DISSERTATION

NEURONAL BASIS OF OLFACTORY IMPRINTING AND KIN RECOGNITION IN THE ZEBRAFISH *DANIO RERIO*

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ABSTRACT

Olfaction plays a fundamental role in detection and discrimination of the environment in all vertebrates, including teleosts, such as the zebrafish, *Danio rerio*. The zebrafish olfactory system is capable to detect a wide range of chemical compounds which trigger or contribute to behaviours crucial for survival such as foraging, migration, intraspecific communication, reproduction and predator avoidance. In contrast to terrestrial vertebrates, the teleost olfactory system lacks a separate vomeronasal organ (VNO), which is known to be involved in pheromone detection. However, although the zebrafish olfactory system consists only of one paired olfactory epithelium (OE), the containing olfactory sensory neurons express olfactory receptors related to those of the main OE and VNO of mammals. Thus, the fish olfactory system is capable to detect and process pheromones and show a context related behavioral response. Beside many olfactory driven social behaviors, kin recognition is of particular relevance to the field of neurobiology, because it depends on an imprinting paradigm which requires a two step learning process of olfactory and visual cues during a defined time window early in life. Zebrafish larvae imprint on the pigmentation pattern and olfactory cue of their kin on the 5^{th} and 6^{th} day of development. The created kin template allows discriminating between kin and non-kin and plays a fundamental role at early stages, as it was shown that zebrafish larvae prefer to group with their kin whereas sexually mature zebrafish use kin recognition to avoid inbreeding. Interestingly, larvae which are exposed to non-kin cues at the appropriate days show neither preference for kin nor for non-kin, suggesting a genetic predisposition for kin cues. However, the neuronal mechanisms underlying olfactory imprinting and kin recognition are unknown so far. Recent studies demonstrated that zebrafish recognize their kin based on Major Histocompatibility Complex (MHC) class II genotype similarity. Zebrafish which share MHC class II alleles show a similar pigmentation pattern (visual cue) as well as chemical signature (olfactory cue) and thus MHC class II genotype similarity may explain the genetic predisposition which prevents larvae to imprint on non-kin cues. Moreover, olfactory stimulation with MHC class II peptide ligands shows spatially overlapping activation of bulbar neurons compared to responses to kin odor, suggesting MHC peptides to be part of kin odor. Presently, the type of olfactory sensory neuron (OSN) which detects a kin odor related signal is unknown. The zebrafish OE bears four different types of OSNs, ciliated- and microvillous OSNs, *kappe* neurons and crypt cells; each type showing morphological- and immunohistochemical characteristics.

Our study combines behavioral, genetic and neuroanatomical methods to investigate the neuronal mechanisms involved in the processes of olfactory imprinting and kin recognition in the zebrafish. The first aim of this study is to provide new insights to the anatomy of the larval and adult zebrafish olfactory system. Combinatorial immunohistological analysis of four different calcium binding proteins (CBPs), Parvalbumin, Calretinin, Calbindin and S100, reveals a differential expression pattern of OSNs and their axonal projections into the olfactory bulb (OB). Combinatorial double immunohistochemistry identifies at least eight subpopulations of OSNs. We report three subpopulations of ciliated OSNs - one major subpopulation expresses Parvalbumin, Calbindin and Calretinin, and two populations are either positive for Parvalbumin and Calbindin or Calretinin only. Furthermore, we identify four subpopulations of microvillous OSNs, one expresses only Parvalbumin, one minor population shows S100 and Parvalbumin positivity, one is positive for Parvalbumin and Calbindin and finally one subpopulation of microvillous OSNs which is immunoreactive for Parvalbumin, Calbindin and Calretinin. Crypt cells, absent in terrestrial vertebrates and only present in teleosts, express only S100 and are negative for all other CBPs. Consistent with other reports, axonal projections of ciliated OSNs terminate into dorso- and ventromedial bulbar fields whereas microvillous OSNs project their axon into the ventrolateral OB. Additionally, we newly describe axonal projections of likewise microvillous OSNs which only express Parvalbumin and terminate into the mediodorsal OB. Moreover, we show S100 positive crypt cells to terminate into one single mediodorsal glomerulus, the mdg2, but also show additional axonal input into this glomerulus from S100 and Parvalbumin expressing microvillous OSNs.

To investigate the type(s) of OSNs which detect a kin odor related signal, we focused on finding a reliable marker for neuronal activity in response to olfactory stimulation. The Extracellular signal Regulated Kinase (ERK) is a member of the ERK / Mitogen Activated Protein Kinase (MAPK) signaling pathway. Activation, for instance by binding of a ligand to an olfactory receptor leads to phosphorylation and therefore activation of ERK (pERK) which in turn translocates into the cell nucleus to modulate gene expression. In mammals, pERK is a common marker for neuronal activity and was previously used in the field of olfaction. Before starting to approach the identity of the OSN type involved in kin recognition, we validated pERK as a reliable marker for neuronal activation in the larval zebrafish after odor exposure. To this aim, we stimulated group raised larvae at the 9th day of development with different odors and analyzed neuronal activation visualized by pERK immunopositivity in the larval zebrafish OE. With the use of accepted morphological criteria, we identified the four different

types of OSNs of the zebrafish OE. Additionally, we used the CBP S100 to mark specifically crypt cells. For the first time in larval zebrafish, we performed a timescale experiment to test best odor exposure duration with the result that detectable pERK levels are recognizable already after 3 minutes of odor exposure. However, prolonging the exposure duration does not lead to better pERK signals in OSNs. Furthermore, we demonstrate that olfactory stimulation with food and non-kin odor (conspecific odor) clearly results in a differential activation pattern of OSNs. Consistent with other studies, we identify activated ciliated and microvillous OSNs after exposure to food odor, whereas only microvillous cells show responses to conspecific odor. Crypt cells show activation neither to food odor nor to non-kin odor. Upon validation of pERK to mark neuronal activation in the larval zebrafish OE, we stimulated imprinted and non-imprinted 9 day old larvae with kin odor and analyzed activated OSNs. In two rounds of stimulation experiments, each with slightly different raising conditions, we provide the first direct evidence for crypt cells as well as a small subpopulation of microvillous cells to be involved in detection of a kin odor related signal. Interestingly, only larvae which were successfully imprinted show activated crypt cells in response to kin odor, whereas crypt cells of non-imprinted larvae show no increase in pERK levels. A difference in crypt cell number does not account for this difference in activation pattern as a comparison of crypt cell quantity reveals no significant difference between imprinted and non-imprinted larvae. Furthermore, we analyzed neuronal activation of bulbar neurons after exposure to kin odor. Consistent with our results on activation at the level of the larval OE, bulbar neurons of imprinted larvae show increased neuronal activation compared to non-imprinted larvae especially around the mediodorsal glomerulus that receives crypt cell input (mdG2) after kin odor exposure.

The final aim of this study is to identify the existence of an accessory olfactory pathway in teleosts. In tetrapods, vomeronasal information is mainly transferred from the VNO to the accessory olfactory bulb (AOB) and from there to the medial amygdala. The medial amygdala is a part of the subpallium and initiates via amygdalo-hypothalamic pathways behavioral and also hormonal responses to incoming signals. Moreover, besides an involvement in fear and associative learning, the medial amygdala is also known to be involved in processing of conspecific odors in rodents. Although a separate VNO is absent in teleosts, we newly identify an accessory olfactory pathway in the zebrafish. By injection of DiI tracer into the mediodorsal OB, which is the target region of crypt cells and some microvillous OSNs, we demonstrate a neuronal circuit running from the mediodorsal OB to the medial amygdala and from there to the tuberal hypothalamus. Interestingly, non-imprinted

zebrafish larvae show increased activity of neurons in the medial amygdala compared to imprinted larvae. Finally, we demonstrate for the first time the OSN type which is involved in the detection as well as processing targets of a kin odor related signal in larval zebrafish.

1. INTRODUCTION

1.1. Olfaction in vertebrates: overview

Olfaction or the sense of smell is an important window for detection and discrimination of the environment in all vertebrates. The competence to detect and be responsive to chemical signals (chemosensation) of the external environment is of benefit for most animal species (including invertebrates) and is vitally important for survival and reproduction (Ache and Young 2005). Chemosensation is evolutionarily very old, as it is even present in bacteria ("quorum sensing"), slime molds and protozoans, illustrating the significance for every organism to sense its environment and communicate via chemicals (Ache and Young 2005). The general mechanism which enables an organism to identify and react on the chemical composition of its environment relies on the interaction of a chemical stimulus and its receptor expressed by chemosensory cells. Focusing on the sense of smell (olfaction), the olfactory system evolved a complex repertoire of receptors to enable an organism to detect and discriminate between numerous structurally different odor molecules (Buck and Axel 1991, Menini et al. 2004). Although the olfactory sense is of fundamental importance for most vertebrates, the degree to which an organism depends on olfaction as well as the anatomy of olfactory structures varies between vertebrate species (Hoover 2010). However, although each organism is well adapted according to its needs to its terrestrial or aquatic olfactory environment the general principle of olfactory organization and its molecular mechanisms are evolutionarily conserved across vertebrates (Zippel 1982, Hoover 2010). Odorant detection occurs at the level of the olfactory epithelium, by binding of an odorant (ligand) to its receptor expressed by receptor neurons which selectively forward the olfactory information via their axons to the olfactory bulb (OB), the first station for odor processing. In general, tetrapod vertebrates sense their olfactory environment via two anatomically distinct olfactory organs: The main olfactory epithelium (MOE) and the vomeronasal organ (VNO) (Dulac and Torello 2003). Both olfactory organs have been traditionally considered as functionally independent, with the MOE detecting common (airborne) odorants and the VNO responsible for detection of (less volatile) pheromones (social odorants), but recent studies refuted this hypothesis of "dual olfaction" by showing that both systems play synergistic roles in odor detection and subsequent olfactory-guided behaviors (Buck 2000, Dulac and Torello 2003, Ache and Young 2005, Spehr et al. 2006, Baum 2012, Suarez et al. 2012). The ability to detect and distinguish between numerous airborne odors which are structurally complex and rarely comprised of a

single compound is mediated by olfactory receptors expressed by olfactory sensory neurons (OSNs) which reside in the olfactory epithelium (OE). The discovery of a large multigene family of olfactory-specific G-protein-coupled receptors (GPCRs) in the rat, now referred to as the OR family of odorant receptors (Mombaerts 2004a) led to the Nobel-prize award for Linda Buck and Richard Axel (Buck and Axel 1991). In the vertebrate genome the OR gene family is known to be the largest gene family although some variability in size of functional OR genes is found between species, ranging between 50 to 100 in teleosts to over 1000 functional OR genes in rodents, demonstrating evolutionary variability to a great extent (Ache and Young 2005, Alioto and Ngai 2005, Niimura and Nei 2005, 2007). A second class of olfactory receptors, trace amine-associated receptors (TAARs), is present in the vertebrate main olfactory epithelium (Borowsky et al. 2001, Liberles and Buck 2006, Shi and Zhang 2009). Like the OR gene family, the TAAR gene repertoire varies in size among vertebrates, but the TAAR gene family is much smaller in tetrapods than in teleosts, such as the zebrafish *Danio rerio*, which possesses the largest TAAR gene repertoire among vertebrates (Hashiguchi and Nishida 2007).

However, in addition to ORs and TAARs, further receptor families, also belonging to GPCRs, are found in the vertebrate olfactory system (Table 1). In the tetrapod vomeronasal epithelium, olfactory sensory neurons express mainly three distinct subtypes of vomeronasal receptors (VRs), V1Rs, V2Rs and formyl peptide receptors (FPRs), all of them also GPCRs (Dulac and Axel 1995, Mombaerts 2004a, Liberles et al. 2009, Riviere et al. 2009). Vomeronasal olfactory sensory neurons expressing these receptors project olfactory information to the accessory olfactory bulb (AOB), which resides at the dorsal posterior region of the OB (Hayden and Teeling 2014). All vomeronasal and main olfactory epithelial OSNs express selectively only a single olfactory receptor allele, which is called the “one receptor - one neuron” hypothesis and, furthermore, neurons expressing the same receptor converge to the same discrete neuropil structure (glomerulus) within the OB (Vassar et al. 1994, Mombaerts 2004a, b).

As mentioned above, not all vertebrates do possess a vomeronasal organ separate from a main olfactory epithelium. Most tetrapods (amphibians, reptiles and mammals) sense their olfactory environment via a dual olfactory system (MOE and VNO). Unlike those tetrapods, a morphologically separate VNO is absent in fish, birds and their closest relatives, crocodiles and adult humans (Suarez et al. 2012). However, homologous genes to the mammalian vomeronasal receptor genes (termed VR-like, both V1R and V2R) are present in teleosts and V1R genes are even found in basal vertebrates such as the lamprey (Grus and Zhang 2009). In

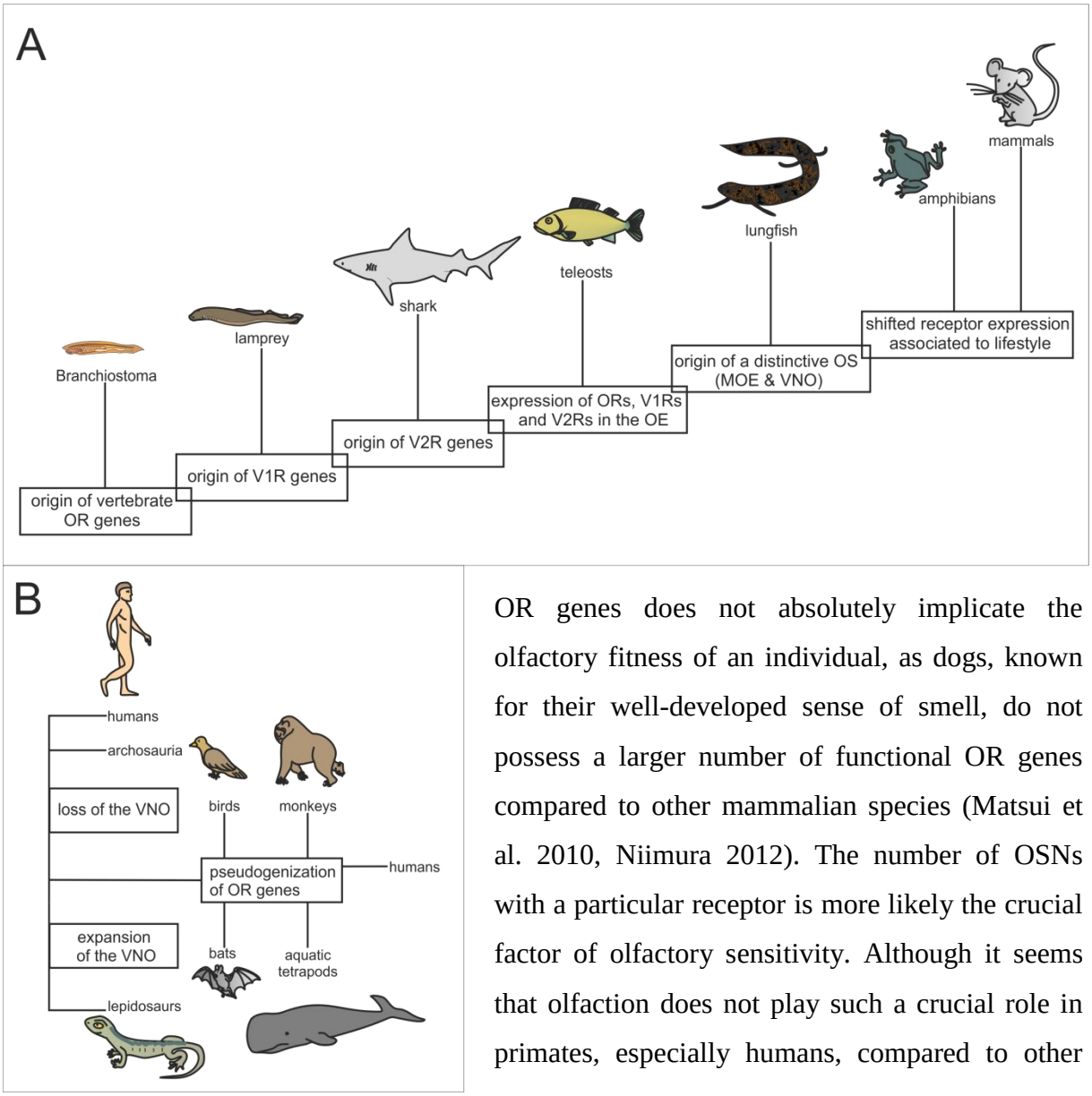
teleosts, both, main olfactory- (ORs & TAARs) (Ngai et al. 1993a, Ngai et al. 1993b) and VR-like (V1Rs, V2Rs & FPRs) (Cao et al. 1998, Asano-Miyoshi et al. 2000, Pfister and Rodriguez 2005) receptors are expressed by OSNs located in one olfactory epithelium (OE). Although fish lack a separate VNO, they indeed use pheromones primarily for social behaviors such as predator avoidance, intraspecific (social) communication and reproductive behavior, hinting at a functional VNO in fishes (Hoover 2010). Early tetrapods were aquatic before they entered terrestrial habitats. Thus, the amphibian olfactory system displays a main OE and a separate VNO (Hayden and Teeling 2014). Nevertheless, the evolution of the VNO seems not uniquely associated to a terrestrial and therefore aerial olfactory lifestyle, as aquatic larva of the African clawed frog *Xenopus laevis* as well as fully aquatic salamanders already display a MOE and a VNO (Freitag et al. 1995, Eisthen 1997).

Overall, the evolution of vertebrate olfactory organs, sensory neurons and receptors shows a remarkable diversity among various groups and results as much from adaptations to changing environments as from different needs (Figure 1). Frequent gene duplications, gene deletions and inactivating mutations (pseudogenes) reduce or expand gene repertoires - the combinations of such events are known as the birth-and-death process of evolution (Nei et al. 1997). Evolutionary plasticity of vertebrate olfaction explains such variability of olfactory gene repertoires among species and gene gains and losses coincide with evolutionary events, such as the transition from water to land. In addition, olfactory subgenomes are directly linked to the habitat of a given species, thus aquatic, terrestrial and flying animals display different olfactory gene repertoires (Hayden et al. 2010, Hayden and Teeling 2014). A phylogenetic analysis of vertebrate olfactory receptor gene families elucidates the high diversity among vertebrates. The vertebrate OR gene family is classified into class I and class II, which are thought to have different affinities to water-soluble and volatile molecules, respectively. Thus, fully aquatic animals, such as the teleosts, mostly express class I ORs whereas amphibians do express both classes and mammals possess mainly class II OR genes (Freitag et al. 1998, Niimura and Nei 2005, 2006). A study in the African coelacanth, *Latimeria chalumnae*, an extant lobe-finned fish, shows that this “living fossil” expresses class I and class II receptor genes, the latter nonfunctional, indicating a possible branch between both classes of ORs (Freitag et al. 1998, Picone et al. 2014). Moreover, an expansion of VNO specific genes similar to those of tetrapods, especially of those of amphibians, is observed in this lobe-finned fish, suggesting an advanced development of the VNO, as recently identified in the African lungfish *Protopterus dolloi* (Gonzalez et al. 2010, Picone et al. 2014). As aforementioned, a MOE and a VNO as well as their characteristic receptors are present in amphibians, which are

well adapted to both aquatic and terrestric life. Thus, phylogenetically positioned in between teleosts und mammals, amphibians already show a segregation of their olfactory system as well as distinct expression zones of olfactory receptors (Syed et al. 2013). A specialization of both subsystems (MOE & VNO) to aerial and underwater olfaction is present in *Xenopus laevis*. Besides the separated VNO, the MOE of *Xenopus* is divided by a valve-like structure into two chambers, the lateral (LD) and medial diverticulum (MD), which express ORs associated to hydrophil (class I) and volatile (class II) odorants, respectively (Freitag et al. 1995, 1998, Syed et al. 2013). The mammalian olfactory receptor repertoire displays a high diversity according to the animal's habitat and lifestyle and thus reflects its extent of reliance on the sense of smell. Interestingly, whereas olfaction plays an important role for most terrestrial mammals, secondarily adapted aquatic vertebrates tend to lose their olfactory systems. For example, in baleen whales (Mysticeti), olfactory structures, such as OE and OB, albeit small as well as functional OR genes are present and are used in foraging. In contrast, their sistergroup, toothed whales (Odontoceti), such as dolphins, have completely lost their olfactory structures and more than 75% of their OR genes are non-functional (Kishida et al. 2007, Thewissen et al. 2011, Kishida et al. 2015). Similarly, most non-terrestrial amniotes (arboreal, aquatic or flying mammals and birds) seem to rely on other senses than olfaction, such as hearing and echolocation. Birds for example mainly communicate vocally and use visual cues, e.g. their plumage to find suitable partners for reproduction. Nevertheless, some studies showed that olfaction does play a role for example in homing behavior in birds (Gagliardo et al. 2013, Hayden and Teeling 2014). Like other mammals, bats possess a functional olfactory system but the degree to which they rely on olfactory cues varies highly between suborders. A VNO for example is not present in all taxa but present in most frugivorous taxa (Bloss 1999). However, whether the absence of a VNO in bats is a primitive trait and reevolved numerous times or existed and got lost or reduced in many chiroptera families is under debate (Wible and Bhatnagar 1996).

Another example for a shift of extent of reliance from the olfactory sense towards others is present in primates. The number of OR genes of primates is much smaller than that of other mammals. Primates, including humans, rely more extensively on vision rather than on olfaction. Gilad and colleagues suggested that the retrogression of primate olfactory ability results from the occurrence of trichromacy vision (color vision), since most remaining mammals are color-blind (dichromatic) (Gilad et al. 2004). However, recent studies on OR gene losses in each lineage of the primate evolution and whole genome analysis do not support this "color vision priority hypothesis" (Niimura 2012). There is a high variation in the

OR gene repertoire within primate lineages and moreover the number of OR genes varies between different individuals and within and between culturally distinct populations (Hoover 2010, Niimura 2012). In humans, the variation of OR gene number, resulting from deletions and duplications of DNA segments, is known as the copy number variation (CNV). In such CNV regions, OR genes are enriched and differ between individuals which explains the variation of olfactory sensitivity to some odors among individuals. Moreover, OR gene loci show high number of single nucleotide polymorphisms (SNPs) which might lead to inactivation of OR genes resulting in a non-functional pseudogene (Niimura 2012). In general, approximately 50% of human OR genes are pseudogenes. However, the number of



OR genes does not absolutely implicate the olfactory fitness of an individual, as dogs, known for their well-developed sense of smell, do not possess a larger number of functional OR genes compared to other mammalian species (Matsui et al. 2010, Niimura 2012). The number of OSNs with a particular receptor is more likely the crucial factor of olfactory sensitivity. Although it seems that olfaction does not play such a crucial role in primates, especially humans, compared to other

Figure 1 Evolution of the vertebrate main olfactory system and vomeronasal system Relevant events related to the evolution of main olfactory system (MOS) **(A)** and vomeronasal system (VNS) **(B)** in vertebrates. *Adapted from* (Suarez et al. 2012)

senses such as hearing or vision (or taste), it is an important source of environmental information which might be perceived consciously or unconsciously. Interestingly, similar to fish, humans are another example for pheromone detection without possessing an anatomical distinct VNO. By expressing vomeronasal receptors (only V1Rs) in the olfactory epithelium, humans are able to detect pheromones and thus react unconsciously to physiological, behavioral and emotional aspects (Hoover 2010).

Olfaction is, together with vision, gustation, somatosensation and mechanosensory lateral line sense, one of the evolutionary old senses among vertebrates and plays an important role for most species. Regarding its habitat, each organism is well adapted to its olfactory environment and uses odorants for foraging, communication, reproduction and predator avoidance. Analysis of olfactory receptor gene classes and their expression shows quite clearly that vertebrate olfaction originated in primarily aquatic living species and is conserved as well as diversified among vertebrates. However, the extent to which an organism relies on olfactory cues varies between species and seems to decline during vertebrate evolution with relying more on other senses such as hearing or vision. Nevertheless, although modern molecular genetic research provided new insights in development and function of olfaction in vertebrates, further comparative studies on gene expression coupled with functional research on molecular mechanisms are necessary to understand and answer those numerous remaining open questions on vertebrate olfaction.

1.2. The teleost olfactory system

Teleosts are the largest infraclass within ray-finned fishes (Actinopterygii) and account, with over 26.000 species, for more than 50% of all extant vertebrate species (Volff 2005). As other vertebrates, teleosts are capable to sense their olfactory environment and use olfactory cues for several important issues such as feeding, homing, kin recognition, reproduction and predator avoidance (Hansen and Reutter 2004). Compared to terrestrial vertebrate olfaction, teleost olfactory odors are dissolved in water and the active odorant detection space of fishes is restricted due to a 10.000 times slower diffusion transmission in water than in air (Rosenthal and Lobel 2006). In addition, a regulation of odor perception, such as the mammalian "sniffing" is not present in teleosts. Instead, teleosts overcome this constrained odor detection space by either pumping water through the nostrils using cilia or if existent, by contraction of the accessory sacs. In addition, fast swimming and the use of natural currents also facilitate the detection of certain odor sources (Cox 2008). However, just as the olfactory gene repertoire, the anatomy of the olfactory organs shows considerable variation among teleosts, but most of them exhibit (at least) a paired peripheral olfactory organ, the olfactory epithelium (OE), each located within an olfactory chamber at the anterodorsal part of the head. The olfactory chamber is connected to the exterior environment by anterior and posterior nostrils which provide a waterflow which flushes the OE with odorants diluted

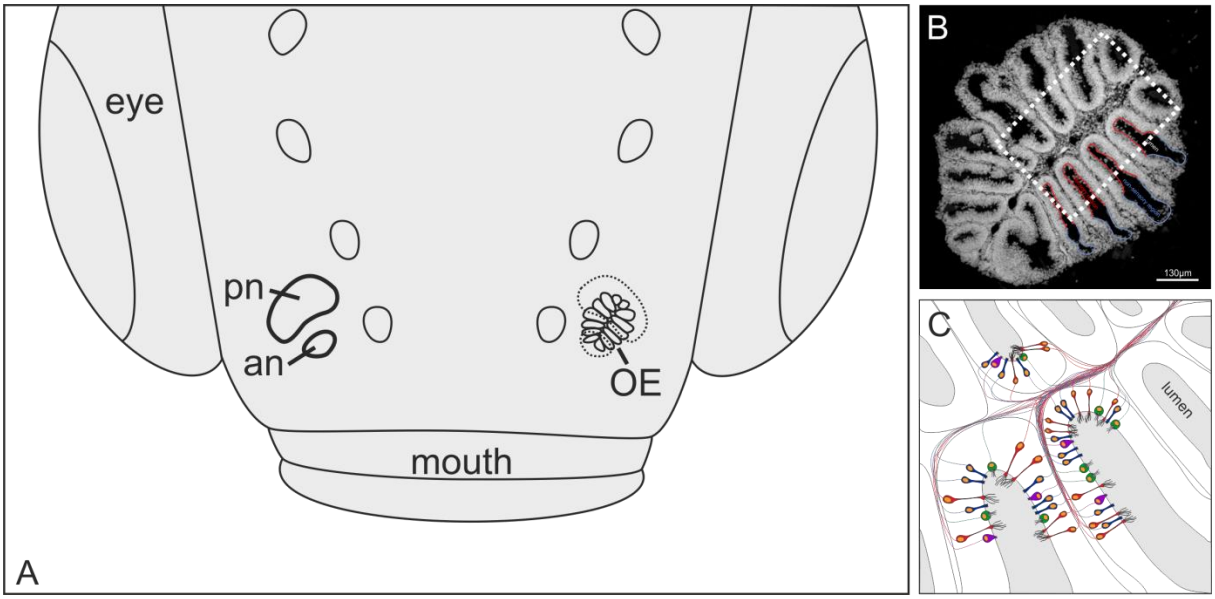


Figure 2 The teleost olfactory epithelium (OE). **(A)** Dorsal view of a zebrafish head shows spatial organization of the OE. The paired OE lies at the dorsal part of the snout beneath the anterior (an) and posterior (pn) nostril. **(B)** Cross section of one adult zebrafish OE stained with DAPI. Red line outlines sensory region and blue line non-sensory region. White dashed box marks area shown in **(C)** in higher magnification. **(C)** Schematic representation of organization of olfactory sensory neurons (OSNs) within a cross section of a adult zebrafish OE. The four types of OSNs are intermingled within the sensory region of the zebrafish OE. Cilliated OSNs are shown in red, microvillous OSNs in blue, crypt cells in green and kappe neurons in purple. Scale bar in **(B)** 130µm.

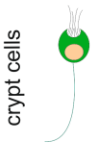
OSN type	Receptor / G-protein	zebrafish	mouse	human
ciliated OSNs 	ORs + $G\alpha_{olf}$	143	~1100	~400
	TAARs + $G\alpha_{olf}$	112	15	6
microvillous OSNs 	V1R + $G\alpha_{i}$	5	~240-300	4-5
	V2R + $G\alpha_{o}$	45	120	?
crypt cells 	V1R + $G\alpha_{i}$ (ora4)	1	✗	✗
kappe neurons 	? + $G\alpha_{o}$	?	✗	✗

Table 1 Comparison of receptor expression and associated G-proteins on olfactory sensory neurons (OSNs) in zebrafish, mouse and human. *See text for details and references.*

within the surrounding water (Figure 2). In most teleosts, the shape of the OE is folded into lamellae whose numbers vary from a few to about 300 depending on the species (Hansen and Reutter 2004). Each lamella consists of a nonsensory region and a sensory region, containing nonsensory cells and olfactory sensory neurons, respectively. Depending on the species, the thickness of the olfactory epithelium ranges from about 15µm (zebrafish, *Danio rerio*) to 110µm (pike, *Esox lucius*) (Holl 1965). The nonsensory region is comprised of supporting cells, nonsensory ciliated cells and basal cells. Basal cells are mitotically active and differentiate into new OSNs, therefore they are competent for a life-long renewal of the OE. At least three types of olfactory sensory neurons (OSNs) have been identified in actinopterygian (ray-finned) fishes, which are intermingled within the sensory region of the teleost olfactory epithelium and show different morphological appearance as well as different expression patterns of olfactory receptors (explained in detail in 1.2.1). Ciliated (cOSNs) and microvillous (mOSNs) olfactory sensory neurons, also present in the rodent MOE and VNO, respectively, make up the most prominent populations of receptor neurons. A third OSN type, named crypt cells, represent a comparatively small population of OSNs and are absent in the mammalian olfactory system. Recently, a fourth OSN type, kappe neurons was identified in the zebrafish OE (Ahuja et al. 2014). Every OSN expresses one type of olfactory receptor at its apical dendritic protrusions, which might be cilia or microvilli, or in the case of crypt cells both (Table 1). The binding of an odorant will lead to a change of the OSNs membrane potential and therefore triggering action potentials which propagate the information along the axon. OSN axons, which express the same receptor, project into the olfactory bulb (OB) into the same defined neuropil structures, named glomeruli, and make synaptic contacts onto projection neurons which in turn process and mediate olfactory information to higher brain areas such as the telencephalon (explained in detail in 1.2.2.).

1.2.1. Odor detection: The zebrafish olfactory epithelium

Odor detection occurs at the level of the olfactory epithelium (OE). Odorants bind to olfactory receptors expressed on the apical dendritic protrusions of olfactory sensory neurons (OSNs) which relay the olfactory information to the olfactory bulb (OB). In the zebrafish, *Danio rerio*, the olfactory epithelium with its sensory and non-sensory cells develops from the olfactory placode, an ectodermal thickening from cells of the anterior neural plate at already 24 hours post fertilization (pf) (Hansen and Zielinski 2005). The first olfactory receptor cells are detectable about 30 hours post fertilization with simultaneous opening of the nares (Moorman 2001). The zebrafish OE lies between the anterior and posterior nostril on each side of the head (Fig. 2A). Compared to other species (e.g. pike 110 µm), the OE of a an adult zebrafish is with its 15-20 µm thickness considerably thin (Hansen and Zeiske 1998). Four different types of OSNs are intermingled randomly within the sensory region of the zebrafish OE (Figure 3). Ciliated (cOSNs) and microvillous olfactory sensory neurons (mOSNs) represent the two main types of vertebrate OSNs. Furthermore, two additional minor populations of OSNs are present in the zebrafish OE, crypt cells and kappe neurons (Ahuja et al. 2014). All four types of OSNs are well definable by morphological criteria, such as cell shape, position of their soma within the OE or by their apical dendritic protrusions.

In addition, there are several immunohistochemical markers available which are beneficial in discriminating the four types of OSNs. As in mammals, **ciliated OSNs** appear spindle shaped with a soma located most basally within the OE. Their long slender dendrites end in a so called olfactory knob, from which a few cilia sprout into the lumen of the olfactory cavity. Zebrafish cOSNs express odorant receptors (OR), homologous to the mammalian OR class receptors, coupled to the olfactory specific G protein α-subunit $G\alpha_{olf/s}$ which activates cyclic AMP during signal-transduction (Jones and Reed 1989, Buck and Axel 1991, Hansen et al. 2003, Hansen and Reutter 2004, Sato et al. 2005, Saraiva et al. 2015). Fish express exclusively class I OR genes whereas tetrapods exhibit both, OR gene class I and class II (see 1.1.) (Freitag et al. 1998, Niimura and Nei 2005). Furthermore, cOSNs express another class of olfactory receptors, trace amine associated receptors (TAARs), which are also found in mammals. However, the teleost TAAR gene repertoire, especially that of the zebrafish, represents the largest repertoire with 112 functional receptor genes among vertebrates so far (Gloriam et al. 2005, Liberles and Buck 2006, Hashiguchi and Nishida 2007, Korsching 2009).

Another major population of olfactory sensory neurons is represented by **microvillous OSNs.** This OSN type appears plumper and its cell body localizes more apically than those of cOSNs. Numerous microvilli extend from the apical surface at the end of a short dendrite (Hansen and Zielinski 2005). Unlike tetrapods whose mOSNs are only present within the vomeronasal epithelium, the teleost mOSNs are intermingled with the other types of OSNs within the single main olfactory epithelium. Nevertheless, teleost mOSNs express homologues to the mammalian VR-type odorant receptors (Cao et al. 1998, Hansen et al. 2003, Sato et al. 2005). The zebrafish genome contains 56 vomeronasal receptor (VR) genes, most of them belong to the V2R gene family but six are V1Rs (Hashiguchi and Nishida 2006, Saraiva and Korsching 2007, Yoshihara 2009). Due to an absence of a separate VNO in teleosts, V2Rs are called OLfC, belonging to the class C of GPCRs, characterized by a large N-terminal extracellular ligand binding region (Okamoto et al. 1998, Hino et al. 2009). Teleost V1R genes are called *ora* genes, which stands for olfactory receptors related to class A GPCRs (Saraiva and Korsching 2007). The *ora* gene family is with its 6 members very small but highly conserved across teleosts. Moreover, the presence of direct orthologues in lamprey indicates that this gene family is evolutionarily very old (Saraiva and Korsching 2007, Korsching 2009). Currently the OSN type on which ORA receptors are expressed is not known but it is presumed that they are involved in pheromone detection whereas V2Rs detect peptides (Leinders-Zufall et al. 2009, Behrens et al. 2014) and both are likely to be expressed on mOSNs.

So far, only one ORA receptor, ORA4, is found to be expressed on a small population of OSNs – the **crypt cells**. In contrast to mOSNs and cOSNs, crypt cells amount for just a minor population in the teleost OE, explaining why this cell type was identified very late (Bazaes et al. 2013). Nevertheless, as an exclusive feature of the fish OE, crypt cells are present in both ray-finned (bony) and cartilaginous fish and share morphological characteristics (Hansen and Finger 2000, Ferrando et al. 2006). Crypt cells appear egg shaped; the non-dendritic cell body is located most superficially in the olfactory epithelium. As the name implies, an apical invagination, the "crypt" opens towards the olfactory lumen from which about 7 cilia as well as numerous microvilli protrude (Hansen and Zeiske 1998, Hansen and Zielinski 2005). Every crypt cell is surrounded by at least one but in most cases two supporting cells. Supporting cells mainly function as supportive elements like releasing detoxifying enzymes, but among other duties they secrete components into the mucus which overlays the OE-lamina, and electrically isolate the OSNs (Hansen and Zeiske 1998, Hansen and Reutter 2004). As mentioned above, all crypt cells express a single V1R- homologue

receptor encoded by the *ora4* gene which may signal olfactory information via the inhibitory Gi_{1b} protein. The expression pattern of crypt cells according to a "one cell type- one receptor" rule represents a unique style of expression pattern different from the mosaic expression of different receptor types a la "one neuron- one receptor" seen in cOSNs and mOSNs (Oka et al. 2012).

Recently, the Korsching Lab (University of Cologne) identified a novel population of olfactory sensory neurons in the zebrafish, *Danio rerio*. **Kappe neurons** represent the fourth OSNs type of the zebrafish OE (Ahuja et al. 2014). Like for the crypt cells, these pear-shaped OSNs with a characteristic cap at their apical end are very scarcely distributed within the OE and although very similar in appearance with crypt cells, kappe neurons show specific characteristics. In contrast to crypt cells, kappe neurons are more basally located within the OE and only bear microvilli on their apical end. So far, the olfactory receptor which is expressed on kappe neurons is not known but it was shown that these cells express the G-protein G_o and are negative for all usual markers for the other OSN types of the zebrafish OE (Ahuja et al. 2014). Beyond supporting cells and the four OSN types of the zebrafish OE mentioned above, the OE bears also basal cells and ciliated non-sensory cells (for an overview see Fig. 3). Roundish **basal cells** lie between the axons of the OSNs and the basal parts of the supporting cells. Because olfactory sensory neurons have a limited life span they are substituted continuously by mitotically active basal cells which differentiate into new OSNs (Hansen and Zeiske 1998, Hansen and Reutter 2004). Ciliated nonsensory cells are intermingled between all other cell types within the OE and bear kinocilia at their apical endings towards the lumen. These cilia are motile and competent to propel odorants containing mucus and/or water forward (Sleigh 1989). Furthermore, **goblet cells** are present,

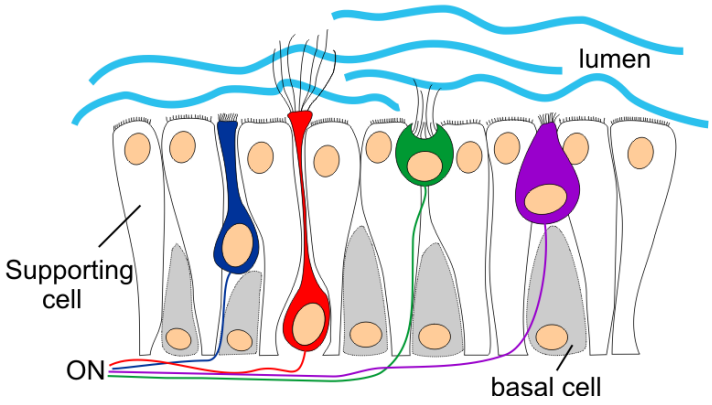


Figure 3 The zebrafish olfactory sensory neurons (OSNs). Four types of OSNs are intermingled within the zebrafish olfactory epithelium (OE). Axonal projections of OSNs form the olfactory nerve (ON) and mediate olfactory information into the brain. **Microvillous OSNs** (blue) appear more plump. A short dendrite bears microvilli and their cell soma lies at intermediate depths. **Ciliated OSNs** (red) are located most basal within the OE. Cilia at the end of their long dendrite are extended towards the lumen. **Crypt cells** (green) are most apically positioned and appaer egg-shaped. The typical crypt bears cilia and microvilli. **Kappe neurons** (purple) are egg-shaped. Their soma is located more basally than that of crypt cells. Kappe neurons express only microvilli at their apical, cap- formed end. Supporting cells and basal cells are intermingled between the four types of OSNs.

but restricted to the nonsensory area of the zebrafish OE. These oval cells are surrounded by ciliated nonsensory cells and secrete granules into the lumen of the OE (Hansen and Zeiske 1998).

In addition to morphological characteristics, the four types of OSNs are distinguishable by several immunohistochemical markers which are often specifically expressed by different OSN types. The olfactory marker protein (OMP) as well as the transient receptor potential channel C2 (TRPC2) are characteristic markers for cOSNs and microvillous OSNs in the zebrafish OE, respectively (Sato et al. 2005). In addition, there are primary antibodies and/or *in situ* probes against Gα subunits available to distinguish between the four OSN types. In the zebrafish, calcium binding proteins (CBPs) are shown to be expressed in a selective combinatorial manner by cOSNs, mOSNs and crypt cells as well as in their axonal projections into the olfactory bulb (OB). Interestingly, our combinatorial expression analysis of four CBPs in the adult and larval zebrafish olfactory system brought at least eight subpopulations of zebrafish OSNs to light (Kress et al. 2015). In this study, the four CBPs, Parvalbumin (PV), Calbindin (CB), Calretinin (CR) and S100 were used in a combinatorial fashion to investigate different CBP expression profiles of the zebrafish OSN types. The CBP Parvalbumin is expressed with Calretinin and Calbindin in at least two of three subpopulations of cOSNs, the third cOSNs subpopulation mainly expresses Calretinin only. In addition, this study identified four subpopulations of mOSNs, which express either only PV, PV and S100, PV and CB and a subpopulation which is PV, CB, and CR positive. All crypt cells are negative for PV, CB and CR but express S100 homogenously in their cell bodies as well as their axonal projections (Oka et al. 2012, Kress et al. 2015). Additionally to the identification of at least eight subpopulations of OSNs in the zebrafish OE, the CBP analysis demonstrated that the axons of these OSN subtypes are also distinguishable in the OB to which they project (see 1.2.2.).

1.2.2. Odor processing: The zebrafish olfactory bulb and its targets

Olfactory information is mediated by reams of olfactory sensory neurons (OSNs) (see 1.2.1) located within the olfactory epithelium (OE). The zebrafish unmyelinated axons of OSNs form the first cranial nerve (I), the olfactory nerve (ON), which terminates in the olfactory bulb (OB), a (sessile) paired structure seated on the telencephalon (Hansen and Reutter 2004). Like in other vertebrates, the OB is the brain structure presumed to be the first

station of odor processing. Each zebrafish OB contains approximately 20,000 neurons which are organized in four distinct layers, olfactory nerve layer, glomerular layer, external layer and internal cellular layer (Friedrich et al. 2009) (Figure 4). The **olfactory nerve layer (ONL)** is the outermost layer of the OB and is formed by axons of OSNs. The axons of OSNs branch at their end in specific neuropil structures called glomeruli, where they make synaptic contacts to second order olfactory neurons. The **glomerular layer** (GL) is comprised of approximately 140 glomeruli per OB which are arranged in a stereotyped pattern, bilaterally symmetric to the other OB (Braubach et al. 2012). Interestingly, each glomerulus is innervated by one or a few OSN subtypes expressing the same receptor (Sato et al. 2005, 2007). Moreover, glomeruli which are activated by similar odorant classes are arranged in defined zones, therefore forming a chemotopic map (Friedrich and Korsching 1997, 1998). Several immunohistochemical studies and research using transgenic lines unraveled the differential projection pattern of OSNs into the OB. In zebrafish, cOSNs are shown to innervate mainly dorsal and a few ventromedial glomerular domains whereas mOSNs send their axons mainly to glomeruli of the ventrolateral OB. Interestingly, all crypt cells are shown to project their axon towards one singular glomerulus within the mediodorsal field of the zebrafish OB (mdg2) (Sato et al. 2005, Gayoso et al. 2012, Ahuja et al. 2013, Kress et al. 2015). Ahuja and colleagues (2014) revealed that also the fourth zebrafish OSN type, the kappe neurons project their axon to a singular mediodorsal glomerulus (mdg5) previously reported to be the sole projection site of a mOSN subtype exclusively positive for $G\alpha_o$ (Braubach et al. 2012) . However, the projection pattern of all OSN types gets more complicated focusing on OSN subtypes with each subtype consequently innervating specific glomeruli as well. Our own study using four calcium binding proteins (CBPs) in a combinatorial pattern reported that at least 8 subpopulations (Figure 6) are present in the zebrafish OE (Kress et al. 2015). Although some of these subpopulations show overlapping bulbar projection targets, single projection profiles could be elucidated. A single subpopulation of mOSNs, for example, sends their Calbindin/Parvalbumin positive axons into a mediodorsal glomerulus (likely mdg5) close to the mdg2. Whereas the latter, the mdG2, is only innervated by S100 positive crypt cells and a small subpopulation of S100/Parvalbumin positive mOSNs. An additional ventral glomerulus (vpG) with Parvalbumin/Calbindin positive axons likely from mOSNs is also reported (Kress et al. 2015). Glomeruli located medial and/or ventral are of special interest because they are thought to be involved in processing of social and reproductive behaviors whereas lateral glomeruli are shown to mediate food odor related signals (Li et al. 2005, Koide et al. 2009).

As mentioned above, each glomerulus provides synaptic contact of OSN axons with dendrites of second-order neurons, such as mitral cells. The **external cell layer (ECL)** contains the somata of glutamatergic mitral and ruffed cells. Mitral cells are large glutamatergic neurons ranging in size from 4 – 18 µm in diameter and located mainly at the superficial edge of the ECL (Fuller et al. 2006). These major output neurons of the olfactory bulb are innervated at their apical dendrites by axonal projections of a single OSN subtype mostly within a single glomerulus but also receive synaptic input via dendritic contacts from bulbar interneurons of the internal cell layer. Additionally, glutamatergic ruffed cells occur between mitral cells which are innervated by OSN axons, mitral cells and bulbar interneurons (Kosaka and Hama 1982). **The internal cell layer (ICL)** is the deepest layer of the OB and contains interneurons and granule cells. GABAergic granule cells lack an axon but they extend dendritic processes to mitral and ruffed cells (Kosaka and Hama 1982, Kermen et al. 2013). Additionally, lateral interactions within neurons of the OB are mediated by glutamatergic juxtaglomerular cells as well as by dopaminergic periglomerular neurons, which extend their processes into the glomerular layer (Byrd and Brunjes 1995, Edwards and Michel 2002).

