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Heterogeneous Distribution of G Protein Alpha Subunits in the Main Olfactory and Vomeronasal Systems of *Rhinella* (*Bufo*) *arenarum* Tadpoles

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We evaluated the presence of G protein subtypes $G\alpha_o$, $G\alpha_{i2}$, and $G\alpha_{olf}$ in the main olfactory system (MOS) and accessory or vomeronasal system (VNS) of *Rhinella (Bufo) arenarum* tadpoles, and here describe the fine structure of the sensory cells in the olfactory epithelium (OE) and vomeronasal organ (VNO). The OE shows olfactory receptor neurons (ORNs) with cilia in the apical surface, and the vomeronasal receptor neurons (VRNs) of the VNO are covered with microvilli. Immunohistochemistry detected the presence of at least two segregated populations of ORNs throughout the OE, coupled to $G\alpha_{olf}$ and $G\alpha_o$. An antiserum against $G\alpha_{i2}$ was ineffective in staining the ORNs. In the VNO, $G\alpha_o$ neurons stained strongly but lacked immunoreactivity to any other $G\alpha$ subunit in all larval stages analyzed. Western blot analyses and preabsorption experiments confirmed the specificity of the commercial antisera used. The functional significance of the heterogeneous G-protein distribution in *R. arenarum* tadpoles is not clear, but the study of G- protein distributions in various amphibian species is important, since this vertebrate group played a key role in the evolution of tetrapods. A more complete knowledge of the amphibian MOS and VNS would help to understand the functional organization and evolution of vertebrate chemosensory systems. This work demonstrates, for the first time, the existence of a segregated distribution of G-proteins in the OE of *R. arenarum* tadpoles.

Key words: *Bufo arenarum*, amphibian, olfactory neurons, vomeronasal organ

INTRODUCTION

The nasal chemical senses in most terrestrial vertebrates are represented primary by the main olfactory system (MOS) and accessory or vomeronasal system (VNS) (Bargmann, 1997). In the MOS, olfactory receptor neurons (ORNs) lie in the olfactory epithelium (OE) of the nasal cavity. These bipolar ORNs project axons to the main olfactory bulb (MOB). The VNS contains vomeronasal receptor neurons (VRNs) in the sensory epithelium of the vomeronasal organ (VNO), and their axons project to the accessory olfactory bulb (AOB) (Halpern, 1987). Odor recognition in the OE is mediated by specific olfactory receptors (ORs) located on the apical surface of dendritic ORN processes (Buck and Axel, 1991). These ORs are G-protein-coupled receptors that contain seven transmembrane α-helical regions. Heterotrimeric GTP-binding proteins (G proteins) play a major role in determining the specificity of the interaction of the receptors and a particular specific effector system. Similar to ORs in the MOS, another two distinct families of G-protein-coupled receptors (V1Rs and V2Rs) were identified in rodent VNOs (Dulac and Axel, 1995; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). These receptor gene families were subsequently described in a variety of vertebrates (Dryer, 2000).

Neither olfactory sensory system shows a homogeneous distribution of sensory components; these systems are heterogeneous in molecular, anatomical, and physiological parameters in all vertebrates studied to date. This heterogeneity has been demonstrated in G-protein expression (Hansen et al., 2004; Wekesa and Anholt, 1999; Wakabayashi and Ichikawa, 2008), lectin histochemistry (Lipscomb et al., 2002), differences in neuronal apical process (Elsaesser and Paysan, 2007; Wakabayashi and Ichikawa, 2008), and the distribution patterns of other olfactory markers identified immunohistochemically (Shnayder et al., 1993; Halpern and Martinez-Marcos, 2003). Among these parameters, hetero-

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geneity in the presence of different G proteins in different neuronal subpopulations has been a useful tool for understanding the organization and evolution of vertebrate chemodetection organs.

