Chem. Senses 37: 219–227, 2012

doi:10.1093/chemse/bjr095
Advance Access publication October 29, 2011

Crypt Neurons Express a Single V1R-Related ora Gene

Yuichiro Oka[1,2], Luis R. Saraiva[1,3] and Sigrun I. Korsching[1]

[1]Institut für Genetik, Universität zu Köln, Zülpicher Strasse 47a, 50674 Köln, Germany
[2]Present address: Division of Cell Biology and Neuroscience, Department of Morphological and Physiological Sciences, Faculty of Medical Sciences, University of Fukui, 23-3 Matsuoka-shimoaizuki, Eiheiji, Fukui 910-1193, Japan
[3]Present address: Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, WA 98109-1024, USA

Correspondence to be sent to: Sigrun I. Korsching, Institut für Genetik, Universität zu Köln, Zülpicher Strasse 47a, 50674 Köln, Germany. e-mail: sigrun.korsching@uni-koeln.de

Accepted September 12, 2011

Abstract

Both ciliated and microvillous olfactory sensory neuron populations express large families of olfactory receptor genes. However, individual neurons generally express only a single receptor gene according to the "one neuron–one receptor" rule. We report here that crypt neurons, the third type of olfactory neurons in fish species, use an even more restricted mode of expression. We recently identified a novel olfactory receptor family of 6 highly conserved G protein–coupled receptors, the *v1r*-like *ora* genes. We show now that a single member of this family, *ora4* is expressed in nearly all crypt neurons, whereas the other 5 *ora* genes are not found in this cell type. Consistent with these findings, *ora4* is never coexpressed with any of the remaining 5 *ora* genes. Furthermore, several lines of evidence indicate the absence of any other olfactory receptor families in crypt neurons. These results suggest that the vast majority of the crypt neuron population may select one and the same olfactory receptor gene, a "one cell type–one receptor" mode of expression. Such an expression pattern is familiar in the visual system, with rhodopsin as the sole light receptor of rod photoreceptor cells, but unexpected in the sense of smell.

Key words: ciliated, crypt cell, inhibitory G protein, microvillous, olfaction, zebrafish

Introduction

The complex task of making sense of a multitude of olfactory stimuli is made more manageable in mammals by partitioning the sense of smell in several spatially segregated and functionally different subsystems. These are populated by 2 morphologically and molecularly different olfactory neuron populations: the ciliated and the microvillous neurons (cf. Mombaerts 2004). Individual neurons express only a single receptor, but overall, large receptor families are expressed in each olfactory neuron type: odorant receptors (ORs) and trace amine-associated receptors (TAARs) in ciliated neurons; type 1 and 2 vomeronasal receptors (V1Rs and V2Rs, respectively) in microvillous neurons (Mombaerts 2004; Liberles and Buck 2006).

The fish olfactory sense is no less complex, even though fish show no spatial segregation in subsystems. Their single olfactory epithelium contains both ciliated and microvillous neurons that express the corresponding receptor gene families, OR-, TAAR-, and the V2R-related OlfC genes, respectively (Sato et al. 2005; Hussain et al. 2009; Korsching 2009). However, it has been unclear, which cell type may express the *v1r*-like ora (olfactory receptor class A-related) genes, a small family of 6 highly conserved receptor genes found in teleost fish (Saraiva and Korsching 2007) that shows the typical sparse expression pattern of olfactory receptor genes.

Intriguingly, fish feature a third olfactory sensory neuron type, the crypt neurons, in their olfactory epithelium (Hansen and Finger 2000; Hamdani el and Doving 2007). Crypt neurons were originally identified at the electron microscopic level by virtue of their unusual morphology with both cilia and microvilli, the former emerging from the bottom of the crypt that gave these cells their name, and the latter lining the rim of the crypt (Hansen and Zeiske 1998). Crypt neurons are conspicuously globose cells that are present already in cartilaginous fish, primitive bony fish, and also many modern fish species (skate, sturgeon, and e.g. zebrafish, respectively; Hansen and Zielinski 2005; Ferrando

et al. 2006). Like the other 2 cell types, crypt neurons possess separate and distinct target regions in the olfactory bulb (Morita and Finger 1998; Hansen et al. 2003; Sato et al. 2005; Hamdani el and Doving 2006, 2007). Thus, it is expected that crypt neurons correspond to another olfactory subsystem functionally different from those formed by either ciliated or microvillous neurons.

We report here that of all olfactory receptor gene families only a single gene from a single family, the *v1r*-like receptor *ora4*, was found to be expressed in crypt neurons and describe an inhibitory G protein, Gi1b, through which ORA4 may signal. This could imply a homogeneous expression of a single receptor in the entire cell type, which is starkly different from the familiar mosaic expression of large olfactory receptor gene families in ciliated and microvillous neurons according to the "one neuron–one receptor" rule of expression. The even more restricted "one cell type–one receptor" mode of expression we suggest here constitutes an interesting parallel to the visual system.

Materials and methods

Antibodies

Primary antibodies used are rabbit anti-S100 antibody (catalog no. Z0311, Dako) and rabbit anti-Gαi3 antibody (sc-262, Santa Cruz Biotechnology). According to the supplier, the latter also shows cross-reactivity with mammalian Gαi1 and to a lesser extent with Gαi2. We thus refer to this antibody as anti-Gi. Secondary antibody used is donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (A21206, Invitrogen).

Tissue preparation

Adult wild type zebrafish (Ab/Tü, 8–12 months old) were anesthetized with MS-222 (ethyl 3-aminobenzoate, Sigma) and decapitated. Olfactory epithelia were dissected out, embedded in TissueTek O.C.T. compound (Sakura), and frozen at –20 °C. Eight-micrometer-thick horizontal cryosections were thaw mounted onto Superfrost Plus slide glasses (Thermo). For paraformaldehyde (PFA)-fixed tissues (prefixed), dissected olfactory epithelia were fixed in 4% PFA in phosphate-buffered saline (PBS, pH 7.5) at 4 °C for 2 h before embedding.

