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Goα Expression in the Vomeronasal Organ and Olfactory Bulb of the Tammar Wallaby

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Abstract

The vomeronasal organ (VNO) detects pheromones via 2 large families of receptors: vomeronasal receptor 1, associated with the protein Giα2, and vomeronasal receptor 2, associated with Goα. We investigated the distribution of Goα in the developing and adult VNO and adult olfactory bulb of a marsupial, the tammar wallaby. Some cells expressed Goα as early as day 5 postpartum, but by day 30, Goα expressing cells were distributed throughout the receptor epithelium of the VNO. In the adult tammar, Goα appeared to be expressed in sensory neurons whose nuclei were mostly basally located in the vomeronasal receptor epithelium. Goα expressing vomeronasal receptor cells led to all areas of the accessory olfactory bulb (AOB). The lack of regionally restricted projection of the vomeronasal receptor cell type 2 in the tammar was similar to the uniform type, with the crucial difference that the uniform type only shows expression of Giα2 and no expression of Goα. The observed Goα staining pattern suggests that the tammar may have a third accessory olfactory type that could be intermediate to the segregated and uniform types already described.

Key words: Goα-protein, marsupials, pheromone, vomeronasal receptors

Introduction

The vomeronasal organ (VNO) is a paired tubular organ that is found in the nasal cavity of most tetrapods (Halpern 1987; Halpern and Martinez-Marcos 2003). In mammals, it lies in the tissue on either side of the nasal septum, stretching along its sides toward the back of the nasal cavity. The mammalian VNO detects pheromones or pheromone blends (Johnston 2000) and some general odors (Sam et al. 2001; Trinh and Storm 2003). The lumen of the VNO is lined with 2 types of epithelium: the medial receptor epithelium and the lateral nonreceptor epithelium (Takami 2002). Receptor cells are only found in the receptor epithelium of the VNO and are absent from the nonreceptor epithelium. In a functional VNO, these receptor cells connect to the olfactory bulb in the area of the accessory olfactory bulb (AOB). Two families of vomeronasal receptors (VNRs)—VN1Rs and VN2Rs—are specific to the VNO and the number of family members varies greatly between species (e.g., Bjarnadóttir et al. 2005; Grus et al. 2005; Shi and Zhang 2007; Young and Trask 2007), making comparative studies difficult. Not all mammals have intact receptors of both families. The dog and the cow, for example, only have intact VN1R genes and lack functional VN2R genes (Shi and Zhang 2007; Young and Trask 2007).

Each of the VNR families is coexpressed with a specific G-protein. VN1Rs are associated with the G-protein subunit Giα2, whereas VN2Rs are associated with Goα (Dulac and Torello 2003). VN1R and VN2R genes are expressed in different receptor cells that have their nuclei in distinct layers of the vomeronasal receptor epithelium. The nuclei of the VN1R-expressing cells are found in the medial layer of the receptor epithelium, whereas nuclei of the VN2R-expressing cells are found in a more basal layer (Pantages and Dulac 2000). In addition, the VN1R- and VN2R-expressing cells project to different areas in the AOB. In the mouse, the VN1R-expressing cells project to the rostral parts of the vomeronasal nerve cell layer and glomerular layer of the AOB, whereas the VN2R-expressing cells project to the caudal parts (Brennan and Keverne 2004). This arrangement of the

vomeronasal receptor nerves is found in rats, mice, guinea pig, degus, lesser tenrec, and the gray short-tailed opossum (*Monodelphis domestica*) (Shinohara, Asano, et al. 1992; Jia and Halpern 1996; Jia et al. 1997; Sugai et al. 1997; Takigami et al. 2004; Suárez and Mpodozis 2009; Suárez et al. 2009). In the lesser tenrec, this arrangement of VN1R- and VN2R-expressing cells is not as strictly separated with some glomeruli of both populations locate within the adjacent subdomain (Suárez et al. 2009). A different pattern is found in the goat, which shows Giα2 expression but not Goα expression throughout the vomeronasal nerve cell layer and glomerular layer of the AOB or in the VNO, and it has functional VN1Rs but no functional VN2Rs (Takigami et al. 2000). The same projection pattern to the AOB has been found in the dog, horse, musk shrew, and common marmoset (Takigami et al. 2004). Thus, 2 types of mammalian accessory olfactory systems have been described. One, the segregated type expresses both Goα and Giα2 in vomeronasal neurons projecting to distinct parts of the AOB. In the other, uniform type only Giα2 is expressed in the vomeronasal axons, and these axons terminate throughout the AOB.

As yet, the distribution of Goα and Giα2 has been studied in the VNO of only one marsupial, the gray short-tailed opossum (Halpern et al. 1995), although some Goα-positive cells have been identified in the receptor epithelium of the neonatal tammar wallaby (*Macropus eugenii*) (Schneider et al. 2009). To better understand the evolution of the accessory olfactory system, it is important to investigate the projection pattern of the vomeronasal receptor cells in as many different mammalian species as possible. We therefore examined the distribution of Goα in the adult VNO, AOB, main olfactory bulb (MOB), and in the developing VNO of a marsupial, the tammar wallaby.

Materials and methods

Animals

Tammar wallabies from our breeding colony were killed by an overdose of sodium pentobarbitone or by cervical dislocation after stunning. Pouch young younger than 40 days were cooled then decapitated. Swiss mice (*Mus musculus*) were obtained from the animal breeding facility at the Department of Zoology, University of Melbourne. Care and treatment of animals conformed to the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council 2004). All experimental procedures were approved by The University of Melbourne Animal Experimentation Ethics Committees.

