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RESEARCH ARTICLE

Mixed input to olfactory glomeruli from two subsets of ciliated sensory neurons does not impede relay neuron specificity in the crucian carp

Kenth-Arne Hansson*, Kjell B. Døving‡ and Frode M. Skjeldal

ABSTRACT

The consensus view of olfactory processing is that the axons of receptor-specific primary olfactory sensory neurons (OSNs) converge to a small subset of glomeruli, thus preserving the odour identity before the olfactory information is processed in higher brain centres. In the present study, we show that two different subsets of ciliated OSNs with different odorant specificities converge to the same glomeruli. In order to stain different ciliated OSNs in the crucian carp *Carassius carassius* we used two different chemical odorants, a bile salt and a purported alarm substance, together with fluorescent dextrans. The dye is transported within the axons and stains glomeruli in the olfactory bulb. Interestingly, the axons from the ciliated OSNs co-converge to the same glomeruli. Despite intermingled innervation of glomeruli, axons and terminal fields from the two different subsets of ciliated OSNs remained mono-coloured. By 4–6 days after staining, the dye was transported trans-synaptically to separately stained axons of relay neurons. These findings demonstrate that specificity of the primary neurons is retained in the olfactory pathways despite mixed innervation of the olfactory glomeruli. The results are discussed in relation to the emerging concepts about non-mammalian glomeruli.

KEY WORDS: Teleost, Olfactory bulb, Bile salt, Olfactory glomeruli, Alarm substance, Olfaction, Trans-synaptic staining

INTRODUCTION

In all animals, olfactory sensitivity and specificity are based upon the unique structure of the olfactory system, where knowledge of the functional relationship between receptors and secondary neurons is a prerequisite for understanding the processing mechanisms of the olfactory system. Axons of the olfactory sensory neurons (OSNs) converge onto relay neurons (mitral cells) in synaptic structures called glomeruli. This anatomical feature has been a focus of interest throughout the history of neuroscience, and the glomeruli were central in the debate between followers of Cajal and Golgi concerning the neuron doctrine (Cajal, 1955; Golgi, 1875). The axons of OSNs branch several times as they enter the glomeruli (van Gehuchten and Martin, 1891) and make synapses en passant (Pinching and Powell, 1971). These features, and the fact that the ratio between the number of OSNs to relay neurons is about 1000:1, documented in rabbits (Allison and Warwick, 1949), bats (Bhatnagar and Kallen, 1975) and fish (Gemne and Døving, 1969), add to the complexity. Several techniques have been utilized to demonstrate the projection pattern: localized degeneration (Land, 1973), amino acid transport (Land and Shepherd, 1974), horse radish peroxidase (HRP) (Astic et al., 1987; Dubois-Dauphin et al., 1981; Jastreboff et al., 1984; Oakley and Riddle, 1992), lectin (Riddle et al., 1993), the neurotracer DiI (Hamdani et al., 2001; Morita and Finger, 1998) and viral tracing techniques (Ghosh et al., 2011; Miyamichi et al., 2011).

A new era in studies of the olfactory system was initiated by the discovery of the genetic basis for odorant receptors (Buck and Axel, 1991) and the demonstration that all sensory neurons expressing a particular odorant receptor project to a small subset of olfactory glomeruli in mammals (Ressler et al., 1994; Vassar et al., 1994) and in fish (Yoshihara, 2009). In mammals, the olfactory receptors may be encoded by as many as 1000 genes (Levy et al., 1991; Parmentier et al., 1992) and only one receptor type is expressed in each cell (Malnic et al., 1999). In fish, the receptor gene family is less extensive (Ngai et al., 1993) and some olfactory sensory neurons can express more than one type of receptor protein (Sato et al., 2007).

In teleosts, three different types of OSNs are embedded in the olfactory sensory epithelium. Based upon morphology, they are classified as: ciliated sensory neurons with long dendrites that end in a dendritic knob with cilia, microvillous sensory neurons with shorter dendrites that end with microvilli (Ichikawa and Ueda, 1977; Thommesen, 1983) and crypt neurons containing both cilia and microvilli (Hansen and Finger, 2000). The somata of the three morphologically different sensory neurons are located in specific layers of the sensory epithelium. The olfactory epithelium can be divided into five layers, from the surface to the basal membrane: lamina 1 is adjacent to the apical surface, and lamina 5 is bordering the basal membrane. The ciliated sensory neurons are located in lamina 4–5, the microvillous neurons in lamina 3–4 and crypt cells are embedded in lamina 1–2 (Hamdani and Døving, 2007).