Probes for in situ hybridization

Digoxigenin (DIG)-labeled probes for 6 *ora* genes were generated as described (Saraiva and Korsching 2007). The fluorescein (FLU)-labeled *ora4* probe was synthesized like the DIG probe except using FLU-UTP instead of DIG-UTP. The cDNA fragments for olfactory marker protein (OMP), transient receptor potential channel C2 (TRPC2), and *Gi1b* (also known as Gαia, GenBank accession NM_198805) were amplified from the olfactory epithelium cDNA with specific primer pairs (OMP-fw 5′-CAAGGACACACAGTAGACGC-3′, OMP-rv 5′-GGAACAGACTG-ACCAGAAGAG-3′, KpnI-TRPC2-fw 5′-aaaggtaccTCAGAAGGGTCACGAAATCC-3′, SacI-TRPC2-rv 5′-aaagagctcGCAGAGGAAAGGCATAGTCAGAAAGATC-3′, Gi1b-fw 5′-GAGTAATCAAGCGGCTCTGG-3′, and Gi1b-rv 5′-TTCGGTTCATCTCCTCATCC-3′) and cloned into pGEM-T (Promega) or pBluescript vector. The resulting plasmid was linearized with *Spe*I or *Kpn*I, purified with PCR purification kit (QIAGEN), and transcribed with T7 RNA polymerase (Roche) in the presence of DIG-UTP following manufacturer's instruction.

Immunohistochemistry combined with in situ hybridization

A rabbit polyclonal antibody to mammalian S100A, S100B proteins, calcium-binding proteins with EF-hand motifs, has been shown to label zebrafish crypt neurons as well as some slender cells with less intensity (Germana et al. 2004; Sato et al. 2005). Using prefixed tissue, we could reproduce this staining pattern, but when fresh-frozen tissue was used, the S100 antibody stains exclusively large globose cells, that is, crypt neurons (Supplementary Figure 1). Quantification of globosity confirmed the existence of a population labeled in prefixed tissue that is clearly distinct from the large globose cells, which are the only population detected in fresh-frozen tissue (Supplementary Figure 2). We could identify *s100z* as the *s100* gene responsible for labeling the slender cell population in prefixed tissue (Supplementary Figure 3), but none of the plausible candidates among the 14 zebrafish *s100* genes (Kraemer et al. 2008) is responsible for the globose cell labeling (Supplementary Figure 3). Consequently, we use the term S100-ir (immunoreactivity) for the S100 staining. In summary, we resolve the previous ambiguity surrounding the use of S100 as marker for crypt neurons and demonstrate S100-ir in fresh-frozen tissue to be a reliable specific marker for cells with crypt neuron morphology.

Pretreatment of sections, probe hybridization, and stringent washing were performed as described (Weth et al. 1996), except omitting Proteinase K digestion. After stringent washing at 65 °C, sections were blocked in 1% blocking reagent (Roche) in PBS for 1 h. The slides were then incubated with sheep anti-DIG F_{ab} fragments conjugated with alkaline phosphate (AP, Roche, 1:1000) together either with rabbit anti-S100 antibody (1:1000) or with rabbit anti-Gi antibody (1:200) in the blocking solution at 4 °C overnight. After washing 3 times in PBS, sections were reacted with the secondary antibody (1:200) in PBS for 2 h at room temperature. Hybridized probes were visualized by enzymatic reaction of AP with HNPP Fluorescent Detection Set (Roche). After evaluating the success of the staining, slides were washed in PBS, mounted with VectaShield with DAPI (Vector), and observed and photographed with a fluorescent microscope (E6000FN, Nikon, attached to a cooled CCD camera, Imago, TILL Photonics, or a BZ-8100E, Keyence).

Double in situ hybridization

Pretreatment of PFA-fixed sections, hybridization with FLU-labeled *ora4* probe together with DIG-labeled *ora*

probes, stringent washing and blocking were as described (Weth et al. 1996). Hybridized FLU probe was first visualized by binding anti-FLU F_{ab} fragments conjugated with AP (1:1000, Roche) for 2 h and AP enzymatic reaction with HNPP and then photographed as above (without mounting). After quenching AP in 100 mM Glycine–HCl (pH 2.2) for 30 min, hybridized DIG probes were visualized by binding anti-DIG conjugated with AP (1:1000) at 4 °C for overnight and AP enzymatic reaction with NBT/BCIP (Roche). After mounting with VectaMount (Vector), bright field pictures were taken with the same microscope. Bright field images were inverted, pseudocolored, and overlaid onto respective HNPP images using Photoshop (Adobe).

Quantification of cell shapes

Fluorescent in situ hybridization with probes for *ora1-6* was performed as described above, and HNPP signals were photographed. Brightness and contrast were optimized for visualization of cell shapes using Photoshop. Each cell shape was saved in a separate file, and file numbers were randomized before evaluation. The transverse and conjugate diameters of each cell (major and minor diameter, respectively) were measured with Image J (National Institutes of Health) by drawing corresponding lines.

Results

Crypt neurons express *ora4* but none of the other *ora* genes

While ciliated neurons express members of the OR and TAAR families and microvillous neurons express members of the V2R/OlfC family (Hansen et al. 2004; Mombaerts 2004; Sato et al. 2005; Liberles and Buck 2006; Hussain et al. 2009), the receptors for crypt neurons were not known so far. Here, we have tested the hypothesis (Korsching 2009) that *v1r*-like *ora* genes might be expressed in crypt neurons.

