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# Projection Pattern of Vomeronasal Neurons to the Accessory Olfactory Bulb in Goats

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## Abstract

Goats have a well-developed vomeronasal (VN) system and exhibit pheromone-induced reproductive facilitation, but there are no reports on the projection pattern of VN neurons in this species. Rodent, guinea pig and opossum accessory olfactory bulbs (AOBs) have been shown to have a segregated pattern of projection of the VN neurons, which express the two α-subtypes of the G-protein, namely Gi2 and Go, to the rostral and caudal regions of the AOB, respectively. In this study we investigated the projection pattern of VN nerve terminals by immunocytochemical staining of the goat vomeronasal organ (VNO) and the AOB with antibodies to Gi2 and Go. Gi2-immunoreactivity was found on the luminal surface of the sensory epithelium of the VNO, and in the VN nerve and glomerular layer throughout the AOB. On the other hand, Go-immunoreactivity was not identified in either the VNO or the VN nerve layer of the AOB. These results indicate that the projection pattern of VN neurons from the VNO to the AOB in the goat is considerably different from that in rodents which show a distinct segregated pattern.

## Introduction

Most mammalian vomeronasal (VN) systems are thought to function as the pheromone-receptive system and to be concerned with reproductive and social behaviors (Wysocki, 1979). Pheromonal information is received by VN neurons in the vomeronasal organ (VNO) and then transmitted to the accessory olfactory bulb (AOB) via the VN nerves, thereby influencing the neuroendocrine system of the recipient animal (Ichikawa, 1996). Dulac and Axel (Dulac and Axel, 1995) reported a family of genes encoding putative pheromone receptors (V1Rs), seven transmembrane receptors coupled with G-proteins, in the rat, and then another type of putative pheromone receptors (V2Rs) was found (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). A recent study has suggested that VN neurons express Gi2 and Go (Berghard and Buck, 1996), and that the V1Rs and V2Rs are expressed on Gi2-expressing and Go-expressing VN neurons, respectively (Dulac and Axel, 1995; Herrada and Dulac, 1997). The V1R-Gi2 neurons are located in the apical layer of the VN sensory epithelium and the V2R-Go neurons in the basal layer. These two types of VN neurons are known, in rodents and some other animals, to project their axons to segregated regions in the AOB. The V1R-Gi2 neurons project to the glomeruli in the rostral region of the AOB, whereas the V2R-Go neurons project to those in the caudal region (Shinohara *et al.*, 1992b; Jia and Halpern, 1996). Shinohara *et al.* argued that the projection of VN neurons to segregated regions in the AOB could be a feature common to mammalian VN systems (Shinohara *et al.*, 1992b). In fact, in all the mammalian species studied to date, including the rat and opossum, immunocytochemical evidence for the segregated projection of VN neurons has been accumulated (Ichikawa *et al.*, 1992; Takami *et al.*, 1992; Shnayder *et al.*, 1993; von Campenhausen *et al.*, 1997; Halpern *et al.*, 1998). In these species, the two VN neural pathways are speculated to be functionally independent and to process different information, although their specific functions still remain largely unknown.

Goats have a VNO and exhibit pheromone-induced reproductive responses (Chemineau, 1987; Ichikawa *et al.*, 1999). The pheromone-induced reproductive response is known as the ‘male effect’, in which ovulation is induced in seasonally anovulatory females by exposure to the male or its odor. This transmission of the pheromonal information to the central nervous system has been demonstrated by monitoring the characteristic pattern of multiple-unit activity (MUA volleys) which reflects the gonadotrophin-releasing hormone pulse generator activity from the medial

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basal hypothalamus (Hamada *et al.*, 1996; Iwata *et al.*, 2000). The projection pattern of the VN neurons to the AOB has not been studied yet in the goat. The aim of the present study was therefore to compare the projection patterns of the VN neurons between goats and rats by immunocytochemically examining the distribution of Gi2 and Go in the VNO and the AOB. Preliminary results from this study have been reported previously in abstract form (Takigami *et al.*, 1999b).

## Materials and methods

### Preparation of the VNO sections

Sprague–Dawley rats (adult: >8 weeks old) and Shiba goats (adult, male and female), which were obtained from a closed colony in the University of Tokyo, were deeply anesthetized with sodium pentobarbital and sacrificed by transcardial or transcarotid vein perfusion of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The VNOs were removed and immersed overnight in 4% PFA. They were then immersed in 30% sucrose/phosphate-buffered saline (PBS) solution for cryoprotection and cut into 20 μm coronal sections with a Cryostat (JUNG CM3000, Leica). The sections were mounted on gelatin-coated slides.

