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Research Article

THE SPATIAL ORGANIZATION OF THE PERIPHERAL OLFACTORY ORGAN IN THE ROUND GOBY (*NEOGOBIUS MELANOSTOMUS*)

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Abstract

Round goby (*Neogobius melanostomus*) spawning behaviour and parental care by males may be mediated by pheromones. We examined the spatial organization of the peripheral olfactory organ in round goby. Olfactory receptor neurons were visualized in cryostat sections stained for acetylated tubulin immunocytochemistry and by transmission electron microscopy. The olfactory epithelium is located adjacent to the tentacular anterior nostril and extends ventrocaudally along the relatively flat surface of the nasal cavity. Numerous nonmyelinated nerve fascicles formed in the lamina propria and converged into a prominent olfactory nerve. A sac-like enclosure was found on the posterior ventral surface that may regulate flow through the nares.

INTRODUCTION

In North America, the round goby (*Neogobius melanostomus*) was first reported in the St. Clair River in 1990 and is now found in all of the Laurentian Great Lakes (Jude *et al.* 1995). These bottom-dwelling fish are presumed to have entered the Great Lakes via ballast water of ships originating in the Ponto-Caspian region (Charlebois *et al.* 1997). The round goby also entered the Gulf of Gdańsk in 1990 and may have arrived along river routes from the Black Sea (Skóra and Stolarski 1995). Reasons for the proliferation of the round goby in the Great

Lakes and in the Gulf of Gdańsk include its broad diet, aggressiveness, high fecundity, repetitive annual spawns, and male parental care (MacInnis and Corkum 2000). The reproductive success of the round goby may be a consequence of pheromonally mediated spawning behaviour. Experiments conducted 45 years ago by Dr. W. N. Tavolga on the frillfin goby (*Bathygobius soporator*) showed that gravid females and extracts of the mature female goby urogenital system attracted mature males and caused them to exhibit reproductive behaviours (Tavolga 1956). Over 40 years later, the findings of C. Murphy (1998) suggested that in the round goby, steroidal compounds function as reproductive pheromones. Two estrones, 17ß-estradiol-3ß-glucuronide, and etiocholanolone, elicited electro-olfactogram responses and gill ventilation rate increases (Murphy *et al.* 2001). One of the constraining factors in the study of pheromonally mediated reproductive behaviour in the goby is the lack of information available on its olfactory system. Although the family Gobiidae is the largest group of marine teleosts, the fundamental organization of the peripheral olfactory organ is unknown.

The nasal cavity of teleost fishes is relatively shallow and lined by a ciliated pseudostratified epithelium. Water enters through an anterior naris, flows over olfactory sensory neurons and leaves through the posterior naris. In many teleosts, an accessory nasal sac facilitates the passage of water through the nasal cavity (Parker 1910, Pipping 1926, Lierman 1933). The olfactory receptor neurons are located on the peripheral olfactory organ, a folded lamellar mucosal structure, on the ventral surface of the nasal cavity. In teleost fishes, olfactory receptor neurons are either ciliated or microvillar (Evans *et al.* 1982, Zielinski and Hara 1988, Hansen *et al.* 1999). Olfactory receptor neuron dimorphism may be related to odourant quality distinction, but the functional distinction between teleost microvillar and ciliated olfactory receptor neurons is yet to be clearly defined. Although a single early study has shown that the olfactory organ in Gobiidae is a uni-lamellar structure (Yamamoto 1982), basic characteristics of the overall organization of the nasal cavity, including olfactory receptor neuron distribution and the types of olfactory receptor neurons, are unknown in the gobiids.

Our objective was to investigate the spatial organization of the peripheral olfactory organ using acetylated tubulin immunocytochemistry to visualize microtubule containing structures: cilia of nonsensory cells and ciliated olfactory receptor neurons, as well as, neuronal dendrites and axons. Transmission electron microscopy was used to confirm the presence of olfactory receptor neurons.

MATERIALS AND METHODS

Neogobius melanostomus adults were collected between August and October 2000 by angling and trawling in the Canadian waters of the Detroit River and the

western basin of Lake Erie. All animals were transported to the University of Windsor and maintained under constant photoperiod (16L:8D) in holding tanks with a flowing dechlorinated tap water system (10 - 15°C). Aquaria contained gravel, an air stone and shelters. Fish were fed Nutrafin® staple fish flakes and zebra mussels (*Dreissena polymorpha*).

