Chapter 5
Zebrafish Olfactory System

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Abstract Similar to other animal species, fishes efficiently use the sense of smell for locating food, detecting danger, communicating social information, and memorizing beneficial and detrimental conditions. This review summarizes recent advances in our knowledge of the olfactory system in the zebrafish (*Danio rerio*), which has become one of the most useful and important model organisms in neurobiology. Olfactory receptors belonging to the OR, V1R, V2R, and TAAR families are differentially expressed in three types of the olfactory sensory neurons (ciliated, microvillus, and crypt) in the olfactory epithelium. In the olfactory bulb, nine glomerular clusters are clearly delineated by anatomical features and molecular markers, serving as functional units important for odor information categorization, coding, and processing. Individual output neurons of the olfactory bulb project axons to a combination of four major target regions in the forebrain: the posterior zone of dorsal telencephalon, the ventral nucleus of ventral telencephalon, the posterior tuberculum, and the right habenula. Distinct modes of odor information decoding are employed by the individual olfactory centers: either nonselective or biased as well as either diffuse or convergent, which contribute to eliciting different physiological and behavioral responses. By taking advantage of its small brain, transparency of larvae, and amenability to various genetic and imaging techniques, zebrafish will pave the way toward understanding the functional organization of the olfactory system as a whole.

Keywords Alarm pheromone • Foraging behavior • Glomerular clusters • Higher olfactory centers • Odor map • Olfactory conditioning • Olfactory imprinting • Sex pheromone • Zebrafish

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5.1 Introduction

Many chemical cues pervade the aquatic environment of fish, activate the olfactory system, and elicit various physiological and behavioral responses. Fish can detect a huge variety of odorants that are emitted from objects and dissolved in the water, such as amino acids, nucleotides, bile acids, amines, steroids, and prostaglandins. The fish olfactory system is highly elaborated to receive and discriminate these odorant molecules, to transmit their signals to the brain, and to evoke fundamental behaviors important for survival of individuals and preservation of species, including food finding, predator avoidance, social communication, mate choice, and spawning migration (Sorensen and Caprio 1998; Zielinski and Hara 2007; Yoshihara 2009).

Zebrafish, a freshwater small teleost fish commonly available in pet shops, offers numerous advantages over other vertebrates for biological studies. Zebrafish are easy to grow and produce large clutches of eggs (100–200 per mating) through external fertilization (Westerfield 1995). The embryos develop quickly, hatching as early as 3 days post fertilization (dpf), and start to swim at 5 dpf. The zebrafish embryos are optically transparent throughout early developmental stages, enabling us to observe organogenesis and morphogenesis in vivo. In particular, transgenic expression of green fluorescent protein (GFP) and its derivatives in selected cell types greatly facilitates the live imaging of dynamic developmental events such as cell division, cell migration, and neural circuit formation. Furthermore, it has recently become possible to image functional neural activities in living transgenic embryos in which genetically engineered, highly sensitive Ca^{2+} indicators are expressed (Ahrens et al. 2013; Muto et al. 2013).

Another major advantage of using the zebrafish is its amenability of various genetic engineering techniques in both forward and reverse directions, including mutagenesis, transgenesis, gene knockdown, and gene knockout. Most recently, in particular, the whole genome sequence of zebrafish has been reported (Howe et al. 2013), and disruptive mutations in more than 38 % of all known protein-coding regions were identified (Kettleborough et al. 2013). These mutant fish will become available to the scientific community, which undoubtedly accelerate the zebrafish research in all fields of biology. In addition to these basic techniques, more advanced genetic methods have been developed in the zebrafish, such as the Tol2 transposon-mediated gene trap approach combined with the Gal4/UAS system (Asakawa et al. 2008: Koide et al. 2009), retrovirus-mediated large-scale enhancer trap screening (Ellingsen et al. 2005), Cre/loxP- and/or Gal4/UAS-mediated single-cell mosaic labeling analysis (Sato et al. 2007a; Miyasaka et al. 2009; Miyasaka et al. 2014), and TALEN- or CRISPR/Cas-mediated genome editing (Bedell et al. 2012; Hwang et al. 2013; Zu et al. 2013). Thus, the zebrafish is one of the most useful vertebrate species with which we can perform both forward and reverse genetic analyses, similar to *Drosophila melanogaster* and *Caenorhabditis elegans*.

This review highlights recent progress in our knowledge on the zebrafish olfactory system with special emphasis on neuroanatomical and functional correlates.

5.2 Olfactory Sensory Neurons

In many mammals, two functionally distinct classes of chemicals (odorants and pheromones) are detected by different types of sensory neurons located in two anatomically segregated olfactory organs in the nose: the olfactory epithelium (OE) and the vomeronasal organ (Buck 2000; Mombaerts 2004). Volatile odorants are received by a huge repertoire of odorant receptors (ORs: ~1,200 genes in mice) expressed by ciliated olfactory sensory neurons (OSNs) in the OE, and the information is transferred to the main olfactory bulb (OB). On the other hand, pheromones are mostly received by two families of vomeronasal receptors, V1Rs and V2Rs (each ~150 genes in mice), expressed by microvillus sensory neurons in the vomeronasal organ, which project axons to the accessory OB. In addition, recent studies have identified trace amine-associated receptors (TAARs) as the fourth family of olfactory receptors that are expressed by ciliated OSNs and take charge of specific pheromone or kairomone signaling (Liberles and Buck 2006; Ferrero et al. 2011; Li et al. 2013; Dewan et al. 2013).

In contrast, the anatomical organization of the olfactory system in fish species is different from that of mammals. Teleost fishes including zebrafish possess only a single type of olfactory organ called the olfactory rosette that contains three morphologically distinct types of OSNs: ciliated, microvillus, and crypt OSNs (Fig. 5.1) (Hansen and Zeiske 1998; Hansen et al. 2003, 2004). All these OSN types innervate the same OB via a tightly fasciculated bundle of olfactory nerves. Two major types of OSNs are the ciliated and microvillus neurons that differ from one another with respect to morphology and their relative positions in the OE. The ciliated OSNs are situated in the deep layer of the OE, project a long dendrite, and extend several long cilia into the lumen of the nasal cavity. The microvillus OSNs are located in the superficial layer, project a shorter dendrite, and emanate tens of short microvilli. The third OSN type is crypt cells, which account for only a small population in the OE, are located in the most superficial part of the OE, and have unique ovoid cell bodies bearing microvilli as well as submerged short cilia.

