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Morphological Evidence for Two Types of Mammalian Vomeronasal System

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Abstract

The vomeronasal (VN) systems of rodents and opossums are of the segregated type, i.e alpha-subtype G protein Gi2- or Go-expressing VN neurons, which are sensory cells, project discretely to the rostral or caudal region of the accessory olfactory bulb (AOB). Although this zone-specific projection is believed to be a common feature for processing pheromones in mammals, we previously found a uniform-type VN system in goat in which only Gi2-expressing VN axons terminate at the AOB. In most mammals, it remains unclear whether their VN systems are of the segregated or uniform type. Therefore, we investigated morphologically the VN systems of different mammalian species (dog, horse, musk shrew and common marmoset). Consequently, all VN axons of the examined animals were positively stained with immunohistochemistry for Gi2 in the same way as that in the goat. On the other hand, we observed immunoreactivities against Go in the olfactory axons, but not in the VN axons. These results suggest that many mammals have uniform-type VN systems, and at least two types of VN systems exist in terrestrial mammals. This morphological evidence will help us determine the processing function of VN systems.

Key words: accessory olfactory bulb, G proteins, olfaction, pheromone, vomeronasal organ

Introduction

It was first reported that a family of genes encoding candidate pheromone receptors is expressed in rat VN neurons by Dulac and Axel (1995). These receptors (V1Rs) consist of seven transmembrane receptors (Dulac and Axel, 1995) and are expressed in microvilli and the knoblike structure exposed in the VN lumen (Takigami *et al.*, 1999). Subsequently, another type of pheromone receptor (V2R) was reported (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). V1R-expressing VN neurons are distributed in the apical layer of the VN sensory epithelium (VSE) where Gi2-expressing VN neurons (Gi2-VN neurons) are located and V2Rs are distributed in the basal layer where the Go-VN neurons are located. Therefore, V1Rs were thought to be co-expressed in Gi2-VN neurons and V2Rs in Go-VN neurons (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Two populations of VN neurons are known to project their axons to segregated (rostral or caudal) regions of the AOB (Shinohara *et al.*, 1992a; Jia and Halpern, 1996; Halpern *et al.*, 1998). Furthermore, the two neural pathways, namely the rostral and caudal VN pathways, are independent of each other (Berghard *et al.*, 1996; Sugai *et al.*, 1997; Kumar *et al.*, 1999; Halem *et al.*, 2001). This segregated projection of VN neuron has been reported to play a critical role in pheromonal information processing (Brennan *et al.*, 1999; Inamura *et al.*, 1999; Krieger *et al.*, 1999; Leinders-Zufall *et al.*, 2000; Fieni *et al.*, 2003). Although this segregated projection was until now believed to be a common feature in mammals, recently we have shown that the goat VN system has a pattern different from that of the rodent VN system (Takigami *et al.*, 2000). In the goat VN system, only Gi2-VN neurons project throughout the glomeruli of the AOB (Takigami *et al.*, 2000). Immunoreactivity (iR) for Go was not observed on the surface of VSE or in the VN axons. Thus, in that report, we suggested that the segregated

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projection is not a common feature of the mammalian VN system.

The pheromonal influences on endocrine regulation via the VN system are expected to enable synthetic pheromones to control the mammalian endocrine system. However, there are few studies on the VN systems of mammals other than experimental animals such as the mouse, rat, rabbit and opossum. Therefore, it is necessary to determine whether most mammals have the same type of VN system as that of rodents. The aim of the present study was to examine AOBs in widely divergent mammalian species.

Materials and methods

All experiments were conducted in accordance with the *Guidelines for the Care and Use of Animals* (Tokyo Metropolitan Institute for Neuroscience, 2000). Five mammalian species, namely guinea pig (Brown Takei, 650–700 g, six males; Seasco), beagle, (five males >1 year old; Veterinary Pharmacological Laboratory, Tokyo University of Agriculture and Technology), horse (thoroughbred, two 2-year-old females, one 13-year-old female; Japan Racing Association Equine Research Institute), musk shrew (five 8-week-old females, five 5-week-old males; Clea Japan Inc.) and common marmoset (3-year-old and 12-year-old females, one 16-year-old male, Central Institute for Experimental Animals) were used in the present study. Dogs are classified under the order Carnivora, and are known to have a keen sense of hearing and smell. Musk shrews are primitive animals that are classified under the order Insectivora. Horses are classified under the order Perissodactyla. In this study, we examined a strain of thoroughbred whose family has good running potential and is known to perform flehmen, which was originally proposed to facilitate pheromone testing (Ladewig and Hart, 1980; Melese-d'Hospital and Hart, 1985). Common marmosets are classified under the order Primates. They are known to have specialized glands and exude characteristic odor.

Western blot analysis

In all of the examined animals, the unfixed olfactory bulbs consisting of the AOB were removed immediately after killing and stored at –80°C until analysis. These olfactory bulbs were then cut into pieces in phosphate-buffered saline (PBS) containing several protease inhibitors and homogenized by sonication. All homogenates of the olfactory bulbs were denatured in sodium dodecyl sulfate under reducing conditions at 95°C and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were blotted onto nitrocellulose membranes and incubated overnight with BlockAce for blocking at 4°C. The blots were incubated with the primary antibody—Gi2 diluted 1:1000 (Wako), or Go diluted to 0.25 μg/ml (MBL)—for 1 h at room temperature. After washing in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20), they were incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG (1:5000 dilution) for 1 h at room temperature. Bound antibodies were visualized by employing a Western-blue-stabilized substrate (Promega).

Preparation of AOB and VNO sections

The guinea pigs, musk shrews and common marmosets were deeply anesthetized with sodium pentobarbital. They were then killed by transcardial perfusion with 0.9% saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The fixed olfactory bulbs, which contain the AOB, were removed and immersed in 4% PFA overnight. After the horses and dogs were killed, their brains and VN organs (VNO) were removed without perfusion and immersed in 4% PFA. Then they were placed in 30% sucrose/PBS solution for cryoprotection. AOBs were cut into 50 μm sagittal sections (horizontal sections in the guinea pigs and dogs) using a freezing microtome. These sections were suspended in 0.1 M PBS.