Immunoblotting

Protein for immunoblotting was extracted from snap-frozen mouse VNO and forebrain as well as tammar VNO and olfactory bulb using a protease inhibitor cocktail (Calbiochem; #535141) in RIPA buffer (50 mM Tris base, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM ethylenediaminetetraacetic acid, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin, 1 mM activated sodium orthovanadate, and 200 mM NaF). The protein concentration was determined by the Ponceau S method as described in Thompson Hayner et al. (1982) with a spectrophotometer (NanoDrop ND-1000) using bovine serum albumin (BSA) standards. Fifty microgram of each mouse forebrain and tammar olfactory bulb and 100 μg of tammar VNO protein were diluted in 1.25% β–mercaptoethanol in Laemmli sample buffer (250 mM Tris base; 40% Glycerol; 5% sodium dodecyl sulphate, 0.005% Bromphenol Blue) and boiled. The protein samples and 5 μL of Precision protein standards (Precision Plus Protein Dual Color Standard; Bio-Rad; #161-0374) were electrophoresed by standard 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis for 50 min at 150 V followed by transfer to a Hybond-P membrane (Amersham). The membrane was blocked overnight in 5% skim milk in Tris-base buffer containing 0.05% Tween-20 (TTBS) at 4 °C. The membrane was then incubated with the primary antibody anti-Goα (rabbit polyclonal antibody against bovine Goα; Upstate, USA; #07-634) for 1 h at 1/1400 dilution at room temperature. The membrane was incubated with the secondary antibody with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1/20 000; #SC-2004) and developed by chemiluminescence (ECL Western Blotting Detection Reagent; Amersham Biosciences; #RPN2106).

Tissues

VNO tissue from adult female ($n = 3$) and male tammar wallabies ($n = 3$) and 5 Swiss mice were collected. Olfactory bulbs from 4 adult female tammars and 1 female Swiss mouse were also used in this study. Whole nose samples were collected after birth from day 5, 10, 15, and 30 (each $n = 3$) pouch young. All adult tissues were fixed for at least 2 weeks and then overnight under vacuum in 10% neutral buffered formalin. The VNO tissue (except one tammar VNO that was collected without the cartilage as a control for the possible effect of decalcifier solution on staining) was then decalcified in Rapid Decalcifier solution (RDO, Grale Scientific) for 3 days under vacuum. Pouch young tissue was fixed in 4% paraformaldehyde for 24 h, washed twice with phosphate buffered saline (PBS), and stored in 70% ethanol. Samples of pouch young older than 5 days were decalcified in 10% formic acid for 2 days under vacuum after fixation. All samples were embedded in paraffin, and serial sections 7 μm thick were cut (Microm, Zeiss). Adult VNO was cut in coronal sections, whereas olfactory bulbs were cut in sagittal sections. The pouch young samples were cut in serial coronal sections. Sections were mounted on glass slides for staining. Some sections were mounted on polylysine-coated slides (Menzel-Gläser; #J2800AMNZ) for immunohistochemistry. Light micrographs of sections were taken with an Olympus

BX51 light microscope mounted with an Olympus DP70 camera. The length of the pouch young VNOs was determined by counting the coronal sections on which the VNO was visible (including sections that were not stained) and multiplying by the section thickness. The diameter of the VNO lumen was measured as its maximum from sections midway through the organ (Figure 1; Table 1). Light micrographs of the olfactory bulb were used to compare the anatomical structure of the MOB and AOB with that of other species in order to identify the different cell layers present in the tammar wallaby.

Immunohistochemistry

An antibody raised against bovine Goα (rabbit polyclonal antibody; #07-634) was used to label receptor cells expressing VRN2. Coronal sections of 5 adult tammar VNOs (from section plane B, area shortly caudal to the entrance of the VNO, as described in Schneider et al. 2008), sagittal sections of 4 adult tammar olfactory bulbs, and coronal sections of the middle of the VNO tube of each 3 pouch young aged 5, 10, 15, and 30 days were used for Goα immunohistochemistry. A tammar VNO that had not been decalcified and sections of mouse olfactory bulb and VNO were, respectively, used as controls for the effect of decalcification and as tissue controls for the antibody. All sections were dewaxed and rehydrated and transferred to PBS. The sections were then incubated with 5% hydrogen peroxide for 5 min. Each section was treated with 10% goat serum to block nonspecific binding of antibodies. The sections were incubated with primary antibody (1/200 for VNO and whole nose sections of pouch young, and 1/300 for olfactory bulbs [both including 2% goat serum]) in 0.1% BSA in PBS overnight at 4 °C. Control sections for Goα from each animal were incubated at 4 °C overnight with rabbit immunoglobulin G (rabbit IgG; Dako, # X0903) diluted in 0.1% BSA in PBS and 2% goat serum. The control IgG was applied at the same dilution as the experimental antibody. A second section was incubated at 4 °C overnight with 0.1% BSA in PBS plus 2% goat serum as negative controls. All sections were incubated with secondary antibody (biotinylated goat anti-rabbit IgG [Dako, #E0432] diluted 1/500) for 30 min. Signals were amplified with Strept ABComplex/HRP kit (Dako, #K0377) and visualized with diaminobenzidine (Dako, #S3000) in 0.03% H_2O_2. Harris' hematoxylin was used as a counter stain.

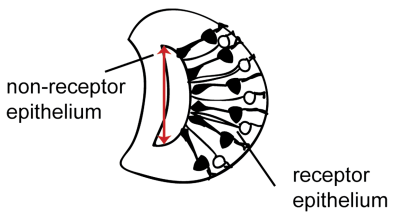

Figure 1 Measurements of the diameter of the vomeronasal lumen. The graphic shows a coronal section through the VNO. The arrow shows how measurements of the diameter of the VNO lumen were taken.

Results

Expression of Goα in the tammar wallaby was investigated using a polyclonal antibody raised against bovine Goα. There was a single band of around 39 kDa for Goα in the immunoblot of protein extracts from mouse and tammar tissue, confirming specificity of this antibody (Figure 2).

