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The Somatotopic Organization of the Olfactory Bulb in Elasmobranchs

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ABSTRACT The olfactory bulbs (OBs) are bilaterally paired structures in the vertebrate forebrain that receive and process odor information from the olfactory receptor neurons (ORNs) in the periphery. Virtually all vertebrate OBs are arranged chemotopically, with different regions of the OB processing different types of odorants. However, there is some evidence that elasmobranch fishes (sharks, rays, and skates) may possess a gross somatotopic organization instead. To test this hypothesis, we used histological staining and retrograde tracing techniques to examine the morphology and organization of ORN projections from the olfactory epithelium (OE) to the OB in three elasmobranch species with varying OB morphologies. In all three species, glomeruli in the OB received projections from ORNs located on only the three to five lamellae situated immediately anterior within the OE. These results support that the gross arrangement of the elasmobranch OB is somatotopic, an organization unique among fishes and most other vertebrates. In addition, certain elasmobranch species possess a unique OB morphology in which each OB is physically subdivided into two or more "hemi-olfactory bulbs." Somatotopy could provide a preadaptation which facilitated the evolution of olfactory hemibulbs in these species. J. Morphol. 274:447–455, 2013.

KEY WORDS: elasmobranch; olfactory bulb; somatotopy; chemotopy; olfaction

INTRODUCTION

The elasmobranch olfactory system is morphologically and physiologically similar to that of teleost fishes, although elasmobranchs possess only two (microvillous and crypt) of the three morphologically different types of olfactory receptor neurons (ORNs) found in bony fishes (Northcutt, 1978; Theisen et al., 1986; Takami et al., 1994; Hansen and Zielinski, 2005; Schluessel et al., 2008). Further, the olfactory system of elasmobranchs demonstrates a similar specificity and sensitivity to amino acid odorants as teleosts (Silver, 1979; Zeiske et al., 1986; Hara, 1994; Tricas et al., 2009; Meredith and Kajiura, 2010). However, notable differences occur in the olfactory systems of these sympatric species. For example, the olfactory bulb (OB) in teleosts typically has a round shape and is located either adjacent to the rest of the forebrain (i.e., a sessile OB) or immediately adjacent to the olfactory organ (a pedunculated OB); however, the elasmobranch OB has only a pedunculated arrangement (Northcutt, 1978; Zielinski and Hara, 2006). Also, in some elasmobranch species, the OB is physically partitioned into either two distinct hemibulbs, as exemplified in the lemon shark (*Negaprion brevirostris*) and the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*) or as a succession of connected bulbar swellings as seen in the bonnethead shark (*Sphyrna tiburo*) (Northcutt, 1978). Although the functional significance of this hemi-OB morphology is currently not understood, it may indicate either a functional segregation of olfactory projections from the OE to the separate hemi-OBs, similar to the medial and lateral portions of the teleost OB which process social and feeding cues, respectively (Nikonov and Caprio, 2001; Hamdani and Døving, 2007). Alternatively, it could signify a spatial segregation of projections to maintain a spatial component in the processing of olfactory information.

A previous study examining three elasmobranch species (*Dasyatis sabina*, *R. terraenovae*, and *S. tiburo*) found that ORNs in the medial half of the OE projected immediately posterior to glomeruli in the medial half of the OB, and ORNs in the lateral

