Chem. Senses 37: 357–369, 2012

doi:10.1093/chemse/bjr109
Advance Access publication December 13, 2011

Crypt Cells of the Zebrafish *Danio rerio* Mainly Project to the Dorsomedial Glomerular Field of the Olfactory Bulb

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Accepted November 11, 2011

Abstract

The olfactory mucosa of the zebrafish consists of 3 morphological types of olfactory receptor neurons (ORNs): ciliated, microvillous, and crypt cells. Previous studies in the zebrafish have revealed differential projections of ciliated and microvillous ORNs, which project to different glomerular fields. However, the bulbar targets of zebrafish crypt cells were not identified. Here, we analyze the relationship between crypt cells of the olfactory epithelium and dorsal glomerular fields of the zebrafish olfactory bulbs, as wells as the connections between these bulbar regions and forebrain regions. For this purpose, a lipophilic carbocyanine tracer (DiI) was used in fixed tissue. Application of DiI to the dorsomedial glomerular field mainly labeled crypt cells in the zebrafish olfactory epithelium. By contrast, application of DiI to the dorsolateral glomerular fields mainly labeled bipolar ORNs and only occasionally crypt cells. Bulbar efferent cells (mitral cells) contacting these dorsal glomerular fields project to different telencephalic areas, with the posterior zone of the dorsal telencephalic area (Dp) as the common target. However, dorsomedial and dorsolateral glomerular fields projected differentially to the ventral telencephalon, the former projecting to the ventrolateral supracommissural region. Retrograde labeling from the ventrolateral supracommissural region revealed mitral cells associated with 2 large glomeruli in the bulbar dorsomedial region, which putatively receives inputs from the crypt cells, indicating the existence of a crypt cell olfactory subsystem with separate projections, in the zebrafish. The comparative significance of the secondary olfactory pathways of zebrafish that convey information from crypt cells is discussed.

Key words: connections, crypt cells, *Danio rerio*, olfactory glomeruli, olfactory organ, telencephalon

Introduction

Olfaction is an important sense for vertebrates and other animals. The olfactory system of teleosts can discriminate among a variety of odorant molecules dissolved in water. The odorants detected by teleost fishes include amino acids, bile salts, nucleotides, polyamines, prostaglandins, and sex steroids. Chemical information is transmitted from the olfactory organ to the brain, mediating behavior such as food finding, alarm response, predator avoidance, social communication, reproductive activity, and homing migration (Sorensen and Caprio 1998; Eisthen and Polese 2007).

The olfactory organ (olfactory rosette) of zebrafish contains 3 morphological types of olfactory receptor neurons (ORNs): ciliated, microvillous, and crypt cells (Hansen and Zeiske 1998). The 2 major morphological types of ORNs, ciliated and microvillous, differ from one another in their relative positions in the olfactory epithelium (deep and superficial, respectively) and exhibit different molecular markers (Sato et al. 2005; Gayoso et al. 2011). The third type of ORN, called crypt cells, forms a small population in the superficial region of the olfactory epithelium (Hansen and Zeiske 1998). These cells have ovoid cell bodies bearing microvilli and short cilia submerged in an apical crypt and are always associated with 1 or 2 supporting light cells. Although it has been reported that the zebrafish crypt cells display immunoreactivity to S100 (a calcium-binding protein) (Germanà et al. 2004, 2007), more recent studies indicate that this characteristic is not unique to this cell type (Gayoso et al. 2011). The types of chemosensory receptors expressed by crypt cells of zebrafish have not been identified (Yoshihara 2009).

Axons of ORNs course in the olfactory nerve to the olfactory bulb where they contact dendrites of mitral cells and local interneurons in the glomerular layer, forming complex neuropil structures (glomeruli), which exhibit a stereotyped organization and bilateral symmetry in the zebrafish (Baier and Korsching 1994). Olfactory glomeruli represent the first relay station in the olfactory pathway. In zebrafish, the different amino acids and bile acids predominantly activate overlapping glomeruli in the lateral and medial regions of the olfactory bulbs, respectively, whereas the pheromones and saponin activate the single large ventral glomeruli (Friedrich and Korsching 1997, 1998; Fuss and Korsching 2001; Li et al. 2005). Thus, chemotopic maps in the zebrafish olfactory bulb reveal the use of both combinatorial (affecting multiple glomeruli) and noncombinatorial representations of odorant molecules. Studies in the zebrafish have also demonstrated a relationship between the major ORN types and their glomerular fields in the olfactory bulb. Studies in transgenic zebrafish have indicated that the ciliated ORNs mainly project to the dorsal and medial bulbar regions, whereas the microvillous ORNs project to the lateral region (Sato et al. 2005). Differential projections from major cell types to various glomerular fields have also been visualized by the use of immunocytochemical markers, such as calcium-binding proteins (calretinin, S100) and G proteins (Golf) (Gayoso et al. 2011). However, projections of the crypt cells have not been observed in the zebrafish. Odorant maps in the olfactory bulb and the relation between the ORN types of the olfactory epithelium and the main functional glomerular regions have also been studied in catfish and crucian carp (Morita and Finger 1998; Hamdani et al. 2001; Hamdani and Døving 2002; Hansen et al. 2003, 2005). With regard to the projections of crypt cells, experimental studies in the catfish and crucian carp have traced these cells from the ventral glomeruli of the olfactory bulb (Hansen et al. 2003, 2005; Hamdani and Døving 2006).

The bulk connections of the olfactory bulbs have previously been studied by different tract-tracing techniques in several teleosts (winter flounder: Prasada Rao and Finger 1984; goldfish: von Bartheld et al. 1984; Levine and Dethier 1985; cod: Rooney et al. 1992; *Apteronotus*: Sas et al. 1993; and salmonids: Matz 1995; Folgueira et al. 2004). Efferents from the olfactory bulb course via the medial and lateral olfactory tracts and mainly reach the different nuclei and divisions of the ventral and dorsal telencephalic areas. Retrograde labeling of mitral cells from the precommissural ventral telencephalic area has also been reported in the zebrafish (Rink and Wullimann 2004). In turn, the olfactory bulbs receive afferents from the contralateral olfactory bulb, different nuclei of the ventral telencephalon, some zones of the dorsal telencephalic area, and the preoptic region (Prasada Rao and Finger 1984; von Bartheld et al. 1984; Levine and Dethier 1985; Sas et al. 1993; Folgueira et al. 2004). In carp and catfish, inputs from the different glomerular regions are conveyed by the medial, intermediate, and lateral olfactory tracts, that is, there are 3 parallel secondary olfactory pathways (see Hamdani and Døving 2007). Physiological evidence in catfish indicates that the odorant mapping is maintained beyond the level of the olfactory bulb, and the 3 classes of odorants that are biologically relevant to the catfish are processed in distinct regions of the forebrain (Nikonov et al. 2005). As regards the differential bulbar projections from the immunocytochemically identifiable glomerular fields in zebrafish, the connections from the ventromedial and caudoventral glomerular fields, which receive projections of ORNs expressing the calcium-binding proteins calretinin (CR) and S100, respectively, have been established by DiI methods (Gayoso et al. 2011). A genetic study using transgenic zebrafish with lhx2a gene promoter, mainly performed in larvae, has reported forebrain projections from single mitral cells, mostly with dendrites contacting particular glomeruli of the medial glomerular cluster (Miyasaka et al. 2009).