Goα expression in the VNO

There was Goα protein detected in the cytoplasm of receptor cells in the receptor epithelium of the VNO from adults as well as pouch young (Figure 3A,B and 4). The nuclei of these cells were located at the base of the epithelium (Figure 3B). This localization was especially pronounced in the adult VNO (Figure 3A,B). There was very strong staining in the knob-like structures that protrude into the VNO lumen from the receptor cell endings (e.g., marked with arrows in Figure 3B). Although staining was observed in cells throughout the receptor epithelium in the adult VNO, only

Table 1 Length of the VNO, diameter of the VNO lumen, and head length of tammar pouch young of different ages

Age (pp)	Length of VNO ± SD (μm)	Diameter of lumen ± SD (μm)	Head length ± SD[a] (mm)
Day 5	1064 ± 225	143 ± 32	9.2 ± 0.28
Day 10	1317 ± 136	209 ± 43	11 ± 0.01
Day 15	1692 ± 154	248 ± 63	12.9 ± 0.09
Day 30	2514 ± 139	387 ± 16	18.2 ± 0.04

SD, standard deviation.
[a]Measured in the live animal.

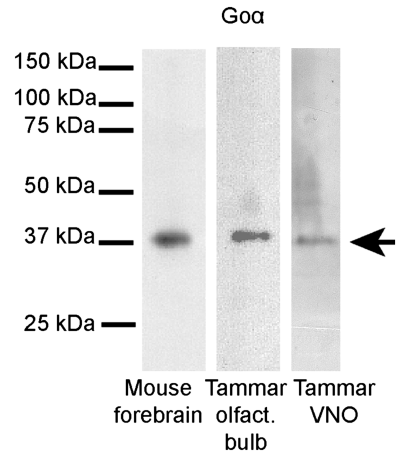

Figure 2 Immunoblot with Goα-antibody. Immunoblots for Goα show a single band at around 39 kDa for mouse forebrain as well as tammar olfactory bulb and VNO.

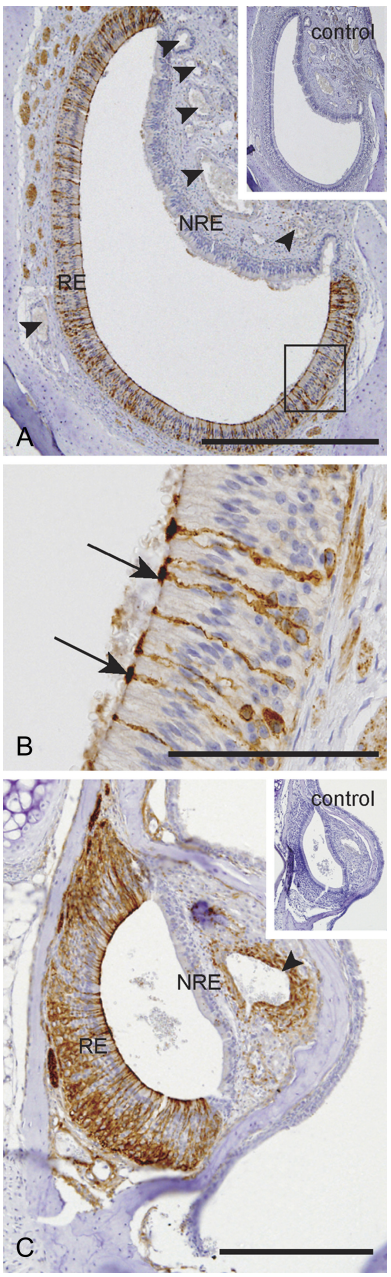

Figure 3 Immunostaining for Goα of adult tammar and mouse VNO. (**A**) Goα-positive cells are also found in the tammar's receptor epithelium. The cell nuclei of the Goα-positive cells are mainly situated in the basal part of the epithelium. Nerve bundles stretching along side the VNO are also Goα-positive (all other not epithelium staining; blood vessels are marked by arrow heads). (Box marks area of close-up shown in **B**) No staining was seen in the IgG control (right hand corner) and the no primary antibody control (not shown). (B) Goα-positive receptor cells are found all throughout the tammar's receptor epithelium of the VNO. Arrows mark the knob-like ending of the receptor cells, which are strongly Goα-positive. Some stained nerve bundles are visible underlining the receptor epithelium. (**C**) In the mouse, tissue control staining for Goα is found in cells mainly situated in the basal part of the VNO epithelium, being especially strong at the receptor cell endings at the epithelium surface. Nerve bundles (all other not epithelium staining) and cells surrounding the blood vessel (marked by an arrow head) are also stained. No staining was seen in the IgG control (right hand corner) and the no primary antibody control (not shown) (RE—receptor epithelium; NRE—nonreceptor epithelium; scales for A 500 μm, B 100 μm, and for C 200 μm; immunostaining in black (brown) with hematoxylin counterstaining).

a few cells were stained in the day 5 postpartum (pp) pouch young VNO (Figure 4A). The proportion of immunoreactive to nonimmunoreactive cells in the receptor epithelium increased in the day 10 pp pouch young. These cells were mainly situated near the boundary between the receptor epithelium and the nonreceptor epithelium (Figure 4B), where the receptor epithelium height was maximal. A further rise in immunoreactive cells in these areas and a further increase in epithelial height was seen in the day 15 pp pouch young (Figure 4C). In contrast to the earlier stages (day 5 and day 10), however, there were some stained cells in the receptor epithelium that lines the medial part of the VNO lumen. The height of the epithelium in the medial part had also increased but did not reach that of the receptor epithelium near its boundary with the nonreceptor epithelium. By day 30 pp, the receptor epithelium was of uniform height, and stained cells were evenly distributed throughout (Figure 4D). The proportion of stained to nonstained cells in the receptor epithelium increased toward day 30 pp, as did the length of the VNO and the diameter of the VNO lumen (Figure 1; Table 1). Large nerve bundles underlying the receptor epithelium, especially at the ventral and dorsal sides of the lumen, also stained darkly. There was no staining in negative controls for Goα using IgG or no primary antibody (Figure 3A). The pattern of staining was not dependent on decalcification of the samples. Control tissues from the mouse showed similar staining of cells (Figure 3C), as found previously (Pantages and Dulac 2000).