Immunocytochemistry (ICC): tricaine methane sulphonate (MS-222) was used to anaesthetize round gobies in preparation for cardiac perfusion fixation using 6.3% saline followed by Zamboni's fixative (2% paraformaldehyde, 1.2% picric acid, 0.1 M phosphate buffer PB). Gross dissection of the peripheral olfactory organ was followed by cryoprotection using a sucrose gradient. The tissue was then frozen and sectioned using a cryostat. Immunocytochemistry was performed as described in Zaidi *et al.* (1998) using acetylated tubulin (anti-mouse acetylated tubulin 1:100, Sigma, St. Louis, MO, USA) and Alexa (fluorescein anti-rabbit and mouse IgG; diluted 1:100; Vector Labs, Burlingame, CA, USA) as the secondary antibody. Images were obtained using fluorescence microscopy (post-acquisition software: Northern Eclipse) and by confocal microscopy (BioRad MRC 1024).

Transmission electron microscopy (TEM): Fish, anaesthetized with MS-222, were weighed and measured. Cardiac perfusion was performed using saline (6.3%) followed by Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, in 0.07 M cacodylate buffer, 0.25% $CaCl_2$). The nostrils were removed and stored overnight in Karnovsky's fixative. The tissue was then immersed in 1% osmium tetroxide buffered with cacodylate buffer (pH 7.4) and dehydrated using ice cold ethanol. Tissue was embedded into an epoxy resin as previously described by VanDenbossche *et al.* (1995). Sections of 90 nm were stained using 2% uranyl acetate and olfactory receptor neurons (ORNs) viewed using transmission electron microscopy (Philips EM201).

RESULTS

Acetylated tubulin immunocytochemistry of the nasal cavity showed the location of cilia and neuronal cell processes within the nasal cavity. Ciliated, acetylated tubulin immunoreactive surfaces lined the entire nasal cavity from the tentacular anterior nostril to the posterior nostril. A sac-like enclosure, the accessory nasal sac was identified on the posterior ventral surface of the nasal cavity (Fig. 1A). Epithelium with numerous acetylated tubulin immunoreactive nerve fascicles in the underlying lamina propria was recognized as olfactory epithelium. Immunoreactive staining was located on the ventral surface of the nasal cavity, and absent from dorsal or posterior areas. Two small depressions were seen in the anterior region of the olfactory epithelium. When the nasal cavity was viewed in cross-section, olfactory epithelium could be seen on the lateral surface as well as

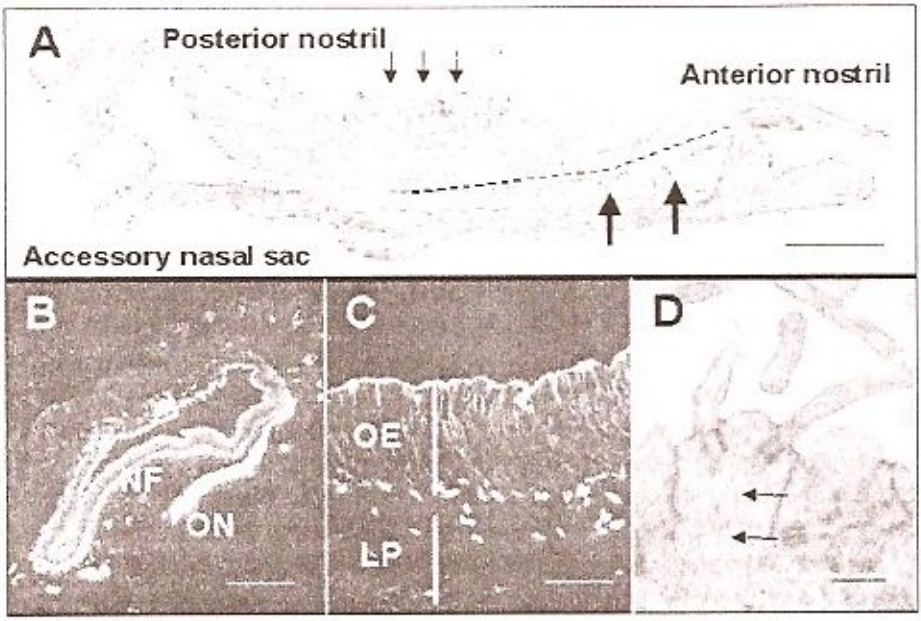

Fig. 1. The location of olfactory receptor neurons in the nasal cavity of the round goby:

A. diagram showing a longitudinal view through the nasal cavity; the anterior nostril is tubular while the posterior nostril is wide and prominent (bar equals 500 μm); several ridges (small arrows) are found on the dorsal epidermal surface; the nasal cavity floor is relatively flat, with the exception of small ridges close to the anterior nostril (wide arrows); the area beneath the dashed line is olfactory epithelium; a sac-like infolding (accessory nasal sac) is found in the posterior portion of the nasal cavity,

B. this cross-sectional view of a confocal micrograph shows acetylated tubulin immunoreactivity in the olfactory muscosa; nerve fasicles (NF) are visible beneath the epithelium, on the medial, lateral, and ventral surfaces (bar equals 50 μm); nerve fasicles converge medially, forming a prominent olfactory nerve (ON),