The organization in the adult zebrafish olfactory bulb, of glomerular fields that are positive for several immunocytochemical markers, has been described (Gayoso et al. 2011). In the latter study, connections with the olfactory mucosa and telencephalon of ventral bulbar glomerular fields were also explored using tracing methods. However, when studying the retrogradely labeled cells in this study from the caudoventral and lateral glomerular fields, we were struck by the scant labeled cells with clear crypt cell morphology in the mucosa, which was in contrast with the tracing results obtained in other fish species from similar regions. This prompted us to investigate the possible targets of crypt cells in the zebrafish. The aim of the present tract-tracing study in adult zebrafish was to determine: 1) the ORN type(s) that project to the dorsomedial and dorsolateral glomerular fields, regions that receive fibers with different immunocytochemical signatures and 2) the organization of the secondary olfactory projections derived from these bulbar regions. The results of this study indicate that the dorsomedial glomerular field is a preferential target of the enigmatic crypt cells and also provide a more complete view of the organization of the olfactory system in the zebrafish, a model organism in genetic and developmental studies of the vertebrates.

Materials and methods

Animals

Thirty-three adult wild-type specimens (of both sexes) of zebrafish (*Danio rerio*; Cyprinidae) were used in the present study. The estimated age of the fish, obtained from local suppliers, was 3–5 months. All specimens were deeply anesthetized with 0.1% 3-aminobenzoic acid ethyl ester methane sulfonate salt (MS-222; Sigma) in fresh water and fixed transcardially by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 (PB). Samples were left in 4% paraformaldehyde until DiI application. All experiments were approved by the Ethics Committee of the

University of A Coruña and conformed to the European Community guidelines on animal care and experimentation.

Tract-tracing experiments from the olfactory bulb

For this tract-tracing study, the fluorescent carbocyanine dye 1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) was applied to 2 selected regions of the olfactory bulb of paraformaldehyde-fixed zebrafish. After fixation, the dorsal side of the olfactory bulbs was exposed and a small DiI crystal, placed on the tip of a sharpened minute insect pin, was applied unilaterally on the surface of the olfactory bulbs, where previous studies have reported dorsomedial and dorsolateral glomerular fields that exhibit different immunocytochemical signatures (Gayoso et al. 2011). The area was then sealed with melted agarose, and the zebrafish heads were left in the dark for 60–90 days in 4% paraformaldehyde in PB at 37 °C. The fixative was changed periodically (each 2–3 days). After this period, the olfactory rosettes, together the olfactory bulbs and brain, were carefully dissected out, embedded in 3% agarose, and cut transversely (50–100 μm thickness) on a vibratome (Campden Instruments). Sections were mounted on gelatin-coated slides.

Tract-tracing experiments from the caudal telencephalon

The results of anterograde tracing suggested that the dorsomedial and dorsolateral glomerular fields have different targets in the ventral telencephalic area (V). In order to assess the existence of differential secondary olfactory projections, 2 regions of the ventral telencephalon were selected for DiI application, a region ventrolateral to the supracommissural nucleus (Vs) and a periventricular region of the ventral nucleus (Vv) (see Figure 5J). For these experiments, the brain and olfactory bulbs of the zebrafish, fixed as above, were embedded in agarose. The agarose blocks were then cut on a vibratome from the caudal pole to the desired level under a stereomicroscope, and minute DiI crystals were applied to the regions. After application of the DiI, the brains were photographed with the stereomicroscope and the exposed surface of the brain was covered with melted agarose and left in fresh fixative in the dark for 30 days, as indicated above. The entire olfactory bulbs were then extracted from the agarose blocks, mounted on slides with PB and photographed under a confocal microscope.

Additional material

Series of Nissl-stained transverse sections of the zebrafish brain from our collection were also used to illustrate the cytoarchitecture of the adult zebrafish telencephalon (see Supplementary Figure 1).

Photomicrography and measurements

Sections were photographed with an epifluorescence microscope equipped with a rhodamine filter set (Olympus) and/or with a spectral laser confocal microscope (TCS-SP2, Leica). Photomicrographs were adjusted for brightness and contrast with Photoshop CS (Adobe). Projections of confocal stacks, brightness/contrast adjustments and measurements were made with LITE confocal software (Leica) or Fiji (ImageJ) free software. Well-labeled rosettes were used for the measurement of cells. For cell measurements, the mean ± standard deviation is indicated. In addition, the XY plots showing the size distribution of typical examples are provided for better characterization of the cell types. Plates were composed with Photoshop CS (Adobe).

Results

Application of minute DiI crystals to the surface of the dorsolateral and dorsomedial glomerular fields of the zebrafish olfactory bulbs resulted in retrograde labeling of ORNs in the ipsilateral olfactory epithelium and also anterogradely labeled efferent bulbar fibers in different areas of the telencephalic hemispheres, most densely on the ipsilateral side (Figures 1 to 5). These experiments with minute crystals (see Figures 1A and 4A) mainly labeled the glomerular layer, as observed in transverse sections of the bulbs (Figures 1B and 4B), thus revealing probably only the bulbar efferents from mitral cells of these regions, as indicated by the small number of bulbar afferent neurons labeled in the telencephalon. Some experiments in which the DiI crystal affected deeper regions of the bulb (as in Figure 5B) led to labeling of more abundant afferent neurons than with more superficial application of DiI.

Connections of the dorsolateral glomerular field in zebrafish

After application of DiI to the dorsolateral glomerular field (Figure 1A,B), 2 types of labeled ORNs were apparent in the olfactory epithelium. Most of the labeled ORNs (about 95% of cells; 91 of 96 cells) were slender spindle-shaped bipolar cells located in the proximal half of all the olfactory lamellae (the region near the rosette raphe), either scattered or in groups (Figure 1C–EE). Most of these cells showed slender apical dendrites running from a perikaryon located in the middle-lower two-thirds of the epithelium and a thin basal axon. The minor diameter of the cell perikarya was 5.0 ± 0.5 μm, and the length of perikarya plus apical dendrites was 22.9 ± 6.1 μm ($n = 91$). In some cells, the presence of labeled cilia extending from the apex of the dendrite was clearly discerned (Figure 1F). A few labeled cells with a more or less bottle-shaped appearance and short apical dendrites were observed in the upper half of the epithelium (Figure 1G). In 3 applications of DiI to the dorsolateral glomerular field, a total of 5 crypt cells were observed in the mucosa (Figure 1H). These showed ovoid perikarya (7.2 ± 0.3 μm × 10.8 ± 0.8 μm; $n = 5$) with a bean-shaped nucleus, a conical or dome-shaped apical region that lacks a dendritic knob protruding toward the lumen and ends apparently (with the