5.3 Olfactory Receptors

The ciliated, microvillus, and crypt OSNs display distinct profiles of functional molecular expression (Yoshihara 2009). The most noteworthy and functionally important is the expression of different families of olfactory receptors. The zebrafish genome harbors ~140 OR-type, 6 V1R-type, ~50 V2R-type, and ~100 TAAR-type olfactory receptor genes (Alioto and Ngai 2005, 2006; Hashiguchi and Nishida 2006, 2007; Ngai and Alioto 2007; Saraiva and Korsching 2007). The expression of OR-type olfactory receptors is observed in ciliated OSNs in teleost fishes, whereas V2R-type olfactory receptors are found in the microvillus OSNs (Cao et al. 1998; Speca et al. 1999; Hansen et al. 2004; Sato et al. 2005). It has been

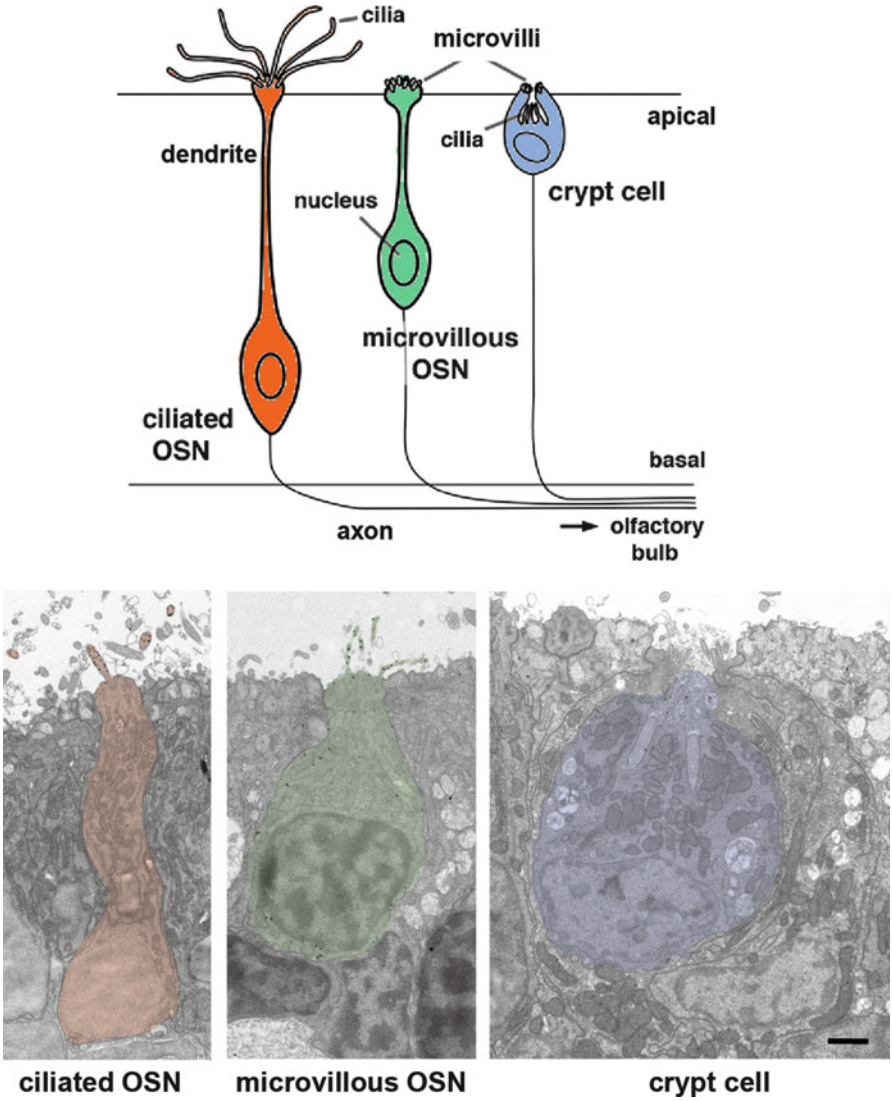

Fig. 5.1 Three types of olfactory sensory neurons (*OSN*s) in zebrafish. The *upper drawing* depicts morphological features of ciliated (*orange*), microvillus (*green*), and crypt (*blue*) OSNs. *Lower panels* show representative electron microscopic images of three OSNs. (Courtesy of Drs. Takumi Akagi and Tsutomu Hashikawa, RIKEN Brain Science Institute)

reported that mammalian ORs detect hydrophobic, volatile molecules, and V2Rs recognize hydrophilic, highly water-soluble compounds (Mombaerts 2004). Therefore, it is likely that the ciliated and microvillus OSNs in fish also take charge of detecting chemical compounds with different physical properties (e.g., hydrophobic bile acids vs. hydrophilic amino acids) through the two distinct families of olfactory

receptors (ORs andV2Rs). Several lines of evidence support this notion, based on molecular biological, electrophysiological, and activity-dependent labeling experiments (Michel and Derbidge 1997; Speca et al. 1999; Michel 1999; Lipschitz and Michel 2002; Nikonov and Caprio 2007). In contrast, V1R-type olfactory receptors are expressed in either the crypt cells or a small subset of the microvillus OSNs (unpublished observations). Intriguingly, the crypt cells express only one of the six V1R-type olfactory receptors, V1R4 (Oka et al. 2012).

The gene repertoires of OR-, V1R-, and V2R-type olfactory receptors in zebrafish are significantly smaller than those in mouse by an order of magnitude (Shi and Zhang 2009). In contrast, the zebrafish genome is equipped with as many as ~100 genes for TAAR-type olfactory receptors that far exceed TAAR genes in any other organisms examined (e.g., 6 in human, 17 in rat, 16 in mouse, 13 in Fugu fish) (Gloriam et al. 2005; Korsching 2009; Shi and Zhang 2009). Such a huge diversity of TAAR genes in the zebrafish suggests the possibility that this fish species can detect and discriminate various amine compounds. Although it remains unknown what physiological and behavioral responses are induced by amines in the environmental water, it is likely that these amines play some important roles as odorants, pheromones, or kairomones in the zebrafish.

In mouse, each OSN expresses only one type of OR gene of a repertoire of ~1,200 genes equipped in the genome (Chess et al. 1994; Serizawa et al. 2003; Mori and Sakano 2011). This "one neuron–one receptor" rule enables individual OSNs to respond to a range of odorants that bind to the expressed ORs. In other words, OSNs expressing a given OR are tuned to a particular molecular receptive range. Is the one neuron–one receptor rule applicable also to the zebrafish olfactory system? Individual OR-type olfactory receptor genes are expressed in a small population of OSNs, ranging from 0.5 % to 2 % (Barth et al. 1996). Double-fluorescence in situ hybridization experiments revealed that most combinations of two OR-type receptor probes label nonoverlapping populations of OSNs (Barth et al. 1997; Sato et al. 2007b). These results support the notion that the zebrafish OSNs fundamentally obey the one neuron–one receptor rule. However, two exceptional cases have been reported for particular olfactory receptors, in which "one neuron–multiple receptors" is true. One is the case for a subpopulation of ciliated OSNs expressing the OR103 family members: OR103-1-positive OSNs simultaneously express OR103-2 and/or OR103-5 (Sato et al. 2007b). Coexpression of multiple chemosensory receptors has been shown in several populations of OSNs in *C. elegans* and *Drosophila* (Troemel et al. 1995; Goldman et al. 2005). For example, a single AWC neuron in *C. elegans* expresses multiple olfactory receptors, responds to various odorants without discrimination, and mediates attractive behavior to all these odorants (Bargmann et al. 1993; Troemel et al. 1995). By analogy, it is likely that zebrafish do not need to discriminate a range of odorants received by the individual OR103 subfamily members. These OSNs expressing multiple OR103 members thus may integrate odor information at the most peripheral level, leading to particular behavioral or endocrine responses. The other case is a broad expression of a V2R-type receptor, OlfCc1 (VR5.3; V2rl1), in almost all microvillus OSNs (Sato et al. 2005). This situation is reminiscent of *Drosophila* Orco (Or83b) and mouse V2R2 olfactory receptors (Larsson et al. 2004; Martini

et al. 2001). *Drosophila* Orco is broadly expressed in almost all OSNs together with a selectively expressed OR and plays a general role as a hetero-dimerization partner for the selected regular OR to constitute a cation channel (Sato et al. 2008; Wicher et al. 2008). In conclusion, both "one neuron–one receptor" and "one neuron–multiple receptor" cases are observed in zebrafish, probably depending on the divergence of relevant functions in distinct types of OSNs.

