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A putative functional vomeronasal system in anuran tadpoles

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Abstract

We investigated the occurrence and anatomy of the vomeronasal system (VNS) in tadpoles of 13 different anuran species. All of the species possessed a morphologically fully developed VNS with a highly conserved anatomical organisation. We found that a bean-shaped vomeronasal organ (VNO) developed early in the tadpoles, during the final embryonic stages, and was located in the anteromedial nasal region. Histology revealed the presence of bipolar chemosensory neurones in the VNO that were immunoreactive for the Gαo protein. Tract-tracing experiments demonstrated that chemosensory neurones from the VNO reach specific areas in the brain, where a discernible accessory olfactory bulb (AOB) could be observed. The AOB was located in the ventrolateral side of the anterior telencephalon, somewhat caudal to the main olfactory bulb. Synaptophysin-like immunodetection revealed that synaptic contacts between VNO and AOB are established during early larval stages. Moreover, using lectin staining, we identified glomerular structures in the AOB in most of the species that we examined. According to our findings, a significant maturation in the VNS is achieved in anuran larvae. Recent published evidence strongly suggests that the VNS appeared early in vertebrate evolution and was already present in the aquatic last common ancestor of lungfish and tetrapods. In this context, tadpoles may be a good model in which to investigate the anatomical, biochemical and functional aspects of the VNS in an aquatic environment.

Key words: accessory olfactory system; chemical sensing; G protein; pheromones; vomeronasal organ.

Introduction

In most terrestrial vertebrates, there are two anatomically distinct olfactory organs: the olfactory epithelium (OE) and the vomeronasal organ (VNO). The olfactory sensory neurones lining the OE and the vomeronasal sensory neurones lining the VNO are characterised as bipolar neurones that project to two different specific telencephalic areas: the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB), respectively. The two sensory systems have anatomical, physiological, and molecular differences, which suggest different behavioural functions (Halpern & Martinez-Marcos, 2003). The VNO is commonly assumed to be specialised for detecting pheromones, whereas general odorants would be detected by the OE. However, this hypothesis is simplistic and not supported by the available behavioural and physiological data (Baxi et al., 2006). For example, the vomeronasal system (VNS) is crucial in mediating responses to foraging cues in snakes and salamanders (Alving & Kardong, 1996; Placyk & Graves, 2002; Halpern & Martinez-Marcos, 2003), whereas in mammals, some pheromones are detected by the OE (Dorries et al., 1997; Swann et al., 2001; Xu et al., 2005).

An anatomically discernible VNS, formed by a VNO in the nasal cavity and an AOB in the anterior brain, has not been recognised in fishes and it is generally believed that this accessory olfactory system exists only in tetrapods (Eisthen, 1992). This prompted the idea that the VNS arose over the course of tetrapod evolution as an adaptation to terrestrial life (Bertmar, 1981). Nevertheless, a growing body of evidence suggests that the VNS appeared in aquatic environments during vertebrate evolution (Eisthen, 2000; Grus & Zhang, 2009; Gonzalez et al., 2011). For example, VNS-specific genes have been identified in bony fishes, elasmobranches and lampreys, indicating that at least genetic components of the VNS arose early on in vertebrate evolution (Grus & Zhang, 2009). Moreover, the presence of a separate diverticulum in the nasal cavity that contains a

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sensory vomeronasal epithelium and an AOB in the telencephalon has been described in permanently aquatic amphibians (Eisthen, 2000). In addition, it was recently shown that the African lungfish *Protopterus dolloi* has epithelial crypts at the base of the lamellae of the OE; chemosensory neurones of these crypts express markers specific to the vomeronasal sensory neurones in tetrapods. Moreover, the projections of the chemosensory cells lining these crypts allowed the authors to identify an AOB on the lateral margin of the MOB (Gonzalez et al., 2011). These findings suggest that the VNS may play a role in chemosensory detection in aquatic environments, but nothing is known about the function of the VNS in water.

Among tetrapods, amphibians are unique in having an aquatic larval stage, followed by metamorphosis to a terrestrial adult. Thus, anuran larvae are excellent models in which to investigate the anatomical, biochemical and functional aspects of the VNS in an aquatic environment. However, there has been little research regarding the nasal chemosensory epithelia of tadpoles. Most classical and some recent works are restricted to descriptions of the general anatomy of the nasal cavity and do not analyse axonal projections to the brain or biochemical aspects of the olfactory and VNSs (Tsui, 1946; Yvroud, 1966; Taniguchi et al., 1996; Jermakowicz et al., 2004; Wang et al., 2008; Jungblut et al., 2011). By contrast, research that integrates anatomical, biochemical and molecular data has generally focused on the main olfactory system and overlooked the VNS (Reiss & Burd, 1997; Nezlin & Schild, 2005).