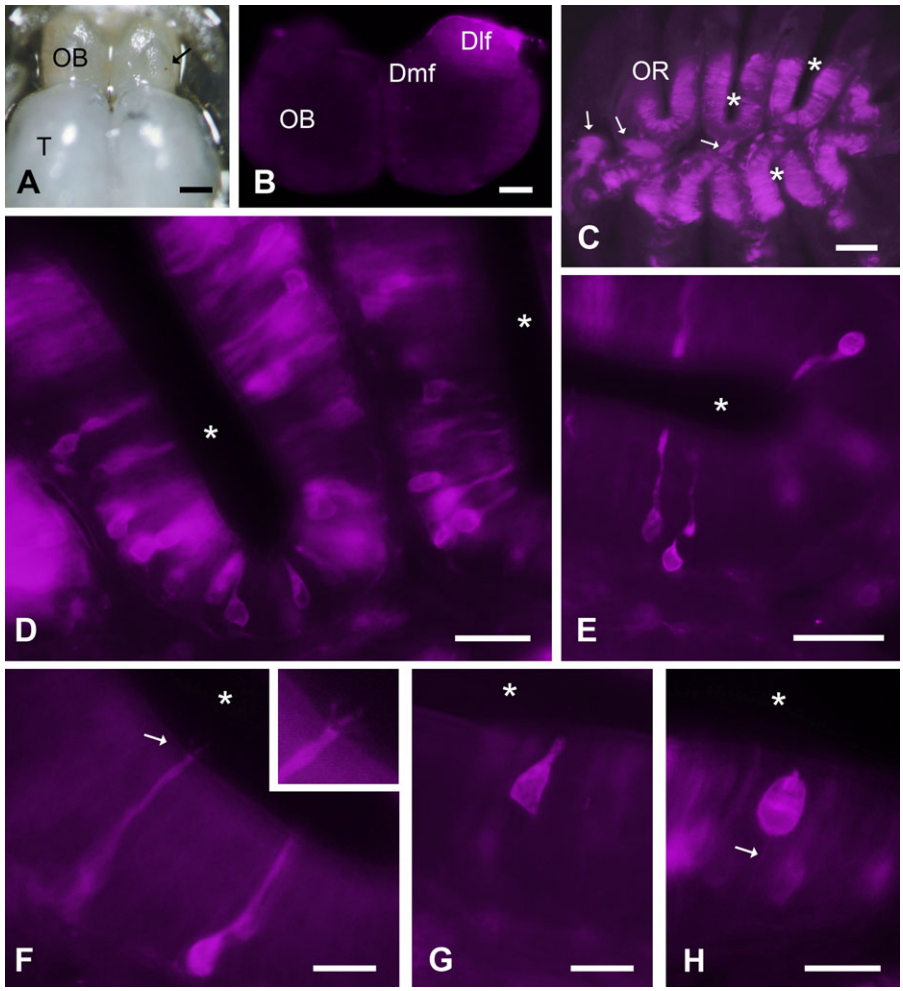

Figure 1 Photomicrographs of the olfactory bulb and epithelium in the typical dorsolateral glomerular field experiments. (**A**) Photograph of the dorsal aspect of the forebrain of a zebrafish showing a minute crystal of DiI applied to the surface of the dorsolateral glomerular field (arrow). This field forms a conspicuous bulge in the dorsal surface of the bulbs. OB, olfactory bulb; T, telencephalic lobe. (**B**) Fluorescence photomicrograph of a transverse section of the olfactory bulbs showing that the typical area of tracer diffusion of DiI after incubation is restricted to the dorsolateral glomerular field (Dlf). Dmf, dorsomedial glomerular field; OB, olfactory bulb. (**C–H**) Fluorescence photomicrographs of the olfactory rosette ipsilateral to the point of DiI application. (**C**) Equatorial section through the olfactory rosette (OR) showing the distribution of labeled olfactory receptor cells in the proximal two-thirds of the mucosa of all lamellae. Asterisks indicate recesses of the lumen of the rosette; Arrows indicate olfactory nerve bundles. Midline is on the left. (**D**) Olfactory epithelium showing DiI-labeled ORNs. Most of the labeled neurons were slender cells with basal perikarya and long apical dendrites. (**E**) Olfactory mucosa, after application of minute DiI crystals showing the slender morphology of most labeled neurons (**F**). Detail of the dendrite of a DiI-labeled slender cell showing cilia at the apical pole (inset). Arrow indicates apical cilia. (**G–H**) A few receptor cells labeled from the dorsolateral glomerular field showing perikarya located in the apical region of the mucosa. The cell shown in (**G**) has a short apical dendrite. The cell shown in (H) has the typical appearance of a crypt cell, with the apical pole below the epithelium surface and cilia-like structures inside the invagination. The axon of this cell is indicated with an arrow. Asterisks in (C–H), rosette luminal recesses. Scale bars = 200 μm (**A**), 100 μm (**B,C**), 50 μm (**D,E**), and 25 μm (**F,G,H**). This figure appears in color in the online version of *Chemical Senses*.

microscopical methods used here) below the surface level of the epithelium. The invagination or "crypt" apical end of the crypt cell bearing short cilia is visible in the dome-shaped region of these cells (Figure 1H). These cells also exhibit a thin basal axon (for an electron microscopic description of these cells in zebrafish, see Hansen and Zeiske 1998). These cells, which were located in the upper third of the olfactory epithelium, were most often observed in the proximal regions of the olfactory lamellae (toward the rosette raphe). Plotting XY graphs of cell sizes shows that the scarce neurons morphologically characterized as crypt cells were closely grouped by size (Figure 2A). Moreover, they were well separated, by the combined width and height, from the much more numerous group of slender neurons, in which the height of the perikarya plus the apical dendrites was widely dispersed.

Application of DiI to the dorsolateral glomerular field labeled secondary olfactory fibers that coursed along the ipsilateral lateral olfactory tract to innervate the posterior zone of the ipsilateral dorsal telencephalic area (Dp; for Dp cytoarchitecture, see supplementary Figure 1B–D), where they formed conspicuous fields of beaded terminal fibers (Figure 3A–D). Labeled fibers also coursed in the medial olfactory