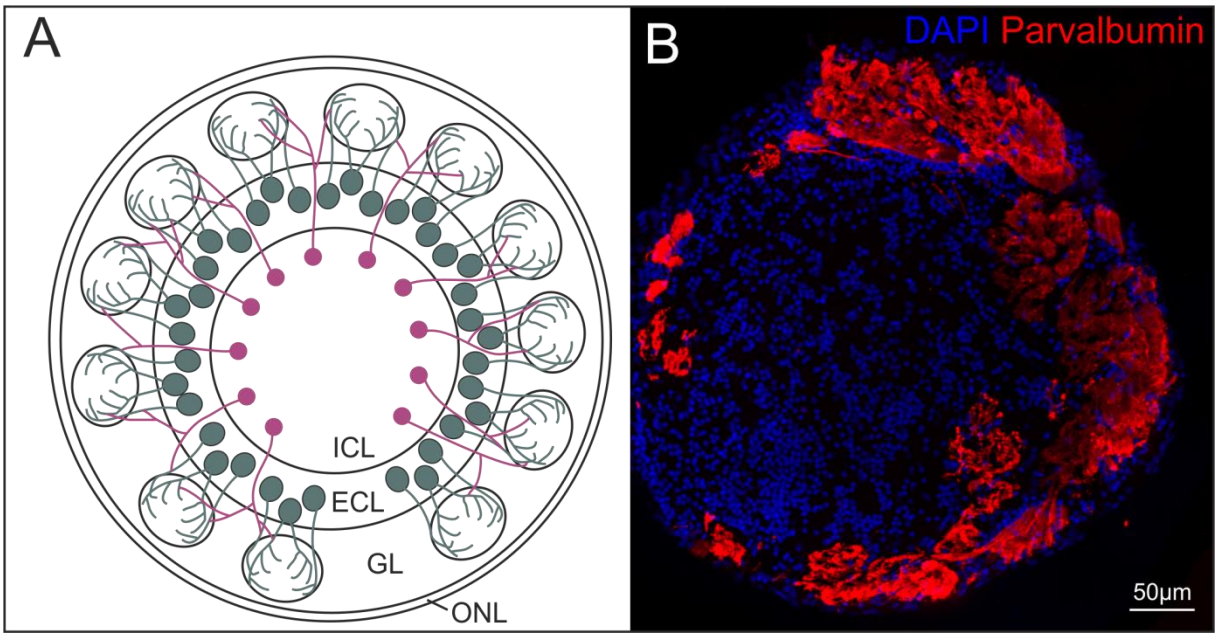


Figure 4 The zebrafish olfactory bulb (OB). **(A)** Schematic representation of arrangement of the four OB layers in the zebrafish. Olfactory sensory neurons (OSNs) project via the olfactory nerve into the OB thereby forming the primary olfactory nerve layer (ONL). The different types of OSNs target their axon to distinct glomeruli within the glomerular layer (GL). The external cell layer (ECL) contains mitral cells (glaucous) and ruffed cells (not shown), the output cells of the OB. Mitral cells make synaptic contacts onto OSN axon endings within glomeruli. The internal cell layer (ICL) contains cell bodies of granules cells (purple) and other inhibitory interneurons which modulate the activity of mitral cells and ruffed cells. **(B)** Cross section of an adult zebrafish OB stained with the Calcium binding protein Parvalbumin (red) and DAPI (blue) for nuclear stain. Parvalbumin visualizes axonal projections of different OSN types into different glomeruli of the ventral, dorsal and lateral OB. *Adapted from (Kermen et al. 2013).*

As mentioned above, a chemotopic map is represented by OSN subtypes expressing the same receptor and projecting their axon into the same glomerulus. Furthermore, glomeruli activated by similar molecular features are organized in defined zones. Because an odor is composed of different odorants, different odorant receptors are activated upon odor stimulation, therefore resulting in activation of spatially distributed glomerular domains (Friedrich and Korsching 1997, 1998, Fuss and Korsching 2001). In the zebrafish, large glomerular domains encode first-order chemical features such as the molecular category of an odorant. Furthermore, second-order-features like molecular structure (e.g. chain-length and branching) is encoded by local differences in activity pattern of cells within a glomerular domain (Friedrich and Korsching 1997, 1998, Fuss and Korsching 2001). Moreover, studies in zebrafish suggest that mitral cell firing carries multiplexed information about an odorant. Therefore, action potentials (APs) provide information about the odor category whereas the remaining mitral cell activity encodes the precise identity of a given odorant (Friedrich and Laurent 2004, Yaksi et al. 2007). Upon odor stimulation, activated OSNs trigger mitral cell firing, whereas spontaneous ruffed cell activity is inhibited by granule cells which are activated by mitral cells (Zippel et al. 1999).

Olfactory information is mediated via mitral cell axons which run through the medial and lateral olfactory tracts to different higher brain centers. Both tracts are separate and anatomically well definable axon tracts. **The medial olfactory tract (MOT)** contains mainly fibers originating from the medial OB whereas **the lateral olfactory tract (LOT)** is comprised mainly of fibers of mitral cells located in the lateral OB (Sheldon 1912). Tracing studies in various teleosts have shown that both tracts are subdivided into medial and lateral regions (Sheldon 1912, Finger 1975, Bass 1981, von Bartheld et al. 1984). The medial part of the MOT (mMOT) as well as the LOT contains mitral cell fibers projecting to the telencephalon and diencephalon. Moreover, some mitral cell fibers of the mMOT also project to the contralateral OB, thus connecting both OBs (von Bartheld et al. 1984). The lateral part of the medial olfactory tract (lMOT) conveys synaptic input into the OB by centrifugal fibers originating from the telencephalon which are supposed to synapse with granule cells, thus providing a cortical feedback in order to modulate bulbar processing (Munz et al. 1982, Stell et al. 1984, Zucker and Dowling 1987, Kermen et al. 2013).

A lot of work in different teleost species gained insights in the physiological role of bulbar efferent neurons and their projection targets of the two tracts. Tracing studies showed that LOT fibers specifically innervate the habenula (Hb) whereas MOT fibers also project to the ventral nucleus of the ventral telencephalon (Vv) and the posterior zone of the dorsal

telencephalon (Dp). The mammalian primary olfactory cortex (pyriform cortex) is believed to correspond to Dp in teleosts whereas Vv, a subpallial region, is linked to the septal area in mammals (Meek and Nieuwenhuys 1998, Kermen et al. 2013). Additionally, studies in zebrafish showed that mitral cell fibers which run through the MOT project specifically to the right dorsal habenula (dHb), the postcommissural and supracommissural ventral telencephalic nuclei (Vp; Vs, respectively), the intermediate nucleus of the ventral telencephalon (Vi) as well as to the hypothalamus in the diencephalon (Levine and Dethier 1985, Rink and Wullimann 2004, Miyasaka et al. 2009, Turner et al. 2016, Biechl et al. 2017). Presently, whether a chemotopic map, as present in the OB, is maintained in OB targets such as Dp is unknown. However, single cell recordings in the channel catfish indicate a chemotopical telencephalic organization thus showing that distinct pallial areas respond highly specific to distinct odorant classes (Nikonov and Caprio 2007, Kermen et al. 2013). On the other hand, studies in zebrafish could not provide evidence for a chemotopical organization in the telencephalon. Yaksi and colleagues showed that Dp and Vv neurons respond in an overlapping manner to various odorant classes, which is in line with studies in rodents, where a spatially segregated activity is absent in the primary olfactory cortex following odor stimulation (Stettler and Axel 2009, Yaksi et al. 2009). As aforementioned, projection neurons from the OB also send axons into distinct regions within the **diencephalon**. Due to their connectivity and functional heterogeneity, the teleost **habenula (Hb)** is divided in dorsal and ventral parts, both homologous to the mammalian medial and lateral habenulae, respectively (Amo et al. 2010). The ventral habenula (vHb) projects to serotonergic raphe nuclei and the dopaminergic neurons containing ventral tegmental area, thus the vHb is shown to be involved in control of motor behavior, motivation and reward-error prediction (Matsumoto and Hikosaka 2007, Stamatakis and Stuber 2012). Furthermore, the dorsal habenula (dHb) is subdivided into medial (dHb_M) and lateral (dHb_L) domains, and each domain expresses a distinct type of neurotransmitter, receives a distinct input and is involved in a distinct pathway to the interpeduncular nucleus of the brainstem (Agetsuma et al. 2010, Krishnan et al. 2014). Studies in the dHb indicate a possible role for the dHb_M to be involved in experience-dependent modulation of fear responses (Agetsuma et al. 2010).

1.3. Odorants sensed by fish: Representation and behavior

Three fundamental processes are essential for survival of every organism - reproduction, feeding and predator avoidance. Additionally, social interactions and migration are of great importance for many species as well. All of these tasks can be mediated by different classes of molecules via the olfactory system. In contrast to tetrapod olfaction, fish olfaction has to overcome the task that odorants are suspended in their aquatic environment (see 1.2.). How do fish discriminate between these complex mixtures of odorants and how do they know what behavior is suitable at this moment? Like the odorous environment, the mechanisms which determine the resulting behavior patterns are highly complex.

There are four major classes of odorants which are known to be detected by fish. **Amino acids** are the prototypical food odorant class and vary in their structure as well as in their olfactory potency (Caprio and Byrd 1984, Valentincic et al. 2000, Nikonov and Caprio 2007). In several teleost species, **amino acids** and **nucleotides** are shown to signal the presence and the quality of food, with detection thresholds of 10^{-6} to 10^{-8} M and around 10^{-6} respectively (Sutterlin and Sutterlin 1971, Suzuki and Tucker 1971). Nucleotides, like adenosine- and inosinetriphosphate (ATP and ITP) reach detection limits from feeding behavior and are characterized by arousal and appetitive swimming behavior by increasing swimming speed with many turns (Valentincic and Caprio 1997, Lindsay and Vogt 2004). Another class of food related odorants are **polyamines**. Cadaverine, putrescine and spermine are biogenic polyamines which are mainly released by decaying tissue. Cadaverine as well as putrescine is a very attractive odor for necrophagous animals such as rat or goldfish whereas this death-associated odor strongly elicits an avoidance response in zebrafish (Heale et al. 1996, Michel et al. 2003, Rolen et al. 2003, Hussain et al. 2013). Although amino acids mainly signal the presence of food in many teleosts, recent electrophysiological and behavioral studies demonstrated amino acids to be involved in behaviors other than feeding. Studies in salmon for example observed that combinations of amino acids dissolved in their natal stream water participate in their homing behavior (Shoji et al. 2000, Yamamoto and Ueda 2009). Additionally, there is evidence that amino acids are involved in reproductive behavior. Thus, polar amino acids are shown to increase attraction of conspecific males by acting as co-factors of the fish reproductive pheromone prostaglandin F2α ($PGF_{2\alpha}$) in the common carp (*Cyprinus carpio*) (Lim and Sorensen 2011). It was shown in salmon that the tryptophan metabolite, L-Kynurenine, which is released by females with their urine into the water initiates sexual behavior in conspecific males (Yambe et al. 2006). However, while

amino acids are shown to be involved in many fish behaviors, such as feeding, reproduction, kin recognition, migration and predator avoidance, the roles for F-series **prostaglandins** (PGFs) are limited only to reproduction within all teleosts so far examined (Kitamura et al. 1994, Sveinsson and Hara 1995, Moore 1996, Laberge and Hara 2001). Besides PGFs, **steroids** play a major role as pheromones by affecting the endocrine status as well as behaviors involved in reproduction in some teleost fish (Sorensen and Scott 1994). Thus, steroids regulate oocyte maturation and ovulation in female goldfish and are released via the urine into the surrounding water to induce sperm production und reproductive behavior in conspecific males (DeFraipont and Sorensen 1993, Poling et al. 2001, Kobayashi et al. 2002). Furthermore, male goldfish release the steroid adrostenedione (AD) and testosterone into the water to induce ovulation in female conspecifics and also increases aggressive behavior in other male goldfish (Sorensen et al. 2005b). Many studies have shown that the olfactory system of goldfish and other numerous teleosts is highly sensitive to sex pheromones like PGF, steroids and testosterone with detection thresholds in a picomolar range (Sorensen and Scott 1994, Stacey and Sorensen 2009). Another class of odorants with importance for social interactions and behaviors between conspecifics are **bile salts** (Doving et al. 1980, Hara 1994). Like, pheromones as mentioned before, bile salts are mainly released into the surrounding water through urine and feces (Zhang et al. 2001). The function of bile salt olfaction in fish is not well understood but electroolfactogram (EOG) recordings revealed that numerous teleost species are highly sensitive to various mixtures of bile salts with detection thresholds of even diluted bile salts in nanomolar concentrations in the lake char (Giaquinto and Hara 2008, Bazaes et al. 2013). Moreover, studies on bile salt olfaction proposed the ability of the olfactory system to distinguish between free and conjugated bile salts at the level of olfactory receptors in lake char as well as in zebrafish (Michel and Derbidge 1997, Zhang and Hara 2009). Bile salts released by pacific salmon and absorbed by rocks and organic material within their home stream have been implicated to be used by the olfactory system to guide returning salmon home (Doving et al. 1980). Additionally, bile salts also may be involved in migration to spawning sites in sea lampreys (Sorensen et al. 2005a). However, recent findings indicate bile acids to be involved in species-specific interactions as they reveal highly similar bile salt profiles within species of the same family and order irrespective of their diet (Hagey et al. 2010). Another example for a social odorant is **skin extract**. Skin extract, liberated for example through injury or parasite infestation signals imminent danger and triggers a specific and stereotyped alarm or flight reaction characterized by rapid swimming, grouping, freezing or hiding in numerous species tested (Frisch 1938, Speedie and

Gerlai 2008, Doving and Lastein 2009). It was thought that club cells, abundant in the epidermis of fish, excrete the alarm substance or "Schreckstoff" which is in charge for the alarm reaction in conspecifics (Brown and Smith 1998, Poulin et al. 1999, Speedie and Gerlai 2008). However, whether club cells release the alarm substance is not clear to date since they are shown to be involved in healing mechanisms of fish skin upon injury. The ingredients and its molecular identity of the alarm substance is not known yet, but recent findings identified chondroitin fragments secreted from shaken zebrafish skin which triggers alarm response in conspecifics (Mathuru et al. 2012). Although conspecific skin extract is a potent odorant for teleosts with response thresholds up to a dilution of $1{:}10^{-6}$ in crucian carp (Hamdani el and Doving 2003), the exact molecular and neuronal mechanisms which mediate this specific olfactory driven behavior is currently unknown.

1.3.1. Odorant tuning of the zebrafish olfactory system

The teleost olfactory system is capable to distinguish between vast numbers of odorants *(for an overview of odorants sensed by fish see 1.3.).* How such large varieties of stimuli are encoded and evoke a wide range of behaviors is an intriguing issue. Especially the study of detection of odorants from the initial event at the level of the olfactory epithelium (OE) upon processing the olfactory information in the olfactory bulb (OB) and higher order neuronal circuits involved in execution and modulation of different behaviors is of special interest in the field of neuroscience.

According to the one receptor - one neuron rule, all vertebrate olfactory sensory neurons (OSNs) express only one receptor (Sato et al. 2007). As a consequence, a given odor activates a set of different receptors and OSNs convey the olfactory information to respective glomerular fields, forming a chemotopic map accordingly to the one receptor – one glomerulus rule (Friedrich and Korsching 1997, 1998) (see 1.2.2.). Many studies in various teleost species using different methods, provided great insight into the repertoire of odorants that bind to olfactory receptors and therefore to the different types of OSNs. However, very little is known about distinct ligands or an olfactory code. In addition, the response profiles or tuning of OSNs towards different classes of odorants is somewhat ambiguous across teleost species. Early studies in salmonids concluded that microvillous OSNs (mOSNs) are tuned towards amino acids whereas ciliated OSNs (cOSNs) detect bile salts (Thommesen 1983). Later studies in different species using patch clamp and calcium imaging allowed more insight into response profiles of OSNs (even though single OSN types are very difficult to

identify) and concluded that all OSN types respond to amino acids and bile salts without featuring any sharp odorant tuning (Lipschitz and Michel 2002, Schmachtenberg and Bacigalupo 2004, Bazaes and Schmachtenberg 2012, Meredith et al. 2012). Analyzing glomerular fields in which the different types of OSNs project to, Hansen et al. (2003) concluded in the channel catfish, using EOG recordings and pharmacology, that cOSNs are tuned mainly towards bile salts but also respond to amino acids whereas mOSNs preferentially respond to amino acids and nucleotides. In rainbow trout, Sato and Suzuki (2001) revealed with whole cell patch recordings that cOSNs respond to a wide variety of odorants, acting as "generalists" whereas mOSNs respond as "specialists" mainly to amino acids.

The role of crypt cells remained elusive since their discovery. At first crypt cells were supposed to be involved in pheromone detection since their amount varies with seasonal changes. Most evidence for crypt cells involved in reproduction comes from a study in the crucian carp in which the number of crypt cells reaches its maximum during spawning season (Hamdani el et al. 2008). Most crypt cells show responses to reproductive pheromones of the opposite sex in mature trout whereas crypt cells in juvenile specimens are tuned to various odorants, indicating a specialization in their response profile while attaining sexual maturity (Bazaes and Schmachtenberg 2012).

In goldfish, it was shown that cOSNs recovered faster than mOSNs after axotomy. Interestingly, olfactory nerve sectioned goldfish showed in behavioral tests sensitivity for food related odorants before responses to pheromones (Zippel et al. 1996). As mentioned above, various studies across many teleost species using different techniques revealed discordant results without making a general conclusion of the response profiles of the different types of olfactory sensory neurons.

In the zebrafish *Danio rerio,* the tuning of OSNs towards different classes of odorants is not resolved as well. Koide et al. (2009) indicated mOSNs to be involved in food odor related behavior. By expression of the tetanus neurotoxin in mOSNs they could abolish attractive behavioral responses to amino acids in transgenic zebrafish. In addition, an immunocytochemical analysis using the ion channel permeant probe agmatine (AGB) also showed a preference for amino acids by mOSNs in the zebrafish (Lipschitz and Michel 2002). Recently, Hussain et al. (2013) uncovered an olfactory receptor which binds with high affinity the polyamine cadaverine. They showed that cadaverine is a major product of zebrafish tissue decay and elicits a strong odor driven avoidance behavior in conspecifics. Furthermore, they stated that cadaverine binds with high affinity to the trace amine associated receptor 13c

(TAAR13c) expressed on a small population of cOSNs. So far, kappe neurons are only identified in zebrafish but it is conceivable to find this cell type in other teleosts as well (Ahuja et al. 2014). Little is known about the possible function of this cell type but it could be supposable that they play a role in detection of social odorants since they project into a mediodorsal glomerulus. Our own study using immunohistochemical methods investigated the differential activation pattern OSNs of zebrafish larvae in response to different odors (Biechl et al. 2016b). By stimulating zebrafish larvae with food- and conspecific odor we showed that both cOSNs and mOSNs respond to a food related odor (containing amino acids) whereas mOSNs also responded to conspecific (social) odor. Activation of OSNs was measured by an increase of the activity marker pERK (phosphorylated extracellular signal regulated kinase). Interestingly, crypt cells showed no activation to these given odors. Moreover, stimulation experiments with kin odor provided strong evidence for crypt cells to be involved in kin recognition in larval zebrafish (Biechl et al. 2017) (see 1.3.2). Zebrafish are able to distinguish behaviorally between kin and non-kin using olfactory cues of their kin (Gerlach and Lysiak 2006, Gerlach et al. 2008). However, the molecular nature of kin odor is not known yet but our studies clearly indicate a role of crypt cells to be involved in detection of a social and kin-related signal in the zebrafish *Danio rerio* (Biechl et al. 2016b, Biechl et al. 2017).

1.3.2. Olfactory imprinting & kin recognition in the zebrafish (Danio rerio)

Recognition of individuals or the discrimination of various categories of conspecifics, also termed **social recognition** is of great importance for living in a stable social group in which individuals may interact with each other or simply to differentiate between “friend and foe”. As a part of social recognition, **kin recognition** allows an individual to distinguish between genetically related conspecifics, therefore between kin and non-kin. The discrimination as well as the differential treatment of kin and non-kin provide the organism as well as its relatives benefits and are considered stable strategies in evolution. Hamilton (1964a, b) states that helping within kinship increases the success of reproduction and fitness of the individual itself. Parental investment, alarm calls or grooming are such social interactions which strengthen and may increase survival of a kinship. In addition, kin recognition also assists in choosing the appropriate mate, thus avoid inbreeding, and producing healthy offspring (Gerlach and Lysiak 2006, Milinski 2014). Kin discrimination and favouring close relatives has been shown in invertebrates (Breed et al. 1988), as well as numerous vertebrate species

such as fish (Russell et al. 2004, Gerlach and Lysiak 2006), frogs (Pfennig et al. 1993), birds (Pravosudova et al. 2001) and mammals (Mateo and Johnston 2003). Besides, kin recognition is also found in plants as they are shown to alter their root growth in the presence of kin or non-kin, probably via chemical cues released by the roots (Pfennig and Sherman 1995, Dudley and File 2007).

But how may an individual recognize its kin? One mechanism, **familiarity**, is suggested to be used to recognize possible relatives by social learning. Familiarity means that an organism is associated with family members early in life and remembers specific circumstances, such as a location (e.g. nest or lair) or characteristics of its conspecifics. This memory is used later to recognize possible relatives. Importantly, familiarity does not constrain genetic relatedness, as cross-fostering experiments in mice show that non-kin pups which are raised together treat themselves like they were full siblings later in life (Kareem and Barnard 1982, Tang-Martinez 2001). However, an experiment in which lambs were separated at birth, but recognized their twins as kin, shows evidence that there must be another mechanism of kin recognition based on their phenotype (Ligout and Porter 2003). Such a mechanism is termed **phenotype matching**. In this case, young individuals learn cues, such as odor (Mann et al. 2003), acoustic signals or appearance (Cooke et al. 1972, Hauber et al. 2000) of conspecifics and of itself and generate a kin template. This template is used later in life and compared to putative kin (Holmes 1986, Mateo and Johnston 2003). However, both mechanisms are often used by the same individual to discriminate between kin and non-kin. Importantly, such life-long memory only occurs if appropriate cues are presented within a critical developmental period. Such form of phase sensitive learning is termed **imprinting**. One of the first and best known examples of imprinting is reported in most nidifugous birds, such as chicken, ducks and geese. Konrad Lorenz (1937) reported that newly born goslings imprint on the first object they see and follow them around. This behavior resulting from visual imprinting persists even into adulthood. Imprinting clearly differs from other forms of learning and memory as it is rapid, robust, limited to a small time window and independent of resulting behaviors. Furthermore, imprinting on parental appearance, such as plumage color influences preferences in mate choice in the lesser snow goose (Cooke et al. 1972). Acoustic stimuli have been shown to improve visual imprinting when presented prior the visual stimulus. Auditory together with visual cues play an important role for creating a strong mother – offspring bond in many avian species. Newly hatched chicks imprint on maternal feeding and distressing calls and learn associated behaviors which increases growth and chances of survival (Tefera 2012).

Several studies in various species reported imprinting based on olfactory cues. **Olfactory imprinting** is not restricted to a critical phase in juvenile specimen as two of the most prominent olfactory imprinting paradigms involve adult vertebrates. The so called `pregnancy block´ or `Bruce effect´ was first noted by Hilda M. Bruce (1959). Female mice imprint on urinary odors released by the mating partner during or shortly after mating. If the female is exposed to the male olfactory cues (pheromones) during the critical period, which is 4 hours after mating, the female forms a long term olfactory memory of this individual male odor. Interestingly, exposure to pheromones from other males, for which the female has formed no olfactory memory, will activate neuroendocrine pathways which terminate the female´s pregnancy (Kaba et al. 1989, Brennan et al. 1990). MHC (Major Histocompabitility Complex) class I molecules, contained in the male´s urine, are detected by the female vomeronasal organ (VNO) which transmits the odorant information, specific to the male, to the accessory olfactory bulb (AOB). Repeated exposure of this learned male odor activates noradrenalin release in the AOB and decreases the receptivity to this odor and pregnancy will continue (Zufall and Leinders-Zufall 2007, Becker and Hurst 2008). Another prominent example of olfactory imprinting is known from sheep. The ewe becomes sensitive to the smell of amniotic fluid during parturition. Within this short, about 2 hours' time window while giving birth, an exclusive bond between mother and lamb is established. Importantly, pregnant ewes show no reaction to new born lambs. Moreover, accordingly to this imprinting definition, ewes which are not exposed to their own lamb within this sensitive period will fail to develop this mother – lamb bond and will reject its offspring (Poindron et al. 1988, Poindron et al. 1993). In the European rabbit (*Oryctolagus cuniculus*), it was shown that pups imprint in utero on chemical cues associated on the mothers nutrition and show preference for this food later in life (Bilkó et al. 1994).

Olfactory imprinting is also reported in several teleosts and is widely accepted to explain natal homing behavior in salmonids (Cooper and Hasler 1974, Cooper et al. 1976, Dittman et al. 1996, Bett and Hinch 2015). Juvenile salmonids are anadromous because they imprint on olfactory cues of their natal stream, migrate to the sea until adulthood and return for spawning guided by the olfactory memory formed early in life (Dittman et al. 1996, Hasler and Scholz 2012). The so called parr-smolt transformation (PST) is the change from young salmon `parr´ (about one to four years old salmon) to `smolt´; a transformation which prepares the young salmon for its emigration to the sea. During the PST, the young animal undergoes several hormonal changes, such as a dramatic increase in thyroid hormone which is supposed to be crucial in the formation of olfactory memory (Dickhoff et al. 1978, Morin et

al. 1989b, a). Therefore, the PST is believed to be the critical period of olfactory imprinting even though the exact or precise point in time at which imprinting occurs is not identified yet (Bett and Hinch 2015). However, there are anadromous fish which do not return to their natal water for spawning. For example, the migration strategy of lamprey is considered as a `non-specific homing´, thus they migrate from the sea to a `general home area´ indicated by olfactory cues released by larval conspecifics (Waldman et al. 2008, Moser et al. 2015). However, most anadromous fish, such as salmonids use predominantly olfactory cues of their natal stream for homing and conspecific cues may play a secondary role for navigation (Bett and Hinch 2015). Another example of olfactory imprinting is shown in many coral reef fish. Reef fish larvae imprint on distinct odor cues from their settlement reef and prefer this odor instead of water from a nearby reef, thus limiting dispersal of reef populations through ocean currents (Atema et al. 2002, Lecchini et al. 2005, Gerlach et al. 2007a). It is supposed that odor cues released by island vegetation might assist reef fish larvae to navigate and find appropriate settlement habitat (Dixson et al. 2008).

In the zebrafish *Danio rerio*, it was shown that visual and olfactory cues are essential to create a kin template which is used to discriminate between kin and non-kin (Gerlach and Lysiak 2006). At larval stages, zebrafish preferences for conspecific odor increases with relatedness. Grouping with kin is shown to increase growth of juvenile zebrafish, whereas zebrafish grouped with unrelated conspecifics showed retarded growth (Gerlach et al. 2007b). Consequently, staying with kin provides benefits such as earlier fertility by accelerated growth and better chances to survive. However, by attaining fertility, zebrafish avoid kin odor, most possibly for preventing inbreeding. In an effort to unravel the critical time window in which zebrafish larvae imprint on their kin, Gerlach and colleagues (2008) reported by cross fostering and behavioural tests that larvae imprint on day 6 post fertilization (dpf) on olfactory cues of their full siblings. Interestingly, zebrafish raised with non-kin do not imprint on non-kin cues nor do they show preference for non-kin odor in behavioural tests. Moreover, if exposed to kin odor on developmental days other than day 6, zebrafish larvae failed to imprint and therefore were unable to distinguish between kin and non-kin. Later, this imprinting paradigm was expanded by adding an essential additional cue involved in zebrafish imprinting. Hinz et al. (2013a) reported that zebrafish larvae raised in semitranslucent glass beakers, thus unable to identify more than the silhouette of surrounding kin, and zebrafish only exposed to olfactory cues of kin did not lead to successful imprinting. Thus, surprisingly, larvae which are not exposed to visual kin cues on day 5 failed to imprint even if exposed to kin odor on 6 dpf. Therefore, imprinting of zebrafish larvae on their kin

involves two sensory cues, vision and olfaction, and both cues have to be presented on appropriate developmental days which are day 5 and day 6, respectively (Hinz et al. 2013a). These findings raise the question why zebrafish larvae only imprint on visual and olfactory cues of their kin and not on non-kin cues, suggesting a genetic predisposition for kin related signatures. By comparing the pigment pattern of several zebrafish families with Major Histocompability Complex (MHC) class genotype similarity, it was shown that iris pigmentation and morphometry of zebrafish larvae relies on MHC class II alleles (Hinz et al. 2012). MHC class II genes, especially MHC peptides were also shown to be involved as a kin related signal in olfactory imprinting in zebrafish (Hinz et al. 2013b). Olfactory choice tests of 6dpf old zebrafish larvae indicate that MHC class II allele similarity is correlated with imprinting on kin. Moreover, MHC class II peptides are sufficient to evoke neuronal activity in the zebrafish olfactory bulb partially overlapping with responses to kin odor shown by calcium imaging (Hinz et al. 2013b). However, MHC class II peptides may serve as species identity cues involved in the process of olfactory imprinting as well as kin recognition later in life, anyway the kin odor itself consists likely of more components than MHC peptides.

Recently, our own studies using a common neuronal activity marker (**pERK**; see next paragraph), showed strong evidence for crypt cells together with a small subpopulation of mOSNs to detect a kin odor specific signal in larval zebrafish. Importantly, only crypt cells of imprinted larvae showed neuronal activation in response to kin odor containing water, suggesting changes already at the level of the olfactory epithelium, especially at the level of receptor expression due to olfactory imprinting (Biechl et al. 2016b, Biechl et al. 2017).

Several methods are available to detect neuronal activity in the peripheral as well as the central nervous system, however, all of them with pros and cons. The extracellular signal regulated kinase (ERK) is a member of the mitogen activated protein kinase (MAPK) family; protein kinases are involved in cell communication in response to different stimuli. ERK is catalytically inactive but upon cell activation, ERK gets activated by phosphorylation by its upstream kinase MEK (ERK kinase). The MAPK signalling pathways are involved in regulation of several cell functions such as proliferation, gene expression, cell survival and apoptosis in response of diverse stimuli. Phosphorylation dependent activation events might be triggered by osmotic stress, heat shock, mitogens or other ligands (e.g. transmitter signalling molecules) which bind on receptors expressed on the cell membrane (Widmann et al. 1999). Such receptors might be G-protein coupled receptors (GPCRs) such as olfactory receptors expressed on OSNs. Upon phosphorylation, phosphorylated ERK (pERK) is translocated into the nucleus of the activated cell to activate transcription factors such as

immediate early genes (e.g. *egr-1* or *c-fos*) which in turn regulate gene expression involved in neuronal and synaptic plasticity underlying learning and memory (Figure 5) (Gao and Ji 2009). pERK is a widely accepted marker for neuronal activity (Randlett et al. 2015) and also used in the field of olfaction because of its rapid activation followed (olfactory) stimulation (Miwa and Storm 2005, Biechl et al. 2016b, Biechl et al. 2017).

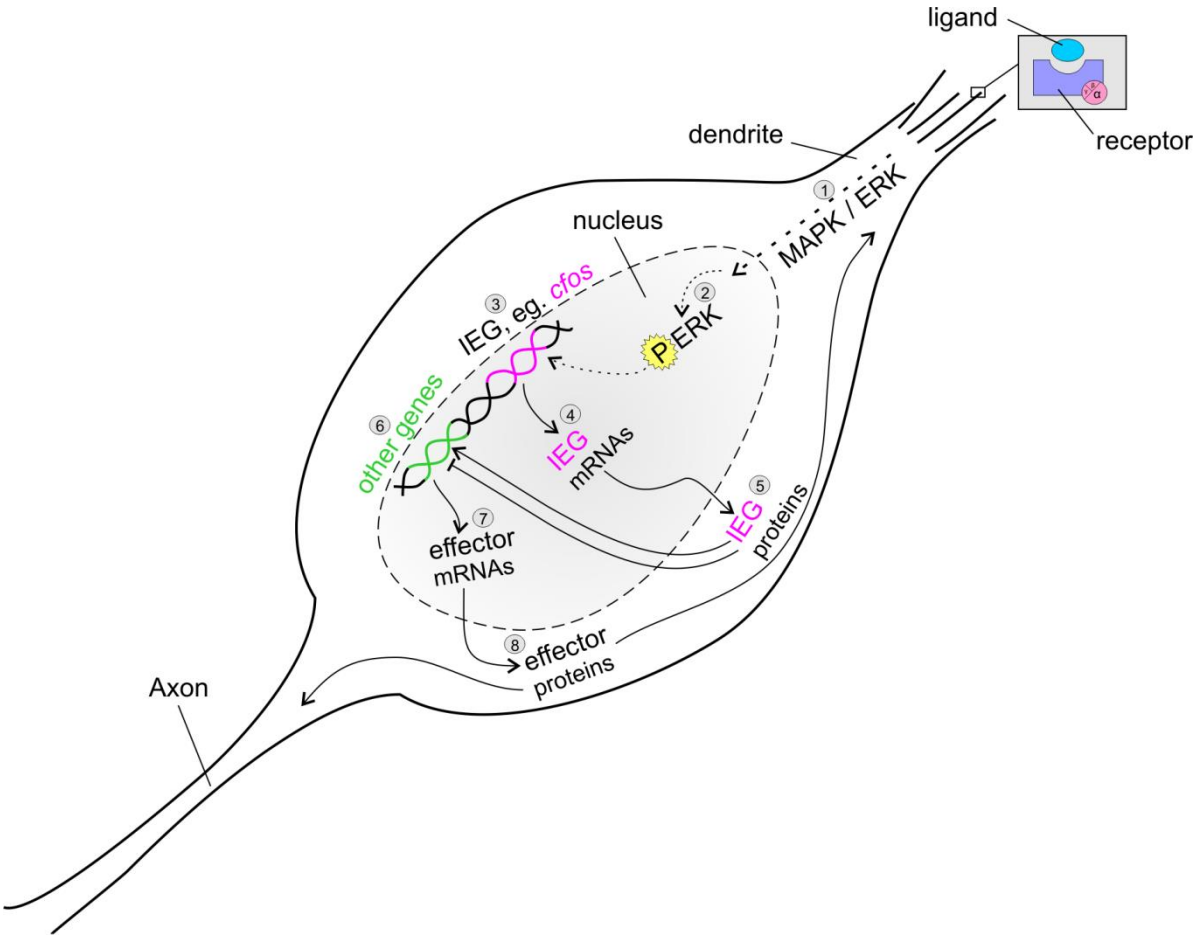


Figure 5 Activation of an olfactory sensory neuron. **(1)** Binding of a ligand to its receptor leads to a cascade of phosphorylation events which finally leads to the **(2)** phosphorylation of the extracellular signal regulated kinase (pERK). pERK translocates into the nucleus and **(3)** promotes expression of immediate early genes (IEGs) such as *cfos*. **(4)** IEG messenger RNA (mRNA) translocates out of the nucleus where it is translated into IEG proteins **(5)** IEG proteins often act as transkription factors and return into the nucleus and promote or inhibit **(6)** the expression of other genes. **(7)** Effector mRNAs are translated to **(8)** effector proteins which lead to intracellular adaptions (synaptic plasticity). *For references see text.*

2. AIM OF THE STUDY

The formation of long lasting olfactory memories as resulting from olfactory imprinting is conserved across animals, in invertebrates as well as in vertebrates (see examples above); however, the underlying mechanisms are poorly understood. Previous work in zebrafish gained much insight into the processes involved in visual and olfactory imprinting resulting in kin recognition later in life (see 1.3.2).

The zebrafish, *Danio rerio*, is widely used in developmental and neurobiological studies. As a model organism, the zebrafish is advantageous for its small size, low cost and rapid reproduction. To study the mechanisms involved in olfactory imprinting, the zebrafish suits especially because of its easy experimental manipulation of breeding, for instance in isolation or kin groups. Additionally to these handling benefits, several well established immunohistochemical methods, involving many primary antibodies, are available to study underlying mechanisms on the neuronal basis of olfactory imprinting in the zebrafish, *Danio rerio*. In addition, although teleosts, such as the zebrafish, lack a separate vomeronasal organ (VNO), receptor expression displays a high degree of molecular conservation between zebrafish and mouse olfactory system (Saraiva et al. 2015).

Based on what is known so far, the aim of this thesis is to obtain more insights into neuronal mechanisms involved in olfactory imprinting, starting at the level of the olfactory epithelium (OE), followed by the level of first odor processing, the olfactory bulb (OB) and finally showing evidence of OB targets involved in this special form of olfactory driven long-term memory.

(1) The first aim of my study is to provide more insights into different subpopulations of zebrafish olfactory sensory neurons (OSNs) characterized with calcium binding proteins (CBPs) in adult and larval zebrafish. In a comprehensive combinatorial immunohistochemical study, I demonstrate different expression patterns of Calbindin, Calretinin, Parvalbumin and S100 in OSNs as well as their differential bulbar targets with special emphasis of filling gaps in the knowledge of OE projections to the dorsomedial olfactory bulb (Kress et al. 2015).

(2) Odor detection occurs at the level of the olfactory epithelium (OE). Olfactory receptors expressed on four distinct types of olfactory sensory neurons (OSNs) bind different odorants with variable affinity (see 1.2.1). The ability to discriminate between thousands of different odorants relies on differential activation of these receptors expressed on OSNs. So far, little is known on odorant specification of the four types of OSNs in the zebrafish, *Danio rerio*. To identify the type or types of OSNs which are activated in response to different odor

exposures, I choose the neuronal activity marker pERK (phosphorylated extracellular signal regulated kinase; see above). Additionally, I validate the expression of the neuronal activity marker pERK in the larval zebrafish OE in response to different odors as well as different odor exposure durations. With the use of accepted immunohistochemical markers (antibodies) as well as morphological characteristics of the four zebrafish OSNs (described in 1.2.1), I demonstrate pERK as a reliable marker for spatial activation of OSNs after odor stimulation in the larval zebrafish. Moreover, with the knowledge of the exact time point of olfactory imprinting, I stimulated imprinted as well as non-imprinted larvae with kin odor and analysed the different response profiles of the four distinct types of zebrafish OSNs (Biechl et al. 2016b).

(3) The final aim of my study is to describe for the first time an accessory olfactory system, as present in mammals, in the zebrafish, *Danio rerio*. By application of DiI tracer into the medial OB or tuberal hypothalamus, I show secondary olfactory projections of the zebrafish OB into distinct telencephalic areas. Furthermore, I describe the presence of a teleostean medial amygdala characterized by Otpa positivity and receiving olfactory input from dorsomedial OB glomerular fields. Moreover, I present evidence for the teleostean medial amygdala to be involved in kin recognition in the larval zebrafish by analyzing neuronal activity in the medial OB and intermediate ventral telencephalic nucleus (medial amygdala) in response to kin odor exposure (Biechl et al. 2017).

3. RESULTS

3.1. Kress, S., D. Biechl and M. F. Wullimann (2015). "Combinatorial analysis of calcium-binding proteins in larval and adult zebrafish primary olfactory system identifies differential olfactory bulb glomerular projection fields." Brain Struct Funct 220(4): 1951-1970.

Contributions:

The study was designed by SK, DB and MFW. The immunohistochemical processing was performed by SK and DB. Analysis of data was done by SK, DB and MFW. The first version of the manuscript was written by SK, DB and MFW. The final version was written by SK, DB and MFW. SK and DB share first authorship.

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ORIGINAL ARTICLE

Combinatorial analysis of calcium-binding proteins in larval and adult zebrafish primary olfactory system identifies differential olfactory bulb glomerular projection fields

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Abstract In the zebrafish (*Danio rerio*) olfactory epithelium, the calcium-binding proteins (CBPs) calretinin and S100/S100-like protein are mainly expressed in ciliated or crypt olfactory sensory neurons (OSNs), respectively. In contrast parvalbumin and calbindin1 have not been investigated. We present a combinatorial immunohistological analysis of all four CBPs, including their expression in OSNs and their axonal projections to the olfactory bulb in larval and adult zebrafish. A major expression of calretinin and S100 in ciliated and crypt cells, respectively, with some expression of S100 in microvillous cells is confirmed. Parvalbumin and calbindin1 are strongly expressed in ciliated and microvillous cells, but not in crypt cells. Moreover, detailed combinatorial double-label experiments indicate that there are eight subpopulations of zebrafish OSNs: S100-positive crypt cells (negative for all other three CBPs), parvalbumin only, S100 and parvalbumin, parvalbumin and calbindin1, and parvalbumin and calbindin1 and calretinin-positive microvillous OSNs, as well as a major parvalbumin and calbindin1 and calretinin, and minor parvalbumin and calbindin1 and calretinin-only-positive ciliated OSN populations. CBP-positive projections to olfactory bulb are consistent with previous reports of ciliated OSNs projecting to dorsal and ventromedial glomerular fields and microvillous OSNs to ventrolateral glomerular fields. We newly describe parvalbumin-positive fibers to the mediodorsal field which is calretinin free, with its anterior part showing additionally calbindin1-positive fibers, but absence thereof in the posterior part, indicating an origin from microvillous OSNs in both parts. One singular glomerulus (mdG2) exhibits S100 and parvalbumin-positive fibers, apparently originating from all crypt cells plus some microvillous OSNs. Arguments for various olfactory labeled lines are discussed.

Keywords Calbindin · Calcium-binding proteins · Calretinin · Parvalbumin · S100

Abbreviations

ac	Anterior commissure
c	Cilia
CB(ir)	Calbindin1 (immunoreactive)
CBP(s)	Calcium-binding protein(s)
CeP	Cerebellar plate (larvae)
Cr	Crypt OSN
CR(ir)	Calretinin (immunoreactive)
CZ	Central zone
D/d	Dendrite
Dp	Posterior zone of dorsal telencephalic area
DT	Dorsal thalamus (thalamus)
DZ	Dorsal zone
EmT	Eminentia thalami
gl, Gl	Glomerular layer of olfactory bulb
Glo	Glomeruli
GPCR	G-protein-coupled receptor
H	Hypothalamus
Ha	Habenula
Had	Dorsal habenular nucleus

S. Kress and D. Biechl share first authorship.

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Hav	Ventral habenular nucleus
icl	Internal cellular layer of olfactory bulb
lfb	Lateral forebrain bundle
LG1–4	Lateral glomeruli 1–4 (larvae)
LOT	Lateral olfactory tract
m	Microvilli
MC	Mitral cell
Mi	Microvillous OSN
MG1–4	Medial glomeruli 1–4 (larvae)
MOE	Main olfactory epithelium
MOS	Main olfactory system
MOT	Medial olfactory tract
N	Region of the nucleus of medial longitudinal fascicle
OB	Olfactory bulb
oc	Optic chiasma
OE	Olfactory epithelium
on	Optic nerve
ON	Olfactory nerve
OSN(s)	Olfactory sensory neuron(s)
OR	Olfactory receptor
P	Pallium
Po	Preoptic region
poc	Postoptic commissure
Pr	Pretectum (larvae)
PT	Posterior tuberculum
PV(ir)	Parvalbumin (immunoreactive)
S	Subpallium
T	Midbrain tegmentum
Tel	Telencephalon
TeO	Optic tectum
Va	Valvula cerebelli
Vs	Supracommissural nucleus of ventral telencephalic area
VT	Ventral thalamus (prethalamus)
Vv	Ventral nucleus of ventral telencephalic area

Abbreviations for identified glomeruli in adult zebrafish (adapted from Baier and Korsching 1994; Braubach et al. 2012)

a–e	Dorsal cluster-associated glomeruli 1–5
ap	Anterior plexus
dc	Glomeruli of the dorsal cluster
k	Medial elongated glomerulus
l	Glomerulus of the lateral chain 1
lc	Lateral chain
m	Glomerulus of the lateral chain 2
$mdG_{1–6}$	Mediodorsal posterior glomeruli 1–6
n	Glomerulus of the lateral chain 3
p	Glomerulus of the lateral chain 5
q	Lateroventral posterior glomerulus
u–w	Glomeruli of the ventral triplet 1–3
vpG	Ventroposterior glomerulus

Introduction

Calcium-binding proteins (CBPs) such as parvalbumin, calretinin, calbindin1 (calbindin D28k), and S100, have roles in intracellular signaling and/or buffering of calcium during neuronal activity (Rogers 1987; Bastianelli 2003; Cheron et al. 2008; Kraemer et al. 2008; Alpár et al. 2012). CBPs are often expressed selectively or in a combinatorial manner in subtypes of a given neuronal class and, thus, are useful to define neuronal subpopulations (Baimbridge et al. 1992), for example in GABAergic interneurons of the mammalian cortex (Hof et al. 1999; Zaitsev et al. 2005; Druga 2009; Barinka and Druga 2010), cerebellar cortex (Floris et al. 1994; Bastianelli 2003) or in brainstem auditory nuclei (Henkel and Brunso-Bechthold 1998; Bazwinsky et al. 2003). Another example are zebrafish primary olfactory sensory neurons (OSNs) which express calretinin predominantly in ciliated cells (Castro et al. 2006; Germana et al. 2007; Koide et al. 2009; Gayoso et al. 2011; Braubach et al. 2012) and S100 mainly in crypt (and in a subpopulation of microvillous) cells (Germana et al. 2004, 2007; Sato et al. 2005; Sandulescu et al. 2011; Oka et al. 2012; Braubach et al. 2012). This is of particular interest because teleosts—in contrast to tetrapods—lack a separate vomeronasal organ (VNO) in addition to a main olfactory epithelium (MOE; Eisthen 1997; 2004; Hansen et al. 2003; 2004). Nevertheless, teleosts do have ciliated olfactory sensory neurons (typical for tetrapod MOE) and microvillous sensory cells (typical for tetrapod VNO), plus a third category of so-called crypt cells only present in ray-finned (Hansen and Finger 2000; Vielma et al. 2008) and cartilaginous fishes (Ferrando et al. 2006). These three cell types occur in different depths of the main (and only) olfactory epithelium, with ciliated cell somata located basally, extending a long dendrite to the surface of the epithelium, whereas microvillous cells are usually situated in intermediate depths with shorter apical dendrites, and crypt cells lying superficially (Hansen et al. 2004). As the names imply, the deep cells have cilia and the intermediate cells microvilli protruding from their dendrites into the nasal cavity, whereas crypt cells have an indentation at their apical pole from which both microvilli and cilia extend towards the olfactory pit lumen. As similarly known from studies in mouse, also teleost ciliated OSNs carry olfactory receptor proteins (ORs) associated with the G-protein Gαolf (Hansen et al. 2004; Oka and Korsching 2011) as well as trace amine receptors (TAARs; Gloriam et al. 2005; Hussain et al. 2009); whereas, teleost microvillous OSNs express V2R receptor proteins (DeMaria et al. 2013) associated with Gαo and, likely, V1R receptor proteins (Pfister and Rodriguez 2005; Saraiva and Korsching 2007; Oka and Korsching 2011). Zebrafish crypt cells apparently express only one V1R type (ora4) receptor

gene and associate with Gαi (Oka and Korsching 2011; Oka et al. 2012).

Since only calretinin and S100 have been investigated in the zebrafish olfactory system, analysis of parvalbumin and calbindin1 might complement the recognition of subtypes of OSNs. Moreover, immunohistochemical visualization of CBPs does not only reveal the olfactory sensory cell somata, but in addition their axonal projections to the olfactory bulb glomeruli (Braubach et al. 2012), likely revealing differential projections patterns of OSNs defined by CBPs. Thus, we studied the expression of these four CBPs in OSN somata and their projection during zebrafish larval development and in adult brains in single and double-label immunohistochemical preparations.