In the VNO of rodents, the V1R receptors family is associated with $G\alpha_{i2}$ in VRNs located in the most apical zone of the sensory epithelium, whereas members of the V2R family are coexpressed with $G\alpha_o$ proteins in VRNs located in the most basal region of the sensory epithelium (Jia and Halpern, 1996; Dulac, 2000). These two subpopulations of VRNs project axons to topographically distinct areas of the AOB, suggesting functional differences between them (Halpern and Martinez-Marcos, 2003). Wekesa and Anholt (1999) showed a partial segregation pattern in the mouse MOB using antibodies against the G_{i2} and G_o α subunits. Although, the distribution of both G proteins overlapped in the MOB and OE, no clear distribution pattern was observed. Moreover, an olfactory-specific G protein α subtype termed $G\alpha_{olf}$, which shares extensive amino acid identity with $G\alpha_s$, is massively expressed in ORNs of the OE of rodents (Jones and Reed, 1989).

In teleost fishes, which lack a VNO and have all their ORNs in a single sensory epithelium, a laminar segregation of sensory cells was observed (Belanger et al., 2003; Hansen et al., 2004). Moreover, Hansen et al. (2004) showed that, in goldfish, ciliated ORNs have OR-family receptors along with $G\alpha_{olf}$, whereas microvillous ORNs express V2R-family receptors along with either $G\alpha_{i3}$ or $G\alpha_o$.

In amphibians, the distribution of the ORs and G proteins has been poorly analyzed, and limited data have been obtained only from *Xenopus laevis*. This amphibian is unusual among anurans in being secondarily aquatic as an adult. *Xenopus laevis* has two separate OEs in different nasal cavities: the principal cavity (PC) and the middle cavity (MC). Most anurans, including *Rinella (Bufo) arenarum*, have only a main OE (located in the principal cavity) and vomeronasal epithelium (Scalia, 1976), as in the majority of other tetrapods (Eisthen, 1992).

Freitag et al. (1995) found two classes of olfactory receptors in *X. laevis:* class-I receptors (most similar to fish receptors) are expressed in the middle cavity along with $G\alpha_o$, while class-II receptors (closely related to mammalian receptors) are expressed in the principal cavity along with $G\alpha_{olf}$ (Mezler et al., 2001). OR expression during larval development confirmed two OR gene families in this amphibian (Mezler et al., 1999); however, there have been no studies on the expression of G protein subtypes during larval development in *X. laevis* or any other amphibian species.

Among vertebrates, amphibians are a very interesting group in which to study the olfactory system for two principal reasons. First, amphibians are the most basal group among tetrapods in which an anatomically separated dual olfactory system (MOS and VNS) appears (Eisthen, 1992; 1997). Second, most anuran amphibians have both: an aquatic period of life (larval) and a more terrestrial life period (post metamorphic), so the amphibian olfactory system has to be able to sense water-soluble odor molecules during larval stages and volatile odor molecules during post-metamorphic stages. Analysis of the amphibian MOS and VNS would help us to understand the functional organization and evolution of vertebrate chemosensory systems.

Since there have been no detailed studies on G protein expression in the olfactory system from amphibian tadpoles, in the present study we analyzed the distribution of G protein subtypes $G\alpha_o$, $G\alpha_{i2}$, and $G\alpha_{olf}$ in the MOS and VNS during post embryonic development in *R. arenarum*. In addition, we examined the fine structure of ORNs and VRNs using electron microscopy. Our findings demonstrate for the first time the existence of a segregated distribution of G-proteins in the OE of amphibian tadpoles. Bipolar ORNs expressing $G\alpha_o$ were located in the basal third of the OE, whereas bipolar ORNs expressing $G\alpha_{olf}$ were located in the middle third of the OE. In the VNO only the $G\alpha_o$ protein subtype was expressed by bipolar VNRs, without a discernable distribution pattern.

MATERIALS AND METHODS

Animals

Rhinella (Bufo) arenarum tadpoles were obtained by in-vitro fertilization according to the method of Paz et al. (1995). Larvae were maintained in dechlorinated tap water, with a constant photoperiod (12:12 h, dark:light) and temperature (24 C). Prometamorphic tadpoles ranging from stages 32 to 38 (staging according to Gosner, 1960) were used. All experiments were performed in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA Res CD: 140/00, and the principles of the NIH (Publication 8523, revised 1985).

Light microscopy

Tadpoles were anesthetized by immersion in 0.1% MS222 (tricaine methanesulfonate, Sigma, St. Louis, MO, USA) and fixed in Bouin's solution for 24 h at 4 C. They were then dehydrated, cleared in xylene, and embedded in Histoplast (Biopack, Buenos Aires, Argentina). Serial paraffin sections (thickness 7 m) were cut, mounted on HiFix glass slides (HF-5001, InProt, TNT, Buenos Aires, Argentina), and subjected to immunohistochemistry or stained with cresyl violet for histological studies.

