sensory epithelium of either only MC or PC or both.

In situ hybridization

For *in situ* hybridization, tissue blocks containing MOE and vomeronasal organ were cut horizontally, fixed in 4% (wt/vol) formaldehyde solution for 2 h at room temperature, equilibrated in 30% saccharose, and embedded in Jung tissue-freezing medium (Leica, Bensheim, Germany). Cryostat sections of 10–12 µm (Leica CM1900) were dried at 55 °C and postfixed in 4% (wt/vol) paraformaldehyde for 10–15 min at room temperature. Hybridizations were performed overnight at 60°C in 50% (vol/vol) formamide using standard protocols. Anti-DIG primary antibody coupled to alkaline phosphatase and NBT-BCIP (4-nitro blue tetrazolium chloride, and 5-bromo-4-chloro-3-indolyl-phosphate, both from Roche Molecular Biochemicals) were used for signal detection. For each stage and gene the olfactory tissue of ten animals was used for *in situ* hybridization, amounting to over 300 sections.

The following primers were used to generate probes: *v2r-C* 5′-CGCACAATAGCCAGTGA-3′ 5′-CTGAACTGCAAAGCCAA-3′, *v2r-A1a* 5′-GCCTTCTCCTGCTTTCC-3′ 5′-TGTCAGGGAGGCGTCT-3′, *v2r-A1b* 5′-CTTCTCATCTCCCTCATG-3′ 5′-AAATGTGTCAGGGAGCT-3′, *omp2* 5′-CTTTCTTAGATGGCGCTGACC-3′ 5′-ACACACTTTTTTGTCTTGGG-3′, *v2r-A3 E-1* 5'-TGAGCTTCCTCCTCCTTGTC-3' 5'-GGTAATGTCCGAGCTAAAAATGC-3' [6] and *trpc2* 5′-*AAGGGATTAAGATGGACATCAA*-3′ 5′-*GCAATGCCCTTGTAGGTGTT*-3′ [7].

Quantification of the spatial expression pattern

The position of cells was evaluated in the basal-to-apical dimension according to [6]. The relative height of the cell was defined as distance of the cell soma center from the basal border of the epithelium divided by total thickness of the epithelial layer at the position of the cell (h_{rel} = h_{cell}/h_{layer}). Cell positions were measured using ImageJ (http://rsbweb.nih.gov/ij/). Distributions are visualized as histograms with 10 bins (x value given corresponds to the bin center), or unbinned as empirical cumulative distribution function (ECDF). Median, skewness, and half-width of the spatial distributions were calculated from unbinned values using Open Office (version 3.2; www.openoffice.org/). Half-width of a height distribution was defined as difference between the height values for the upper quartile and the lower quartile. The peak value was taken from the graphical representation of the histograms. To estimate whether two spatial distributions were significantly different, we performed Kolmogorov–Smirnov tests on the unbinned distributions (see [6]). This test is particularly suitable for continuous distributions and makes no assumptions about the nature of the distributions investigated, which is essential because the observed distributions are not Gaussian. Due to the sensitive nature of the test for large distributions (n > 100), we selected $p < 0.01$ as cutoff criterion for significant difference (see [6]). To count total cells per section, slides were mounted with VectaShield containing DAPI (Vector). Fluorescence was analysed using a Keyence BZ-9000 fluorescence microscope.