In the present work, we looked for the presence of VNO and AOB in tadpoles of 13 different anuran species. We found that a morphologically fully developed VNS was present in all of the species that we examined. The anatomical organisation of the VNS was highly conserved among anuran tadpoles and quite similar to the anatomy described in the VNS of adult anurans. To assess the degree of maturity of the VNS in tadpoles, we looked for expression of the G protein Gαo, a selective marker for vomeronasal sensory neurones in tetrapods (Halpern & Martinez-Marcos, 2003; Hagino-Yamagishi et al., 2004). Gαo protein was expressed in the VNO of tadpoles from the late embryonic stages onward, during organogenesis of the OE and the VNO. Moreover, tract-tracing techniques and synaptophysin immunoreactivity demonstrated that sensory neurones from the VNO are in synaptic contact with specific telencephalic areas, forming a histologically discernible AOB.

Materials and methods

Animals

Tadpoles of *Rhinella arenarum*, *Hypsiboas pulchellus* and *Xenopus laevis* were obtained by *in vitro* fertilisation according to methods previously described (Paz et al., 1995). Larvae were maintained in dechlorinated tap water with a constant photoperiod and temperature (12 : 12 h, dark : light; 22 °C) and fed *ad libitum*. *Lithobates catesbeianus* tadpoles were obtained from a local supplier and tadpoles of the other species that we examined were obtained from the Herpetological Collection of the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia'-CONICET (see Appendix 1). Animals were staged according to a generalised table for anuran embryos (Gosner, 1960) except for *X. laevis* tadpoles, which were staging according to a specific developmental table (Nieuwkoop & Faber, 1994) (Table 1). All of the 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 United States National Institutes of Health (Publication 8523, revised 1985).

Light microscopy

Tadpoles were anaesthetised by immersion in a 0.1% solution of tricaine methanesulfonate (MS222; 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 (7 μm thick) were cut, mounted on HiFix glass slides (HF-5001; InProt, Buenos Aires, Argentina), and subjected to immunohistochemistry and lectin histochemistry or stained using classical histological techniques (cresyl violet).

Immunohistochemistry and lectin histochemistry

General procedures for immunohistochemistry were followed as in our previous report (Jungblut et al., 2009). The primary antibodies used was rabbit anti-Gαo, 1 : 12 000 (sc-387; Santa Cruz). After primary antibody incubation (overnight at 4 °C), sections were treated with the appropriate biotinylated secondary antibody (Vector

Table 1 Specimens analysed in the study.

Species	Family	Developmental stage used *
Rhinella arenarum	Bufonidae	25–39
Scinax acuminatus	Hylidae	37
Hypsiboas curupi	Hylidae	29–30
Hypsiboas pulchellus	Hylidae	24–39
Phyllomedusa azurea	Hylidae	36–37
Limnomedusa macroglossa	Cycloramphidae	38
Dermatonotus muelleri	Microhylidae	28–30
Crossodactylus schmidti	Hylodidae	36–38
Physalaemus sp.	Leiuperidae	39
Lepidobatrachus llanensis	Ceratophryidae	36–37
Leptodactylus latrans	Leptodactylidae	37–38
Lithobates catesbeianus	Ranidae	33–36
Xenopus laevis	Pipidae	46–56

*Larval stages are according to Gosner (1960), except *X. laevis,* which was staged according to Nieuwkoop & Faber (1994).

Laboratories, Burlingame, CA, USA), followed by 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). Omission of the primary antiserum (negative control) produced negligible background staining (data not shown).

To facilitate the identification of glomerular structures in the AOB, lectin histochemistry staining was performed using lectin soybean agglutinin (SBA), which has been shown to stain the AOB in several amphibian species (Meyer et al., 1996). After deparaffinisation and rehydration, tissue sections were blocked for nonspecific binding, as described above, incubated with biotin-labelled SBA (Vector Labs) overnight at 4 °C, and treated with streptavidin–FITC complex (Sigma).

FITC-treated sections were counterstained with propidium iodide (P-1304; Molecular Probes, Eugene, OR, USA), and images were captured with a confocal laser microscope (Olympus FV-300 attached to an Olympus Bx-61 microscope).