C. this high power confocal micrograph shows acetylated tubulin immunoreactive olfactory receptor neurons (bar equals 5 μm); the olfactory epithelium (OE) contains olfactory receptor neurons, cilia, dendrites, cell bodies and axons; axons form small nerve fasicles in the lamina propria (LP),

D. transmission electron micrograph of a ciliated olfactory receptor neuron (bar equals 0.2 μm); two cilia protrude from the prominent olfactory knob; microtubules are abundant in the dendritic cytoplasm (arrows)

the ventral surface. The olfactory mucosa was clearly distinguished from non-olfactory surfaces by the presence of numerous acetylated tubulin-immunoreactive nonmyelinated nerve fascicles in the lamina propria. In the medial margin, fascicles converged into a prominent olfactory nerve (Fig. 1B). High power views of the olfactory mucosa showed abundant bipolar olfactory

receptor neurons with acetylated tubulin immunoreactive dendrites, cell bodies, and axons (Fig. 1C). Transmission electron microscopy confirmed the presence of ciliated olfactory receptor neurons within the olfactory mucosa (Fig. 1D).

DISCUSSION

The peripheral olfactory organ of the round goby contains a continuous surface of olfactory epithelium with two small anterior depressions along the floor of the nasal cavity. This structure varies from that seen in most teleost fishes. Usually the peripheral olfactory organ is a lamellar (folded) structure (Yamamoto 1982). Olfactory ability is not directly related to the number of lamellae present in the nasal cavity and there is no simple relationship between the number of lamellae and the acuity of olfactory sense (Pipping 1926, 1927). Yamamoto (1982) showed one olfactory lamellae on gross dissection of the peripheral olfactory organ in gobiids. He listed other species deficient in olfactory lamellae (*e.g.*, *Oryzias latipes*, *Fluta alba*, *Omobranchus elegans*, *Rudarius arcodes*, *Ostracion tuberculatus*) and confirmed that olfactory ability is not directly related to the surface area in the olfactory organ.

In fish, water enters the nose through the anterior nares and exits through the posterior nares. This may happen passively through locomotion of the fish in water or actively by ciliary action within the pits. Intense acetylated tubulin immunoreactivity along the epithelial surface of the entire nasal cavity indicates that water flows through the nasal cavity of the round goby with the assistance of ciliary action. Passive flow of water through the nostril is not likely in the round goby because the anterior nostril opening is narrow, with a narrow tubular opening. This skin covering may have evolved to keep benthic debris out of the nose.

Some fish contain an accessory nasal sac in the posterior region of the nasal sac cavity proper that provides a pumping action of water through the nostril (Hara 1975). A sac-like enclosure found on the posterior ventral surface of the nostril was identified in the round goby. This is a novel finding, as previous research on gobiids has not identified this structure. Other fish species (*e.g.*, sticklebacks, blennies, and catfish) also possess accessory nasal sacs (Parker 1910, Pipping 1926, Lierman 1933). The accessory nasal sac in the round goby may regulate flow through the nares. A tendon or other connective tissue may attach the accessory nasal sac to the gills, so that gill movements control expansion and contraction of the sac. With increased gill movement, more water would be available for "sniffing" olfactory stimuli (Nevitt 1991). Increases in gill movement following the application of steroidal compounds to the round goby nasal cavity, previously seen by Murphy (1998), may have originated from this motor reflex.

In the round goby, olfactory receptor neuron cilia, dendrites, cell bodies, and axons were strongly immunoreactive to the acetylated tubulin antibody. Closely packed ORNs were visualized on ventral, lateral, and medial surfaces of the peripheral olfactory organ. Axons from ORNs formed numerous nonmyelinated nerve fascicles, which formed in the lamina propria and converged into a prominent medial olfactory nerve. Olfactory epithelium extended ventrocaudally from the anterior nostril and was not present in the dorsal or posterior areas of the nostril. Transmission electron microscopy confirmed the presence of ciliated ORNs on the ventral surface of the peripheral olfactory organ in the round goby. Microvillar ORNs were not observed in this preliminary examination of olfactory epithelium by TEM, but use of other antibodies and further TEM analysis will help determine if microvillar ORNs are present in the peripheral olfactory organ in the round goby.

The spatial structure of the round goby olfactory system was discerned for the purpose of relating behavioural responses of the round goby to sex pheromones perceived during reproductive activities. Knowledge of the organization will allow for olfactory deprivation experiments and research showing cellular responses to sex pheromones released by conspecifics. Eventually, a sex pheromone may be used to disrupt the reproductive habits of the round goby, a Great Lakes fish invader that threatens the reproductive success of smallmouth bass (*Micropterus dolomieui*), lake trout (*Salvelinus namaycush*), and lake sturgeon (*Acipenser fulvescens*).

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