Calcium imaging, solutions, staining protocol and stimulus application

Amphibian Ringer's consisted of (in mM): 98 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 glucose, 5 Na-pyruvate, 10 HEPES (pH 7.8; osmolarity 230 mOsmol/l). Tissue slices (see above) were stained with the Ca^{2+}-sensitive dye Fluo-4/AM (Molecular Probes, Leiden, The Netherlands) as described in our previous work [23]. The slices were then placed in a recording chamber, which was constantly perfused with amphibian Ringer's solution applied by gravity feed from a storage syringe through a funnel applicator placed directly above the olfactory epithelia. Amino acid odors and forskolin were applied into the funnel without stopping the flow. Amphibian Ringer's solution was constantly removed from the recording chamber through a syringe needle. All experiments were conducted at room temperature. The reproducibility of the responses was verified by regularly repeating the applications at least twice. The minimum interstimulus interval was at least two minutes in all of the experiments. Amino acid odors were applied as a mixture of 19 L-amino acids (for a detailed list of the amino acids see [10]), all purchased from Sigma (Deisenhofen, Germany). Forskolin was also purchased from Sigma. The amino acids and forskolin were dissolved in amphibian Ringer's solution and DMSO (10 mM stock),

respectively, aliquoted and frozen. Aliquots were thawed only once and the working solution (mixture of amino acids, 100 μM; forskolin, 50 μM) was freshly prepared before performing the experiments.

Calcium imaging and data evaluation

Changes of intracellular calcium concentrations of individual ORNs of the epithelia of the principal cavity (PC) and middle cavity (MC) of the olfactory organ were monitored using a laser-scanning confocal microscope (LSM 510/Axiovert 100M, Zeiss, Jena, Germany). Fluorescence images (excitation at 488 nm; emission > 505 nm) of the epithelia of the principal and middle cavity were acquired at 1 Hz, with about 10 images taken as control before the onset of stimulus delivery. The thickness of the optical slices excluded fluorescence detection from more than one cell layer. The data were analyzed using custom written programs in MATLAB (Mathworks, Natick, USA). To facilitate selection of regions of interest, a "pixel correlation map" was obtained (see [24]). The fluorescence changes for individual ORNs are given as ΔF/F values. For more detailed information, see our previous work [23].

Competing interests

The authors declare no competing financial interests.

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Table 1 Specificity of olfactory receptor neuron (ORN) responses during metamorphosis

Developmental stage	# ORNs only responsive to amino acids total (MC, PC)	# ORNs only responsive to forskolin total (MC, PC)	# ORNs responsive to both total (MC, PC)	# slices evaluated total (MC, PC)
57/58	24 (14, 10)	43 (21, 22)	0 (0, 0)	19 (10, 9)
61/62	22 (21, 1)	61 (37, 24)	0 (0, 0)	13 (7, 6)
66+	26 (26, 0)	35 (23, 12)	2 (2, 0)	16 (10, 6)

Figure legends

Fig. 1 Metamorphotic shift of the sensitivity to amino acid odors from the principal cavity to the newly formed middle cavity

The metamorphotic stages examined for *Xenopus laevis* are visualized as schematic drawings to the left. Calcium transients induced by amino acid mix (100 µM) and forskolin (50 µM) in acute slices are represented as ∆F/F, with responses of three representative ORNs overlayed for each stimulus (amino acid mix, forskolin) and olfactory cavity (middle and principal cavity). Shades of magenta are used for traces of middle cavity neurons, whereas shades of green are used for neurons located in the principal cavity. Within one panel, traces with the same color originate from the same neuron. The relative abundance of neurons analysed is given as horizontal bar graphs below the representative traces; dark shades, amino acid responses; light shades, forskolin responses. **A**, late prometamorphotic stage, note the forming MC (magenta), the PC (green) and the vomeronasal organ (VNO; grey). At this stage ORNs of the MC already show responsiveness to amino acid odors and forskolin, while the PC still shows responses characteristic for the PC of premetamorphotic larvae (see [10]). Amino acid odor and forskolin-sensitive ORNs are almost equally distributed between the epithelia of the two cavities. **B**, mid-metamorphosis stage, MC (magenta), PC (green) and VNO (grey) have grown in size. In this stage the responsiveness to amino acid odors has almost completely shifted to the epithelium of the MC (see bar diagram). Forskolin-sensitive ORNs still coexist in both the epithelia of the MC and PC. **C**, post-metamorphotic froglet, all three epithelia have further grown in size and the olfactory nerve (ON) has become shorter. The responsiveness to amino acid odors has completely shifted to the epithelium of the MC. Forskolin-sensitive ORNs continue to coexist in both the epithelia of the MC and PC (see bar diagram). Abbreviations: AOB, accessory olfactory bulb; MOB, main olfactory bulb; ON, olfactory nerve; ORNs, olfactory receptor neurons; PC, principal cavity; MC, middle cavity; VNO, vomeronasal organ; AAmix, amino acid mixture.