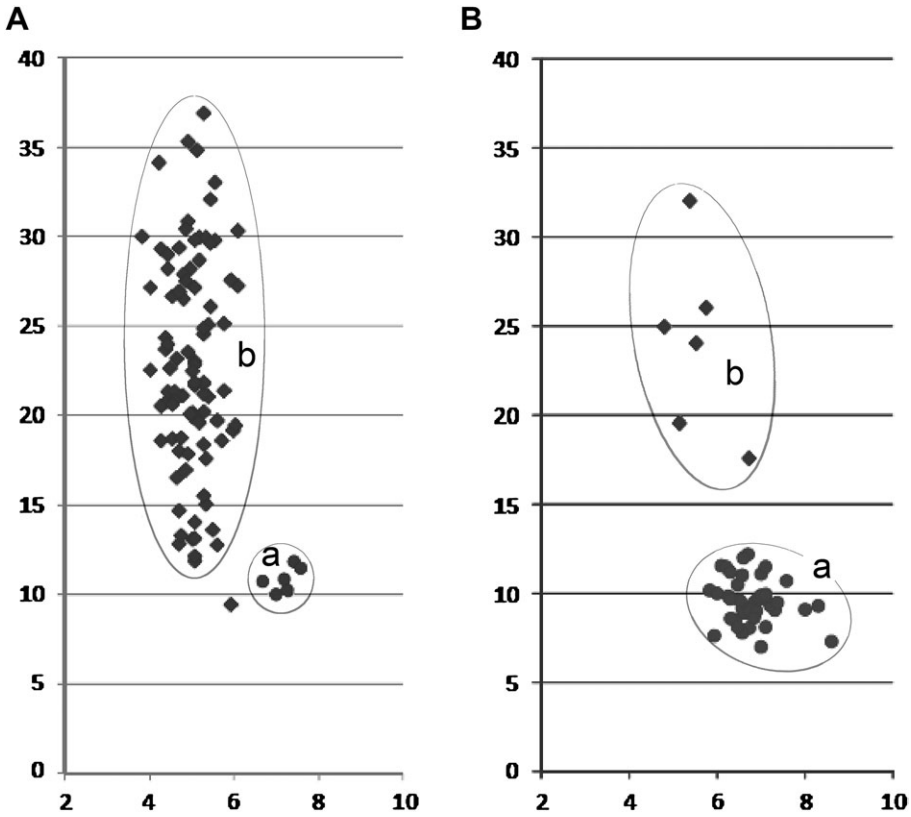

Figure 2 XY plotting of the width (abscissas) and height (ordinates) of olfactory cells retrogradely labeled from the dorsolateral (**A**) and dorsomedial (**B**) glomerular fields. All cells closely grouped in the plotting (encircled as "a") have crypt cell morphology (circles) and are clearly different from the cells with bipolar morphology (lozenges, encircled as "b"), which are variable in shape. Most cells labeled from the dorsomedial field are crypt cells. Measures are expressed in μm.

tract until the level of the anterior commissure (Figure 3A,G,H). Fibers from this tract crossed the midline in the anterior commissure (Figure 3I) and innervated the contralateral Dp (Figure 3E,F) in an ascending direction. In addition, labeled fibers of the medial olfactory tract coursed ventrally to an intermediate neuropil region of the ventral nucleus of the ventral telencephalic area (Vv), both ipsilaterally and contralaterally (Figure 3B,G,H; for Vv cytoarchitecture, see Supplementary Figure 1A–C). In this region, they formed rich terminal fields, denser on the ipsilateral side, which extend to the transition region, to the preoptic region situated below the anterior commissure, where both fields join in the midline forming a rich single terminal field (Figure 3I). In typical experiments in which the minute DiI crystals only affected the dorsolateral glomerular region superficially (as seen in control sections of the olfactory bulb), only some perikarya were retrogradely labeled in the ipsilateral telencephalon, mostly in Dp (Figure 3C,D).

Connections of the zebrafish dorsomedial glomerular field

After application of a minute DiI crystal to the dorsomedial glomerular field of the olfactory bulb (Figures 4A,B and 5B), most of the retrogradely labeled ORNs observed in the olfactory rosette (more than 95% of cells; 91 of 96 cells) were ovoid and rather uniform in size (6.8 ± 0.6 × 9.6 ± 1.2 μm in transverse and major diameter, respectively; n = 45). These cells were identified as crypt cells, using the criteria indicated below (Figures 4C–F and 5A). This result is in striking contrast with the major cell type labeled from the dorsolateral glomerular field. Labeled crypt cells contained a bean-shaped nucleus located in the basal region of perikarya below a conical or dome-shaped region that lacked an apical dendritic knob but had a "vacuolar" structure that probably represents the invaginated region (crypt) containing cilia characteristic of crypt cells. Crypt cells were located in the upper third of the olfactory epithelium (Figures 4D–F and 5A). These cells were scattered in the lamellae throughout more than half of its extension (from the raphe to the external surface) but were more abundant near the raphe of the rosette (Figure 4C,D). In addition, scarce bipolar ORNs (less than 5% of the labeled cells) were labeled in a more proximal position of the sensory epithelium, mainly when DiI application partially affected the dorsolateral glomerular field (not shown). These scarce cells measured 5.6 ± 0.7 μm × 24.0 ± 5.7 μm (n = 5) and were similar to the main cell type labeled after DiI application to the dorsolateral glomerular field. Plotting XY graphs of cell sizes revealed that

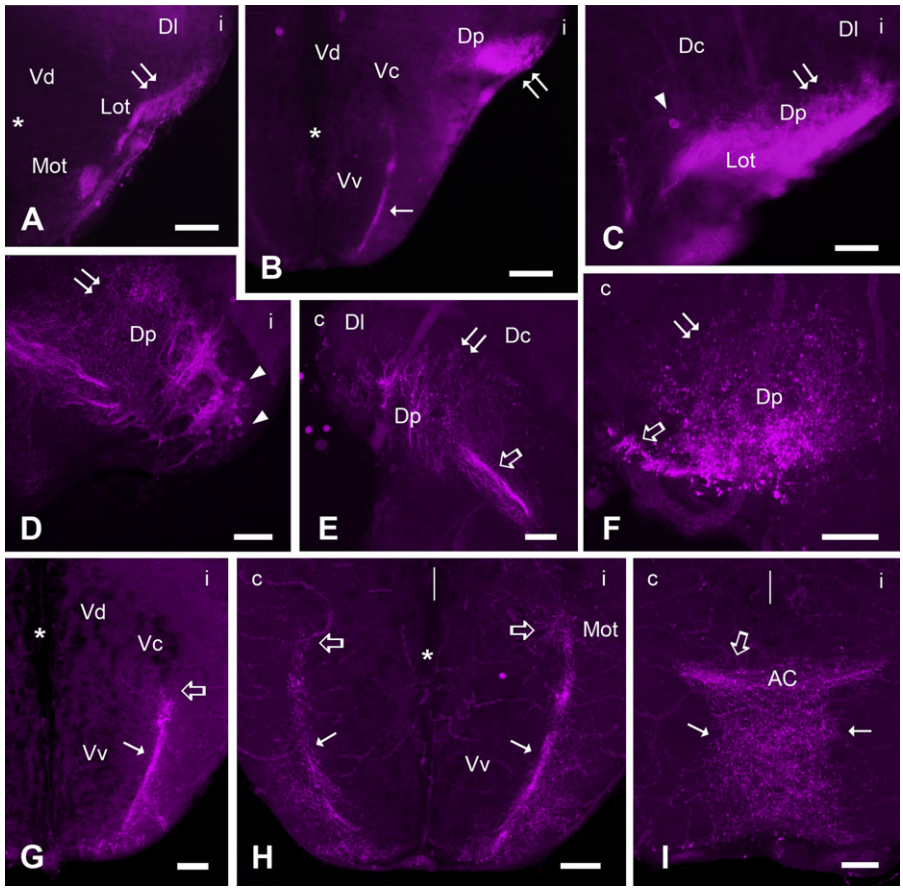

Figure 3 Fluorescence photomicrographs (**A–C**) and confocal projections (**D–I**) of transverse sections of the telencephalon from the same zebrafish as in Figure 1A, showing secondary olfactory projections from cells contacting the dorsolateral glomerular field. Most labeled fibers project to the posterior zone of the dorsal telencephalic area (double arrows indicate Dp) and the intermediate neuropil region (thin arrows) of the ventral nucleus of the ventral telencephalic area (Vv), extending in this nucleus to levels below the anterior commissure rostrally to the preoptic recess. Projections to these areas are both ipsilateral and contralateral. The sides ipsilateral (i) and contralateral (**c**) to the point of DiI application in the bulb are indicated in the panels. In (**C**) and (**D**), note retrogradely labeled neurons in Dp (arrowheads). Outlined arrows in (E,F,I) indicate the tract from the anterior commissure, those in (**G**) and (**H**) indicate the medial olfactory tract. Some blood vessels exhibit autofluorescence. AC, anterior commissure; Asterisks, telencephalic ventricle; Dc, central zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Lot, lateral olfactory tract; Mot, medial olfactory tract; Outlined arrows, medial olfactory tract; Vd, dorsal nucleus of the ventral telencephalic area; Vc, central nucleus of the ventral telencephalic area; Vv, ventral nucleus of the ventral telencephalic area. Midline is on the left in (**A–D**) and (**G**), and on the right in (**E**) and (**F**). In (**H**) and (**I**), the vertical bar indicates the midline. Scale bars = 100 μm (**A**,**B**), 50 μm (**C–I**). This figure appears in color in the online version of *Chemical Senses*.

the short wide ovoid neurons characterized as crypt cells formed a dense cluster that was well separated from the scarce slender labeled neurons (Figure 2B).