VNOs were removed from the fixed tissues of all the animals and immersed in decalcifying solution B (Wako). They were then immersed in 30% sucrose/PBS solution for cryoprotection. The sections were cut into 20 μm coronal cryosections using a Cryostat (Jung CM3000; Leica) and mounted on gelatin-coated slides. After drying overnight, they were stained with hematoxylin and eosin or Nissl's stain and observed using an Axiophot (Zeiss).

Immunohistochemistry for Gi2 or Go

The AOB sections were incubated in a blocking solution containing 10% BlockAce (Dainippon Pharmaceutical), 3% goat serum and 0.05% Triton X-100 in PBS for 1 h. The sections were then incubated for 3 days with antibodies against Gi2 (diluted to 1:800; Wako) or against Go (diluted to 1 μg/ml; MBL) in PBS containing 10% BlockAce and 0.05% Triton X-100 at 4°C. After washing in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector) in PBS with 10% BlockAce for 2 h at room temperature. After washing with PBS again, the sections were incubated with streptavidin–fluorescein (Amersham) in PBS for 1 h and then rinsed four times with PBS prior to observation under a fluorescence microscope (Axioplan 2; Zeiss).

Results

Cross-reactivity of anti-Gi2 or anti-Go antibody among the examined animals

In the guinea pig, the AOB has a five-layer structure and a segregated-type VN system similar to that in rodents (Figure 1A). The distinct five-layer structure consists of a VN nerve (VNL), glomerulus (GL), mitral/tufted cell (MTL), myelinated nerve (MNL) and granule cell layer (GRL). By immunohistochemistry, Gi2-iR was found in a VN axon projecting to the rostral region of the AOB (Figure 1B) and Go-iR in a VN axon projecting to the caudal region (Figure

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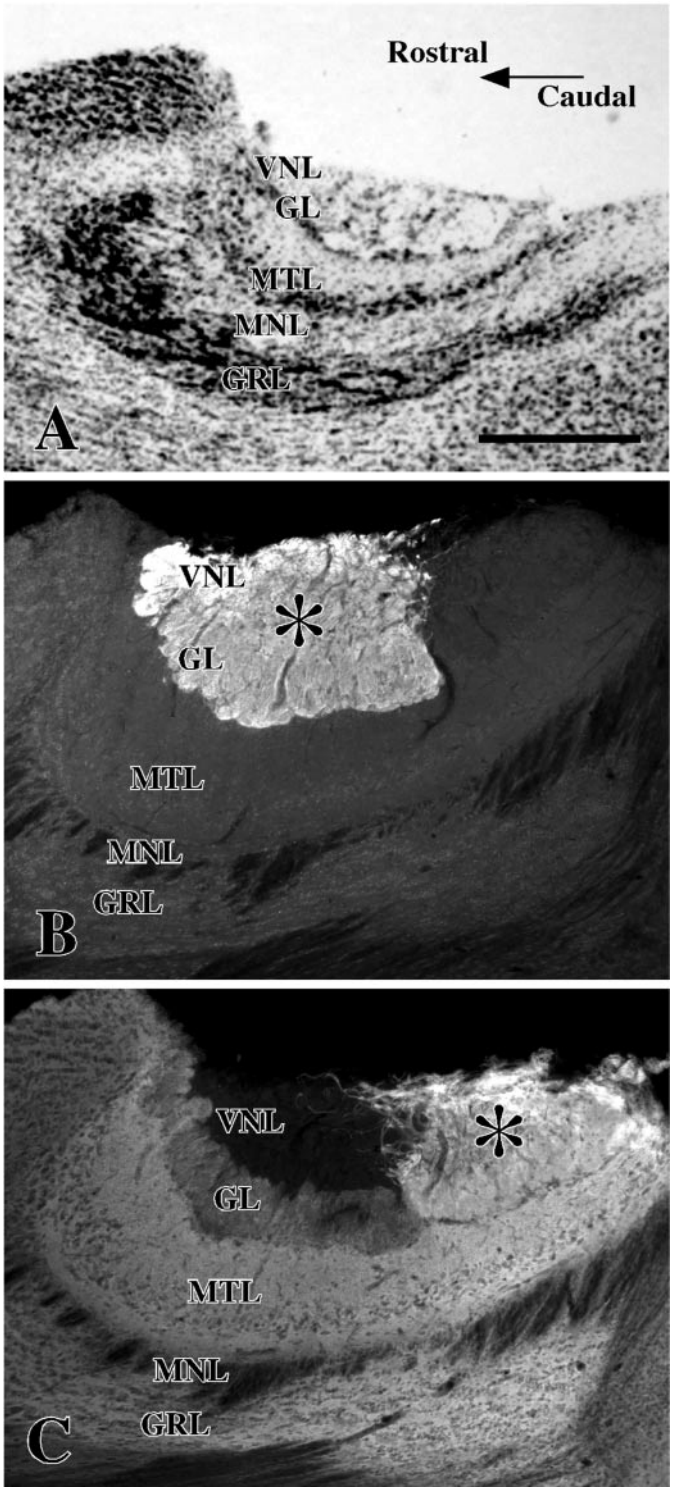

Figure 1 **(A)** Light micrograph of horizontal section (thickness = 50 μm) of guinea pig AOB (bar = 500 μm). VNL, vomeronasal nerve layer; GL, glomerulus layer; MTL, mitral tufted cell layer; MNL, myelinated nerve layer; GRL, granule cell layer. **(B)** Immunoreactivity for Gi2 (*) in AOB. Gi2-VN axons were found in the rostral region. **(C)** Immunoreactivity for Go (*) in AOB. Go-VN axons were found to terminate at the caudal region.