In the present study, we tested two different chemical odorants, a bile salt (taurolithocholate, TLC) and an alarm agonist (hypoxanthine-3-N-oxide, H3NO). In both salmonids (Døving et al., 1980) and the sea lamprey *Petromyzon marinus* (Fine and Sorensen, 2005), bile salts are a putative social pheromone (Døving et al., 1980; Fine and Sorensen, 2005). H3NO is a potent odorant that evokes an alarm reaction in minnows (Brown et al., 2000), black tetra (Pfeiffer et al., 1985) and zebrafish (Mathuru et al., 2012; Parra et al., 2009). Although the odorants are different their putative role as olfactory cues in crucian carp is not yet proved, both TLC and H3NO induce activity-dependent uptake of fluorescent dye into ciliated OSNs (Døving et al., 2011). Previous studies in crucian carp have shown that the ciliated OSNs project to the medial olfactory bulb and synapse with relay neurons with axons in the medial part of

Department of Biosciences, University of Oslo, Oslo N-0316, Norway.
‡Dedicated to Professor Kjell B. Døving (1936-2014).

*Author for correspondence (k.a.hansson@medisin.uio.no)

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the medial olfactory tract (Hamdani and Døving, 2002). This part of the olfactory tract also mediates the alarm reaction (Hamdani et al., 2000).

An accumulating body of evidence suggests that odour signalling in the non-mammalian olfactory glomeruli is different from the mammalian glomeruli; in zebrafish, some OSNs can express more than one type of receptor, and glomeruli are not innervated by different types of OSNs (Sato et al., 2005, 2007). Because of the broad tuning properties of olfactory receptors (Malnic et al., 1999) and their bimodal expression pattern in fish (Sato et al., 2007), some subsets of either microvillous or ciliated OSNs may share type-specific glomeruli. Furthermore, some glomeruli are activated in a combinatorial manner (Friedrich and Korsching, 1997, 1998) and in amphibians, axons from OSNs tend to innervate more than one glomerulus (Hassenklover and Manzini, 2013). However, most studies have not combined physiological and anatomical methods to investigate odour mapping in the olfactory bulb while identifying activated primary OSNs. Thus, as previous findings revealed that dextran conjugated with Alexa dye could stain a specific set of primary OSNs (Døving et al., 2011) as a result of activity-dependent uptake of the fluorescent dye, the present study was undertaken with the intention of clarifying whether there are two separate subsets of ciliated OSNs responding to TLC and H3NO, respectively, with axons projecting to different glomeruli in the olfactory bulb. The TLC was applied with a pseudo-green dye and H3NO with a pseudo-magenta dye. As expected, these two odorants stained two different subsets of ciliated OSN ensembles. Unexpectedly, the H3NO- and TLC-stained axons frequently terminated in the same glomeruli. However, the axons of relay neurons in the medial olfactory tract were trans-synaptically stained either magenta or green. Therefore, despite the intermingled and complex association of H3NO- and TLC-stained axons in the glomeruli, the two subsets of axons still make synaptic contact with dendrites of different and specific relay neurons. Thus, the specificity of connections between primary OSNs and relay neurons is retained.

MATERIALS AND METHODS

Experimental procedures were approved by the Norwegian Animal Research Authority and were conducted in accordance with the Norwegian Animal Welfare Act of 1974, and the Regulation of Animal Experimentation of 1996. Crucian carp (20–45 g body mass) were caught in a lake close to Oslo and kept in holding tanks at the University of Oslo.

Chemicals and odorants

We used dextrans conjugated with the Alexa dyes 10 kDa anionic fixable Alexa fluor 488 (D 22910) and 10 kDa anionic fixable Alexa fluor 647 (D22914). Bile salt taurolithocholic acid (TLC) was prepared as a stock solution at a concentration of 1 mmol l^{-1} in DMSO and applied at 10 nmol l^{-1} with the final DMSO concentration at 0.001% (v/v). Hypoxanthine-3-N-oxide (H3NO) was obtained from Menai Organics Limited (Bangor, UK) and prepared by dissolving H3NO in 5 mmol l^{-1} NaCl and 2 mmol l^{-1} HEPES, pH 5.3. H3NO was diluted to 0.1 mmol l^{-1} in artificial pond water (APW): (mmol l^{-1}) NaCl (0.5), KCl (0.05), $CaCl_2$ (0.4), $NaHCO_3$ (0.2), pH 7.4. The final H3NO concentration was 10 nmol l^{-1}. All solutions were stored at −20°C.