We identify crypt neurons by antibody labeling for S100-ir, a crypt neuron marker (Germana et al. 2004; Sato et al. 2005; see also Materials and methods). S100-ir was observed exclusively in a sparse population of large ovoid apical cells, sometimes with a visible crypt (Figure 1), that is, with the hallmarks of crypt neurons in regard to morphology, position in the epithelium, and frequency. Both ciliated and microvillous neurons are much more slender and are found in deeper layers, mid-apical for microvillous neurons, and below these mid-basal for ciliated neurons (Sato et al. 2005).

We performed in situ hybridization for all 6 *v1r*-like *ora* genes combined with antibody labeling for S100-ir. Nearly, all S100-ir-positive cells, 90%, are labeled by the *ora4* probe (Figure 1 and Supplementary Table 1). In stark contrast, the other 5 *ora* genes (*ora1–3*, *ora5–6*) were either absent in S100-ir-labeled cells or found only very rarely, and all 5 together amount to less than 2% of all S100-ir-positive cells (Supplementary Table 1), that is, much less than the already small number of S100-ir-positive cells that are *ora4* negative. The latter could theoretically constitute either immature crypt neurons or a separate population of crypt neurons not expressing *ora* genes but some other unidentified receptors,

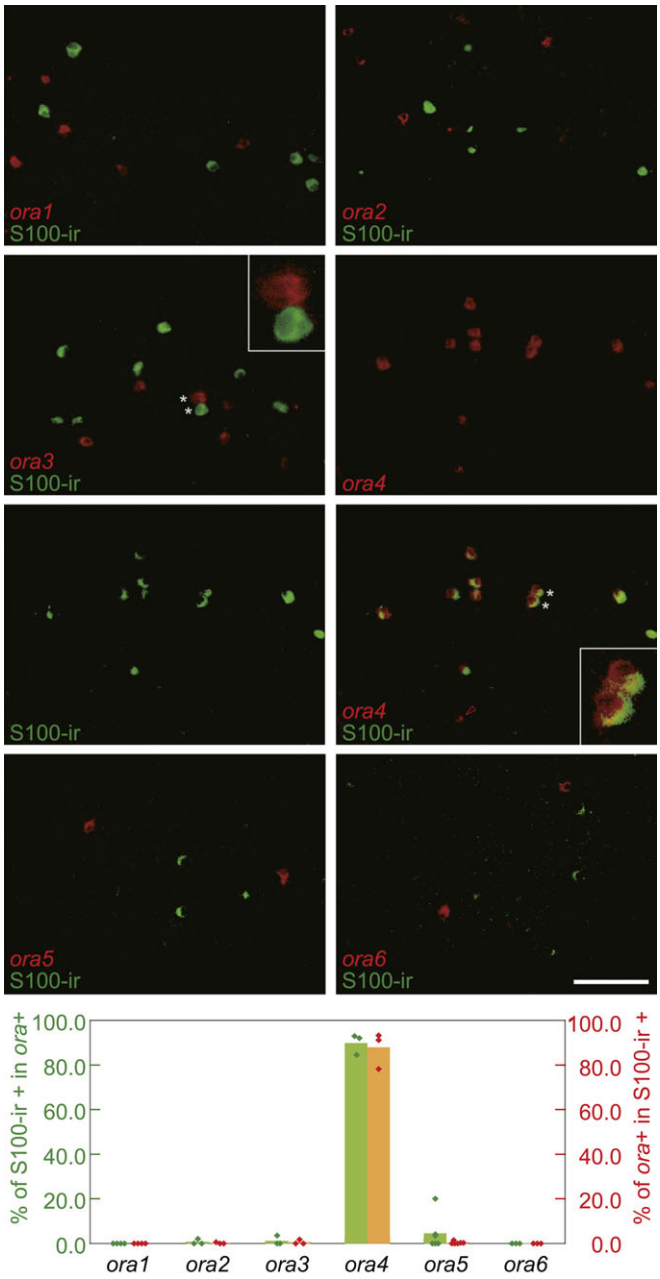

Figure 1 Of all 6 *ora* genes only *ora4* is expressed in S100-ir-positive crypt neurons. Fresh-frozen cryostat sections of adult zebrafish olfactory epithelium were hybridized with probes for all 6 *ora* genes and double labeled with anti-S100 antibody. For *ora1–3* and *ora5–6*, only the overlay of in situ hybridization and immunohistochemical staining is shown, for *ora4*, in addition, the individual stainings are shown. Only the *ora4* probe labels S100-ir-positive cells. Nearly all *ora4*-positive cells are S100-ir-positive and vice versa, rare exceptions, see arrowhead. Asterisks point to cells shown in enlarged insets. Scale bar, 50 μm. Bottom panel, quantitative evaluation of *ora* and S100-ir coexpression. Mean values are given as bars, individual results as small diamonds. The corresponding numbers are given in Supplementary Table 1.

but conceivably may be due to technical reasons only (e.g., below threshold levels for *ora4* detection).

The lack of expression of the other 5 *ora* genes in crypt neurons is unexpected and one might hypothesize an S100-ir-negative crypt cell population expressing them. However, in this case, one would expect expression of these 5 *ora* genes in globose cells, as this peculiar morphology is the hallmark of crypt neurons (Hansen et al. 2004). Although *ora4* indeed is expressed in globose cells, all 5 other *ora* genes (*ora1–3*, *ora5–6*) are expressed in slender cells with significantly different morphology from the *ora4*-expressing cells (Supplementary Figure 5). Thus, absence of S100-ir-colabeling and non-crypt neuron morphology both show *ora* genes 1–3, 5–6 not to be expressed in crypt neurons to a noticeable extent.