Electron microscopy

Tadpoles were anesthetized as described above; the OE and VNO were dissected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4 C, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated, and embedded in Durcupan resin (Fluka). Ultrathin sections (thickness 70–90 nm) were cut on a Reichert-Jung ultramicrotome (Model Ultrcut E). Ultrathin sections were counterstained with lead citrate, and observed and photographed with a Zeiss 10C transmission electron microscope.

Immunohistochemistry

Fourteen tadpoles were analysed. General procedures for immunohistochemistry were as in our previous report (Pozzi et al., 2006). The primary antibodies used were rabbit anti-$G\alpha_o$, 1/12,000 (sc-387, Santa Cruz), rabbit anti-$G\alpha_{olf}$, 1/1000 (sc-385, Santa Cruz), and rabbit anti-$G\alpha_{i2}$ (sc-7276, Santa Cruz). After primary antibody incubation, sections were treated with an anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) followed by the avidin–biotin horseradish peroxidase complex (Vectastain ABC Kit, Vector Laboratories). The reaction was developed with the 3,3'-diaminobenzidine tetrahydrochloride (DAB) Staining Kit (Dako, Glostrup, Denmark). Specificity control was determined by preabsorption of the primary antiserum with synthetic specific antigen (cat. sc-387 P and sc-385 P, Santa Cruz) in a 1:10 antiserum:antigen ratio. Additional controls were made by omission of the primary

antiserum. All negative control sections produced negligible background staining (data not shown).

For immunofluorescence detection, sections were incubated with anti-Gα_o or Gα_{olf} followed by the appropriate FITC-labeled secondary antibodies (Vector Labs) and counterstained with propidium iodide (P-1304, Molecular Probes, Eugene, Oregon, USA). Images were captured with a confocal laser microscope (Olympus FV-30 attached to an Olympus Bx-61 microscope).

Western blots

Olfactory systems (the OE, VNO, nerves, and main and accessory bulbs) from twenty tadpoles were homogenized in Tris-buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.5% NP40, 1% Triton, 1 mM PMSF) with 1X protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Sample proteins (20 l) were separated on 15% sodium dodecyl sulfate polyacrylamide gels by electrophoresis (SDS-PAGE). Proteins and molecular markers (SeeBlue Plus2 PreStained Standard, Invitrogen) were then transferred onto a PVDF membrane (Amersham Biosciences, Arlington Heights, IL, USA) as previously described (Pozzi et al., 2006). Detection of proteins was carried out after blocking the membranes overnight with a 5% solution of non-fat dry milk and incubating for 2 h at room temperature with the primary antibody: anti-Gα_o (1/5000) or anti-Gα_{olf} (1/1000). After incubation with the primary antibody, the membranes were washed with Tris-buffer saline–Tween (TBST) and incubated with a secondary antibody coupled to horseradish peroxidase (Chemicon, Millipore, Billerica, MA). Membranes were then developed by using an enhanced chemiluminescence detection system to visualize peroxidase activity (Chemicon), and images were captured with a Luminescent Image Analyzer LAS-1000 Plus (Fuji Photo Film).