Experiments

The crucian carp were anesthetized by an intra-peritoneal injection of 2.5 mg kg^{-1} Alfaxan (Vétoquinol Ltd, Buckingham, UK) and placed in a cradle with tap water irrigating the gills. Solutions were led to the olfactory cavity via a catheter to the anterior naris. For all experiments, the odorants were dissolved in APW.

We stained two subsets of ciliated sensory neurons of the olfactory epithelium of crucian carp with the aid of odorant-induced uptake of fluorescent dye (Døving et al., 2011). To this end we perfused the ipsilateral olfactory epithelium, first with 10 nmol l^{-1} of the bile salt taurolithocholate (TLC) together with 10 µmol l^{-1} of a dextran conjugated with Alexa 488 in APW for 5 min, rinsed with APW for 5 min, and subsequently exposed the epithelium with 10 nmol l^{-1} of the alarm substance hypoxanthine-3-N-oxide (H3NO) together with 10 µmol l^{-1} of a dextran conjugated with Alexa 647 in APW for 5 min. In the contralateral olfactory epithelium we reversed the staining protocol. After a variable time period, from 1 h to 6 days, the fish were re-anesthetized and perfused via the heart with 4% buffered paraformaldehyde (phosphate buffer 0.1 mol l^{-1}, pH 7.2). After fixation, the olfactory organs with bulb and tracts were dissected free and left in fixative. The lamellae of the olfactory rosette were dissected free and placed in Fluoromount G (EMS, Hatfield, PA, USA) under a coverslip on a microscope slide. For detailed studies of the epithelium, bulb and olfactory tract, the specimens were embedded in 12% gelatine solution in 0.1 mol l^{-1} phosphate buffer cooled and solidified at 4°C, and sectioned in a vibratome. Controls were performed by exposing the epithelium to APW with 10 µmol l^{-1} dextran-conjugated with Alexa dye, with or without DMSO at its final concentration. DMSO did not affect the uptake of dye.

Optical imaging

A confocal microscope (Olympus FluoView 1000, BX61W1, Olympus, Japan) was used to observe the details of single lamellae or sections in the preparations. Pictures were taken in confocal planes, separated by *z*-axis steps varying between 0.4 and 2 µm. Confocal microscope images were processed and analysed using Imaris®. ImageJ (NIH, Bethesda, MD, USA) was used to handle information from photographs taken at different depths. Images were imported and stacks added in a *Z*-projection with standard deviation as a parameter. The following water immersion objectives were used: 10× NA 0.30; 20× NA 0.50; 40× NA 0.80; and 60× NA 0.90. Imaging of lamellae and whole *in situ* preparations was performed using a conventional fluorescence microscope (Olympus, BX50WI) equipped with a digital camera (ProgRes, Jena, Germany). For this microscope, a 2× objective with NA 0.05 was also used.

Analysis

For vibratome sections of the olfactory rosette, the position of the cell body of each stained sensory neuron was categorized by the location of its nucleus within the epithelium. The sensory epithelium was divided into five equal zones (layers) from the surface to the basal lamina, zone 1 being the uppermost and zone 5 the region closest to the basal membrane. Thus, the position of each cell soma was assigned to a particular zone as in Hamdani et al. (2007). Counting of stained sensory neurons was made from confocal images of olfactory lamellae using a 40× objective taking a *z*-series of 10 images, each with a 1 µm *z*-step, stacked in the *z*-plane and covering a width of 315 µm and a depth in the *z*-axis of 50 µm, giving an area of 15,750 $µm^2$. The distribution of cell soma of five such regions was determined. For analysis of the olfactory bulb, a circumference region of interest at the external border of each glomerulus was selected and analysed with a colour histogram in ImageJ. Each colour channel was subtracted for background fluorescence. Results are represented as relative numbers of mean±s.d.