### Preparation of the AOB sections

The olfactory bulbs containing the AOB were removed from the sacrificed rats and goats, and immersed overnight in 4% PFA. They were then immersed in 30% sucrose/PBS solution for cryoprotection. The AOBs were cut into 50 μm sagittal sections using a freezing microtome, and the sections were floated in 0.1 M PBS.

### Immunocytochemistry

The AOB and VNO sections were incubated in a 10% blocking solution of BlockAce (Dainippon Pharmaceutical) in PBS for 1 h and thereafter for 3 days with the antibody to Gi2 (Wako, diluted 1:800) or Go (MBL, diluted to 1 μg/ml) in PBS containing 10% BlockAce and 0.05% Triton X-100 at 4°C. After being washed in PBS, the sections were incubated with normal goat serum followed by incubation with biotinylated goat anti-rabbit immunoglobulin G (IgG) (Vector) in PBS with 10% BSA for 2 h at room temperature. After being washed again, the sections were incubated with streptavidin–fluorescein (Amersham) in PBS for 1 h and then rinsed four times with PBS prior to observation by using Axiophot (Zeiss).

### Western blotting analysis

Adult goats were deeply anesthetized with sodium pentobarbital and sacrificed by being bled via sectioning of the carotids and jugular veins. The olfactory bulbs including the AOBs were then removed and stored in PBS containing protease inhibitors at –80°C until analysis. The total homogenates of the olfactory bulb were denatured in sodium dodecyl sulfate (SDS) under reducing conditions at 95°C and subjected to SDS–polyacrylamide gel electrophoresis. Proteins were blotted onto nitrocellulose membranes and incubated overnight with BlockAce at 4°C. The blots were incubated with the primary antibody (Gi2 or Go; 1:1000) at room temperature for 1 h. After being washed in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20), the blots were incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG (1:5000 dilution) at room temperature for 1 h. Bound antibodies were visualized by employing a Western blue-stabilized substrate (Promega).

## Results

### Western blotting analysis and immunohistochemical analysis in the goat main olfactory bulb (MOB)

Single bands, each corresponding to Gi2 ($M_r$ 40 000) and Go ($M_r$ 39 000), were detected (Figure 1). The sizes of these immunoreactive proteins were in agreement with the reported molecular masses of Gi2 and Go in the rat. The Western blot analysis indicated that these antibodies specifically interacted with the Gi2 or Go of goats (Figure 1). In the goat MOB, olfactory nerves, which were terminated at glomeruli in the MOB, were positively stained with antibody to Go but not to Gi2 (Figure 2). In the rat MOB, Go expresses in the olfactory nerves (Shinohara *et al.*, 1992a). Thus, we used these antibodies for examing the distribution of Gi2- and Go-expressing VN fibers in the goat AOB.

### General histology and immunocytochemical analysis of the VNO

In the goat VNO, the sensory epithelium was observed on the side of the nasal septum and the nonsensory epithelium was seen on the opposite side (Figure 3E). The sensory and nonsensory epithelia formed the VN lumen. There was no difference in their histological component parts of the VNO between goats and rats, but the morphological degree of every tissue appears to be different (Figure 3A,E). In the goat VNO, the sensory epithelium was relatively flatter than that in the rat (Figure 3B,F). In both the goat and the rat, blood vessels were observed to run along the nonsensory epithelium (Figure 3A,E). In the goat, a number of veins were noted to run along the lumen (Figure 3E), while a big blood vessel was observed at the center of the VNO in the rat (Figure 3A). We could observe numerous VN glands in both goat and rat VNO. In the goat, the VN glands were observed around the VN lumen and the veins, while in the rat they were lateral to the blood vessel. The goats VN glands were developed greater than those of the rat (Figure 3A,E). The sensory epithelium was composed of three kinds of cells, namely, the VN neurons, supporting cells and basal cells. The basal cells could be identified easily along the basal region of the VN epithelium in the goat. In the rat, the location of the basal cells is still unclear (Figure 3B,F).