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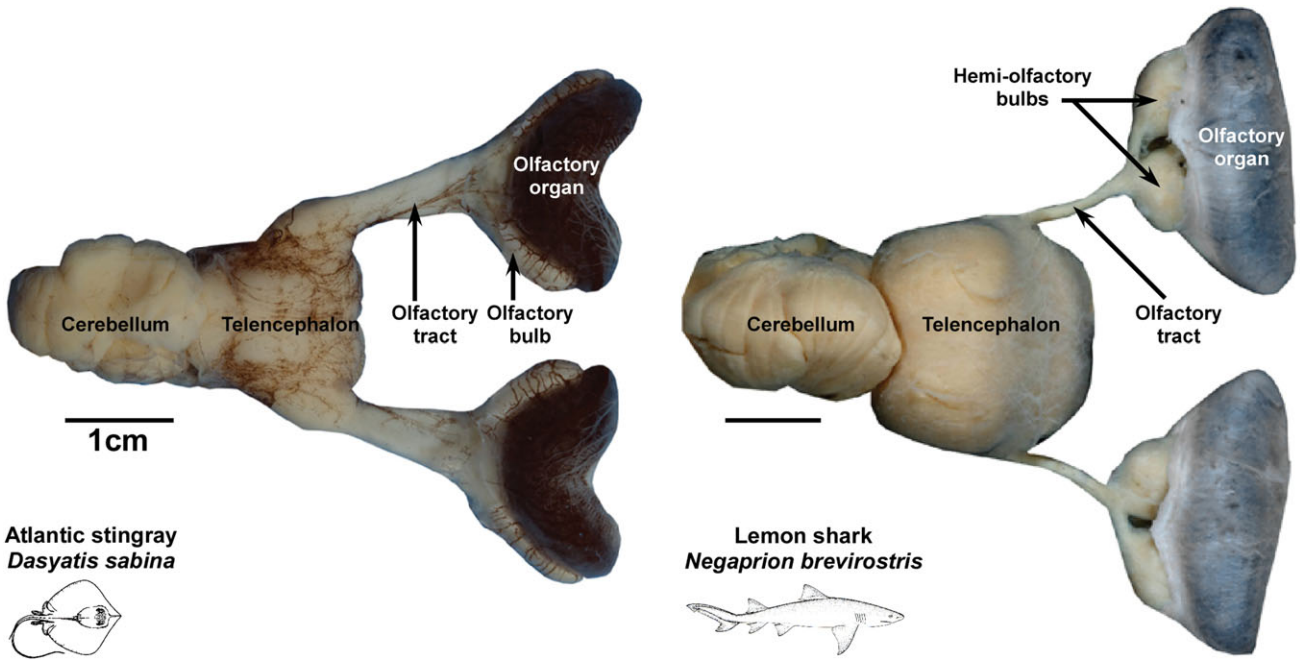

Fig. 1. Photographs of the brains of the Atlantic stingray (*D. sabina*) and lemon shark (*N. brevirostris*) illustrate their differing OB morphologies. The OBs of *D. sabina* and *D. say* (the bluntnose stingray) both occur as a cohesive unit, whereas the OB of *N. brevirostris* occurs as two physically separate hemi-OBs. The Line drawing of the Atlantic stingray is modified from Bigelow and Schroeder (1953), and the line drawing of the lemon shark is modified from Compagno (2002).

OE projected to the lateral OB (Dryer and Graziadei, 1993). These data suggested a somatotopic arrangement, in which sensory projections maintain their spatial organization from the peripheral to the central nervous system as opposed to a functional, or chemotopic, arrangement as in teleost fishes and other vertebrates. Given the limitations of their methodology, their goal was to determine the segregation of input between the medial and lateral halves of the OB, rather than to define precise projection regions. In this study, we examined the morphology and organization of ORN projections in three elasmobranch species, *D. sabina, D. say,* and *N. brevirostris*, that possess differing OB morphologies to more precisely assess the extent of somatotopy in the elasmobranch OB. Specifically, we tested the hypothesis put forth by Dryer and Graziadei (1993) that axons projecting from the OE to the OB exhibit a somatotopic arrangement, which may be related to the hemi-OB morphology.

MATERIALS AND METHODS

Sample Collection

We examined the morphology and organization of the OE and OBs of two stingray species, the Atlantic stingray *Dasyatis sabina* (Lesueur, 1824) and the bluntnose stingray *D. say* (Lesueur, 1817), and one shark species, the lemon shark *Negaprion brevirostris* (Poey, 1868), representing two elasmobranch orders, Rajiformes and Carcharhiniformes (Fig. 1). Adult stingray samples were acquired during the collections of the Florida Fish and Wildlife Conservation Commission in the Indian River Lagoon in Florida. Samples from juvenile lemon sharks were obtained from other investigators at the Elasmobranch Research Laboratory at Florida Atlantic University in Boca Raton, FL. All animals were humanely euthanized, the cranial cavity carefully exposed, and the head immediately fixed by immersion in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer for a minimum of 48 h. Fixed specimens were then transferred to the University of Colorado Denver for sample processing.

Histological Staining

The organization of the OB and OE of a single *D. say* sample was examined by treating serial cryosections (30–40 μm) of the whole olfactory organ and the OB with the histological stain Kernechtrot-Lichtgrün-Orange (KLO; nuclear red-light green-orange). The slides containing the olfactory structures were washed in dH_2O for 2 min, immersed in 0.1% nuclear red (C.I. 60760, Merck, Darmstadt, Germany) in 5% aluminum sulphate for 10 min, washed in dH_2O for 5 s, and immersed in a mixture of 0.2% light green (C.I. 42095, Allied Chemical, NY) and 1% orange G (C.I. 16230, Allied Chemical, NY) in 0.5% phosphotungstic acid in 1% acidic acid for 2 min. After staining, the slides were dehydrated in ethanol (96% for 10 s, 96% for 20 s, 100% for 5 min, and 100% for 5 min) and xylene (twice for 10 min each). Slides were then sealed with coverslips using Permount mounting medium (Fisher Scientific, Pittsburgh, PA) and examined under a light microscope (Olympus, Center Valley, PA). KLO stains nuclei red, the collagenous connective tissue and basal lamina were stained green, erythrocytes stained yellow to orange, neuron somas appear red (due to nuclear staining), and neuropil (neuron processes) stain slightly greenish-gray.