RESULTS

To examine the staining of the OSNs, we used specimens that were sacrificed minutes to 1 h after exposure. In these preparations, we observed two separate subsets of cells, with the initial part of the axons stained either magenta or green. Fig. 1A shows an example of the separate staining of the two subsets of the ciliated OSNs in an overview of an olfactory lamella. In specimens sacrificed only minutes after exposure, the dye appeared in vesicular structures (Fig. 1B, insert), but the staining became homogeneous as the vesicles accumulated after prolonged survival time (Fig. 1B).

Morphometric quantification of the H3NO- and TLC-sensitive OSNs, and the position of their cell soma in discrete areas (315×50 $µm^2$) of the sensory epithelium, revealed that 80% of the OSNs had their cell soma in layers 4 or 5, indicating that they were

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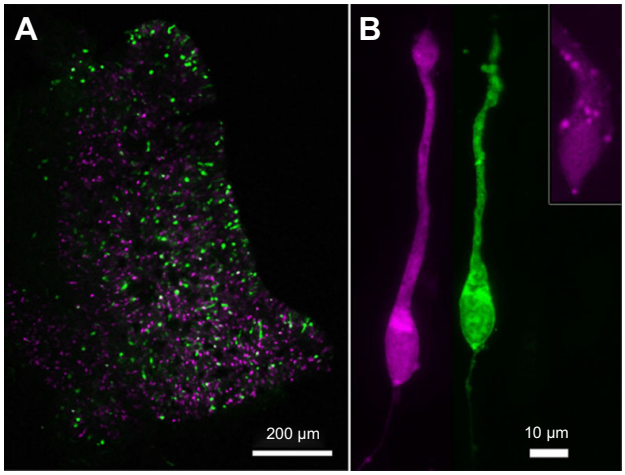

Fig. 1. Sensory neurons in the olfactory epithelium of crucian carp (*Carassius carassius*). (A) Confocal image from the middle of a lamella of the olfactory rosette with stained sensory neurons. (B) *Z*-projection of 40 images taken with a *z*-distance of 0.4 µm, thus covering a depth of 16 µm, demonstrating that the stain is distributed differently in the ciliated sensory neuron. Insert is an image taken minutes after odorant exposure. At this stage, the dextran dye is observed to be transported in vesicle-like structures, with a perinuclear distribution at the interface between soma and axon.

ciliated OSNs (Hamdani and Døving, 2007). The total density of cells was approximately $8.8\pm1.3\times10^3$ cells mm^{-2}, of which H3NO stained $4.8\pm1.3\times10^3$ cells mm^{-2} and TLC stained $3.4\pm0.9\times10^3$ cells mm^{-2}.

In preparations from crucian carp that were sacrificed 2 or 3 days after exposure, axons from ciliated OSNs fasciculate as they leave the sensory epithelium (Fig. 2). The axons projecting from the soma of sensory neurons were stained either magenta or green, and the mean diameter of these axons was 0.14 µm (Gemne and Døving, 1969). Owing to the different pseudo-colours of the two dyes, it was easy to distinguish between the two subsets of axons in the olfactory nerve (Fig. 3). Because there were co-stained OSNs in the epithelium, we specifically looked for co-stained axons in fasciculi, the distal nerve and the bulb. Co-stained axons were seldom observed, and then only for short distances as they were leaving the neuron.

The terminal fields of both the H3NO- and TLC-stained axons were found mainly in the dorsal and medial olfactory bulb, which is congruent with previous findings related to the odotopy for bile acid (Døving et al., 1980; Friedrich and Korsching, 1997; Hamdani and Døving, 2007; Sato et al., 2005) and H3NO (Mathuru et al., 2012) in the olfactory bulb. To our surprise, the magenta and green labelled axons were observed intermingled (18 olfactory bulbs) as they terminated in the same glomeruli (Fig. 4). In 3 of the 18 bulbs examined, mixed innervation was only observed in glomeruli restricted to the medial part of the olfactory bulbs, and one bulb showed mixed innervation in the dorsal region only. The remaining 14 bulbs showed heterogeneous innervation of glomeruli both in the medial and dorsal part of the bulb. The morphology of glomeruli in one olfactory bulb was not always repeated in the contralateral bulb; however, structures in the dorsal region of the olfactory bulb resembling glomerular plexi (Fig. 4A–C) were frequently observed and canonical glomeruli were prominent in the medial region (Fig. 4D–I). The diameters of different glomeruli vary between 15 µm and 100 µm.