1C). Also, by immunohistochemistry of the main olfactory bulb (MOB) of all the examined animals, we detected iR for Go in their olfactory axons (Figure 2). It has previously been reported that Go is expressed in olfactory axons (Shinohara *et al.*, 1992b).

The Gi2 antiserum (Wako) was prepared from rabbit immunized with a synthetic peptide, LERIAQSDYI (160–169) of human. The Go-antiserum (MBL) was prepared from rabbit immunized with affinity-purified Go from a bovine brain. These antisera are not known to interact with the antigens of other species. Then, to confirm the interaction between the commercial antibodies used and Gi2 or Go in the examined animals, we performed Western blot analysis of anti-Gi2 and anti-Go antibodies using the olfactory bulbs of all the examined animals. In all cases, we detected single bands each corresponding to Gi2 (M_r = 40 000) and Go (M_r = 39 000; Figures 4B, 5B, 6B and 7B). The positions of these single bands were in agreement with the reported molecular masses of Gi2 (Graziano and Gilman, 1987) and Go (Sternweis and Robishaw, 1984). Because these results show that the antibodies interact with Gi2 or Go in all the examined mammalian species, we used both polyclonal antibodies in the comparative observation by immunohistochemistry for Gi2 and Go.

Comparative observation in VNO

In all the examined animals, VNOs were located in the basal regions of the nasal septum. In the coronal sections of the VNOs, VSE was located in the medial part (Figure 3A–E). On the opposite side of the VSE, a nonsensory epithelium was located. In the circumference of the VN lumen there were numerous VN glands. Blood vessels were localized at the lateral part of the lumen. The VNO consisted of the same components in all the examined animals, but the lumen form, the number of VN glands and the development of blood vessels varied.

In the dog, the VN lumen was large and surrounded by many blood vessels (Figure 3B). In the horse, the VN lumen had a complicated form and numerous VN glands existed around it (Figure 3C); blood vessels were small in diameter, but numerous. In the musk shrew, we observed few VN glands and a blood vessel with a large diameter (Figure 3D). In the common marmoset, the VN lumen formed an ellipsoid (Figure 3E) and the VN glands were randomly distributed around the lumen with few blood vessels.

In the guinea pig VSE, many VN neurons were observed (Figure 3F). In the apical region, supporting cells were aligned. Basal cells, which were believed to be stem cells, were not observed in the guinea pig VSE. In the VSEs of the dog, horse, musk shrew and marmoset, VN neurons were located in two or three rows (Figure 3G–J,). Supporting cells were aligned in the same manner as that observed in rodents. Although no basal cells were found in the VSEs of the dog, musk shrew and marmoset, basal cells were observed in the VSE of the horse (Figure 3H).

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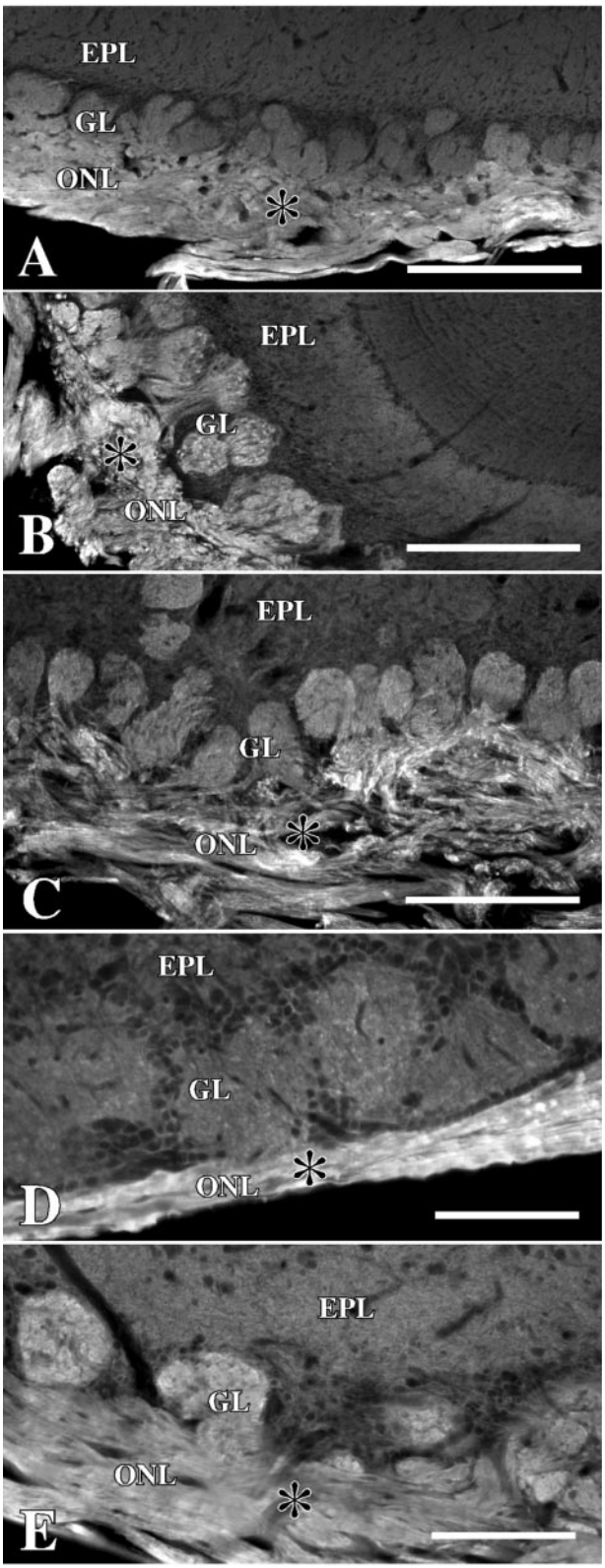

Figure 2 Immunohistochemistry for Go in MOBs of (A) guinea pig (bar = 500 μm), (B) dog (bar = 500 μm), (C) horse (bar = 500 μm), (D) musk shrew (bar = 100 μm) and (E) common marmoset (bar = 200 μm). Immunoreactivity (*) for Go was observed on olfactory axons in all the examined animals (A–E). ONL, olfactory nerve layer; GL, glomerulus layer; EPL, external plexiform layer.