Secondary olfactory fibers labeled after application of DiI to the dorsomedial glomerular region coursed in the lateral and medial olfactory tracts. Some labeled fibers reached the caudal region of ipsilateral Dp through the lateral olfactory tract (Figure 5C,F,G). Labeled fibers running in the medial olfactory tract formed terminal fields in the dorsal and caudal regions of Vv (Figure 5C,F,I). Interestingly, numerous labeled fibers of the medial olfactory tract coursed to a neuropil region with scattered neurons located laterally to the supracommissural nucleus of the ventral telencephalic area (Vs; for Vs cytoarchitecture, see Supplementary Figure 1C–D), where they form a conspicuous terminal field (Figure 5F,G). This field was caudally continuous with that of Dp (Figure 5G). Some labeled fibers of the medial olfactory tract crossed the midline in the anterior commissure and coursed to the lateral region of Vs and contralateral Dp (Figure 5F,G).

In cases where application of DiI superficially affected the dorsomedial glomerular field (as seen in control sections of the olfactory bulb), only occasional bulbar afferent neurons were labeled in the telencephalon. When the DiI diffusion area also affected deeper bulbar regions, as in the experiment shown in Figure 5B, some bulbar afferent neurons were observed in the medial, central, and posterior parts of the dorsal telencephalic area (Figure 5C,D,F; for cytoarchitecture, see Supplementary Figure 1A–D), in dorsal Vv (Figure 5C,E) and the region lateral to Vs (Figure 5F,H).

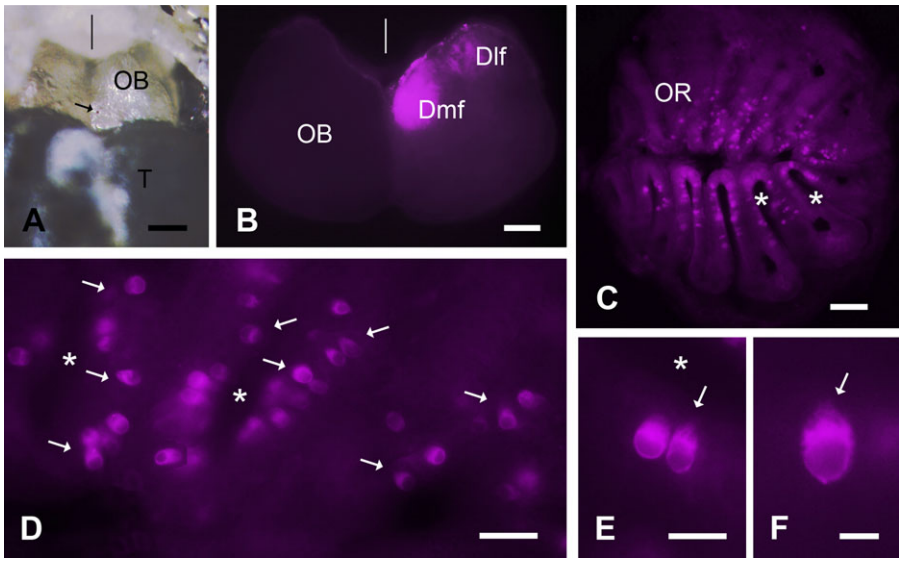

Figure 4 Photomicrographs of the olfactory bulb and epithelium in typical dorsomedial glomerular field experiments. (**A**) Photograph of the dorsal aspect of the forebrain of a zebrafish, showing the minute crystal of DiI applied to the surface of the dorsomedial glomerular field (arrow). OB, olfactory bulb; T, telencephalic lobe. (**B**) Transverse section of the olfactory bulbs, showing the restricted area of tracer diffusion of DiI after incubation in the dorsomedial glomerular field (Dmf). Dlf, dorsolateral glomerular field; OB, olfactory bulb. (**C**) Photomicrograph of an equatorial section through the olfactory rosette (OR) ipsilateral to the point of DiI application in the bulb (same experiment as in A and B), showing the distribution of retrogradely labeled crypt cells in the mucosa of all lamellae after the application of DiI to the dorsomedial glomerular field. Asterisks indicate the lumen of the rosette. (**D**) Olfactory lamellae, showing that DiI-labeled cells after application of tracer to the dorsomedial glomerular field are crypt cells (arrows). Asterisks, rosette luminal recesses. (**E,F**) Detail of retrogradely labeled crypt cells (arrows) from the dorsomedial glomerular field. Asterisk, lumen. In (**A**) and (**B**), the vertical line indicates the midline. In (**C**), midline is on the left. Scale bars = 200 μm (**A**), 100 μm (**B,C**), 50 μm (**D**), 25 μm (**E**), 10 μm (**F**). This figure appears in color in the online version of *Chemical Senses*.

Comparison of projections from the dorsomedial and dorsolateral glomerular fields

The pattern of projections to the ventral nucleus was quite different in the 2 types of experiments, with projections from the dorsolateral glomerular field region forming a conspicuous arch-shaped terminal field in Vv, which was not observed after application of DiI to the dorsomedial field. Projections from both glomerular regions reached the midregion of caudal Vv at commissural levels, although the observed patterns again appear somewhat different (compare Figure 3I with Figure 5I). The regions of Vv to which the secondary olfactory neurons project are also quite different from the medial neuropil region receiving extrabulbar primary olfactory projections (see Gayoso et al. 2011). Our previous and present results suggest that the secondary olfactory targets of the dorsomedial glomeruli receiving the majority of primary projections from the crypt cells, and those of the other glomerular fields receiving projections from other ORN types, may be segregated in the ventral telencephalic area of the zebrafish. Such a segregation may be important for understanding the functional significance of circuits conveying odor signals received by these enigmatic cells.

In order to confirm these differences, additional experiments in which minute DiI crystals were applied to putatively differential targets of the dorsomedial and dorsolateral glomerular fields were carried out (Figure 5J). Although a large number of glomeruli were labeled after application of DiI to the periventricular Vv, application of DiI to a region ventrolateral to Vs led to specific labeling of mitral cell dendrites in only 2 large dorsomedial glomeruli in whole mounts of olfactory bulbs (compare Figure 5K with Figure 5L). These glomeruli were probably those identified by Baier and Korsching (1994) in this field (mdpG1 and mdpG2). The results of crypt cell labeling from the dorsomedial glomerular region, together with the specific retrograde labeling of mdpG2 and mdpG1 mitral cells from ventrolateral Vs receiving afferent fibers from the dorsomedial glomerular field, identify these glomeruli as the probable primary targets of the crypt cells in zebrafish.