Analysis of the differential innervation of the glomeruli with axons stained with either H3NO (0.56±0.039) or TLC (0.44±0.072) based on a colour histogram, revealed a minor bias of glomeruli innervated by H3NO axons independent of glomerular size, structure and position. Because olfactory glomeruli in teleosts are much less well defined than those in mammals (Satou, 1990), axons could simply be passing glomeruli en route to juxtaposed glomeruli. We searched for terminal fields with only magenta or green axons, but did not find any. Hence, axons from the two stained subsets of ciliated OSNs seemed to coincide in the glomeruli, and we therefore conclude that the olfactory glomeruli received a merged projection of H3NO- and TLC-labelled ciliated OSNs.

Dextran dyes are transported in axons in both anterograde and retrograde directions (Glover et al., 1986). Although stained dendrites and soma of relay neurons were difficult to detect in the olfactory bulb by our present methods, distinctly magenta or green axons were seen in the medial olfactory tract in 3 of 5 preparations that were made more than 4 days after exposure (Fig. 5), and one preparation showed unilateral staining in the olfactory tract. In all preparations, stained axons were found in the medial olfactory tract, demonstrating that the ciliated OSNs make synapses with relay neurons with axons in this region, in agreement with previous findings (Hamdani and Døving, 2002; Hamdani et al., 2000). As teleosts and amphibians have an additional extrabulbar projection that extends from the periphery and traverse the olfactory bulb before ending in different regions of the telencephalon (Kermen et al., 2013), we explicitly searched for axons crossing the olfactory bulb without finding any. These findings demonstrate that the specificity of TLC- and H3NO-sensitive ciliated OSNs is retained in the olfactory pathways despite mixed innervations of the olfactory glomeruli.

DISCUSSION

The present study is a functional mapping of primary neurons responding to two different odorants: a bile salt and a substance

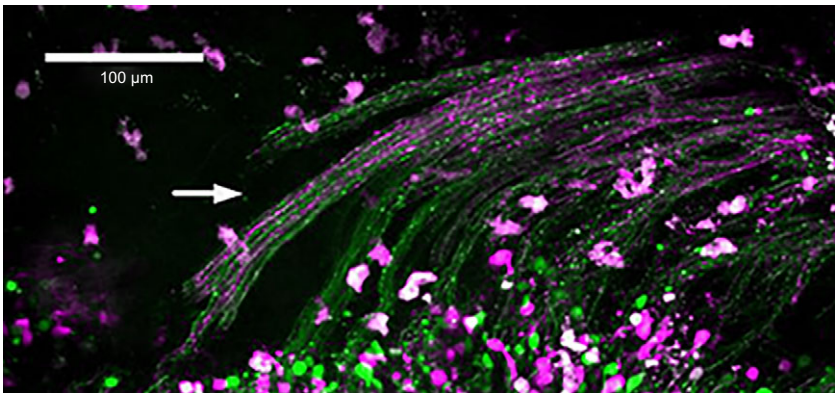

Fig. 2. Fasciculating axons from ciliated sensory neurons of crucian carp. *Z*-projection of 160 images taken with a *z*-distance of 1 µm, covering a depth of 160 µm. Axons from H3NO- and TLC-sensitive ciliated sensory neurons fasciculate before they form bundles in the proximal part of the olfactory nerve (arrow).

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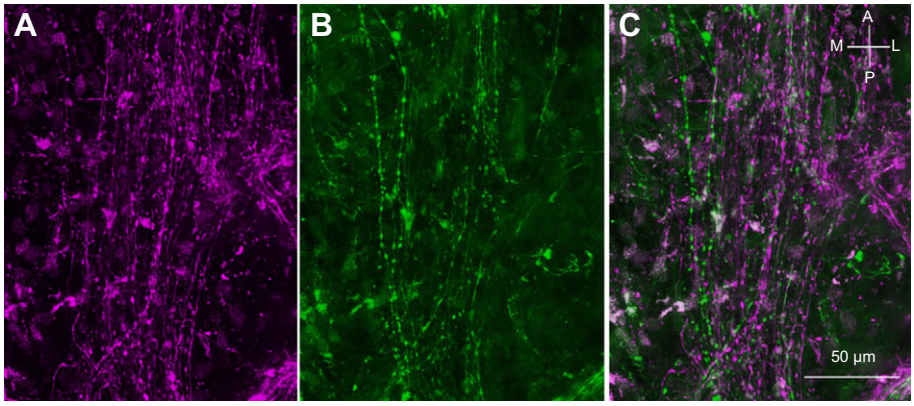