The nearly complete colabeling of *ora4*-positive cells by S100 antibody (Figure 1 and Supplementary Table 1) indicates that nearly all *ora4*-expressing cells are crypt neurons. Again the difference to 100% may have technical reasons (incomplete retention of the antigen under the demanding conditions for in situ hybridization in fresh-frozen tissue sections). Thus, crypt neurons as identified by S100-ir- and *ora4*-expressing cells appear to be by and large the same cell population.

ora4 follows the monogenic rule of expression

Mammalian olfactory receptor genes generally conform to the "one neuron–one receptor" rule, also referred to as monogenic expression (Mombaerts 2004; Serizawa et al. 2004). Initial studies in zebrafish showed that OR genes obey the same rule, although closely related subfamily members may be coexpressed (Sato et al. 2007). The colabeling of S100-ir only with *ora4* and not with the other *ora* genes suggests that *ora4* may obey the monogenic rule of expression as well.

We have investigated the monogenic expression hypothesis directly by double in situ hybridization of *ora4* with all other *ora* genes. No overlap of *ora4* expression with any other *ora* gene was detected, not even in a single cell (Figure 2 and Supplementary Table 2). The mutually exclusive expression of o*ra4* and all other *ora* genes verifies the monogenic expression hypothesis for the *ora4* gene in respect to the complete *ora* gene family.

Crypt neurons do not appear to express members of other olfactory receptor gene families

Next, we asked whether any olfactory receptor genes from other families are expressed in crypt neurons, again using S100-ir as crypt neuron marker (Germana et al. 2004; Sato et al. 2005; see also Materials and methods). When we examined several members of each of the other 3 known receptor gene families, ORs, TAARs, and V2R-like OlfC receptors, expression for all of them was found to be completely exclusive of S100-ir-positive neurons (Figure 3). To further confirm the exclusion of other receptor families from crypt neurons, we took advantage of cell type–specific marker genes, broadly expressed in ciliated or microvillous neurons. The S100-ir-positive cell population was completely segregated from both ciliated neurons expressing *OMP* and microvillous neurons positive for *TRPC2* (Figure 3). This suggests that other olfactory receptor gene families such as ORs, TAARs, and V2R-like OlfCs known to be coexpressed with *OMP* and *TRPC2*, respectively (Sato et al. 2005, 2007; Liberles and Buck 2006), likely are absent from crypt neurons. Furthermore, a particular OlfC receptor, *OlfCc1*, also called zVR5.3, which is broadly expressed in microvillous receptor neurons (Sato et al. 2005), also segregates completely from S100-ir-positive cells (Figure 3). Taken together, these results are consistent with the hypothesis that the *v1r*-like *ora4* gene may be the sole olfactory receptor expressed by crypt neurons.

An inhibitory G protein is coexpressed with ORA4, suggesting a role in ORA4 signal transduction

ORA4 is a G protein–coupled receptor, suggesting that signal transduction of olfactory cues detected by crypt neurons proceeds via trimeric G proteins. In fact, alpha subunits Go and Gq have been found previously in crypt neurons from catfish and goldfish, using antibodies against mammalian G alpha proteins (Hansen et al. 2003, 2004). However, some species differences appear to exist, because in zebrafish some of the same antibodies stain other cell populations, for example, Gq antibody only labels cells outside the sensory area (Supplementary Figure 4).

Among several antibodies against mammalian G proteins, only one against inhibitory G proteins (Gi) labeled a sparse pattern of isolated cells reminiscent of the *ora4* labeling pattern (data not shown). Double-labeling experiments using the Gi antibody and the *ora4* probe showed a high degree of coexpression (Figure 4). Thus, ORA4 may signal through an inhibitory G protein.

Because the antibody cross-reacts with all 3 mammalian Gi proteins, we performed in situ hybridization with all 26 zebrafish G alpha genes (Oka et al. 2009) to identify the particular G protein responsible for the signal. Only *Gi1b*, also known as *Gαia*, was expressed in a sparse pattern of infrequent cells within the olfactory epithelium, reminiscent of the S100-ir, and the *ora4* labeling patterns (cf. Figure 1 and Oka and Korsching 2011).

Double labeling with the anti-Gi antibody and the *Gi1b* probe showed considerable overlap (Figure 4) and suggests that the inhibitory G protein present in *ora4*-expressing neurons is *Gi1b*. This is supported by results of double-labeling experiments with *Gi1b* and S100-ir that show the expression of *Gi1b* in most S100-ir-positive cells (Figure 4 and Supplementary Table 3), that is, crypt neurons. It should be noted that *Gi1b* is expressed not only in crypt neurons, as there appears to be a population of *Gi1b*-positive/S100-ir-negative cells (Figure 4 and Supplementary Table 3). These *Gi1b*-positive/S100-ir-negative cells are more slender than