Retrograde Tracing

The ORN projections from the OE to the OB of *D. sabina* (n = 2), *D. say* (n = 4), and *N. brevirostris* (n = 12) were visualized using retrograde tracing techniques. Small crystals of 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate

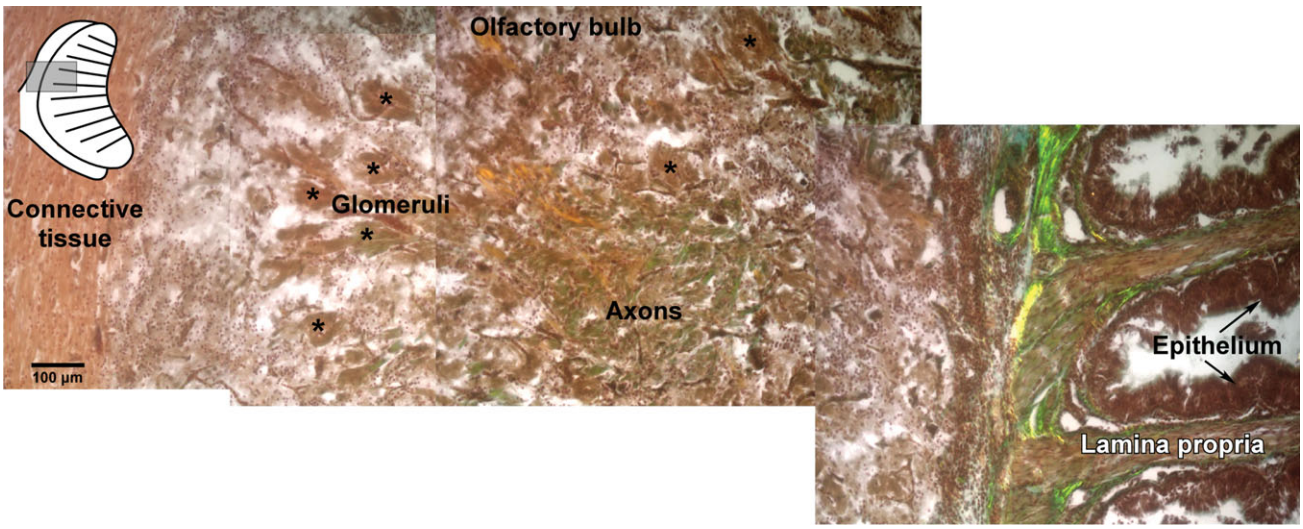

Fig. 2. Morphology of the OB and OE of *D. say* visualized using KLO (nuclear red-light green-orange) staining. An inset in the top left corner illustrates the area of the OB and organ being examined (gray shaded area). The axons of the ORNs situated in the folds of OE project through the lamina propria of each lamella and to the OB where they synapse with mitral cells at glomeruli. A superficial connective tissue layer occurs at the posterior aspect of the OB. Deep to that layer, glomeruli (*) are distributed in a diffuse layer. Note the proximity of the OE to the OB.

(DiI) and/or PTIR271 (Far Red; both dyes a gift of Dr. Brian Gray at Molecular Targeting Technologies, West Chester, PA), two lipophilic carbocyanine dyes that fluoresce at different wavelengths, were placed on the tip of a needle and inserted into various locations on the dorsal surface of the OBs of each sample. As these dyes do not cross synapses, only cells whose axons reside at the labeling site and came into contact with the dye were labeled. Brain samples were then covered in 3% agar and reimmersed in 4% paraformaldehyde for 4 to 9 months to allow the dye to diffuse throughout the brain and OE. The olfactory organ and OB were dissected from the head of each specimen and embedded in either egg yolk or 15% gelatin. The OB and OE-containing gelatinous block was then fixed overnight in 4% paraformaldehyde. The following day, 50–150 μm sections were cut on a vibratome (PELCO, Redding, CA), mounted on slides, and examined with epifluorescence using a Zeiss microscope (Thornwood, NY) or an Olympus confocal laser scanning microscope (Center Valley, PA).