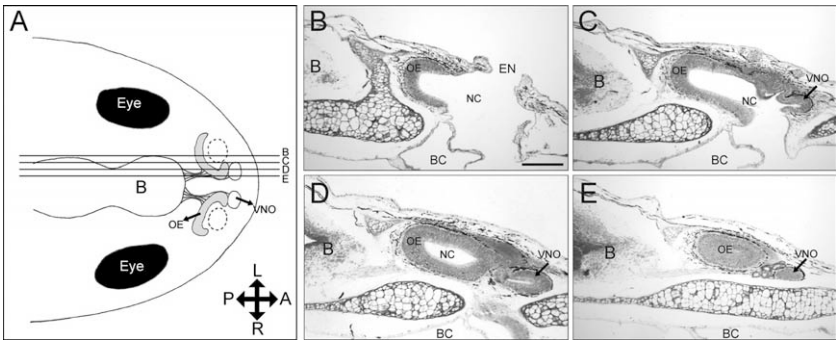

Fig. 1. Topographic organization of the olfactory system in *R. arenarum* tadpoles. **(A)** Schematic diagram showing a horizontal section through the head of a representative prometamorphic tadpole. Lines B–E indicate the planes of the parasagittal sections shown in panels B–E. **(B–E)** Parasagittal sections (cresyl violet staining) showing the distribution of the olfactory epithelium (OE) and vomeronasal organ (VNO) in a stage-36 tadpole (anterior is to the right and top is dorsal). BC, bucal cavity; B, brain; EN, external naris; NC, nasal cavity; A, anterior; P, posterior; L, left; R, right. Scale bar, 200 m.

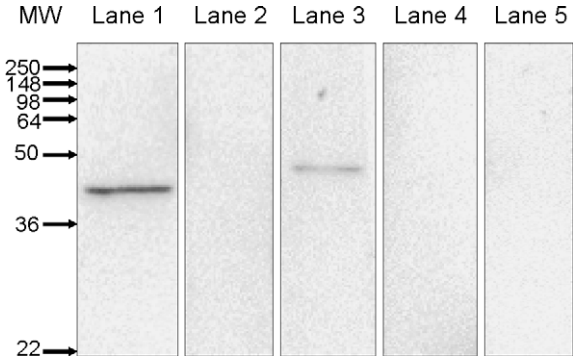

Fig. 2. Western blot analysis of G-proteins confirming the specificity of the antisera used. Lane 1, Gα_o; lane 2, Gα_o preabsorption; lane 3, Gα_{olf}; lane 4, Gα_{olf} preabsorption; lane 5, primary antiserum omitted; MW, molecular weight standards (kDa).

RESULTS

Cresyl violet-stained parasagittal sections showed the topographic organization of the dual olfactory system of *R. arenarum* tadpoles (Fig. 1B–E). The VNO was unmistakably identified as a sensory epithelium anatomically separated from the OE, ensuring that the heterogeneity observed was not due to a misinterpretation of different sensory epithelia. The VNO is situated antero-ventrally to the nasal cavity (NC) (Fig. 1).

The specificity of the commercially obtained antisera used in immunohistochemistry was confirmed by western blots. The immunoblots showed a specific band with the molecular weight expected from the literature for both the antiserum against Gα_o (44 KDa) (Fig. 2, lane 1) and that against Gα_{olf} (46 KDa) (Fig. 2, lane 3). Moreover, neither of the bands was observed in preabsorbed controls (i.e., the antiserum was previously incubated with the specific antigen) (Fig. 2, lanes 2 and 4, respectively). On the other hand, no specific band was observed when membranes were incubated with the antiserum against Gα_{i2} (data not shown).

Clear immunostaining of ORNs was observed with the antisera against Gα_o and Gα_{olf} in the OE of stage-36 tadpoles (Fig. 3A, B). The antiserum to Gα_o stained the membranes of bipolar neurons whose cell bodies were located in the basal third of the OE, stained the neuronal axons that form bundles in the lamina propria, and faintly stained dendrites and the luminal terminations of neurons (Fig. 3A). In contrast, the antiserum against Gα_{olf} immunostained a different population of bipolar neurons with cell bodies located in the middle third of the OE. The axons and dendrites of these neurons were also well stained (Fig. 3B). Comparison of the Gα_o and Gα_{olf} immunoreactivity (-ir) in the OE of consecutive sections indicated that they were distributed in complementary layers, and that the boundary between the Gα_{olf} neuronal layer and the Gα_o deep layer showed an undulating rather than a straight pattern. Similar immunohistochemical results were observed in all larval stages analyzed. The antiserum against Gα_{i2} was ineffective in staining ORNs in all developmental stages analyzed (data not shown), which was congruent with the results of the western blot experiments.