5.4 Glomeruli

Glomeruli are spherical neuropils deployed on the surface of the OB where odor information is transmitted across a synapse from OSNs to the second-order neurons in the OB. Orderly arranged glomerular architecture is observed in fishes, similar to mammals and insects. There are about 140 glomeruli in zebrafish, among which 27 glomeruli are clearly identifiable whereas others are ambiguous, tiny, or sometimes fused (Baier and Korsching 1994; Braubach et al. 2012). Although the boundaries of individual glomeruli in zebrafish are, in most cases, not so clear as those in mice and *Drosophila*, a unique feature of glomerular organization is observed in the zebrafish: the presence of easily discernible glomerular clusters (Fig. 5.2). Based on their spatial locations, shapes, and molecular markers, nine glomerular clusters can be delineated and designated as dorsal glomerular cluster (dG), dorso-lateral (dlG), lateral (lG), medio-anterior (maG), medio-posterior (mpG), medio-dorsal (mdG), ventro-anterior (vaG), ventro-medial (vmG), and ventro-posterior (vpG) (Braubach et al. 2012). Individual glomerular clusters display characteristic molecular receptive ranges and play crucial roles as functional units for coding of structurally and functionally different odor categories (see following).

A recent study revealed that the zebrafish OB glomeruli can be classified into two distinct groups with respect to developmental process, anatomical size, and structural/functional stability: early-generated, highly stereotypic, large, stable glomeruli versus later-developing, smaller, plastic glomeruli (Braubach et al. 2013). The maturation of small glomeruli is heavily dependent on olfactory experience, and they are variable across individuals, whereas large and identifiable glomeruli grow steadily irrespective of sensory inputs. Thus, the two types of glomeruli form at different times and display distinct maturation mechanisms in either sensory input-dependent or input-independent manners, probably reflecting their involvement in different types of olfactory outputs: experience-dependent plastic responses versus hard-wired innate responses.

5.5 Olfactory Axon Projection

A number of neuroanatomical tracing studies were conducted for analysis of neural circuitry in the fish olfactory system (Morita and Finger 1998; Hamdani et al. 2001; Hamdani and Doving 2002, 2006; Hansen et al. 2003). For example, a lipophilic

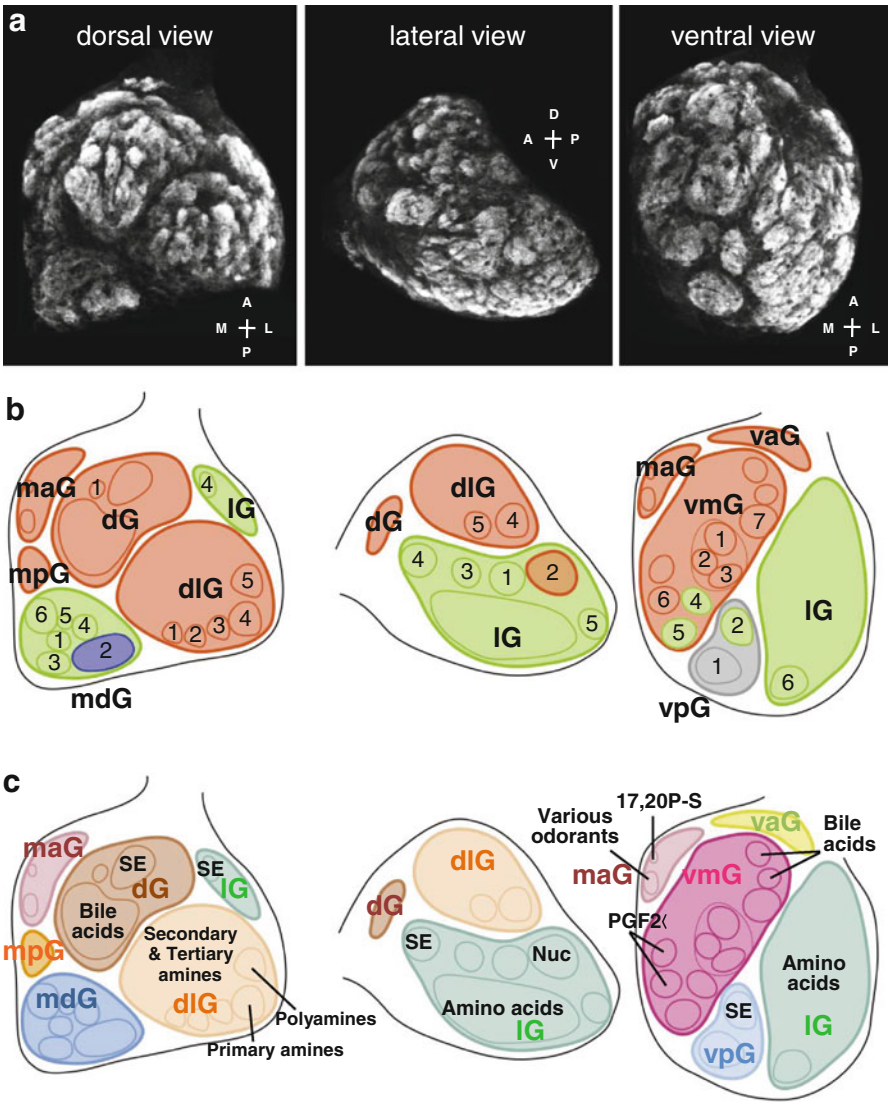

Fig. 5.2 Glomerular clusters and odor map in the zebrafish olfactory bulb (*OB*). (**a**) Whole-mount OB immunostained with anti-SV2 antibody, viewed from dorsal, lateral, and ventral sides. (**b**) Eight glomerular clusters and 29 identifiable glomeruli. (**c**) Odor map. *Nuc* nucleotides, *SE* skin extract, *dG* dorsal glomerular, *dlG* dorso-lateral, *lG* lateral, *maG* medio-anterior, *mpG* medio-posterior, *mdG* medio-dorsal, *vaG* ventro-anterior, *vmG* ventro-medial, *vpG* ventro-posterior

fluorescent tracer, DiI, was injected into a small area of the OB, taken up by olfactory axon terminals in glomeruli, and retrogradely transported to the OE. Subsequently, the types of DiI-labeled OSNs were determined on the basis of cellular morphology and location in the OE. Their results implied a tendency of

axonal segregation from the distinct types of OSNs to different regions of the OB. However, it was impossible with such a conventional tracing method to elucidate detailed patterns of axon projection from the distinct types of OSNs to individual glomeruli.