RESULTS

Morphology

Using both, KLO and carbocyanine dye-labeled samples, the morphology of the OB in three elasmobranch species representing two OB morphologies was examined. A laminar organization was observed in the OB of all three species with a superficial, thick fibrous layer at the posterior of the OB (Fig. 2). At the anterior face of the OB, adjacent to the OB/OE interface, is the olfactory nerve layer in which axons enter the OB from the OE and exhibit considerable divergence before synapsing at their target glomeruli (Fig. 3A). Deep to the fibrous and olfactory nerve layers is a wide, ill-defined glomerular layer (Figs. 2, 3A, and 4B). For all three species, the glomeruli in the OB were distributed throughout and not limited to a distinct layer at the outer rim of the OB. The OE of each species exhibited secondary folding with sensory epithelium located in the "troughs" and nonsensory epithelium in the "ridges" of the tissue folds (Figs. 2, 3B–D).

The two stingray species possess elongate, cohesive OBs, whereas the lemon shark possesses OBs divided into two physically distinct units or hemi-OBs (Fig. 1). Secondary OE folding throughout all lamellae was seen in the two dasyatid species, whereas in the lemon shark the secondary folding was absent from the more dorsal base of the lamellae located in the middle of the olfactory organ. We found microvillous ORNs (Figs. 3E, 4D,E) and few egg-shaped crypt ORNs, but no ciliated ORNs.

Organization of ORN Projections

All three elasmobranch species exhibited a similar pattern of ORN projections from the OE to the OB (Figs. 3 and 4). Dye placement in the OB resulted in labeling of the glomeruli and axons in the OB near the labeling site. The adjacent olfactory nerve fascicles and axon bundles in the lamina propria of the three to five lamellae immediately anterior to the OB labeling site were retrogradely labeled (Figs. 3A,B and 4A–C). Labeling extended from those axon bundles into individual axons innervating the ORNs in the OE (Figs. 3C–E, 4D,E). No labeling was ever seen in lamellae distant from the labeling site (i.e., ORNs project only to a very limited area in the OB). In samples where DiI and Far Red were placed in separate hemi-OBs, distinct labeling of the hemibulbs (Fig. 4A), axons, lamellae (Fig. 4C), and ORNs in the OE were always observed. In samples where both DiI and Far Red were placed in two locations