Tract-tracing techniques

Animals were transferred to a humidified chamber; under anaesthesia (see above), the remaining water in the olfactory cavity was carefully dried prior to the application of neuronal tracers. A dorsal approach through the external nares was used. Alexa Fluor 680 conjugated to dextran amine (680DA 3 kD; Molecular Probes) was applied by impaling the VNO with a glass micropipette (680DA had been recrystallised from a saturated solution in distilled water onto the tip of the micropipette). Tetramethylrhodamine-conjugated dextran amine (TMRDA 3 kDa; Molecular Probes) was applied to the contralateral OE following the methods described above. Dye-injected animals were maintained individually for 24 h at room temperature. After this survival time, tadpoles were fixed in Bouin's solution and processed for paraffin sections (15 μm thick), as described earlier. Images were captured with a confocal laser microscope (Olympus FV-300 attached to an Olympus Bx-61 microscope).

Combined tract-tracing/immunohistochemical techniques

Under anaesthesia, 680DA 3 kDa (Molecular Probes) was applied by impaling the VNO with a glass micropipette following methods described above. After 24 h at room temperature animals were fixed in Bouin's solution and processed for paraffin sections (15 μm thick) as described earlier. Sections were deparaffinised, rehydrated and incubated overnight at 4 °C with primary antibody (mouse anti-Synaptophysin 1/100; Sigma). Then, sections were treated with the appropriate biotinylated secondary antibody (Vector Laboratories) followed by incubation with FITC-labelled streptavidin (Sigma), and mounted for confocal analysis.

Results

Nasal region and vomeronasal organ

We analyzed the entire nasal region in tadpoles of 13 anuran species performing serial sections in the transverse and sagittal planes. In all 13 of the species that we examined, the VNO was a bean-shaped structure in the anteromedial nasal region (Fig. 1). Schematic illustration in Fig. 1A summarizes the general anatomical organization of the chemosensory nasal structures (OE and VNO) in a tadpole. The sensory epithelium of the VNO (composed of supporting, basal, and sensory cells) lay in a separate accessory

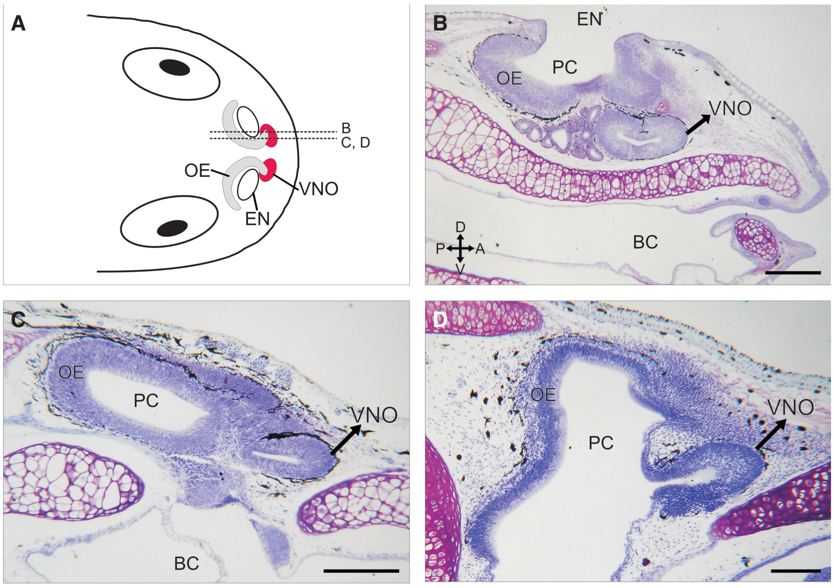

Fig. 1 Nasal region in anuran tadpoles. (A) Schematic illustration of the nasal region of a generalised tadpole (dorsal view). Dotted lines (B, C, and D) indicate the estimated planes of the parasagittal sections shown in panels B–D. (B–D) Histological parasagittal sections (stained with cresyl violet) through the nasal region of *X. laevis* (B, stage N/F54), *R. arenarum* (C, stage G35), and *L. catesbeianus* (D, stage G34) showing the relative position of the bean-shaped VNO anteroventral to the olfactory epithelium in the principal chamber (PC). BC, buccal cavity; EN, external naris. Axes indicate: A, anterior; P, posterior; D, dorsal; V, ventral. Scale bar, 200 μm.

chamber, or diverticulum, of the nasal cavity, located anteroventral to the OE of the principal chamber (Fig. 1A–D). Notably, the positioning of the VNO within the nasal region was remarkably conserved among the tadpoles. In a rostrocaudal direction, the VNO was the first nasal chemosensory structure and was located dorsolateral to the cornua trabeculae. As an example, histological parasagittal sections of the mesobatrachian *X. laevis* and the neobatrachians *R. arenarum* and *L. catesbeianus* are shown in Fig. 1B,C,D, respectively. The VNO lumen opened into the anterior wall of the principal nasal chamber (Fig. 1D).