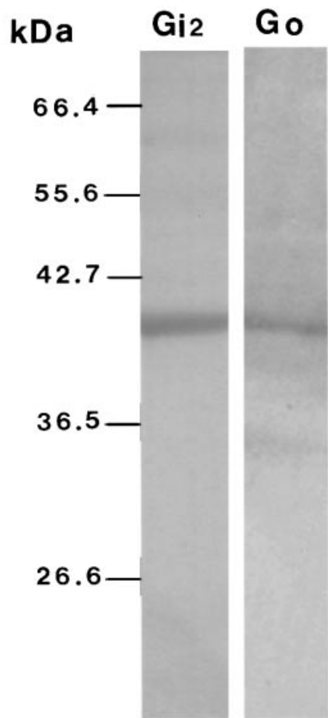


**Figure 1** Western blot analysis in the goat olfactory bulb including the AOB to examine the cross-reactivity with antibodies for rat Gi2 and Go. Major bands were detected in both Gi2 ($M_r$ 40 000) and Go ($M_r$ 39 000) lanes.

In the rat VNO, the immunoreactivities for both Gi2 and Go were detected as dots along the luminal surface of the sensory epithelium (Figure 3C,D). In the goat VNO, although the immunoreactivity for Gi2 was observed as clearly as in the rat, no immunoreactivity for Go was detected on the luminal surface of the sensory epithelium (Figure 3G,H).

## General histology and immunocytochemical analysis in the AOB

In both the goat and the rat, the AOB were located in the dorso-caudal region of the olfactory bulb (Figure 4A,E), and sagittal sections distinctly identified five laminar layers, namely, the VN nerve, glomerular, mitral/tufted cell, myelinated nerve and granule cell layers (Figure 4B,F). However, in the goat, the myelinated nerve layer was found under the granule cell layer, and thus the laminar pattern was different from that in the rat AOB (Figure 4B,F).

In the rat AOB, Gi2-immunoreactivity was found mainly in the VN nerve and glomerular layers of the rostral half region (Figure 4C). In the goat, positive immunoreactivity for Gi2 was identified on the VN nerve and glomerular layers throughout the AOB (Figure 4G). On the other hand, the Go-immunoreactivity was observed in the VN nerve and glomerular layers of the caudal part in the rat AOB (Figure 4D), but reactivity for Go could not be recognized in the VN nerve layer in the goat AOB (Figure 4H). In addition, no difference in the distribution pattern of the G-proteins in the