Comparative immunohistochemistry of AOBs

In the guinea pig, the AOB is located at the dorsocaudal position of the olfactory bulb. The five-layer structure (VNL, GL, MTL, MNL and GRL) was distinctly observed (Figure 1A). Gi2-iR was observed in the rostral VNL and GL of the AOB (Figure 1B) and Go-iR was observed in the caudal VNL and GL (Figure 1C). The guinea pig was reconfirmed to have the segregated-type VN system (Sugai *et al.*, 1997).

The dog AOB was located on the medial surface of the olfactory peduncle at the caudal margin of the MOB (Figure 4C). In these sections, three-layer structures—VNL, GL and MTL (or GRL)—were observed (Figure 4A). It was difficult to distinguish MT cells from GR cells. By immunohistochemistry, Gi2-iR was observed in the VNL and GL throughout the AOB (Figure 4D). Go-iR was not observed in the VNL in the AOB (Figure 4E). The dog was confirmed to have the uniform-type VN system.

The horse AOB was located in the dorsocaudal position of the olfactory bulb (Figure 5C). In the sagittal AOB sections of the horse, three-layer structures—VNL, GL, MTL (or GRL)—were observed (Figure 5A). VNL was thick in the horse AOB. Gi2-iR was observed in VNL and GL (Figure 5D), but not Go-iR (Figure 5E). The horse was found to have the uniform-type VN system.

The AOB of the musk shrew was located in the dorsocaudal region of the olfactory bulb (Figure 6C). In the sagittal AOB sections, four-layer structures—VNL, GL, MTL and GRL—were recognized (Figure 6A). Gi2-iR VN nerves were observed throughout VNL and GL (Figure 6D). In contrast, Go-iR VN nerves were not observed in VNL (Figure 6E). The musk shrew was confirmed to have the uniform-type VN system.

The common marmoset AOB was located on the olfactory peduncle (Figure 7C). In the sagittal AOB sections of the common marmoset, three-layer structures—VNL, GL and MTL (or GRL)—were identified (Figure 7A). The boundary between MTL and GRL could not be distinguished. All VN axons were stained with the anti-Gi2 antibody, but not with the anti-Go antibody (Figure 7D,E). Although iR for Go was found in the caudal part of VNL and GL (Figure 7E), the region was not stained with the anti-neural cell adhesion molecule antibody used as a marker of olfactory and vomeronasal nerves (data not shown). The common marmoset was confirmed to have the uniform-type VN system.

Discussion

In the previous study using rats and goats (Takigami *et al.*, 2000), the immunohistochemical results for Gi2 and Go in AOB were consistent with those in VSE. Although immunoreactivity for Gi2 or Go was detected in both VSE and AOB of rats, only nerve terminals with immunoreactivity for Gi2, but not Go, were observed in the goat VSE and AOB (Takigami *et al.*, 2000). Therefore, immunohistochemistry results

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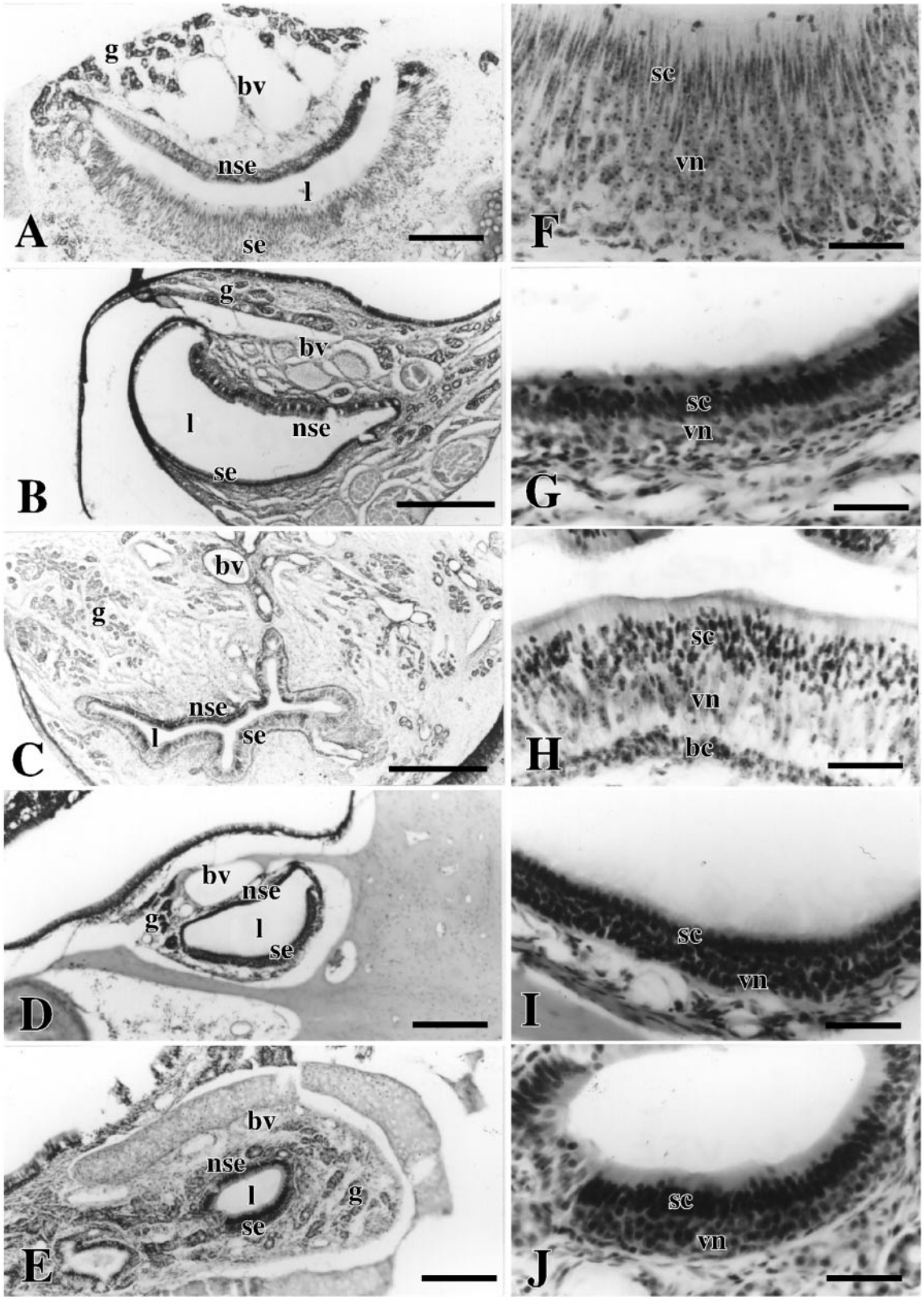