The introduction of genetic technology such as transgenesis and gene trap approaches opened a new avenue in the zebrafish olfactory research and unambiguously solved the issue on axonal wiring from the OE to the OB (Miyasaka et al. 2005, 2007; Sato et al. 2005, 2007b; Koide et al. 2009). The two major types of OSNs, ciliated and microvillus OSNs, can be differentially labeled with spectrally distinct fluorescent proteins (e.g., RFP and Venus) under the control of zebrafish OMP and TRPC2 gene promoters, respectively (Sato et al. 2005). In these double transgenic zebrafish (OMP-RFP; TRPC2-Venus), fluorescence images of whole-mount OB clearly show that the ciliated OSNs project axons mostly to the dorsal and medial regions of the OB, whereas the microvillus OSNs project axons to the lateral region. A careful histological analysis of OB sections indicates that the two distinct types of OSNs innervate different glomeruli in a mutually exclusive manner. Importantly, there is no double-positive glomerulus that receives convergent inputs from both types of OSNs. Together with immunohistochemical results with several marker antibodies (Braubach et al. 2012) and transgene expression patterns in specific subsets of OSNs in several gene trap lines (Koide et al. 2009), the primary olfactory projection in the zebrafish is summarized in Fig. 5.2b. According to the nomenclature of identifiable glomeruli and glomerular clusters in the zebrafish OB by Braubach et al. (2012), the ciliated OSNs project their axons to the maG, vaG, dG, and dlG clusters, mpG glomerulus, lG2 glomerulus, and most of vmG glomeruli, whereas the microvillus OSNs innervate all glomeruli in the lG cluster except for lG2, one of the two vpG glomeruli, and several mdG glomeruli. The third minor type of OSNs, crypt cells, send axons to at least one particular glomerulus mdG2, as demonstrated by the immunohistochemical staining of the OB with antibody against crypt cell-specific S100 calcium-binding protein (Germana et al. 2004, 2007; Oka et al. 2012; Braubach et al. 2012). These segregated neural pathways are important prerequisites for representation of distinct olfactory information on the OB as an "odor map" (see following).

5.6 Odor Map

The odor map is a central representation of chemical structural features in odorants that are systematically arranged on a two-dimensional glomerular sheet of the first relay station along the olfactory neural circuitry (Mori et al. 1999; Mori and Sakano 2011). In other words, each glomerulus represents a single olfactory receptor and is tuned to specific molecular features of odorants that can activate the receptor. The concept of the odor map was first described in the rabbit OB by electrophysiological single-unit recording of spike discharges from mitral and tufted cells to odor stimuli (Mori et al. 1992) and subsequently confirmed in various mammalian and

insect species upon the emergence and refinement of neural activity imaging techniques (Rubin and Katz 1999; Uchida et al. 2000; Wang et al. 2003; Mori et al. 2006; Vosshall and Stocker 2007; Mori and Sakano 2011).

A series of pioneering studies measuring glomerular activities with conventional voltage-sensitive dyes or Ca^{2+} indicators demonstrated the existence of an odor map also in the zebrafish OB (Friedrich and Korsching 1997, 1998; Fuss and Korsching 2001). Thereafter, genetically engineered Ca^{2+} probes (e.g., Inverse Pericam; GCaMP) were introduced to analyze the developmental and functional aspects of the zebrafish OB odor map in a more detailed and comprehensive manner (Li et al. 2005; unpublished observation). Furthermore, an immunohistochemical analysis using anti-phosphorylated Erk (MAP kinase) antibody has recently turned out to be a convenient and powerful tool to visualize glomerular activation upon odorant stimulation (unpublished observation). These findings revealed that various kinds of water-soluble compounds are represented on the surface of the OB in a highly systematic fashion and that the glomerular clusters play important roles as functional units for coding of different categories of odorants (Fig. 5.2c). For example, the dG cluster responds predominantly to bile acids, whereas the dlG cluster is exclusively devoted to amines (see following). Although the majority of glomeruli display specific activation to particular odorants, there is one glomerulus in the maG cluster that responds to various different odorants without selectivity. This glomerulus might function as a "generalist" to elicit olfactory alertness, responding to the presence of any odor stimuli (unpublished observation).

5.6.1 *Amino Acids*

Amino acids strongly attract fishes as food-derived odorants (Steele et al. 1990, 1991; Koide et al. 2009). Zebrafish are capable of discriminating between different amino acids (Miklavc and Valentincic 2012). Amino acids are detected mostly by microvillus OSNs through binding to V2R-type olfactory receptors (Speca et al. 1999; Hansen et al. 2003; Luu et al. 2004) and activate multiple glomeruli in the lG cluster (Friedrich and Korsching 1997, 1998; Fuss and Korsching 2001). Structural features of side chains in individual amino acids (e.g., long or short; hydrophilic or hydrophobic; acidic, neutral, or basic) are represented as a combinatorial code in spatially confined glomerular groups in the lateral cluster.

5.6.2 *Bile Acids*

Bile acids are biliary steroids synthesized in the liver, stored in the gallbladder, secreted into the intestine, and reabsorbed by the enterohepatic system. Interestingly, various fishes produce species-specific bile acid derivatives, such as cyprinol sulfate in carp (*Cyprinus carpio*), petromyzonol sulfate in sea lamprey

(*Petromyzon marinus*), and myxinol disulfate in hagfish (*Mixni*) (Hagey et al. 2010), suggesting their potential roles in olfactory-mediated social interaction. In the sea lamprey, for example, specific bile acids are released into the environment to act as sex and migratory pheromones, respectively (Li et al. 2002; Sorensen et al. 2005). In zebrafish, taurocholic acid activates a population of OSNs (Michel and Lubomudrov 1995) and induces a significant attractive response (Koide et al. 2009). Bile acids activate ciliated OSNs possibly via the OR-type olfactory receptors -G_{olf}- cyclic AMP signaling cascade (Hansen et al. 2003; unpublished observation). In the zebrafish OB, various bile acids elicit strong responses in the dG cluster and the anterior part of the vmG cluster (Friedrich and Korsching 1998; unpublished observation). The activity patterns induced by different bile acids show a similar but not identical distribution, indicating that distinct molecular features in bile acids are represented in the dG and vmG clusters in a combinatorial manner.