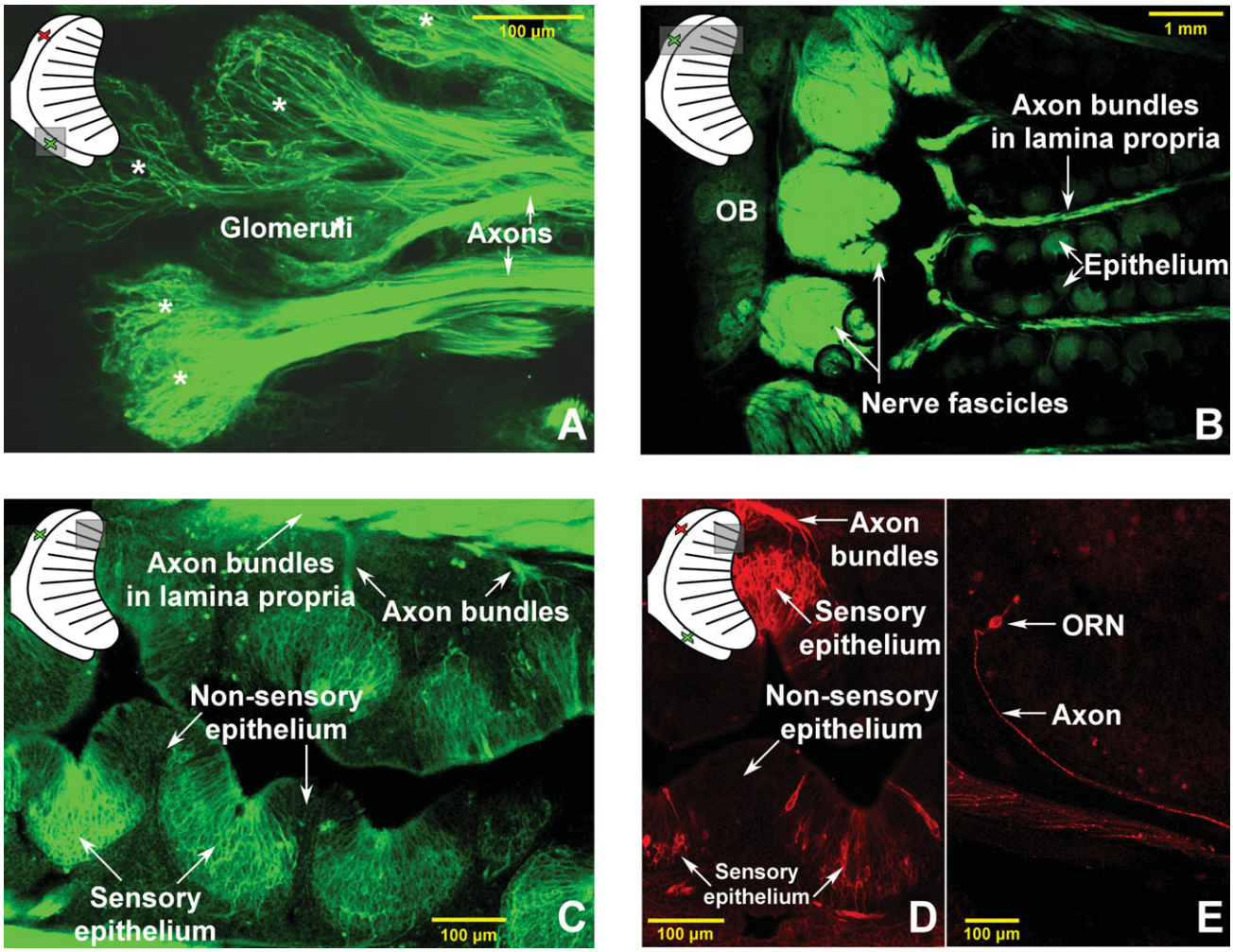

Fig. 3. Retrograde labeling from the OB to the OE in both stingray species. An inset in the top left corner of each panel illustrates the dye type (green = Far Red and red = DiI), general dye placement location, and the area of the OB and organ being examined (gray shaded area). Panel **A** shows labeling of the glomeruli (*) and axons in the OB near the labeling site. Far Red in the OB spread into the adjacent nerve fascicles and the axon bundles in the lamina propria of the three to five lamellae immediately anterior to the labeling site in the OB (Panel **B**). Labeling extended from those axon bundles into axons innervating the ORNs in the secondary folds of the OE (Panels **C** and **D**). Panel **E** shows a single microvillus ORN labeled with DiI. No labeling was seen in distant lamellae.

within the same hemi-OB, labeling of distinct glomeruli occurred and axons were traced retrogradely into separate lamellae by each dye (Figs. 4D and 5A). Double labeling was only observed when the two dyes were inserted into the OB in such close proximity that they labeled overlapping sets of axons (Fig. 5B).

DISCUSSION

This study examined the morphology of the olfactory structures and organization of the ORN projections in three elasmobranch species with varying OB morphologies to test the hypothesis that axons projecting from the OE to the OB exhibit a somatotopic arrangement. We found in all three species that glomeruli in the OB received projections from ORNs located only on the three to five lamellae situated immediately anterior (Figs. 3 and 4). These results support our hypothesis, and previous results from Dryer and Graziadei (1993), that the OB of elasmobranchs demonstrates a unique somatotopic arrangement unlike that of teleost fishes and most other vertebrates.

Organization of ORN Projections

Two previous studies have shown that ORNs in the OE project immediately posterior to glomeruli in the OB (Daniel, 1934; Dryer and Graziadei, 1993). This suggests a somatotopic arrangement in which sensory projections maintain their spatial organization in the CNS, which is congruent with