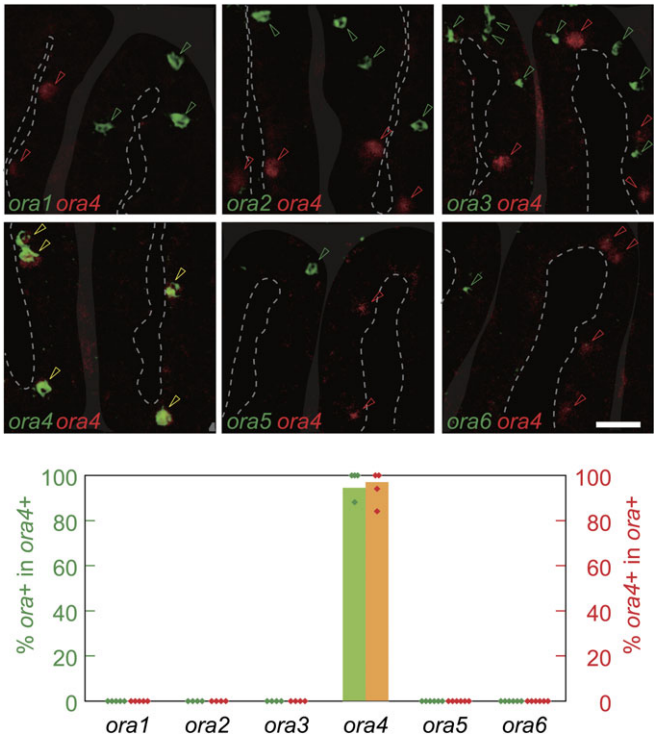

Figure 2 *ora4* is not coexpressed with any other *ora* gene. Double in situ hybridization with probes for *ora1–6* (green) and *ora4* (red). Colored arrowheads point to the respective stained somata. The basal lamina is overlaid with transparent gray and the lumen of the epithelium is delimited by dashed lines to help visualization of the morphology. Without exception, overlap is only seen for the 2 differently labeled *ora4* probes. Scale bar, 20 µm. Bottom panel, quantitative evaluation of coexpression of *ora4* with *ora1–6*. Mean values are given as bars, individual results as small diamonds. The corresponding numbers are given in Supplementary Table 2.

the globose S100-ir-positive cells and therefore unlikely to be crypt neurons. They are located apically and medially (i.e., closer to the median raphe) in the epithelium but are generally *TRPC2*-negative (unpublished observation), suggesting they might constitute an as yet undescribed cell type.

Taken together, these results suggest that Gi1b is expressed in crypt neurons and therefore could serve as signal transduction molecule for the ORA4 receptor.

Discussion

Crypt neurons, the third type of olfactory sensory neurons, are evolutionary old, occurring already in cartilaginous fish, but so far little is known about their role in olfaction. We have "deorphanized" zebrafish crypt neurons by showing their expression of a single *v1r*-like gene, *ora4*. No evidence for expression of other *v1r*-like *ora* genes, *v2r*-like *olfC*, *taar*, and *or* genes in crypt neurons was found. In particular, we could show that neurons expressing the 5 other *ora* genes do not express the crypt neuron marker (S100-ir) to a noticeable extent and do not exhibit the crypt neuron morphology (large globose somata). It is somewhat unexpected that the 5 other *ora* genes would be expressed in another neuronal population than the *ora4* gene, but not unprecedented, compare the recently discovered FPR family of olfactory receptor genes (Liberles et al. 2009; Riviere et al. 2009). The cell types expressing these 5 *ora* genes are not known, but *ora1* and *ora2* might be expressed in microvillous neurons, based on their mid-apical positions and generally slender soma shapes. This would constitute a conserved feature across the teleost/tetrapod divide because the mammalian V1R family, which is derived entirely from the ora1/ora2 subfamily (Saraiva and Korsching 2007), is expressed in a subgroup of microvillous olfactory sensory neurons (Mombaerts 2004).

Interestingly, the vast majority of all crypt neurons as defined by S100-ir appeared to express *ora4*, leaving nearly no room for (monogenic expression of) any other olfactory receptor gene even beyond those examined here. In other words, nearly the whole crypt neuron population as defined by S100-ir appears to express a single olfactory receptor gene. Although it is theoretically possible—and indeed with current knowledge impossible to exclude—that another crypt neuron population not expressing S100-ir might exist, there is also currently no evidence for such a population. With this caveat we are, to the best of our knowledge, the first to report that nearly all olfactory sensory neurons of a certain type express the same G protein–coupled olfactory receptor gene.

This situation should not be confused with previous reports of ubiquitous receptors in insects and mammals (*or83b* and *v2r2*, also known as *v2r83*, Martini et al. 2001; Larsson et al. 2004). In all these cases, the (nearly) ubiquitous receptors are coexpressed with other variable, sparsely expressed receptors, for which they serve as chaperone or subunit (Martini et al. 2001; Larsson et al. 2004; Sato et al. 2008; Wicher et al. 2008). These coreceptors are from the same receptor gene family as the receptors they complement. In contrast, for *ora4*, we have shown that other *ora* genes are not expressed together with *ora4*.

One might designate the expression of a single olfactory receptor in an entire olfactory sensory neuron type the "one cell type–one receptor" mode of gene expression. This mode comprises the well-known "one neuron–one receptor" rule but goes far beyond it. In the visual system, such a mode has long been known for rhodopsin, which is the sole light receptor of rod photoreceptors. This expression mode is distinctly different from the expression of all other olfactory receptor gene families in ciliated and microvillous neuron populations, whose mode of expression corresponds to that of color opsins in the visual system (mosaic and monogenic expression of several opsins in one cell type, cone photoreceptors). The mechanism responsible for this extreme specificity is unknown but may just involve a strong bias for selection of *ora4* within crypt neurons and vice versa against *ora4* in non-crypt neurons. However, as far as neural coding is concerned, such a biased mode of expression can make a significant difference from the strategy employed by