5.6.3 Amines

Although the physiological functions as odorants are enigmatic, amines should be definitely important olfactory stimuli to zebrafish from the following three reasons. First, the zebrafish genome is equipped with the largest number of amine receptors, TAARs, in all animal species examined (Gloriam et al. 2005; Korsching 2009; Shi and Zhang 2009). Second, several amine compounds induce strong electro-olfactogram responses in the zebrafish OE (Michel et al. 2003). Third, the dlG cluster in the OB is almost completely devoted to amine responses (unpublished observation). The dlG is composed of several tens of small glomeruli, among which five glomeruli (dlG1-5) are identifiable based on their unique position, morphology, and molecular expression (Braubach et al. 2012). Distinct glomeruli in the dlG tend to be activated by structurally different categories of amines: dlG4 by primary amines, dlG5 by polyamines, and many glomeruli in the anterior part of dlG by secondary and tertiary amines. Thus, there is a clear topographic map for structural features of amines in the dlG.

5.6.4 Nucleotides

Nucleotides such as ATP, IMP, and ITP induce excitatory responses in fish OE and bulbar neurons (Kang and Caprio 1995; Nikonov and Caprio 2001), possibly acting as feeding cues together with amino acids (Carr 1988). An immunohistochemical analysis with anti-phospho-Erk antibody revealed that nucleotides activate a small population of OSNs bearing a short dendrite and locating in the apical portion of OE (unpublished observation). However, these OSNs are positive for OMP promoter-driven RFP, but negative for TRPC2 promoter-driven GFP (Sato et al. 2005). Thus, nucleotides appear to activate a peculiar subset of ciliated OSNs whose morphology is similar to that of microvillus OSNs. Because the amine moiety is

contained in structure of purines and pyrimidines, it is likely that nucleotides are detected by ciliated OSNs expressing TAARs. In the OB, nucleotides activate a specific single glomerulus lG2 belonging to the lG cluster (Koide et al., in preparation). Although all other glomeruli in the lG cluster are innervated by microvillus OSNs, only the lG2 is innervated by G_{olf}-positive ciliated OSNs (Braubach et al. 2012). However, it remains largely unknown what physiological or behavioral responses are induced by nucleotides as olfactory stimulants in zebrafish.

5.6.5 Sex Pheromones

Two classes of sex pheromones, primers and releasers, acting on different steps of reproductive responses have been identified in various teleost fishes: steroid derivatives and prostaglandins, respectively (see following for details). In zebrafish OB, two sex pheromones evoke neural responses in only one or two glomeruli (Friedrich and Korsching 1998; Koide et al., in preparation). A primer pheromone, 17α,20β-dihydroxy-4-pregnen-3-one-20-sulfate (17,20P-S), activates a single or few glomeruli in the maG cluster, whereas a releaser pheromone, prostaglandin F2α (PGF2α), activates two glomeruli in the vmG cluster.

5.6.6 Skin Extract

In various fish species, putative alarm pheromones released from the injured skin of conspecifics induce robust aversive responses of other nearby fish (see following). Although two candidate molecules were reported as alarm pheromones in zebrafish (Pfeiffer et al. 1985; Mathuru et al. 2012), their validity still remains controversial. When the conspecific skin extract, a mixture of various compounds including a putative alarm pheromone, is applied to the OE, three glomerular foci are specifically activated in the OB: the anterior part of the dG cluster, the most anterior glomerulus (lG4) in the lG cluster, and one glomerulus (vpG2) in the vpG cluster (unpublished observation). At present, however, it remains unknown which glomerulus (or glomerular combination) is responsible for mediating the aversive responses to the alarm pheromone.

In addition to the aforementioned spatial representation of odorant structural features on the OB, several electrophysiological and activity imaging studies proposed the temporal coding of odor quality and intensity and the odor information processing by neuronal populations in the fish OB (Kang and Caprio 1995; Friedrich and Laurent 2001; Friedrich et al. 2004; Niessing and Friedrich 2010; Wiechert et al. 2010). For details, see reviews by Laberge and Hara (2001), Friedrich (2006), and Friedrich et al. (2009).

5.7 Higher Olfactory Centers

Odorant and pheromone information represented on the glomerular map of the OB is next transferred via the second-order projection neurons, mitral cells, to several distinct regions in the forebrain. In these higher olfactory centers, the information is decoded and processed in different manners to perceive, discriminate, and memorize odorants, to change hormonal secretion and reproductive activity, and to elicit various olfactory behaviors such as attraction to foods, escape from predators, social communication with company, and spawning migration to home rivers. Compared with a wealth of knowledge on functional correlates in the OE and OB, little has been elucidated on the molecular, cellular, and circuit mechanisms underlying odor coding and processing in higher olfactory centers in fish (Nikonov et al. 2005). However, physiological and anatomical studies have begun to shed light on basic principles of odor information representation and computation in the secondary olfactory circuitry of zebrafish (Yaksi et al. 2009; Miyasaka et al. 2009; Blumhagen et al. 2011). In particular, the most recent study combining a genetic single-neuron labeling method with the image registration system has uncovered a nearly comprehensive axon projection map from the OB to higher bran centers in zebrafish larvae (Miyasaka et al. 2014).

The OB output neurons project axons to the four major target regions in the forebrain: the posterior zone of dorsal telencephalon (Dp), the ventral nucleus of ventral telencephalon (Vv), the posterior tuberculum (PT), and the right habenula (rHb) (Fig. 5.3). In addition, approximately one-third of OB output neurons send axonal branches back into the OB ipsilaterally, contralaterally, or both. The higher olfactory centers receive odor information from OB glomeruli (and glomerular clusters) in a highly specific manner, either nonselective or biased as well as either diffuse or convergent, which is important for eliciting different olfactory outputs.

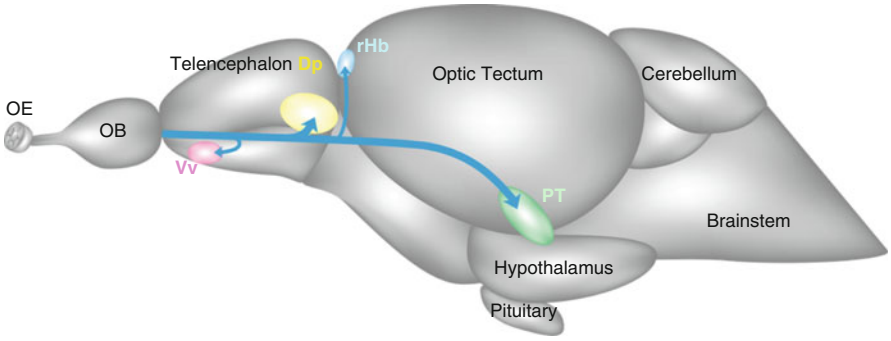

Fig. 5.3 Secondary olfactory pathway from the *OB* to higher brain centers. The four major targets of OB output neurons are highlighted: Dp (*yellow*), Vv (*pink*), rHb (*light blue*), and PT (*green*)