The developing VNO differed from the adult VNO in the shape of its lumen, which was crescent-shaped in the adult but oval-shaped in the pouch young stages investigated. Another notable feature was the pronounced difference in height between the receptor and the nonreceptor epithelia in the VNO of pouch young: with a higher stratified columnar receptor epithelium and a simple columnar nonreceptor epithelium. In the adult, both epithelia were stratified columnar, with the receptor epithelium only slightly higher.

Goα expression in the olfactory bulb

The tammar wallaby olfactory bulb could be divided into the MOB that comprises most of the bulb and the AOB that was found on its caudal medial surface. The tammar AOB size was around a tenth of the MOB (Figure 5). The MOB showed the following different layers from external to internal: olfactory nerve cell layer, glomerular layer, external plexiform layer, mitral cell layer, granule cell layer (Figure 5A). The 3 layers of the AOB from external to internal were: vomeronasal nerve cell layer, glomerular layer, and a mitral tufted cell layer and granule layer which could not be separated into 2 as

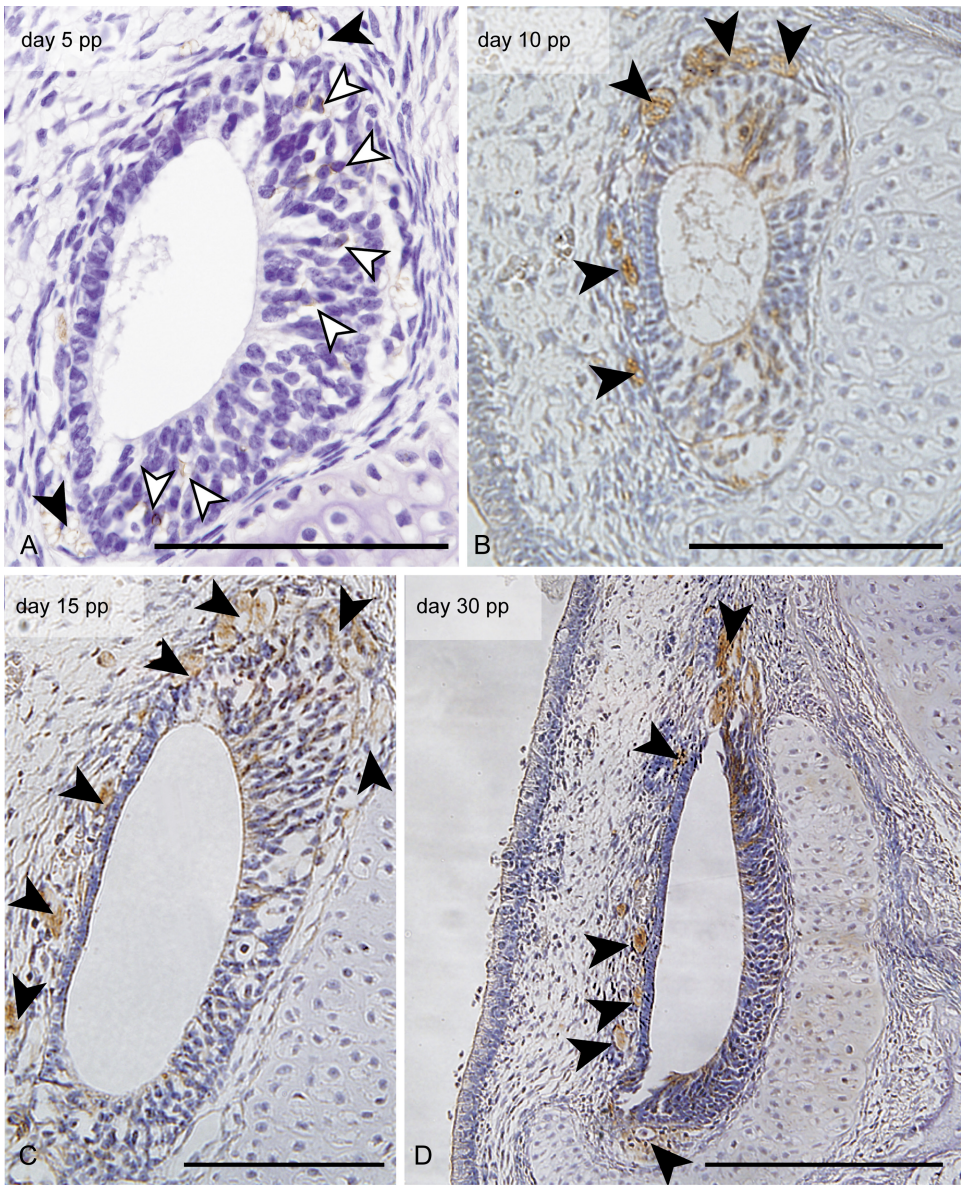

Figure 4 Immunostaining for Goα of the developing tammar VNO. (**A**) At day 5 pp, only a few cells in the receptor epithelium of the VNO were Goα-positive (marked by white arrow heads). The number of cells increased with the age and the size of the VNO (**B**—day 10 pp; **C**—day 15 pp; **D**—day 30 pp). Labeled cells are mainly situated near the boundary between receptor and nonreceptor epithelia. Nerve bundles that run along the sides of the VNO are also stained (black arrow heads) (Scales for A to C are 100 μm and for D is 200 μm; immunostaining in dark (brown) with hematoxylin counterstaining).