Materials and methods

Animal maintenance and tissue preparation

Zebrafish were kept in mixed-sex groups of 5–15 animals in 8-l aquaria in Aquatic Habitats (Zeb TEC, Standalone Aquatic System, Tecniplast) at Ludwig Maximilians-Universität Munich (LMU). Animals were kept at 28 °C water temperature and a 14:10 h light/dark cycle and fed twice daily with standard fish food flakes. Animals used in this study were treated according to the German regulations on Animal Protection (Deutsches Tierschutzgesetz).

Adult zebrafish (total of 36) were deeply anesthetized in tricaine methanesulfonate (MS222; Sigma-Aldrich; 500 mg/l) in tank water. They lost control over balance after around 15 s, and did not react to tail pinching with forceps after around 40 s. The animals were then immediately killed by decapitation. Brains were exposed dorsally by removing the skull and fixed with 4 % paraformaldehyde (PFA) in Sörensen phosphate buffer at 4 °C overnight before being removed from the skull.

Larvae (total of 64) were killed with an overdose of MS222 and fixed with cold 4 % PFA overnight. Following cryoprotection in 30 % sucrose solution overnight, adult brains and whole larvae were embedded in Tissue-Tek (A. Hartenstein GmbH). Adult brains were cut into 30-µm cryosections for single-label immunohistological analyses of calcium-binding proteins (CBPs). For confocal microscopy of CBP and GFAP expression, olfactory bulbs and epithelia of adult zebrafish, as well as whole larvae, were cut into 14-µm sections on a freezing microtome.

Antibody characterization

Table 1 lists used primary (1st) antibodies and provides information on antigens, antibody description (incl. species in which the antibody was raised, manufacturer, catalog number), dilution used, Western blot information and specificity tests when available.

Various secondary (2nd) antibodies to determine the location of the 1st antibodies were used with specific corresponding conjugates for immunofluorescence. In Table 2, the different conjugates, antibody description (incl. species in which antibody was raised, manufacturer, catalog number) and dilution used is shown. Included are also Fab-fragments. For double-immunolabeling with two primary antibodies from the same host species (i.e., mouse

Table 1 Overview of 1st antibodies used in this study

Antigen	Antibody description	Dilution used	Western blots	Specificity tests (manufacturer)
Recombinant human calretinin	Rabbit polyclonal, SWANT, # 7699/3H	1:500	Zebrafish Brain: Manufacturer/present report (29–30 kDa)	No cross reaction with calbindin D-28k (=calbindin1). No staining in calretinin knock-out mouse cortex
Recombinant human calbindin D28 k (calbindin1) (aa 3-251)	Mouse monoclonal, SYSY, # 214011	1:200	Rat brain: Manufacturer Zebrafish brain: present report (ca 28 kDa)	Recognizes calbindin1 in human, rat and mouse neurons
Parvalbumin purified from frog muscle	Mouse monoclonal, Millipore, # MAB1572	1:2,000	Mouse/rat/human Brain: Manufacturer; Zebrafish brain: present report (12 kDa)	Recognizes parvalbumin in brain and muscle
S100 isolated from cow brain	Rabbit polyclonal, DAKO, # Z0311	1:400	Recombinant human S100B: strong label (Manufacturer); Zebrafish brain: present report (13 kDa)	Other S100 proteins not recognized in Western blots. Recognizes S100 in various mammalian brain tissue
GFAP purified from cow spinal cord	Rabbit polyclonal, DAKO, # Z 0334	1:500	Cow brain: strong label	Recognizes GFAP in cat, mouse, rat, dog, human and sheep

Table 2 Overview of 2nd antibodies used in this study

Conjugate	Antibody description	Dilution
Alexa 488	Donkey antimouse, Mol Probes, # A21202	1:200
Alexa 488	Donkey anti-rabbit, Mol Probes, # 21206	1:200
DyLight 488	Donkey anti-rabbit, dianova, # 711-486-152	1:400
Cy3	Donkey anti-rabbit, dianova, # 711-165-152	1:200
Cy3	Donkey anti-mouse, dianova, # 711-166-151	1:200
Cy3	Donkey anti goat, dianova, # 705-166-147	1:200
Alexa-488	Donkey anti-goat, Invitrogen, Mol Probes, # A-110-55	1:400
AffiniPure Fab-Fragment	Goat anti-mouse-IgG (H + L), dianova, # 115-007-003	1:100

antibodies against parvalbumin-calbindin1), Fab-fragments (Fab Fragment Goat Anti-Mouse IgG, dianova; see Table 2) were used. Sections were incubated with first primary antibody followed by incubation with Fab-fragments diluted in IS-block solution (1:100) overnight at 4 °C in order to bind to the host species IgG and present these primary binding sites as goat-Fab. Subsequently, sections were incubated with the secondary (actually tertiary) antibody directed against the host species of the Fab antibody overnight at 4 °C. The subsequent immunofluorescence procedure was as usual (see below).

As a control, we combined goat-anti mouse Fab fragments after the (first primary) parvalbumin antibody in co-stainings with ZebrinII antibodies (also raised in mouse) on zebrafish cerebellar tissue to show Purkinje cells and these are indeed double-labeled, because ZebrinII is a specific Purkinje cell marker. However, in corresponding stains of the olfactory epithelium, where no ZebrinII is present, this procedure revealed, as expected, only parvalbumin-positive sensory cells. Because ZebrinII is absent in the olfactory epithelium, neither the second primary antibody (ZebrinII), nor the second secondary antibody bind anywhere in the tissue and this demonstrates that no mouse antigen is recognized. Thus, this shows the reliability of the Fab fragments used.

Further, the extensive, but not ubiquitous, parvalbumin positivity in olfactory epithelium sensory cells prompted us to cross-confirm selectivity in OSNs by performing immunostainings of the zebrafish cerebellum (data not shown). Our cerebellar stains show parvalbumin positivity selectively in Purkinje cells—as confirmed by co-stainings with ZebrinII—next to solely calretinin-positive eurydendroid cells, and solely calbindin1–positive posterior lobe granule cells. This complete non-overlap of parvalbumin-positive Purkinje cells with the other two cerebellar cell types as defined by calcium-binding proteins has previously been shown by Bae et al. (2009). Thus, as we can repeat these data in the cerebellum, the partial overlap of calretinin, calbindin1 and parvalbumin observed in zebrafish olfactory sensory neurons can not be the result of antibody cross-reactivity.

Immunofluorescence

Incubations were done in a humid chamber. After washing off TissueTek in cryosections in PBS, endogenous peroxidase activity was first blocked with 0.3 % H_2O_2 in PBS for 30 min at room temperature (RT) and the sections were washed in PBT (PBS + 0.1 % Tween 20). The sections were then incubated with blocking buffer (2 % normal goat serum, 2 % bovine serum albumin, 0.2 % Tween 20, 0.2 % Triton X-100 in PBS) for 1 h at RT before exposition to a primary antibody diluted in blocking buffer at 4 °C for 1–3 days (dilution see Table 1). After washing, the sections were again incubated with blocking buffer for 1 h at RT before the secondary antibody (see Table 2) was applied at 4 °C overnight. In case of double-immunofluorescence, a second primary antibody was applied after washing in PBT and blocking. Subsequently, a corresponding secondary antibody diluted in blocking buffer was applied overnight, again after washing in PBT and blocking (see above for details). Finally, sections were washed in PBT and counterstained with DAPI (4′-6-diamidino-2-phenylindole; Carl Roth) for 3 min at RT and washed in PBT. Slides were then mounted with Vectashield (Vectorlabs) and coverslipped.

Western blot analysis

An adult wild-type zebrafish was anesthetized and killed as described above. After decapitation, the brain tissue was dissected and immediately transferred to RIPA lysis buffer (Sigma-Aldrich) followed by mechanical homogenization on ice. Sample was centrifuged for 20 min/1,000*g* at 4 °C. Afterwards, the supernatant was centrifuged for 20 min/10.000*g* at 4 °C. Supernatant containing proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane by electrophoretic transfer (250 mA/2 h).

Success of blot was checked with Ponceau solution (Sigma-Aldrich) following washing steps with dH_2O and blocked using 0.5 % milk powder (Roth) dissolved in TBST for 1 h at 4 °C in a humid chamber. Respective primary antibodies were diluted in 0.5 % milk powder

solution and incubated overnight at 4 °C. Afterwards, membrane was washed 3 × 20 min with TBST and incubated in secondary antibody diluted in 0.5 % milk solution for 2 h at RT. After 3 washes for 20 min each in TBST following a short wash with TBS, air-dried membranes were scanned using the Typhoon Imager (GE Healthcare) and analyzed with ImageJ. All four assays for CBPs showed one band in the expected molecular weight range (see Supplementary Figure 1) using always 10 and 20 µl protein extract at two antibody concentrations.

Photomicrography

Furthermore, the immunofluorescence of triple (two antibodies plus DAPI) labeled adult and larval zebrafish brain sections were captured with a Leica TCS SP-5 confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with Plan Fl25×/0.75 NA and Plan 63×/1.32 NA oil immersion objectives. Fluorochromes were visualized by using an argon laser with excitation wavelengths of 488 nm, emission 510–540 nm for Alexa 488 and a DPSS laser with a laser line of 561 nm, emission 565–600 nm for Cy3 and a diode laser with a laser line of 405 nm, emission 420–470 nm for DAPI. Stacks of eight-bit grayscale images were obtained with axial distances of 0.3–1 µm between optical sections and pixel sizes of 0.12–1.2 µm depending on the selected zoom factor and objective. After stack acquisition, Z chromatic shift between color channels was corrected. The RGB stacks, montages of RGB optical sections, and maximum-intensity projections were assembled into tables using ImageJ 1.37k plugins and Adobe Photoshop 8.0.1 (Adobe Systems, San Jose, CA) software. Some photomicrographs of sectioned adult olfactory epithelia were taken with a light microscope (Nikon Eclipse 80i; Nikon Instruments Inc.) equipped with Nikon Plan Fluor 10×/0.30 and Plan Fluor 20×/0.50, a Nikon Digital Sight DS-U1 Photomicrographic Camera (Nikon Instruments Inc.) and LUCIA-G5 software.

All microscopic images used in this study were slightly adapted for brightness and contrast with Adobe Photoshop 8.0.1 (Adobe Systems, San Jose, CA) software and photographic plates were mounted and further processed with CorelDraw 9.532 (Corel Corporation).

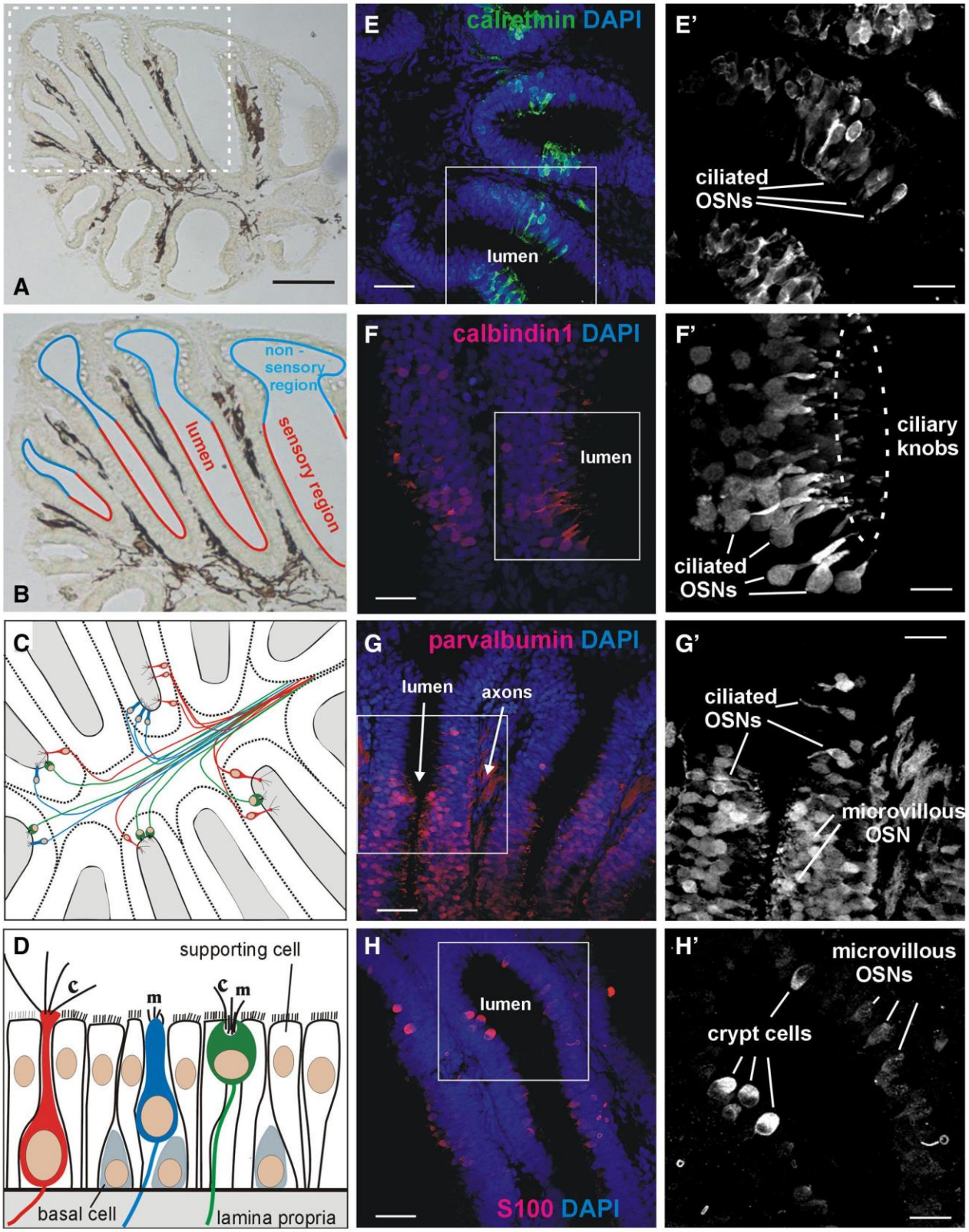
Results

Calcium-binding protein expression in adult zebrafish olfactory epithelium

The accepted criteria for identifying the three zebrafish olfactory sensory neuron (OSN) types are as follows: cell soma position (basal for ciliated cells, intermediate for microvillous cells, and superficial for crypt cells), cell soma shape (ciliated cells, stout with one long dendrite towards luminal surface; microvillous cells, somewhat elongated with basal and superficial dendrite; crypt cells, round with acentric nucleus, no dendrites, but superficial indentation), membranous protrusions (cilia in ciliated cells, microvilli in microvillous cells, both in crypt cells). This is schematically shown in Fig. 1c, d, although it should be kept in mind that the depth of microvillous cells within the epithelium may vary as to make individual cell identifications sometimes difficult. Overall, however, using these criteria, zebrafish olfactory epithelia immunolabeled for a single calcium-binding protein (CBP) nicely show the general picture of the relationship of CBPs with OSN cell type (Fig. 1e–h). While calretinin (CR; Fig. 1e, e′) and calbindin1 (CB; Fig. 1f, f′) are both ubiquitously expressed in ciliated OSNs, parvalbumin (PV) is present equally abundantly in ciliated and microvillous OSNs (Fig. 1g, g′). In contrast, S100 characterizes most if not all crypt cells with strong expression and a subpopulation of microvillous cells with weaker expression (Fig. 1h, h′). Especially in CB immunostains, the so-called ciliary knobs are clearly visualized (Fig. 1f′). As a rule, when observed in single-label visualization, the olfactory nerve fibers issued by labeled OSN somatas are also positive for any single CBP and, thus, olfactory bulb glomerular staining is to be expected in all cases (see below). These results are summarized in Table 3. Because of difficulties regarding the identity of the molecule recognized by the anti-S100 polyclonal antibody (see "Discussion"), we will refer in the following to S100-like immunopositivity.

In order to analyze co-localization of the four CBPs under investigation, double-labelings of each possible combination (plus DAPI as a nuclear background stain) of the four CBPs were generated (Figs. 2, 3). Sections of entire olfactory epithelia reveal CBP-positive OSNs within the inner sensory part (see Fig. 3c) of the U-shaped folds of the olfactory epithelial rosette and absence of immunostain in the outer non-sensory epithelium (Figs. 1a, b, 2, 3). Large mucus-producing goblet cells are present in the zebrafish non-sensory epithelium (Fig. 1a, b), as seen in goldfish (Hansen et al. 1999). Further, there is a conspicuous concentric ring of very dense immunoreactivity at the edge of the sensory towards the non-sensory epithelium for CR, CB and PV (Figs 2a–c, 3a–c). In contrast, S100-like immunoreactivity is present more homogenously within the sensory epithelium (Fig. 2a–c). The S100-like positivity in the non-sensory epithelium (Fig. 3a–c) is an extracellular immunoreactivity not related to crypt or microvillous cells (see inset in Fig. 3a).

Upon magnification of the sensory epithelium, our double-labeled preparations confirm previous reports and


A
B
non - sensory region
lumen
sensory region
C
D
supporting cell
c
m
c
m
basal cell
lamina propria
E
calretinin DAPI
lumen
E'
ciliated OSNs
F
calbindin1 DAPI
lumen
F'
ciliary knobs
ciliated OSNs
G
parvalbumin DAPI
lumen
axons
G'
ciliated OSNs
microvillous OSN
H
lumen
S100 DAPI
H'
microvillous OSNs
crypt cells

◀**Fig. 1** Overview of calcium-binding protein expression in adult zebrafish olfactory sensory neurons (OSNs; regarding criteria for their identification, see text). **a** Unstained section of zebrafish olfactory epithelium with pigment cells in abluminal side of U-shaped folds of olfactory rosette. **b** Enlargement indicates lumen and sensory vs. non-sensory regions. **c** Schema with three olfactory sensory neuron (OSN) types. *Red* ciliated OSNs, *blue* microvillous OSNs, *green* crypt OSNs. **d** Schema shows known cytology of teleost olfactory epithelium. **e**–**h** Immunostained sections of adult zebrafish olfactory epithelia. Calretinin (**e**, **e′**) and calbindin1 (**f**, **f′**) are predominantly expressed in basally located ciliated cells with long dendrites (but see double-labels for some microvillous cells in Figs. 2, 3), whereas (**g**, **g′**) parvalbumin is equally abundant in ciliated and microvillous cells as visualized in magnifications (*right panels*). **h** S100-like immunopositivity is strong in superficially located crypt cells and weak in a fraction of microvillous cells, but never in ciliated cells. Middle panels are co-stained with nuclear marker DAPI and right panels show calcium-binding protein expression monochromatically. Note position of cell somata relative to lumen and other cytological details (compare with **c**, **d** and text). Note also that axons are labeled. *c* cilia, *m* microvilli. *Scale bar* in **a** 100 μm, *scale bars* in **e**–**h** 20 μm and in **e′**–**h′** 10 μm

our own monolabelings. Thus, CR/CB versus S100-like positivity is predominantly seen in ciliated versus crypt cells, respectively, with a subpopulation of microvillous OSNs additionally positive for S100 (Fig. 2b‴). S100-like-positive crypt or microvillous cells are never double-labeled with CB or CR (Fig. 2a, b). In PV-S100-like double-stainings, most microvillous cells are PV positive, and some microvillous cells are faintly double-labeled for S100-like signals (Fig. 2c″, c‴).

As expected from our single labelings, CB is co-localized with CR in most if not all ciliated OSNs (Fig. 3a–a‴), and additionally in a fraction of microvillous cells (see yellow arrows in Fig. 3a″, a‴). In contrast, PV is confirmed in double immunolabels with CR or CB to be equally abundantly present in ciliated and microvillous cells, but like CR and CB never seen in crypt cells, as has been indicated in single immunolabelings already. Also as expected, PV-CR double labelings show that most ciliated OSNs are double-positive, while most microvillous cells were only PV positive, with some being also double-labeled (yellow arrows in Fig. 3b″, B‴). However, both single PV-positive ciliated and microvillous OSNs (red arrows in Fig 3b″) do occur, while only single CR-positive ciliated (not microvillous) OSNs were seen (green arrows in B‴). The situation is similar in CB/PV double-labels with the exception that single CB or PV labeled ciliated

Table 3 Summary of CBP expression in larval and adult zebrafish OSNs revealed in the present study compared to previous literature

	ciliated cells 	microvillous cells 	crypt cells 
parvalbumin	our data	our data	---
calretinin	our data our data° Braubach et al., 2012 Castro et al., 2006 Gayoso et al., 2011 Germana et al., 2007 Koide et al., 2009°	our data our data° Braubach et al., 2012 ? Koide et al., 2009° Duggan et al., 2008°	---
calbindin1	our data our data°	our data our data°	---
S100	? ?	our data ? Gayoso et al., 2011	our data Braubach et al., 2012 Gayoso et al., 2011 Germana et al., 2004 Germana et al., 2007 Oka et al., 2012 Sandulescu et al., 2011 Sato et al., 2005

small subpopulation (and weakly stained)
main expression

° in embryos [48 h]
°in larvae

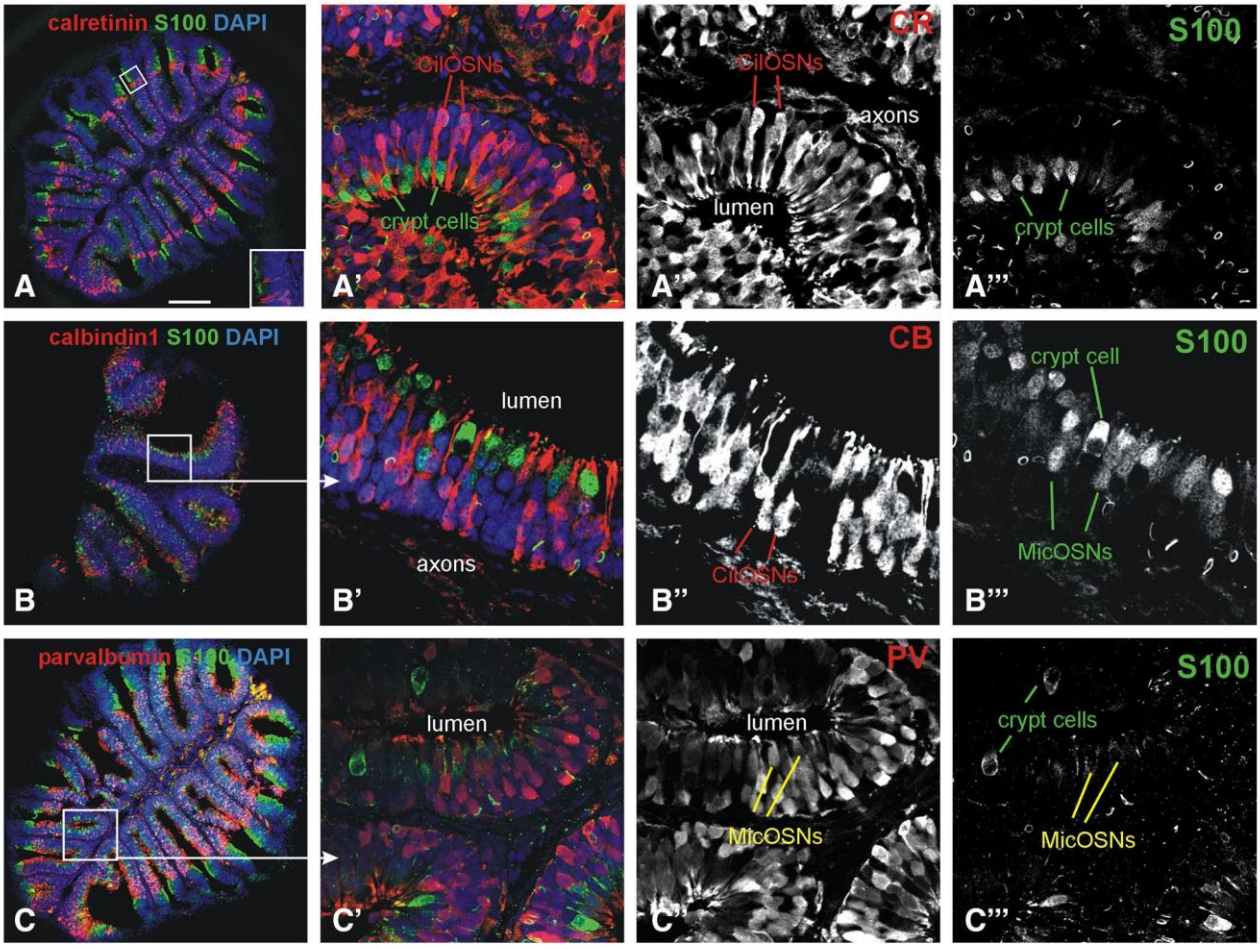


Fig. 2 Detailed double-label analysis of adult zebrafish olfactory sensory neurons: immunostained sections of olfactory epithelia show calretinin-S100-like (*row* **a**), calbindin1-S100-like (*row* **b**), parvalbumin-S100-like (*row* **c**). *Rows* **a** and **b** demonstrate that calbindin1 and calretinin-positive-ciliated OSNs are clearly distinguishable from S100-like-positive crypt and microvillous OSNs. *Row* **c** shows that parvalbumin is co-localized with S100 in some microvillous cells. *Inset* in **a** shows S100-like-positivity in an extracellular compartment of the non-sensory epithelium. **a**, **a′** through **c**, **c′** are co-stained with nuclear marker DAPI. **a′**–**a‴** through **c′**–**c‴** show enlargements of the squares in the double-immunostained olfactory epithelia shown in **a** through **c**, plus two monochromatic pictures of this same enlargement. *Scale bar* in **a** 100 μm, applies to **b**–**c**

OSNs were absent, whereas single PV microvillous OSNs were present; red arrows in Fig. 3c″. Often, strong CB staining goes along with weak PV stain in ciliated cells and the opposite is seen in microvillous OSNs.

In summary, all crypt cells apparently express S100-like positivity. As for microvillous OSNs, the data show a minor S100-like/PV double-positive population separate from an S100-like negative/PV-positive subpopulation. Apparently all microvillous OSNs show PV positivity, including single PV-labeled cells (i.e., CR or CB negative; Fig. 3). Further, the double-label preparations reveal that most ciliated, but also a fraction of microvillous cells express both CR/CB or CR/PV or CB/PV. Thus, whereas the S100-like negative/PV-positive microvillous cells consist for sure of a PV singe-labeled population; in addition, separate PV/CR and PV/CB subpopulations as well as a CR/CB subpopulation of microvillous OSNs might exist. However, triple-labeling may well reveal that there is a large CR/CB/PV-positive microvillous OSN subpopulation. As the projection patterns to the olfactory bulb will reveal, solely CB/PV-positive (i.e., CR negative) and solely PV-positive populations must exist. Thus, our CBP analysis indicates that microvillous cells form at least four subpopulations, namely a CR/CB/PV-positive one, a CB/PV-positive one, a PV/S100-like-positive one and a PV-only-positive one. Regarding ciliated OSNs, the majority appears to express at the same time PV, CR and CB. Since CB is always co-localized with PV in ciliated cells, single PV-positive ciliated OSNs likely do not exist. However, CR only and PV/CB-positive ciliated OSNs may

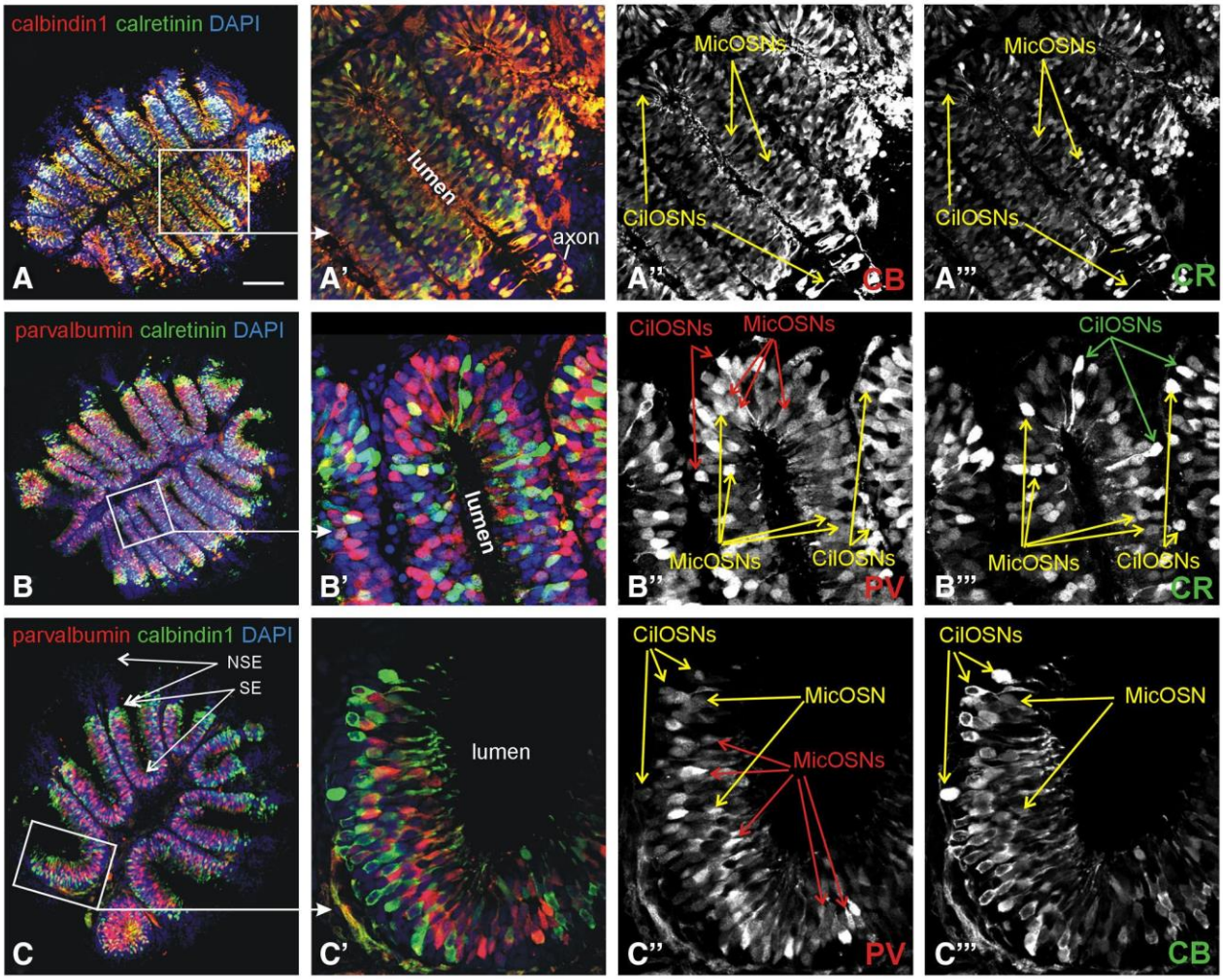


Fig. 3 Detailed double-label analysis of adult zebrafish olfactory sensory neurons: immunostained sections of olfactory epithelia show calbindin1-calretinin (*row* **a**), parvalbumin-calretinin (*row* **b**), and parvalbumin-calbindin1 (*row* **c**). Note spatial separation of sensory (SE) and non-sensory olfactory epithelium (NSE; indicated in **c**). While calretinin, calbindin1 and parvalbumin-positive OSNs form dense clusters at the outer edge of the sensory epithelium compared to the inner sensory epithelium (**a**–**c**), S100-like-positive OSNs are more equally distributed within the entire sensory epithelium (compare with Fig. 2a–c). **a** Demonstrates ubiquitous co-localization of calbindin1 and calretinin in most ciliated and some microvillous cells. For **b** calretinin and parvalbumin and **c** calbindin1 and parvalbumin, see text for details. **a**, **a**′ through **c**, **c**′ are co-stained with nuclear marker DAPI. **a**′–**a**‴ through **c**′–**c**‴ show enlargements of the squares in the double-immunostained olfactory epithelia shown in **a** through **c**, plus two monochromatic pictures of this same enlargement. *Scale bar* in **a** 100 μm, applies to **b**–**c**

exist. This would result in at least three subpopulations of ciliated OSNs, namely a large CR/CB/PV-positive one and two minor CR only and PV/CB-positive ones.

Calcium-binding protein expression in adult zebrafish olfactory bulb

Because the CBP immunolabel extends into the axons of OSNs, the data allow for analysis of selective projections of the adult zebrafish primary olfactory system into olfactory bulb glomeruli. We use the terminology of Baier and Korsching (1994) for most zebrafish olfactory bulb glomeruli and in addition that of Braubach et al. (2012) for mediodorsal glomeruli. In the olfactory bulbs, there is extensive overlap of CR, CB and PV fibers in various glomerular fields (Fig. 4a–c) consistent with the characteristic expression of all three CBPs in most ciliated and in many microvillous OSNs. These fields include the dorsal cluster (dc), lateroventral posterior glomerulus (q), and glomeruli of the ventral triplet (u–w). However, the lateral bulbar area (lateral chain and glomerulus m) is very faintly CR positive, but shows strong PV and CB positivity. In contrast, the lateral glomerulus p is also strongly CR positive. Furthermore, strong PV—but without any CR—positivity is

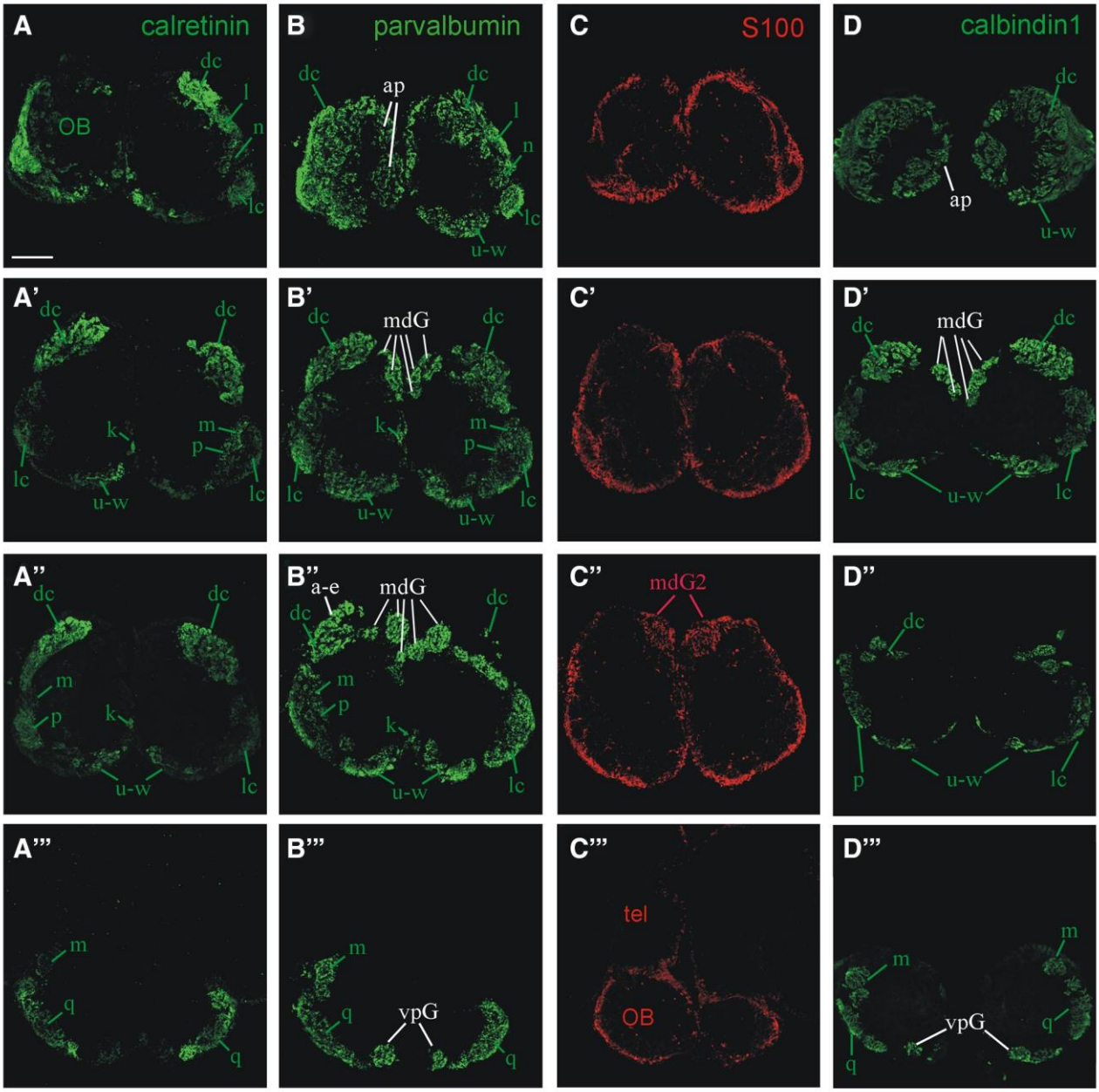


Fig. 4 Projection patterns in adult zebrafish olfactory bulb shown in transverse sections for **a**–**a**‴ calretinin, **b**–**b**‴ parvalbumin, **c**–**c**‴ S100-like, and **d**–**d**‴ calbindin1-positive fibers. Sections run from rostral (*top*) to caudal (*bottom*) in single immunostains. **a**–**a**‴, **b**–**b**‴, and **c**–**c**‴ are from one single adult zebrafish brain showing adjacent sections for each CBP at four selected levels. Note in particular the parvalbumin-positive mediodorsal region which is entirely free of calretinin and free of calbindin1 positivity in addition caudally (compare **a**″, **b**″, **d**″) and the single S100-like-positive glomerulus mdG2 (seen in **c**″). All calretinin-negative glomeruli are lettered in white. For abbreviations: see list. *Scale bar* in **a** 100 μm (applies to all *panels*)

present in anterior plexus (ap), the dorsal cluster-associated glomeruli (a–e), glomeruli of the mediodorsal group (mdG) and the ventroposterior glomerulus (vpG). CB-positive fibers are absent in a–e and the posterior part of mdG, but present in the anterior part of mdG, in the ap and the vpG. Thus, the solely PV-labeled projections (a–e; posterior part of mdG) apparently originate from solely PV-positive microvillous OSNs; whereas, overlapping PV/CB-positive projections stem from microvillous OSNs positive for these two CBPs. The remaining PV/CB/CR-positive projections then stem from triple-labeled ciliated and/or microvillous cells (see "Discussion").

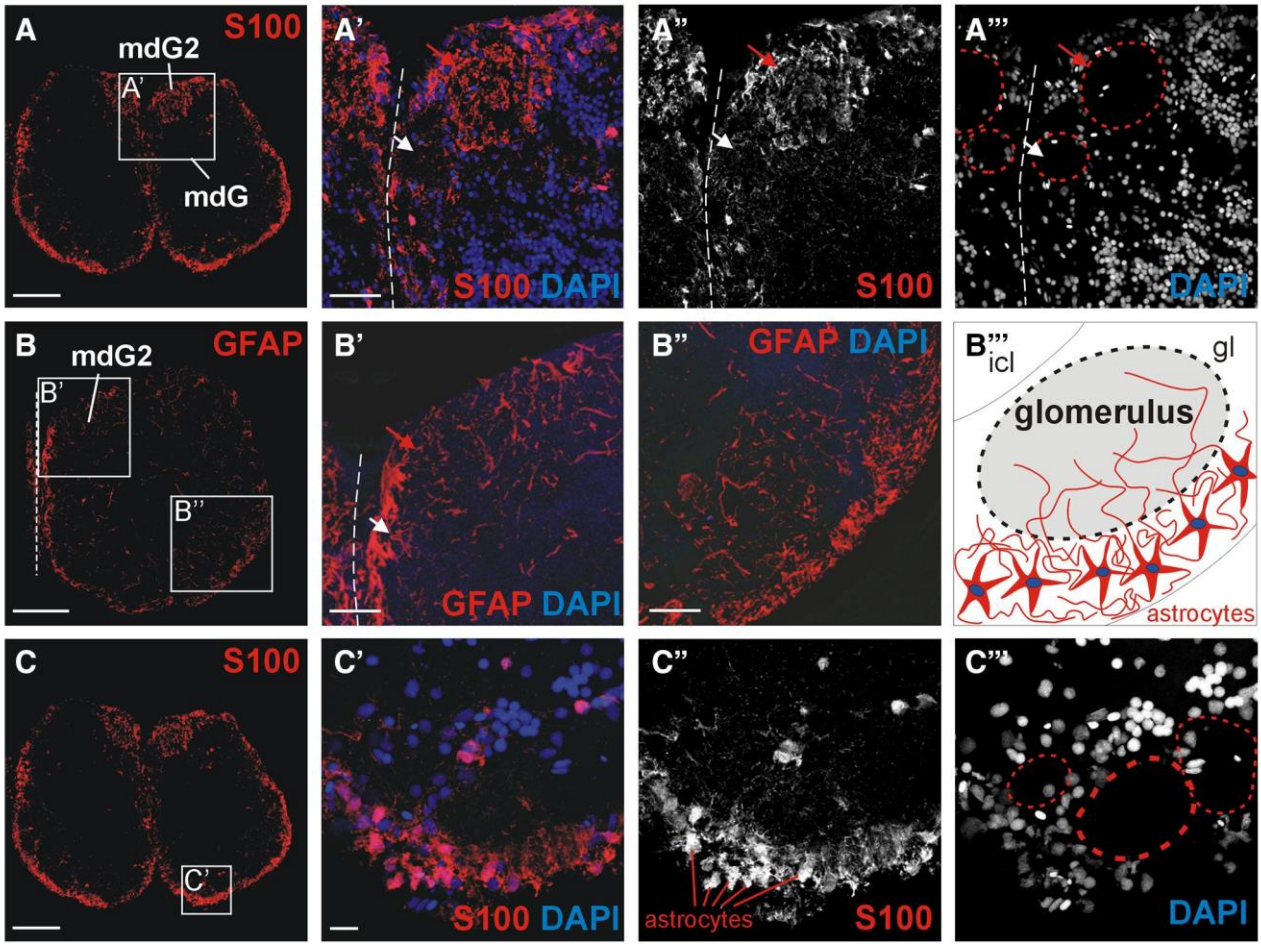


Fig. 5 Analysis of S100-like-positive astroglia-type elements versus S100-like-positive OSN axons. Rows **a**–**c** show adult zebrafish olfactory bulb transverse sections. Row **a**–**a**‴ shows the singular mediodorsal glomerulus mdG2 (*red arrow*) positive for dense S100-like fibers in total view olfactory bulb cross sections (**a**), at higher (**a**′) magnification counterstained with DAPI, in monochromatic views for S100 (**a**″) and DAPI (**a**‴), demonstrating that the dense fiber net is in one glomerulus (mdG2). Row **b**–**b**‴ shows astroglial marker GFAP in peripheral fiber net in total view (**b**), magnified in the region of mdG2 (**b**′) and in a ventrolateral bulb area (**b**″), both counterstained with DAPI. **b**‴ is an interpretative drawing of the findings shown in this plate. Row **c**–**c**‴ compares S100-like-positive astroglial-type cell bodies (**c** total view, **c**′–**c**‴ magnifications) with all bulbar cells visualized using DAPI. Especially the monochromatic pictures for S100-like (**c**″) and DAPI (**c**‴) demonstrate many peripheral and less central S100-like-positive astroglia-type somata compared to all somata which include within the bulb only some glial cells but many more neurons (see text). *gl* glomerular layer, *icl* inner granule cells, *mdG* mediodorsal glomerular field, *mdG2* mediodorsal posterior glomerulus 2. *Scale bars* in **a**–**c** 100 µm and in **a**′–**c**′ 50 µm

The S100-like-positive fiber projections only label one particular glomerulus in the posterior mdG area which is also PV positive (mdG2; Fig. 4c″). In addition, the S100-like antibody also visualizes cell somata and an associated fiber net surrounding the entire olfactory bulb (Fig. 5a–a‴, c–c‴). Closer examination reveals that these S100-like-positive cell somata at the olfactory bulb periphery also occur to some extent in the glomerular layer of the bulb and that fibrous S100-like-positive elements extend moderately into all glomeruli. However, only the mdG2 glomerulus shows a distinctly denser fiber net of S100-like positivity (Fig. 5a–a‴, compare to c–c‴). Therefore, we investigated whether the stained cell bodies and associated fiber net surrounding the olfactory bulb represent astroglial-type cells since these are in general known to be S100 positive (Lillo et al. 2002; Donata et al. 2013; Li et al. 2013).

Indeed, the said fiber net also stains with an antibody against the astroglial marker glial fibrillary acidic protein (GFAP; Fig. 5b–b″, schema in B‴). Whereas GFAP is known not to be present in astrocytic cell somata, the dense fiber net surrounding the olfactory bulb plus finer fibers in all glomeruli are GFAP positive, similar to what is seen with the S100-like immunostains. In contrast, the dense S100-like-positive fibers in the dorsomedial glomerulus are absent in the GFAP staining (compare Fig. 5a′, b′).

Furthermore, comparisons with DAPI stains show that the peripheral S100-like-positive somata and fewer more centrally located somata stain for S100-like signals (Fig. 5c′, c″), but not the many additional olfactory bulb neuronal cell bodies, for example the periglomerular neurons (compare Fig. 5c″, c‴). Thus, we conclude that in the zebrafish olfactory bulb, S100-like positivity labels ubiquitously astroglial-type cell bodies and their fibers and the axonal terminals of S100-like-positive OSNs (i.e., crypt and some microvillous OSNs), the latter very selectively converging in the mediodorsal posterior glomerulus 2 (mdG2; see "Discussion").

Adult teleost brains have many ependymoglial glial cells or tanycytes (which resemble embryonic radial glia because they make peripheral contacts with the pial surface and within the brain parenchyma; Manso et al. 1997; Arochena et al. 2004; Kálmán 1998) and fewer mammal-typical astrocytes with somata remote from the ependyma (Derouiche et al. 2012). Ma (1993) described in addition to such tanycytes at the mediodorsal border of the olfactory bulb of the sunfish also extraependymal tanycytes (i.e., astroglial-type) somata throughout the olfactory bulb internal granular cell layer. The pattern of processes emanating from both of these cell types resembles very much our pattern of GFAP fibers in the zebrafish olfactory bulb.