To evaluate the maturational state of the VNS in tadpoles, we assessed the expression of the second messenger protein Gαo, a marker specific to vomeronasal neurones in tetrapods (Fig. 2). The VNO originated as a ventral evagination of the rostral portion of the olfactory pit during the late embryonic stages, around the time of development of the operculum. From this early stage, developing sensory neurones from the VNO exhibited strong Gαo expression (Fig. 2A–C). Moreover, at the first larval stages (G26), these Gαo-expressing cells acquired the typical bipolar morphology of chemosensory neurones (Fig. 2D,E). Dendrites of the vomeronasal sensory neurones projected toward the luminal surface of the VNO, whereas axons ran toward the basal lamina of the VNO, forming obvious axon bundles at the lamina propria. As the larvae developed, the VNO continued growing and expanding medially, forming a medial diverticulum at the rostral end of the nasal region (Fig. 2G–L).

Accessory olfactory bulb

The general morphology of the anterior portion of the telencephalon was very similar in all 13 species. The olfactory

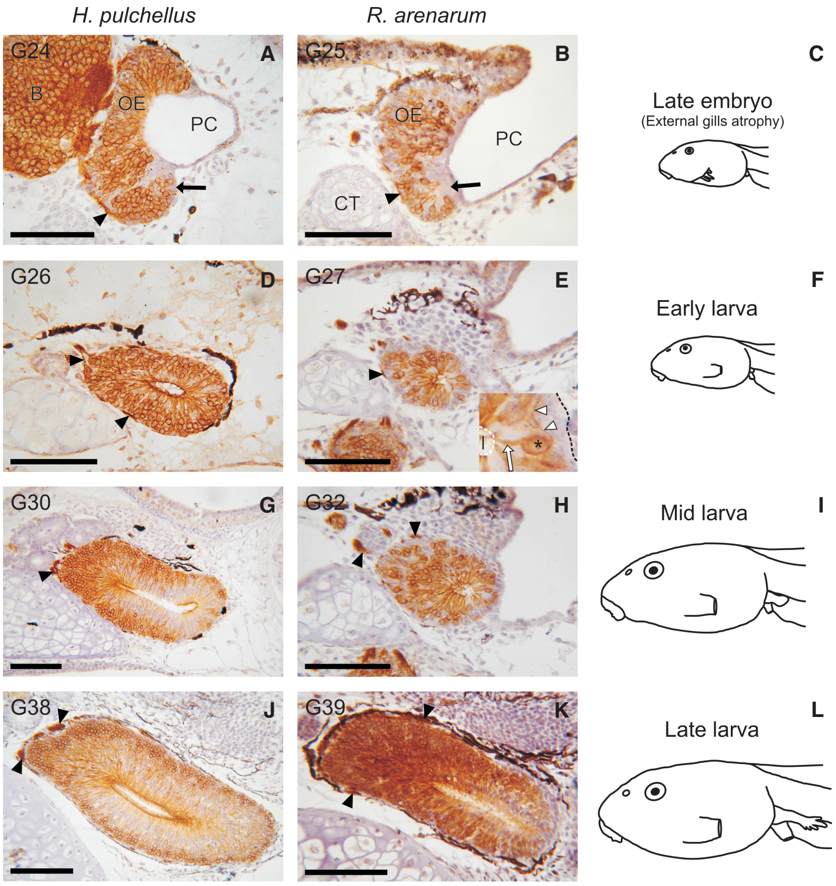

Fig. 2 Gαo protein expression in the vomeronasal organ (cross sections, left side) of *H. pulchellus* (A, D, G, and J) and *R. arenarum* (B, E, H, and K) throughout larval development. Panels in the right column (C, F, I, and L) show a schematic lateral view of the estimated developmental stages used in both species. The specific developmental stage is shown in the top-left corner in each panel. (A and B) At the final embryonic stages (C), the vomeronasal organ (VNO) (arrows) evaginates from the rostral portion of the olfactory pit. Axons of the Gαo-expressing cells in the VNO project to the anterior telencephalon and form axon bundles in the lamina propria (arrowheads). (D, E) At early larval stages (F), the Gαo-expressing cells in the VNO acquire a bipolar morphology. Inset in (E) shows a higher magnification of a bipolar vomeronasal chemosensory neurone. Asterisk, cell body; white arrow, dendrite; white arrowheads, axon; black dotted line, basal lamina; white dotted line, luminal surface; l, lumen of the VNO. (G, H) VNO at mid-larval stages (I). (J, K) At late larval stages (L), Gαo protein is strongly expressed in the conspicuous VNO. B, brain; CT, cornua trabeculae; PC, principal chamber. Top is dorsal and left is medial. Scale bar, 100 μm.