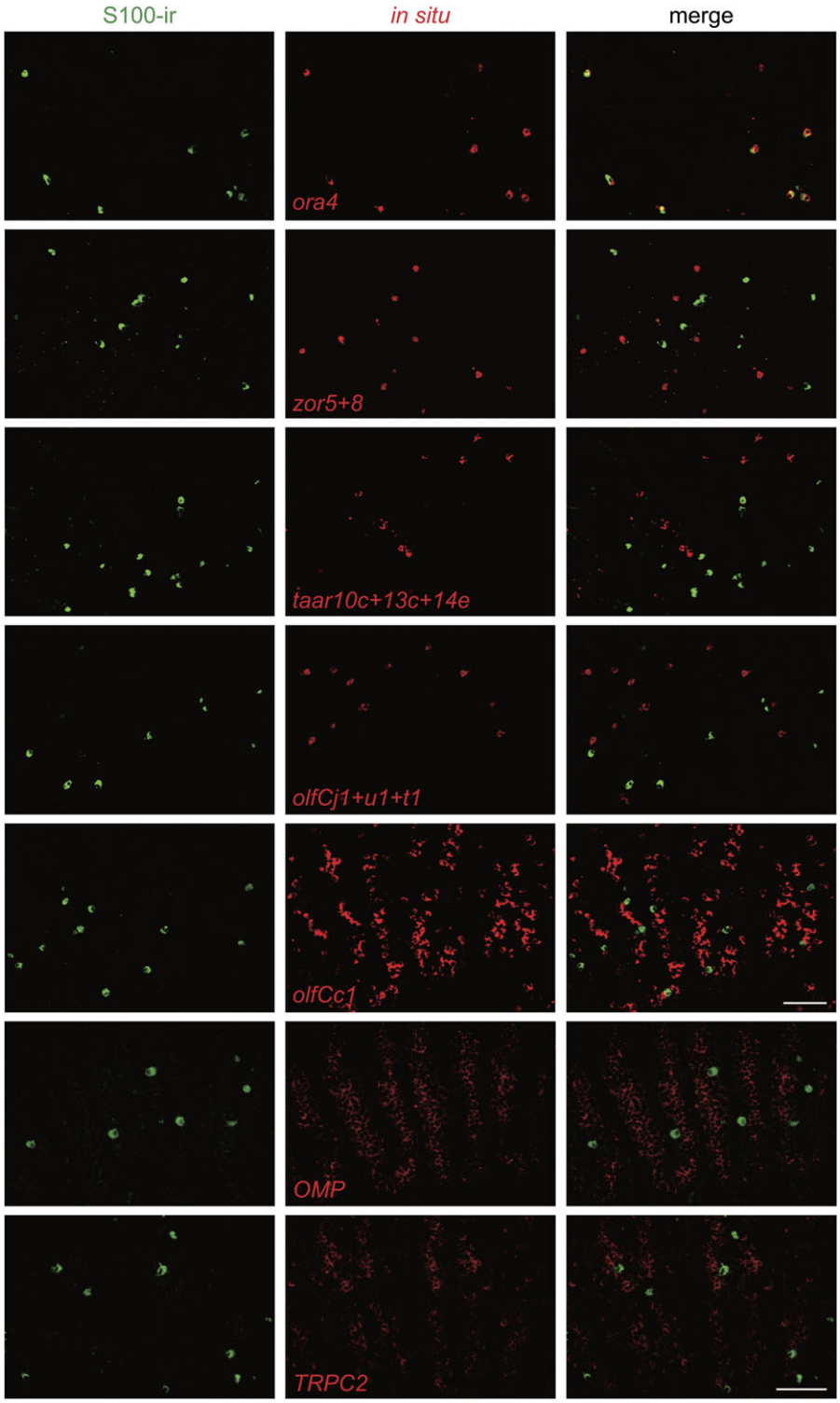

Figure 3 No evidence for expression of OR-, TAAR-, and V2R-like OlfC genes in S100-ir-positive crypt neurons. Fresh-frozen cryostat sections of zebrafish olfactory epithelium were stained with S100 antibody (left column) and hybridized (middle column) either with mixtures of probes specific for olfactory receptor genes from the OR- (*zor5* and *zor8*; Weth et al. 1996), TAAR- (*taar10c*, *taar13c*, and *taar14e*; Hussain et al. 2009), and V2R-like OlfC (*olfCj1*, *olfCu1*, and *olfCt1*) families, with a probe for a broadly expressed *olfCc1*, *OMP*, and *TRPC2* or with a probe for the *ora4* gene (positive control for colocalization). Nomenclature for OlfCs follows Alioto and Ngai (2006). The overlay is shown in the right column. S100-ir showed no overlap with any of the olfactory receptor genes other than *ora4*. Scale bars, 50 μm.