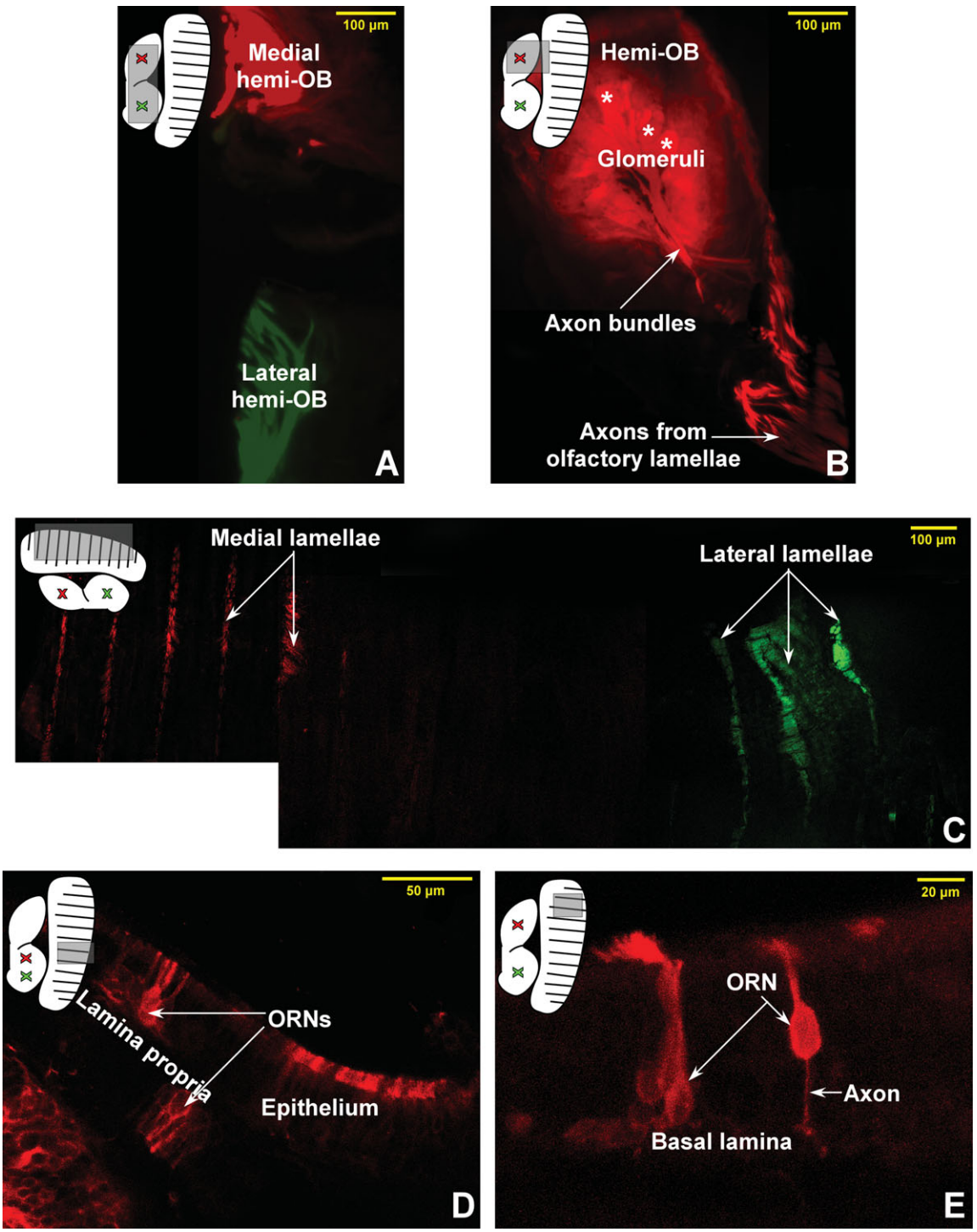

Fig. 4. Retrograde labeling from the OB to the OE in the lemon shark, *N. brevirostris*. An inset in the top left corner of each panel illustrates the dye type (green = Far Red and red = DiI), general dye placement location, and the area of the OB and organ being examined (gray shaded area). Panel **A** shows distinct labeling of the medial and lateral hemi-OBs. Panel **B** shows a DiI labeled medial hemi-OB, including axon bundles from the adjacent olfactory lamellae leading to several glomeruli (*). Panel **C** is a composite image of multiple olfactory lamellae labeled on the medial side with DiI and the lateral side with Far Red as a result of the initial OB labeling with DiI on the medial side and Far Red on the lateral side (shown in the inset). Both dyes spread anteriorly, and no double labeling of lamellae was seen. Panels **D** and **E** show the OE and individual ORNs labeled with DiI. The sample in panel D was labeled with both dyes in the lateral hemi-OB; however, we saw no double labeling of the OE.