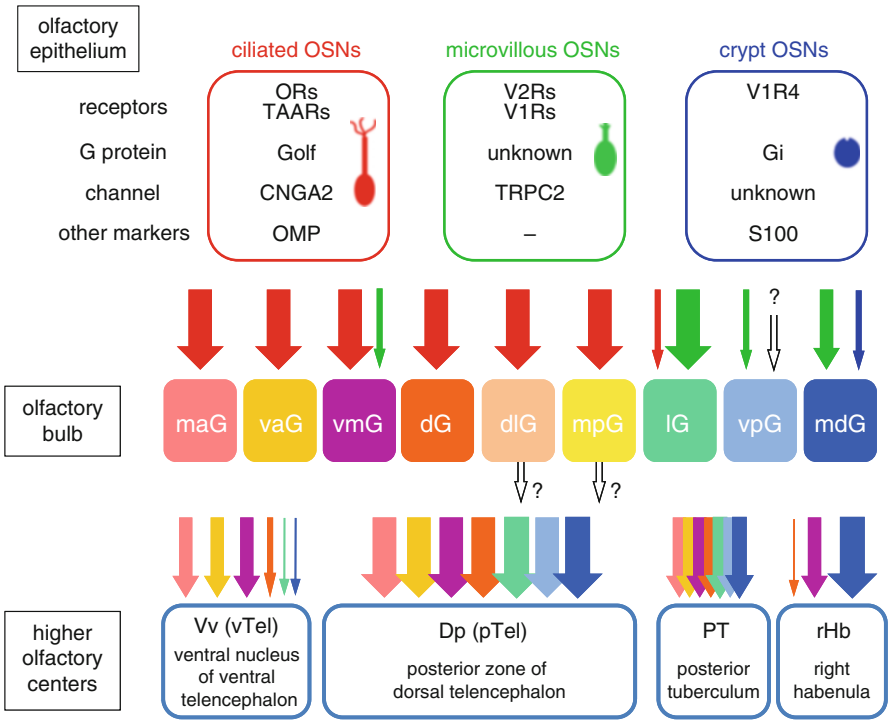

Fig. 5.4 Odor information flows from the olfactory epithelium (*OE*) to the OB and further to higher olfactory centers. Three types of OSNs express distinct receptors and other functional molecules and project axons to different sets of glomerular clusters. The odor information received by distinct glomerular clusters in the OB is next transferred to the four brain regions in higher olfactory centers, where different modes of odor information decoding are performed: either nonselective or biased and either sparse or convergent

5.7.1 Posterior Zone of the Dorsal Telencephalon (Dp)

The Dp, a pallial structure located in the dorsoposterior telencephalon, constitutes the largest part of the secondary olfactory centers in zebrafish. Genetic single-neuron visualization revealed that all the labeled OB output neurons project axons to the Dp with extensive overlap. Within the Dp, the large core region samples intermingled inputs from all the glomerular clusters (Fig. 5.4), thus transforming topographic information in the OB to broad and sparse representations (Miyasaka et al. 2014). An optical imaging study showed that individual Dp neurons extract information about discrete combinations of odorant molecular features from ensembles of glomeruli to establish representations of higher-order olfactory objects (Yaksi et al. 2009). These anatomical and functional features of the central Dp indicate that it may correspond to the piriform cortex in mammals (Ghosh et al. 2011; Miyamichi et al. 2011; Sosulski et al. 2011; Igarashi et al. 2012) and the mushroom body in *Drosophila* (Jefferis et al. 2007; Lin et al. 2007;

Caron et al. 2013), where information from the OB and the antennal lobe is computed for odor discrimination and olfactory memory. In contrast, the marginal portion of Dp is divided into several subregions that receive biased inputs from particular glomerular clusters. Thus, these Dp subregions could specifically respond to distinct categories of odorants (Miyasaka et al. 2014). This notion is supported by the fact that the anterior and posterior parts of Dp show biased responses to bile acids and amino acids, respectively (Yaksi et al. 2009).

5.7.2 Ventral Nucleus of the Ventral Telencephalon (Vv)

Another telencephalic target of OB output neurons is the Vv, a subpallial region in the ventro-anterior telencephalon. Although the Vv is thought to be equivalent to the septum and striatum in mammals, based on the expression patterns of molecular markers (Ganz et al. 2012; Wullimann and Mueller 2004), it remains largely unknown what brain functions the Vv neurons exert in fish. In contrast to the Dp, which is innervated by all the OB output neurons, the Vv receives massive inputs from particular glomerular clusters: maG, vaG, vmG, and dG (Fig. 5.4) (Miyasaka et al. 2014). These four glomerular clusters are innervated by ciliated OSNs expressing OR-type olfactory receptors that detect socially relevant odor cues such as bile acids and prostaglandins. Because the Vv neurons are reciprocally connected with the preoptic and hypothalamic areas (Rink and Wullimann 2004), the Vv might have some role in transformation of the odor and pheromone information into various social behaviors and endocrine responses.

5.7.3 Posterior Tuberculum (PT)

In addition to the two major telencephalic targets, the OB output neurons directly send axons to two diencephalic regions in zebrafish. One is the posterior tuberculum (PT), a hypothalamus-related region containing groups of dopaminergic neurons (Schweitzer et al. 2012). One or two axon branches of OB output neurons emanate from the posterior telencephalon, extend a long distance through the medial forebrain bundle, and finally reach the PT (Miyasaka et al. 2014). These axons appear to make close contacts with the dopaminergic neurons in the PT. The PT receives convergent inputs from all the glomerular clusters (Fig. 5.4), suggesting a wide range of responsiveness of PT neurons to various odor stimuli. In the sea lamprey, a group of dopaminergic neurons in the PT mediates olfactory-locomotor transformation by relaying the odor information from the OB to the reticulospinal neurons via the mesencephalic locomotor region (Ren et al. 2009; Derjean et al. 2010). Therefore, it is likely that the OB–PT pathway drives the descending neural circuitry for locomotion also in zebrafish, irrespective of odor classes and output

responses, either attraction or aversion. A small population of dopaminergic neurons in the zebrafish PT sends ascending projection to the ventral telencephalon (Tay et al. 2011), which is reminiscent of dopaminergic neurons in the ventral tegmental area and the substantia nigra in mammals. Thus, the OB–PT pathway might be also involved in controlling brain functions such as motivation, reward, and emotions.