in other species (e.g., guinea pigs; Takigami et al. 2004; Figure 5B). To identify the projection of the receptor cells from the VNO to the AOB, we also performed immunostaining for Goα-protein of the AOB and as a control for immunostaining specificity of the MOB. All layers were intensely stained for Goα in the MOB (Figure 6A). The darkest staining was found in the olfactory nerve cell layer and the glomerular layer. Mitral cells did not stain for Goα. The dark staining right at the border of the olfactory bulb tissue may result from artefactual staining caused by the ruptured olfactory nerve cells (Figure 6A,B). In the control tissue of the mouse, the staining resembled that found in the tammar and the literature (Wekesa and Anholt 1999; Figure 6B). The controls for the antibody showed no staining (Figure 6C,D).

Goα was detected in the tammar AOB in some areas of the vomeronasal nerve cell layer and the glomerular layer and all throughout the third layer which comprises the mitral tufted and granule cell layer (Figure 6E; Supplementary Figure S1). The staining in the vomeronasal nerve cell layer and the glomerular layer was not restricted to the posterior part of the AOB as seen in the mouse control tissue (Figure 6F), as found previously (Jia and Halpern 1996). However, the

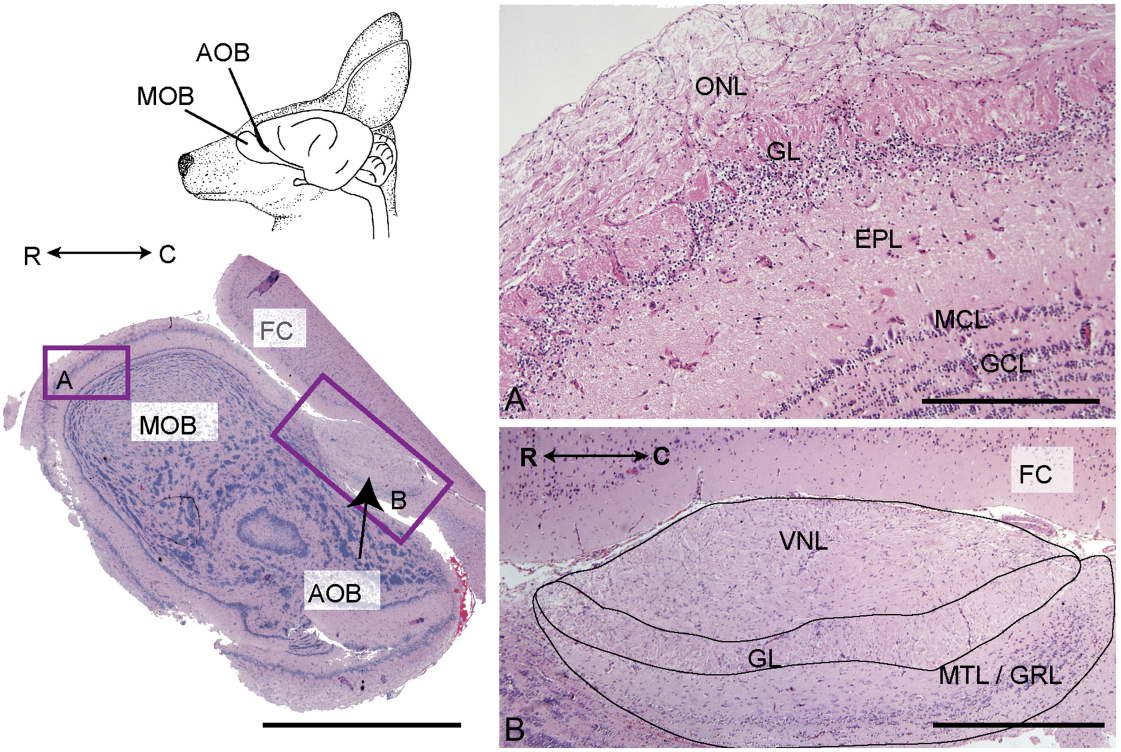

Figure 5 Structures of the tammar olfactory bulb. The olfactory bulb can be divided in to MOB that most of the bulb consists of and the AOB that is found on its caudal medial surface (see overview on the left side). All boundaries of layers were based on detailed anatomical analysis of the structures visible. (**A**) shows a close-up of the different layers of the MOB. The first layer consists of the olfactory nerve cell layer (ONL) under which the glomerular layer (GL) lies. This layer is followed by the external plexiform layer (EPL), the mitral cell layer (MCL), and the granule cell layer (GCL) (scale 500 μm). (**B**) The AOB can only be divided in 3 layers. The vomeronasal nerve cell layer (VNL), the GL, and a third layer which could not be distinguished into mitral tufted cell layer and granulus layer (MTL/GRL) (scale 1 mm) (All sections stained with H. & E.; R—rostral, C—caudal).

staining of the vomeronasal nerve cell layer was restricted to only few cells of which distribution varied between the sections of different individuals. No staining was found in negative controls using IgG or no primary antibody on olfactory bulb tissue (Figure 6G,H).