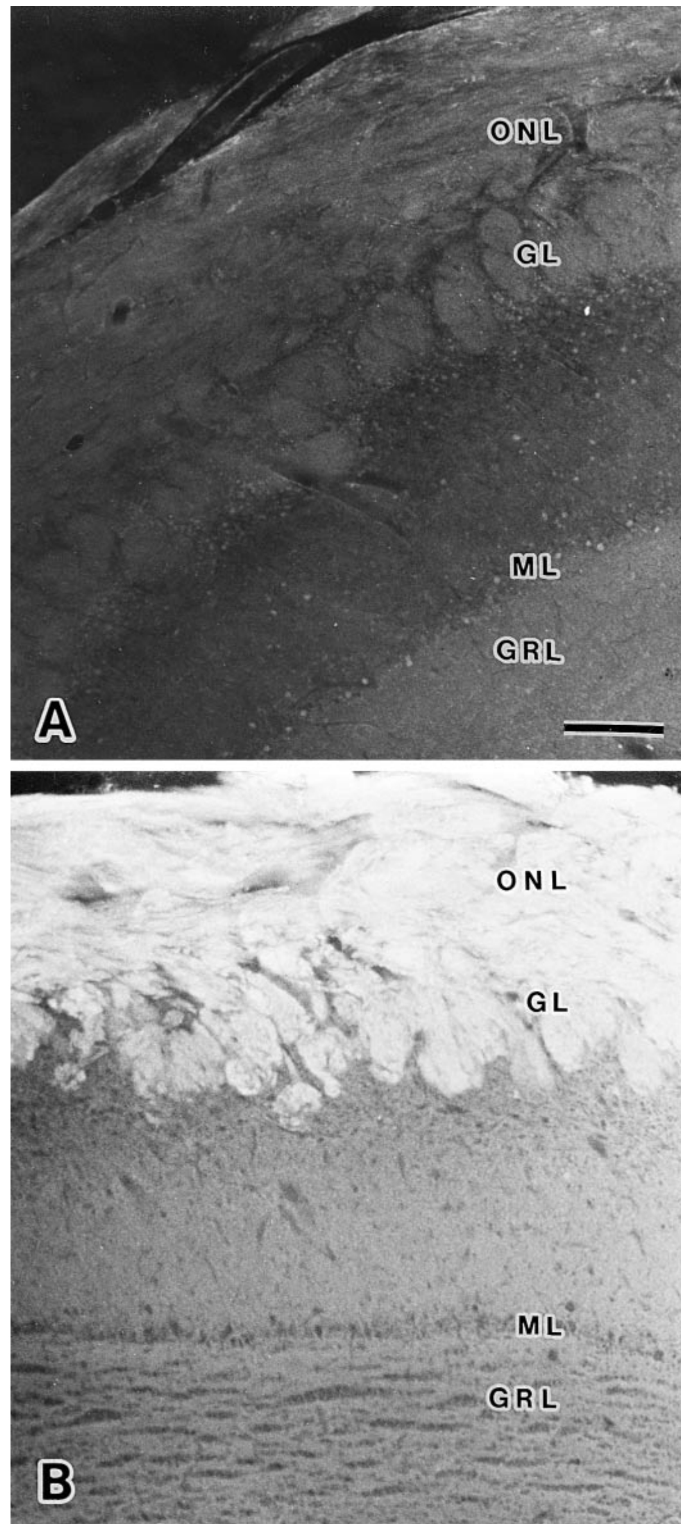


**Figure 2** Immunocytochemical analysis with antibody for Gi2 **(A)** and Go **(B)** in the main olfactory bulb of the goat. Positive immunoreactivity for Go was found in the ONL and GL. Bar = 100 μm.