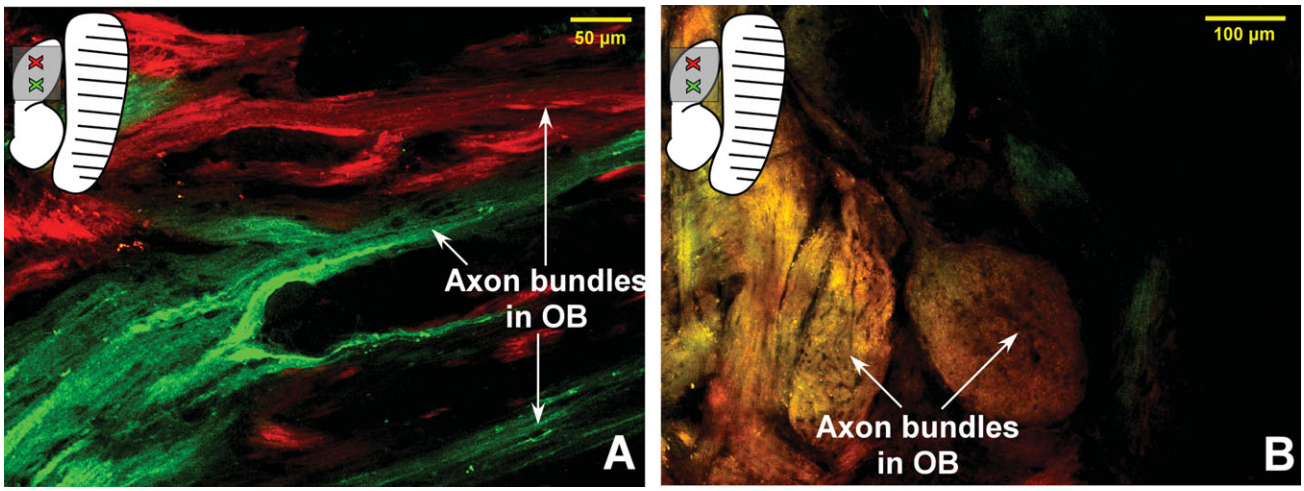

Fig. 5. Retrograde labeling from the OB to the OE in lemon shark (*N. brevirostris*) samples that were labeled using both dyes in a single hemi-OB. An inset in the top left corner of each panel illustrates the dye type (green = Far Red and red = DiI), general dye placement location, and the area of the OB and organ being examined (gray shaded area). Although the lateral hemi-OB was labeled with both dyes in the sample in Panel **A**, there was distinct labeling of axon bundles by each dye. In contrast, only when the two dyes were placed so closely that the same axons were affected did we observe double labeling of axons in the OB, indicated by the yellow hue (Panel **B**). In samples where both dyes were placed in the same hemi-OB, we did not see double labeling of any lamellae or ORNs.

the results of this study. However, Ferrando et al. (2009) examined the immunolocalization of G-protein α-subunits in the small-spotted catshark OB and concluded that the pattern of immunoreactivity suggested a topographic organization where each ORN type (microvillous and crypt) projects to a localized region of the OB, similar to that in teleosts. Our study supports the results of Dryer and Graziadei (1993) and Daniel (1934).

Teleost fishes, along with most other vertebrates, exhibit a functional or chemotopic OB organization in which ORNs widely distributed over the epithelium converge to particular glomeruli in the OB based on the ORN morphotype (Riddle and Oakley, 1991; Baier et al., 1994; Sato et al,, 2005; Hamdani and Døving, 2007; Sato et al., 2007). As a result, the OB possesses separate functional zones that process different types of odorants (Nikonov and Caprio, 2001, 2004). For example, certain microvillous ORNs respond primarily to amino acids and their axons project to specific regions in the dorsolateral and ventral OB (Nikonov and Caprio, 2001; Sato and Suzuki, 2001; Hansen et al., 2003; Døving et al., 2011). This chemotopic organization of the OB is thought to play a significant role in encoding olfactory information; odor identity and concentration are represented in the OB by the particular patterns of glomerular activity (Hildebrand and Shepherd, 1997; Strausfeld and Hildebrand, 1999; Nikonov and Caprio, 2001; Johnson and Leon, 2007; Caprio and Derby, 2008).

The OE of elasmobranchs is situated in an oval, laterally elongated olfactory organ adjacent to a similarly elongate OB and both are quite large compared to the olfactory organ and OB of most teleost species, which are more spherical and compact. In addition, numerous elasmobranch species (e.g., the lemon shark) exhibit a unique, hemi-OB morphology (Tester, 1963; Northcutt, 1978). The pervasiveness of this morphology in elasmobranch clades and how it relates to the processing of odor information is not fully understood. Based on our findings, two main hypotheses emerge. Given the elongated nature of the olfactory structures, it may be inefficient for ORNs to extend their axons from the medial side of the OE to synapse at distant glomeruli on the lateral side of the OB, for example; as a result, each half or section of the OB effectively became functional units that receive projections from the adjacent portion of the OE. With each half or portion of the OB handling a local, independent subset of ORNs, the OB may have become increasingly compartmentalized over time potentially leading to the development of the hemibulbs seen in certain elasmobranch species. Alternatively, the elasmobranch olfactory system may possess a topographical organization of ORNs in the OE based on their specificity to different odorant classes. In this scenario, ORNs would project to the OB based on both location within the OE and olfactory receptor type, yielding both a somatotopic and chemotopic map. Future studies should examine the odorant specificities across the length of the OE to test for a potential topographic map and determine the detailed projections of the different ORN types to the OB.

Morphology

To further understand the arrangement of the elasmobranch OB, we examined the gross morphology and fine structure of the olfactory system.