Fig. 2 Gradual transition from the MOE to the middle cavity for the broadly expressed v2r-C gene

Expression of *V2R-C* was monitored by *in situ* hybridization of cryostat sections throughout metamorphosis. A schematic representation of the ontogenetic stages is shown to the left (see also Fig. 1), with quantitative results for the respective stage shown as bar graphs just below. Labelled cells were counted in MC/PC in 6/6 sections (stage 57/58), or 4/4 sections (stage 61/62) and (stage 66+), and frequency is given as mean+/-SEM of labelled cells per 1000 total

cells (counted as DAPI signals in three sections). Total cell numbers roughly double in the observed time window, with about fourfold more cells in PC compared to MC. Color code is identical to that used in Fig. 1 for schemes and bar graphs, respectively. Middle column, representative tissue sections are shown, with magnified subregions shown to the right. In the prometamorphotic stage (top row), similar numbers of labelled cells are present in principal and middle cavity. In mid-metamorphosis (middle row), very few *V2R-C*-expressing cells remain in the principal cavity. Post-metamorphosis (bottom row), not a single *V2R-C*-expressing cell is detected in the principal cavity, with over 15% of total cells concomitantly expressing *V2R-C* in the middle cavity. Bottom right subpanel, the post-metamorphotic VNO also does not contain *V2R-C* -expressing cells. Scale bar = 100 µm. Abbreviations: PC, principal cavity; MC, middle cavity; VNO, vomeronasal organ.

Fig. 3 Post-metamorphosis ancient v2r genes and a component of their signal transduction cascade are completely absent from the principal cavity

Principal and middle cavity as well as VNO were examined for expression of *v2r-A1a*, *v2r-A1b*, *v2r-A3 E-1*, *trpc2*, and *omp2* at stage 66+, using *in situ* hybridization. Left column, low magnification view shows both principal and middle cavity; second column, middle cavity at higher magnification; third column, principal cavity at higher magnification; right column, VNO. Scale bar = 100 µm. Abbreviations: PC, principal cavity; MC, middle cavity; VNO, vomeronasal organ.

Fig. 4 Ancient V2Rs form a basal expression domain in the post-metamorphotic middle cavity

A, quantification of cells expressing *v2r-A1a*, *v2r-A1b*, *v2r-C*, *trpc2*, and *omp2* as well as cells responding to forskolin and amino acids at stage 66+. Numbers of labelled cells per section are given as mean +/- SD for *v2r* genes and *trpc2* (MC: n=4 and n=3, respectively; PC: n=10). Numbers for OMP are from three sections (mean+/-SD); *, cumulative values (from 10 sections for MC, and 6 for PC). At this stage total cells per section in the middle cavity are about one fourth of those in the principal cavity (990 +/- 120 vs. 4040 +/- 190; mean +/- SD, n=3). Note the broad distribution of *omp2*-expressing cells and of forskolin-responsive cells, in contrast to the ancient *v2r* genes, *trcp2* and amino acid responses, which are restricted to the middle cavity.

B, height distribution of cells expressing *v2r-A1a*, *v2r-A1b*, *v2r-C*, *trcp2*, and *omp2* within the middle cavity at stage 66+. Top panel shows data as histogram, bottom panel as empirical cumulative distribution function. Note the apical OMP peak and distribution clearly segregating from the more basal distributions for *v2r* genes and *trcp2,* whereas all *v2r* gene distributions

are not significantly different from each other; ***, $p < 10^{-10}$ as evaluated by Kolmogorov-Smirnov test (see [6]).