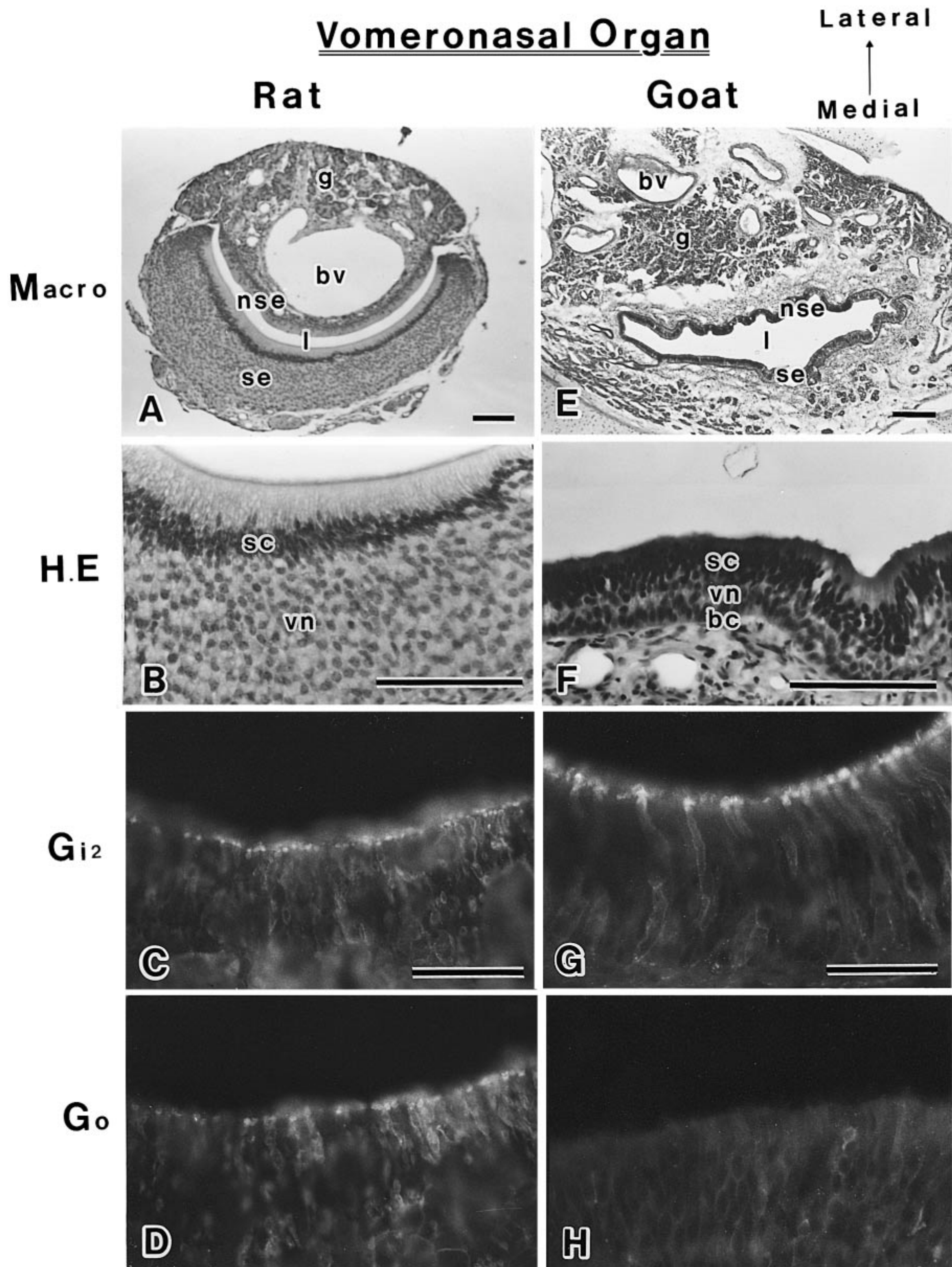


**Figure 3** **(Top)** Light micrograph of a coronal section (thickness = 20 μm) of the vomeronasal organ of the rat **(A)** and the goat **(E)**. se, sensory epithelium; nse, nonsensory epithelium; l, vomeronasal lumen; bv, blood vessel; g, vomeronasal gland. Bar = 200 μm. **(Second row)** A high magnification (thickness = 20 μm) of the sensory epithelium in the rat **(B)** and the goat **(F)**. vn, vomeronasal neuron; sc, supporting cell; l, vomeronasal lumen; bc, basal cell. Bar = 100 μm. **(Third row)** Immunocytochemical analysis with antibody for Gi2 in the rat **(C)** and the goat **(G)**. The luminal surface of the sensory epithelium was positively stained as indicated by the dots. Bar = 50 μm. **(Fourth row)** Immunocytochemical analysis with antibody for Go in the rat **(D)** and the goat **(H)**.