nerve entered the forebrain at the ventral zone in the rostral end of the telencephalon. The MOB was present as a laminar structure, as in all vertebrates (nerve, glomerular, external plexiform, mitral cell, internal plexiform, and granular layers). An AOB was apparent in the ventrolateral side of the anterior telencephalon, somewhat caudal to the MOB. The presence of an AOB was assessed by histological staining of the anterior brain serial sections (Fig. 3A,B). The AOB was represented as an anteroposteriorly elongated, ovoid nucleus. As in the MOB, the AOB was organised in layers. The glomerular layer was located anterolaterally in the AOB, whereas bodies of the mitral cells were localised in the mid-caudal portion of the AOB.

The use of lectin histochemistry has proven to be an excellent tool for the study of morphological aspects in the olfactory system of vertebrates (Endo et al., 2011). In particular, soybean agglutinin (SBA) has been used to stain specifically the AOB in several amphibian species (Meyer et al., 1996). SBA histochemistry revealed spherical neuropil structures, called glomeruli, in the AOB (Fig. 3C–E). Well-developed glomeruli were observed in the AOB of all of the tadpole species, except for *Scinax acuminatus* and *Dermatonotus muelleri,* in which the glomerular layer of the AOB appeared as a nonstructured fiber meshwork (Fig. 3F,G).

To characterise the VNS in tadpoles more thoroughly, we analysed the efferent projections of the VNO. Tract-tracing experiments demonstrated that axons of the vomeronasal sensory neurones reached the AOB in the anterior telecephalon, whereas sensory neurones from the OE projected their axons to the MOB (Fig. 4A–C). Axons of vomeronasal sensory neurones entered the AOB by the frontal side and formed spheroidal arborisations (glomeruli) in the glomerular layer. In some specimens, a few tract-positive glomeruli were observed in the MOB when the ipsilateral VNO was impaled (see Fig. 4E). Nevertheless, we do not consider that these are vomeronasal sensory neurones projecting to the MOB. Instead, this unexpected result may have been an artefact associated with the method of impaling we used. The anterior border of the OE is closely related to the VNO (see the schematic illustration of the nasal region in Fig. 1A); thus, the olfactory neurones of this zone are extremely difficult to avoid when the VNO is impaled in intact animals.

To evaluate synaptic connections between vomeronasal sensory neurones and the brain, we performed double labelling experiments using tract-tracing and synaptophysin-like immunodetection techniques. This experiment demonstrated that axons from the VNO had established synaptic contact with telencephalic neurones (mitral cells in the AOB) during the larval phase (Fig. 4F–H). As in the results obtained using lectin histochemistry, tract tracing and synaptophysin immunoreactivity revealed the presence of glomerular structures in the AOB (Fig. 4G,H).

Discussion

We found that tadpoles of all 13 of the species that we examined possess an anatomically fully developed VNS,