Discussion

The vomeronasal system of the tammar was typically mammalian because Goα-positive receptor cells in the adult and developing tammar were detected in the VNO and all parts of the AOB. However, the projection pattern of the receptors suggests that the tammar may represent a different type of mammalian accessory olfactory system, intermediate to the segregated and uniform types previously described (Figure 7). Although Goα staining was specific, high background staining made interpretation of Giα2 staining not possible (Schneider NY, unpublished data). However, the tammar vomeronasal 1 receptor family has functional VN1R genes (Young et al. 2010), suggesting that Giα2 expressing receptor cells may also project to the AOB. Giα1, 2, and 3 are highly conserved, and all have high sequence identity (ENSMEUT00000005451, ENSMEUT 00000011250, ENSMEUT00000009117; see supplement for sequences and alignment Supplementary Figure S2 and S3) making the design of a specific antibodies or in situ hybridization probes to Giα2 difficult. Using the expression of another protein such as Ocam or Pde4a as observed in mice, VN1R cells (Yoshihara et al. 1997; Lau and Cherry 2000) would not clarify the tammar situation because it is not known whether these proteins are coexpressed with Giα2 and VN1Rs in marsupials. In addition, earlier studies showed that coexpression of the olfactory marker protein and other VNO-specific proteins with any one of the G-proteins varies between species (Halpern et al. 1998). Therefore, the projecting pattern of VN1R-expressing cells and the number of VN2R genes still remains to be established in the tammar.

We detected Goα-positive receptor cells as early as day 5 pp in this study and even at birth in a complementary study (Schneider et al. 2009). This makes it likely that the VNO is already functional at this stage even though it may only be able to detect few molecules. In the gray short-tailed opossum during neurogenesis, cells from the basal lamina of the VNO receptor epithelium develop into receptor cells while moving vertically into the receptor cell layer (Jia and Halpern 1998). In the developing VNO of the tammar, receptor cells were mostly found near the boundary between the receptor and the nonreceptor epithelia. In the day 10 pp gray short-tailed opossum, receptor cells are present all throughout

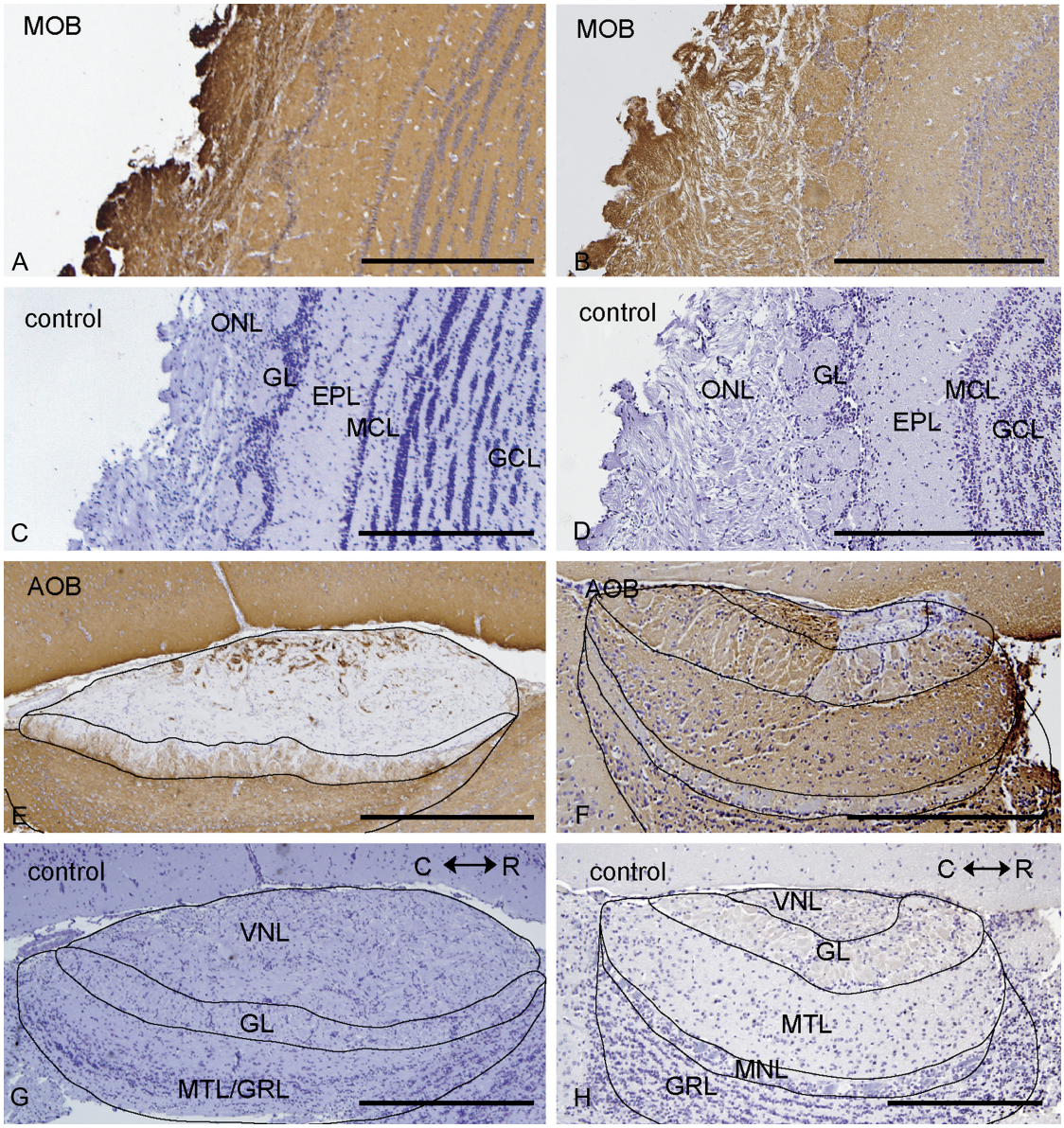

Figure 6 Immunostaining for Goα of adult tammar and mouse MOB and AOB. (**A**) Goα staining was strong all throughout the tammar MOB with the strongest staining occurring in the olfactory nerve cell layer (ONL) and the glomerular layer (GL). No staining is seen in the controls (**C** and **D**). (**B**) The mouse MOB stains strongly for Goα with the strongest staining occurring in the ONL and the GL as in the tammar. (**E**) Goα staining is not specific to certain areas of the vomeronasal nerve cell layer (VNL) of the tammar AOB. The GL was partly stained for Goα while the whole mitral tufted cell layer and granule cell layer (MTL/GRL) appeared immunopositive. (**F**) Goα staining is found in the caudal half of the VNL, the GL, and MTL, myelinated nerve layer (MNL) and GRL of the mouse AOB. No staining was found in the controls (**G** and **H**) (EPL—external plexiform layer, MCL—mitral cell layer, GCL—granule cell layer; A to D scales 500 μm and E to H scales 1 mm; immunostaining in black (brown) with hematoxylin counterstaining).