Development of zebrafish olfactory sensory neurons and their projections

Similar to the adult situation, parvalbumin is strongly expressed in larval (9 dpf) zebrafish ciliated and microvillous OSNs (Fig. 6a, b). Also as in adult specimens, strong ubiquitous expression of S100-like positivity in larval crypt cells is accompanied by limited and weaker expression of S100-like signals in some microvillous OSNs (Fig. 6c–e). Parvalbumin/S100-like double-immunolabelings reveal that larval microvillous cells are also already divided at least in solely parvalbumin positive and parvalbumin/S100-like double-positive subpopulations (Fig. 6c; white arrows point to S100-like-positive crypt cells, red arrowhead to double-labeled microvillous cell). Single S100-like label in crypt cells versus double-label for S100-like/parvalbumin in some microvillous OSNs is confirmed in enlarged maximum intensity projections (Fig. 6d) and optical sections (Fig. 6e). Combinatorial analysis of the CBPs parvalbumin/S100 and calretinin/calbindin1 in larval (9 dpf) zebrafish olfactory epithelium reveals that calretinin and calbindin1 are strongly expressed in ciliated and microvillous OSNs (Fig. 7c–c″), similar to the situation in adults (Fig. 3a–c). Also similar in larvae compared to adults is that CR, CB and PV are all not present in crypt cells (Figs. 6, 7).

Primary projections of different OSNs into the larval zebrafish (9 dpf) olfactory bulb were also visualized with CBP immunohistochemistry. For larval glomeruli, we used the nomenclature proposed by Dynes and Ngai (1998). Combinatorial analysis of axonal projections in the larval olfactory bulb reveals different bulbar targets of CBP-immunoreactive axons correlated with different expression patterns of corresponding CBP expression in OSNs (Fig. 7). Consistent with strong expression of parvalbumin in ciliated and microvillous OSNs, parvalbumin-positive axons also show numerous glomerular targets in olfactory bulb. In fact, almost all larval glomeruli are innervated by parvalbumin-positive axonal projections (Fig. 7a, a′, b). In contrast, double-labeling of parvalbumin and S100 reveals that S100-like immunopositive projections only innervate a defined glomerulus in the medial region (medial glomerulus 2 = MG2) in the larval olfactory bulb which is also innervated by parvalbumin-positive axonal projections (Fig. 7b, b′). This closely resembles the adult situation (see above).

Double-labeling of calretinin and calbindin1 visualizes that both CBPs are expressed in many larval ciliated and microvillous OSNs in the olfactory epithelium and largely overlap in bulbar targets in olfactory bulb (Figs. 7c–c″). But in comparison, calbindin1 is less strongly expressed than calretinin in the olfactory epithelium (white arrows in Fig. 7c–c″ show calbindin1 negative OSNs) and olfactory bulb. Importantly, calretinin- and calbindin1-immunopositive fibers do not project to the MG2. Calretinin-immunopositive projections innervate lateral glomeruli (LG) and glomeruli of the central zone (CZ) in the olfactory bulb (Fig. 7c″). Expression of calbindin1 is only visualized in lateral glomerli (LG) (Fig. 7c′), not in the central zone.

Thus, differences between calretinin and calbindin1-immunopositive projections correlate with the observation that calbindin1 is less strongly expressed than calretinin in the olfactory epithelium as this is also reflected in the extent of bulbar targets of these two CBP immunopositive axons. For a more detailed description of the projection differences of calbindin1 and calretinin, additional confocal microscopical analyses are necessary.

Analysis of parvalbumin versus S100-like-positive axonal projections in the developing larval olfactory bulb between 3 and 9 dpf reveals differing time courses of different primary projections defined by CBPs (Fig. 8). At 3 dpf, parvalbumin-positive projections are already seen to invade the olfactory bulb (Fig. 8a–a″). However, S100-like-positive fibers are completely absent at this early larval stage despite the fact that both (many) immunoreactive parvalbumin and (few) S100-like-positive OSNs are present at this age (Fig. 8a′, a″). From 6 dpf on, the S100-like-positive fibers are beginning to be visualized in the medial glomerulus 2 (MG2) and increase constantly up to 9 dpf

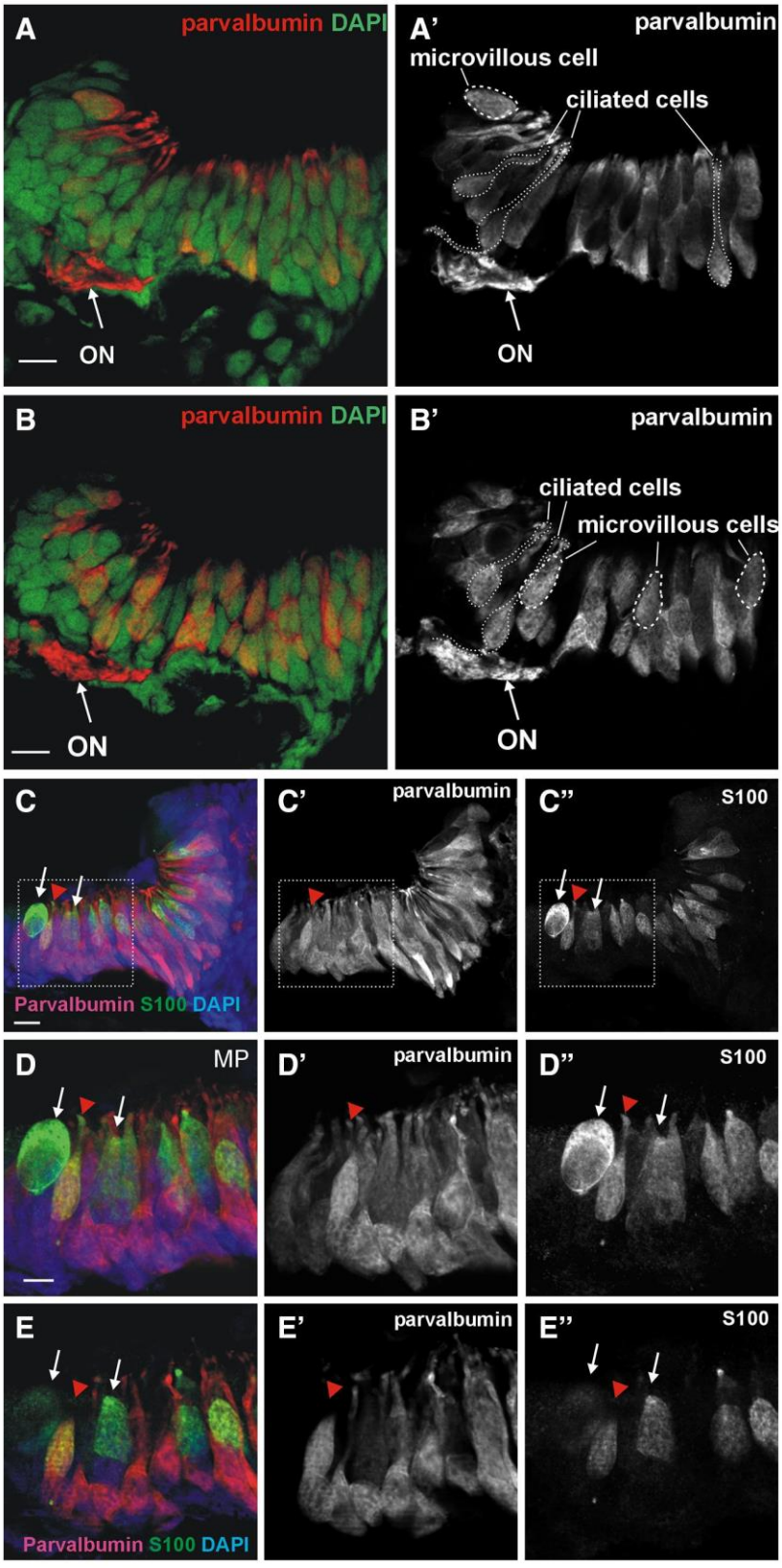


Fig. 6 Double-label analysis of parvalbumin and S100-like positivity in larval (9 dpf) zebrafish olfactory sensory neurons shown in transverse sections. **a**, **b** Parvalbumin labels both microvillous and ciliated OSNs. **a′**, **b′** Monochromatic pictures for parvalbumin show both OSN types. **c** Examples of double-labeled microvillous and single S100-like labeled crypt OSNs, with DAPI counterstain. **d** Enlarged maximum projection shows S100-like-positive crypt cells (*white arrows*) and double-labeled microvillous cell (*red arrowhead*). **e** Optical section confirms single vs double-label. **c**, **c″** through **e′**, **e″** Monochromatic pictures. *MP* maximum intensity projection, *ON* olfactory nerve. *Scale bars* in **a**–**c** 20 μm and in **d** 10 μm

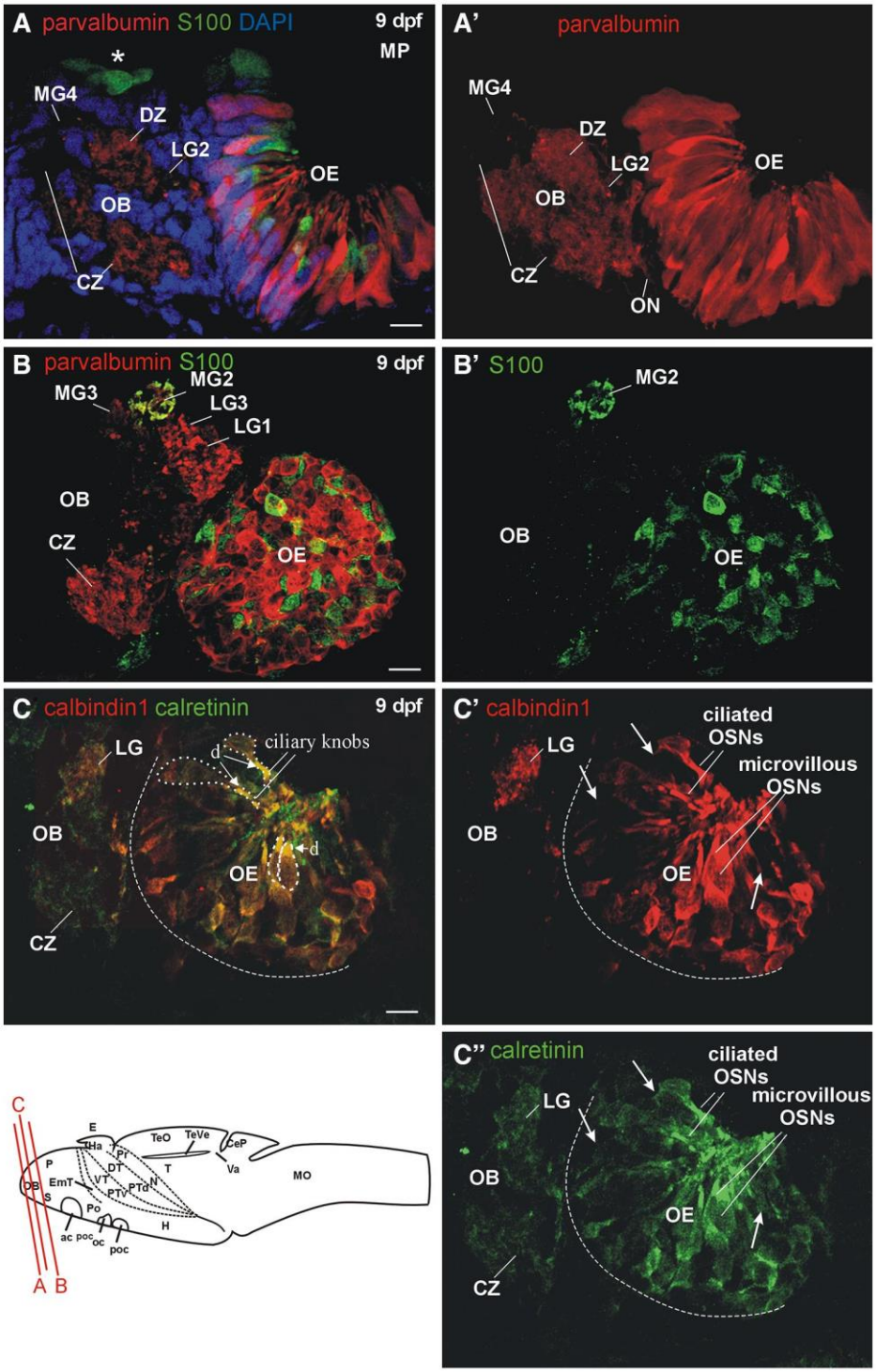


Fig. 7 Double-label analysis of parvalbumin/S100-like and calbindin1/calretinin in larval (9 dpf) zebrafish olfactory sensory neurons and their olfactory bulb projections shown in transverse sections. Parvalbumin/S100-like double-label at rostral (**a**) and caudal (**b**) levels. **a**, **a**′ Extent of parvalbumin-positive OSNs and their bulbar projection fields. **b**, **b**′ Extensive parvalbumin-positive projections contrast with restricted S100-like-positive projections to MG2. **c** Calbindin1/calretinin double-label shows OSNs and primary projection fields which are more extensive for calretinin (**c**″) than calbindin1 (**c**′; *white arrows* point to calbindin1 negative OSNs). Note that glomerulus MG2 is not calbindin1/calretinin positive. *Asterisk* in **a** denotes S100-like-positive head lateral line neuromast. For abbreviations see list. *Scale bars* in **a** through **c** 20 μm

(Fig. 8b–d). In contrast, parvalbumin-positive fibers appear to increase at a much slower rate during this time frame, and apparently reach various glomeruli much earlier (Fig. 8a–d).

Discussion

Our study demonstrates that calcium-binding proteins (CBPs) may indeed be used to characterize combinatorially

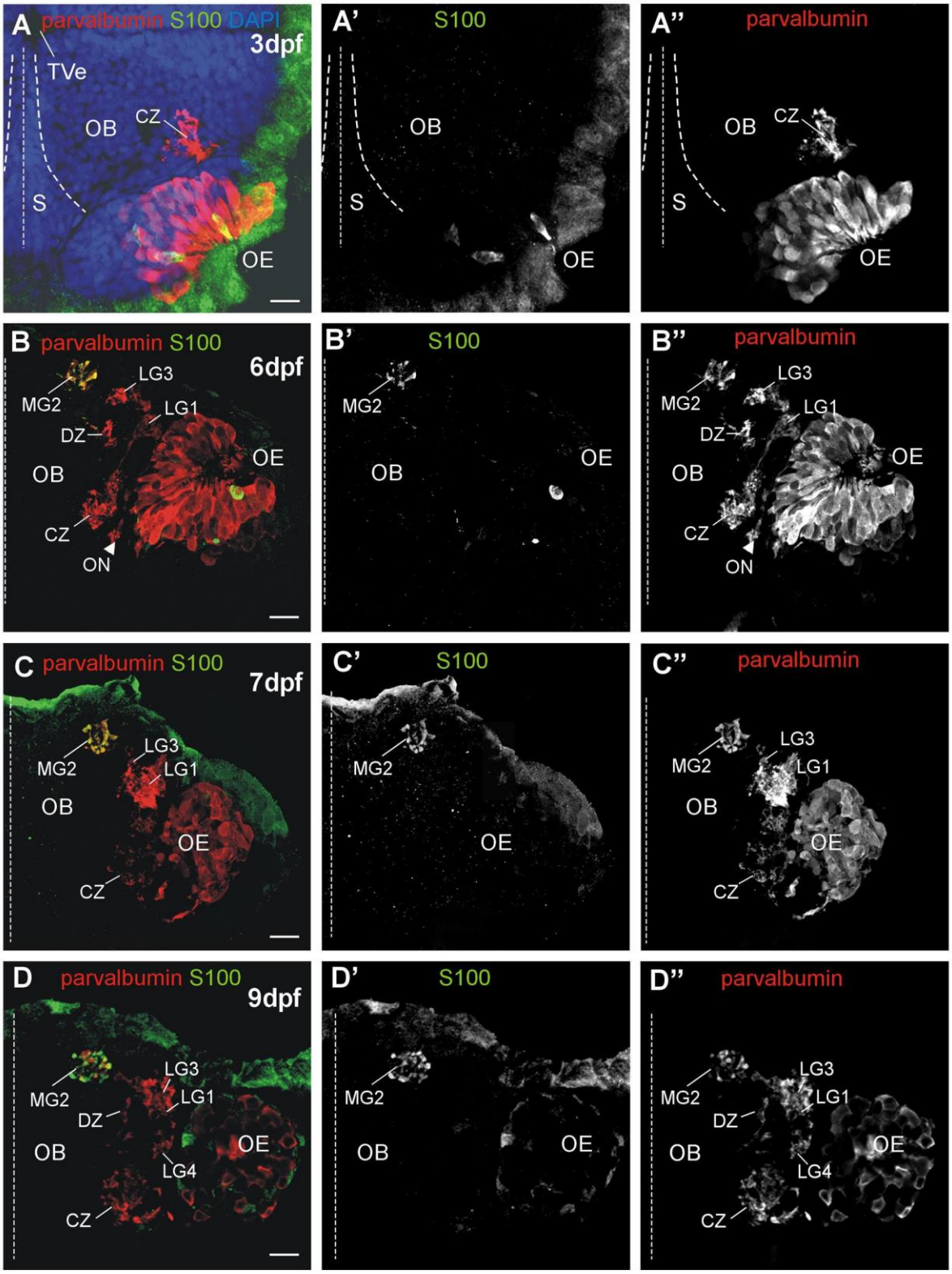


Fig. 8 Double-label analysis of parvalbumin and S100-like in a time series of larval (3, 6, 7, 9 dpf) zebrafish olfactory sensory neurons and their olfactory bulb projections shown in transverse sections at **a** 3 dpf, **b** 6 dpf, **c** 7 dpf, and **d** 9 dpf. Note absence of S100-like-positive projections at 3 dpf despite presence of few crypt cells and presence of parvalbumin projections in **a**. Note also steady increase of strength of S100-like-positive projections to MG2 between 6 and 9 dpf. For abbreviations see list. *Scale bars* in **a** through **d** 20 μm

different olfactory sensory neuron (OSN) types in the zebrafish, as has analogously been done in central nervous areas (e.g., mammalian cortex or cerebellum, see "Introduction"). Furthermore, since axons are nicely visualized by CBP expression, primary olfactory projection patterns of OSNs in the zebrafish olfactory bulb are visualized very selectively in our preparations. While S100-like and calretinin (CR)-positive OSNs and partially their primary projections have been described in various papers in the zebrafish before (Germana et al. 2004, 2007, Castro et al. 2006; Gayoso et al. 2011; Sandulescu et al. 2011; Braubach et al. 2012), parvalbumin (PV) and calbindin1 (CB) positive OSNs (see Table 3) and their projections are described here for the first time. Moreover, the present report is the first attempt to use four CBPs in single and double-label preparations of each possible combination in order to characterize subpopulations of OSNs and their primary projections to the olfactory bulb in a teleost. Of note, the extensive immunopositivity for PV in the zebrafish primary olfactory system may be special for some cypriniform teleosts only, since this has been neither reported in basal ray-finned fishes (Graña et al. 2012) nor in other cypriniform teleosts (Crespo et al. 1999). However, our double-labelings reveal not only that crypt OSNs are negative for PV, but also that various subpopulations of PV-positive microvillous OSNs lack calbindin1/calretinin or calretinin immunopositivity (see next paragraph). This is different from general primary olfactory system immunopositivity observed with an antibody against keyhole limpet hemocyanin (KLH) where all olfactory bulb glomeruli, and presumably all OSN subtypes, are labeled (Gayoso et al. 2011; Braubach et al. 2012) and demonstrates that no cross-reactivity occurs between the used markers.

Our data indicate that there are at least eight subpopulations of zebrafish OSNs (see "Results"; Figs. 1, 2, 3): We identify one homogeneous S100-like-positive crypt cell population (negative for the other three CBPs investigated) and four parvalbumin-positive microvillous OSN subpopulations (a PV only-positive one, an S100-like and PV-positive one, a PV and CB-positive one and a PV and CB and CR-positive one). In addition there are three subpopulations of ciliated OSNs, the majority consisting of a PV and CB and CR-positive one, plus minor PV/CB and CR-only-positive ones. These data on the CBP phenotype of OSNs are consistent with what we see in primary projection patterns in the olfactory bulb which will be considered in the following.

The zebrafish olfactory bulb mediodorsal glomerular field can be newly defined using CBPs

Sato et al. (2005) generated a double-transgenic zebrafish line targeting olfactory marker protein (OMP)-expressing ciliated OSNs (visualized by RFP) and transient receptor potential channel 2 (Trpc2)-expressing microvillous OSNs (visualized by Venus). In this way, these two classes of cells were demonstrated to have non-overlapping primary projections to the olfactory bulb. The transgenically labeled ciliated OSNs project to dorsal and ventromedial glomerular fields, whereas the microvillous OSNs terminate in ventroateral glomerular fields. This is in line with our findings that almost all ciliated as well as one large subpopulation of microvillous cells are CR/CB/PV positive and that all fields outlined by the transgenic label show extensive overlap of these three markers. Intriguingly, a mediodorsal field remained unstained in said study (Sato et al. 2005) and, thus, seems neither to receive fibers from those transgenically labeled ciliated nor microvillous OSNs. We demonstrate here that the entire mediodorsal glomerular field (mdG) receives parvalbumin-positive fibers and that it is at the same time calretinin free (see Fig. 4). The posterior part of this parvalbumin-positive mediodorsal field is also calbindin1 negative. Similar to this posterior mdG, also the dorsal cluster-associated glomeruli (a–e) are solely parvalbumin positive. These findings corroborate our data on a PV-only-positive population of microvillous OSNs. However, parvalbumin and calbindin1-positive (but no calretinin positive) projections reach the anterior part of this mediodorsal field [including the anterior plexus (ap)].

Recognition of singular mediodorsal glomerulus mdG2

We furthermore show a selective dense S100-like-positive glomerulus (mdG2) within the posterior parvalbumin only-positive mdG field, apparently representing converging projections from all crypt cells. This glomerulus neither shows overlap with CB nor with CR-positive fibers, but—as mentioned—does show overlap with PV-positive fibers. Thus, the latter finding is most consistently interpreted with an origin of these PV-positive fibers in S100-like/parvalbumin-positive microvillous cells seen in the olfactory epithelium which must possess double S100-like/parvalbumin-positive axons. The anterior PV-positive mediodorsal field (including the anterior plexus), which is additionally CB positive, likely are also subserved only by microvillous OSNs, because most ciliated OSNs contain CR and PV/CB-positive ciliated OSNs are rare. Thus, our findings in the zebrafish olfactory epithelium and bulb suggest consistently that three CR-free but PV-positive microvillous OSN subpopulations project into the mediodorsal field (i.e., PV-only, S100 and PV, PV and CB-positive microvillous OSNs). In mammals, microvillous OSNs are associated with Trpc2 (Spehr et al. 2006) and this applies to teleosts such as the zebrafish (Sato et al. 2005) as well. Thus, the microvillous OSNs which project to the

mediodorsal field either do not express the Trpc2 (Sato et al. 2005) or they might express an undescribed paralogue of it.

Braubach et al. (2012) have reported six glomeruli in the zebrafish mediodorsal olfactory bulb area defined now in addition with differential parvalbumin/calbindin1-positive primary projections in the present study. This study showed that only one of them (mdG2) receives S100-like-positive projections. We confirm that this glomerulus represents the only S100-like-positive glomerulus within the entire zebrafish olfactory bulb here and show that crypt OSNs are the cellular origin of these S100-like-positive fibers. However, we further demonstrate that a subpopulation of microvillous OSNs is also weakly stained for S100 and their projections apparently also converge in mdG2. This microvillous OSN subpopulation is at the same time parvalbumin positive, but not calretinin/calbindin1 positive. Therefore, one can exclude that (CR positive) ciliated OSNs or CB-positive microvillous OSNs project to mdG2. Although it has been reported that some crypt OSNs might express V2R type receptors (Cao et al. 1998), a recent report suggested that crypt OSNs express only one—out of totally six (Saraiva and Korsching 2007)—V1R type receptor gene (*ora4*) which is associated with the G-protein Gαi1b (Oka and Korsching 2011; Oka et al. 2012). Assuming that the latter is true, the corresponding converging projection pattern into mdG2 is highly reminiscent of a labeled line. Possibly then, the S100-like-positive microvillous OSNs might also express *ora4*, or alternatively, another receptor, likely a V1R type receptor protein.

Ahuja et al. (2013) recently further substantiated the claim of converging crypt OSN projections into one mediodorsal glomerulus (mdG2). Using paraformaldehyde-fixed versus fresh-frozen tissue they could either label crypt plus some microvillous OSNs versus only crypt OSNs, respectively. Further they provide TrkA (a neurotrophin—NGF—receptor) immunohistology as a selective crypt cell marker, including selective projections into mdG2. Further, Ahuja et al. (2013) argue that crypt cells do not express any S100 gene based on in situ hybridization data (Oka et al. 2012; see also Kraemer et al. 2008 for the teleost S100 gene family) and that the TrkA antibody does not recognize the neurotrophin receptor and cross-reacts with an unknown protein. In contrast, the S100-positive microvillous cells apparently do express a S100 gene (i.e., S100z). Also, S100β is firmly established to be present in mammalian astrocytes (e.g., Li et al. 2013) and, as reported above, we show S100-like immunoreactivity in astroglia-like cells in the present manuscript. These data indicate that the S100-like antibody used here does recognize this CBP at least in astroglial cells and likely in microvillous OSNs, but also that a similar molecule, not related to a known S100 protein, is recognized in crypt cells. We use therefore the term S100-like to describe the immunopositivity in this manuscript. In any case, all this does not invalidate our results regarding the characterization of selective OSNs and their bulbar projections.

Irrespective of the true molecular nature of the immunostains, Ahuja et al. (2013) rightly argue that TrkA immunostains reliably visualize crypt cells and their projections to mdG2, but also that S100 (under fixed conditions) reveals false-positive projections of microvillous OSNs to this glomerulus. Our results are consistent with these findings regarding crypt cells. However, we believe that the S100/parvalbumin-positive microvillous cells do indeed also project to the mdG2, since we do not see S100-like fibers outside the mdG2 glomerulus. Moreover, we also see parvalbumin in this glomerulus, which can only be explained by additional input from microvillous cells since crypt cells lack parvalbumin positivity. Both facts speak independently for the double-labeled microvillous OSN subpopulation to have projections into mdG2. Our claim is furthermore consistent with subtle details of the tracing data in Ahuja et al. (2013) who confirm their TrkA immunolabeling with DiI application to selective glomeruli, including the mdG2. Interestingly, they find in addition to crypt cells also some backlabeled microvillous cells after injections into mdG2 (apparently those which we describe as S100-like/PV positive to project to mdG2), whereas after tracing OSNs from another nearby glomerulus, only microvillous cells (as expected) were seen (see their Fig. 5). There is no logical reason why spread of injected DiI outside of the targeted glomerulus would only occur in one but not the other case.

Recognition of singular mediodorsal glomerulus mdG5

Another curious convergence of olfactory axons is seen in mdG5, a second mediodorsal glomerulus in the zebrafish olfactory bulb (Braubach et al. 2012). The latter study reports that mdG5 is the only glomerulus with positive fibers originating in Gαo-positive microvillous OSNs (although they also report some crypt cells as Gαo positive). Since this G protein is associated with microvillous cells (Oka and Korsching 2011), and lies in the anterior part of mdG (Braubach et al. 2012), it seems plausible—judged from our data—that parvalbumin/calbindin1 double-positive microvillous ONSs project there. Thus, there are four more (calretinin-negative) glomeruli within mdG which do receive parvalbumin only (one glomerulus) or parvalbumin/calbindin1-positive projections (three glomeruli) from microvillous ONSs. Likely only microvillous cells with a particular receptor (likely V2R type) project to mdG5. Thus, possibly two labeled lines exist, one converging in mdG2 (originating in crypt and microvillous OSNs) the other in mdG5 (originating in microvillous

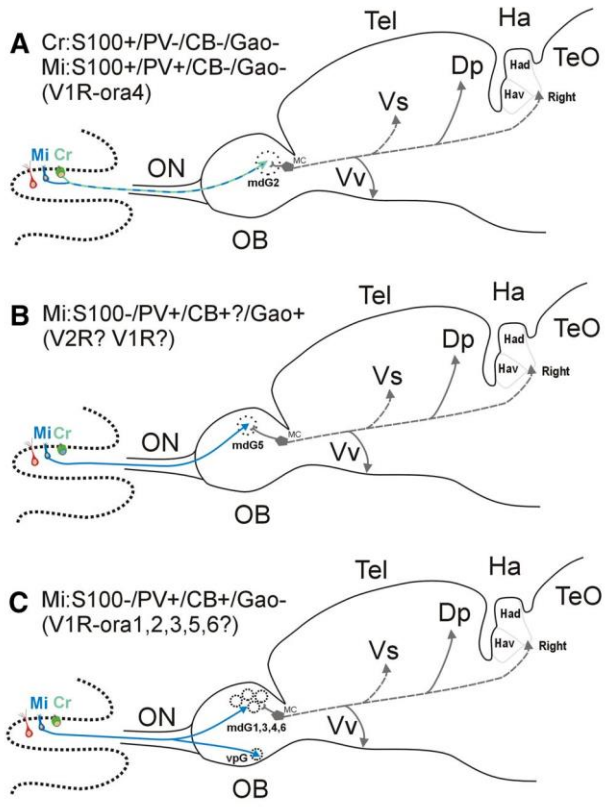


Fig. 9 Differential projections of crypt and microvillous OSNs in zebrafish olfactory system. Based on our data on CBP distribution in crypt and microvillous OSNs we propose that the mediodorsal region of the zebrafish olfactory bulb may harbor two labeled lines (**a**, **b**). Additional microvillous OSN projections reach mediodorsal glomeruli and the ventral posterior glomerulus (**c**). Higher order (i.e., secondary) olfactory projections of mediodorsal area are indicated with solid gray lines (targets shared with projections of entire olfactory bulb) and dashed lines (targets specifically attributed to mediodorsal bulbar area (see text for more details). Data on CBPs and connections from this study, with additional data from Braubach et al. (2012), Ahuja et al. (2013), Gayoso et al. (2012), Miyasaka et al. (2009) and Sato et al. (2005). During the publication process, Ahuja et al. (2014) reported a specific Gαo-positive subpopulation of microvillous cells (Kappe neurons), confirming our PV+/S100- subpopulation shown in **b**

OSNs) and maybe another five parvalbumin/calbindin1 or parvalbumin only positive microvillous OSN populations each carrying another of the remaining five V1R type receptor proteins project to the remaining four mediodorsal glomeruli and the additional ventral glomerulus (vpG) that is also solely parvalbumin/calbindin1 positive (Figs. 4a″–d‴, 9). However, the dorsal cluster-associated glomeruli (a–e) with parvalbumin only positive fibers and the anterior plexus with parvalbumin/calbindin1-positive fibers would then be hypothesized to be subserved from microvillous OSNs with V2R type receptors. Clearly, the recognition of parvalbumin and parvalbumin/calbindin1-positive microvillous populations with regard to their specific receptors as well as their primary projections to these calretinin-negative glomeruli needs further investigation.

The fourth large category of microvillous OSNs which are PV/CB and CR-positive would apparently project to the ventrolateral glomerular field and it is plausible then that they carry V2R type receptors (and as mentioned above, express Trpc2). Lastly, the overwhelming majority of ciliated OSNs express PV/CR/CB and project to the dorsal and ventromedial area. Both are in line with CR and Gαolf (the G-protein associated with ciliated OSNs) double-immunostaining in these two areas, but not in CR-positive ventrolateral areas (lc; Gayoso et al. 2011). Curiously, Gayoso et al. (2011) apparently label with the S100 antibody (same as used here) mostly microvillous OSNs and consequently show projections into ventrolateral glomeruli, clearly different from other reports (Braubach et al. 2012, present report), but consistent with the finding in transgenically labeled projections of microvillous cells (Sato et al. 2005).

Higher order connections

In addition to these molecular genetic and immunohistological studies, tracing studies support these analyses. Gayoso et al. (2012) report in a DiI tracing study the zebrafish mediodorsal olfactory bulb area to be recipient mostly of crypt cell projections and to project in turn to the supracommissural nucleus of the ventral telencephalic area (Vs), in addition to secondary olfactory projections shared with the dorsolateral bulb area (recipient of ciliated OSN input) to the ventral nucleus of ventral telencephalic area (Vv) and to the (pallial) posterior zone of area dorsalis (Dp). This is interesting because Vs is possibly homologous to the medial amygdala of other vertebrates and thus, might be processing socially relevant signals (Mueller et al. 2008; see discussion in Kress and Wullimann 2012). Furthermore, Miyasaka et al. (2009) have described three populations of larval and adult efferent olfactory bulb cells (possibly mitral cells), one of which is restricted to the mediodorsal bulb region (the OMP-RFP and Trpc2-Venus negative area discussed above). These mitral cell bodies were characterized by an lhx2a:GFP line and their axonal projections by an lhx2a:gap-YFP line. The latter transgenic line visualizes secondary olfactory projections to expected telencephalic targets (such as Dp), but also reach (asymmetrically) the right (dorsal) habenula which is not usually revealed in studies on adult secondary olfactory projections and is involved with regulation of anxiety (see discussion in Mathuru and Jesuthasan 2013).

Thus, the data discussed above speak for the presence of a labeled-line olfactory subsystem running from crypt cells and likely a fraction of microvillous OSNs to mdG2 and possibly a second such system from different microvillous

OSNs to mdG5. In addition, both systems might have distinctive higher order secondary olfactory connectivity, likely to the supracommissural nucleus of area ventralis (Vs) or the right habenula (Fig. 9). While the receptor protein/G-protein involved in the first case is ora4/Gαi1b, the second system's receptor protein is likely a (unidentified) V2R receptor as these are usually associated with Gαo (Oka and Korsching 2011). As discussed above in detail, there are four more mediodorsal glomeruli at least one PV only and three PV/CB-positive ones, plus the ventroposterior glomerulus (vpG) which is PV/CB positive. It is tempting to speculate that they may be related to the five more V1R type receptor proteins in a one to one fashion. However, the PV-only-positive dorsal cluster-associated glomeruli (a–e) and the anterior plexus (ap) would then have to be associated with V2R type receptors as they almost certainly also receive microvillous OSN input. In any case, the identity of specific ligands which possibly relate to a particular pheromone sensing function in the zebrafish mdG2 and mdG5—or additional labeled lines—remains to be shown. A pioneering start in this respect is the recent elucidation in zebrafish of the ligand cadaverine (an aliphatic diamine indicative of dead organic material) for the trace-amine-associated receptor 13c (TAAR13c) carried by ciliated OSNs and parallel olfactory-mediated avoidance behavior (Hussain et al. 2013).

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Contributions:

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Crypt cells are involved in kin recognition in larval zebrafish

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Zebrafish larvae imprint on visual and olfactory kin cues at day 5 and 6 postfertilization, respectively, resulting in kin recognition later in life. Exposure to non-kin cues prevents imprinting and kin recognition. Imprinting depends on MHC class II related signals and only larvae sharing MHC class II alleles can imprint on each other. Here, we analyzed which type of olfactory sensory neuron (OSN) detects kin odor. The single teleost olfactory epithelium harbors ciliated OSNs carrying OR and TAAR gene family receptors (mammals: main olfactory epithelium) and microvillous OSNs with V1R and V2R gene family receptors (mammals: vomeronasal organ). Additionally, teleosts exhibit crypt cells which possess microvilli and cilia. We used the activity marker pERK (phosphorylated extracellular signal regulated kinase) after stimulating 9 day old zebrafish larvae with either non-kin conspecific or food odor. While food odor activated both ciliated and microvillous OSNs, only the latter were activated by conspecific odor, crypt cells showed no activation to both stimuli. Then, we tested imprinted and non-imprinted larvae (full siblings) for kin odor detection. We provide the first direct evidence that crypt cells, and likely a subpopulation of microvillous OSNs, but not ciliated OSNs, play a role in detecting a kin odor related signal.

Olfaction is an important sense for detection and discrimination of the environment in all vertebrates, including teleosts, such as the zebrafish, *Danio rerio*. In addition to information on the location and composition of food, this sense mediates the recognition of objects of an aversive nature, such as predators, and, most importantly, of social cues such as pheromones[1].

Olfactory imprinting is a specific learning process during early development that occurs in a short period of time. The life-long memory of the learned cue influences environmental (see for review)[2], social[3], dietary[4] and mating[5] preferences in a wide variety of species. These memories are critically important, for example young salmon imprint on their natal stream odors and use these memories for spawning migration[6]. Furthermore, some coral reef fish larvae memorize olfactory cues of their natal environment which allows them to return and settle at their home reefs[7].

Imprinting on olfactory cues plays an important role in the discrimination between 'own' and 'foreign' in terms of social behavior such as altruism and inbreeding avoidance. The ability to distinguish between kin and non-kin is defined as kin recognition and has been shown in many species (for review see[8,9]) including in amphibians[10],reptiles[11], birds[12], mammals[13] and fish[2].

Previous studies have shown that zebrafish larvae imprint on visual and olfactory cues of their immediate kin (siblings) during a 24 h time window at 6 days postfertilization (dpf)[14–16]. Larvae can use the learned cues to differentiate between kin and non-kin later in life (kin recognition)[16]. However, imprinting and, consequently, kin recognition does not occur when larvae experience cues of non-kin during the imprinting phase, suggesting a genetic predisposition for kin odor[17]. Further investigations revealed major histocompatibility complex (MHC) peptide ligands to be the underlying chemical cues triggering olfactory imprinting[18]. *In vivo* calcium imaging showed responses to MHC peptides in olfactory bulb neurons to be spatially overlapping with responses to kin odor but not food odors, suggesting MHC peptides to be part of kin odor[18].

While imprinting is a critical process for salmon (see above), it is still not fully understood when in development imprinting occurs, which cues trigger imprinting or what the underlying genetic basis of imprinting is (reviewed in)[19]. Furthermore, captive rearing changes brain development in salmonids[20] which might negatively affect the imprinting process[19]. In contrast, the timing of imprinting, the required cues and the genetic basis are already known for zebrafish. In addition, kin recognition, as a result of olfactory imprinting, can be easily

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detected in laboratory reared animals at 10 days post-hatching. These traits combined make zebrafish an ideal model for studying the mechanisms of imprinting and kin recognition.

The teleost olfactory system lacks a separate vomeronasal organ (VNO) in addition to a main olfactory epithelium. Instead, teleosts possess a single olfactory epithelium (OE) embedded in the nostrils dorsally on each side of the head. Odorants are detected by thousands of different types of olfactory sensory neurons (OSNs) which mediate odor information via the olfactory nerve into the olfactory bulb, the first central nervous station for odor processing.

The two main types of vertebrate OSNs are ciliated (cOSNs) and microvillous olfactory sensory neurons (mOSNs) which in teleosts and mammals express olfactory receptors of the OR and TAAR gene families or V1R- and V2R-type genes, respectively. In addition, teleosts feature two more minor groups of OSN types. Crypt cells, which apparently express only a single olfactory receptor, the V1R-related ORA4[21] and the recently identified *kappe* neurons[22], both believed to be absent in tetrapods[23,24].

All four OSN types are recognizable by morphological characteristics like cell- shape, nuclear position within the olfactory epithelium and sometimes by their cell extensions. The cOSN somata are located most basally and extend a long slender dendrite towards the olfactory pit lumen. Cell bodies of mOSNs appear plumper with short dendrites and their nuclei are located at intermediate depths of the OE. Dendrites of cOSNs and mOSNs end in a so-called olfactory knob from which either cilia or microvilli protrude into the olfactory lumen. Compared to cOSNs and mOSNs, crypt cells and *kappe* neurons represent only a small population amongst OSNs but are morphologically well definable as being different from the two main OSN types. Both crypt and *kappe* neurons are apically positioned within the OE directly facing the lumen of the olfactory organ. Crypt cells are ovoid-shaped with a large apical positioned soma and a typical crypt on their apical pole bearing microvilli and cilia[25]. The *kappe* OSN type recently described by the Korsching lab[22] are somewhat similar to crypt cells but appear more pear-shaped and are positioned even more apical than crypt cells. Moreover, *kappe* neurons do not possess cilia, but only microvilli that protrude on their apical end which is formed like a cap[22].

Additionally to these morphological characteristics, the use of immunohistochemical markers, such as calcium-binding-proteins, which are often expressed in a cell-type selective manner, facilitates the identification of OSNs. In the zebrafish olfactory system, various calcium-binding-proteins show expression in OSNs as well as in their axonal projections into the olfactory bulb. Moreover, a combinatorial immunohistological expression analysis of four calcium-binding-proteins, that is calbindin, calretinin, parvalbumin and S100, reveals at least eight subpopulations of zebrafish OSNs[26]. As shown before, the calcium binding protein S100 is a marker for zebrafish crypt neurons and a small subpopulation of mOSNs. Although this immunopositivity results mostly from a cross-reaction with an unknown protein[21,27], the S100-like antibody can be used to detect selectively zebrafish crypt cells and a small subpopulation of mOSNs[26]. In addition, the projections of S100 positive OSNs into the olfactory bulb are visualized and are restricted to one single mediodorsal glomerulus (mdG2)[26–28]. Since crypt cells are only present in teleostean and cartilaginous fishes and not in land vertebrates, these cells may have a special role in odor detection and olfactory processing in fish. Because teleosts lack a separate vomeronasal organ, this special olfactory cell type might be involved in recognition of social odorants and resulting behavior.

However, presently the type(s) of OSN(s) detecting kin specific odor in zebrafish are unknown. Therefore, we stimulated the OE of imprinted and non-imprinted larval zebrafish with various odors (food, conspecific odor, kin odor) in a series of experiments. This resulted in differential activation of OSNs which is shown by an increase in the activity marker pERK (phosphorylated extracellular signal regulated kinase) after exposure to the stimuli mentioned. Thus, knowing already the time window of imprinting, some of the likely involved signals and the genetic basis for imprinting, we investigated in the present study which type of olfactory sensory neuron (OSN) detects kin odor.

Results

pERK is a reliable marker for showing differentially activated zebrafish olfactory sensory neurons in response to different stimuli. The phosphorylated extracellular signal regulated kinase pERK is used for marking neuronal activity in mammalian olfactory systems[29]. Presence of pERK indicates neuronal activation of the extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signaling pathway caused by a binding of signaling molecules or a synaptic transmitter release[30,31].

To validate pERK as a neuronal activity marker in the larval zebrafish olfactory epithelium, we performed a temporal analysis of pERK upregulation with two different olfactory stimuli in comparison to control stimulation with E3 medium. This experiment should give information on best duration of stimulation and additionally on the question whether pERK immunofluorescence shows different activation patterns due to different stimuli. We stimulated 9 day old larvae (group reared; see Methods and Fig. 1a) with either non-kin conspecific larvae odor, food odor or, for controls, with E3 medium for 3, 7, 11 and 15 minutes. Afterwards, we used an antibody against pERK to mark activated OSNs within the olfactory epithelium and counted those OSNs using the accepted morphological criteria for the different types of OSNs (see Methods).

Intensity of pERK labeled OSNs does not seem to depend on stimulus duration. Equally strongly pERK upregulated OSNs were observed with all four stimulus durations using two odor stimuli and control stimulation (Fig. 2a–c). However, we observed the best signal to noise ratio at stimulus durations of 7 minutes (data not shown). In addition, the duration of stimulation did not show an effect on the number of activated OSN types, because within each OSN type, no significant differences were observed between the four stimulation durations for all three stimuli (Fig. 2a–c).