Discussion

In the zebrafish, the glomerular layer of the olfactory bulb is organized into stereotyped glomerular terminal fields, which may reflect the functional parcellation of the olfactory information in the glomerular layer (Baier and Korsching 1994). The use of immunocytochemical markers such as calcium-binding proteins (CR, S100) and G proteins (Gayoso et al. 2011), as well as the use of transgenic animals expressing reporter fluorescent proteins coupled with the expression of the olfactory marker protein (OMP) and the transient receptor potential C2 (TRPC2) channel (Sato et al. 2005), have enabled different glomerular fields to be distinguished. The above studies also revealed the relationship between the

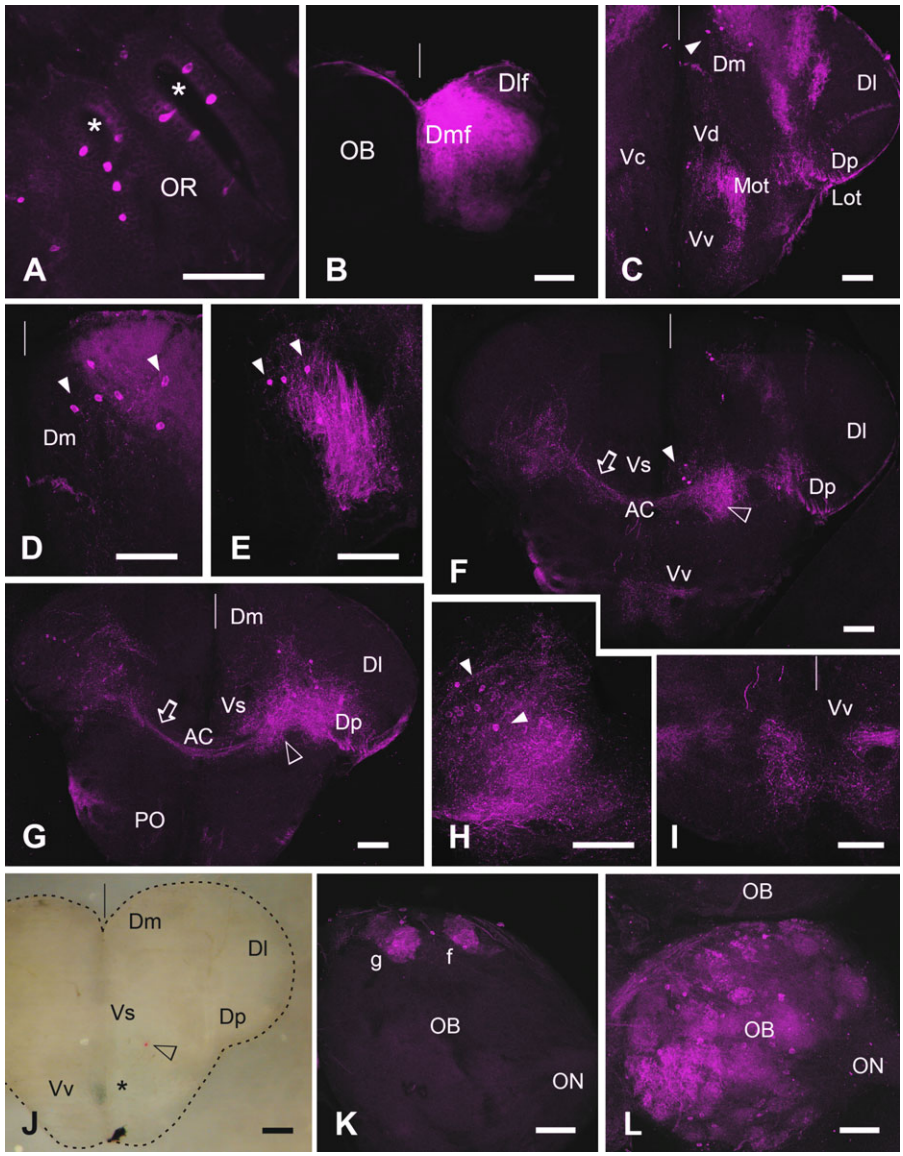

Figure 5 Confocal photomicrographs showing retrograde and anterograde labeling following application of DiI to the dorsomedial glomerular field. (**A**) Crypt cells labeled in the olfactory mucosa. Asterisks, luminal recesses. OR, olfactory rosette. (**B**) Transverse section of the bulbs showing the region of diffusion of DiI mainly centered in the dorsomedial glomerular field (Dmf). Dlf, dorsolateral glomerular field; OB, olfactory bulb. (**C–I**) Photomicrographs of transverse sections of the zebrafish telencephalon shown in (**B**), showing labeled fibers and cells. Secondary olfactory projections from cells contacting the dorsomedial glomerular field mainly project to the caudomedial part of posterior zone of the dorsal telencephalic area (**F, G**; Dp) and to an intermediate neuropil region adjacent to the supracommissural nucleus (Vs) of the ventral telencephalic area (**F, G**; outlined arrowhead). The outlined arrows in (F) and (**G**) indicate labeled fibers crossing in the anterior commissure. Secondary olfactory projections to Vv (**C, E, I**) and bulbar afferent neurons in Dm (**C, D**), dorsal Vv (**E**), and lateral supracommissural region (**F, H**) are also shown. White arrowheads indicate labeled perikarya. (**J**) Posterior pole of a block of the forebrain-olfactory bulb, showing a minute DiI crystal (outlined arrowhead) applied to the putative target of dorsomedial field of olfactory glomeruli. The asterisk indicates the typical location of DiI crystals in experiments such as that in (**L**). (**K**) Confocal projection of an entire olfactory bulb showing the 2 glomeruli and mitral cells labeled from the region in (**J**). "*f*" and "*g*" indicate the medial glomeruli mdpG2 and mdpG1 of Baier and Korsching (1994), respectively. ON indicates the olfactory nerve. The arrows "m" and "r" indicate medial and rostral regions, respectively. (**L**) Confocal projection photomicrograph through the whole olfactory bulbs (**OB**) after application of DiI to the region labeled with an asterisk in (**J**). Note that this led to the labeling of a large number of glomeruli. Arrows "m" and "r" indicate medial and rostral regions, respectively. **AC**, anterior commissure; **Dl**, lateral zone of the dorsal telencephalic area; Dm, medial zone of the dorsal telencephalic area; Dp, posterior zone of the dorsal telencephalic area; Lot, lateral olfactory tract; Mot, medial olfactory tract; **PO**, preoptic region; Vc, central nucleus of the ventral telencephalic area; Vd, dorsal nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area; Vv, ventral nucleus of the ventral telencephalic area. In (E) and (H), the midline is on the left. In (**B–D, F, G, I, J**), vertical lines indicate the midline. Scale bars = 100 μm (**A–J**), 50 μm (**I, K, L**). This figure appears in color in the online version of *Chemical Senses*.

major ORN types in the mucosa and the different glomerular fields. In the present study, DiI tract-tracing methods were used to analyze the afferent and efferent connections of 2 regions of the dorsal glomerular field of the olfactory bulb, in order to extend our understanding of the regional organization of bulbar circuits in the adult zebrafish. The morphological ORN types projecting to 4 different glomerular fields revealed in the present and in a previous study (Gayoso et al. 2011) are compared in Figure 6.