In the VNO, clear immunostaining of the VRNs was observed with the antiserum against Gα_o. These Gα_o-ir neurons were distributed in the sensory epithelium, without a discernable pattern. As with the ORNs in the OE, axons and dendrites of the VRNs were clearly stained. Moreover, the luminal terminations of the neurons were even more strongly stained in the VRNs (Fig. 3C). The antiserum against Gα_{olf} was ineffective in staining VRNs in the VNO in

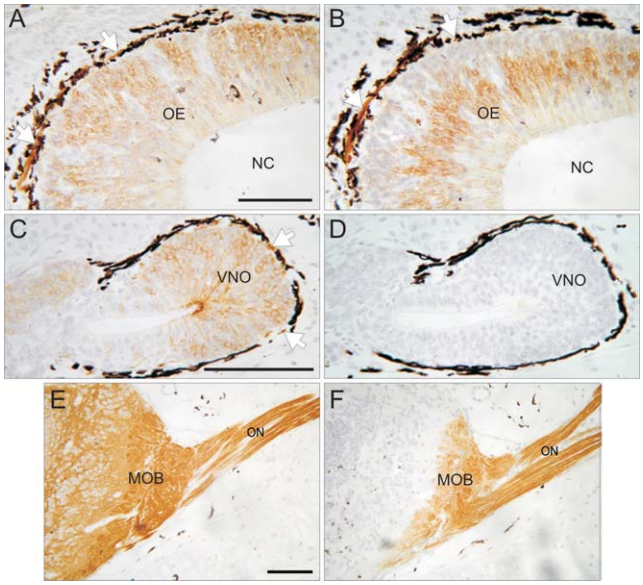

Fig. 3. Immunohistochemical detection of $G\alpha_o$ (**A, C, E**) and $G\alpha_{olf}$ (**B, D, F**) in parasagittal sections of the olfactory system of *R. arenarum* tadpoles (stage 36). **(A)** $G\alpha_o$ is present in olfactory neurons in the basal third of the olfactory epithelium (OE). **(B)** $G\alpha_{olf}$ is present in olfactory neurons in the middle third of the OE. **(C)** $G\alpha_o$ in the vomeronasal organ (VNO). **(D)** Absence of $G\alpha_{olf}$ in the VNO. **(E)** $G\alpha_o$ in the main olfactory bulb (MOB). **(F)** $G\alpha_{olf}$ in the MOB. The arrows indicate stained olfactory axons bundles (A, B) and stained vomeronasal axons bundles (C) running in the lamina propria. NC, nasal cavity; ON, olfactory nerve. Scale bars, 100 m.

all developmental stages analyzed (Fig. 3D). Both the $G\alpha_o$ and $G\alpha_{olf}$ antibodies stained the olfactory nerve (ON) and MOB, without an apparent heterogeneous distribution along the dorsoventral axis (Fig. 3E, F).

To evaluate more accurately whether the heterogeneous distribution of ORNs was topographically represented in the MOB, a detailed serial immunofluoresce analysis was made. Horizontal sections of the forebrain were incubated with the $G\alpha_o$ antiserum, and the immediately following sections were incubated with the $G\alpha_{olf}$ antiserum. The $G\alpha_o$ and $G\alpha_{olf}$ antisera clearly stained the ON, nerve layer, and glomerular layer of the MOB (Fig. 4A, B), but no clear pattern of immunostained glomeruli was observed in the MOB. Moreover, some glomeruli seemed to be shared by $G\alpha_o$-ir neurons and $G\alpha_{olf}$-ir neurons (compare Fig. 4Ai with Bi, and 4Aii with Bii). Neuronal tracing experiments using fluorescent dyes combined with immunohistochemistry would be helpful in confirming this observation.

Stained glomeruli were observed in the AOB with the $G\alpha_o$ antiserum (Fig. 4C), but no glomeruli were stained in the AOB in sections incubated with the $G\alpha_{olf}$ antiserum (Fig. 4D), as also observed in the VNO.

Transmission electron microscopy showed that the ORNs in the OE were ciliated on the dendritic apical surface. These cilia were embedded in the mucous sheet on the surface of the OE, secreted by adjacent supporting cells. The ORNs contained ciliary basal bodies, mitochondria, and numerous neurotubules in the dendrites. Supporting cells in the OE were characterized by a large number of vesicles, which correspond to secretory granules, in the apical cytoplasm (Fig. 5A). The dendrites of the VRNs contained microvilli on the apical surface, and these sensory cells alternated with nonsensory ciliated supporting cells (Fig. 5B). The VRNs contained centrioles and numerous neurotubules in the dendrites (Fig. 5C).