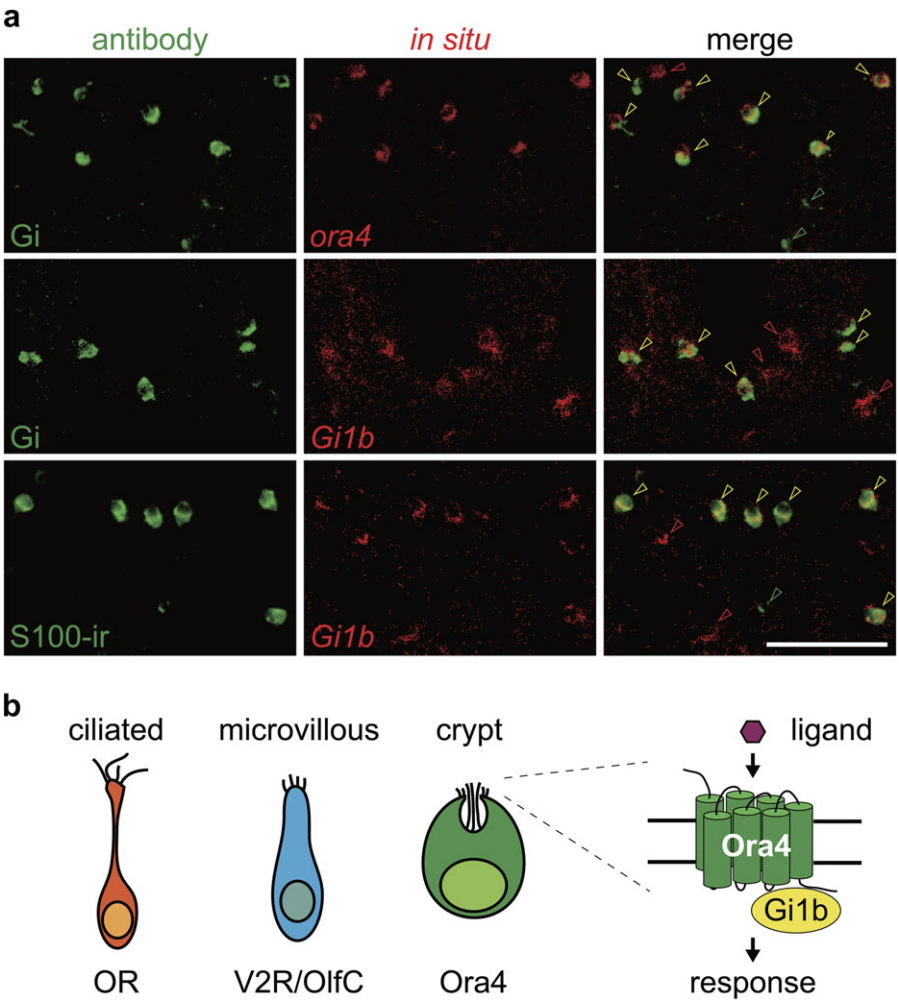

Figure 4 The G alpha protein Gi1b is specifically expressed in *ora4*-positive crypt neurons. (**a**) Top row, double labeling of sections from fresh-frozen olfactory epithelium with anti-Gi antibody (left panel) and in situ probe for *ora4* (middle panel), overlay (right panel). Middle row, double labeling of olfactory epithelium sections with anti-Gi antibody (left panel) and in situ probe for *Gi1b* (middle panel), overlay (right panel). Bottom row, double labeling of olfactory epithelium sections with anti-S100 antibody (left panel) and in situ probe for *Gi1b* (middle panel), overlay (right panel). Colored arrowheads point to the respective stained somata. Scale bar 50 μm. (**b**) Schematic representation of all 3 olfactory sensory neuron types and corresponding olfactory receptors.

ciliated and microvillous neurons. While these 2 neuronal types are thought to embody a combinatorial code of odorants, all crypt neurons would send the same information to the olfactory bulb, equivalent to a "one receptor–one glomerulus" rule. Such a coding strategy could enable crypt neurons to be dedicated to a particular innate behavioral response, similar to the "labeled lines" for pheromone perception in insects (Touhara and Vosshall 2009).

As expected from S100-ir being colocalized with *ora4* but no other olfactory receptor genes and additionally shown directly by absence of any coexpression of *ora4* with all 5 other *ora* genes, *ora4* appears to conform to the monogenic expression pattern generally found for olfactory receptor genes including the *ora*-related *v1r* genes (Roppolo et al. 2007).

The frequency of *ora4*-expressing neurons is in the range of that observed for other individual olfactory receptor genes (cf. Weth et al. 1996; Saraiva and Korsching 2007; Hussain et al. 2009), which is consistent with crypt neurons being a much smaller population than microvillous or ciliated neurons (Hansen and Zeiske 1998; Germana et al. 2004).

The *ora4* gene has been lost during evolution upon the transition from water to land-living organisms (Saraiva and Korsching 2007), possibly together with the cell type expressing it. Crypt neurons have not been described in mammals, see, however, the Grueneberg ganglion for olfactory neurons with a similar globose morphology (Brechbuehl et al. 2008). It is conceivable that the so far unknown ligand(s) activating the ORA4 receptor may be restricted to the aquatic environment. Mackerel crypt neurons have been shown to respond to amino acids (Vielma et al. 2008), which signal the presence of food for fish. On the other hand, several lines of evidence have pointed to reproductive

pheromones as activators of crypt neurons in crucian carp (Hamdani el and Doving 2006; Lastein et al. 2006). We observed no sex difference in number and position of crypt neurons and degree of coexpression, but such differences may arise in the pheromone-processing pathway at a later stage (cf. Lastein et al. 2006).

We have begun to examine the signal transduction pathway of ORA4 by identifying an inhibitory G protein, Gi1b, as being coexpressed with ORA4 in zebrafish crypt neurons. Interestingly, this is similar but not identical to the situation for mammalian V1Rs, which are coexpressed with Gi2 (Mombaerts 2004). The further signal processing steps in crypt neurons and their subsequent neuronal network are not known so far. The high degree of ORA4 conservation, unprecedented for an olfactory receptor (cf. Saraiva and Korsching 2007), would be consistent with an evolutionary conserved neuronal circuit among jawed fish, possibly dedicated to a particular innate behavioral response.

In conclusion, we have provided evidence that crypt neurons, the third type of olfactory sensory neurons, appear to express a single olfactory receptor gene. We have identified the crypt neuron receptor as the highly conserved *v1r*-like *ora4* gene, which may signal through the inhibitory G protein Gi1b. Our identification of this ''one cell type–one receptor'' mode of expression in the olfactory system demonstrates a closer parallelism of the receptor expression modes in the visual and olfactory senses than previously suspected.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

Funding

This work was supported by research grants from the German Research Foundation [KO1046/3 and KO1046/5 to S.I.K.].

Acknowledgements

We thank Mehmet Saltürk for taking good care of the fish. Yen Yen Kwan graciously provided larval expression data for comparison purposes and Walter Nadler gave expert advice on statistical analyses.

References

Alioto TS, Ngai J. 2006. The repertoire of olfactory C family G protein-coupled receptors in zebrafish: candidate chemosensory receptors for amino acids. BMC Genomics. 7:309.

Brechbuehl J, Klaey M, Broillet MC. 2008. Grueneberg ganglion cells mediate alarm pheromone detection in mice. Science. 321:1092–1095.