The zebrafish crypt cells preferentially innervate the dorsomedial glomerular field

The zebrafish dorsomedial glomerular field was immunonegative to the 2 calcium-binding proteins expressed by most primary sensory fibers, the CR (mainly expressed by ciliated ORNs) and S100 proteins (mainly expressed by microvillous ORNs) (Gayoso et al. 2011). Moreover, this field also lacks fibers that express Golf (Gayoso et al. 2011). With regard to the retrogradely labeled cells from the caudoventral glomerular field (Gayoso et al. 2011), which receives S100-immunoreactive fibers, all cells observed in the typical tracing experiments showed a short thick dendrite passing the apical border of the epithelium, corresponding to the "plump cells" observed in other immunocytochemical experiments with anti-S100 antibodies. Interestingly, the present results with DiI tracing from the bulbs suggest that zebrafish crypt cells preferentially innervate the dorsomedial glomerular field. This is in contrast with the findings of Hamdani and Døving (2006) in the crucian carp, and with those of Hansen et al. (2003) in the channel catfish, which show that in these species crypt cells project to ventral glomerular fields. Crypt cells of most bony fish species are recognized by their morphological appearance (globular appearance and the presence of cilia and microvilli in an invaginated region of the perikaryon), their apical location in the olfactory epithelium, and by the light supporting cells surrounding them (Hansen and Zeiske 1998; Hansen and Finger 2000; Hansen et al. 2003, 2004; Zeiske et al. 2003). Although it has been reported that the crypt cells are the only cells of the olfactory epithelium showing S100 immunoreactivity in the zebrafish (Germanà et al. 2004, 2007), other studies have cast doubts on this because most S100-immunoreactive (ir) cells do not belong to this cell type (Sato et al. 2005; Gayoso et al. 2011). Moreover, the observation that the dorsomedial glomerular field was not innervated by S100-ir or CR-ir fibers (Gayoso et al. 2011) is intriguing because, of the 4 glomerular fields examined by tract-tracing methods (Gayoso et al. 2011; present results), this field was the only one that mainly received afferents from the crypt cells. Moreover, olfactory fibers ending in the dorsomedial glomerular field are also negative for OMP and TRPC2 (Miyasaka et al. 2009). The molecular expression profile of the zebrafish crypt cells has not been described, and in particular, it is not known what type(s) of chemosensory receptors are expressed in this unique cell type (Sato et al. 2005; Yoshihara 2009). As regards the other types of ORN labeled after the application of DiI in the dorsomedial glomerular field

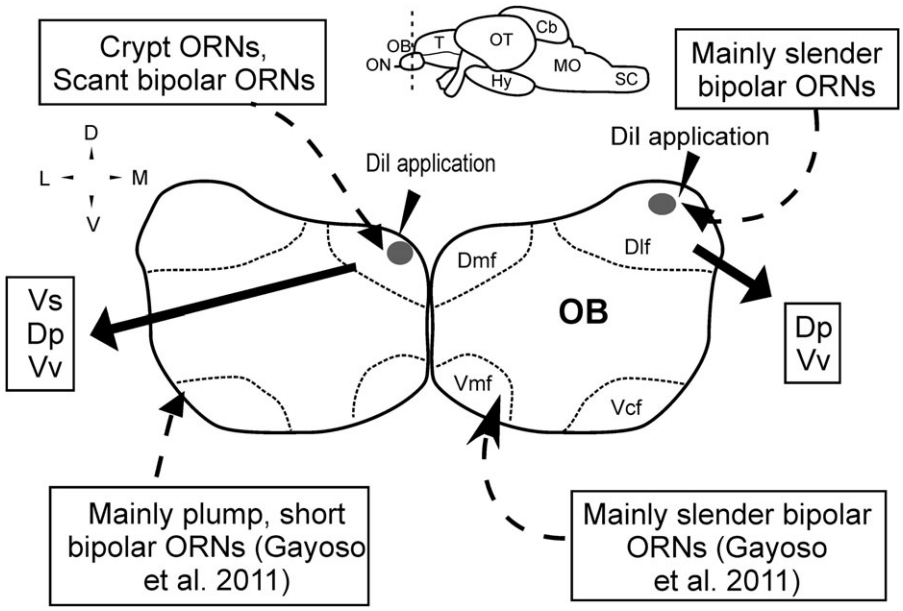

Figure 6 Schematic drawing of a section of the olfactory bulbs (at the level indicated in the schematic drawing of the brain) showing the differential projection of main morphological types of ORNs to bulbar glomerular regions in zebrafish, based on the present observations and the previous results (Gayoso et al. 2011). The main target regions of the dorsomedial and dorsolateral glomerular fields are also indicated. Cb, cerebellum; Dlf, dorsolateral glomerular field; Dmf, dorsomedial glomerular field; Dp, posterior zone of the dorsal telencephalic area; Hy, hypothalamus; MO, medulla oblongata; OB, olfactory bulb; ON, olfactory nerve; OT, optic tectum; SC, spinal cord; T, telencephalon; Vcf, ventrocaudal glomerular field; Vmf, ventromedial glomerular field; Vs, region lateral to the supracommissural nucleus of the ventral telencephalic area; Vv, intermediate neuropil of the ventral nucleus of the ventral telencephalic area. The compass card indicates the dorsal (D), lateral (L), medial (M), and ventral (V) sides.

(i.e., bipolar cells), the low number of labeled cells in the dorsomedial field is consistent with the results showing that these glomeruli are also CR negative.

Studies in the crucian carp suggest that crypt cells are a type of ORNs that respond to sex pheromones (Hamdani and Døving 2002, 2006; Hamdani et al. 2008). However, activity studies in isolated crypt cells of the Pacific jack mackerel indicate that bile salts and putative fish pheromones do not elicit responses, although the cells frequently respond to amino acids (Schmachtenberg 2006; Vielma et al. 2008). Immunohistochemical studies in catfish and goldfish have shown that crypt cells express the G proteins Gαo and Gαq (Hansen et al. 2003, 2004). It is not known what type(s) of receptors are expressed or which odorants are detected by the zebrafish crypt cells.

The zebrafish dorsolateral glomerular field is preferentially innervated by slender bipolar ORNs

Application of DiI to the dorsolateral glomerular fields led to the retrograde labeling of 2 distinct morphological classes of ORNs in the zebrafish olfactory epithelium: most cells were slender bipolar ORNs, and a few were crypt cells. Although the morphology of the apical dendrites of most labeled bipolar ORNs was not assessed, at least some had cilia. The dorsolateral glomerular field of the zebrafish olfactory bulb is mainly innervated by olfactory fibers expressing OMP, calretinin and/or Golf (Miyasaka et al. 2009; Gayoso et al. 2011). Thus, olfactory projections to this glomerular field are mostly originated from the ciliated slender ORNs (Gayoso et al. 2011), which is consistent with the present observations. As regards the microvillous ORNs, immunolabeled by S100 immunocytochemistry (short bipolar cells), they appear to project mainly to the lateral region of the zebrafish olfactory bulb (Sato et al. 2005; Gayoso et al. 2011).