Figure 3 Light micrographs of coronal VNO sections (thickness = 20 μm) of **(A)** guinea pig (bar = 200 μm), **(B)** dog (bar = 500 μm), **(C)** horse (bar = 500 μm), **(D)** musk shrew (bar = 200 μm) and **(E)** common marmoset (bar = 200 μm). se, sensory epithelium; nse, nonsensory epithelium; l, vomeronasal lumen; bv, blood vessel; g, vomeronasal gland. High-magnification micrographs of sensory epithelia (thickness = 20 μm) of **(F)** guinea pig (bar = 50 μm), **(G)** dog (bar = 50 μm), **(H)** horse (bar = 100 μm), **(I)** musk shrew (bar = 50 μm) and **(J)** common marmoset (bar = 50 μm). vn, vomeronasal neuron; sc, supporting cell; l, vomeronasal lumen; bc, basal cell.

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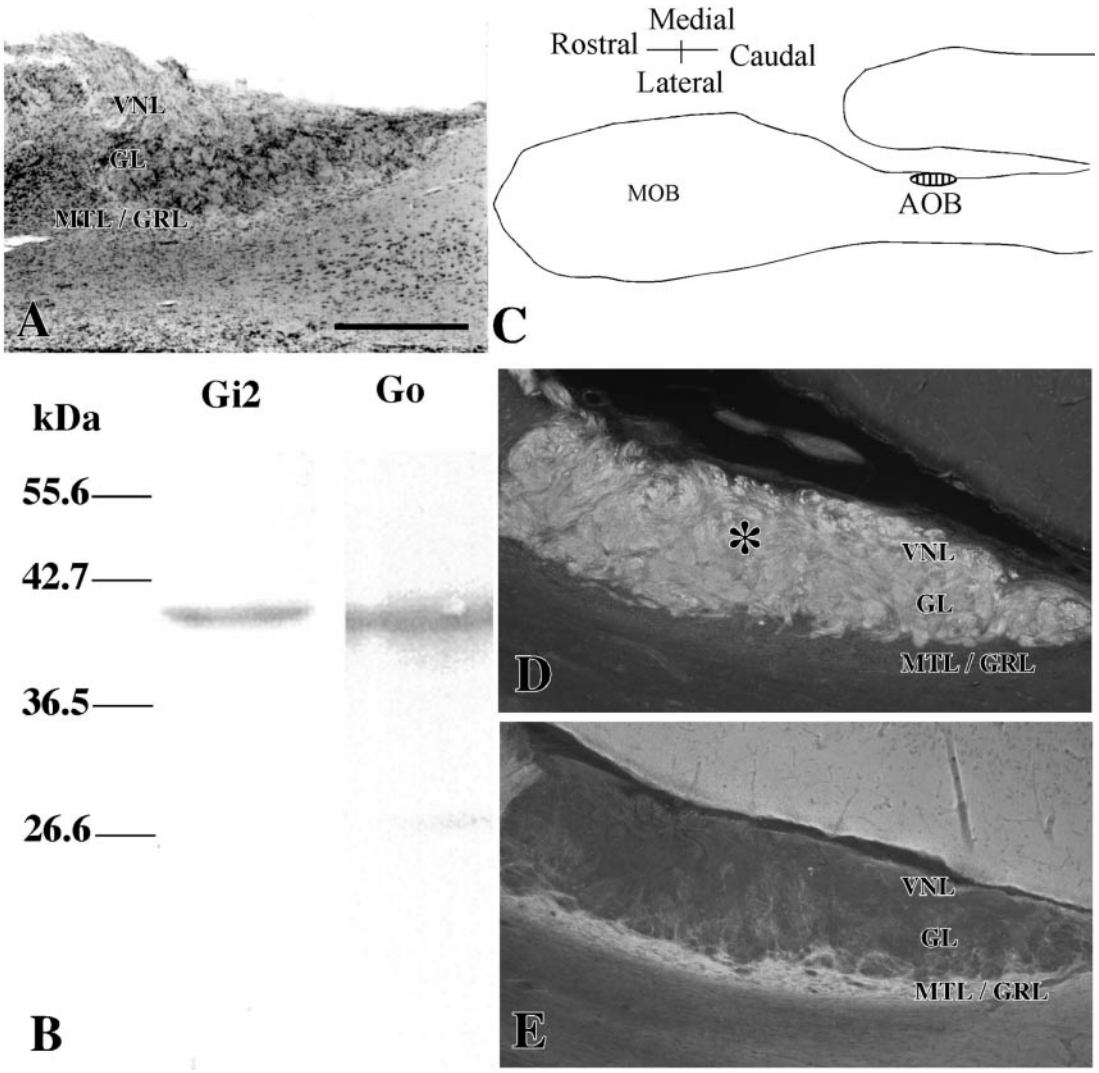