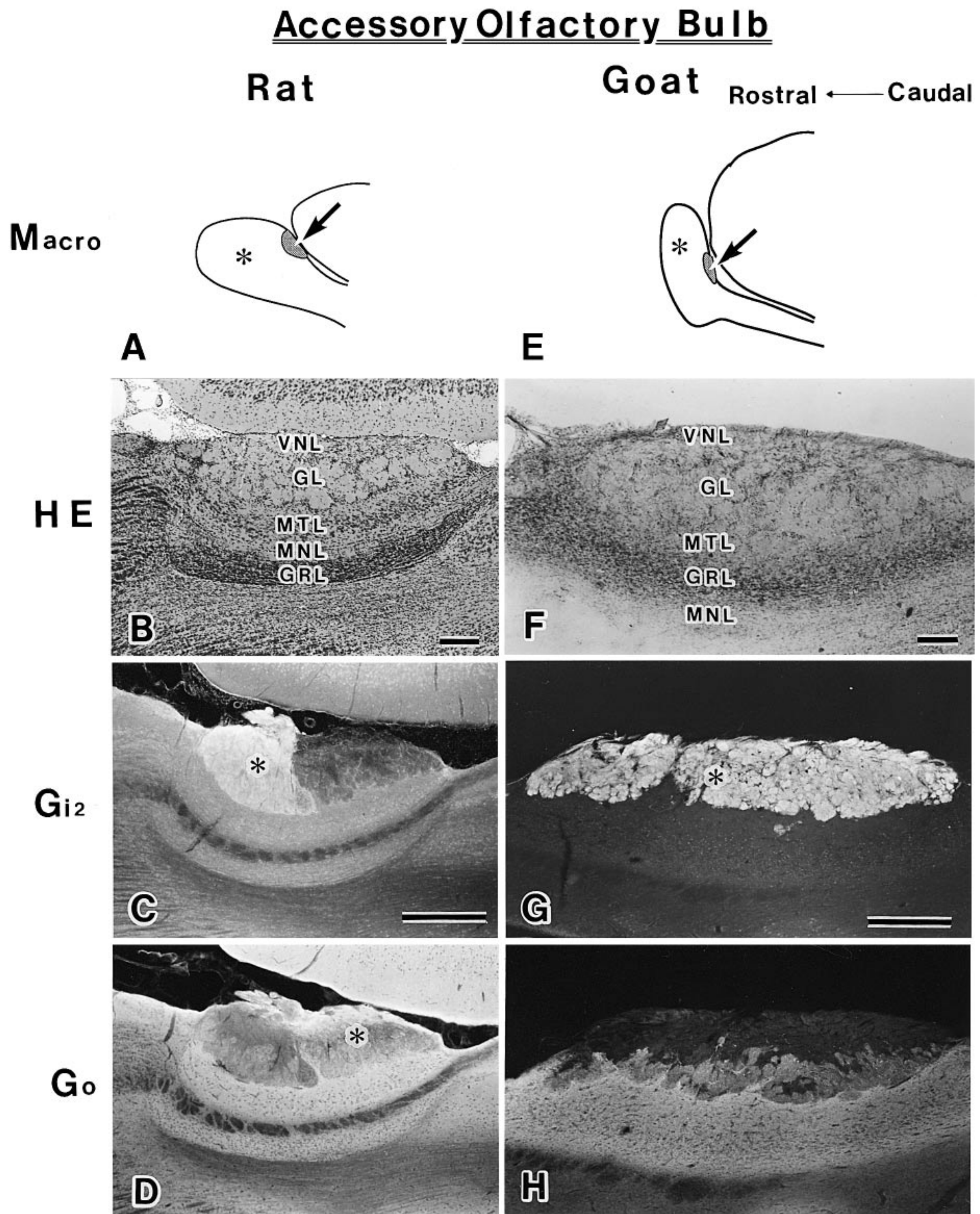


**Figure 4** **(Top)** An illustration of the main (*) and accessory (arrow) olfactory bulb in the rat **(A)** and the goat **(E)**. **(Second row)** Light micrograph of a sagittal section (thickness = 40 μm) of the accessory olfactory bulb in the rat **(B)** and the goat **(F)**. Bar = 200 μm. VNL, vomeronasal nerve layer; GL, glomerulus layer; MTL, mitral/tufted cell layer; MNL, myelinated nerve layer; GRL, granule cell layer. **(Third row)** Immunocytochemical analysis with antibody for Gi2 in the rat **(C)** and the goat **(G)**. Positive immunoreactivity (*) was found in the VNL and GL. Bar = 500 μm. **(Fourth row)** Immunocytochemical analysis with antibody for Go in the rat **(D)** and the goat **(H)**.

AOB was found between males and females in either goats or rats (data not shown).

## Discussion

It is known that the AOB is anatomically divided into rostral and caudal regions in rodents, guinea pigs and opossums (Shinohara *et al.*, 1992b; Jia and Halpern, 1996). A recent study has shown that the centrifugal inputs are also segregated in the AOB (Martinez-Marcos and Halpern, 1999). In the present study, the rostro-caudal segregation in the projection patterns of VN nerves observed in the AOB of rodents and other animals could not be identified in the goat. Gi2-immunoreactive VN nerve fibers, distributed in the rostral region of the rat AOB, were found throughout the AOB in the goat. Moreover, Go-immunoreactive VN nerve fibers, distributed in the caudal regions of the rat AOB, did not exist at all in the goat AOB. These results indicate that the AOB of the goat has an unsegregated projection pattern of the VN neurons, and that the segregated VN nerve projections reported in the rat might not be a common feature to all mammals, as was postulated based on earlier findings in rodents and other species. Immunoreactivities for both Gi2 and Go could be found in the rat VNO. In the goat VNO, however, only Gi2 immunoreactivity was found along the luminal surface of the sensory epithelium, which appears to be in accordance with the observed Gi2 and Go immunoreactivity patterns in the AOB. Our results shows that Gi2-expressing VN neurons were shown to project to the AOB in both rats and goats. If Gi2-expressing VN neurons exist commonly in mammalian VN systems, they may play an essential role in the processing of pheromones. Recent studies reveal that, in the mouse, Gi2-expressing VN neurons receive low-volatility substances concerned with strain recognition (Brennan *et al.*, 1999). Krieger *et al.* (Krieger *et al.*, 1999) suggested that V1Rs co-expressed with Gi2 may be activated by lipophilic probably volatile substances in the rat. Our results together with these findings suggest the possibility that Gi2-expressing VN neurons may have the common function of identifying differences among individuals, strains and species. It has been reported recently that an ion channel of the transient receptor potential family, which mediates G-protein-regulated and cyclic-nucleotide-independent signaling pathways, is expressed in rat VN neurons (Liman *et al.*, 1999). We have also been shown that putative pheromone receptors exist at the luminal surface of the VN sensory epithelium (Takigami *et al.*, 1999a). Immunocytochemical analysis of the VNO in both rats and goats revealed Gi2-immunoreactivity along the luminal surface of the sensory epithelium, which indicates the involvement of the Gi2 system in pheromonal signal transduction.