Fig. 3. Axons in the olfactory nerve of crucian carp. *Z*-projection of 96 confocal images taken with a *z*-step of 0.4 µm, covering a depth of 38 µm of the olfactory nerve. Images are taken at the distal part of the olfactory nerve. Note that axons are stained with H3NO (A) or TLC (B) and that the merged image (C) does not show any co-labelled axons. Note also the occurrence of spots with high fluorescence. M, medial; L, lateral; A, anterior; P, posterior.

that evokes an alarm reaction. The chosen odorants stained two distinct subsets of ciliated OSNs as a result of activity-dependent uptake of fluorescent dextran. Despite the broad tuning properties of the olfactory receptors (Malnic et al., 1999), most of the ciliated OSNs were mono-coloured, showing that the chemoresponsiveness differed in the two subsets of ciliated OSNs. Furthermore, afferent axons from differently stained neurons frequently merged into common glomeruli, but such mixed sensory projection to glomeruli probably does not impede the specificity of the relay neurons.

The experiments revealed a small percentage of OSNs that co-stained with both the bile salt and the alarm substance. These neurons may have been in a state of apoptosis since they had shorter dendrites than the OSNs that stained either green or magenta, and because none of the axons in the olfactory nerve or bulb co-stained. The crypt cells in the olfactory system of crucian carp disappear during the winter season to reappear in the spring (Hamdani et al., 2008). Application of the neurotracer DiI in the bulb revealed that the first reappearing crypt cells were found deep in the epithelium, emerging from the progenitor cells at the basal membrane (Hamdani