Because we observed that stimulus duration did not affect the number of activated OSNs, we plotted pERK activated cells independent of stimulus durations against the two different odors food and non-kin conspecific larvae odor and compared it with controls (Fig. 3). The activation profile of the different OSNs revealed a highly significant activation in response to food odor compared to controls in ciliated and microvillous OSNs (cOSNs,

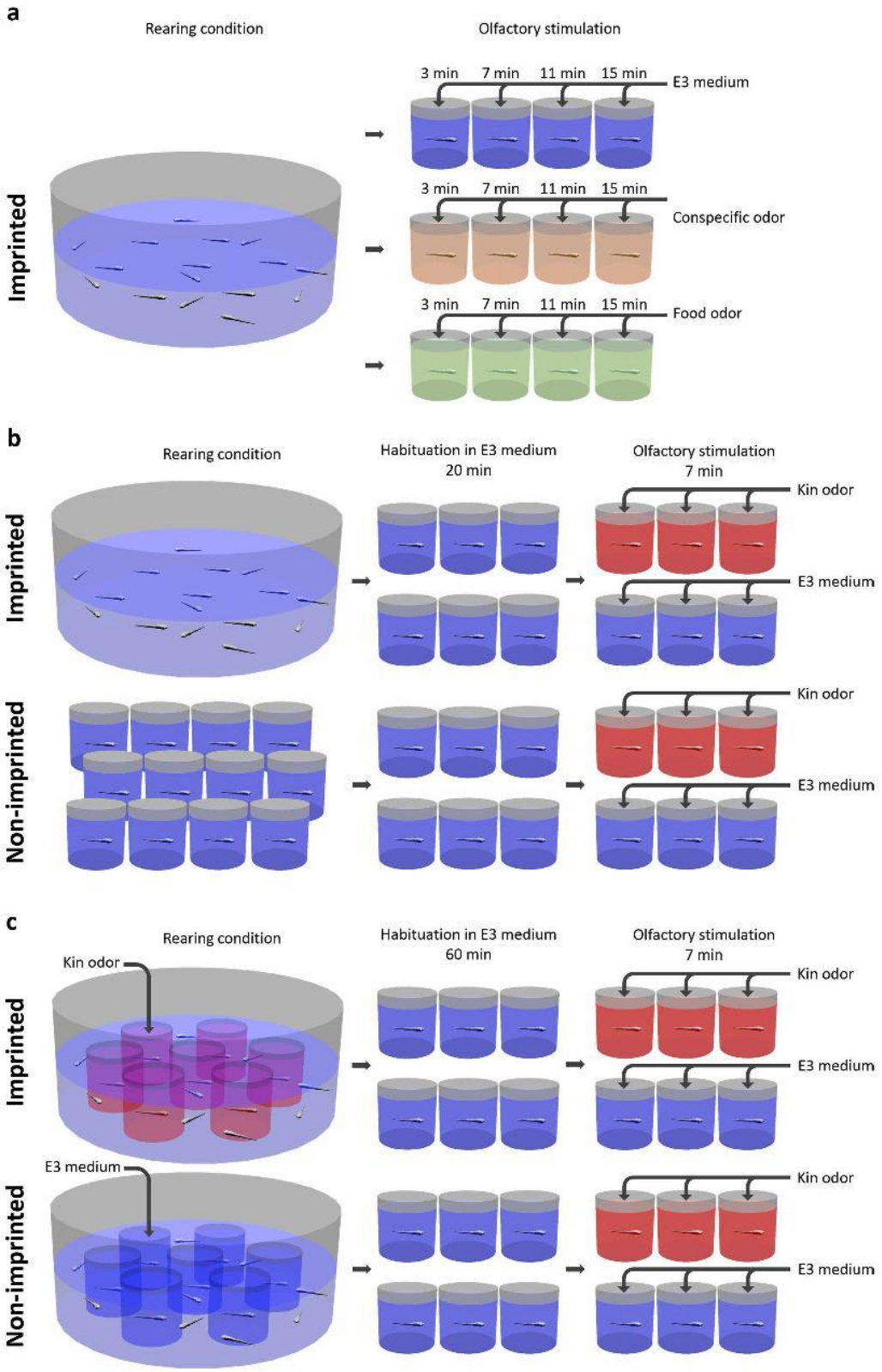

Figure 1. Schemes show three setups of performed experiments. (**a**) Validation of pERK as a marker for olfactory sensory neuron activity. (**b**) Kin odor test I. (**c**) Kin odor test II.

mOSNs) (Fig. 3a,b). Stimulation with non-kin conspecific larvae odor did not show a significant difference in number of activated neurons compared to control stimulation in both mOSNs and cOSNs. In contrast, crypt cells did not show any significant activation in response to both stimuli compared to controls (Fig. 3c). While mOSNs

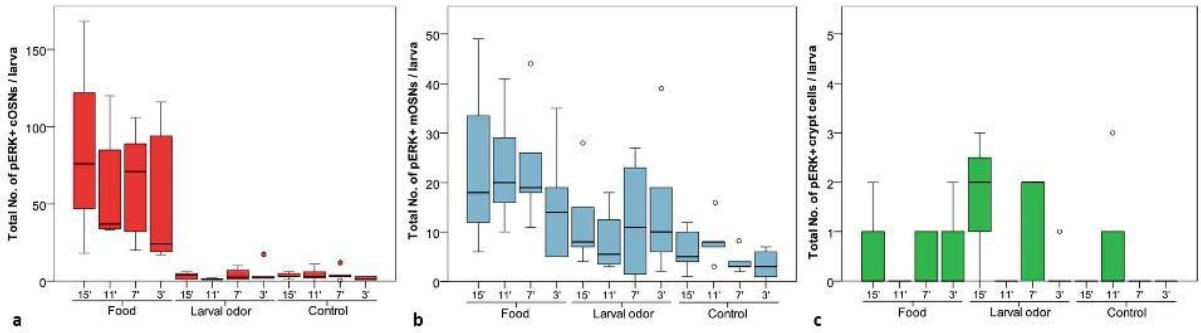

Figure 2. Effect of exposure duration of different stimuli on activity of cOSNs, mOSNs and crypt cells. 9 day old zebrafish larvae were exposed to either food odor, non-kin larvae odor or E3 medium as control (ctr) for either 3, 7, 11, or 15 minutes (min). The total number of pERK+activated cOSNs, mOSNs, and crypt cells was counted per larva and statistically analyzed. Box plots show median, upper and lower quartile and whiskers (maximum interquartile range: 1.5). (**a**) Stimulus duration does not affect number of pERK + cOSNs in larvae stimulated with food odor (Kruskall-Wallis test: $H(2) = 0.794$, $p = 0.851$, $n_{3,7,11\,min} = 5$, $n_{15\,min} = 3$), larvae odor ($H(2) = 3.030$, $p = 0.387$, $n_{3,15\,min} = 5$, $n_{7,11\,min} = 4$), or in controls ($H(2) = 2.866$, $p = 0.413$, $n_{3,7,11,15\,min} = 5$). (**b**) Number of pERK + mOSNs does not alter at different stimulus durations when stimulated with food odor ($H(2) = 1.714$, $p = 0.634$), larvae odor ($H(2) = 0.964$, $p = 0.810$), or in controls ($H(2) = 4.779$, $p = 0.189$). For n values: see (**a**). (**c**) No significant difference in number of pERK+ crypt cells at different stimulus durations when stimulated with food odor ($H(2) = 2.488$, $p = 0.478$), larvae odor ($H(2) = 6.685$, $p = 0.083$), or in controls ($H(2) = 6.316$, $p = 0.097$). For n values: see (**a**).

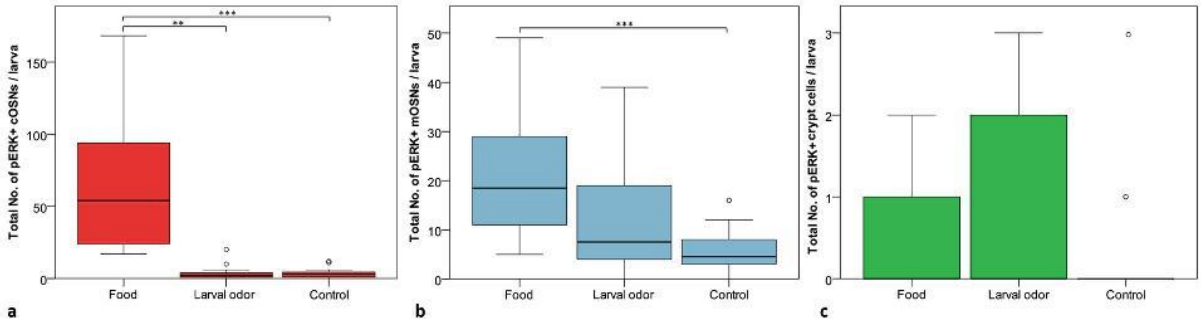

Figure 3. Differential activation of cOSNs, mOSNs and crypt cells by stimulation with different odors. 9 day old zebrafish larvae were exposed to either food odor, non-kin larvae odor or E3 Medium (control) (pooled data of Fig. 2). The total number of pERK+activated cOSNs, mOSNs, and crypt cells was counted per larva and statistically analyzed. Box plots show median, upper and lower quartile and whiskers (maximum interquartile range: 1.5). *indicates statistical significance p: ***$p < 0.001$. (**a**) cOSNs are strongly activated by food odor. Significantly more pERK+cOSNs were counted in larvae stimulated with food compared to larvae odor (Mann-Whitney U: 4.0, $p < 0.001$, median $(Mdn)_{food} = 54$, $Mdn_{larvae} = 2$, $n = 18$) and to control ($U < 0.0$, $p < 0.001$, $Mdn_{food} = 54$, $Mdn_{ctr} = 3$, $n_{food} = 18$, $n_{ctr} = 20$). (**b**) mOSNs show the highest activation when stimulated with food odor. Significantly more mOSNS were activated by food odor compared to control stimulation (U: 33.5, $p < 0.001$, $Mdn_{food} = 19$, $Mdn_{ctr} = 4.5$, $n_{food} = 18$, $n_{ctr} = 20$). pERK + mOSNs stimulated with larvae odor do not differ in numbers compared to controls (U: 116.5, $p = 0.062$, $Mdn_{larvae} = 7.5$, $Mdn_{ctr} = 4.5$, $n_{larvae} = 18$, $n_{ctr} = 20$). (**c**) Crypt cells show no significant difference in pERK+cell numbers due to stimulation with different odors (Kruskall Wallis test: $H(2) = 3.197$, $p = 0.202$, $n_{food} = n_{larvae}$ odor $= 18$, $n_{ctr} = 20$).

and cOSNs did show a significant activation in response to food but not to non-kin conspecific larvae odor, crypt cells did not respond to either of the stimuli. These results clearly show (a) that within the temporal range tested, stimulus duration has no effect and (b) that pERK is a reliable marker for activated OSNs in zebrafish larvae specific for different odor stimulations.

Finally, a comparison of activated OSN types depending on different odor stimulation shows the following (Supplementary Fig. 1). After food stimulation, significantly more activated cOSNs and mOSNs are seen compared to crypt cells. There are also higher numbers of activated cOSNs versus mOSNs. After non-kin conspecific larvae odor stimulation, significantly more activated mOSNs and cOSNs are seen compared to crypt cells. Also, a significant higher number of mOSNs were activated in comparison to cOSNs. Somewhat surprisingly (but see discussion), within control stimulations, activated cOSN and mOSN numbers are significantly higher than crypt cell numbers.

Exposure to kin odor indicates a role of crypt cells and possibly of mOSNs in olfactory kin recognition (and maybe in imprinting) in zebrafish. The results of the following two test series show a role of mOSNs and crypt cells in detecting kin specific odor. Because of the high number of activated crypt cells in imprinted larvae of the *control group* in the first kin odor test (kin odor test I), it can be speculated whether this high activation is due to a higher spontaneous firing rate or, alternatively, to the rest activity of these OSNs resulting from previous kin exposure resulting from the group rearing condition prior to the relatively short adaptation phase in the glass beaker containing E3 medium (Fig. 1b). To answer this question we decided to repeat the experiment (i.e. kin odor test II) with identical rearing conditions of imprinted and non-imprinted groups and with extension of the adaptation period to 1 h before starting the odor stimulation to allow the pERK signal to return to baseline (Fig. 1c). By rearing both groups under the same conditions except for the presence or absence of the olfactory kin-related signals, we also ascertain that differing physiological stress factors did not influence our results. As expected, in this second kin odor test, the high activation of the imprinted control group was eliminated (see discussion).

Kin odor test I. In this experiment we investigated which OSN type(s) respond to a kin odor produced by full siblings and therefore play a role in olfactory kin recognition and maybe imprinting. To this aim, we stimulated 9 dpf old imprinted (group reared, see Methods) and non-imprinted (isolated reared; see Methods and Fig. 1b) larvae with kin odor for 7 minutes, and compared these two groups with equally reared control groups stimulated with E3 medium. In this experiment, we used the anti-pERK antibody together with an established crypt cell marker, an antibody against the calcium binding protein S100[26], in order to examine a possible overlap between these two markers (Fig. 4) and to differentiate S100-positive (S100 +) from S100-negative pERK activated OSNs.

First, we were interested if there was a quantitative difference of S100 + mOSNs and crypt cells between imprinted and non-imprinted larvae (Fig. 5a). When plotting all detected S100 + mOSNs and crypt cells independent of pERK immunopositivity, no significant difference between imprinted and non-imprinted larvae was observed. Imprinted as well as non-imprinted larvae show nearly the same amount of S100+ mOSNs or crypt cells. Therefore, we can exclude that preventing olfactory imprinting in zebrafish larvae has an effect on total OSN cell number (i.e. S100 + mOSN and crypt cell) development within the olfactory epithelium.

Focusing on the two populations of S100 + OSNs, we plotted the percentage (Fig. 5b,c) of activated (pERK+) S100 positive mOSNs and crypt cells per larva. Regarding S100+ mOSNs as well as crypt cells, all S100 + OSNs of each larva were counted and therefore the percentage of activated S100 + OSNs can be specified. The number of double labelled mOSNs was significantly higher in imprinted larvae than in non-imprinted larvae after exposure to E3 medium (see Fig. 5b). This might result from group rearing conditions and time of adaptation before stimulation (see Discussion).

The pERK+/S100+ double labelled crypt cells showed a clearer picture of differential activation after kin odor versus E3 medium exposure (Fig. 5c). Stimulation with kin odor showed considerable numbers of activated crypt cells in imprinted larvae whereas in non-imprinted larvae only few activated crypt cells were counted. However, again, as for double labelled mONSs, also imprinted control larvae stimulated with E3 medium showed considerable numbers of activated crypt cells and the difference is highly significant compared to non-imprinted control larvae (Fig. 5c), likely for the same reasons as indicated for double labelled mOSNs (group rearing and adaptation time before testing; see Discussion).

Finally, we counted all other pERK+cell types within the olfactory epithelium which were S100 negative using the accepted cytological and morphological criteria for OSNs (see Methods). Exposure to either kin odor or E3 medium showed no difference in pERK activation of ciliated S100 negative OSNs in imprinted and non-imprinted larvae (Fig. 5d). In S100 negative mOSNs of imprinted and non-imprinted larvae olfactory stimulation with kin odor or E3 medium showed no significant effect on activation of OSNs. As expected, no S100 negative crypt cells were detected which were pERK immunoreactive. In summary, there are no significant differences in S100 negative OSNs between kin odor stimulated imprinted and non-imprinted larvae in this experiment.

Kin odor test II. As in the first experiment using kin odor stimulation, there is no significant difference in total quantity of S100+mOSNs and crypt cells between imprinted and non-imprinted larvae (Fig. 6a).

The small S100+subpopulation of mOSNs and crypt cells which are also positive for the activity marker pERK are shown as percentage of all S100+OSNs counted (Fig. 6b,c). In the case of S100+/pERK + mOSNs, the generally very low cell numbers reveal a significant difference between imprinted and non-imprinted larvae when exposed to kin odor (Fig. 6c). In contrast to S100+/pERK + mOSNs, crypt cells of imprinted larvae show high numbers of pERK activated cells in response to the kin odor (Fig. 6c). In imprinted larvae exposed to kin odor, more than 90% of all S100+crypt cells were activated, which is highly significantly more than in non-imprinted larvae exposed to kin odor and than in imprinted larvae exposed to E3 medium. Little activation was also observed in non-imprinted larvae exposed to E3 medium. In addition, extending the adaptation time in E3 medium before starting the stimulation experiments in all groups reduced greatly the high amount of activated crypt cells (and presumably also mOSNs) in imprinted control larvae as seen in the experiment before (see Fig. 5c). Therefore we can exclude a higher spontaneous activity of crypt cells in imprinted versus non-imprinted larvae.

Comparing data on S100 negative OSNs that were activated in response to kin water or E3 medium in this and the previous experiment revealed a similar picture (Figs 5d and 6d). As expected, no S100 negative/pERK positive crypt cells were observed, confirming that all of them are S100 positive. Ciliated S100 negative OSNs of imprinted larvae showed only slight activation in response to the kin odor. However, a certain number of cOSNs of non-imprinted larvae were also activated in response to the kin odor. Also imprinted and non-imprinted control larvae show small numbers of activated cOSNs. However, there were no significant differences between

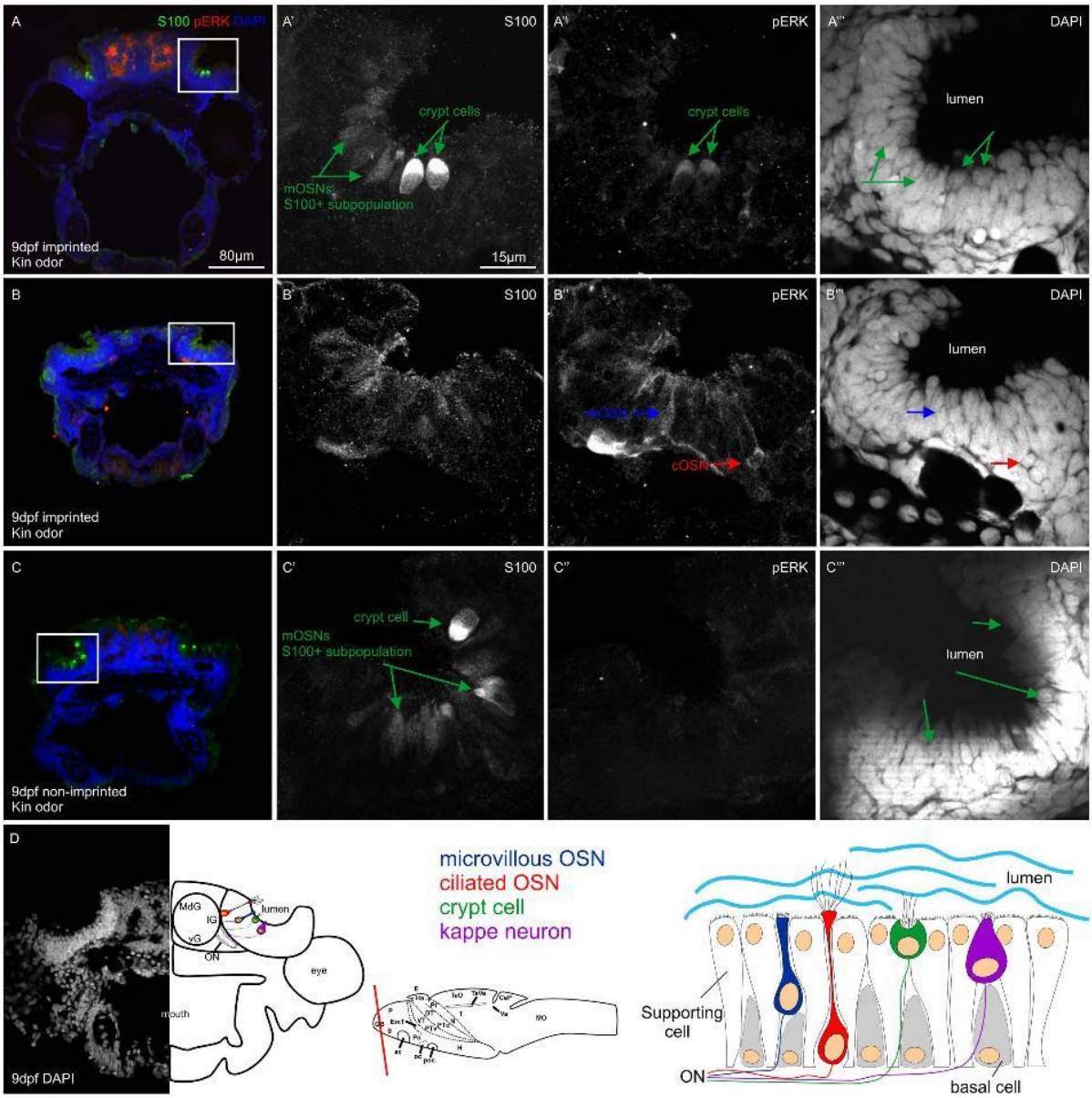

Figure 4. Examples of activated OSN identification and counting. All photographs shown are confocal optical sections. (**A–C**) Overviews of 9 dpf larval zebrafish cross-sections triple-stained for DAPI, S100 and pERK. (A'-A'''), (B'-B'''), and (C-C''') show magnified monochromatic pictures of each marker in the olfactory epithelium. Note examples of activated crypt cells in imprinted larvae tested with kin odor (A-A''') as well as some mOSNs and cOSNs (B-B'''). In non-imprinted larvae tested with kin odor, crypt cells are not activated (C-C'''). (**D**) shows a DAPI view of the position of the olfactory epithelium relative to eye and olfactory bulb with a corresponding explanatory drawing. Larval brain outline indicates the level of section of (**D**). Drawing at right bottom gives an overview on the cytoarchitectonic organization of the olfactory epithelium. Abbreviations: ac anterior commissure, CeP cerebellar plate, DT dorsal thalamus, E epiphysis, EmT eminentia thalami, H hypothalamus, Ha habenula, lG lateral glomeruli, MdG mediodorsal glomeruli, MO medulla oblongata, N region of the nucleus of the medial longitudinal fascicle, OB olfactory bulb, oc optic chiasma, ON olfactory nerve, P pallium, Po preoptic region, poc postoptic commissure, PTd dorsal part of posterior tuberculum, PTv ventral part of posterior tuberculum, S subpallium, T tegmentum, TeO tectum opticum TeVe tectal ventricle, Va valvula cerebelli, vg ventral glomeruli, VT ventral thalamus.

cOSNs among all four tested groups (Fig. 6d). Regarding mOSNs, higher numbers of S100 negative mOSNs are pERK activated in imprinted as well as non-imprinted larvae in response to kin odor stimulation or E3 medium stimulation. In this second experiment, there is a significant difference in numbers between the imprinted kin and imprinted control group (see Discussion).

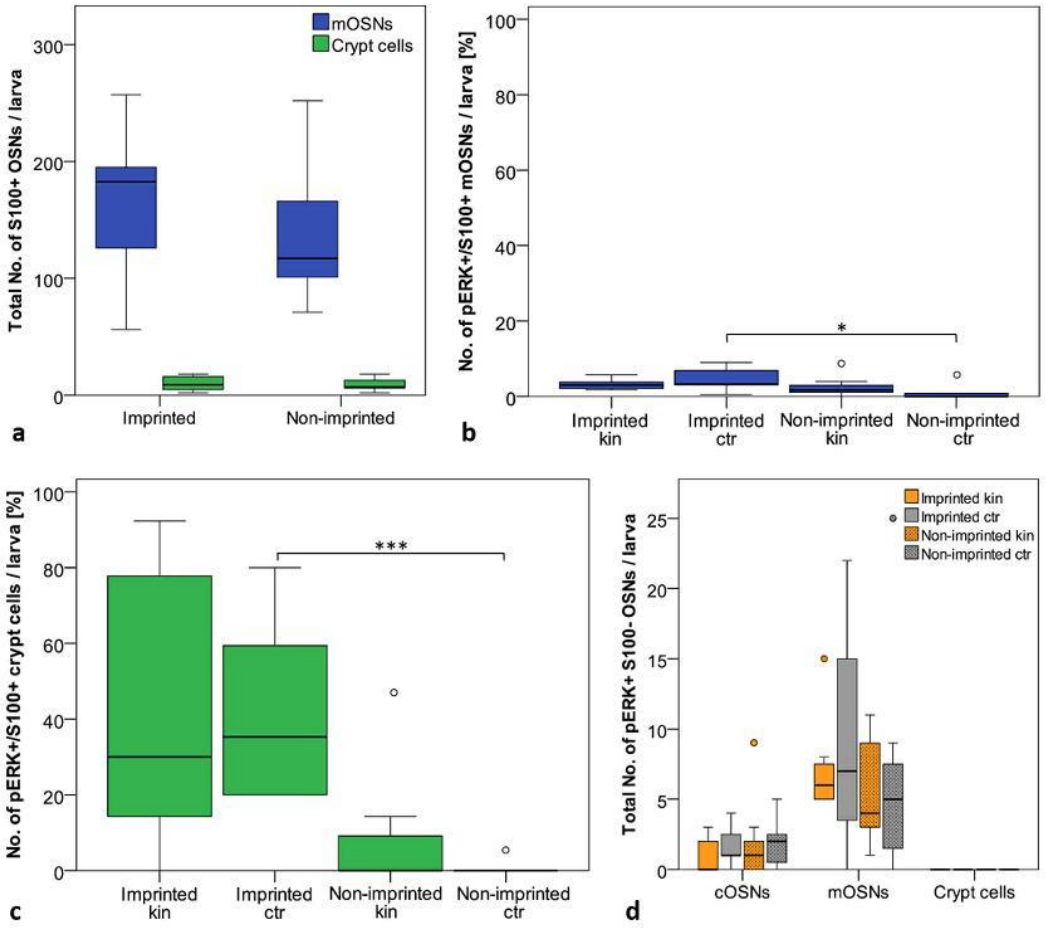

Figure 5. Kin odor test I (see Fig. 1b): Effects of olfactory imprinting. (**a**) Total cell quantity of S100+mOSNs and crypt cells. Box plots show median, upper and lower quartile and whiskers (maximum interquartile range: 1.5). No significant difference in total number of mOSNs and crypt cells was found (mOSNs Mann-Whitney U: 104.5, $p = 0.109$, $Mdn_{impr} = 182.5$, $Mdn_{non\ impr} = 117$; crypt cells U: 149, $p = 0.894$, $Mdn_{impr} = 9$, $Mdn_{non\ impr} = 7$, $n_{impr} = 18$, $n_{non\ impr} = 17$). (**b**) S100+/pERK+mOSNs shown as percentage of all S100+ mOSNs per larva. Box plots show median, upper and lower quartile and whiskers (maximum interquartile range: 1.5). *indicates statistical significance p: $*p < 0.05$, $*p < 0.01$, $***p < 0.001$ (also applies to (**c**) S100+mOSNs show no difference in activation between imprinted and non-imprinted larvae after kin stimulation. Number of activated mOSNs is significantly higher in imprinted larvae versus non-imprinted control larvae (Mann-Whitney $U < 0.001$, $p < 0.001$, $Mdn_{impr} = 3.25$, $Mdn_{non\ impr} = 0$, $n_{impr} = 11$, $n_{non\ impr} = 7$). (**c**) S100+/pERK+crypt cells shown as percentage of all S100+ crypt cells per larva. S100+ crypt cells show no difference in activation between imprinted and non-imprinted larvae after kin stimulation U: 15, $p = 0.035$ [Bonferroni correction], $Mdn_{impr} = 30$, $Mdn_{non\ impr} = 0$, $n_{impr} = 7$, $n_{non\ impr} = 10$). A significant difference between imprinted and non-imprinted control larvae exists ($U < 0.001$, $p < 0.001$, $Mdn_{impr} = 35$, $Mdn_{non\ impr} = 0$, $n_{impr} = 11$, $n_{non\ impr} = 7$). (**d**) The total numbers of pERK activated, but S100 negative cOSNs, mOSNs, and crypt cells are shown. Box plots show median, upper and lower quartile and whiskers (maximum interquartile range: 1.5). *indicates statistical significance p: $**p < 0.01$. No difference in cell activation was found in either cOSNs (Kruskall-Wallis $H(2) = 1.729$, $p = 0.630$) or mOSNs ($H(2) = 1.901$, $p = 0.593$) ($n_{impri\ kin} = 7$, $n_{impr\ ctr} = 11$, $n_{non\ impr\ kin} = 10$, $n_{non\ impr\ ctr} = 7$).

Finally, in both kin odor experiments, we tested 11 dpf or 9 dpf old larvae in a 2 channel choice flume (as established in the Gerlach laboratory[15,16,32]; Fig. 7) which showed successful imprinting in both of these group reared larvae taken from the same batch, respectively, as the larvae used for the stimulation experiment.

Discussion

We investigated in zebrafish larvae whether pERK expression in OSNs depends on/differs with olfactory stimulus duration and whether different olfactory stimuli result in a differential activation pattern of pERK. Therefore, in a first experiment, we stimulated group raised zebrafish larvae at 9 dpf (see Methods and Fig. 1a) to validate pERK as a marker of OSN activity in response to a food odor or a non-kin conspecific larvae odor at various exposure times. Our results demonstrate that pERK is a reliable marker to show differentially activated OSN types after exposure to different odors (Figs 2 and 3). The pERK signal was rapidly induced and detectable in different types

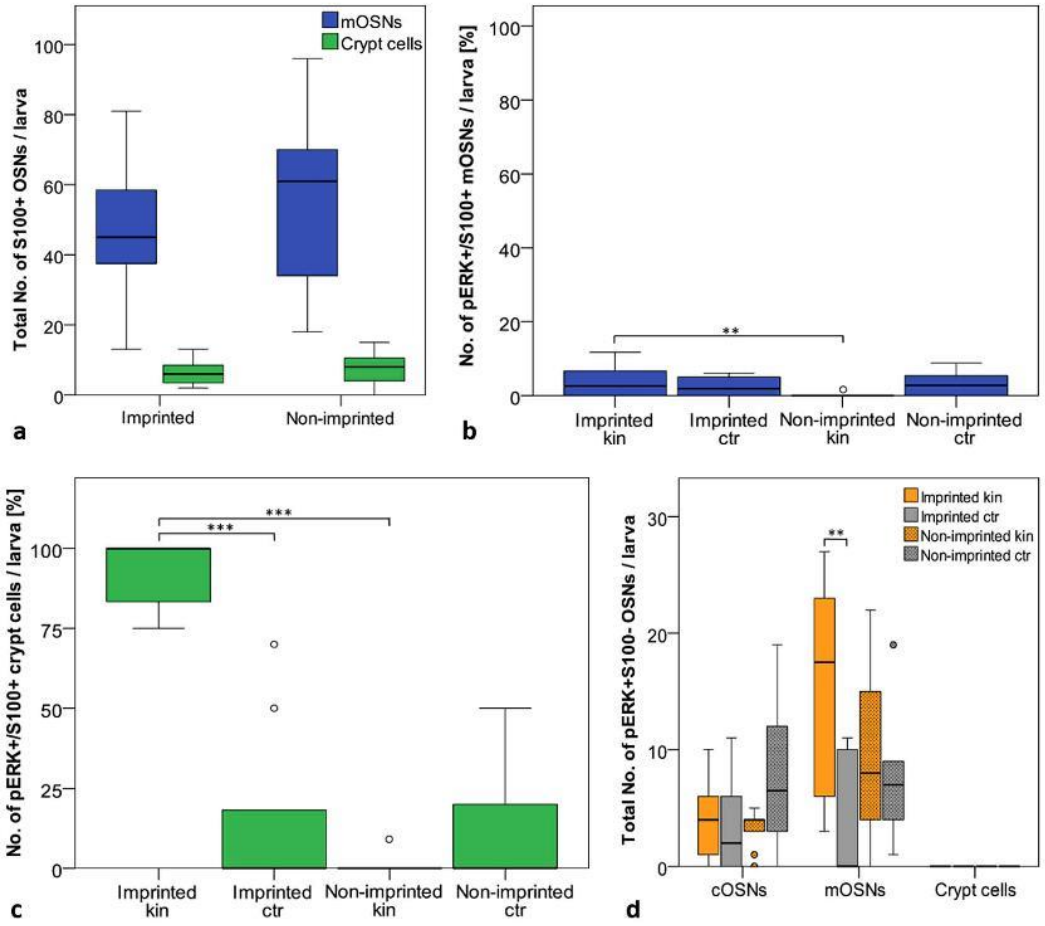

Figure 6. Kin odor test II (see Fig. 1c): Effects of olfactory imprinting. (**a**) Total cell quantity of S100 + mOSNs and crypt cells. Box plots show median, upper and lower quartile and whiskers (maximum interquartile range: 1.5). Imprinting has no effect on total cell numbers (mOSNs U: 147, $p = 0.227$, $Mdn_{impr} = 54$, $Mdn_{non\ impr} = 61$; crypt cells U: 156, $p = 0.351$, $Mdn_{impr} = 6$, $Mdn_{non\ impr} = 8$, $n_{impr} = 19$, $n_{non\ impr} = 20$). (**b**) S100 +/pERK+ mOSNs shown as percentage of all S100 + mOSNs per larva. Box plots show median, upper and lower quartile and whiskers (maximum interquartile range: 1.5). *indicates statistical significance p: $*p < 0.05$, $*p < 0.01$, $***p < 0.001$ (also applies to (**c**)). Significantly more S100 + mOSN are activated in imprinted larvae versus non-imprinted control larvae exposed to kin odor (U: 18, $p = 0.008$, $Mdn_{impr} = 2.6$, $Mdn_{non\ impr} = 0$, $n_{impr} = n_{non\ impr} = 10$). (**c**): A significant higher number of crypt cells are activated after kin odor stimulation in imprinted compared to non-imprinted larvae ($U < 0.001$, $p < 0.001$, $Mdn_{impr} = 100$, $Mdn_{non\ impr} = 0$, $n_{impr\ kin} = n_{non\ impr\ kin} = 9$) and compared to imprinted control larvae stimulation (U: $U < 0.001$, $p < 0.001$, $Mdn_{impr} = 100$, $Mdn_{non\ impr} = 0$, $n_{impr\ kin} = 10$, $n_{impr\ ctr} = 9$). No difference in activation was found within non-imprinted larvae. (**d**) The total numbers of pERK activated, but S100 negative cOSNs, mOSNs, and crypt cells are shown. Box plots show median, upper and lower quartile and whiskers (maximum interquartile range: 1.5). * indicates statistical significance p: $**p < 0.01$. Cell activation was similar for all treatments in cOSNs ($H(2) = 5.405$, $p = 0.144$) ($n_{impri\ kin} = 10$, $n_{impr\ ctr} = 9$, $n_{non\ impr\ kin} = 10$, $n_{non\ impr\ ctr} = 10$). A significantly higher number of mOSNs was found in imprinted larvae stimulated with kin compared to control stimulation (Mann-Whitney U: 13, $p = 0.008$, $Mdn_{impr\ kin} = 17.5$, $Mdn_{impr\ ctr} = 0$). No S100- negative crypt cells were observed.

of OSNs after 3 minutes of odor stimulation (Fig. 2). The intensity of immunofluorescence as well as the numbers of OSNs remained unchanged with prolonged odor exposure times (7, 11, 15 min). Both cOSNs and mOSNs were strongly activated by the food stimulus compared to controls, while crypt cells were not (Figs 2 and 3). The comparison of OSN types convincingly showed that cOSNs were more strongly activated by food than mOSNs, whereas mOSNs were more strongly activated by (non-kin) conspecific larvae odor compared to cOSNs (Supplementary Fig. 1). These two OSN types occur in equally high numbers already in the larvae, whereas the crypt cells form a minor population (see below). Thus, we interpret the apparent significant differences within the controls simply as a consequence of the much higher numbers of both cOSNs and mOSNs compared to crypt cells.

There is great interspecific variability within teleosts regarding the potential roles of OSNs[33]. In channel catfishes, a comparison of OSN olfactory bulb projections and electrophysiological responses to amino acids and nucleotides (both indicative of food) or bile salts (presumably social signals) in the olfactory bulb indicated that

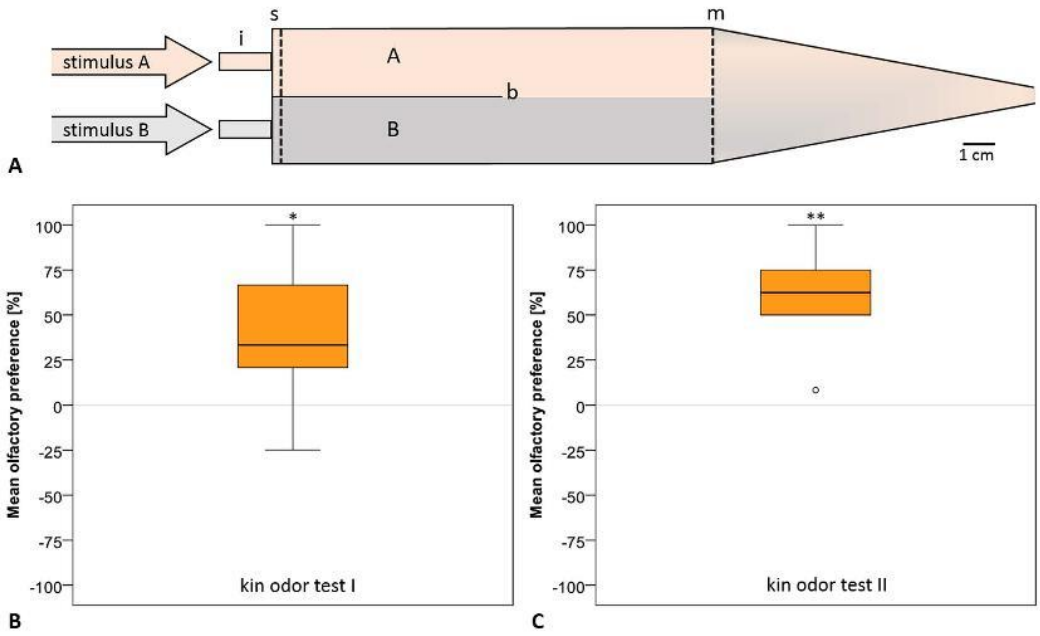

Figure 7. Group reared full-sibling larvae prefer kin odor over non-kin odor in behavior test. Mean olfactory preference of larvae was tested using the two-channel choice flume. (**A**) Two-channel choice flume (after Hinz *et al.*)[18]. Two distinct parallel-flowing water masses A (orange) and B (grey) containing different odors are separated through a glass barrier (b). A sponge (s) reduces pulsation caused by the pump. i: inflow; m: mesh screen to prevent test fish leaving the area of laminar flow. (**B**) Full-siblings of test larvae (11 dpf) of kin odor test I (compare Fig. 1b) significantly prefer the smell of kin over non-kin (Wilcoxon signed-rank test Z: -2.325, p = 0.020, median (Mdn) = 33.3, n = 11). (**C**) Full-siblings of test larvae (9dpf) of kin odor test II (compare Fig. 1c) showed a significant olfactory preference for kin compared to non-kin odor (Wilcoxon signed-rank test Z: -3.065, p = 0.002, Mdn = 62.5, n = 12).

cOSNs respond to amino acids and bile salts, mOSNs to amino acids and nucleotides, and crypt cells to amino acids[34]. Studies in carp indicate that mOSNs are related to feeding, cOSNs to alarm reaction and crypt cells to reproduction[35]. In goldfish, mOSNs expressing V2R-type odorant receptors are best tuned to amino acids[36]. In zebrafish, cOSNs are associated with sensing bile salts and prostaglandins, mOSNs with sensing amino acids and nucleotides, and crypt cells with sensing skin extract (reviewed in[33,37]). Koide *et al.*[37] found in transgenic zebrafish lines visualizing different OSN types that only ablation of mOSNs through genetically encoded tetanus toxin abolished behavioral responses to amino acids. Physiological preference for amino acids by mOSNs was also found in zebrafish[38]. In salmon, mOSNs have also been related to amino acids, while cOSNs sense bile salts[39,40]. Trout crypt cells have been related to sensing gonadal extracts[41]. However, in other teleost species, amino acid sensing was clearly also seen in cOSNs[33,42]. Thus, a general conclusion that teleost mOSNs mediate food-related olfactory cues based on amino acid detection and cOSNs detect social signals through bile salt sensing is too simplified, because studies in different teleost species show that cOSNS, mOSNs as well as crypt cells respond to amino acids. Our results are consistent with an activation of both cOSNs and mOSNs through food stimulus which contains a variety of chemicals including amino acids. Moreover, non-kin conspecific odor additionally activated mOSNs. In any case, our first experiment ascertains that pERK is a reliable marker for OSN activity in zebrafish larvae after odor stimulation.

In order to investigate which OSNs are involved in kin odor detection, we stimulated in two additional experiments imprinted and non-imprinted larvae (see Methods and Fig. 1b,c) with kin odor containing E3 medium. Here we used an anti-S100 antibody to mark specifically all crypt cells as well as a small subpopulation of mOSNs[26] in addition to the anti-pERK antibody to stain neuronal activation after odor stimulation. Imprinted zebrafish larvae recognize their kin siblings while non-imprinted larvae do not ([15,16]; see Introduction). This difference between imprinted and non-imprinted larvae might depend on activity at the level of the olfactory epithelium. Indeed, our results show a great difference between imprinted and non-imprinted larvae with regard to crypt cell activation in response to kin odor in both experiments (Figs 5 and 6). The S100 staining allows for counting all crypt cells and the subpopulation of S100 positive mONS. Since these cell numbers are the same (9 dpf larvae), we can exclude that this highly significant difference is due to a dissimilar number of crypt cells between imprinted and non-imprinted larvae (Fig. 5).

Regarding to the role of crypt cells in olfactory imprinting we compare now in more detail the differences of OSN activation responses of imprinted and non-imprinted larvae after kin odor stimulation between the two experiments using kin odor stimulation. First, high numbers of activated crypt cells were seen in both experiments in kin odor stimulated imprinted fish. However, the first kin odor experiment indicated a possible difference of crypt cells as well as S100 + mOSNs in their spontaneous activity also in control conditions (neutral

E3 medium as stimulus) between imprinted and non-imprinted larvae (Fig. 5b,c). To test this possibility we performed a second experiment in which both larvae groups (imprinted and non-imprinted fish) were raised in the same way in glass beakers. Further, the adaptation time was extended (to 1 h) before starting the experiment. Thus, we excluded the possibility that group reared and isolated reared larvae undergo different stress levels (e.g. through water changes using pipettes). Furthermore, the prolonged adaptation time was introduced to make sure that all fish reached baseline levels regarding OSN activity. The data showed clearly that the increased activity seen in crypt cells and mOSNs in imprinted controls in the first experiment (Fig. 5b,c) is eliminated by these changes (Fig. 6b,c). This demonstrates even more explicitly the role of crypt cells in kin recognition.

Besides crypt cells, we marked a significant activation of S100 negative mOSNs (Fig. 6d) in imprinted larvae in response to the kin odor which indicates also an involvement of mOSNs in kin recognition. A collaboration between two OSN types conveying a kin-related signal with subsequent behavioral response is more likely than the involvement of one cell type and is similarly seen in rodents[43]. In contrast, cOSNs did not show significant responses to kin odor (Figs 5d and 6d). Moreover, the kin odor containing E3 medium (see Methods) is doubtlessly comprised of many odor cues, some of which may not be kin-related. Apparently, crypt cells express only a single V1R homologue odorant receptor, encoded by the *ora4* gene[21]. There is strong evidence from other studies in teleosts which implicate a role of crypt cells in reproductive behavior[35,44]. In the crucian carp, the number of crypt cells varies during the year, with a dramatic increase during the spawning season[44]. Similar studies in adult zebrafish and guppies did not indicate a seasonal change in crypt cell quantity which might be related to year round reproductive behavior[23,45,46]. However, the kin-specific ligand(s) and its (their) molecular nature by which crypt (or other) cells are activated is unknown.

Sandulescu and colleagues[47] report that the zebrafish crypt cell population undergoes nonlinear growth during larval development. This study reports a linear increase of zebrafish crypt cell numbers from day 2 until day 7 of larval postembryonic development, followed by a rapid decrease of crypt cell numbers around 8-9 dpf. Thus, a peak in crypt cell number is reached at 7 dpf with an average of 7.8 cells per larva, with numbers decreasing at 8 dpf and 9 dpf to finally 2.2 cells per larva. At 12 dpf a rebound of crypt cell numbers is seen[47]. Our high crypt cell numbers at 9 dpf (average of 7 cells per larva) may at first glance seem to disagree with these results of a time point of extreme reduction of crypt cells. However, larvae of the other study were maintained at 28 °C while we raised the larvae at 26 °C. Since the development of larvae is temperature dependent[48], our larvae at 9 dpf are likely delayed in development which might explain our higher cell numbers. We are confident about our numbers of counted crypt cells since they originate from 40 larvae (80 olfactory epithelia) compared to 6 specimens (12 olfactory epithelia) used in the study of Sandulescu and colleagues. Thus, the crypt cell population likely grows linearly until the critical period of imprinting to ensure an adequate amount of cells expressing specific receptors for binding of kin-specific ligands.

Together, these results provide the first direct evidence that clearly crypt cells play a role in detecting a kin odor related signal. They also harbour the possibility that a subpopulation of mOSNs might be involved in kin recognition. The data show that the total numbers of S100 positive mOSNs and crypt cells do not differ between imprinted and non-imprinted fish. Furthermore, there are different quantitative patterns of how cOSNs, mOSNs and crypt cells are activated in response to food and non-kin (conspecific) odor.

Methods

Study animals and rearing conditions. Adult zebrafish wildtype were obtained from different commercial breeding facilities (Germany, Vietnam, Sri Lanka) and maintained in 3 liter aquaria per breeding pair at 26 °C under a 13 h:11 h light:dark cycle. Fish were fed daily, alternating with commercial flake food, *Artemia salina* and white mosquito larvae. For breeding spawning trays were used. Eggs were kept in E3 medium[49] in an incubator at the same temperature and light conditions as the adults. Larvae hatched at 3-4 day post fertilization (dpf). After depletion of the yolk (on 5 dpf) larvae were fed with commercial fry food and *Paramecium spec*. Eggs and larvae were reared according to experiment conditions (see Fig. 1).

Animal Use and Care Protocols were approved by the Institutional Animal Care and Use Committee of the University of Oldenburg and the government of the state Niedersachsen, Germany (18.01.2013-17.01.2016). All experiments were carried out in accordance with the approved guidelines. After the experiment, larvae were killed by an overdose of MS222 (see below).

Odor choice test. Olfactory preference tests were conducted in a two-channel choice flume (Fig. 7A) with a steady driven flow (30 ml/min per channel; approx. 2.5 cm/sec) generated by a peristaltic pump. Regular dye tests ensured that the flume maintained two distinct parallel-flowing water masses (A and B), which remained entirely separated up to the downstream mesh screen.

For the tests, single fish were placed into the flume with both water sources (kin odor and non-kin odor) running and were allowed to acclimate and swim freely. The test period started directly after the fish experienced both water masses (i.e. entered once both A and B).

We recorded the position of the fish's head and nose in one or the other water flow every 10 s during two 2-min periods separated by a 1.5-min transition period to switch water sources as a control for possible (non-olfactory) side bias of the fish. If the larvae swam directly at the center line between both water masses, the location would be recorded as 'unclear' and excluded from the analysis. The tests were run blind, so that the observer did not know on which side the respective odor stimulus was delivered. Olfactory preference is expressed as the percentage of observations spent in kin odor minus non kin odor stimulus. A random distribution across water masses (zero difference) is expected if a fish did not express a preference for one of the odor stimuli; a negative value indicates a preference for non-kin odor, and a positive value for kin odor (Fig. 7B,C).

Kin odor was created by keeping 25 full siblings of the test fish in 250 ml odorless E3 medium overnight, which then was filled up to 5 l (5 larvae/l). Larvae of genetically different families were used to create the non-kin (conspecific) larvae stimulus.

Stimulation experiments. *Validation of pERK as a marker for olfactory sensory neuron activity.* Larvae were reared in a group of full siblings (Fig. 1a). At the age of 9 dpf they were olfactory stimulated either with a non-kin conspecific odor mix (generated from three non-related larvae batches of the same age), food odor (generated from commercial flake food and *Paramecium spec.*), or E3 medium. While stimulated for 3, 7, 11, or 15 minutes, single larvae were kept in small glass beakers in a calm environment.

Kin odor test I. For the first kin odor stimulation experiment larvae were either reared in olfactory isolation to suppress the imprinting process or in a group of full siblings to evoke imprinting on kin (Fig. 1b). For olfactory isolation single eggs were reared in small glass beakers. At 9 dpf larvae were olfactory stimulated. Thus, single larvae were placed into small glass beakers containing pure E3 medium and were allowed to acclimate for 20 minutes. Afterwards, the olfactory stimulus, either kin odor or E3 medium, was added for 7 minutes. To make sure imprinting was successful, some of the group reared larvae underwent the odor choice test at the age of 11 dpf as described above.

Kin odor test II. Larvae were reared in isolation and either visually and olfactory exposed (imprinted) or only visually exposed (non-imprinted) to their kin. Thus, single eggs were placed into small glass beakers. Glass beakers were placed into a larger dish containing 12 eggs from the same batch (Fig. 1c). Larvae that were allowed to imprint on their kin were olfactory stimulated with kin odor at 5 dpf in the evening, at 6 dpf in the morning, noon, and evening and at 7 dpf in the morning. Whereas those larvae, in which imprinting was prevented, were exposed to E3 medium instead of kin odor at corresponding time points. Both groups were able to see their kin during the entire experiment.

Prior to the olfactory stimulation, larvae were placed into fresh glass beakers containing E3 medium and were allowed to acclimate for one hour. Thereafter, they were stimulated for 7 minutes either with kin water or E3 medium. Stimulation took place at 9 dpf. Some of the larvae that were allowed to swim freely in the larger dish, surrounding the small glass beakers, were tested for olfactory preference.

Tissue preparation and immunohistochemical processing. Larvae were killed with an overdose of tricaine methanesulfonate (MS222; Sigma-Aldrich) in E3 medium and cut in halfes. Heads were fixed with cold 4% PFA overnight and tails transferred into 99% ethanol for later genotyping. Following cryoprotection in 30% sucrose solution overnight, heads were embedded in Tissue-Tek (tissue freezing medium, Leica Jung) and horizontal cryosections of 14 µm thickness were thaw mounted onto Superfrost Plus slide glasses (Thermo Scientific).