The two distinct VN nerve distributions in the AOB in rodents have been regarded as a reflection of the presence of two types of G-protein-coupled pheromone receptors in the VN sensory epithelium (Dulac and Axel, 1995; Herrada and Dulac, 1997; Belluscio *et al.*, 1999; Rodriguez *et al.*, 1999). The present results suggest that the goat may have only one family of pheromone receptors and may lack the other type with which rats are equipped. Goats have a VN system which shows some differences in structure from that in rats. The behavior related to pheromonal reception, namely flehmen, in goats (Ladewig and Hart, 1980; Melese-d'Hospital and Hart, 1985) is also different from that in rats, which use VN pumping (Meredith *et al.*, 1980; Meredith, 1994). The difference in the projection patterns of the VN neurons may be related to some species-specific behaviors concerned with pheromone reception. For example, flehmen is thought to be a typical pheromone-receptive behavior in the goat, and is not observed in rodents. On the other hand, rodents have a VNO pump operated with highly developed blood vessels for conveying pheromones into the VNO. This type of behavioral variation may be ascribed to the interspecies differences in the chemical characteristics of the pheromone ligands used for communication. It can be speculated that the available pheromone molecules have evolved in accordance with the ecological circumstances of each species and that the VN system has acquired flexibility in function during adaptation.

The results of this study have demonstrated the existence of different projection patterns of VN neurons to the AOB between goats and rats, including the necessity of further comparative studies of the VN systems in other mammalian species. To clarify the function as well as the evolution of the mammalian VN system, we also need to examine the behavioral and ecological changes associated with or caused by the differences in the projection pattern of VN neurons to the AOB.

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## References

**Belluscio, L., Koentges, G., Axel, R.** and **Dulac, C.** (1999) *A map of pheromone receptor activation in the mammalian brain.* Cell, 97, 209–220.

**Berghard, A.** and **Buck, L.B.** (1996) *Sensory transduction in vomeronasal neurons: evidence for G alpha o, G alpha i2, and adenylyl cyclase II as major components of a pheromone signaling cascade*. J. Neurosci., 16, 909–918.

**Brennan, P.A., Schellinck, H.M.** and **Keverne, E.B.** (1999) *Patterns of expression of the immediate–early gene egr-1 in the accessory olfactory bulb of female mice exposed to pheromonal constituents of male urine*. Neuroscience, 90, 1463–1470.

**Chemineau, P.** (1987) *Possibilities for using bucks to stimulate ovarian and*

*oestrus cycles in anovulatory goats: a review*. Livest. Prod. Sci., 17, 135–147.

**von Campenhausen, H., Yoshihara, Y.** and **Mori, K.** (1997) *OCAM reveals segregated mitral/tufted cell pathways in developing accessory olfactory bulb.* NeuroReport, 8, 2607–2612.

**Dulac, C.** and **Axel, R.** (1995) *A novel family of genes encoding putative pheromone receptors in mammals*. Cell., 83, 195–206.

**Halpern, M., Jia, C.** and **Shapiro, L.S.** (1998) *Segregated pathways in the vomeronasal system.* Microsc. Res. Tech., 41, 519–529.

**Hamada, T., Nakajima, M., Takeuchi, Y.** and **Mori, Y.** (1996) *Pheromone-induced stimulation of hypothalamic gonadotropin-releasing hormone pulse generator in ovariectomized, estrogen-primed goats*. Neuroendocrinology, 64, 313–319.

**Herrada, G.** and **Dulac, C.** (1997) *A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution*. Cell, 90, 763–773.

**Ichikawa, M.** (1996) *Neural development, differentiation, and plasticity in the mammalian vomeronasal system*. Zool. Sci., 13, 627–639.