Figure 4 Immunohistochemistry of dog AOB. **(A)** Light micrograph of horizontal AOB section (thickness = 50 μm; bar = 500 μm). **(B)** Western blot analysis of olfactory bulb comprising AOB for examining cross-reactivity with antibodies for rat Gi2 and Go. Major bands (M_r = 39 000) were detected in Gi2 and Go lanes. **(C)** AOB location. Immunohistochemistry with antibodies for Gi2 **(D)** and Go **(E)**. In VNL and GL, immunoreactivity for Gi2 (*), but not Go, was observed.

for Gi2 or Go in AOB were thought to reflect the existence of Gi2- or Go-VN neurons in VSE. In the present study, only Gi2-VN axons terminated at the AOBs of the dog, horse, musk shrew and marmoset (Figures 4–7). In MOBs of all examined animals, nerve terminals with immunoreactivity for Go were clearly observed (Figure 2). Because olfactory and VN system are anatomically separated (Winans and Scalia, 1970; Scalia and Winans, 1975), these results suggest that only Gi2-expressing VN neuron may exist in the VSE of uniform-type VN systems. Interestingly, although VNO had common structural components such as a sensory epithelium, a nonsensory epithelium, a VN lumen, VN glands and blood vessels, in all the examined animals (Figure 3A–E), thin sensory epitheliums were observed in the examined animals with the uniform-type VN system (Figure 3G–J). In contrast, in the segregated-type VN system, thick VSE was observed (Figure 3F) and many VN neurons were stratified (Figure 3F). The difference in the thickness of sensory epithelium may reflect the number of VN neurons.

In the olfactory sensory epithelium, olfactory neurons are discretely distributed among four zones and they project to four respective zones of MOB (zone-to-zone projections; Mori *et al.*, 1999). The zone-to-zone projections depend on the expression of the olfactory receptor gene and play a critical role in axon guidance (Buck and Axel, 1991; Wang *et al.*, 1998; Mombaerts *et al.*, 1996; Mombaerts, 1999; Zou *et al.*, 2001) and odor discrimination (Uchida *et al.*, 2000). Thereby, mixed odors were expected to be divided into the respective components of the MOB. This anatomical filter is

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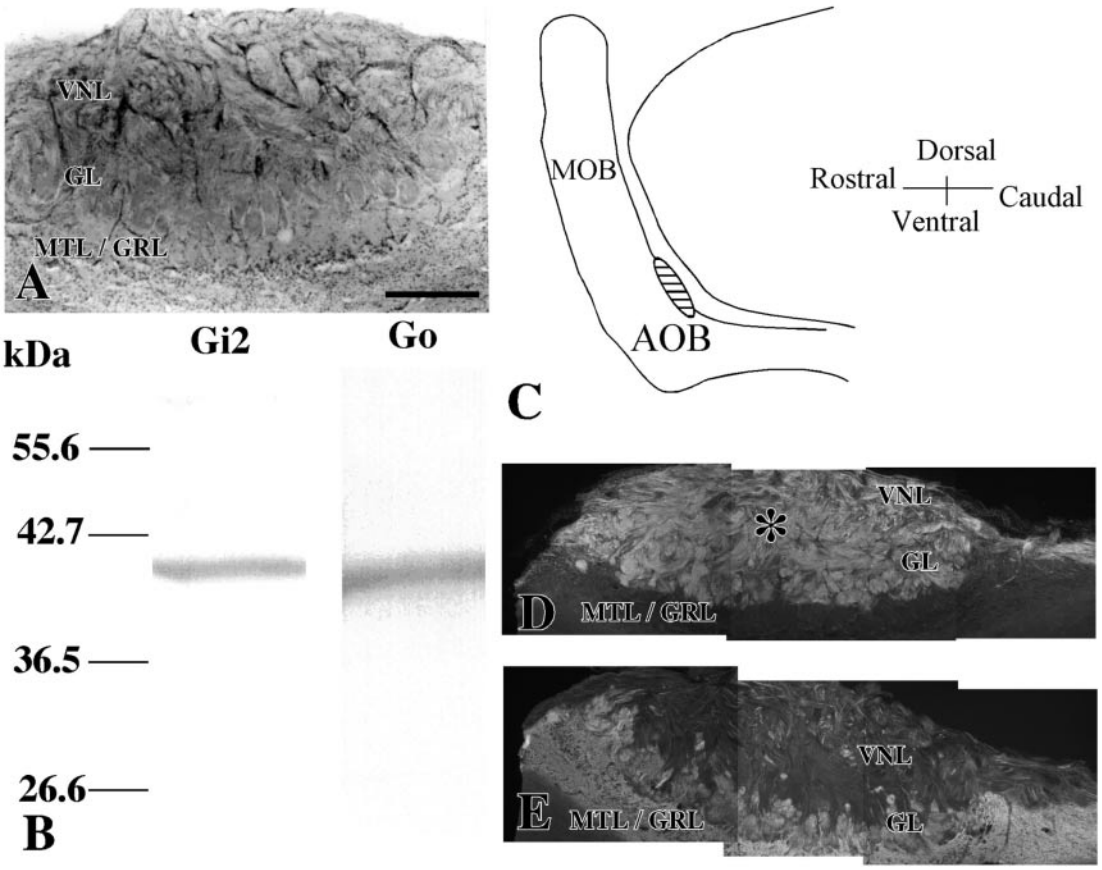

Figure 5 Immunohistochemistry of horse AOB. **(A)** Light micrograph of sagittal AOB section (thickness = 50 μm; bar = 500 μm). Three layer structures—VNL, GL and MTL (or GRL)—were distinctly observed. **(B)** Western blot analysis of olfactory bulb comprising AOB for examining cross-reactivity with antibodies for rat Gi2 and Go. Major bands were detected in Gi2 (M_r = 40 000) and Go (M_r = 39 000) lanes. **(C)** AOB location. Immunohistochemistry with antibodies for Gi2 **(D)** and Go **(E)**. In VNL and GL, immunoreactivity for Gi2 (*), but not Go, was observed.