5.7.4 Right Habenula (rHb)

The habenula is an epithalamic structure conserved among all vertebrate species. In mammals, the habenula relays information from the forebrain to the midbrain nuclei such as the interpeduncular nucleus, the raphe nuclei, the substantia nigra, and the ventral tegmental area to regulate the activities of serotonergic and dopaminergic systems. The mammalian habenula is subdivided into medial and lateral nuclei, which correspond to the dorsal and ventral habenula in zebrafish, respectively (Amo et al. 2010). The dorsal habenula in zebrafish exhibits prominent left–right asymmetry in terms of the developmental timing, molecular expression, and size ratio of medial and lateral subnuclei (Bianco and Wilson 2009; Okamoto et al. 2012). The medial and lateral subnuclei of the dorsal habenula innervate the ventral and dorsal parts of the interpeduncular nucleus, respectively (Aizawa et al. 2005). Two prominent features are observed in the neural connection from the OB to the habenula. First, the habenula receives strongly biased olfactory inputs predominantly from two glomerular clusters, mdG and vmG (Fig. 5.4) (Miyasaka et al. 2014). Therefore, it is likely that the OB–habenula pathway may constitute part of a hard-wired circuit conveying particular odor information to evoke stereotyped responses such as innate olfactory behavior. Second, the OB output neurons project axons only to the right habenula (rHb) but not to the left habenula, displaying clear left–right asymmetry of neural circuitry (Miyasaka et al. 2009). Within the rHb, axon termination is specifically observed in the medial subnucleus, suggesting that the odor information conveyed to the rHb is next transferred to the ventral part of the interpeduncular nucleus. Two recent studies reported that the habenula plays a crucial role in controlling fear responses in zebrafish (Agetsuma et al. 2010; Lee et al. 2010), although the involvement of olfactory inputs has not yet been investigated.

5.8 Olfactory Behaviors in Zebrafish

Finding foods, escaping from danger, and mating with a partner are the most basic behaviors commonly observed in various animal species. Odorants and pheromones in the aquatic environment activate olfactory receptors and neural circuits, mediating these innate behaviors also in zebrafish. In addition, zebrafish can be utilized

for analyses of odor-associated short-term memory (olfactory conditioning), as well as extremely long-lasting memory (olfactory imprinting) reminiscent of salmon homing behavior.

5.8.1 Foraging Behavior

Attraction toward food sources is one of the fundamental behaviors needed for animals to survive. Amino acids contained in the diet are indispensable for fishes not only as nutrients but also as odorants. Zebrafish exhibit robust appetitive behavior to amino acids including attraction and increased turning, recognizing them as potential feeding cues (Steele et al. 1990, 1991: Braubach et al. 2009). When a hungry zebrafish is placed into a tank of water with amino acids pumped into one corner, the fish tend to spend more time near the amino acids (Fig. 5.5c). The primary olfactory circuitry mediating this attractive behavior was elucidated by a combination of genetic, anatomical, and behavioral approaches (Koide et al. 2009). First, three gene trap and transgenic zebrafish lines were established in which the Gal4 transactivator is expressed in three distinct populations of OSNs innervating different glomerular clusters (Fig. 5.5a,b). Next, synaptic transmission from each population of OSNs to the OB neurons was selectively blocked by Gal4/UAS-mediated expression of tetanus neurotoxin that specifically cleaves VAMP2 (synaptobrevin), a synaptic vesicle protein required for exocytosis (Fig. 5.5d). The attractive response of zebrafish to amino acids was completely abolished only when the synaptic transmission to the lG cluster was silenced. These results clearly demonstrate the functional significance of the OSNs innervating the lG in the amino acid-mediated feeding behavior in zebrafish. However, it remains totally unknown how the amino acid information in the lG is read and transformed by neurons in higher olfactory centers to elicit the attractive response.

5.8.2 Alarm Response

In 1938, the Austrian ethologist Karl von Frisch discovered the existence of an alarm substance, the so-called Schreckstoff (German for “scary stuff”), in minnows (von Frisch 1938). When a minnow in a shoal was accidentally injured, von Frisch noticed that the other fish in the same tank displayed conspicuously frightened reactions: darting and freezing. Subsequent experiments demonstrated that putative alarm substances are contained in specialized cells (alarm substance cells or club cells) in the fish skin, released into water upon injury, and activate specific olfactory neural circuitry in its shoaling company to notify the presence of danger (Lebedeva et al. 1975; Pfeiffer 1977; Kasumyan and Lebedeva 1975). The Schreckstoff-induced alarm response is observed in the superorder Ostariophysi that includes approximately two-third of freshwater fish species including zebrafish.