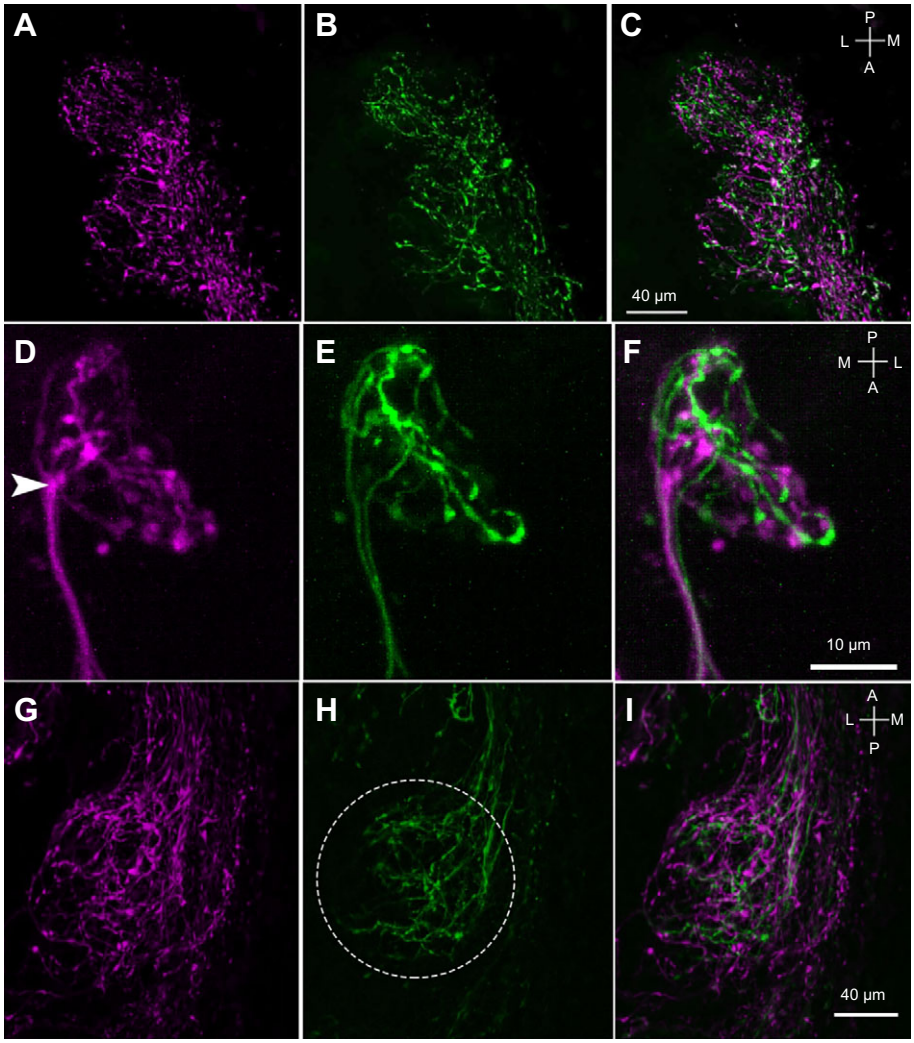

Fig. 4. Axonal terminals in glomeruli of the olfactory bulb of crucian carp. (A–C) Images of a dorsal glomeruli with a multitude of terminating axons. (D–F) Images of a few axons terminating in a small glomerulus in the medial olfactory bulb. (G–I) Images of axons terminating in a medial glomerulus. The confocal images are representatives from a region of the olfactory bulb where axons are stained with either H3NO (A,D and G) or with TLC (B,E and H). The images in C,F and I are merged. A–C are *z*-projections of a stack of 132 images taken at 0.4 µm intervals, thus covering a depth of 52 µm. D–F are *z*-projections of 17 images taken at 0.4 µm intervals, covering 6.4 µm. G–I are *z*-projections of 128 images taken at 0.4 µm intervals, covering 51 µm. The images demonstrate that axons from the two subsets of ciliated sensory neurons merge in the glomeruli. Note that the axons branch (arrowhead) when they enter glomeruli (dashed circle) and that the stain is more intense at certain regions than in others. M, medial; L, lateral; A, anterior; P, posterior.

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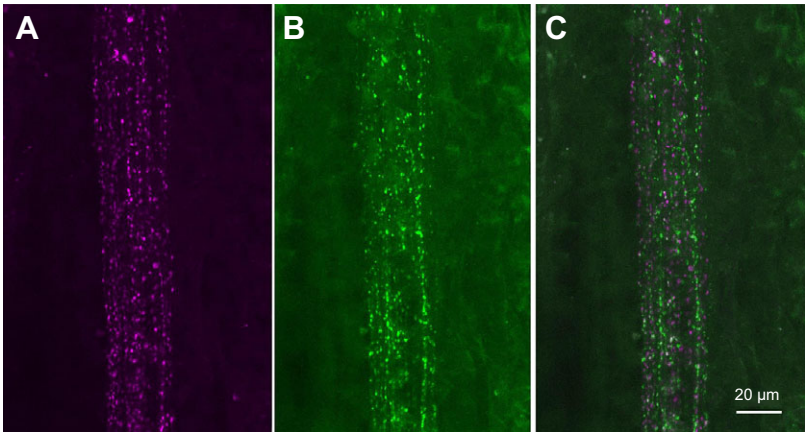

Fig. 5. Axons of the secondary neurons in the olfactory tract. Z-projections of 31 images taken with a *z*-distance of 0.4 μm, thus covering a depth of 12 μm. Axons of secondary neurons are stained either magenta or green. Axons of secondary neurons stained from H3NO-sensitive ciliated sensory neurons (A) and axons of secondary neurons stained from TLC-sensitive ciliated sensory neurons (B) are intermingled in the medial part of the medial olfactory tract (C, merged image).

and Døving, 2006). Thus, the co-stained neurons could be at the stage of being expelled and excluded from the sensory epithelium. If so, it would mean that the OSNs in apoptosis lose their specificity. Obviously, this suggestion can be studied by a variety of different methods.

Fig. 4 shows representative glomeruli with different morphologies that are doubly stained by two different odorants in sequence. Hence, the heterogeneous innervation of glomeruli is coupled to two different odour-induced behaviours. Although the precise function of bile salts (Buchinger et al., 2014) and alarm substances (Døving and Lastein, 2009) as odorants has been debated, the present study shows that TLC and H3NO induce staining in different subsets of ciliated OSNs. Hence, the two ensembles of ciliated OSNs show different chemoresponsiveness and are stained accordingly.

As there are no available data that describe the glomerular organization in the crucian carp, an in-depth analysis of the position of the glomeruli was not further investigated. Although the structure seen in Fig. 4D–F is clearly a single glomerulus, previous studies indicate that larger and diffuse glomeruli (Fig. 4A–C) receive input from more than one population of OSNs (Sato et al., 2005). This structure resembles a glomerular plexus that has been anatomically investigated in zebrafish (Baier and Korsching, 1994) and in the rainbow trout *Oncorhynchus mykiss* (Riddle and Oakley, 1992).