believed to be a critical mechanism of olfactory discrimination. In the VN systems of rodents and opossums, there was a similar segregated projection by the two populations of VN neurons to the olfactory system exists. In previous studies, different responses to various pheromones in the rostral or caudal region of AOB were reported (Brennan *et al.*, 1999; Kumar *et al.*, 1999; Halem *et al.*, 2001). For example, when male mice are exposed to the urine of a diestrus female, increased neural activity is observed in the rostral side of AOB. The rostral side of the female AOB is associated with processing of the male pheromone signal and the strain recognition component (Brennan *et al.*, 1999). Furthermore, the pheromones associated with aggressive behavior against strange males are processed in the caudal region of the male AOB (Brennan *et al.*, 1999) and Gi2- and Go-expressing VN neurons respond to different urinary pheromones (Inamura *et al.*, 1999). A recent study (Fieni *et al.*, 2003) also reported that these VN neurons exhibit distinct electrophysiological properties in the amplitudes of their voltage-gated Na^+ currents. In addition, it was also reported that the two types of VN neurons respond to different chemical stimuli (Leinders-Zufall *et al.*, 2000) and possibly to different types of chemicals (Krieger *et al.*, 1999). These data show that both VN pathways appear to be functional in rodents. In the present study, immunohistochemistry for Gi2 or Go revealed that no Go-VN axons project into AOBs of the dog, horse, musk shrew or marmoset (Figures 4–7). Our results suggest the possibility that the Go-VN pathway does not exist in uniform-type VN systems. Furthermore, genes encoding pheromone receptors have been reported in the goat (Wakabayashi *et al.*, 2002). In goats, two types of V1R homologues and eight kinds of V2R homologues have been identified. The V2R homologues have many pseudogenes. On the other hand, one V1R homologue (gV1R1) has 40–50% homology to V1Rs and has an open reading frame encoding 309 amino acids. This evidence increases the possibility that only one pheromone receptor population is expressed in the uniform-type VN system. Consequently, the processing potentials in the uniform-type VN system are considered to be less than those in the segregated type.

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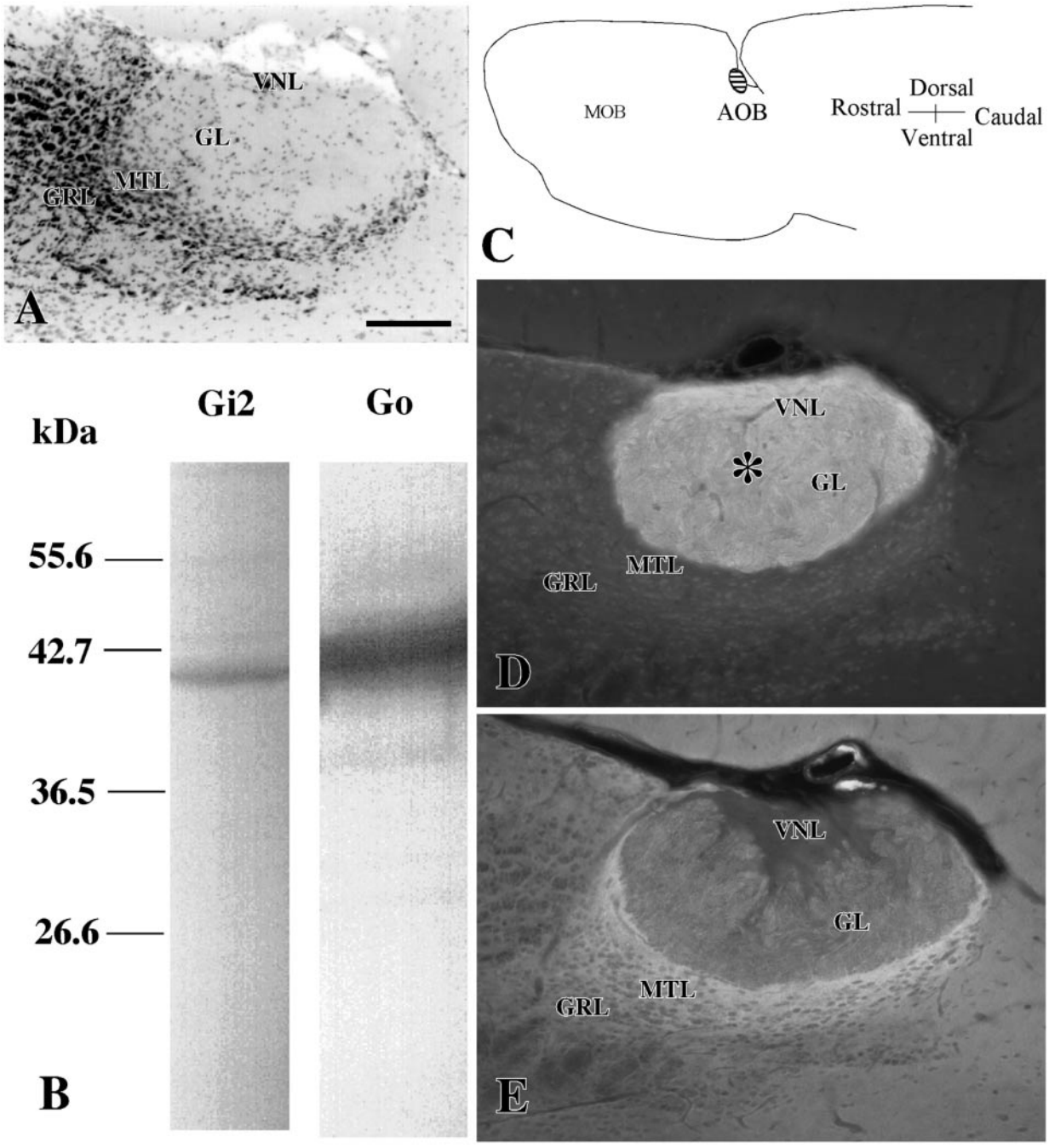

Figure 6 Immunohistochemistry of musk shrew AOB. **(A)** Light micrograph of sagittal AOB section (thickness = 50 μm; bar = 200 μm). **(B)** Western blot analysis of olfactory bulb comprising AOB for examining cross-reactivity with antibodies for rat Gi2 and Go. Major bands were detected in Gi2 (M_r = 40 000) and Go (M_r = 39 000) lanes. **(C)** AOB location. Immunohistochemistry with antibodies for Gi2 **(D)** and Go **(E)**. In VNL and GL, immunoreactivity for Gi2 (*), but not Go, was observed.