Incubations were done in a humid chamber. After washing off TissueTek in PBS, cryosections were incubated in 100% MeOH for 10 minutes at −20 °C, washed several times in PBT and blocked in blocking buffer (2% normal donkey serum, 0.1% fish gelatine, 0.5% Tween 20, 0.5% Triton X-100 in PBS) for 1 h at room temperature. Double labeling with two primary antibodies from same host species, Fab-Fragments (CyTM3-conjugated AffiniPure Fab Fragment, dk-anti-rb IgG (H + L), 1:100 dilution, Jackson Immuno Research) were used.

Slides were incubated with first primary antibody (rabbit anti-pERK, 1:200 dilution, Cell Signaling) diluted in blocking buffer for 2 days at 4 °C followed by incubation with Fab Fragments overnight at 4 °C. Afterwards, slides were incubated with the second primary antibody (rabbit anti-S100, 1:600 dilution, Dako) diluted in blocking buffer for 2 days at 4 °C, following incubation with the second secondary antibody (Alexa 488 anti rabbit, 1:400 dilution, Dianova) diluted in blocking buffer for 2 h at room temperature. Finally, sections were washed in PBT and counterstained with DAPI (40−6-diamidino-2-phenylindole; 1:1000 dilution, Carl Roth) and mounted with Vectashield (Vectorlabs) and coverslipped.

Confocal microscopy. Optical sections were acquired with a Leica TCS SP-5 confocal laser-scanning microscope (Leica Microsystems). All microscopic images used in this study were processed to RGB stacks and projections by using ImageJ and slightly adapted for brightness and contrast with either ImageJ or Corel PHOTO-PAINT. Photographic plates were mounted and further processed into figures with CorelDRAW 12.0 (Corel Corporation).

Quantification of activated cells. Stacks of olfactory epithelia were analyzed by using the RoiManager tool of ImageJ. Activated cells (pERK+) were identified according to accepted criteria for OSNs as follows:

Position of cell-soma: basal for ciliated-, intermediate for microvillous- and superficial for crypt- cells. Shape of cell-soma: ciliated OSNs, stout with one long dendrite towards luminal surface; microvillous OSNs, somewhat elongated with basal and superficial dendrite; crypt cells, round with acentric nucleus, no dendrites, but superficial indentation. In addition to the mentioned criteria, the calcium binding protein S100 was used to label crypt cells and a small subpopulation of microvillous cells (as previously described)[26].

Cell counting for statistical analysis was performed blind, by two observers unknowingly which specimen (imprinted/non-imprinted) and stimulus (control/kin odor) they were evaluating.

Statistical evaluation. To study the effect of exposure duration on the activity of cOSNs, mOSNs and crypt cells (Fig. 2a–c) the number of pERK+activated cells was counted and a Kruskall-Wallis test was performed (H(2): Chi square value; p: significance value; n: sample size). Differences in pERK activation of cOSNs were first analyzed within the group of food stimulated larvae between different stimulation times. The same test was performed with non kin-larval odor and control stimulation. The activity of mOSNs and crypt cells was analyzed likewise.

Data of different stimuli duration were pooled (Fig. 3) due to the fact that no duration-dependent differences in pERK activation was found. A Kruskall-Wallis test was used to analyze differential activation of cOSNs by stimulation with food, larval odor and control. Followed by a pairwise Mann-Whitney U test including Bonferroni correction for multiple comparison (U: Mann-Whitney U value; Mdn: median). The same procedure was implemented for mOSNs and crypt cells.

In kin odor tests I and II (Figs 5 and 6) the cell quantity of S100-positive (s100+) mOSNs and crypt cells between imprinted and non-imprinted larvae (Figs 5a and 6a) was tested by using a pairwise Mann-Whitney U test. A Kruskall-Wallis test followed by a Mann-Whitney U test including Bonferroni correction was used to analyze the activation of S100+mOSNs (Figs 5b and 6b) and crypt cells (Figs 5c and 6c) between imprinted and non-imprinted larvae either stimulated with kin odor or control. S100-negative OSN numbers were tested likewise (Figs 5d and 6d). Note that only S100 negative mOSNs and cOSNs exist, but no crypt cells.

Olfactory preference is expressed as a preference index (Fig. 7B,C). The percentage of time the larvae spend in kin odor was subtracted by the percentage of time spend in non-kin odor. A Wilcoxon signed-rank test (Z: Wilcoxon signed-rank value) was performed to test whether the preference index differs significantly from zero.

All analyses are two-tailed and were done in IBM SPSS statistic 23 for windows.

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Author Contributions

The study was designed by D.B., K.T., G.G. and M.F.W. The behavioral experiments were performed by D.B. and K.T. (Oldenburg). The immunohistochemical processing was performed by D.B (Munich). Analysis of data was done by D.B., K.T., G.G. and M.F.W. The first version of the manuscript was written by D.B. and K.T. The final version was written by D.B., K.T., G.G. and M.F.W. D.B. and K.T. share first authorship.

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Identification of accessory olfactory system and medial amygdala in the zebrafish

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Zebrafish larvae imprint on visual and olfactory cues of their kin on day 5 and 6 postfertilization, respectively. Only imprinted (but not non-imprinted) larvae show strongly activated crypt (and some microvillous) cells demonstrated by pERK levels after subsequent exposure to kin odor. Here, we investigate the olfactory bulb of zebrafish larvae for activated neurons located at the sole glomerulus mdG2 which receives crypt cell input. Imprinted larvae show a significantly increased activation of olfactory bulb cells compared to non-imprinted larvae after exposure to kin odor. Surprisingly, pERK activated Orthopedia-positive cell numbers in the intermediate ventral telencephalic nucleus were higher in non-imprinted, kin odor stimulated larvae compared to control and to kin-odor stimulated imprinted larvae and control. Moreover, DiI tracing experiments in adult zebrafish show a neuronal circuit from crypt/microvillous olfactory sensory neurons via dorsomedial olfactory bulb and intermediate ventral telencephalic nucleus (thus, arguably the teleostean medial amygdala) to tuberal hypothalamus, demonstrating for the first time an accessory olfactory system in teleosts.

Bony and cartilaginous fishes have a single main olfactory epithelium (MOE). Thus, in contrast to most tetrapods, they lack an additional vomeronasal organ[1–3]. Nevertheless, teleost fishes have in addition to ciliated olfactory sensory neurons, which are typical for the tetrapod main olfactory epithelium, also microvillous olfactory sensory neurons, which in tetrapods reside in the vomeronasal organ. These fish microvillous olfactory sensory neurons are intermingled with ciliated olfactory sensory neurons within the unitary main olfactory epithelium[4,5]. The presence of two olfactory sensory neuron types matches with receptor class and associated G-proteins reported in mammals. Teleost olfactory receptors (ORs) and trace amine receptors (TAARs) associated with Gαolf are located on the cilia of ciliated olfactory sensory neurons, while V1Rs (ora) associated with Gαi, and V2Rs (OlfC) associated with Gαo are present on microvilli of microvillous olfactory sensory neurons both in mammals and teleosts[3,6,7]. However, in goldfish a few microvillous olfactory sensory neurons also show Gαi-3 or Gαq[5], while in catfish, microvillous olfactory sensory neurons are associated with Gαq/11[4]. Furthermore, teleosts as well as cartilaginous fishes have a third class of olfactory sensory neurons, the crypt cells[8–10]. In teleosts, they carry the V1R type receptor ora4, associated with Gαi[11] in zebrafish, but both Gαo and Gαq in goldfish[5] and Gαo in catfish[4]. Within the teleostean olfactory epithelium, the cell somata of olfactory sensory neurons are typically positioned at different depths, with ciliated olfactory sensory neurons somata lying basally, crypt olfactory sensory neurons superficially and microvillous olfactory sensory neurons interspersed at intermediate depths. A recently discovered fourth olfactory sensory neuron type, the cap (kappe) cell, lodges also rather superficially in the zebrafish main olfactory epithelium; its receptor type is not known, but cap cells are associated with Gαo[12]. An overview of zebrafish olfactory receptor and G -protein classes in comparison to mouse is shown in Fig. 1B.

In the zebrafish, tracing of projections from olfactory sensory neurons using transgenic labelling of olfactory marker protein (OMP) visualized by RFP for ciliated olfactory sensory neurons and transient receptor potential channel 2 (TRPC2) visualized by Venus for microvillous olfactory sensory neurons[13], as well as using calcium binding protein immunotracing[10], indicate that ciliated olfactory sensory neurons terminate in anterior

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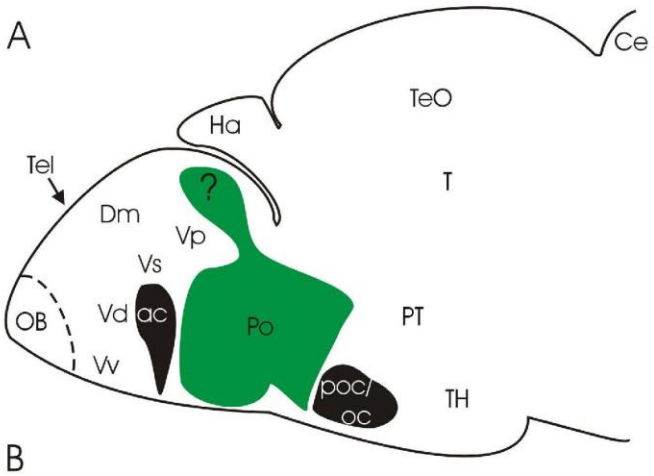
A



B

OSN type	Receptor / G-protein	zebrafish	mouse
ciliated OSNs	ORs + $G\alpha_{olf}$	✓	✓
	TAARs + $G\alpha_{olf}$	✓	✓
microvillous OSNs	V1R + $G\alpha_{i}$	✓	✓
	V2R + $G\alpha_{o}$	✓	✓
crypt cells	V1R + $G\alpha_{i}$ (ora4)	✓	✗
kappe neurons	? + $G\alpha_{o}$	✓	✗

Figure 1. (**A**) The distribution of the transcription factor Otpa (shown in green) in the preoptic region and a posterior ventral telencephalic region (the latter indicated by a question mark) shown in a sagittal view of a 5 dpf zebrafish larva (drawn after[29]). Major telencephalic regions treated in the Introduction are schematically indicated. (**B**) Comparison of receptor molecules and associated G proteins on olfactory sensory neurons in zebrafish and mouse. See text for more details and references. Abbreviations: ac: anterior commissure; Ce: Cerebellum; Dm: medial zone of dorsal telencephalic area (pallium); Ha: Habenula; Po: preoptic region; poc/oc: postoptic commissure/optic chiasma; OB: olfactory bulb; PT: posterior tuberculum; T: midbrain tegmentum; TH: tuberal hypothalamus; Tel: telencephalon; TeO: tectum opticum; Vd, Vp, Vs; Vv: dorsal nucleus, postcommissural nucleus, supracommissural nucleus and ventral nucleus of ventral telencephalic area (subpallium).

dorsal and ventromedial olfactory bulb glomeruli and that microvillous olfactory sensory neurons terminate in ventrolateral bulb glomeruli. Our own studies using calcium binding protein immunoreactivity[10] showed furthermore that the posterior dorsomedial bulb area receives massive additional parvalbumin-positive projections from microvillous olfactory sensory neurons not visualized in the TRPC2-Venus transgenic line (and also not in the OMP-RFP line). Furthermore, crypt cell axons terminate in one single glomerulus within this mediodorsal area[9,10,14]. Secondary olfactory bulb projections, also using transgenic line labelling as well as tract tracing indicate some common projection targets of the entire olfactory bulb (posterior zone of dorsal telencephalon, ventral nucleus of ventral telencephalon), whereas more posterior ventral telencephalic areas (postcommissural nucleus of ventral telencephalon) and the habenula have been implied only to receive inputs from the mediodorsal area[15,16].

The tetrapod amygdala is a complex structure composed of pallial and subpallial areas[17]. The medial amygdala is part of the subpallium and receives input from the tetrapod vomeronasal organ. Considerable progress has been made to differentiate between subpallial and pallial telencephalic areas in teleosts[18–21] which is particularly critical for recognizing their relative contributions to the amygdala in teleosts. The teleostean medial zone of the dorsal telencephalon is generally considered to contain the homologue of the pallial amygdala[21–23]. There is also good evidence for recognizing in the zebrafish ventral telencephalic region (i.e. the subpallium) the septum (ventral nucleus of ventral telencephalon) and the basal ganglia (dorsal nucleus of ventral telencephalon)[24]. However, the more posterior subpallial nuclei, the supracommissural and postcommissural nuclei might correspond to the subpallial amygdala[24]. In the goldfish, but not in the zebrafish, an even more posterior subpallial region, the intermediate nucleus of ventral telencephalic area has been recognized as a separate olfactory bulb projection target in addition to other ventral telencephalic nuclei[25]. However, none of these posterior ventral telencephalic regions (postcommissural, supracommissural, intermediate nuclei of ventral telencephalon) has unequivocally been recognized as the teleostean medial amygdala.

The transcription factor Orthopedia (Otp) has various roles in zebrafish brain development. It is for example involved in the generation of neuropeptidergic cells of the supraopto-paraventricular region (SPV;[26–29]) and in the development of dopaminergic cells with long distance projections to spinal cord and to striatum in both mouse and zebrafish[30,31]. In embryonic mice, the SPV area seemingly contributes *otp*-expressing cells to the medial amygdala[32]. In accordance with their origin in the SPV, these cells are not GABAergic themselves, but become located in a principally GABA cell producing subpallial territory of the medial amygdala[33,34]. Similarly, in the zebrafish a dorsomedial stream of Otpa-positive cells (shown with immunohistochemistry) is seen to extend into a posterior domain of the telencephalon[29] (Fig. 1A). If this Otpa-positive region receives secondary olfactory input, it might correspond to the intermediate nucleus of the ventral telencephalon described in the goldfish brain[25] and, thus, represent the teleostean medial amygdala.

Zebrafish larvae imprint on visual and olfactory cues of their immediate kin (siblings from same batch) on day 5 and 6 postfertilization (dpf), respectively[35–37] resulting in their ability to discriminate kin from non-kin later in life (kin recognition[37]). We use the term imprinting exclusively within this behavioral context. However, imprinting and resulting kin recognition does not occur in larvae that have experienced non-kin cues during the imprinting phase suggesting a predisposition for kin odor[38] (see in this citation for details on the production of kin odor in our experiments). We previously analyzed which type(s) of olfactory sensory neuron(s) are activated by various olfactory stimuli using the activity marker phosphorylated Extracellular Signal Regulated Kinase (pERK) after stimulating imprinted zebrafish larvae with either non-kin conspecific odor or food odor[39]. While food odor activated both ciliated and microvillous olfactory sensory neurons, only the latter were activated by conspecific odor, but crypt cells showed no activation to both stimuli. Furthermore, tests with imprinted and non-imprinted zebrafish larvae (full siblings) for kin odor detection showed that crypt cells (and likely a subpopulation of microvillous olfactory sensory neurons, but not ciliated olfactory sensory neurons) were strongly activated only in imprinted fish and, thus, may play a role in detecting a kin odor related signal[39]. Thus, the sole olfactory bulb glomerulus mdG2 which receives crypt cell input and its likely next synaptic target, the postcommissural nucleus of the ventral telencephalon/intermediate nucleus of ventral telencephalon, might show enhanced or changed activity after kin odor stimulation.

In order to show that the posterior ventral telencephalic region called intermediate nucleus of ventral telencephalon corresponds to the zebrafish medial amygdala, we have used the following two approaches.

1. We investigated whether the known crypt cell projection target in the olfactory bulb (mdG2, identified with S100) and its likely next target, the intermediate nucleus of the ventral telencephalon (identified by Otpa) show increased or changed activity after kin odor stimulation in imprinted compared to non-imprinted zebrafish larvae by using an assay for pERK as performed successfully previously for the olfactory epithelium[39].

2. We also performed tracing experiments in adult zebrafish to show the pathway from crypt cells via the mediodorsal olfactory bulb (incl. mdG2) into the Otpa-positive intermediate nucleus of the ventral telencephalic area, and from it to the tuberal hypothalamus, demonstrating an accessory olfactory system in teleosts.

In conclusion, we suggest that the intermediate nucleus of the ventral telencephalon is the homologue of the tetrapod medial amygdala based on its topology, transcription factor expression (Otpa), its neuronal circuitry (i.e. part of the accessory olfactory system) and, possibly, its changed activity after kin odor stimulation in non-imprinted larval fish (see Discussion).

Results

Neuroanatomical and neurochemical analysis. Sagittal sections of larval zebrafish immunostained for Otpa previously showed that Otpa-positive cells extend from the major Otpa expression domain in the neuroendocrine supraopto-paraventricular region into the posterior telencephalon (Fig. 1A; redrawn from data shown in ref. 29). This is the only Otpa-positive area in the entire telencephalon and is identified here as the intermediate nucleus of the ventral telencephalon which receives secondary olfactory (bulb) projections in the closely related

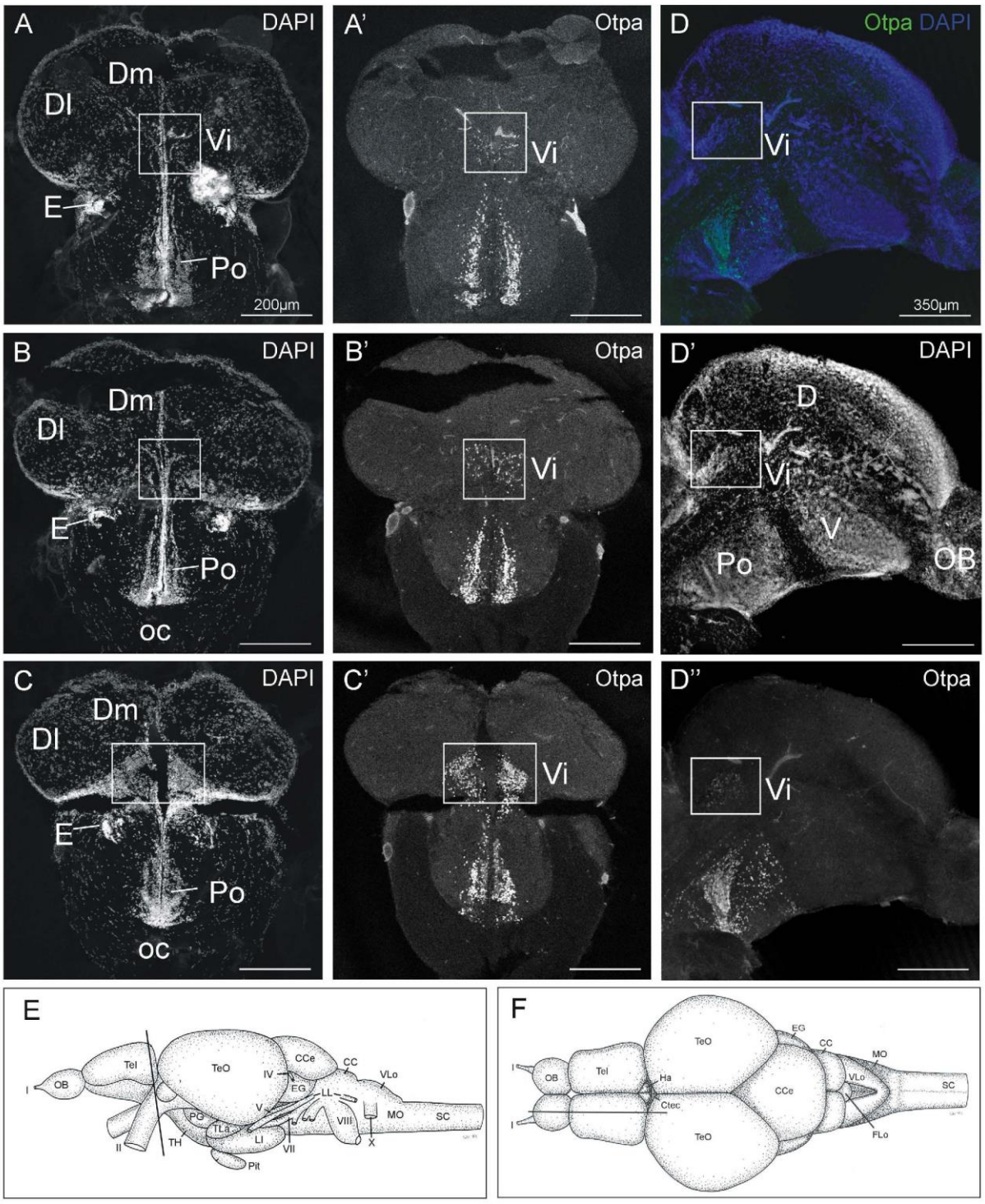


Figure 2. Neuroanatomical analysis and identification of the intermediate nucleus of ventral telencephalon using (**A–C,D,**D') nuclear stain (DAPI) and (A'–C',**D**,D") immunohistochemistry for Otpa. (**E**) Lateral view of adult zebrafish brain shows level of sections shown in (**A-C,**A'–C'). (**F**) Dorsal view of adult zebrafish brain shows level of sections shown in (**D**–D"). Abbreviations: CC: crista cerebellaris; CCe: corpus cerebelli; Ctec: commissura tecti; D: dorsal telencephalic area; Dl: lateral zone of dorsal telencephalic area; Dm: medial zone of dorsal telencephalic area; EG: eminentia granularis; OB: olfactory bulb; LI: hypothalamic lobus inferior; LL: lateral line nerves; MO: medulla oblongata; MS: medulla spinalis; oc: optic chiasma; Pit: pituitary; Po: preoptic region; SC: spinal cord; TeO: optic tectum; TH: tuberal hypothalamus; TLa: torus lateralis; V: ventral telencephalon; Vi: intermediate nucleus of ventral telencephalon; VLo/LX: vagal lobe. I: olfactory nerve; II: optic nerve; IV: trochlear nerve; VII: facial nerve; VIII: octaval nerve; X: vagal nerve.

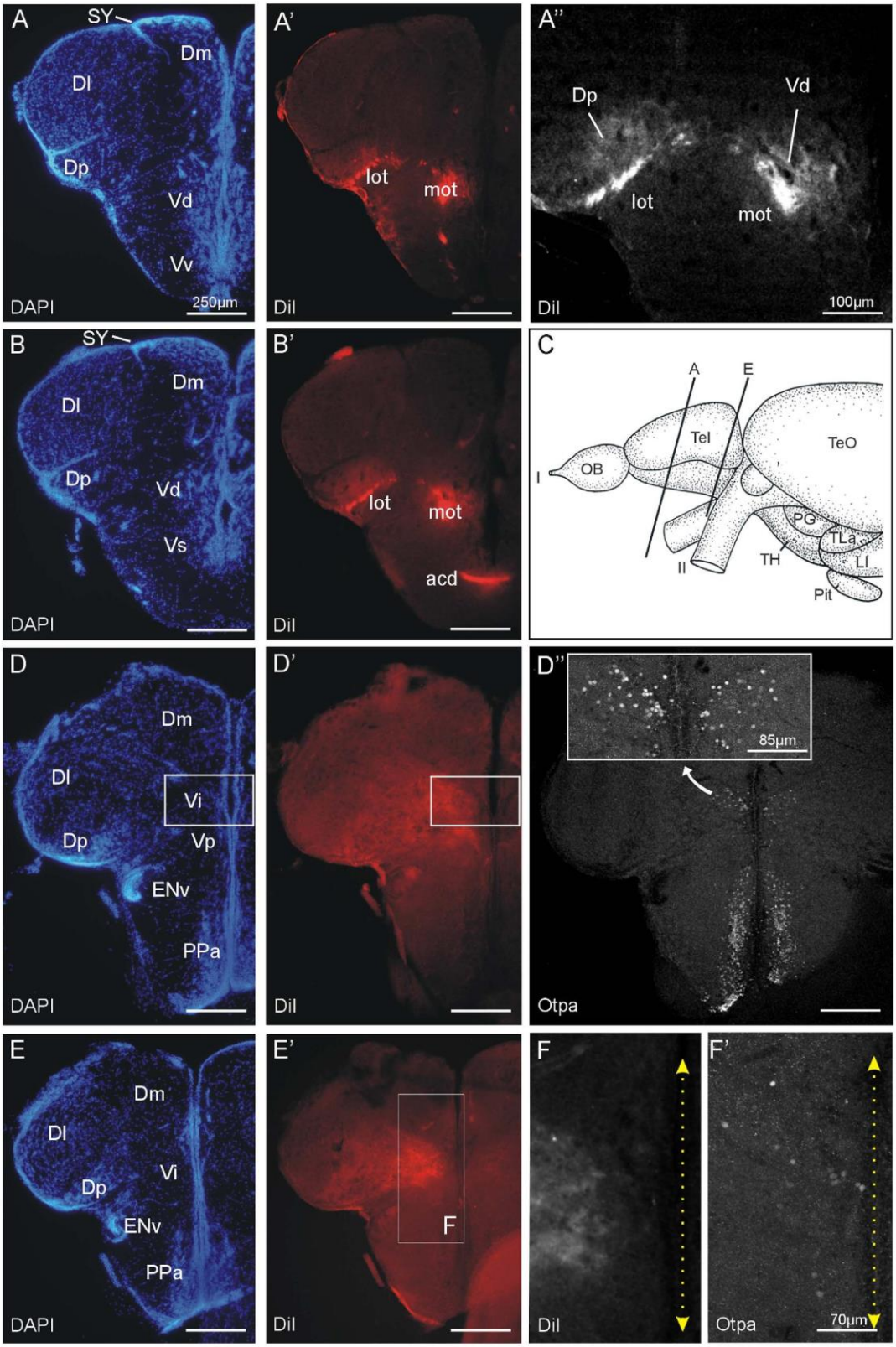


Figure 3. Projections after a unilateral DiI injection into the olfactory bulb of an adult zebrafish shown at four telencephalic levels from anterior (**A,**A') to posterior (**E,**E'), with corresponding DAPI and fluorescent photomicrographs demonstrating tracing results. (A") is a confocal blow-up at anterior levels detailing terminal fields and retrogradely labeled cells in the posterior zone of the dorsal telencephalon (Dp) and the dorsal nucleus of the ventral telencephalon (Vd). (**C**) shows section levels of (**A**) and (**E**). Sections (**B**) and (**D**) are the immediate caudal and rostral sections, respectively, and are not separately indicated. (D") confocal

photomicrograph shows Otpa staining in the preoptic region and the intermediate nucleus of the ventral telencephalon (Vi; insert). (**F**,F') are confocal blow ups of E' showing Di terminals and Otpa-positive cells in Vi. Stippled double arrows indicate midline. Abbreviations: acd: dorsal part of anterior commissure; Dm: medial zone of dorsal telencephalic area; Dl: lateral zone of dorsal telencephalic area; Dp: posterior zone of dorsal telencephalic area; OB: olfactory bulb; ENv: ventral entopeduncular nucleus; LI: hypothalamic lobus inferior; lot: lateral olfactory tract; mot: medial olfactory tract; PG: preglomerular complex; Pit: pituitary; Po: preoptic region; PPa: anterior parvocellular preoptic nucleus; TeO: optic tectum; TH: tuberal hypothalamus; TLa: torus lateralis; Vd: dorsal nucleus of ventral telencephalic area; SY: sulcus ypsiloniformis; Vi: intermediate nucleus of ventral telencephalon; Vp: posterior nucleus of ventral telencephalic area; Vv: ventral nucleus of ventral telencephalic area. I: olfactory nerve; II: optic nerve.

goldfish[25]. Since a similar situation regarding *otp* expression and olfactory input has been reported in the mouse for the medial amygdala[32–34], we undertook the tracing experiments in adult zebrafish brains in order to show that the intermediate nucleus of the ventral telencephalon receives secondary olfactory input. As will be reported below, in addition to the postcommissural nucleus of the ventral telencephalon, also the Otpa-positive intermediate nucleus of ventral telencephalon indeed receives secondary olfactory input from the mediodorsal olfactory bulb region which demonstrates that the intermediate nucleus of the ventral telencephalon qualifies as medial amygdala. The intermediate nucleus of the ventral telencephalon in the adult zebrafish brain lies in the extreme caudal pole of the telencephalon in the position where the telencephalon detaches from the preoptic region. A comparison of DAPI stains and Otpa immunostains visualizes these relationships nicely with various landmarks present at this level, such as the ventral entopeduncular nucleus (Fig. 2). Thus, both in sagittal (Fig. 2D–D") as well as in transverse (Fig. 2A,A',B,B',C,C') adult zebrafish brain sections, the main well known Otpa expression domain known from the preoptic region of larval zebrafish[29] can be seen to extend a thin stalk of Otpa-positive cells into the ventral telencephalic intermediate nucleus of the ventral telencephalon.

Tracing experiments. *Injections into dorsomedial olfactory bulb.* DiI injections into the adult zebrafish dorsomedial olfactory bulb results in massive labelling of medial and lateral olfactory tracts (mot, lot) and of secondary olfactory projections up to the caudal pole of the telencephalon (for an overview see Supplementary Figure S1). The lateral olfactory tract increasingly extends laterally posteriorly and underlies the posterior zone of the dorsal telencephalon issuing many small tracts and terminal fields in dorsal direction into this pallial zone from most anterior to most caudal telencephalic levels (Fig. 3A,A'–E,E'). Furthermore, retrogradely labelled cells are present in the posterior zone of the dorsal telencephalon and the dorsal nucleus of the ventral telencephalon which form the origin of a pallio-bulbar projection (Fig. 3A"). After entering the telencephalon, the medial olfactory tract initially runs laterally to the ventral nucleus of the ventral telencephalon and then remains lateral to the dorsal nucleus of the ventral telencephalon up to commissural levels (see Supplementary Figure S1). The medial olfactory tract may issue terminals to both the ventral and dorsal nuclei of the ventral telencephalon. At commissural levels, some fibers cross the midline via the anterior commissure (Fig. 3B'). At postcommissural levels the medial olfactory tract forms a large terminal field covering the postcommissural and intermediate nuclei of the ventral telencephalon (Vp, Vi; Fig. 3D,D'–E,E',F). The intermediate nucleus of the ventral telencephalon is clearly identifiable at most caudal telencephalic levels where it is detected by Otpa immunohistochemistry subsequently performed on the same sections (3D",F'). There is a dense terminal field of secondary olfactory bulb projections overlying these Otpa-positive cells. More laterally at these caudal levels, the Dp is still visibly covered by fine terminal fields coming from the lateral olfactory tract (Fig. 3E,E').

Injections into tuberal hypothalamus. In order to show that the intermediate nucleus of the ventral telencephalon projects to the tuberal hypothalamus, we performed DiI injections into the area of the anterior tuberal nucleus and lateral hypothalamic nucleus. Because Otpa is also expressed in the tuberal hypothalamus (Fig. 4E,E'), i.e. in the ventral periventricular hypothalamic nucleus (Hv), as well as in the lateral hypothalamic nucleus (LH) and in the midline aspect of the periventricular nucleus of the dorsal hypothalamus (Hd) - but not in its major extent around the lateral hypothalamic recess - we can identify nicely the exact injection site (see two examples, Fig. 4C,C',E"). Many fibers affected by such injections can be traced in anterior direction running laterally to the preoptic area through the preoptic stalk into the telencephalon where they form part of the medial forebrain bundle. The lateral forebrain bundle remains unlabeled (Fig. 4C') after tuberal hypothalamic injections. This fact fits well with anterograde tracing data in the goldfish where it was shown that the medial zone of the dorsal telencephalon (i.e. pallial amygdala homologue) projects via the medial forebrain bundle heavily into the tuberal hypothalamus whereas the lateral zone of the dorsal telencephalon (i.e. hippocampus homologue) projects via the lateral forebrain bundle to targets in the diencephalon other than the tuberal hypothalamus[40].

In our experiments, the DiI labelled fibers can be followed into the postcommissural nucleus of the ventral telencephalon and the intermediate nucleus of the ventral telencephalon where many retrogradely labelled cell bodies are observed (Fig. 4B",D). Interestingly within the pallium, retrogradely labelled cells are also seen in the medial zone of the dorsal telencephalon, but not in the lateral zone of the dorsal telencephalon (Fig. 4B"), which confirms the tracing data mentioned above in goldfish[40]. Immunostains for Otpa on the same DiI section demonstrate the presence of the Otpa-positive intermediate nucleus of the ventral telencephalon (Fig. 4D,D'). It is a hallmark of the mammalian accessory versus the main olfactory system that a part of the olfactory bulb (the accessory one) bypasses the olfactory cortex, but enters a subpallial amygdalar nucleus (the medial one) from which projections reach the tuberal hypothalamus in mammals[17,41]. Since our tracing experiments demonstrate

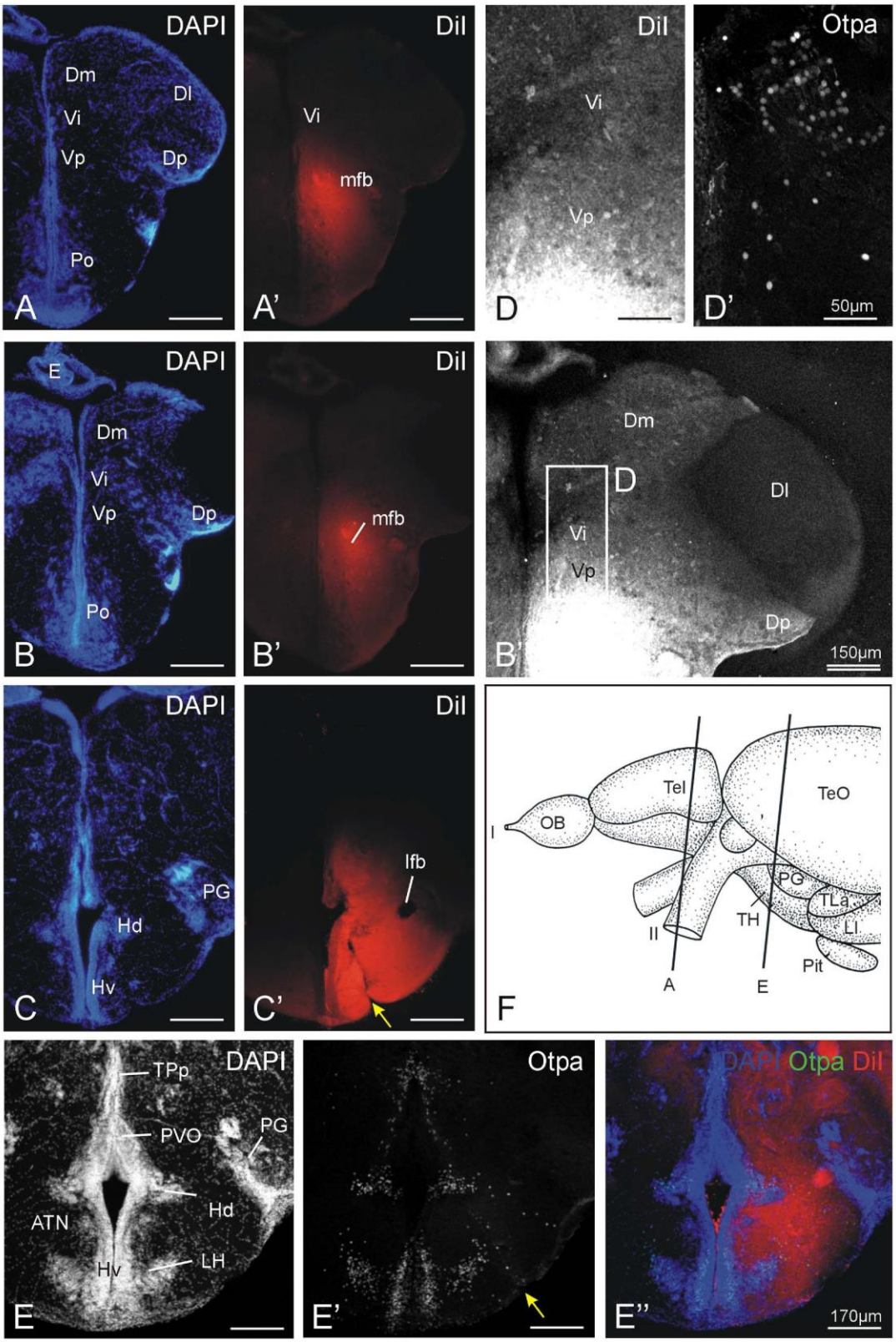


Figure 4. Neuronal connections after a unilateral DiI injection into the tuberal hypothalamus in adult zebrafish shown at three levels from anterior (**A,**A') to posterior (**C,**C'; note yellow arrow at injection site) with corresponding DAPI and fluorescent photomicrographs demonstrating tracing results. (B") Confocal photomicrograph shows retrograde tracing result in the telencephalon at the level of the intermediate nucleus of the ventral telencephalon (Vi). Note also that Dm, but not Dl, has retrogradely labeled cells (see text). (**D,**D') shows confocal blow-up of B" (**D**) and corresponding Otpa stain (D'). (**E**–E") details another injection

site (yellow arrow) which is shown for DAPI, Otpa and DiI in confocal photomicrographs. (**F**) shows section levels of (**A**) and (**E**). Section (**B**) is immediately caudal to (**A**), and section (**C**) is at the same level as (**E**). Abbreviations: ATN: anterior tuberal nucleus; Dm: medial zone of dorsal telencephalic area; Dl: lateral zone of dorsal telencephalic area; Dp: posterior zone of dorsal telencephalic area; OB: olfactory bulb; E: epiphysis (pineal); ENv: ventral entopeduncular nucleus; Hd: dorsal zone of periventricular hypothalamus; Hv: ventral zone of periventricular hypothalamus; lfb: lateral forebrain bundle; LH: lateral hypothalamic nucleus; LI: hypothalamic lobus inferior; lot: lateral olfactory tract; mfb: medial forebrain bundle; mot: medial olfactory tract; PG: preglomerular complex; Pit: pituitary; Po: preoptic region; PPa: anterior parvocellular preoptic nucleus; PVO: paraventricular organ; Vd: dorsal nucleus of ventral telencephalic area; SY: sulcus ypsiloniformis; TeO: optic tectum; TH: tuberal hypothalamus; TLa: torus lateralis; TPp: periventricular part of posterior tuberculum; Vi: imtermediate nucleus of ventral telencephalon; Vp: posterior nucleus of ventral telencephalic area; Vv: ventral nucleus of ventral telencephalic area. I: olfactory nerve; II: optic nerve.

a similar synaptic chain of connections we hypothesize the presence of an accessory olfactory system in teleosts as described in tetrapods leading sequentially via olfactory epithelium, dorsomedial olfactory bulb, and intermediate ventral telencephalic nucleus (i.e., medial amygdala) into tuberal hypothalamus (see Discussion for further reference).

Kin odor stimulation experiment. Two groups of wildtype zebrafish fertilized eggs (siblings) were raised in isolation in separate small glass beakers within two larger dishes each also containing their siblings (see Fig. 5). The latter later provided the visual kin signal to both groups of isolated fish. In contrast, the olfactory kin-related signals were applied during the critical time window individually to the isolated fish only in one group (the imprinted fish) whereas the other group (the non-imprinted fish) received a neutral signal (E3 water; see Fig. 5 and Methods for more details). This experiment has been reported before and demonstrated a role of crypt cells in the detection of kin specific odor by 9 day postfertilization (dpf) old larvae. This was shown through activation in olfactory sensory neurons using a pERK assay[39]. The difference between imprinted and non-imprinted fish was highly significant. However, neuronal activation in postsynaptic central nervous centers such as olfactory bulb and telencephalon had not been investigated in the previous study. Thus, we analyzed here whether there is a difference in activated neuronal cell numbers in the section containing the mdG2 glomerulus in imprinted and non-imprinted larvae (Figs 6 and 7). This section was visualized by using S100 immunohistochemistry which is only present in the olfactory bulb glomerulus mdG2 because all olfactory sensory neuron axons containing it converge there (Fig. 6; see also ref. 10). Similarly, we counted pERK-positive cells in a defined area in the telencephalon section containing the intermediate nucleus of the ventral telencephalon (Figs 8 and 9) which is visualized selectively by Otpa immunohistochemistry (see also previous paragraphs).

Already on first inspection, an olfactory bulb section taken from an imprinted larva tested with kin odor shows more pERK activated cells than the one from a non-imprinted larva tested in the same manner (compare Fig. 6A–A‴ and B–B‴). Cell counting included either all cells in this section or only those in the glomerular layer (GL), the inner cellular layer (ICL) or in the vicinity of mdG2 (Fig. 6A–D). A significant difference between imprinted and non-imprinted larvae (tested for kin odor) in pERK-positive cell numbers is seen for cells surrounding the mdG2 glomerulus and in the inner cellular layer (Fig. 7B–C). Furthermore, also the number of pERK-positive cells of entire sections differs significantly between imprinted and non-imprinted larvae (stimulated with kin odor) (Fig. 7A).

In addition, there is also a highly significant difference in activated cell numbers of imprinted fish tested with kin odor compared to the imprinted control fish (tested with E3 water) for total numbers of pERK bulb cells, as well as for the ICL and the area surrounding mdG2 (Fig. 7A–C). Due to the very conservative Bonferroni correction the significant difference in activated cells for the GL was discarded (for details see legend of Fig. 7D).

Next, activated cell numbers in a restricted telencephalic area defined by Otpa staining, i.e. the area of the intermediate nucleus of the ventral telencephalon (see Fig. 8), were counted, both for all activated (pERK-positive cells) as well as for cells double-labelled for Otpa and pERK (Fig. 9). A significant difference in cell numbers of double-labeled cells for Otpa and pERK between imprinted and non-imprinted larvae, stimulated with kin odor or control stimulus was found (Kruskall-Wallis Test; for details see legend of Fig. 9). The pairwise comparison of cell number revealed no differences in cell number due to a very conservative post hoc test (Mann-Whitney U test and Bonferroni correction; for details see legend of Fig. 9). However, the number of Otpa and pERK double positive cells tends to be increased in non-imprinted fish when tested for kin odor and compared to either control group or imprinted fish tested for kin (Fig. 9, right panel). All other comparisons did not yield differences for cell counts in this experiment.

Finally, we also tested 9 dpf old group-reared larvae taken from the same batch as the larvae used for the stimulation experiments in a 2-channel choice flume (as established in the Gerlach laboratory: see ref. 39) for successful imprinting.

Discussion

The functional roles of teleostean ciliated and microvillous olfactory sensory neurons are somewhat puzzling. Ablation experiments in carp have associated ciliated cells with the alarm reaction response while food sensing has been correlated with microvillous olfactory sensory neurons, and crypt cells with reproduction[42]. Furthermore, while amino acids are sensed by both microvillous olfactory sensory neurons and ciliated olfactory sensory neurons, bile salts, gonadal steroids and prostaglandins are perceived by ciliated olfactory sensory neurons and nucleotides by microvillous olfactory sensory neurons[4,43]. This is somewhat unexpected because

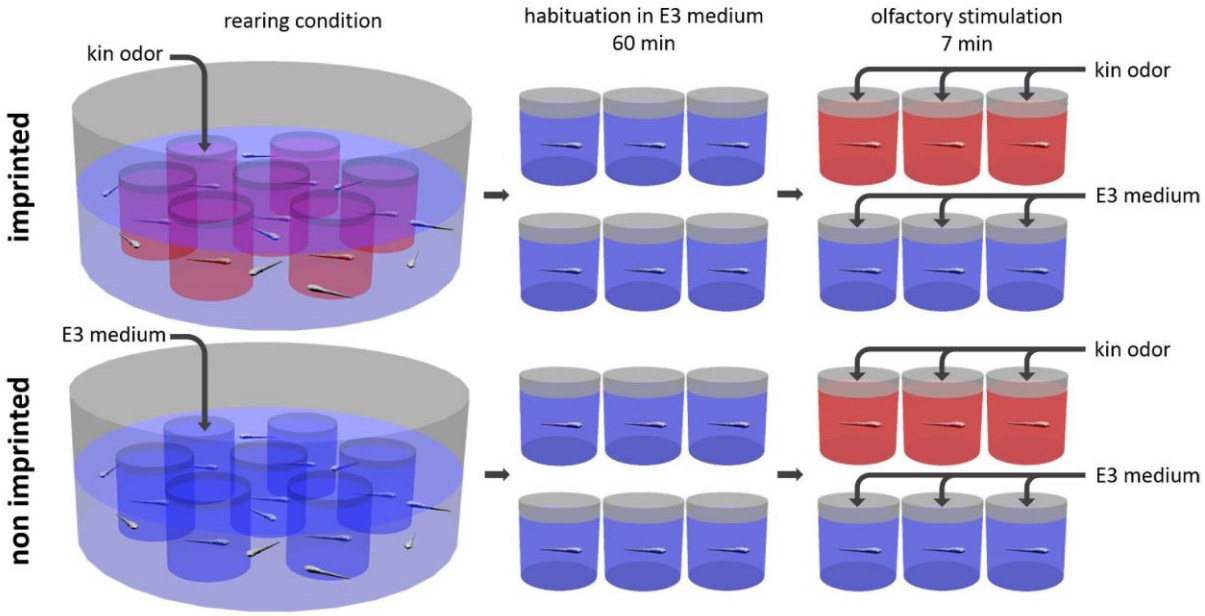


Figure 5. Experimental set-up: Schema shows how imprinted (red) and non-imprinted (blue) larval zebrafish were created and subsequently tested for kin odor activation. Zebrafish larvae were either exposed to kin odor or E3 medium at day 6 and both groups were subsequently tested either for kin odor or E3 medium at 9 days. Then, the larvae were sacrificed immediately after olfactory stimulation and further processed. Previous experiments had established that 7 minutes allow for optimal assay for pERK[39]. E3 medium is a commonly used medium for raising zebrafish eggs[49].

food signals (presumably indicated by nucleotids and amino acids) in mammals are typically sensed by ciliated olfactory sensory neurons.

The function of crypt cells has remained largely unknown (see Discussion in ref. 39). However, it had been established recently in the zebrafish that one receptor is present in all crypt cells (the V1R type receptor ora4 which is associated with Gαi;[11]) and that they project into the single S100-positive glomerulus located in the mediodorsal olfactory bulb, the mdG2[9,10,14] (see Introduction). In addition, we have provided new data on neuronal activation using pERK suggesting a role of crypt cells in kin recognition[39]. These studies involved experimentally raised imprinted and non-imprinted zebrafish larvae (see Fig. 5), which were stimulated at 9 dpf with kin odor and immediately processed for visualization of pERK.