the receptor epithelium of the VNO, but there is a greater number of developing receptor cells near the boundary between the receptor and the nonreceptor epithelia (Jia et al. 1997; Jia and Halpern 1998). The higher numbers of developing receptor cells in this area are thought to be important for the growth of the receptor epithelium. The receptor epithelium of the gray short-tailed opossum is higher in the medial part of the receptor epithelium than at the boundary, as found in the adult opossum, whereas the receptor epithelium of the tammar pouch young was higher near the boundary between the 2 epithelia (Jia et al. 1997). This could explain why there were fewer receptor cells in the medial part of the receptor epithelium in the tammar wallaby up to the age of day 15 pp. At day 30 pp, the receptor epithelium reached uniform height over most of its length as in the adult, and labeled receptor cells could be detected throughout the epithelium. Although the shape of the lumen itself should not change the functionality, the overall size of the lumen as well as the increase of

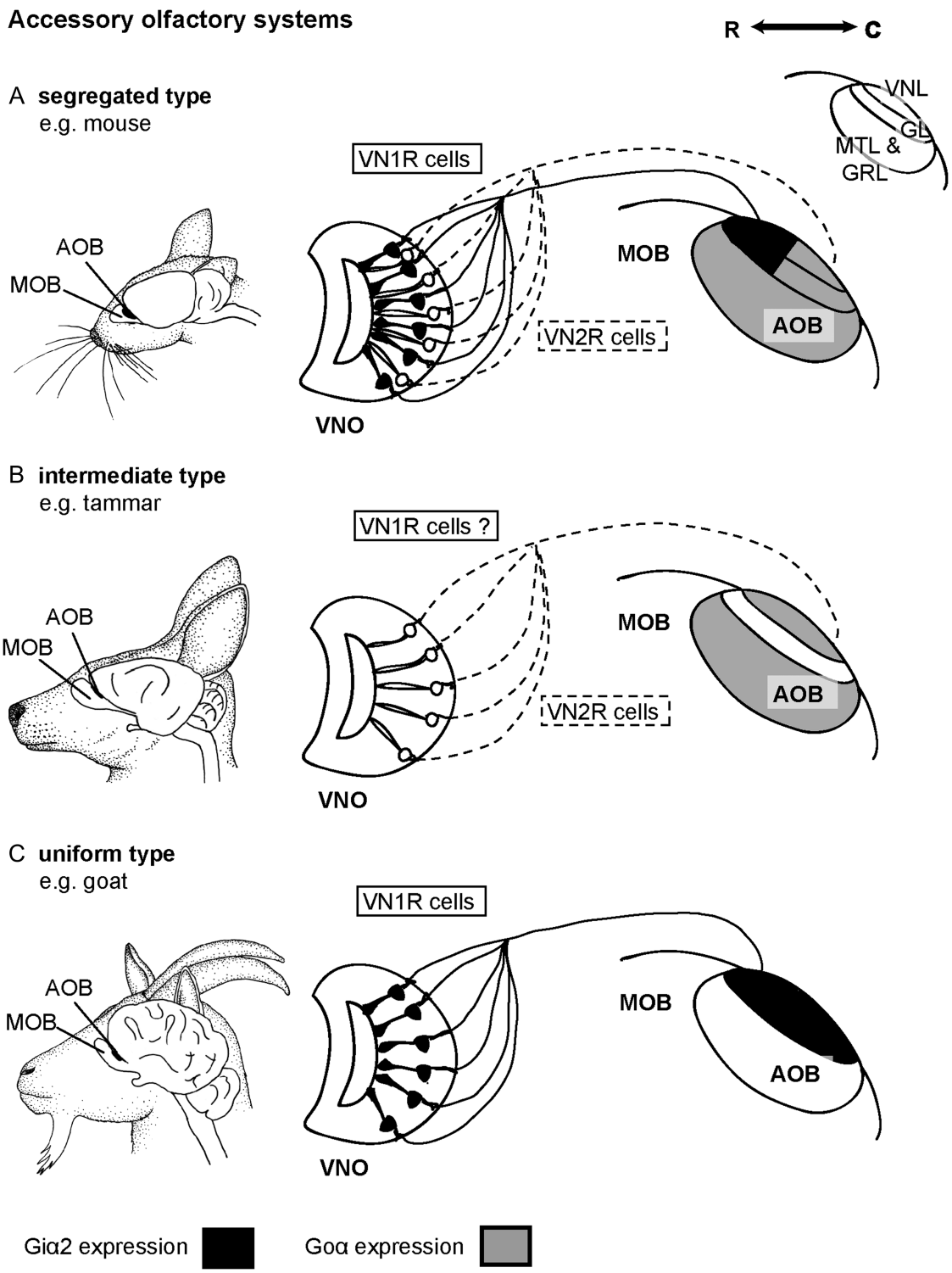

Figure 7 Model for 3 accessory olfactory systems. Two patterns of vomeronasal receptor projections (the segregated and the uniform type) were previously described in the vomeronasal organ of mammals, based on the differing expression patterns of the receptor genes in the VNO and the AOB of different eutherian species. This study suggests that the tammar wallaby may possess a third intermediate type. (**A**) The mouse (Takigami et al. 2004) has the segregated type in which receptor cells that produce Giα2-protein have their cell nuclei in a higher level of the receptor epithelium of the VNO than Goα-protein producing receptor cells. Although the cells expressing Giα2-protein connect to the rostral part of the AOB, the Goα-protein producing cells connect to the caudal part. (**B**) The tammar wallaby may possess an intermediate type in which the vomeronasal nerve cell layer (VNL) is Goα-positive without any restriction to a specific area as found in the segregated type. The Giα2-expression pattern remains to be established. (**C**) The goat (Takigami et al. 2000, 2004) with a uniform type has only Giα2-expressing cells in the VNO that project to both the rostral as well as the caudal AOB (VN1R and VN2R, vomeronasal receptors 1 and 2; GL, glomerular layer; MTL, mitral tufted cell layer; GRL, granule cell layer).

VNO length during growth will increase the surface area covered by receptor epithelium, and therefore, the number of receptor cells which are able to detect any signals.

The MOB of the adult tammar resembled in its structure that of other mammals (e.g., McCotter 1912; Stephan 1965; Salazar et al. 1994; Meisami and Bhatnagar 1998). The expression pattern of Goα-protein in the tammar MOB was similar to that described in rat and mice (Shinohara, Kato, et al. 1992; Wekesa and Anholt 1999). In tammar and rat, all layers were strongly immunopositive for Goα. Takigami et al. (2000) only found Goα expression in the olfactory nerve cell layer of the goat MOB and no Giα2 expression. The localization of Goα in the MOB shows that the species difference in expression pattern of Goα is specific to the AOB.

Goα was detected in cells with basally located nuclei in the VNO receptor epithelium of the tammar, as in other mammalian species studied to date. These Goα-positive receptor cells projected to all parts of the vomeronasal nerve cell layer of the AOB.