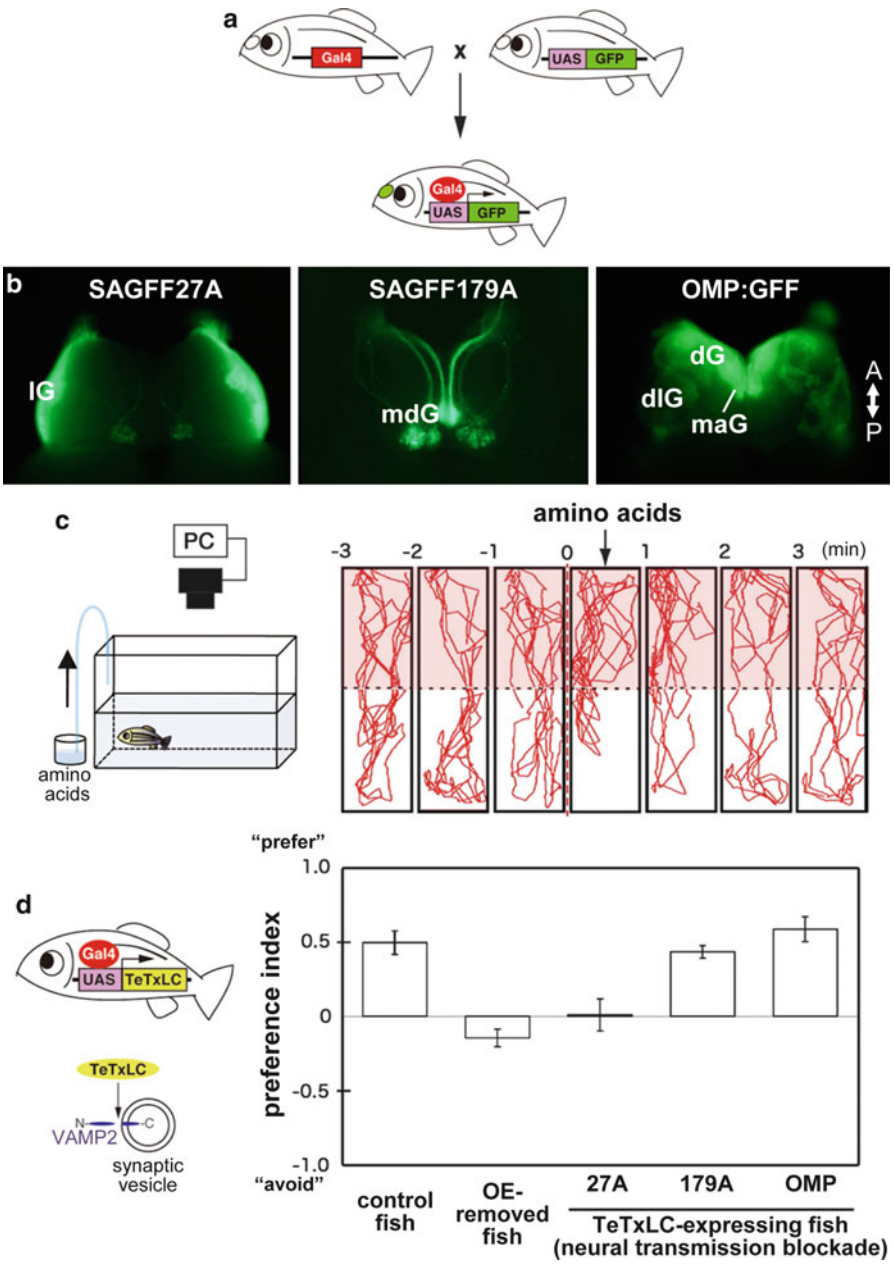

Fig. 5.5 Genetic dissection of olfactory neural circuitry mediating attraction to amino acids. (**a**) A principle of Gal4/UAS system in zebrafish. (**b**) GFP fluorescence in whole-mount OBs from three transgenic lines expressing Gal4 and GFP in different populations of OSNs. Axon innervations of differential glomerular clusters are observed among the three transgenic lines. (**c**) *Left*: A behavioral assay setup. *Right*: Representative swimming paths of zebrafish on amino acid application. Results are presented for every 1 min before and after the application of amino acid mixture. (**d**) Synaptic transmission blockade by forced expression of tetanus neurotoxin (TeTxLC). *Left*: TeTxLC-mediated blockade of synaptic transmission by specific cleavage of VAMP2 in synaptic vesicles. *Right*: The attractive responses to amino acids for individual genotypes are represented by the preference index (*Y*-axis). The OE-removed fish and the SAGFF27A; UAS: TeTxLC double-transgenic fish show no preference, demonstrating the importance of the lateral glomerular cluster in the attraction to amino acids. (Modified from Koide et al. 2009)

Upon application of a conspecific skin extract, most zebrafish display a robust, biphasic response in the bottom of a tank: burst swimming followed by freezing (Speedie and Gerlai 2008). Although two structurally unrelated molecules were reported as candidates of fish alarm substances, hypoxanthine-3*N*-oxide (Pfeiffer et al. 1985) and chondroitin sulfate (Mathuru et al. 2012), the real identity of Schreckstoff is still a mystery. As already mentioned, our calcium imaging and anti-phospho-Erk immunohistochemical experiments have identified three glomerular foci in the zebrafish OB that are strongly activated by the skin extract (unpublished observation). It is conceivable that plural components in the skin extract activating different glomeruli may coordinately evoke the alarm response through some coincidence-detection mechanism in higher olfactory centers.

5.8.3 Reproductive Behavior

Two types of sex pheromones, steroids and prostaglandins, that have been identified in female goldfish are secreted at different steps of the estrus cycle and sequentially act on male fish for successful reproduction (Sorensen et al. 1998; Sorensen and Caprio 1998). Two steroid derivatives, 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) and its sulfated form (17,20P-S), are secreted from female goldfish at a preovulatory stage and act on males as primer pheromones that change the male endocrine-gonadal responses (Stacey et al. 1989). 17,20P and 17,20P-S evoke a rapid increase in luteinizing hormone release from the pituitary, leading to spermatogenesis in several hours (DeFraipont and Sorensen 1993). In zebrafish, only 17,20P-S appears an active pheromone that is sensed by a small subset of ciliated OSNs and activates a single or few glomeruli in the maG cluster (Friedrich and Korsching 1998; unpublished observation). During ovulation in zebrafish as well as goldfish, prostaglandin F2α (PGF2α) and its metabolite 15-keto-PGF2α are synthesized and secreted in female urine, acting on male fish as releaser pheromones (Sorensen et al. 1988). The male sexual behavior upon stimulation with these releaser pheromones includes increased swimming activity, attraction to females, nudging (abdomen touch), and quivering. PGF2α and 15-keto-PGF2α activate a selective olfactory pathway involving two glomeruli in the vmG cluster in zebrafish (Friedrich and Korsching 1998; unpublished observation). Future studies are awaited for the identification of pheromonal receptors for 17,20P-S and PGF2α, and the dissection of higher-order neural circuitry mediating endocrine and behavioral responses evoked by these sex pheromones.

5.8.4 Olfactory Conditioning

Similar to other animal species, fish can be conditioned to associate odors with either aversive or attractive stimuli. The aversive conditioning experiments include electrical shock to catfish (Little 1977), lithium chloride injection to goldfish

(Manteifel and Karelina 1996), and conspecific skin extract (Schreckstoff) exposure to zebrafish (Suboski et al. 1990), all of which were associated with particular odorants. In contrast, fish also can associate odorants with positive reinforcement stimuli such as food rewards (Herbert and Atema 1977; Valentincic et al. 2000; Braubach et al. 2009; Miklavc and Valentincic 2012). Thus, zebrafish can learn and memorize odor-associated behavioral tasks reliably in standard conditioning paradigms. Hence, it is now possible to analyze these learning behaviors with a combination of genetic, optical imaging, electrophysiological, and neuroanatomical methods for elucidation of neural circuit mechanisms underlying olfactory memory and behavioral plasticity.

5.8.5 Olfactory Imprinting

One of the most widely known olfactory imprinting behaviors is homing of salmon to their mother-rivers. Juvenile salmon imprint on the odors of their natal stream, then migrate to sea, and grow up to be adults. After several years in the sea, the adult salmon return to their home river for reproduction by navigating through the environment using various sensory cues including the odors of their natal stream (Scholz et al. 1976; Dittman and Quinn 1996; Yamamoto et al. 2010). Although zebrafish do not display homing behavior in nature, Harden et al. (2006) reported that zebrafish in laboratories can form and retain olfactory memories experienced in juveniles, similar to those observed in salmons. Zebrafish were exposed to an artificial odorant, phenylethyl alcohol (PEA), for the first 3 weeks post fertilization, then raised in ordinary water up to adult stage, and subjected to a preference test in a Y-maze. As a result, the PEA-exposed zebrafish showed significant preference to this odorant whereas the control fish did not. Thus, zebrafish clearly remember the odor to which they were exposed as juveniles, rendering this fish species as an attractive model organism for studying olfactory imprinting or long-lasting olfactory memory.

5.9 Conclusions and Perspectives

These two decades since the discovery of odorant receptor genes by Buck and Axel (1991) have witnessed great advances in our understanding of the functional architecture of the primary olfactory system. Multigene families encoding odorant and pheromone receptors were identified in various animal species (see Chap. 2 by Touhara). The axon guidance mechanism for establishing neural connectivity patterns from the OE to the OB was clarified (see Chap. 3 by Sakano). The concept of the "odor map" was established as the internal representation of odorant molecular features in the OB, demonstrating the importance of glomerular modules as functional units for odor coding and processing (see Chap. 4 by Mori). Therefore, it

is high time for us to contemplate, hypothesize, and investigate the functional architecture of the secondary and tertiary olfactory circuitry from the OB to the cortex and beyond, linking odor inputs to various higher-order brain functions such as perception, emotion, memory, decision making, and consciousness. In our efforts toward understanding the olfactory system as a whole, the zebrafish will undoubtedly become an ideal model vertebrate in the next decade, with its tiny but well-organized brain, sophisticated olfactory circuits, and robust olfactory behaviors, as well as amenability of various state-of-the-art genetic techniques.

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