In the present contribution, we provide additional evidence that also the first central nervous processing station of crypt cell projections to the olfactory bulb glomerulus mdG2 is significantly elevated in activity as measured by the numbers of pERK-positive cells. In fact, comparing the section containing the S100-positive mdG2 glomerulus and counting all olfactory bulb cells in imprinted and non-imprinted 9 dpf larvae reveals that imprinted larvae have significantly more pERK activated cells compared to the imprinted control group (tested with E3 water; Fig. 7A). When certain compartments (see Fig. 6C for explanation of compartments) of the sections are analyzed, such as the inner cellular layer (ICL), the glomerular layer (GL), or the area surrounding mdG2, activated cell numbers are highly significantly increased in imprinted kin odor tested larvae versus controls in the area surrounding mdG2 (Fig. 7B) and in the ICL (Fig. 7C). Additionally, significant differences are also seen in activated, pERK-positive cell numbers of kin odor tested imprinted fish compared to non-imprinted kin odor tested fish in the ICL and in the area surrounding mdG2.

The results for the area of the intermediate nucleus of the ventral telencephalon are somewhat unexpected at first sight because no increase in the numbers of pERK activated cells (neither total pERK cells nor additionally Otpa-positive ones) after kin odor stimulation was seen in imprinted zebrafish larvae when compared to controls (Fig. 9, left panel). However, there was a significant difference in the number of activated cells positive for both Otpa and pERK. Cell numbers in **non**-imprinted fish are higher than in imprinted fish and control groups. This might indicate that non-imprinted fish show a neuronal response in the intermediate nucleus of the ventral telencephalon (the medial amygdala) to the unknown kin odor and that this response is alleviated or absent in larvae that had been imprinted. These activity differences in the zebrafish medial amygdala (intermediate nucleus of ventral telencephalon) in comparisons of imprinted and non-imprinted zebrafish larvae are highly exciting and clearly call for further investigation in particular with respect to additional inputs from microvillous cells. As reported before, in addition to the ubiquitous activation of crypt cells, a small subpopulation of microvillous olfactory sensory neurons was also activated by kin odor[39].

These behavioral and neuronal activation experiments compare imprinted and non-imprinted zebrafish larva with respect to their response to kin odor and allow for tracking neuronal activation of the involved olfactory pathways from the sensory epithelium (crypt cells/some microvillous olfactory sensory neurons) central nervous system i.e. olfactory bulb (mdG2) and intermediate nucleus of the ventral telencephalon. Thus our results extend

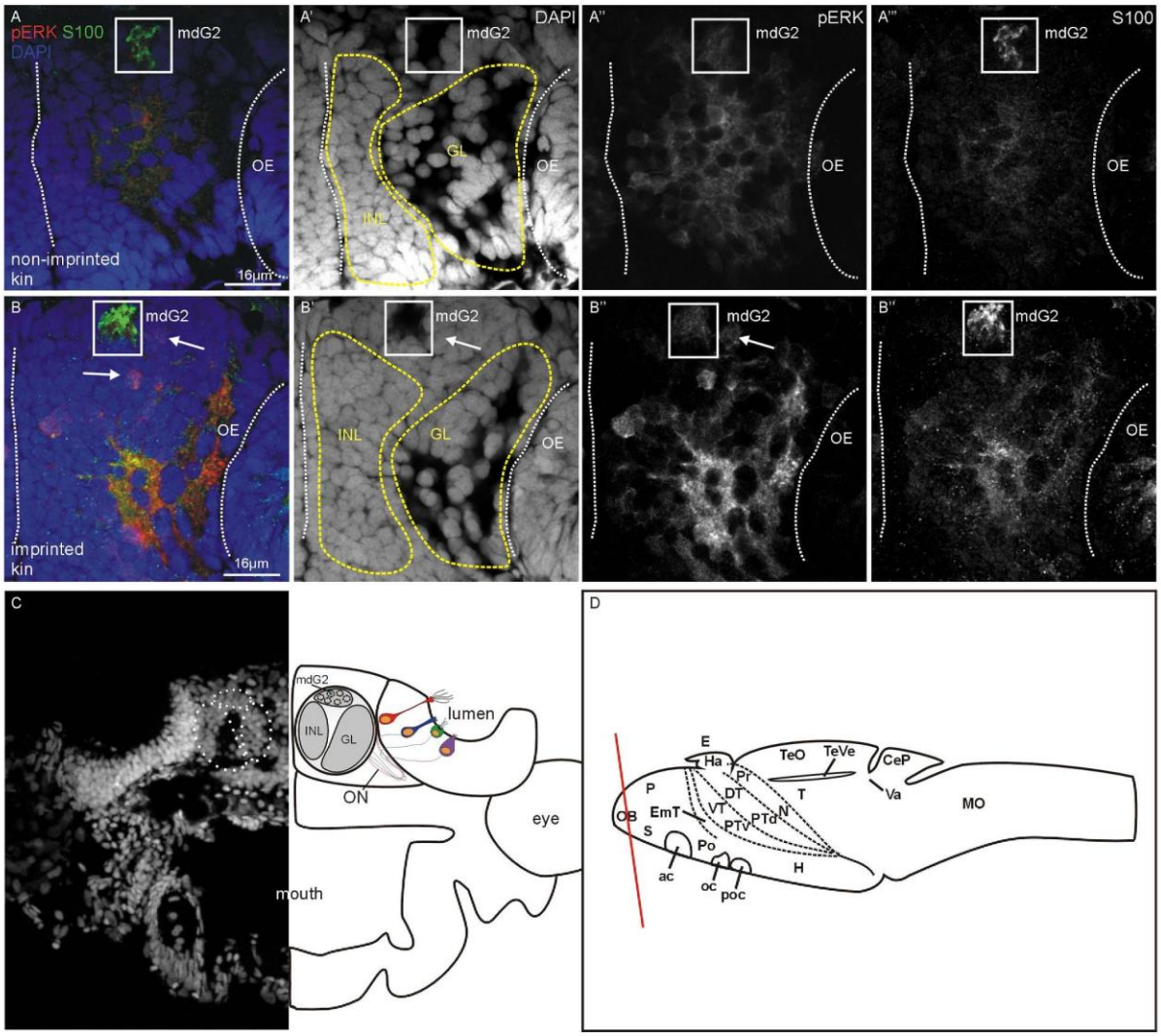


Figure 6. Example of pERK activation in olfactory bulb section containing mdG2 (frame) in imprinted and non-imprinted zebrafish larva. (**A**–A''') confocal photomicrograph of a sectioned imprinted larva. (**B**–B''') confocal photomicrograph of a sectioned non-imprinted larva. Channels comprise in addition to pERK, the nuclear stain DAPI and the calcium-binding protein immunostain S100. (**C**) Shows DAPI (left) and a schema with olfactory bulb fields that were counted (mdG2, INL, GL). (**D**) Larval brain in lateral view shows section level. Abbreviations: ac: anterior commissure; CeP: cerebellar plate; DT: dorsal thalamus (thalamus); E: epiphysis; EmT: eminentia thalami; GL: glomerular layer; H: hypothalamus; Ha: habenula; INL: inner nuclear layer; mdG2: mediodorsal glomerulus 2; MO: medulla oblongata; N: nucleus of the medial longitudinal fascicle; OB: olfactory bulb; ON: olfactory nerve; P: pallium; Po: preoptic region; Pr: pretectum; PTd, PTv: dorsal, ventral part of posterior tuberculum; S: subpallium; T: tegmentum; TeO: optic tectum; TeVe: tectal ventricle; Va: valvula cerebelli; VT: ventral thalamus (prethalamus).

the functional neuroanatomical knowledge about the secondary olfactory circuitry of the mediodorsal olfactory bulb which includes the crypt cell target glomerulus mdG2[9,10,18].

Previous studies had already shown that the zebrafish mediodorsal olfactory bulb receives preferential input from microvillous cells and projects – in addition to olfactory bulb targets common to the entire olfactory bulb (posterior zone of dorsal telencephalon, ventral nucleus of ventral telencephalon) – also to the postcommissural and supracommissural ventral telencephalic nuclei, as well as to the right dorsal habenula (Fig. 10; Vp, Vs, Had;[10,16,44]). Our tracing data presented here demonstrate that the adult zebrafish mediodorsal olfactory bulb area not only projects to the posterior zone of the dorsal telencephalon, as well as to the dorsal, ventral, supracommissural and postcommissural nuclei of the ventral telencephalon, but in addition also to the intermediate nucleus of the ventral telencephalon. We are certain about this projection to intermediate nucleus of the ventral telencephalon because our DiI tracing experiments involved the parallel immunohistochemical visualization of the Otpa-positive cells in the intermediate nucleus of the ventral telencephalon (Fig. 3D-D") which is diagnostic for this nucleus. Since *otp* is also solely expressed in the mammalian medial amygdala within the telencephalon

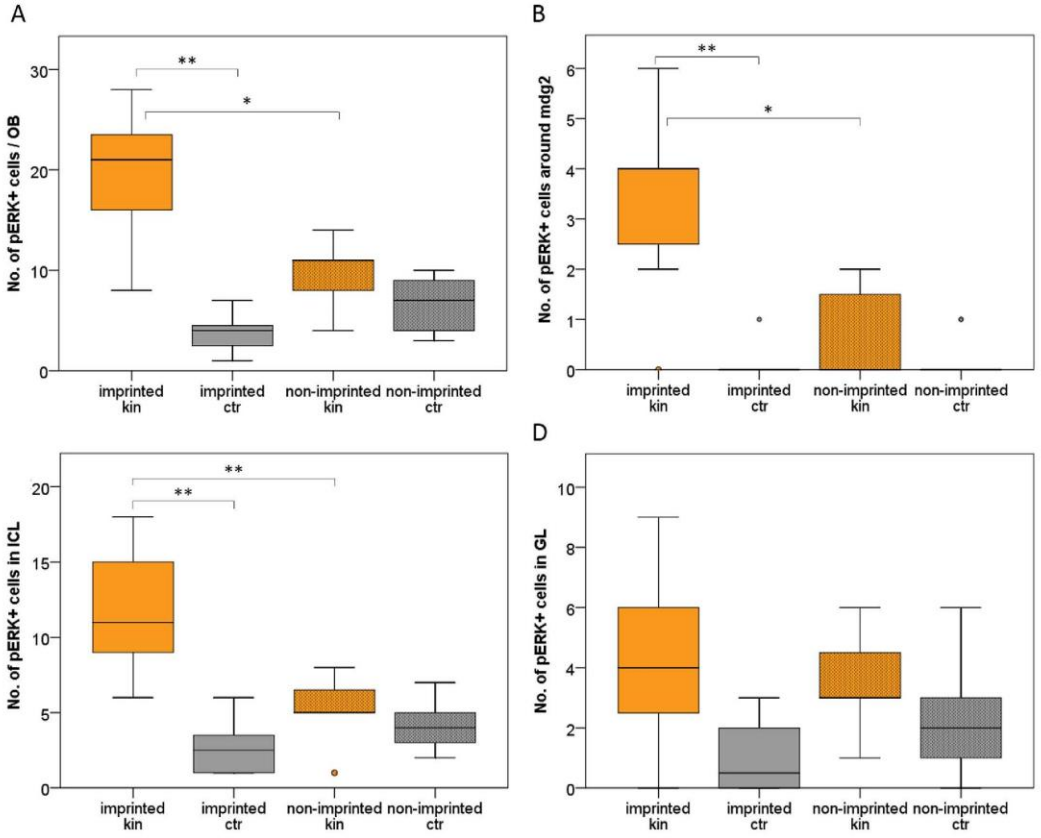


Figure 7. Number of pERK-positive cells in imprinted and non-imprinted zebrafish larvae, stimulated with either kin or control odor, in different olfactory bulb fields. Box plots show median (Mdn), upper and lower quartile and whiskers (maximum interquartile range: 1.5). *Indicates statistical significance p: *$p < 0.05$; **$p < 0.01$. kin = kin odor stimulus; ctr = control stimulus. $n_{imprinted\ kin} = 7$; $n_{imprinted\ ctr} = 8$; $n_{non\text{-}imprinted\ kin} = 7$; $n_{non\text{-}imprinted\ ctr} = 9$. (**A**) Total cell quantity of pERK-positive cells in entire olfactory bulb section at level of mdG2. Number of activated cells is significantly higher in imprinted larvae exposed to kin compared to imprinted larvae exposed to control stimulus (Mann-Whitney $U = 6$, $p = 0.001$, $Mdn_{impr\ kin} = 21$, $Mdn_{impr\ ctr} = 4$). Significant difference in cell number were detected between imprinted and non-imprinted larvae, exposed to kin ($U = 6$, $p = 0.018$, $Mdn_{impr\ kin} = 21$, $Mdn_{non\text{-}impr\ kin} = 11$). (**B**) pERK + cells around mdG2 (see Fig. 6) show a difference in activation between imprinted larvae stimulated with kin odor compared to imprinted larvae exposed to control stimuli ($U = 4.5$, $p = 0.003$, $Mdn_{impr\ kin} = 4$, $Mdn_{impr\ ctr} = 0$) or compared to non-imprinted larvae stimulated with kin odor ($U = 6$, $p = 0.015$, $Mdn_{impr\ kin} = 4$, $Mdn_{non\text{-}impr\ kin} = 0$). The same picture of neuronal activity arises in cells of inner cellular layer (**C**). Cell number differs significantly in imprinted larvae exposed to kin compared to imprinted larvae exposed to control stimulus ($U = 0.5$, $p = 0.001$, $Mdn_{impr\ kin} = 11$, $Mdn_{impr\ ctr} = 3$). pERK + cell number differs between imprinted larvae and non-imprinted larvae exposed to kin ($U = 3$, $p = 0.006$, $Mdn_{non\text{-}impr\ kin} = 5$). (**D**) shows the number of pERK + cells in glomerular layer of the olfactory bulb. A significant difference in pERK-positive cell number was found (Kruskall-Wallis test: $H(2) = 9.357$, $p = 0.025$); but after Mann-Whitney U correction for multiple comparisons (Bonferroni correction; $\alpha = 0.017$) no significant differences could be detected between treatments for glomerular layer pERK + cell numbers ($U = 9$, $p = 0.024$, $Mdn_{impr\ kin} = 4$, $Mdn_{non\text{-}impr\ kin} = 3$).

(see Introduction), we confidently identify the intermediate nucleus of the ventral telencephalon as teleostean medial amygdala. This is further supported by the fact that the intermediate nucleus of the ventral telencephalon projects to the tuberal hypothalamus, more specifically, the region of the ventral periventricular hypothalamic zone (Hv), and the anterior tuberal nucleus and lateral hypothalamic nucleus where our DiI injections were located (Fig. 4C,C,E-E"). The zebrafish Hv has previously been identified as the homologue of the mammalian hypothalamic arcuate nucleus[45].

Such higher order olfactory circuitry is diagnostic in mammals for the accessory olfactory system originating there in microvillous olfactory sensory neurons of the vomeronasal organ and running via the accessory olfactory tract and bulb into the subpallial medial amygdala and from there directly into the tuberal hypothalamus[41] unlike the targets of the main olfactory pathway(s). In fact, a similar situation exists for all tetrapods[17]. What is more, a previous paper in the African lungfish *Protopterus* has shown that a "hidden" accessory olfactory system exists in this basal sarcopterygian clade. Lungfish, like teleosts, have no vomeronasal organ separate from the main olfactory epithelium[46]. However, similar to teleosts, lungfish also have a specialized circuit originating from

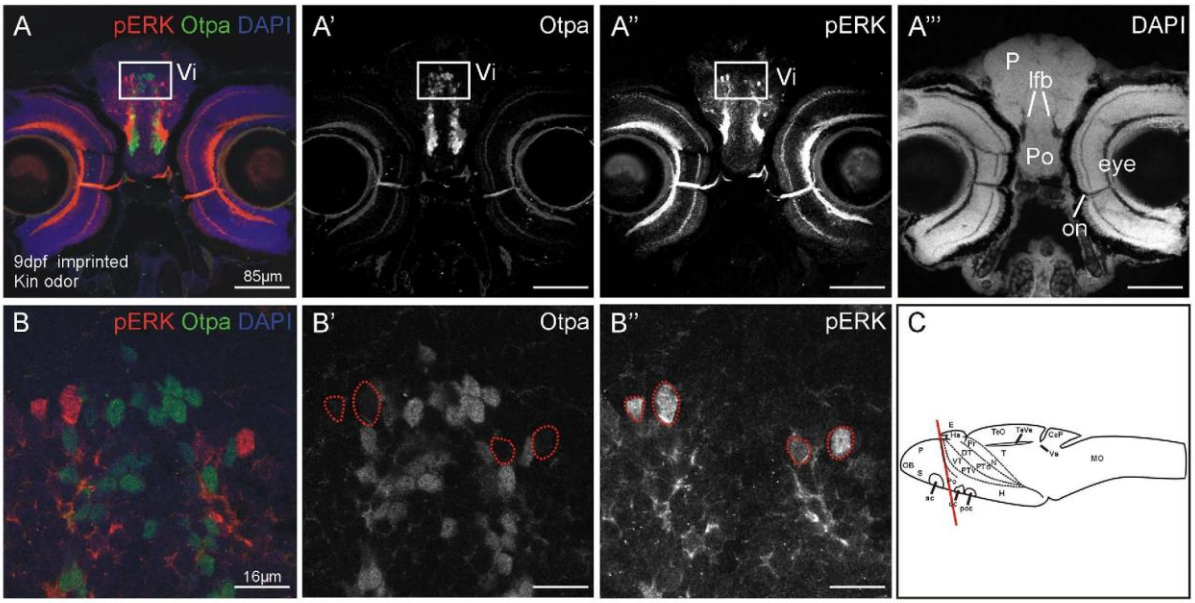


Figure 8. Example of pERK activation and Otpa-positive cells in the intermediate nucleus of the ventral telencephalon (Vi) and indication of the sector that was counted. (**A–A**") Confocal photomicrographs show in addition to pERK, the nuclear stain DAPI and Otpa. (**B**) Higher power details of insert show pERK and Otpa-positive cells in confocal photography in histological material of tested fish. (**C**) Larval brain in lateral view shows section level. Abbreviations: ac: anterior commissure; CeP: cerebellar plate; DT: dorsal thalamus (thalamus); E: epiphysis; EmT: eminentia thalami; GL: glomerular layer; H: hypothalamus; Ha: habenula; INL: inner nuclear layer; lfb: lateral forebrain bundle; mdG2: mediodorsal glomerulus 2; MO: medulla oblongata; N: nucleus of the medial longitudinal fascicle; OB: olfactory bulb; on: optic nerve; P: pallium; Po: preoptic region; Pr: pretectum; PTd, PTv: dorsal, ventral part of posterior tuberculum; S: subpallium; T: tegmentum; TeO: optic tectum; TeVe: tectal ventricle; Va: valvula cerebelli; VT: ventral thalamus (prethalamus).

microvillous "crypts" (containing microvillous cells, not to be confounded with teleostean crypt cells) in the main olfactory sensory epithelium via a special part of the olfactory bulb to medial amygdala and from there to tuberal hypothalamus[46]. This shows, together with our results presented here in the zebrafish, that a dichotomous presence of the main and accessory olfactory system is basal to all bony vertebrates. The condition in cartilaginous fishes remains to be established. However, a possible dichotomy of the olfactory system has recently been reported in agnathans[47,48], although the accessory olfactory system in lampreys may be analogous rather than homologous to the vomeronasal system because it runs from the olfactory bulb directly via the basal diencephalic posterior tuberculum into the rhombencephalon

Thus, the evidence for identifying the intermediate nucleus of the ventral telencephalon as the medial amygdala of teleost fishes is based on the following findings: (A) the intermediate nucleus of the ventral telencephalon lies topologically between pallial amygdala (medial zone of dorsal telencephalon) and the remainder of the subpallial amygdala (supracommissural nucleus of ventral telencephalon, postcommissural nucleus of ventral telencephalon; see Fig. 2). (B) The intermediate nucleus of the ventral telencephalon is the sole expression domain of Otpa in the telencephalon (see Fig. 2). (C) The intermediate nucleus of the ventral telencephalon receives mediodorsal olfactory bulb projections shown with anterograde tracing (see Fig. 3). (D) The intermediate nucleus of the ventral telencephalon projects to the tuberal hypothalamus as shown with retrograde tracing (see Fig. 4). (E) There is phylogenetic continuity (teleosts, lungfish, amphibians, and amniotes) indicating that a medial amygdala exists before the divergence of actinopterygian and sarcopterygian fishes.

In summary, this study shows that imprinted and non-imprinted larval zebrafish differ in neuronal activation after kin odor exposure in three successive synaptic levels along the pathway leading from olfactory epithelium to telencephalon (Fig. 10A). Our tracing experiments furthermore show that an accessory olfactory system indeed does exist in teleosts which originates in crypt and microvillous olfactory sensory cells and runs sequentially via the dorsomedial olfactory bulb and medial amygdala (intermediate nucleus of ventral telencephalon) to the tuberal hypothalamus (Fig. 10B).

Methods

The experimental set-up for creating imprinted and non-imprinted zebrafish larvae and subsequent testing with kin odor (see Fig. 5), followed by histological processing for detection of neuronal activation has been described in detail already[39]. Briefly, we recapitulate these issues below. The tracing study involved different adult zebrafish specimens and the histological processing for visualizing the neuronal tracer as well as the concurrent immunohistological identification of the transcription factor Otpa.

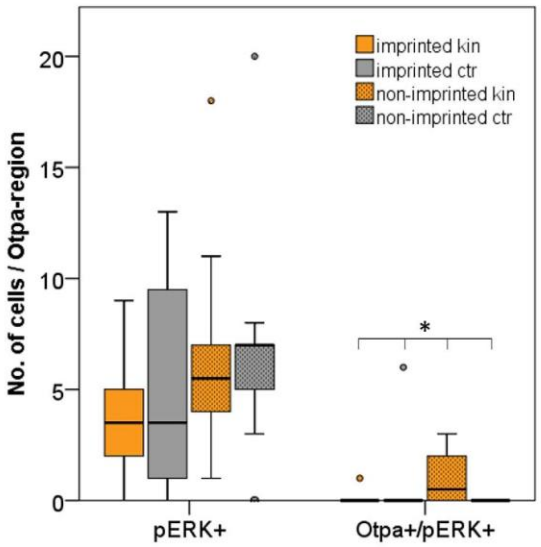


Figure 9. Analysis of pERK activated cell number in a restricted area of telencephalon, defined by Otpa staining. Both, pERK + cells, as well as double-labeled cells for Otpa and pERK were counted and analyzed in imprinted and non-imprinted zebrafish larvae, stimulated with either kin or control odor. Box plots show median (Mdn), upper and lower quartile and whiskers (maximum interquartile range: 1.5). *Indicates statistical significance p: $*p < 0.05$. kin = kin odor stimulus; ctr = control stimulus. $n_{imprinted\ kin}$: 10; $n_{imprinted\ ctr}$: 8; $n_{non\text{-}imprinted\ kin}$: 10; $n_{non\text{-}imprinted\ ctr}$: 9. Total cell quantity of single-labeled pERK-positive cells does not differ significantly between imprinted and non-imprinting larvae, stimulated with either kin odor or control stimulus (Kruskall-Wallis test: $H(2) = 3.78$, $p = 0.295$). Furthermore, pERK-positive and Otpa-positive double-labelled cell quantity was analyzed. A significant difference in cell number was found ($H(2) = 8.579$, $p = 0.035$) between treatments. A Mann-Whitney U test was performed to determine significant differences between two treatments. No significant differences could be detected after performing the Bonferroni correction ($U = 28.5$, $p = 0.044$, $Mdn_{non\text{-}impr\ kin} = 0.5$, $Mdn_{impr\ kin} = 0$; $U = 22.5$, $p = 0.018$, $Mdn_{non\text{-}impr\ kin} = 0.5$, $Mdn_{non\text{-}impr\ ctr} = 0$).

Study animals and rearing conditions. Adult zebrafish wildtype used in Oldenburg were obtained from different commercial breeding facilities (Germany, Vietnam, Sri Lanka) and maintained in 3 liter aquaria per breeding pair at 26 °C under a 13 h:11 h light:dark cycle. Fish were fed daily, alternating with commercial flake food, *Artemia salina* and white mosquito larvae. For breeding spawning trays were used. Eggs were kept in E3 medium[49] in an incubator at the same temperature and light conditions as the adults. Larvae hatched at 3–4 day post fertilization (dpf). After depletion of the yolk (on 5 dpf) larvae were fed with commercial fry food and *Paramecium spec*. Eggs and larvae were reared according to kin odor stimulation experiment conditions (see Fig. 5).

Animal Use and Care Protocols were approved by the Institutional Animal Care and Use Committee of the University of Oldenburg and the government of the state Niedersachsen, Germany (18.01.2013–17.01.2016). All experiments were carried out in accordance with the approved guidelines. After the experiment, larvae were killed by an overdose of MS222 (Sigma-Aldrich).

Adult zebrafish wildtype used in Munich for immunohistochemical and tracing experiments came from the zebrafish facility at the Ludwig-Maximilians-University (LMU) Munich. Zebrafish were kept in a zebrafish housing system (ZebTEC,stand-alone-unit,Tecniplast©) at a temperature of 28 °C and a 12/12 light/dark cycle. Animals used in the tracing study were treated according to the German regulations on Animal Protection (Deutsches Tierschutzgesetz). Tracing experiments conducted in this paper involved no animal experiments in the sense of the German Animal Protection law. We used fixed brain tissue from decapitated adult zebrafish that were killed with an overdose of MS222 (Sigma-Aldrich).

Odor choice test. As described in our previous paper, successful olfactory preference tests were performed on imprinted full siblings of those 9 dpf larvae which were stimulated with kin odor and analyzed for upregulation of pERK in the olfactory epithelium[39] and in the central nervous system (present contribution; see Figs 5 to 9).

Stimulation experiments with kin odor. Imprinted and non-imprinted larvae of the same batch were reared and treated according to our previous study regarding neuronal activation in the olfactory epithelium after kin odor exposure[39].

Tracing Experiments. Adult zebrafish (n = 9) were deeply anesthetized with MS222 (Sigma, Aldrich) before decapitation and fixation of the brains for 48 h in 4% paraformaldehyde (PFA) in Sörensen phosphate buffer (PB). Fine crystals of DiI (D3911; Molecular Probes) were applied with a fine needle to the dorsomedial area of the

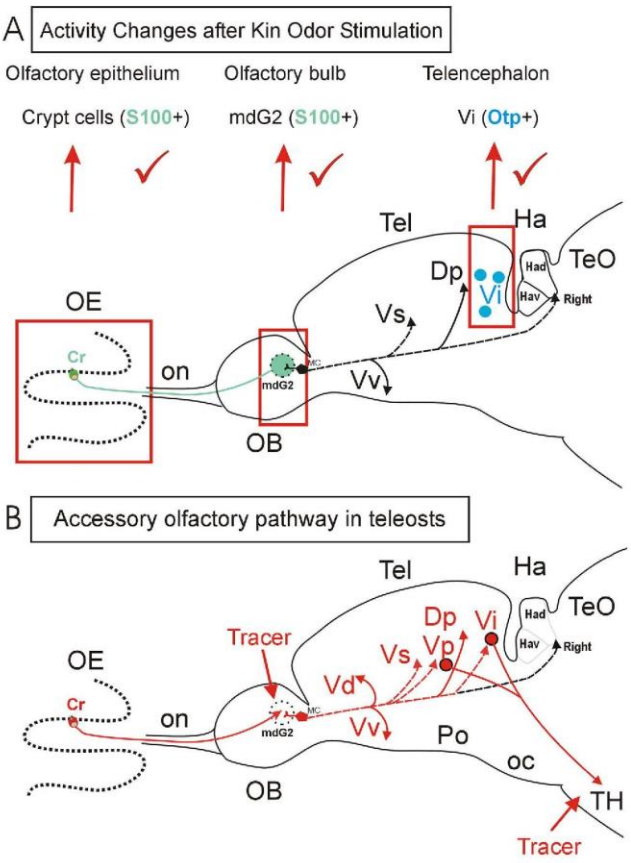


Figure 10. Schema of primary and secondary olfactory pathways in the adult zebrafish. (**A**) Neuronal activity quantified with pERK at three successive synaptic levels from peripheral sensory olfactory sensory neurons to central nervous targets (mdG2, which is immunohistochemically identified with S100 antibody because the projections of S100 immunopositive crypt cells terminate there; Vi, which is immunohistochemically identified with Otpa antibody for many of its cell bodies) after kin odor stimulation of imprinted and non-imprinted larvae. The counted pERK activated cells were located around the mdG2 and within Vi. Red tickmarks indicate significant changes in activated cell numbers seen at each level (see text for details). Higher order (i.e. secondary) olfactory projections of mediodorsal bulb area are indicated with solid black lines (targets shared with projections of entire olfactory bulb) and dashed black lines (targets specifically attributed to mediodorsal bulbar area; see literature below and text for more details). Crypt cells are widely distributed over the entire olfactory epithelium. (**B**) Projections of adult zebrafish mediodorsal olfactory bulb area (incl. mdG2) as shown in the present paper using the lipophilic tracing substance DiI (red lines). Tracer injections into tuberal hypothalamus (TH) demonstrate also a teleostean accessory olfactory pathway via Vp/Vi. Arrowheads indicate where a projection terminates. Olfactory bulb projections shown as dashed lines to telencephalic targets are selective for mediodorsal olfactory bulb (present paper and additional data from[9,10,13–16,44]). Abbreviations: Cr: crypt cells; Dp: posterior zone of dorsal telencephalon; Ha: habenula; Had: dorsal part of Ha; Hav: ventral part of Ha; OB: olfactory bulb; OE: olfactory epithelium; oc: optic chiasma; on: olfactory nerve; Po: preoptic region; Tel: telencephalon; TeO: optic tectum; TH: tuberal hypothalamus; Vd, Vp, Vs, Vv: dorsal, postcommissural, supracommissural, ventral nucleus of ventral telencephalon.

olfactory bulb or the tuberal hypothalamus unilaterally and the injection site was sealed with warm Agar-agar (4% in Aq. dest). After 5 to 6 days of incubation in PFA-PB fixative in an incubation chamber at 37 °C, the brains were rinsed in PB and embedded in Agar-agar (4% in Aq. dest) and cut at 30–50 μm on a vibratome (Leica, VT1000 S). Sections were collected on Superfrost Plus glass slides (Thermo Scientific) counterstained with DAPI (40–6-diamidino-2-phenylindole; 1:1000 dilution, Carl Roth), mounted with Vectashield (Vectorlabs) and cover-slipped. DiI positive neuronal connections were then photographed (see below) before coverslips were removed and sections further processed for Otpa immunohistochemistry (see next section).

Tissue preparation and immunohistochemical processing. Staining for pERK/S100: imprinted and non-imprinted larvae were processed according to our previous study[39].

Staining for Otpa: Rat polyclonal antibodies against the transcription factor Otpa (Immunogen: Synthetic peptide CKKPVHPGDLAPVSDA) were manufactured by Covance (USA) and used on our brain tissue at a dilution of 1:500. The specificity of this custom-made antibody was confirmed in 3dpf *otpa*^{m866} mutant larvae[30] and in 5dpf *otpa*^{m866} mutant larvae[29].

Cryostate sections of imprinted and non-imprinted larvae were additionally incubated with a third primary antibody against Otpa (rat anti Otpa, dilution 1:500) diluted in blocking solution for 2 days at 4 °C in a humid chamber. Afterwards slides were washed with PBS and incubated with the third secondary antibody (AMCA, anti rat, Dianova or 488 anti rat, Dianova, 1:300,) diluted in blocking solution for 3 hours in a humid chamber at room temperature.

Coverslip of adult zebrafish brain vibratome (DiI) sections were washed off for final Otpa immunohistochemical processing in PBS. Additional adult zebrafish brains were cryosectioned at 30 μm for neurochemical/-anatomical analysis. Sections were incubated with proteinase-K (Sigma, P6556, 1:1000, diluted in PBS) for 15 min at 37 °C to facilitate antibody penetration. Afterwards, all adult sections were processed for Otpa immunostaining as described for larval sections above.

Confocal microscopy. Optical larval and adult tissue sections were acquired with a Leica TCS SP-5 confocal laser-scanning microscope (Leica Microsystems). All microscopic images used in this study were processed to RGB stacks and projections by using ImageJ and slightly adapted for brightness and contrast with either ImageJ or Corel PHOTO-PAINT 12.0. Photographic plates were mounted and further processed into figures with CorelDRAW 12.0 (Corel Corporation).

Epifluorescence microscopy. Photomicrographs of sectioned adult brain tissue were taken with a light/fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Inc.) equipped with Nikon Plan Fluor 109/0.30 (10x) and Plan Fluor 209/0.50 (20x) objectives, a Nikon Digital Sight DSU1 Photomicrographic Camera (Nikon Instruments Inc.) and LUCIA-G5 software.

Quantification of activated cells. Stacks of olfactory bulb sections were analyzed by using the RoiManager tool of ImageJ and all activated cells (pERK+) were counted. Position of cell-soma was identified according to assignment to one of three areas shown in Fig. 6C. Appropriate section was identified with immunohistochemistry for the calcium binding protein S100 which labels the neuropil in the center of the mdG2 glomerulus (as described previously[10]).

Similarly, telencephalon sections containing the intermediate nucleus of the ventral telencephalon (Vi, identified with immunohistochemistry for Otpa) were analyzed in the same way for pERK-positive cells and additionally for pERK/Otpa-positive cells in a defined sector (see Fig. 8).

Cell counting for statistical analysis was performed blind, by two observers unknowingly which specimen (imprinted/non-imprinted) and stimulus (control/kin odor) they were evaluating.

Statistical evaluation. After kin odor tests, the quantity of pERK-positive cells was counted in the section with the S100-positive mdG2 glomerulus in the total olfactory bulb (Fig. 7A), the inner cellular layer (B), in the glomerular layer (C) or in the area surrounding the mdG2 (D; see also Fig. 6C for explanation) both in imprinted and non-imprinted larvae. Furthermore, pERK-positive labeled cells and Otpa-positive/pERK-positive double labeled cells were quantified in a defined telencephalic field containing the Otpa-positive intermediate ventral telencephalic nucleus (Vi) in imprinted and non-imprinted larvae, either stimulated with kin or control stimulus.,

Cell quantity was analyzed using a nonparametric Kruskall-Wallis test followed by a pairwise Mann-Whitney U test including Bonferroni correction ($\alpha = 0.017$). All analyses are two-tailed and were done in IBM SPSS statistic 23 for windows.

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Author Contributions

The study was designed by D.B., K.T., G.G. and M.F.W. The behavioral experiments were performed by D.B. and K.T. (Oldenburg). The tracing experiments were done by D.B. and M.F.W. The immunohistochemical processing was performed by D.B. (Munich). Analysis of data was done by D.B., K.T., S.R., B.G., G.G. and M.F.W. The first version of the manuscript was written by D.B., K.T. and M.F.W. The final version was written by D.B., K.T., S.R., B.G., G.G. and M.F.W. D.B. and K.T. share first authorship.

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4. DISCUSSION

Across a wide range of vertebrates, the process of olfactory imprinting on distinct olfactory cues and therefore formation of long lasting olfactory memories is conserved across animals, in invertebrates as well as in vertebrates. Olfactory imprinting is a learning process which is related to a natural, biologically relevant context and occurs at a defined developmental time window (sensitive period). Larvae of zebrafish are known to imprint on visual and olfactory cues of their kin during a 24-hour window and use this olfactory memory for kin recognition later in life (see 1.3.2). Interestingly, larvae do not imprint on non-kin cues on the appropriate days, suggesting a genetic predisposition on kin cues. Although several studies revealed first insights on the processes involved in olfactory imprinting in zebrafish, the underlying mechanisms are poorly understood so far.

The aim of this doctoral thesis was to gain more insight into the process of olfactory imprinting from a neurobiological view. For this, it is absolutely essential to understand and interrelate anatomical, genetic and behavioral factors involved in zebrafish olfaction. For this reason, this thesis was part of a collaboration with the laboratory of Gabriele Gerlach in Oldenburg, Germany (behavioral and genomic experiments) and the Rainer Friedrich lab in Basel, Switzerland (behavior, Ca^{2+} imaging and MHC peptide-stimulation). My thesis begins with a comprehensive anatomical study of elements of the zebrafish olfactory system and proceeds with following analyses involving neuronal activity in response to kin odor exposure in imprinted and non-imprinted zebrafish larvae.

In contrast to other vertebrates, the teleostean olfactory system consists of one paired olfactory epithelium (OE), which bears olfactory sensory neurons (OSNs) mediating olfactory information via the olfactory nerve to the olfactory bulb (OB). The zebrafish OE consists of four different types of OSNs. Like in tetrapods, ciliated and microvillous OSNs (cOSNs and mOSNs), are the two main types of OSNs in teleosts. However, both types of OSNs are intermingled within each single OE and therefore not correlated to separate structures represented by the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) in tetrapods, respectively. Additionally, two further types of OSNs, kappe neurons and crypt cells, even though making up small populations, are intermingled within the zebrafish OE. However, although the teleostean olfactory system displays morphological differences to that of tetrapods, basic essentials, such as expression of odorant receptors, binding and processing of odorants or involved signaling molecules, are comparable to that of tetrapods (see 1.1).

First of all, the data on calcium binding protein expression will be discussed in detail (4.1). Afterwards, an extended discussion will focus on neuronal activity at the level of odor detection (olfactory epithelium) as well as at the level of odor processing (olfactory bulb) (4.2.1). Moreover, the existence of a telostean medial amygdala and its possible involvement in kin recognition will be part of this extended discussion (4.2.2).

4.1. Combinatorial analysis of calcium binding proteins in larval and adult zebrafish reveals distinct subpopulations of olfactory sensory neurons and identifies their differential glomerular olfactory bulb targets

The aim of this study was to define the expression of four different calcium binding proteins (CBPs), Parvalbumin (PV), Calbindin (CB D28k), Calretinin (CR) and S100 in the olfactory system of larval and adult zebrafish (*Danio rerio*). Since only the two CBPs, CR and S100 have been investigated in the zebrafish olfactory system so far, the present study using additional CBPs provided new insights into subpopulations of olfactory sensory neurons (OSNs) as well as their projection targets into the olfactory bulb (OB) in larval and adult zebrafish. In order to investigate a possible differential expression pattern of OSNs, we applied the four different CBPs in a combinatorial fashion and analyzed the expression pattern of single- and double-label preparations of larval as well as adult zebrafish olfactory epithelia (OE) and OB cryostate sections. Altogether, the present study using four different CBPs in combinatorial fashion reveals at least eight subpopulations of OSNs in the zebrafish OE (Figure 6). There is one very obvious S100-like positive crypt cell population which is negative for all other CBPs, projecting into one of overall six glomeruli in the mediodorsal olfactory bulb (see also (Braubach et al. 2012)). Additionally, we report three subpopulations of cOSNs, one major subpopulation positive for PV, CB and CR, and two minor populations either positive for PV and CB or CR only. Furthermore, we identified four subpopulations of mOSNs, one minor population double labeled with S100 and PV, one only PV positive subpopulation, one positive for PV and CB and finally mOSNs immunoreactive for PV, CB and CR.

Calcium-binding proteins (CBPs), such as Parvalbumin, Calretinin, Calbindin (D28k) and S100, belong to a larger group of proteins which are involved in numerous cellular functions across vertebrates. The family of CBPs is a heterogenous group but most CBPs exhibit a characteristic amino acid sequence, which folds up into a helix-loop-helix pattern, the so called EF-hand, in which calcium ions bind (Andressen et al. 1993). CBPs of the EF-hand family may either act as a `trigger´ to induce distinct cellular responses or function as a `buffer´ in the presence of Ca^{2+} (Dalgarno et al. 1984). The four CBPs used in this study are such `buffer´- proteins which, in addition to their role in regulation of Ca^{2+} within cells, are of special interest from a neuroanatomical point of view. Thus, CBPs are shown to be expressed in distinct neuronal subpopulations in various vertebrate species, therefore considered as an excellent marker to study anatomy and distribution of distinct neuronal subsystems in the

vertebrate central nervous system (CNS). Parvalbumin (PV), Calbindin (CB) and Calretinin (CR), for example, reveal consistent cytoarchitectonical distributions of different neocortical cell types in several mammalian species (Celio 1990, Van Brederode et al. 1990, Hendry and Jones 1991). In rats, Celio (1990) stated a general conclusion in which PV is said to be mainly expressed in neurons with thick myelinated axons with restricted projection fields such as GABAergic interneurons of the mammalian cortex. On the other hand, CB is mostly observed in neurons with thin unmyelinated axons with diffuse projection targets such as thalamic projection neurons or spinal-, retinal-, vestibular- or cochlear nuclei. However, although PV and CB mainly show complementary distribution, both CBPs are expressed in cerebellar Purkinje cells and dorsal root ganglia of the spinal cord. In addition, combinatorial analysis of PV, CB and CR in cortices of several mammalian species demonstrates that these three CBPs define non-overlapping distinct subpopulations of GABAergic cortical interneurons (Celio 1990, Hendry and Jones 1991, Résibois and Rogers 1992, Andressen et al. 1993). In the rat main olfactory bulb (MOB), these three CBPs are localized in several classes of bulbar neurons and deprivation experiments demonstrate that CBP expression is regulated by olfactory experience (Philpot et al. 1997).

For example, PV is expressed in a subpopulation of GABAergic bulbar interneurons, however, the morphology and laminar distribution of this PV immunopositive (-ir) subpopulation displays an enormous heterogeneity (Kosaka et al. 1994). PV, CR and S100 are also shown to be located in distinct neuronal populations in the teleost central nervous system. Like in mammals, teleostean cerebellar Purkinje cells are shown to express PV in their soma as well as in their axonal projections (Alonso et al. 1992, Porteros et al. 1998, Crespo et al. 1999). Moreover, CR-ir is seen in the efferent cerebellar system as well as in other specific neuronal populations of the teleost brain (Díaz-Regueira and Anadón 2000, Castro et al. 2006, Biechl et al. 2016a). The CBP S100 selectively labels neuromasts of the lateral line system and taste buds in adult zebrafish. In the zebrafish olfactory system, S100 is expressed exclusively in a small subpopulation of olfactory sensory neurons (OSNs), the crypt cells, whereas CR-ir is seen in very numerous OSNs, mostly ciliated OSNs (Castro et al. 2006, Germanà et al. 2007, Koide et al. 2009, Braubach et al. 2012, Gayoso et al. 2012). Several studies using CR and S100 demonstrated no conclusive expression profiles of the four zebrafish OSNs. This disagreement might result from CBP investigation at different developmental stages, as for example CR is expressed in cOSNs and mOSNs in larval zebrafish (Koide et al. 2009), whereas CR-ir is seen mostly in cOSNs in adult specimen (Castro et al. 2006, Germanà et al. 2007, Gayoso et al. 2011, Braubach et al. 2012, Kress et al.

2015). Another CBP, S100 has been under great debate to be considered as a specific marker for crypt cells. Several studies demonstrated S100 to visualize steadily the entire crypt cell population of larval and adult zebrafish (Germana et al. 2004, Sandulescu et al. 2011, Braubach et al. 2012, Biechl et al. 2016b). Crypt cells are easily distinguishable, because their round shape, their crypt formed indentation, as well as their most apical location within the OE is well distinguishable from the appearance of cOSNs and mOSNs. However, some studies in zebrafish showed that that in addition to crypt cells, also some plump and spindle shaped OSNs, likely mOSNs, show immunopositivity to the S100 antibody (Gayoso et al. 2011, Gayoso et al. 2012, Biechl et al. 2016b). Furthermore, based on *in situ* hybridization data, Oka et al. (2012) stated that in contrast to a small subpopulation of mOSNs, crypt cells do not express any S100 gene and specific crypt cell recognition by S100 immunohistochemistry is only feasible in particular assay conditions. Ahuja et al. (2013) showed that paraformaldehyde (PFA) fixed OE of adult zebrafish resulted in S100 staining of crypt cells and a small subpopulation of mOSNs as reported in other studies mentioned above as well as shown in our present study. In fresh frozen tissue preparations they specifically identify the entire crypt cell population. Based on a study by Catania et al. (2003), Ahuja et al. (2013) tested an antibody against trkA, a neurotrophin-NGF-receptor, to suit as a better marker for crypt cells. Unfortunately, it turned out to be that trkA, such as in the case of S100, cross reacts with an unknown protein instead of the expected antigen.

Given that the S100 antibody recognizes an unknown protein in crypt cells, but nevertheless provides reliable crypt cell identification, we used the term S100-like to describe the immunopositivity of crypt cells from now on. In the present study (Kress et al. 2015), as well as in the following study (Biechl et al. 2016b), we demonstrate S100-like positive crypt cells and their projections into one sole glomerulus (mediodorsal glomerulus; mdG2) in the zebrafish OB, as reported in previous studies (Braubach et al. 2012, Ahuja et al. 2013). Moreover, we show evidence of additional input of S100-like and PV positive mOSNs into this glomerulus. This result is affirmed by tracing data (Ahuja et al. 2013) which shows some backlabeled mOSNs following DiI injection into mdG2 most likely the subpopulation of S100/PV-ir mOSNs described in our study. Furthermore, we are confident that our S100 antibody is a reliable marker for the entire crypt cell population as well as for a small subpopulation of mOSNs, since we do not see any other projection targets of S100-like fibers outside the mdG2 glomerulus. Furthermore, regarding the S100/PV-ir mOSN projection into mdG2, we also see PV-ir within the mdG2 in single antibody staining preparations in larval and adult zebrafish. This label cannot originate from crypt cells since they are negative for

PV. The only plausible explanation is that mOSNs, expressing S100 and PV project their axons into the mdG2 of the zebrafish OB. In addition, our study shows that almost all cOSNs project their axons into dorsal and ventromedial glomerular domains whereas one large subpopulation of mOSNs, expressing PV, CB and CR, terminate into ventrolateral fields of the zebrafish OB. This projection pattern is in line with a study by Sato et al. (2005) who generated double transgenic zebrafish lines expressing distinct fluorescent proteins in cOSNs and mOSNs under the control of regulatory regions of the olfactory marker protein (OMP) and transient receptor potential channel 2 (TRPC2), respectively. Interestingly, in this study, neither cOSNs nor mOSNs showed innervations into mediodorsal bulbar fields of transgenic zebrafish. However, our study demonstrates PV-ir axonal projections innervating the entire mediodorsal bulbar field (mdG). Furthermore, these PV expressing fibers of the mdG only co-express CB at more anterior parts whereas no co-expression is seen for CR in any part within the entire mdG, thus confirming our finding of a PV only positive subpopulation of mOSNs. Regarding to the absence of TRPC2 expressing mOSN projections within the mdG shown in transgenic zebrafish (Sato et al. 2005), we conclude that mOSNs innervations into mdG reported in our study (PV only and PV/CB positive mOSNs) express no TRPC2, but another type of ion channel. In this case, the view on signal transduction in mOSNs has to be reconsidered, since mOSNs have been specifically associated with TRPC2 in mammals as well as in teleosts, such as zebrafish (Liman et al. 1999, Sato et al. 2005). In zebrafish, another protein, $G\alpha_{o,}$ is associated with mOSNs (Oka and Korsching 2011). A comprehensive anatomical study on glomerular organization in zebrafish reported $G\alpha_o$-ir fibers in mdG5, a second mediodorsal glomerulus (Braubach et al. 2012). Up to that time, it was suggested these $G\alpha_o$-ir fibers originate from mOSNs and some crypt cells. However, a previous study unraveled a new OSN type, kappe neurons, as the source of these $G\alpha_o$ positive fibers in mdG5 (Ahuja et al. 2014). However, the receptor which is expressed on this cell type is not known so far. Based on our results on CBP expression within the mdG, it would be possible that kappe neurons correspond to the PV/CB-ir subpopulation of mOSNs, as they also project into mdG5 in our data.