In comparison to the mouse and gray short-tailed opossum, the number of Goα-positive cells was quite low in the VNO of the tammar (Halpern et al. 1995; Jia and Halpern 1996). This could be due to a lower affinity of the antibody to tammar Goα or to a relatively lower number of VN2R cells in the tammar. The number of VN1R/Giα2-expressing cells may be higher than the number of VN2R/Goα-expressing cells and may reflect the number of different receptor genes of both receptor families in the tammar. The tammar wallaby has one of the largest mammalian olfactory receptor families containing up to 1500 olfactory receptor (OR) genes (Delbridge et al. 2010; Renfree et al. 2011). There are 89 VN1R genes in the tammar, but the number of VN2R genes in tammar is unknown (Young et al. 2010). In the gray short-tailed opossum, the number of predicted VN1R (98) and VN2R (86) family members are similar (Jia et al. 1997; Shi and Zhang 2007; Young and Trask 2007). In contrast, the number of VN2R genes have decreased in some mammals to zero (e.g., dog; Young and Trask 2007) compared with amphibian and fish (Grus et al. 2007; Shi and Zhang 2007). VN1Rs appear to bind small volatile chemicals (Emes et al. 2004), whereas VN2Rs bind to water-soluble molecules, such as the peptide ligands of MHC class I molecules (Leinders-Zufall et al. 2004) and exocrine gland peptides (Kimoto et al. 2005). The change of environment from semi- or exclusively aquatic, in which water-soluble pheromones are likely to play a greater role, to landliving, where small volatile pheromones will be of greater use, may have induced a shift toward a greater number of VN1R genes (Shi and Zhang 2007). It is interesting to note, however, that the platypus, an aquatic mammal, has the greatest number of VN1R genes (270) so far found in a mammal (Grus et al. 2007; Warren et al. 2008).

In contrast to the mouse and the gray short-tailed opossum (Halpern et al. 1995; Jia and Halpern 1996), Goα-positive receptor cells in the tammar projected to all parts of the AOB vomeronasal nerve cell layer and not only to the rostral area. So far, this pattern of Goα-positive receptor cells projecting to all parts of the AOB vomeronasal receptor layer has only been shown in the tammar. Although the projection pattern for Giα2 remains to be established, it is clear from genetic studies that functional receptor cells of the VN1R family do exist (Young et al. 2010). The pattern differs from the uniform type in which both types of receptor cells are found and from the segregated type (Figure 7). Only 3 cell layers could be distinguished in the tammar AOB, resembling the AOB of the dog, horse, and common marmoset (Salazar et al. 1994; Takigami et al. 2004). All these animals show a uniform projection pattern of the vomeronasal nerves to the AOB, so the number of layers of the AOB is not linked to the projection pattern. The guinea pig with a segregated projection pattern and the goat with a uniform projection pattern both have an AOB that has 5 cell layers (Meisami and Bhatnagar 1998; Takigami et al. 2000, 2004).

As in other mammals that show the uniform type, the tammar receptor epithelium height was relatively low (around 85–94 μm; Schneider et al. 2008). The goat with its uniform projection type has a receptor epithelium height of around 60–80 μm (Ichikawa et al. 1999), whereas the height is 150 μm in the gray short-tailed opossum and in rodents that show the segregated projection type it is up to 170 μm (Mendoza 1993; Poran 1998). The projection pattern therefore appears to be related to the overall grade of development of the accessory olfactory system. In the tammar, the lack of regionally restricted projection of the vomeronasal 2 receptor cells is novel and has so far only been observed for vomeronasal 1 receptor cells for the uniform type. The possible projection of both receptor cell types to the AOB in the tammar on the other hand resembles the segregated type in that both G-proteins are expressed. The vomeronasal receptor cell projection pattern of the tammar therefore appears to have similarities to both previously described types. We suggest the possibility that it may be a new intermediate type of mammalian accessory olfactory system.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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