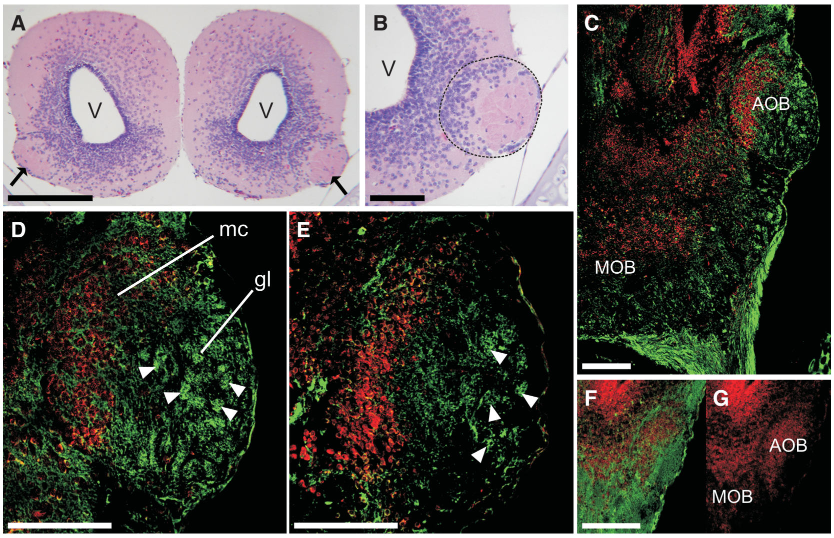

Fig. 3 Accessory olfactory bulb in tadpoles. (A) Histological cross section of the anterior telencephalon of *H. pulchellus* (stage G34) showing a discernible accessory olfactory bulb (AOB) in the ventrolateral side (arrows); top is dorsal. (B) Higher magnification of the left AOB (demarcated by a black dotted line) shown in (A). (C–F) soybean agglutinin (SBA)-lectin staining (green) in tadpole horizontal sections. Nuclei were counterstained with propidium iodide (PI, red). (C) Anterior telencephalon (left side) of *Crossodactylus schmidti*. (D) Higher magnification of the AOB shown in (C). (E) Detail of the AOB of *Leptodactylus latrans*. (F and G) Anterior telencephalon (left side) of *Dermatonotus muelleri*; SBA-lectin and PI staining (F) and PI alone (G). Arrowheads show representative glomerular structures in the AOB. gl, glomerular layer; mc, mitral cell layer; MOB, main olfactory bulb. Scale bars, 200 µm (A and C) and 100 µm (B and D–F).

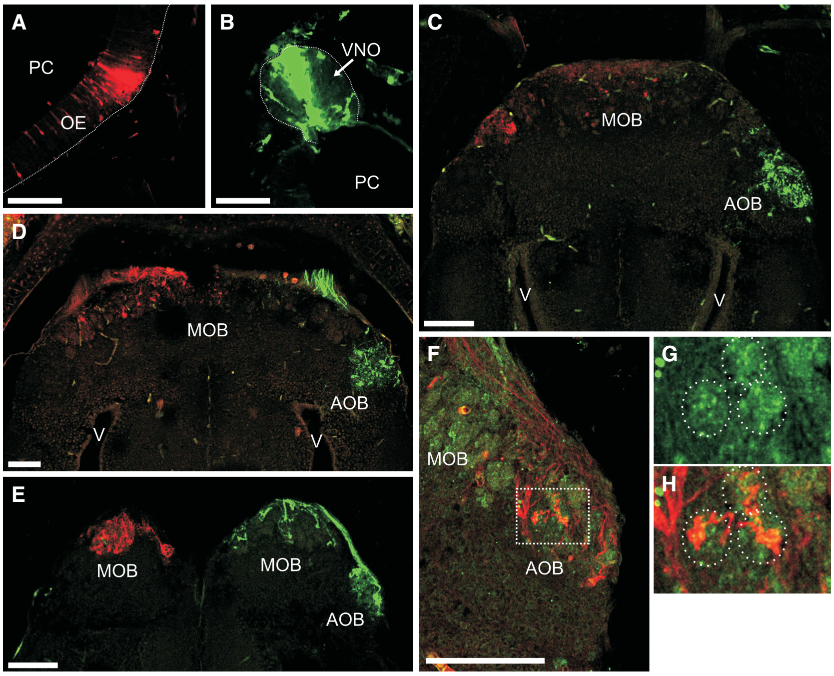

Fig. 4 Vomeronasal sensory neurones establish synaptic contact with the accessory olfactory bulb (AOB). (A–E) Horizontal sections of tadpoles after application of the neuronal tracers TMRDA [left olfactory epithelium (OE), red] and 680DA [right vomeronasal organ (VNO), green]. (A–C) *R. arenarum* (stage G32) showing the injection sites in the OE (A) and VNO (B) and the corresponding projection sites in the anterior brain (the MOB and the AOB, respectively) (C). (D) Anterior brain of *H. pulchellus* (stage G30). (E) Anterior brain of *X. laevis* (stage N/F54). (F-H) Double labelling using 680DA (red) and synaptophysin-like immunodetection (green). (F) Horizontal sections of the anterior brain (right side) of *R. arenarum* at G35. G and H show high-magnification views of the boxed area in (F). The same three glomeruli are identified by circular dotted lines in G (synaptophysin-like immunoreactivity alone) and H (double labelling image). PC, principal chamber; MOB, main olfactory bulb; V, ventricle. Dotted line delimits the basal lamina in (A and B). Top is anterior in all panels, left is lateral in (A, F, and G) and medial in (B). Scale bar, 100 μm.