To recap our results of CBP positivity focused on the mediodorsal field of the zebrafish OB, we identify the cellular origin of (at least) two glomeruli, which are the mdG2 and mdG5, as receiving input from crypt cells and possibly PV/CB positive kappe neurons, respectively. However, four more glomeruli are present in the mediodorsal field of the zebrafish OB, all of them showing PV only (one) or PV/CB (three) positivity. It would be possible that the remaining mediodorsal glomeruli also receive input by at least three distinct

subpopulations of mOSNs, all of them negative for CR expression. If this is the case, these five glomeruli in all would be innervated by distinct populations of mOSNs, each of them expressing one particular receptor. There are numerous vomeronasal type 2 (V2R) genes present in teleosts, about 24 potentially functional in zebrafish (Hashiguchi and Nishida 2005, 2006), whereas only six V1R like genes (*ora* 1-6), all of them highly conserved between several telost families, exist (Saraiva and Korsching 2007). In contrast to teleosts, mammalian V1Rs are numerous, highly divergent and vary between species. In rodents, V1Rs are related to pheromone detection which is also assumed to other species (Boschat et al. 2002, Young et al. 2005). However, based on several behavioral studies, the teleostean medial olfactory tract (MOT), containing fibers originating from the medial OB, is associated with mediating social behaviors, whereas the lateral olfactory tract (LOT) contains mainly fibers of lateral bulbar regions and is shown to mediate behaviors related to feeding (Sheldon 1912, Finger 1975, von Bartheld et al. 1984). Furthermore, both tracts contain mitral cell axons which project to different targets within the telencephalon and diencephalon (see 1.2.2). In general, odors are supposed to be processed in a combinatorial fashion, whereas odors of special biological significance, such as pheromones, are considered to activate a so called `labeled line´ pathway. The `labeled line´ theory defines a coding strategy in which a given odor activates a distinct signaling pathway which immediately leads to a behavioral or physiological response (Touhara and Vosshall 2009). It was shown in insects that a male-specific pheromone elicits and suppresses mating behavior in males and females respectively. Interestingly, both resulting behaviors are mediated by only one class of OSNs, expressing the same receptor ("one neuron - one receptor rule") and project to one glomerulus ("one receptor - one glomerulus rule"). To elicit those two opposed behaviors, two different classes of second-order neurons, one GABAergic and the other cholinergic, are connected to this glomerulus and innervate a putative pheromone sensing center which in turn specifically targets two sexually dimorphic regions (Kurtovic et al. 2007). Similarly, such a `labeled line´ pathway may also be present in the case of crypt cell signaling. In contrast to cOSNs and mOSNs, the entire crypt cell population expresses only one single V1R related receptor encoded by the *ora4* gene, thus extending the "one neuron - one receptor rule" to a "one cell type - one receptor rule" (Oka et al. 2012). Furthermore, consistent with other studies, the present study demonstrates crypt cells to target their axons into one single mediodorsal glomerulus, the mdG2, thus correlating to the "one receptor – one glomerulus rule" which is also present in mammals (Mombaerts et al. 1996, Ahuja et al. 2013). With regard to following studies included within my thesis (see RESULTS 3.2 and 3.3), crypt cells suit very well to be

considered a labeled line, as they are shown to be specifically involved in detecting a kin odor related signal in larval zebrafish (Biechl et al. 2016b). Moreover, increased activity of second-order neurons around mdG2 as well as in a telencephalic region assumed to be related to socially relevant olfactory information in response to kin odor confirms the assumption for crypt cells as a labeled line (Biechl et al. 2017). However, the latter results regarding crypt cells as a labeled line will be discussed in detail in section 4.2.

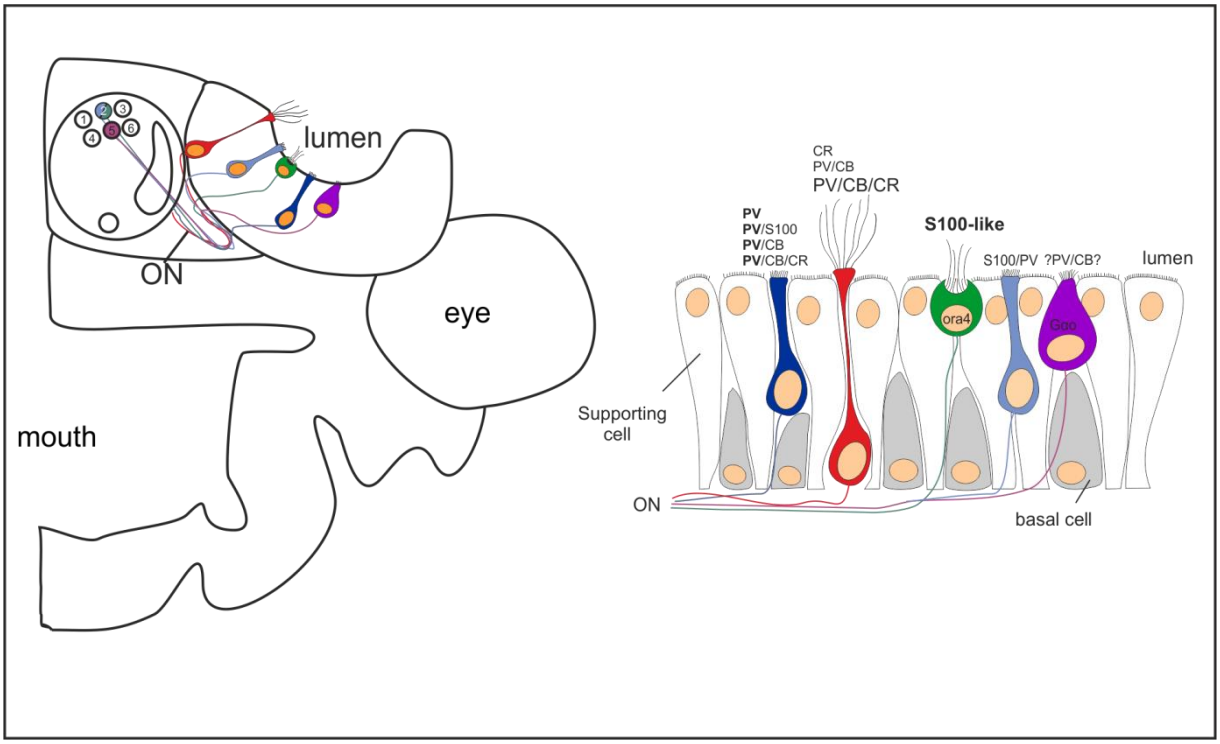

Figure 6 Expression of four different Calcium-Binding Proteins (CBPs) in the zebrafish olfactory system. **Right:** Combinatorial analysis of CBP expression reveals at least eight subpopulations of olfactory sensory neurons (OSNs). Bigger font size means that this (sub)population makes up a major (sub)population. There are four subpopulations of microvillous OSNs (dark-blue), all of them expressing Parvalbumin (PV) and three subpopulations of ciliated OSNs (red). The CBP S100 is specifically expressed in all crypt cells (green) and a minor subpopulation of microvillous OSNs which also express PV (light-blue). Kappe neurons (purple) likely express PV and Calbindin (CB). **Left:** Schematic drawing of a cross section through a larval zebrafish olfactory epithelium (OE) and olfactory bulb (OB). Analysis of the differential expression pattern of CBPs shows evidence for the existence of at least two labeled lines. Crypt cells (green) project their axon into one mediodorsal glomerulus (mdG2). Additional axonal input into this glomerulus comes from S100/PV expressing microvillous OSNs (light blue). Another glomerulus (out of 6) within the mediodorsal domain, mdG5, recieves input from PV/CB expressing OSNs, which represent, according to other studies most likely kappe neurons.

A second possible labeled line is delineated in our study by PV/CB-ir mOSNs projecting to mdG5. This assumption is affirmed by the fact that only $G\alpha_o$-ir OSNs, kappe neurons, send their axon to this glomerulus. However, although it is likely that all kappe neurons express a single receptor, likely a V2R type, proof of the unique expression of one receptor, as seen in crypt cells, is so far missing (Ahuja et al. 2014). As mentioned above, six *ora* genes are present in the zebrafish, one of them expressed by crypt cells (ora4). The remaining five V1R type receptors (encoded by *ora1-3* and *ora6*) might represent labeled lines as well, thus distinct populations of mOSNs positive for PV only or double labeled by

PV and CB may express one particular *ora* gene and project to the four remaining mediodorsal glomeruli as well as to the ventral glomerulus which is also innervated by PV/CB-ir fibers. Regarding the large population of PV/CB/CR-ir mOSNs, they almost certainly innervate the ventrolateral glomerular field and, possibly, according to Sato et al. (2005), likely express V2R related receptors and TRPC2. In conclusion, the present study expands the knowledge of expression of CBPs within distinct subpopulations of OSNs as well as their axonal projections into appropriate bulbar glomeruli. Moreover, combinatorial analysis of CBP expression of OSNs reveals hints for various labeled lines and their possible olfactory role.

4.2. Processing of kin odor in the zebrafish olfactory system

With regard to identify neuronal events involved in the process of kin odor detection we performed a comprehensive study using behavioral and immunohistochemical techniques in larval zebrafish. Foremost, we validated the phosphorylated extracellular signal regulated kinase (pERK) as a reliable marker for neuronal activity in the larval zebrafish olfactory system. Thereby, we performed a temporal analysis of pERK up-regulation in olfactory sensory neurons (OSNs) in response to two different olfactory stimuli (food and conspecific, non-kin larval odor). This resulted in best duration of 7 minutes of odor stimulation in larval zebrafish. These experiments also showed that both, cOSNs and mOSNs are activated by food odor whereas mOSNs mainly responded to conspecific odor (non-kin odor). Interestingly, crypt cells showed no activation to food as well as to conspecific odor.

Furthermore, by manipulation of larval rearing conditions, we created groups of either imprinted or non-imprinted larvae, which enabled us to compare neuronal activation at the level of the olfactory epithelium, olfactory bulb and telencephalic brain centers between these two groups. Based on our data, we show strong evidence of crypt cells and a small subpopulation of microvillous cells to detect a kin odor related signal. Moreover, only crypt cells of imprinted larvae show neuronal activation in response to kin odor exposure whereas crypt cells of non-imprinted larvae do not. Consequently, we also show an increase of neuronal activation of second order neurons around the crypt cell´s target glomerulus (mdG2) in the olfactory bulb of imprinted larvae in contrast to non-imprinted larvae. Tracing experiments in adult zebrafish revealed a neuronal pathway starting at crypt cells and probably microvillous cells to transmit olfactory information, including kin odor, to the mediodorsal olfactory bulb and via the medial olfactory tract to the intermediate ventral telencephalic nucleus (medial amygdala in teleosts; see later) and from there to the tuberal hypothalamus. This olfactory circuit shown in our study demonstrates for the first time an accessory olfactory system in zebrafish as described in tretrapods. Surprisingly, regarding neuronal activation of cells in the medial amygdala, we only find an increase of neuronal activation in non-imprinted larvae when exposed to kin odor.

4.2.1 Crypt cells are involved in kin recognition in larval zebrafish

In order to investigate which type of olfactory sensory neuron (OSN) detects a kin odor related signal, we mapped neuronal activity following olfactory stimulation, indicated by

pERK upregulation, in olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) of 9 days post fertilization (dpf) old zebrafish larvae. pERK is widely used to mark neuronal activity in mammals and is also shown to visualize neuronal responses within the olfactory system following odor stimulation in mice (Mirich et al. 2004). Upon phosphorylation, pERK is translocated into the nucleus of the activated cell to modulate expression of transcription factors which in turn regulate gene expression involved in neuronal and synaptic plasticity underlying learning and memory (Figure 5). It was shown that pERK is preferable to other markers, such as IEGs (e.g *c-fos* or *egr1*), because of its rapid activation and its cellular distribution (soma and cell protrusions) (Gao and Ji 2009, Randlett et al. 2015). IEGs, such as *c-fos*, are also good markers for neuronal activity. However, it takes at least about 1 hour to trigger IEG induction and therefore detect a sufficient IEG protein signal by immunohistochemistry. Although detection of IEG mRNA by *in situ* hybridization (ISH) is possible already around 30 minutes after stimulation, the resulting delay between stimulation and potential response of the tested animal is often too long to link neuronal induction with specific responses (behaviors) (Chaudhuri 1997, Watts et al. 2006). However, we first wanted to investigate pERK as a reliable marker for activation in zebrafish OSNs following olfactory stimulation. Therefore we stimulated zebrafish larvae with either food-, conspecific odor (non-kin) or E3 medium (control) for 3, 7, 11 and 15 minutes. Analysis of activated OSNs reveals that pERK intensity as well as number of activated cells are independent of stimuli duration since a strong pERK signal is observed in all activated OSNs in all exposure durations. However, our perception of signal to noise ratio in the immunostains suggested an exposure time of 7 minutes to give best results. Our results are in line with data from studies in mice, which show that about 10 - minute olfactory stimulation is sufficient to detect neuronal activation in brain regions related to processing of olfactory cues (Dudley et al. 2001, Taziaux et al. 2011). Moreover, consistent with our results on rapid induction of pERK in OSNs, Hussain et al. (2013) reports detectable pERK signals in OSNs following 3-5 minute olfactory stimulation in adult zebrafish. Furthermore, olfactory stimulation to different odors (food, conspecific and E3-medium) reveals a differential pattern of activated OSNs within the larval OE. High numbers of cOSNs and to a much lesser extent mOSNs are activated in response to food odor exposure, whereas almost exclusively mOSNs show pERK induction following exposure to a conspecific odor in addition to food odor. Interestingly, crypt cells show neuronal activation neither to food nor to conspecific odor. These response patterns are consistent with data from other studies, although there is a great interspecific variability regarding the potential tuning of OSNs within teleosts (Bazaes et al. 2013). For example in

carp, mOSNs are mainly tuned towards food related odors, whereas cOSNs play a role in mediating the alarm reaction and crypt cells are suggested to be involved in reproduction (Hamdani el et al. 2001, Hamdani el and Døving 2002, Hamdani el et al. 2006, 2008). Electrophysiological data by, Hansen et al. (2003) in channel catfish suggest all OSN types to respond to amino acids, but with cOSNs additionally responding to bile salts, whereas mOSNs also respond to nucleotides. In goldfish, mOSNs are assumed to respond preferentially to amino acids (Speca et al. 1999). In transgenic zebrafish, blocking of synaptic transmission in distinct populations of mOSN abolished attractive behavioral responses to amino acids (Koide et al. 2009) which is in line with other studies (Lipschitz and Michel 2002), including the study presented here (Biechl et al. 2016b). Amino acids and nucleotides are typically indicative for food whereas bile salts are considered as a social odorant since bile salt profiles within a teleost family and order feature high similarities (Hagey et al. 2010). However, a general conclusion on teleost OSN tuning is hard to make since within all examined teleosts so far, not only the responding profiles of OSNs differ, but also bulbar projection patterns across species show discrepancies (Bazaes et al. 2013).

In any case, stimulation with food odor, as well as with conspecific odor revealed no activation of zebrafish crypt cells. To evaluate a possible role for crypt cells in detection of a kin odor related signal, we performed additional stimulation experiments. In contrast to the previous experiment, we generated two different groups of zebrafish larvae. By rearing larvae isolated in small glass beakers, we either prevented olfactory imprinting on day 6, or allowed the larvae to imprint on their kin by adding kin odor containing water into the beakers. Except for this difference, this rearing condition allowed all larvae to grow up under same conditions (e.g. changing water, feeding, and isolation) and precludes other influences on resulting data. Importantly, despite raising larvae in glass beakers, visual imprinting, which occurs at day 5 and is required for successful olfactory imprinting (see Introduction) is possible because larvae are able to recognize pigmentation of other kin through glass walls in a larger tank which contains the small glass beakers with the isolated fish (Hinz et al. 2013a). Additionally, in these kin odor stimulation experiments we used in the subsequent histological assay the calcium binding protein (CBP) S100 to identify specifically crypt cells. Similar to the previous experiment we used accepted morphological criteria to identify cOSNs and mOSNs. Since their discovery, crypt cells have been suggested to play a role in detection of social olfactory signals, such as pheromone sensing. In the crucian carp, crypt cells project into the ventral OB from where projections neurons terminate into the lateral part of the medial olfactory tract (lMOT), the latter known to mediate reproductive behaviors (Weltzien et al.

2003). Furthermore, crypt cells are shown to vary in their density as well as location within the OE depending on the seasons in sexually mature carp (Hamdani el et al. 2006, 2008). During winter, only some crypt cells are present within the carp OE whereas in spring even more crypt cells are visible at more deep locations within the OE. Interestingly, during the summer, and therefore the spawning season, crypt cells are clearly detectable and positioned at the surface of the OE, reinforcing the hypothesis of crypt cells to be involved in carp reproductive behavior, (Hamdani el et al. 2008). Also, Sandulescu et al. (2011) demonstrated an early increase of zebrafish crypt cell quantity which later decreases at a particular time during OE development. In this study, upon first appearance in the zebrafish OE at day 4 post fertilization (dpf) the number of crypt cells increased steadily until reaching a peak at 7 dpf. From that age on, crypt cell number deceased dramatically over 70% and recovered until larvae reached 12 dpf. Comparing this non-linear growth of crypt cells to the sensitive phase in which olfactory imprinting (from day 6 to 7) occurs, it seems as if the olfactory system, especially crypt cells, prepare for the upcoming imprinting event. Based on these studies, we exposed imprinted and non-imprinted zebrafish larvae with kin odor and demonstrate strong evidence for crypt cells to detect a kin odor related odorant. Interestingly, only crypt cells of imprinted larvae show activation in response to kin odor. In contrast to imprinted larvae, non-imprinted larvae show no response after stimulation with kin odor. Importantly, this is not due to an absence of this cell type, as crypt cell numbers do not differ between non-imprinted and imprinted larvae (Biechl et al. 2016b). Furthermore, quantitative polymerase chain reaction (qPCR) data reveals no evidence for down-regulation of ORA4 receptor expression in non-imprinted zebrafish larvae (unpublished data by Gerlach Lab, Oldenburg). However, it seems that the missing cue, which is obviously contained in the kin odor, changes the responsiveness of crypt cells in an unknown manner. Crypt cells express only one single V1R-like receptor, encoded by the *ora4* gene (Oka et al. 2012). However, the ligand which binds on ORA4 is presently unknown. Based on our data, the ligand of ORA4 is contained in kin odor. Moreover, our study also indicates that olfactory imprinting occurs at the level of the OE, as crypt cells, and therefore ORA4, show no activation in presence of the appropriate odorant when this is not presented during the sensitive phase of olfactory imprinting. However, as mentioned in section 4.1. ORA4 is one of 6 teleostean receptors identified within the highly conserved *ora* gene family (Saraiva and Korsching 2007). So far, only one of those receptors, ORA1, was deorphanized in adult zebrafish and shown by calcium imaging to be highly responsive to 4-hydroxyphenylacetic acid. Moreover, even low concentrations of this compound mediate, via ORA1, an increase in oviposition frequency in zebrafish mating pairs

(Behrens et al. 2014). Thus, similar to the role of mammalian V1Rs, ORA1 represents a putative pheromone receptor involved in modulation of reproductive behavior (Boschat et al. 2002). Therefore, it would be in line with our data that demonstrate crypt cells, and therefore consequently ORA4, to be involved in modulation of reproductive behavior. However, recent studies in zebrafish demonstrated that visual as well as olfactory imprinting is related to similarity of Major Histocompatibility Complex (MHC) class II genes (Hinz et al. 2012). Moreover, based on calcium imaging data and behavioral assays, Hinz et al. (2013b) concluded that MHC class II peptides function as chemical signals. Only zebrafish which share MHC class II alleles, are able to imprint on each other during the sensitive period. However, MHC peptides are recognized by V2R receptors expressing mOSNs of the mice vomeronasal epithelium and are shown to function as individuality signals underlying mate recognition. Furthermore, MHC peptides have been demonstrated to be linked to pregnancy block (also known as `bruce effect´; see 1.3.2) in mice (Leinders-Zufall et al. 2004, Becker and Hurst 2008). Since mammalian V1R and V2R receptors have been shown to be tuned towards molecules of low molecular weight (e.g. steroids) and peptides respectively, it would be unlikely that ORA4 is a specific receptor for MHC peptides in zebrafish (Boschat et al. 2002, Isogai et al. 2011, Behrens et al. 2014). On the other hand, despite the `one neuron – one receptor ´ rule, one cannot rule out that crypt cells express another receptor in addition to ORA4. The idea of a second olfactory receptor expressed on crypt cells arises by a study in mice which demonstrates that parallel expression of two odorant receptors in one neuron is possible if one of these receptors is not active (Mombaerts 2004b). Ferreira et al. (2014) showed in zebrafish that indeed receptor expression depends on receptor activity. However, they demonstrated that signaling through G protein βγ subunits is necessary to maintain expression of only one single receptor per OSN by suppression of other receptor genes. The molecular mechanisms which maintain such a silencing of odorant receptors also in newly developing OSNs is likely due to epigenetic events such as histone methylation.

In addition to crypt cells, a small subpopulation of mOSNs is shown to respond to kin odor. Considering that MHC peptides are a compound of kin odor, possibly these mOSNs express a V2R receptor and bind to such peptides. In any case, kin odor is comprised by a mixture of numerous odorants that signal besides genetic relatedness also other social information such as gender and physiological status. It is maybe too simple if kin recognition would be mediated by only one chemical compound. It is more likely that a receptor code, generated by interaction of multiple receptors, consequently activated by more than one ligand, signals the information of familiarity and unfamiliarity in larval zebrafish. In the same way could

concentration of odorants and possible interactions in-between activated receptors play a role in detection at the level of the OE as well as processing in the OB. The latter idea arises from studies in mice which demonstrate olfactory receptor antagonism between odorants in the mammalian OE. These studies show that the electrophysiological outcome of odorant mixtures was neither additive nor a simple average of its compound and suggest that a masking, counteraction or other interaction of odorant mixtures occurs within the olfactory system (Jinks and Laing 2001, Wiltrout et al. 2003). Oka et al. (2004) demonstrated in mice that odorants are able to activate olfactory receptors as agonists as well as antagonize OSN responses during binding. Since crypt cells, as well as their receptor ORA4 are present in non-imprinted zebrafish larvae, it would be possible that such a receptor antagonism is responsible for the failed activation of crypt cells in response to kin odor. In that case, ORA4 is not activated because the appropriate ORA4 ligand may act as an antagonist to ORA4, resulting from the absence of this ligand during the sensitive phase of olfactory imprinting. Another possibility as mentioned above could be derived from inhibition because of changes at the second messenger transduction pathway or by direct effects of odorants on ion channels which leads to suppression of the inward transduction current in the OSN (Kurahashi et al. 1994). Anyway, molecular modifications regarding crypt cell sensitivity must involve epigenetic mechanisms since OSNs are replaced continuously and newborn crypt cells somehow “know” how to response to kin odor (Ferreira et al. 2014).

Taken together, our study reveals that crypt cells as well as a small subpopulation of mOSNs detect a kin odor related signal. Moreover, we demonstrate that lack of a so far unknown compound contained in kin odor during the sensitive phase of olfactory imprinting (day 6 to 7 post fertilization) results in a failure of crypt cells activation in non-imprinted zebrafish larvae. As a result, the ability of zebrafish larvae to imprint and therefore distinguish kin from non-kin later in life depends on at least one distinct olfactory cue presented during a defined period of time which determines the sensitivity of crypt cells to kin odor.

4.2.2 Identification of the teleostean medial amygdala and its possible role in kin recognition based on neuronal activity in response to kin odor

The olfactory system of most tetrapods, including mammals, reptiles and amphibians, is comprised of two anatomically distinct and segregated olfactory systems: a main olfactory system (MOS) composed of a main olfactory epithelium (MOE) bearing ciliated olfactory sensory neurons (OSNs) projecting odor information into the main olfactory bulb (MOB) and

the accessory olfactory system (AOS) consisting the vomeronasal organ (VNO) with microvillous OSNs targeting their axons into the accessory olfactory bulb (AOB). In both, MOB and AOB, OSNs expressing the same receptor target their axons into one or two glomeruli where they synapse with mitral cells. In general, the MOE is specialized for detection of volatile, generic odorants whereas the VNO is shown to detect mainly non volatile odorants relevant for social and reproductive behaviors. However, both systems are not entirely separated as integration of olfactory information of MOS and AOS occurs in the mammalian amygdala (Licht and Meredith 1987). Furthermore, several studies demonstrated that the response profiles of both systems are not exclusive, as responses of MOE and AOB to both pheromones and general odorants are shown (Baxi et al. 2006, Eisthen and Wyatt 2006). The AOB forms a projection pathway different and independent to that of the MOB. Mitral cells of the MOB target their axons to multiple brain structures forming the primary olfactory cortex; whereas mitral cells of the AOB project to discrete brain areas within the so called vomeronasal amygdala and to specific nuclei of the hypothalamus. The vomeronasal amygdala includes the bed nuclei of the accessory olfactory tract and stria terminalis, posteromedial cortical and medial nuclei of the amygdala (Halpern 1987). In tetrapods, the amygdaloid complex is composed of pallial and supallial areas and shown to be crucial in motivated and emotional behaviors. Regarding olfaction, the amygdala is involved in associative learning between odorants and modulation of olfactory driven behaviors (Ono et al. 1995). In the ventral diencephalon, hypothalamic nuclei play a dominant role in regulation of numerous physiological functions, such as regulation of sleep, blood pressure, temperature, thirst and satiety, stress and social behaviors. The endocrine state of the animal is controlled by hypothalamic areas which regulate the release of various hormones by the pituitary gland. Therefore, projections from the VNO to hypothalamic areas (e.g. preoptic area, the ventromedial hypothalamic nucleus and ventral premammillary nucleus) are involved in reproductive and aggressive behaviors (Halpern 1987).

In contrast to most tetrapods, the teleostean olfactory system lacks a separated vomeronasal organ (VNO). However, expression of corresponding vomeronasal receptors also present in tetrapods (V1R & V2R) as well as detection of and physiological response pheromones is demonstrated in all teleost species investigated (see 1.1 and 1.3.1). In teleosts, the medial zone of the dorsal telencephalon is suggested to contain the homologue of the pallial amygdala (Salas et al. 2003). In vertebrates, including zebrafish, each glomerulus receives convergent olfactory input from OSN types expressing the same olfactory receptor. As mentioned above (see 1.2.2), bulbar mitral cells receive odor information from the

periphery by forming synapses with axonal endings of OSNs. Olfactory information is mediated to different higher brain centers via mitral cell axons forming the medial and lateral olfactory tract (MOT & LOT). Several studies, including our work, demonstrated crypt cells to project their axon into one single defined glomerulus (mdG2) within the OB (see citations above). Moreover, DiI - tracing visualized secondary olfactory projections from the mediodorsal OB into telencephalic areas (Gayoso et al. 2012). These are, the dorsal posterior part of the telencephalon (Dp) which is considered to correspond to the mammalian primary olfactory cortex and receives the strongest olfactory input amongst teleosts. The teleostean ventral nucleus (Vv) as well as the supracommissural nucleus (Vs) of the ventral telencephalon corresponds to the septal area and subpallial amygdala in mammals, respectively (Wullimann and Mueller 2004, Mueller et al. 2008). In goldfish, Levine and Dethier (1985) identified another bulbar projection target which is located even more posterior within the subpallial region, the intermediate nucleus of the ventral telencephalic area (Vi).

Based on our data which show that crypt cells (and a small population of mOSNs) are strongly tuned towards kin odor, we extended our approach on kin odor processing to the next stations of odor processing, that is the OB as well as a distinct subpallial area, Vi - eventually representing the medial amygdala in teleosts. Consistent with other studies, application of DiI into the mediodorsal bulbar field, including mdG2, visualized clearly telencephalic olfactory projections targets in adult zebrafish. Such as other glomerular fields do, projection neurons located in the mediodorsal OB innervate the posterior zone of the dorsal telencephalon (Dp) as well as the ventral nucleus of the ventral telencephalon (Vv). Additionally to those common olfactory bulb targets, neurons of the mediodorsal glomerular field project to the postcommissural (Vp) and supracommissural (Vs) ventral telencephalic nuclei as well as to the right dorsal habenula (dHb) (Gayoso et al. 2012, Turner et al. 2016). The experiments in my thesis combine antero and retrograde tract tracing and immunohistochemical visualization of the transcription factor Orthopedia (Otp) (indicative for the mammalian medial amygdala) and provide for the first time evidence for an accessory olfactory pathway in zebrafish. Besides demonstrating Vi to receive secondary olfactory input, also indicative for the medial amygdala, we further demonstrate that Vi projects to the ventral periventricular hypothalamic zone (Hv) of the tuberal hypothalamus, which has been previously identified in zebrafish as the homologue of the mammalian hypothalamic arcuate nucleus (Forlano and Cone 2007). The fact that mediodorsal bulbar projections run into this posterior subpallial region, more precisely into the Otp positive Vi (medial amygdala) and from there to the tuberal

hypothalamus is diagnostic for an accessory olfactory system in mammals (Dulac and Torello 2003) as well as in all tetrapods examined so far (Martinez-Garcia et al. 2008). Although an accessory olfactory system or vomeronasal system was considered to be exclusive to tetrapods, several data from studies in teleosts, such as goldfish or the present study in zebrafish, indicate that this sensory subsystem involved in processing vomeronasal information is basal to all bony vertebrates. Interestingly, in lungfish, a sarcopterygian clade considered as the closest living relatives of tetrapods, a "hidden" accessory olfactory system was previously discovered. Gonzalez et al. (2010) demonstrated that a olfactory circuitry, as seen in tetrapods, is existing in the African lungfish *Protopterus dolloi*. They showed that `epithelial crypts´ (microvillous cells; not to be confounded with teleostean crypt cells), which express markers of vomeronasal receptors project their axon to an as AOB identified structure at the lateral edge of the MOB. Moreover, secondary projections from the AOB run via a putative medial amygdala and from there to the lateral hypothalamus. Although a separate VNO may be exclusive to all tetrapods, vomeronasal receptors are found in all vertebrates so far. Moreover, based on the latter study in lungfish, together with our results presented here, we clearly demonstrate that the zebrafish *Danio rerio* exhibits all components (except a separate VNO) of a vomeronasal system comparable to that present in tetrapods. In zebrafish, OSNs within the single peripheral OE project into the mediodorsal OB from where projection neurons mediate vomeronasal information via the medial amygdala to the tuberal hypothalamus.

Since we demonstrated an accessory olfactory system in zebrafish, we investigated a possible role of the medial amygdala to be involved in kin recognition (Figure 7). Therefore, we analyzed neuronal activity along the recent identified accessory olfactory pathway, starting in the mediodorsal OB and finally in the medial amygdala in imprinted and non-imprinted zebrafish larvae following kin odor exposure. Additional to a high activation of crypt cells, visualized by an increase of the neuronal activity marker pERK (see above), we demonstrate high activation of second order neurons around the bulbar crypt cell target, mdG2. This increase in neuronal activation matches appropriately to what we found at the level of the OE. In fact, significantly elevated neuronal activation in response to kin odor is seen in crypt cells as well as in their bulbar target glomerulus, mdG2, whereas a contrary situation is seen in non-imprinted larvae (Biechl et al. 2016b). This result, together with data of our previous work corroborates that mdG2 is the exclusive target of crypt cells consistent to the `one receptor – one neuron´ rule. Furthermore, the comparison of neuronal activation

within the area of the intermediate nucleus of the ventral telencephalon (Vi; teleostean medial amygdala) revealed a somewhat unexpected situation.

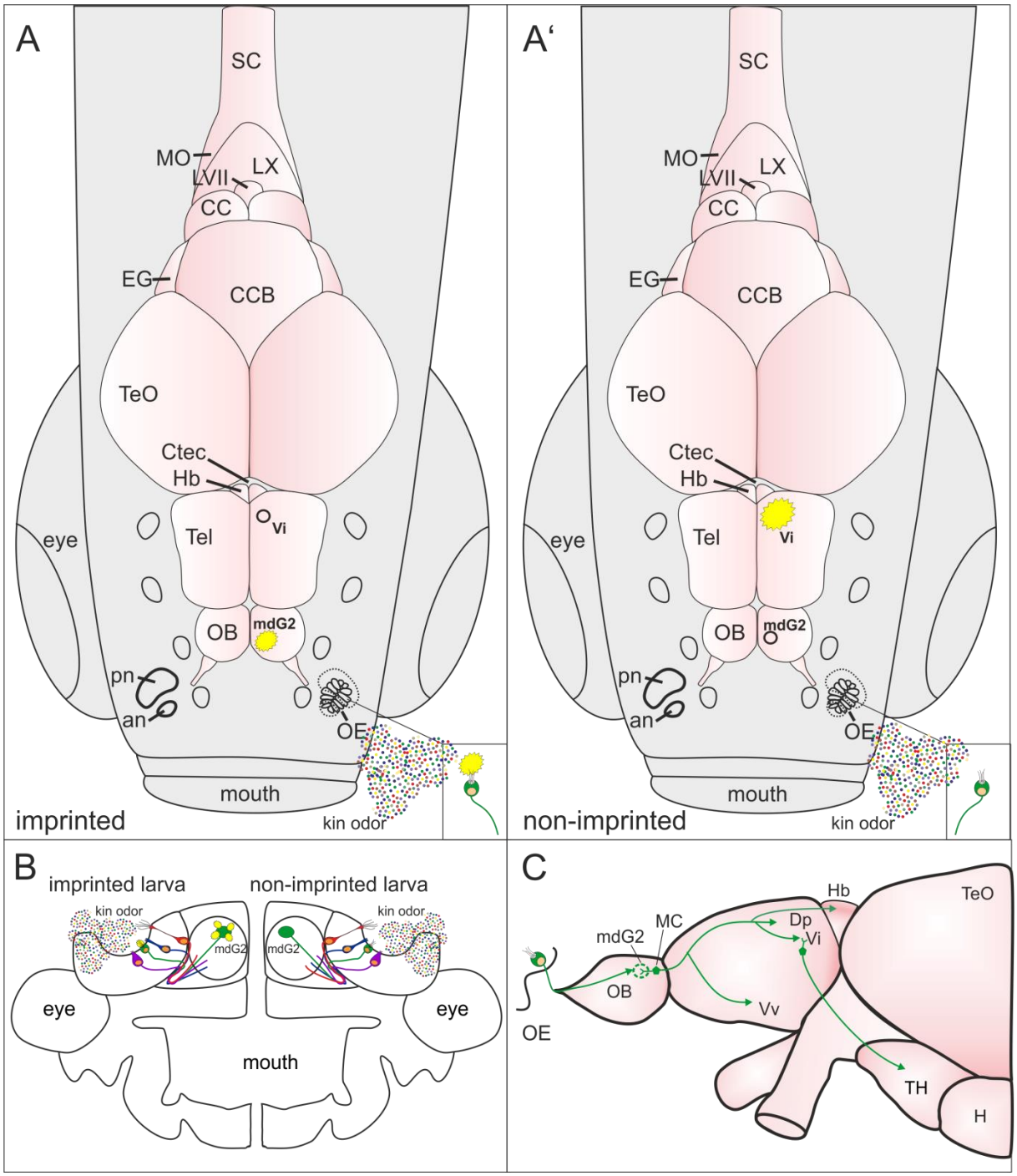

Figure 7 Comparison of neuronal activity in imprinted and non-imprinted zebrafish larvae in response to kin odor. **(A)** and **(B)** Crypt cells of imprinted larvae show increased activity (yellow cloud) following kin odor exposure, whereas **(A')** crypt cells of non-imprinted larvae show no response to kin odor.I (A) Increased activity is also seen in bulbar neurons around the crypt cell projection target, the mediodorsal glomerulus 2 (mdG2) of imprinted larvae compared to non-imprinted larvae **(A')**. In contrast to imprinted larvae **(A)**, neurons of Vi show increased activity after kin odor exposure **(A')**. **(C)** Scheme of the teleostean accessory olfactory pathway demonstrated by injection of DiI into the mediodorsal glomerulus. **Abbreviations:** an: anterior nostril; CC: crista cerebellaris; CCB: corpus cerebelli; Ctec: commissura tecti; Dp: posterior zone of dorsal telencephalon; EG: eminentia granularis; Hb: Habenula; LVII: facial lobe; LX: vagal lobe; MO: medulla oblongata; OB: olfactory bulb; OE: olfactory epithelium; pn: posterior nostril; SC: spinal cord; Tel: telencephalon; Teo: optic tectum; TH: ruberal hypothalamus; Vi: intermediate nucleus of ventral telencephalon; Vv: ventral nucleus of ventral telencephalic area.

Instead of an increase of neuronal activation in imprinted larvae as seen at previous levels of odor detection, only cells of non-imprinted larvae show increased neuronal activation exposed to kin odor within the medial amygdala. The observation of increased neuronal activation in Vi of non-imprinted larvae was puzzling at first sight, but indicates that Vi cells show activation to a new, for the larvae unknown social odor. The amygdaloid nuclei are generally considered as the center of emotional processings, as they are associated with mediating the emotional and hormonal response to sensory information, often related to stress (Davern and Head 2011). Generally, almost all subdivisions of the amygdala are involved in processing of diverse sensory inputs. However, several studies in mammals demonstrate the medial amygdala to be more activated in response to stimuli which causes stress or anxiety than other nuclei. Assuming that stress is defined by distinct brain areas, including the amygdala, into different categories, Dayas et al. (2001) demonstrated a differential sensitivity of central and medial amygdaloid nuclei to physical stress and psychological stress. Visualizing neuronal activation by expression of the immediate early gene *c-fos,* they showed that hemorrhage and immune challenge (physical stressors) activated cells of the central amygdala whereas noise and restraint (psychological stressors) activated more cells in medial nuclei of the amygdala. However, the medial amygdala is also implicated to be a major center for decision making in a social related context. Moreover, a previous study by Samuelsen and Meredith (2009) further subdivided the medial amygdala into distinct regions, each showing differential response to conspecific or heterospecific odors. A categorization of biologically relevant odors within the medial amygdala was demonstrated by presenting male mice either conspecific (male, female urine) or heterospecific (hamster vaginal fluid, steer urine) odors. Conspecific odors increased *c-fos* expression in both anterior and posterior medial amygdala, whereas a heterospecific odor activated cells only in anterior medial amygdala.

This study, as well as similar experiments in hamster, clearly demonstrates that also within the medial amygdala, a decision of odorant relevance (biologically important or not important) is made. The exposure to threatening stimuli, indicating the presence of a predator leads to an increase of neuronal activation in the posterior medial amygdala, similar to conspecific odors. Therefore, biological relevant odors, such as conspecific (reproduction & social behavior) or potentially ominous odors elicit responses in the dorsal and ventral subregions, respectively (Samuelsen and Meredith 2009).

Relating to our finding in zebrafish, which shows increased activation of the medial amygdala after kin odor exposure only in non-imprinted larvae, there are several possible hypotheses. Because of the absence of the necessary olfactory cue during the imprinting

phase, cells of the medial amygdala are activated by the "unknown" kin odor similar as they would be activated by a stressor. This also implicates that non-imprinted larvae recognize kin odor as an potentially conspecific odor, since kin odor most likely contains compounds which signal the presence of another conspecific. However, it would make sense that the presence of conspecifics, which is definitely new to a naive non-imprinted larva, and which did not create any positive or negative memory to this odor (such as imprinted larvae do in a natural environment as well as in our experiment, see below) signals potential danger. In contrast, imprinted larvae may probably store a "nothing bad happened" memory when exposed to kin odor and recollecting this olfactory memory when exposed to kin odor may modulate the release of calming hormones and peptides, such as oxytocin. The peptide hormone oxytocin has been implicated in many behaviors involved in social recognition, social attachment and especially in processes such as social bonding (Carter et al. 1992). In the sheep OB as well as in other brain regions, oxytocin release has been shown to be involved in induction of maternal behavior (Keverne and Kendrick 1992, Da Costa et al. 1996). Moreover, oxytocin knock-out mice fail to recognize their kin, but this social memory is restored after oxytocin treatment. Interestingly, neuronal activation of neurons in the medial amygdala was decreased in this mouse line compared to wildtype mice after exposure, whereas other brain structures like the OB, piriform cortex, cortical amygdala and the lateral septum displayed equal levels of neuronal activation. Moreover, projection targets of the medial amygdala also showed decreased neuronal activation in oxytocin knock-out mice compared to wildtype, thus demonstrating oxytocin receptor activation in the medial amygdala to play a crucial role for social recognition in mice (Ferguson et al. 2001).

In conclusion, at the level of the olfactory epithelium, olfactory imprinting as well as kin recognition depends on neuronal activation of crypt cells as well as a small subpopulation of mOSNs in larval zebrafish. Moreover, in contrast to imprinted larvae, crypt cells of non-imprinted zebrafish larvae fail to show neuronal response after kin odor exposure. This difference between imprinted and non-imprinted larvae indicates changes resulting from the absence of the olfactory kin cue at day 6 post fertilization. However, the molecular mechanisms underlying this difference as well as the crypt cell ligand are unkown so far. Further studies on potential structural changes of the crypt cell receptor, as well as changes involving elements of the downstream signaling pathway or inhibition of crypt cell activity by other OSNs are needed to figure out why crypt cells of non-imprinted larvae change their odorant tuning related to kin odor.

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APPENDIX

List of abbreviations:

AOB	accessory olfactory bulb
CB	Calbindin
CBP	Calcium Binding Protein
cOSN	ciliated olfactory sensory neuron
CR	Calretinin
Dp	posterior zone of the dorsal telencephalon
Dpf	days post fertilization
ECL	external cell layer
GL	glomerular layer
GPCR	G-protein coupled receptor
H	Hypothalamus
Hb	Habenula
HT	tuberal hypothalamus
ICL	internal cell layer
lMOT	lateral part of the MOT
LOT	lateral olfactory tract
mdG	mediodorsal glomerulus
mMOT	medial part of the MOT
MOE	main olfactory epithelium
mOSN	microvillous olfactory sensory neuron
MOT	medial olfactory tract
OB	olfactory bulb
OE	olfactory epithelium
ON	olfactory nerve
ONL	olfactory nerve layer
OR	odorant receptor
OSN	olfactory sensory neuron
pERK	phosphorylated extracellular signal regulated kinase
PV	Parvalbumin
TAAR	trace amine-associated receptor
TRPC2	transient receptor potential channel C2
Vi	intermediate ventral telencephalic nucleus
VNO	vomeronasalorgan
Vp	postcommissural ventral telencephalic nucleus
VR	vomeronasal receptor
Vs	supracommissural ventral telencephalic nucleus
Vv	ventral nucleus of the ventral telencephalon

Appendix

Eidestattliche Versicherung / Statutory declaration:

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation

„Neuronal basis of olfactory imprinting and kin recognition in the zebrafish *Danio rerio*“

selbstständig angefertigt habe. Desweiteren habe ich mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen.

I hereby confirm that the dissertation

“Neuronal basis of olfactory imprinting and kin recognition in the zebrafish *Danio rerio*“

is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den

Munich, date ----------

Daniela Biechl

Author Contributions

Kress, S., D. Biechl and M. F. Wullimann (2015). "Combinatorial analysis of calcium-binding proteins in larval and adult zebrafish primary olfactory system identifies differential olfactory bulb glomerular projection fields." *Brain Struct Funct* 220(4): 1951-1970.

The study was designed by SK, DB and MFW. The immunohistochemical processing was performed by SK and DB. Analysis of data was done by SK, DB and MFW. The first version of the manuscript was written by SK, DB and MFW. The final version was written by SK, DB and MFW. SK and DB share first authorship.

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Appendix

Biechl D, Tietje K, Gerlach G, Wullimann MF (2016). "Crypt cells are involved in kin recognition in larval zebrafish." *Scientific reports* 6, 24590.

The study was designed by D.B., K.T., G.G. and M.F.W. The behavioral experiments were performed by D.B. and K.T. (Oldenburg). The immunohistochemical processing was performed by D.B (Munich). Analysis of data was done by D.B., K.T., G.G. and M.F.W. The first version of the manuscript was written by D.B. and K.T. The final version was written by D.B., K.T., G.G. and M.F.W. D.B. and K.T. share first authorship.

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Biechl D, Tietje K, Ryu S, Grothe B,Gerlach G, Wullimann MF. Identification of accessory olfactory system and medial amygdala in the zebrafish.*Scientific reports* 7, 44295 (2017)

The study was designed by DB, KT, GG and MFW. The behavioral experiments were performed by DB and KT (Oldenburg). The tracing experiments were done by DB and MFW. The immunohistochemical processing was performed by DB (Munich). Analysis of data was done by DB, KT, SR, BG, GG and MFW. The first version of the manuscript was written by DB, KT and MFW. The final version was written by DB, KT, SR, BG, GG and MFW. DB and KT share first authorship.

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2013	SPP Integrative Analyses of Olfaction Annual meeting Jena, Germany. ”Neuronal basis of olfactory imprinting in the zebrafish Danio rerio”.
	SPP Integrative Analyses of Olfaction PhD student meeting Munich, Germany. “What happens when fish smell kin odor?”

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	Biechl, D., Kress S., Wullimann M., SPP Integrative Analyses of Olfaction Annual meeting Jena, Germany. “Combinatorial analysis of zebrafish olfactory sensory neurons and their primary projections shown with Cabinding proteins”.

Biechl, D., Kress S., Wullimann M., SPP Integrative Analyses of Olfaction Annual meeting German Zoollogical Society, Munich, Germany. “Combinatorial analysis of zebrafish olfactory sensory neurons and their primary projections shown with Cabinding proteins”.

Biechl, D. and Wullimann M. F., European Conference on Comparative Neurobiology (ECCN 7) Budapest, Hungary. “Transgenic *shh*-GFP line suggests hedgehog signaling in larval zebrafish optic tectum and cerebellum”

Publications

Kress, S., D. Biechl and M. F. Wullimann (2015). "Combinatorial analysis of calcium-binding proteins in larval and adult zebrafish primary olfactory system identifies differential olfactory bulb glomerular projection fields." Brain Struct Funct **220**(4): 1951-1970.

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