Projections to the subpallium from dorsal glomerular fields receiving fibers from highly different ORN types are different

We observed the secondary olfactory projections arising from the 4 selected glomerular fields, which receive clearly differentiated primary olfactory afferents as regards the morphological and immunocytochemical features (Gayoso et al. 2011; present results). The major pathway from bulbar efferent neurons (mitral cells) contacting these different fields was a bilateral projection to the posterior zone of the dorsal telencephalic area (Dp). In adult zebrafish, bulbar projections to Dp run in both the lateral and medial olfactory tracts (Miyasaka et al. 2009; Gayoso et al. 2011, present results). No clear segregation between fibers arising from the different glomerular fields investigated was observed in Dp, indicating probable convergence on this area of inputs from pathways conducting different olfactory information. These anatomical results are consistent with the results of the functional analysis by optical imaging of the neurons of Dp in the zebrafish (Yaksi et al. 2007).

As regards the secondary olfactory projections to the ventral telencephalic area (subpallium), results obtained by Rink and Wullimann (2004) in the zebrafish reveal retrogradely labeled mitral cells in various glomerular regions after the application of a tracer to a wide precommissural region of Vv–Vd. However, our results from DiI application to the dorsomedial and dorsolateral glomerular fields reveal important differences at the level of the commissural-preoptic region. The most important difference was the finding of the dorsomedial field projections in a region just ventrolateral to Vs, which was not reached by the secondary olfactory fibers labeled from the dorsolateral field. This difference was assessed by retrograde labeling of 2 target areas in the ventral telencephalon. Interestingly, only 2 glomeruli of the dorsomedial region were labeled between the ventrolateral region and Vs, and probably corresponded to the mdpG1 and mdpG2 glomeruli identified by Baier and Korsching (1994). This suggests that the secondary olfactory projections arising from the crypt cells follow different pathways to the subpallium than those followed by the olfactory neurons that contact the other glomerular fields. This is reminiscent of the different projections of the main and accessory olfactory bulbs in tetrapods, which contact lateral and medial regions of the amygdala, respectively. The existence of the differential projections from specialized medial glomeruli in the olfactory bulb has recently been reported in the lampreys (Ren et al. 2009) and the lungfish (González et al. 2010). The present results in the zebrafish provide strong evidence of topographical segregation of high order pathways arising in crypt cells. However, it is not known if these different olfactory targets in zebrafish are associated with specific recruitment of different neural circuits, as recently shown in the sea lamprey (Derjean et al. 2010).

Comparison of zebrafish with other teleosts

Odotopic maps of the olfactory bulb have been constructed for salmonids, catfish and crucian carp by electrophysiological methods (Hara and Zhang 1996, 1998; Nikonov and Caprio 2001, 2004; Hamdani and Døving 2007). These studies have indicated a pattern of segregation of odorant inputs (amino acids, nucleotides, bile salt, and alarm substances) into different olfactory bulb regions. Maps of odor-induced activity in the zebrafish olfactory bulb constructed by optical imaging methods reveal a similar pattern, but are more detailed than in these teleosts, allowing identification of responses in specific glomeruli (Friedrich and Korsching 1998; Fuss and Korsching 2001; Yaksi et al. 2007). Although the odor maps of zebrafish and other teleosts are roughly similar, there are some differences between species. In the zebrafish, a group of ventral glomeruli do not respond to any odorants except for a putative pheromone (Friedrich and Korsching 1998). On the other hand, no bulbar response

to pheromones was recorded in the bulbs of salmonids (Hara and Zhang 1998).

A few tracing studies have reported different projections of ORN types onto the main glomerular regions of the olfactory bulb of catfish and crucian carp (Morita and Finger 1998; Hamdani et al. 2001; Hamdani and Døving 2002, 2006). As far as we are aware, no immunocytochemical studies have been performed in these species to investigate the heterogeneity of glomerular fields. Tracing studies in the crucian carp indicate that microvillous neurons project to lateral glomerular fields, crypt cells to intermedioventral glomerular fields, and ciliated neurons to medial glomerular fields (Hamdani et al. 2001; Hamdani and Døving 2002, 2006). Tracing studies in the channel catfish also reveal that ciliated neurons project to ventral and medial glomeruli, and microvillous cells to dorsal and lateral glomeruli (Hansen et al. 2003, 2005). These results are quite different from those reported for zebrafish (Sato et al. 2005; Gayoso et al. 2011; present results). Although the targets of crypt cells in the crucian carp and the channel catfish are reported to be ventral glomeruli (Hansen et al. 2003, 2005; Hamdani and Døving 2006), the main target is the dorsomedial region, in the zebrafish. These results reveal large differences in projections between the species of the same teleost branch (Otophysi), so that the findings for 1 species cannot be extended to the others, and suggest rapid evolution of navigation clues used by crypt cell axons in the olfactory bulb.

Direct physiological evidence in catfish indicates that spatial mapping of different odorants is maintained above the level of the olfactory bulb, with the 3 classes of biologically relevant odorants for catfish being processed in distinct regions of the forebrain (Nikonov et al. 2005). It has also been shown that in the crucian carp, the main classes of odorants are conveyed to telencephalic targets by 3 parallel systems of fibers (Hamdani and Døving 2007). Secondary olfactory pathways, medial and lateral olfactory tracts are present in the zebrafish, and both tracts are connected to Dp (Miyasaka et al. 2009; Gayoso et al. 2011, present results). Moreover, neurons responding to different combinations of odors are intermingled in Dp (Yaksi et al. 2007). The latter authors also found neurons responding to different odors intermingled in Vv (Yaksi et al. 2007). In the present study, the only clear segregation of secondary projections arising from the glomeruli connected with different cell subtypes of the zebrafish olfactory organ was observed in the subpallium, specifically in neuropil areas ventrolateral to Vv and Vs—to which perikarya of these nuclei send main dendritic trees. On the basis of gene expression patterns during early development, these Vv and Vs nuclei of the zebrafish have been considered homologous to the septal region and subpallial amygdala of mammals, respectively (Mueller et al. 2008). Thus, the putative amygdalar region of the zebrafish (Vs plus associated neuropil) appears to process olfactory information from crypt cells via axons from mitral cells of the dorsomedial glomeruli, which may be relevant in providing behavioral clues for some odorant molecules. In the salmon *Oncorhynchus nerka*, Vs and the medial preoptic area have been shown to play important roles in sexual behavior (Shiga et al. 1985), and this may also occur in the zebrafish.

In summary, the present results in zebrafish reveal that the 2 glomeruli identified by Baier and Korsching (1994) in the bulbar dorsomedial field appear to receive projections specifically from the crypt cells, suggesting for the first time, the existence of separate circuits for these cells that are different from those known for other types of olfactory receptors. The partial anatomical segregation of the higher order pathways of crypt cells and those of the other ORNs, together with previous immunohistochemical distinction in the zebrafish of 3 other primary olfactory subsystems, revealed by the antibodies raised against calretinin, S100, and KLH (Gayoso et al. 2011) suggests differential involvement of the olfactory subsystems in the analysis of olfactory molecules and the initiation of behavioral programs. Further studies of the responsiveness to odorants of the 2 dorsomedial glomeruli that are the putative targets of the crypt cells in zebrafish would reveal the specific roles of these cells in olfaction.

Supplementary material

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

Funding

This work was supported by the Spanish Xunta de Galicia [grant number PGIDIT07PXIB103159PR].

Acknowledgements

We thank M. Rivas Cascallar (Electron Microscopy Service, University of Santiago de Compostela) for provision of confocal microscope facilities and assistance with the study. Experiments comply with applicable law.

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