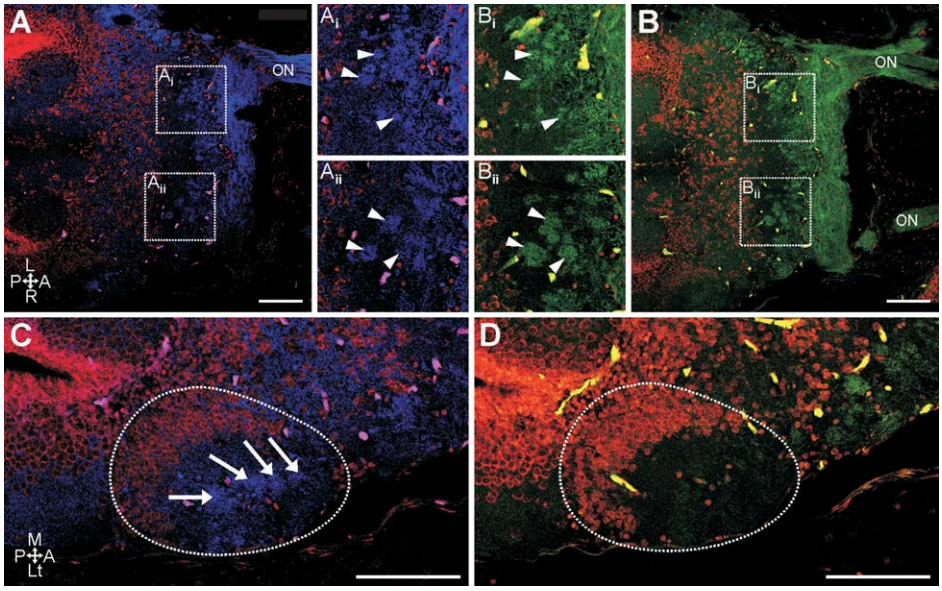

Fig. 4. Immunofluorescence of $G\alpha$ proteins in the forebrain of stage-36 *R. arenarum* tadpoles (horizontal sections). **(A)** $G\alpha_o$ (blue, false-color representation) in the main olfactory bulb (MOB) shows clear staining in the nerve and glomerular layers. The olfactory nerves (ON) are strongly stained as well. Ai and Aii show high-magnification views of the boxed areas in (A). **(A)** $G\alpha_{olf}$ (green) in the MOB. The ON and the nerve and glomerular layers are well stained with $G\alpha_{olf}$. Bi and Bii show high-magnification views of the boxed areas in (B). Arrowheads indicate glomeruli shared by $G\alpha_o$-ir neurons and $G\alpha_{olf}$-ir neurons (compare Ai with Bi, and Aii with Bii). **(C)** AOB (delimited area) stained with $G\alpha_o$ (blue, false-color representation). Arrows indicate G_o-ir glomeruli. **(D)** The AOB incubated with anti- $G\alpha_{olf}$ (green) does not show any stained glumeruli. All sections were counterstained with propidium iodide (red). P, posterior; A, anterior; L, left; R, right; M, medial; Lt, lateral. Scale bars, 100 m.

DISCUSSION

In this study, we used immunohistochemistry to reveal the presence of $G\alpha_{olf}$ and $G\alpha_o$ proteins in the olfactory system of *Rhinella (Bufo) arenarum* tadpoles. We employed antibodies against peptidic epitopes based on mammalian $G\alpha$ protein sequences. We had to assess whether these antibodies reacted specifically with toad counterparts. We first performed a western blotting analysis that produced a clear, single band of the expected molecular weight for each protein. Furthermore, preabsorption of the $G\alpha$ protein antibodies completely abolished