As previously documented, the OB of elasmobranchs is pedunculated (Fig. 1B) in contrast to the sessile OB placement of many teleost species (Northcutt, 1978; Zielinski and Hara, 2006). However, the cytoarchitecture of the OB for all three species was similar to that previously described for teleosts and other elasmobranch species (Tester, 1963; Dryer and Graziadei, 1993; Takami et al., 1994; Ferrando et al., 2009). The teleost OB is divided into four distinct concentric layers: from most superficial to deep there are the olfactory nerve layer, the glomerular layer, the mitral cell layer, and the granule cell layer (Oka et al., 1982; Byrd and Brunjes, 1995; Laberge and Hara, 2001). Although the elasmobranch OB demonstrates many morphological similarities with the teleost OB, the glomerular layer in the teleost OB is thinner and more distinct. In elasmobranchs, the glomerular layer may be interspersed with the mitral cell layer (Takami et al., 1994; Ferrando et al., 2009) instead of occurring as two distinct layers as seen in teleosts (Satou, 1992; Baier and Korsching, 1994; Baier et al., 1994; Byrd and Brunjes, 1995). The deepest OB layer described for elasmobranchs is the granule cell layer, which was not visibly defined with the staining methods used in this study.

In their survey of elasmobranch olfactory morphology, Schluessel et al. (2008) found secondary folding of the OE in all 21 species examined and reported considerable differences in the degree of folding among them. The two dasyatid species in our study exhibited secondary OE folding throughout all lamellae, whereas for the lemon shark the secondary folding was absent toward the more dorsal base of the lamellae that are located in the middle of the olfactory organ. This contrasts with the clearnose skate (*Raja eglanteria*) and the brownbanded bamboo shark (*Chiloscyllium punctatum*) in which the secondary folds are present over most of the OE but disappear toward their ventral free margin (Takami et al., 1994; Schluessel et al., 2008). In all three species examined, sensory epithelium was located in the "troughs" and nonsensory epithelium in the "peaks" of the secondary tissue folds, similar to the spotted eagle ray (*Aetobatus narinari*) and the Pork Jackson shark (*Heterodontus portusjacksoni*; Schluessel et al., 2008). In contrast, the sensory epithelium of the spiny dogfish (*Squalus acanthius*) and the small-spotted catshark (*Scyliorhinus canicula*) was found on both the ridges and the troughs of the secondary epithelial folds (Theisen et al., 1986). The Port Jackson shark demonstrated an irregular and patchy arrangement of sensory and nonsensory epithelium within the OE (Schluessel et al., 2008). This indicates that interspecific variation exists not only in the degree of secondary folding of the OE but also the precise location of the sensory epithelium within those folds.

Nearly all studies on the types of ORNs present in the elasmobranch sensory OE, including this study, have confirmed the presence of microvillous ORNs (Reese and Brightman, 1970; Bronshtein, 1976; Theisen et al., 1986; Takami et al., 1994; Schluessel et al., 2008). Ciliated ORNs found in the OE of teleost fishes and many other vertebrate groups are lacking in the elasmobranch OE (Theisen et al., 1986; Eisthen, 2004; Schluessel et al., 2008). A third ORN type, the crypt ORN, was recently described in elasmobranchs (Ferrando et al., 2006), and in the species investigated in this study, we observed only a few crypt ORNs. Ferrando et al. (2009) suggested that crypt ORNs may project to the ventral OB; as we labeled only the dorsal surface of the OB, we may have missed labeling the majority of crypt ORN axons. More directed studies on the projections of elasmobranch crypt ORNs are needed to confirm this hypothesis.

Morphologically different ORN types are thought to mediate fishes' responses to specific odorant classes. Amino acids, a feeding stimulant to fishes (Zielinski and Hara, 2006), are detected primarily by microvillous ORNs in teleosts (Sato and Suzuki, 2001; Lipschitz and Michel, 2002) and also possibly by ciliated and crypt ORNs (Hansen et al., 2004; Vielma et al., 2008); whereas bile salts are detected only by ciliated ORNs (Sato and Suzuki, 2001; Hansen et al., 2003; Døving et al., 2011). Due to the lack of ciliated ORNs in the elasmobranch OE, it was unknown whether they are able to detect bile salts, although the biological relevance of this odorant class as a potential pheromone for agnathans and teleosts makes it seem likely (Døving et al., 1980; Zhang et al., 2001; Li et al., 2002; Siefkes and Li, 2004; Sorensen and Stacey, 2004; Sorensen et al., 2005). In a recent electrophysiological study, we demonstrated that the olfactory system of two elasmobranch species (*D. sabina* and *S. tiburo*) responds to bile salt odorants, although they must use a different ORN type than teleost fishes (Meredith et al., 2012). This apparently different bile salt detection mechanism coupled with our finding that elasmobranchs possess a somatotopically arranged OB highlights the uniqueness of the olfactory system in this group of basal vertebrates.

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