Ferrando S, Bottaro M, Gallus L, Girosi L, Vacchi M, Tagliafierro G. 2006. Observations of crypt neuron-like cells in the olfactory epithelium of a cartilaginous fish. Neurosci Lett. 403:280–282.

Germana A, Montalbano G, Laura R, Ciriaco E, del Valle ME, Vega JA. 2004. S100 protein-like immunoreactivity in the crypt olfactory neurons of the adult zebrafish. Neurosci Lett. 371:196–198.

Hamdani el H, Doving KB. 2006. Specific projection of the sensory crypt cells in the olfactory system in crucian carp, Carassius carassius. Chem Senses. 31:63–67.

Hamdani el H, Doving KB. 2007. The functional organization of the fish olfactory system. Prog Neurobiol. 82:80–86.

Hansen A, Anderson KT, Finger TE. 2004. Differential distribution of olfactory receptor neurons in goldfish: structural and molecular correlates. J Comp Neurol. 477:347–359.

Hansen A, Finger TE. 2000. Phyletic distribution of crypt-type olfactory receptor neurons in fishes. Brain Behav Evol. 55:100–110.

Hansen A, Rolen SH, Anderson K, Morita Y, Caprio J, Finger TE. 2003. Correlation between olfactory receptor cell type and function in the channel catfish. J Neurosci. 23:9328–9339.

Hansen A, Zeiske E. 1998. The peripheral olfactory organ of the zebrafish, Danio rerio: an ultrastructural study. Chem Senses. 23:39–48.

Hansen A, Zielinski BS. 2005. Diversity in the olfactory epithelium of bony fishes: development, lamellar arrangement, sensory neuron cell types and transduction components. J Neurocytol. 34:183–208.

Hussain A, Saraiva L, Korsching SI. 2009. Positive Darwinian selection and the birth of an olfactory receptor clade in teleosts. Proc Natl Acad Sci U S A. 106:4313–4318.

Korsching S. 2009. The molecular evolution of teleost olfactory receptor gene families. Results Probl Cell Differ. 47:37–55.

Kraemer AM, Saraiva LR, Korsching SI. 2008. Structural and functional diversification in the teleost S100 family of calcium-binding proteins. BMC Evol Biol. 8:48.

Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB. 2004. Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron. 43:703–714.

Lastein S, Hamdani el H, Doving KB. 2006. Gender distinction in neural discrimination of sex pheromones in the olfactory bulb of crucian carp, Carassius carassius. Chem Senses. 31:69–77.

Liberles SD, Buck LB. 2006. A second class of chemosensory receptors in the olfactory epithelium. Nature. 442:645–650.

Liberles SD, Horowitz LF, Kuang D, Contos JJ, Wilson KL, Siltberg-Liberles J, Liberles DA, Buck LB. 2009. Formyl peptide receptors are candidate chemosensory receptors in the vomeronasal organ. Proc Natl Acad Sci U S A. 106:9842–9847.

Martini S, Silvotti L, Shirazi A, Ryba NJ, Tirindelli R. 2001. Co-expression of putative pheromone receptors in the sensory neurons of the vomeronasal organ. J Neurosci. 21:843–848.

Mombaerts P. 2004. Genes and ligands for odorant, vomeronasal and taste receptors. Nat Rev Neurosci. 5:263–278.

Morita Y, Finger TE. 1998. Differential projections of ciliated and microvillous olfactory receptor cells in the catfish, Ictalurus punctatus. J Comp Neurol. 398:539–550.

Oka Y, Korsching SI. 2011. Shared and unique G alpha proteins in the zebrafish versus mammalian senses of taste and smell. Chem Senses. 36:357–365.

Oka Y, Saraiva LR, Kwan YY, Korsching SI. 2009. The fifth class of G{alpha} proteins. Proc Natl Acad Sci U S A. 106:1484–1489.

Riviere S, Challet L, Fluegge D, Spehr M, Rodriguez I. 2009. Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. Nature. 459:574–577.

Roppolo D, Vollery S, Kan CD, Luscher C, Broillet MC, Rodriguez I. 2007. Gene cluster lock after pheromone receptor gene choice. EMBO J. 26:3423–3430.

Saraiva LR, Korsching SI. 2007. A novel olfactory receptor gene family in teleost fish. Genome Res. 17:1448–1457.

Sato K, Pellegrino M, Nakagawa T, Nakagawa T, Vosshall LB, Touhara K. 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature. 452:1002–1006.

Sato Y, Miyasaka N, Yoshihara Y. 2005. Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. J Neurosci. 25:4889–4897.

Sato Y, Miyasaka N, Yoshihara Y. 2007. Hierarchical regulation of odorant receptor gene choice and subsequent axonal projection of olfactory sensory neurons in zebrafish. J Neurosci. 27:1606–1615.

Serizawa S, Miyamichi K, Sakano H. 2004. One neuron-one receptor rule in the mouse olfactory system. Trends Genet. 20:648–653.

Touhara K, Vosshall LB. 2009. Sensing odorants and pheromones with chemosensory receptors. Annu Rev Physiol. 71:307–332.

Vielma A, Ardiles A, Delgado L, Schmachtenberg O. 2008. The elusive crypt olfactory receptor neuron: evidence for its stimulation by amino acids and cAMP pathway agonists. J Exp Biol. 211:2417–2422.

Weth F, Nadler W, Korsching S. 1996. Nested expression domains for odorant receptors in zebrafish olfactory epithelium. Proc Natl Acad Sci U S A. 93:13321–13326.

Wicher D, Schafer R, Bauernfeind R, Stensmyr MC, Heller R, Heinemann SH, Hansson BS. 2008. Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature. 452:1007–1011.