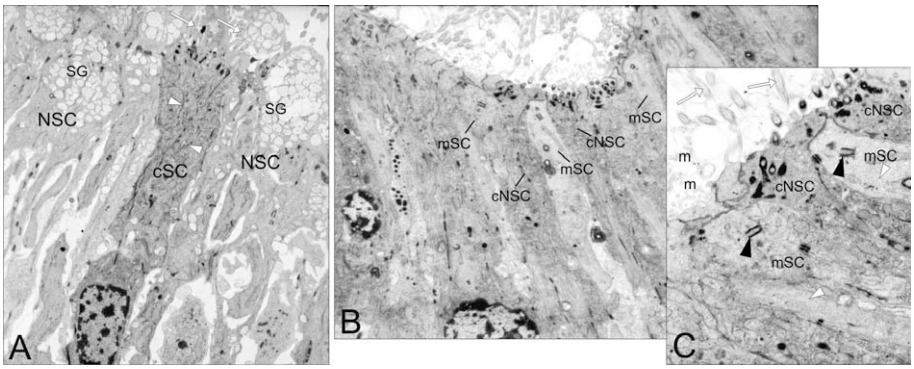

Fig. 5. Transmission electron micrographs of the apical zone of the OE (**A**) and VNO (**B**, **C**). **(A)** The EO contains sensory cells with apical ciliated ends (cSC, ciliated sensory cell) surrounded by nonsensory cells (NSC) filled with secretory granules (SG). **(B)** Microvillous sensory cells (mSC) alternate with ciliated nonsensory cells (cNSC) in the VNO. **(C)** High magnification view of part of (B). Arrows indicate cilia and white arrowheads indicate neurotubules in (A) and (C); black arrowheads indicate centrioles in (C). m, microvilli. Magnification, 4400X (A), 3000X (B), 12000X (C).

the signals from both blotting membranes and tissue sections. Thus, the antibodies against $G\alpha_o$ and $G\alpha_{olf}$ produced specific signals in toad tadpoles. These commercial antibodies have been successfully employed for the immunodetection of G proteins in a variety of vertebrates (Belanger et al., 2003; Hansen et al., 2004; Wakabayashi and Ichikawa, 2008).

In contrast, no $G\alpha_{i2}$ immunoreactivity was found in MOS or VNS tissue sections, and no clear band was detected by western blotting in homogenates from the olfactory system. Furthermore, we performed a western blot analysis using whole-body homogenates from tadpoles, but no clear band was identified (data not shown). Therefore, we suspect that in this case the mammalian commercial antibody against $G\alpha_{i2}$ did not recognize the *R. arenarum* counterpart.

The goal of this study was to demonstrate that there are at least two segregated populations of ORNs in the OE of *R. arenarum* tadpoles: $G\alpha_o$-ir ORNs were located in the basal layers of the OE, while $G\alpha_{olf}$-ir ORNs were located in the medial layers of the OE. In fishes, a segregated pattern of $G\alpha_{olf}$- and $G\alpha_o$-expressing neurons has been observed in the OE. Moreover, ciliated ORNs that express rodent-type ORs (Buck and Axel, 1991) and $G\alpha_{olf}$ are distributed more basally in the OE, whereas microvillous ORNs expressing V2R-like receptors and $G\alpha_o$ are distributed more apically in the OE (Belanger et al., 2003; Hansen et al., 2004). On the other hand, a segregated pattern, with $G\alpha_o$- and $G\alpha_{olf}$-expressing ORNs, has been recently observed in the sensory epithelium lining the olfactory middle cavity of *X. laevis* (Date-Ito et al., 2008). This amphibian has two separate OEs in different nasal cavities: the principal cavity (PC), which responds to air-borne odorants, and the middle cavity (MC), which responds to water-soluble odorants (Freitag et al., 1995; Hagino-Yamagishi et al., 2004). A sensory epithelium appears to be uniquely present in the MC in members of the family Pipidae (Paterson, 1951), and is functionally correlated with the aquatic lifestyle of adult *Xenopus*. Taken together, these results suggest that the segregation pattern determined by $G\alpha_o$- and $G\alpha_{olf}$-expressing neuronal subpopulations would be a common characteristic of sensory epithelia involved in the detection of odors in water. It would thus be interesting to evaluate the distribution of G proteins in the OE of *R. arenarum* in post-metamorphic stages (when chemoreception of volatile ligands takes place), to assess whether the segregation pattern is retained or lost.

Interestingly, research on the the semi-aquatic Reeve's turtle has demonstrated that the two signal transduction pathways (via $G\alpha_o$ and via $G\alpha_{olf}$) are present in neurons of the OE and the VNO of these animals. Moreover, an ultrastructural analysis showed that $G\alpha_o$ is present in the microvilli, whereas $G\alpha_{olf}$ is present in the cilia, of the same sensory neuron (Wakabayashi and Ichikawa, 2008).