with morphological and biochemical characteristics similar to those observed in adult anurans and other tetrapods. To the best of our knowledge, this is the first comparative study of the VNS in anuran larvae that integrates morphological and biochemical data. The general anatomical organisation of the VNS (summarised in Fig. 5) appears to be highly conserved among anuran tadpoles. It is represented by the VNO (bean-shaped structure), located in the medial nasal wall anteroventral to the OE, and the AOB, ventrolaterally positioned in the anterior telencephalon, somewhat caudal to the MOB. Interestingly, tadpoles of the basal anuran *Ascaphus truei* do not show this generalised organisation in the VNS (Benzekri & Reiss, 2011). In that species, the VNO has a completely different anatomical position; it is located in the ventrolateral side of the mid-nasal region. *Ascaphus truei* belong to the anuran family Leiopelmatidae, the most basal of extant anurans (Frost et al., 2006). They have various specific characteristics in the nasal organs that have not been observed in other anurans (Benzekri & Reiss, 2011).

Development of the VNO occurs very early in *H. pulchellus* and *R. arenarum*, during the final embryonic stages, meaning that the VNO, functional or not, is present during the entire larval phase. The early appearance of the VNO during development is a widespread characteristic of most tadpoles described to date (Cooper, 1943; Tsui, 1946; Nieuwkoop & Faber, 1994; Taniguchi et al., 1996; Jermakowicz et al., 2004; Wang et al., 2008; Jungblut et al., 2011). Moreover, the early appearance of the VNO during development seems to be a shared characteristic with other vertebrates, such as mammals (Garrosa et al., 1998) and reptiles (Holtzman & Halpern, 1990). Apparently, tadpoles of the amphibians *Bufo americanus* and *Bufo regularis* would be exceptional cases, in which the VNO appears during mid-larval stages, at G34 and G30–32, respectively (Khalil, 1978; Jermakowicz et al., 2004).

Robust evidence of the early appearance and maturation of the VNO in tadpoles was provided by Hansen et al. (1998), who performed an ontogenetic ultrastructural analysis of *X. laevis* olfactory organs. They found that differentiated neurones are present in the VNO as early as stage N/F42. Moreover, whereas neurones of the principal chamber dramatically change at metamorphosis (changing from water-sensitive to air-sensitive epithelium), the ultrastructural characteristics of the larval VNO remain in the adult (Hansen et al., 1998). This result strongly suggests that significant maturation of the VNO is achieved during the larval period in *X. laevis.*

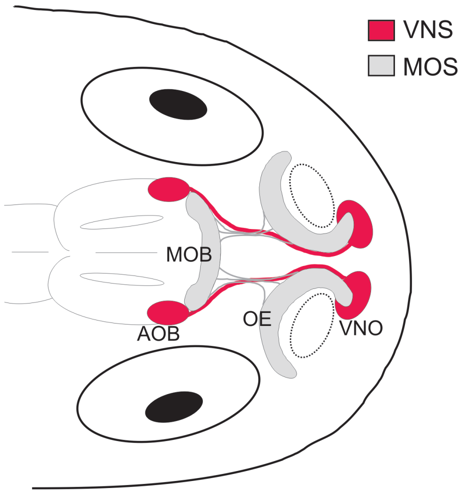

Fig. 5 Schematic drawing representing the anatomical organisation of the vomeronasal system (VNS) in a generalised tadpole. The main olfactory system (MOS) is also shown. AOB, accessory olfactory bulb; OE, olfactory epithelium; MOB, main olfactory bulb; VNO, vomeronasal organ. Dotted-line circles indicate the position of the external nares.

A particularly interesting finding of the present work is the occurrence of the second messenger protein Gαo in the VNO of tadpoles, starting at early stages of development. Moreover, shortly after organogenesis of the VNO, Gαo-expressing cells acquire the typical bipolar morphology of chemical sensory neurones (olfactory and vomeronasal). These data indicate that a significant maturation of the transduction system occurs in larval vomeronasal sensory neurones.