Studies using voltage- and calcium-sensitive dyes in the zebrafish olfactory bulb indicate a combinatorial and non-combinatorial representation of natural odorants (Friedrich and Korsching, 1997, 1998). However, these studies were not undertaken to demonstrate the identity of primary neurons. Although, the latter study showed that increasing odour concentration facilitated combinatorial activation in the olfactory bulb, and as our present study revealed minor inconsistency in morphology of glomeruli, this is most likely due to the broad tuning properties, specificity, and expression of olfactory receptors (Sato et al., 2007). Hence, the staining in ciliated OSNs and in the olfactory bulb reflects the odorant-induced neural activity *pro tempore*, as opposed to neuroanatomical tracing studies that saturate the input to glomeruli (Baier and Korsching, 1994; Braubach et al., 2012).

The present study shows that TLC- and H3NO-sensitive ciliated OSNs share glomeruli. In mammals, all OSNs expressing a particular receptor protein converge to innervate the same olfactory glomeruli. Within the glomeruli, axons of OSNs make synapses with dendrites of mitral cells, implying that all mitral cells and OSNs connecting to the same glomerulus convey the same odour identities. Thus, mitral cells related to a given glomerulus and the associated OSNs constitute a functional unit, which represent a certain odour specificity (Bozza et al., 2002; Kajiya et al., 2001; Wachowiak et al., 2004). Interestingly, transgenic studies in zebrafish revealed that ciliated OSNs do not share glomeruli with microvillous OSNs (Sato et al., 2005, 2007) and recently, chondroitins were shown to be potent alarm substances that activate a mediodorsal locus in the zebrafish olfactory bulb (Mathuru et al., 2012), which is also activated by sex pheromones (Friedrich and Korsching, 1998). The mediodorsal region is innervated by crypt neurons expressing one type of receptor (Ahuja et al., 2013), which was reported to be activated by multiple ligands (Bazaes and Schmachtenberg, 2012; Vielma et al., 2008). Therefore, it might be that H3NO- and TLC-sensitive neurons express the same type(s) of receptor, but differentiate with respect to function and subcellular machinery.

In the present study, we demonstrate that the dyes are conveyed from the OSNs to the relay neurons. This feature raises the question of how the dye is transported across the synapse. The dye transfer occurs from primary neurons in the synaptic region with retained specificity, even if axon terminals carrying either magenta or green dye terminate side by side in the glomeruli, as shown in Fig. 4. A probable scenario for the dye transfer is as follows: because fixable dextran has lysine residues, which are suitable for conjugation to other biomolecules and processing in the Golgi apparatus (Broadwell and Balin, 1985), the dye could be situated in either the interior or exterior of the synaptic vesicles or at the presynaptic membrane, and thus could be released with the neurotransmitter. At the postsynaptic site, the dye is again taken up by endocytosis (Lu et al., 2007). As the relay neurons are stained either magenta or green, spread of dye by diffusion is sufficiently restricted to prevent dye uptake by neighbouring neurons. Such restricted sub-compartmentalization within a glomerulus has been observed both in mammals (Treloar et al., 1996) and fish (Friedrich and Korsching, 1998), implying functional segregation within the glomeruli. Thus, the re-sorting concept of efferent axons to the telencephalon (Miyasaka et al., 2014) might be established at the site of olfactory glomeruli, as shown by the present study.

By using a confocal microscope to visualize the internalization of two fluorescent dyes into functionally distinct ciliated OSNs by odorant-induced uptake of fluorescent dextran, we demonstrate that two different subsets of ciliated OSNs are stained, and that their corresponding axons converge onto the same glomeruli. As the two subtypes of ciliated OSNs show different chemoresponsiveness, we conclude that physiologically different ciliated OSNs share glomeruli, implying functional subdivisions within a glomerulus. However, the specificity of relay neurons is retained. It should be recognized that the stimuli were tested in one species of fish only,

and that their precise function as odorants is as yet unresolved; glomerular function should be confirmed by electrophysiological recording.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
K.-A.H., K.B.D. designed research; K.-A.H., K.B.D. and F.M.S. performed research; K.-A.H. contributed new reagents/analytic tools; K.-A.H., K.B.D. and F.M.S. analysed data; and K.-A.H., K.B.D. and F.M.S. wrote the paper.

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