Our ultrastructural analysis showed that only ciliated ORNs are present in the OE of *R. arenarum* tadpoles. Data from fishes and the *X. laevis* middle cavity show that at least two types of ORNs occur in the same sensory epithelia. Some ORNs have cilia at the dendritic apical end, whereas others have microvilli (Ichikawa and Ueda, 1977; Hansen et al., 1998; Belanger et al., 2003). However, the presence of neurons with different signal transduction systems (i.e., different associated G proteins) in the same sensory epithelium does not necessarily mean that these neurons are morphologically different. For example, all VRNs in rodents have microvilli, although the basally located VRNs express $G\alpha_o$, whereas the more apical VRNs express $G\alpha_{i2}$. The ultrastructural study of the VNO showed that the VRNs of *R. arenarum* are covered with microvilli, similarly to other species studied (Oikawa et al., 1998; Halpern and Martínez-Marcos, 2003).

In vertebrates, neuronal turnover of ORNs takes place from undifferentiated stem cells located at the basal end of the OE (Graziadei and Metcalf, 1971; Graziadei and Monti Graziadei, 1979). These stem cells divide to give rise to daughter cells that migrate apically in the OE and gradually differentiate into ORNs. So, it is possible that the more basally located $G\alpha_o$-ir neuronal population differs in maturational stage from the more apically located $G\alpha_{olf}$-ir neuronal population. In rodents, it has been demonstrated that during embryonic development, progenitor cells and immature and mature ORNs express $G\alpha_s$ (which is involved in axon guidance), whereas ORNs in post-embryonic animals express $G\alpha_{olf}$ (which is involved in odor-evoked transduction) (Chesler et al., 2007). It is unlikely that the $G\alpha_o$-ir cell population observed in *R. arenarum* comprises undifferentiated stem cells, since immunostaining for $G\alpha_o$ revealed axon bundles reaching the glomerular layer of the MOB, an exclusive characteristic of neurons. Nevertheless, we cannot rule out the possibility that the $G\alpha_o$-ir neuronal population is "younger" than the $G\alpha_{olf}$-ir neuronal population. It could thus be possible that the segregation pattern observed in the tadpole OE represents two processes taking place simultaneously: on one hand, apical $G\alpha_{olf}$-ir ORNs could represent "mature" neurons participating (via $G\alpha_{olf}$) in odor-evoked transduction; on the other hand, basal $G\alpha_o$-ir ONRs could

represent "immature" neurons undergoing an active axonal sorting process that adds newly generated neurons to the circuit. This hypothesis could explain why some $G\alpha_{olf}$-ir ONRs and $G\alpha_o$-ir ONRs reach a common glomerulus in the MOB (see Fig. 4). Nevertheless, further studies using species-specific markers are necessary to discern between immature and mature neurons in *R. arenarum*.

We found that the $G\alpha_o$ protein was extensively expressed in the VNO of *R. arenarum* tadpoles. $G\alpha_o$-ir VRNs were distributed throughout the sensory epithelium of the VNO, without a clear distribution pattern, while $G\alpha_{olf}$ was not detected in the VNO. A study of the expression of G protein genes showed that only $G\alpha_o$ is extensively expressed in the VNO of *X. laevis* (Hagino-Yamagishi et al., 2004), in accordance with our results. Interestingly, sensory neurons that utilize the $G\alpha_o$ signal transduction system express V2R receptor family genes. This association between $G\alpha_o$ and V2Rs has been observed in VRNs in *X. laevis* (Hagino-Yamagishi et al., 2004) and rodents (Jia and Halpern, 1996). So, it would be interesting to study which family of vomeronasal receptors is expressed in the VRNs of *R. arenarum*, to evaluate whether $G\alpha_o$ and V2Rs are associated in this amphibian.

Finally, the phylogenetic aspects of the amphibian dual olfactory system have been recently discussed (Taniguchi et al., 2007). This work pointed out that amphibians are the key phylogenic group in studying chemodetection organs, because they are the first animals in phylogeny to live on land, and the appearance of the dual olfactory system may have derived from this change in life style. So, research on this group using both molecular and morphological approaches closely connected with each other would help us to understand the functional organization and evolution of vertebrate chemodetection organs.

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