Crypt Neurons Express a Single V1R-Related ora Gene

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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, microvillus, 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 microvillus 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 microvillus 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 microvillus 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 Zielske 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 vlr-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 suggests here constituting 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 Gzia, GenBank accession NM_198805) were amplified from the olfactory epithelium cDNA with specific primer pairs (OMP-fw 5′-AAAGGACACCA-GTAGACGC-3′, OMP-rv 5′-GAACAGACTG-ACCAG

**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 s100z1 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)2 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 fluorescence 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 oral-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 (oral-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,
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 ora4 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 OlfC receptors known to be coexpressed with OMP and TRPC2, respectively (Sato et al. 2005, 2007; Liberes 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 vlr-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 Gilb, also known as Gaia, 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 Gilb probe showed considerable overlap (Figure 4) and suggests that the inhibitory G protein present in ora4-expressing neurons is Gilb. This is supported by results of double-labeling experiments with Gilb and S100-ir that show the expression of Gilb in most S100-ir-positive cells (Figure 4 and Supplementary Table 3), that is, crypt neurons. It should be noted that Gilb is expressed not only in crypt neurons, as there appears to be a population of Gilb-positive/S100-ir-negative cells (Figure 4 and Supplementary Table 3). These Gilb-positive/S100-ir-negative cells are more slender than
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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 (ora83b 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- (zo5 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.
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

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.
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, G11b, 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 G12 (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 G11b. 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/

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References