The segregated-type VN system has been reported mainly in rodents and opossum. Rodents are the most common experimental animals and they have the highest number of strains in the Eutheria, thus it is generally believed that the results obtained using rodents are applicable to all mammals. In the present and previous studies (Takigami *et al.*, 2000), mammals other than rodents were investigated. All the examined animals belonged to the Eutheria and their VN systems were found to be of the uniform type. Our results suggest that the VN systems of many terrestrial mammals are of the uniform type rather than the segregated type. In addition, because Gi2-VN neurons definitely exist in both types of VN systems, they have been conserved in most mammalian species during evolution. Gi2-VN neurons are

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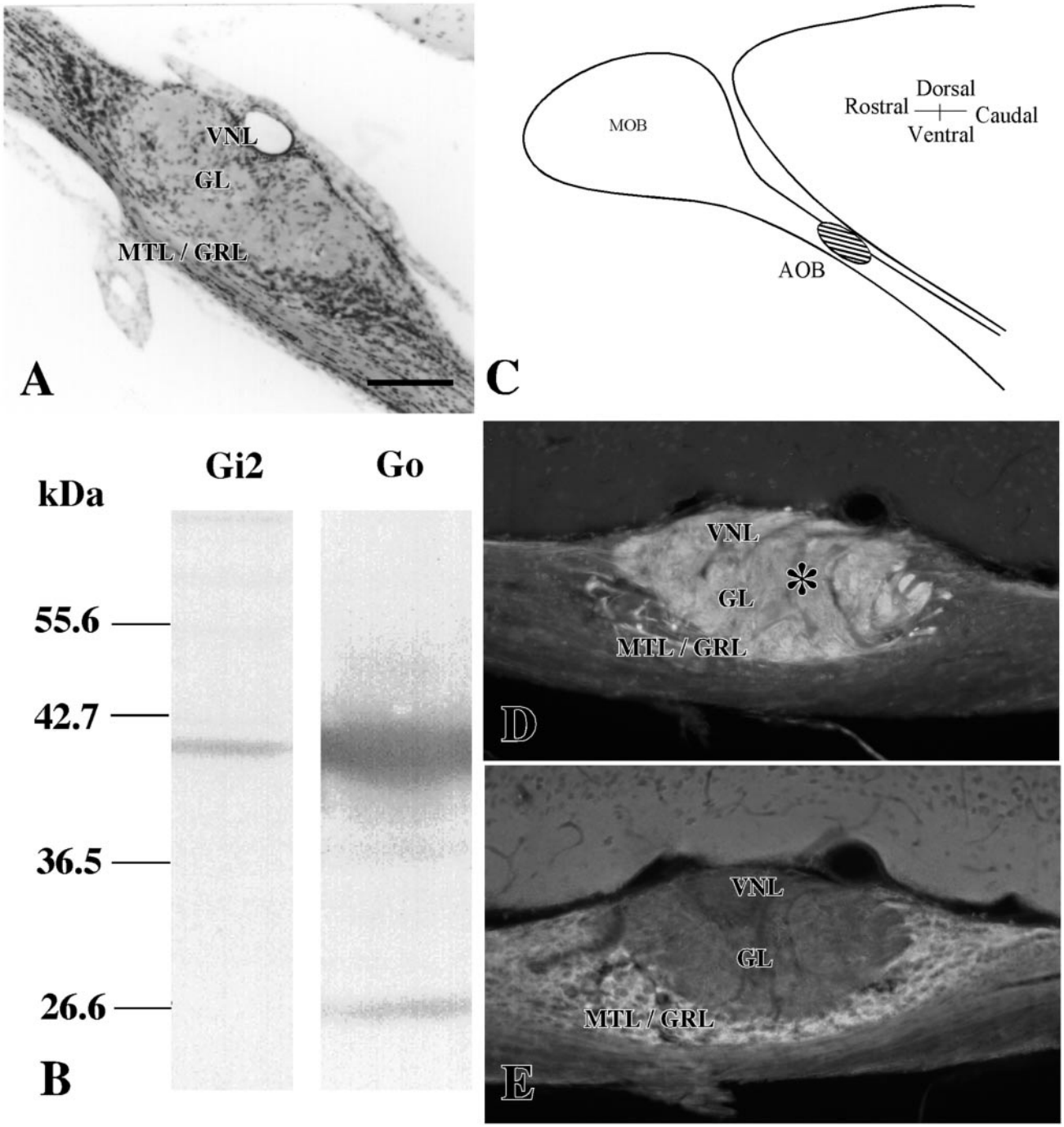

Figure 7 Immunohistochemistry of common marmoset AOB. **(A)** Light micrograph of sagittal AOB section (thickness = 50 μm; bar = 200 μm). **(B)** Western blot analysis of olfactory bulb comprising AOB for examining cross-reactivity with antibodies for rat Gi2 and Go. Major bands were detected in Gi2 (M_r = 40 000) and Go (M_r = 39 000) lanes. **(C)** AOB location. AOB was found on the olfactory peduncle. Immunohistochemistry with antibodies for Gi2 **(D)** and Go **(E)**. In VNL and GL, immunoreactivity for Gi2 (*), but not Go, was observed.

expected to receive pheromones with a common effect in the mammalian VN system. To elucidate the function of the VN system in mammals, further comparative studies are necessary. In our next study, we will examine ontogenetically animals with the uniform-type VN system in an attempt to understand differences and similarities in features between the segregated- and uniform-type VN systems.

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