Gαo-expressing neurones have been described in the VNO of mammals (Jia & Halpern, 1996; Halpern & Martinez-Marcos, 2003), reptiles (Luo et al., 1994; Murphy et al., 2001) and adult amphibians (Hagino-Yamagishi et al., 2004; Date-Ito et al., 2008; Hagino-Yamagishi & Nakazawa, 2011). Moreover, Gαo protein expression was described previously in the VNO of tadpoles of *X. laevis* and *R. arenarum* (Hagino-Yamagishi et al., 2004; Jungblut et al., 2009). Gαo-expressing neurones have even been found in the lungfish *P. dolloi,* in structures identified as 'vomeronasal-like' (Gonzalez et al., 2011).

G proteins play an important role in signal transduction in chemosensory neurones (Jia & Halpern, 1996) and different G protein transduction systems are coupled to different receptor families (Halpern & Martinez-Marcos, 2003). In the mammal VNO, two large and divergent families of receptors have been identified: vomeronasal receptor type I (VR1) and vomeronasal receptor type II (VR2), which encode G protein-coupled seven-transmembrane proteins (Matsunami & Buck, 1997; Ryba & Tirindelli, 1997). Neurones that express either V1R or V2R also coexpress Gαi2 or Gαo, respectively. V1R and Gαi2 coexpressing neurones have been described only in the VNO of mammals (Jia & Halpern, 1996; Halpern et al., 1998), whereas V2R and Gαo coexpressing neurones are a common feature in the VNO of mammal and non-mammal tetrapods (Hagino-Yamagishi et al., 2004; Date-Ito et al., 2008). Our findings suggest that V2R receptors (Gαo-expressing cells) are a common characteristic in the VNO of anuran larvae. This hypothesis has been corroborated in *X. laevis* tadpoles (Hagino-Yamagishi et al., 2004).

Tract-tracing experiments demonstrated that chemosensory neurones from the VNO reach specific areas in the telencephalon, in which there is a discernible AOB. Positioning of the AOB in the anterior brain was highly conserved among the anuran tadpoles included in our study. Moreover, this anatomical organisation is maintained in adult anurans (Meyer et al., 1996).

In the present study, we demonstrated the presence of glomerular structures in the AOB of tadpoles. Glomeruli are formed by synaptic contacts between axons of the chemosensory neurones and dendrites of the mitral/tufted cells. They represent the first relay station in the olfactory pathway and are suggested to represent functional units in olfactory information processing. In *X. laevis* tadpoles, specific glomeruli are activated in the MOB when the OE is exposed to amino acids, whereas only spontaneous activity has been reported in glomeruli of the AOB (Manzini & Schild, 2010). Interestingly, structural analysis of the olfactory bulb in premetamorphic tadpoles of *X. laevis* showed that minor differences exist between glomeruli in the AOB and MOB. Moreover, the total number of glomeruli in the AOB is higher than in the MOB (approximately 350 and 234, respectively) (Manzini & Schild, 2010). In our opinion, glomerular activity in the AOB has not been characterised in tadpoles because nothing is known about the natural odorants/pheromones that stimulate it, rather than because of physiological immaturity of the sensory system.

In conclusion, our work demonstrates that an accessory olfactory system that is anatomically fully developed is present in anuran larvae. Moreover, our results indicate that a significant degree of maturation is achieved in the larval VNS, which suggests that it could be a functional sensory system in tadpoles. Recent molecular data strongly suggest that the VNS appeared early in vertebrate evolution (Grus & Zhang, 2009). Moreover, the presence of a VNS in lungfish indicates that this sensory system was already present in the aquatic last common ancestor of lungfish and tetrapods (Gonzalez et al., 2011). Within this context, tadpoles could be helpful models with which to investigate the anatomical, biochemical and functional aspects of the VNS in an aquatic environment.

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Appendix 1

Specimens examined and collection data

JF1598: *Scinax acuminatus* from Laguna Yema, Formosa, Argentina. JF326: *Hypsiboas curupi* from Campo Experimental INTA 'Cuartel Rio Victoria', San Vicente, Departamento Guarani, Misiones, Argentina. JF1765: *Phylomedusa azurea* from Resistencia, Chaco, Argentina. JF220: *Limnomedusa macroglossa* from Misiones, Argentina. JF1153: *Crossodactylus schmidti* from Campo Experimental INTA 'Cuartel Rio Victoria', San Vicente, Departamento Guarani, Misiones, Argentina. JF1622: *Physalaemus* sp. from Laguna Yema, Formosa, Argentina. Not classified: *Dermatonotus muelleri* from Laguna Yema, Formosa, Argentina. JF1498: *Lepidobatrachus llanensis* from Laguna Yema, Formosa, Argentina. Not classified: *Leptodactylus latrans*, locality unknown.