**Ichikawa, M., Osada, T.** and **Ikai, A.** (1992) Bandeiraea simplicifolia *lectin I and* Vicia villosa *agglutinin bind specifically to the vomeronasal axons in the accessory olfactory bulb of the rat*. Neurosci. Res., 13, 73–79.

**Ichikawa, M., Shin, T.** and **Kang, M.S.** (1999) *Fine structure of the vomeronasal epithelium of Korean goat (*Capra hircus*).* J. Reprod. Dev., 45, 81–89.

**Iwata, E., Wakabayashi, Y., Kakuma, Y., Kikusui, T., Takeuchi, Y.** and **Mori, Y.** (2000) *Testosterone dependent primer pheromone production in the sebaceous gland of male goat.* Biol. Reprod., 62, 806–810.

**Jia, C.** and **Halpern, M.** (1996) *Subclasses of vomeronasal receptor neurons: differential expression of G proteins (Gi alpha 2 and G(o alpha)) and segregated projections to the accessory olfactory bulb*. Brain Res., 719, 117–128.

**Krieger, J., Schmitt, A., Lobel, D., Gudermann, T., Schultz, G., Breer, H.** and **Boekhoff, I.** (1999) *Selective activation of G protein subtypes in the vomeronasal organ upon stimulation with urine-derived compounds.* J. Biol. Chem., 274, 4655–4662.

**Ladewig, J.** and **Hart, B.L.** (1980) *Flehmen and vomeronasal organ function in male goats.* Physiol. Behav., 24, 1067–1071.

**Liman, E.R., Corey, D.P.** and **Dulac, C.** (1999) *TRP2: a candidate transduction channel for mammalian pheromone sensory signaling.* Proc. Natl Acad. Sci. USA, 96, 5791–5796.

**Martinez-Marcos, A.** and **Halpern, M.** (1999) *Differential centrifugal afferents to the anterior and posterior accessory olfactory bulb*. NeuroReport, 10, 2011–2015.

**Matsunami, H.** and **Buck, L.B.** (1997) *A multigene family encoding a diverse array of putative pheromone receptors in mammals*. Cell, 90, 775–784.

**Melese-d'Hospital, P.Y.** and **Hart, B.L.** (1985) *Vomeronasal organ cannulation in male goats: evidence for transport of fluid from oral cavity to vomeronasal organ during flehmen*. Physiol. Behav., 35, 941–944.

**Meredith, M., Marques, D.M., O'Connell, R.O.,** and **Stern, F.L.** (1980) *Vomeronasal pump: significance for male hamster sexual behavior.* Science, 207, 1224–1226.

**Meredith, M.** (1994) *Chronic recording of vomeronasal pump activation in awake behaving hamsters*. Physiol. Behav., 56, 345–354.

**Rodriguez, I., Feinstein, P.** and **Mombaerts, P.** (1999) *Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system.* Cell, 97, 199–208.

**Ryba, N.J.** and **Tirindelli, R.** (1997) *A new multigene family of putative pheromone receptors*. Neuron, 19, 371–379.

**Shinohara, H., Kato, K.** and **Asano, T.** (1992a) *Differential localization of G proteins, Gi and Go, in the olfactory epithelium and the main olfactory bulb of the rat*. Acta Anat. Basel, 144, 167–171.

**Shinohara, H. Asano, T.** and **Kato, K.** (1992b) *Differential localization of G-proteins Gi and Go in the accessory olfactory bulb of the rat*. J. Neurosci., 12, 1275–1279.

**Shnayder, L., Schwanzel-Fukuda, M.** and **Halpern, M.** (1993) *Differential OMP expression in opossum accessory olfactory bulb*. NeuroReport, 5, 193–196.

**Takami, S., Graziadei, P.P.** and **Ichikawa, M.** (1992) *The differential staining patterns of two lectins in the accessory olfactory bulb of the rat.* Brain Res., 598, 337–342.

**Takigami, S., Osada, T., Yoshida-Matsuoka, J., Matsuoka, M., Mori, Y.** and **Ichikawa, M.** (1999a) *The expressed localization of rat putative pheromone receptors.* Neurosci. Lett., 272, 115–118.

**Takigami, S., Mori, Y.** and **Ichikawa, M.** (1999b) *Different pattern of vomeronasal nerve projections from rodents in caprine and feline.* Soc. Neurosci. Abstr., Part 1, 29, 155.3.

**Wysocki, C.J.** (1979) *Neurobehavioral evidence for the involvement of the vomeronasal system in mammalian reproduction*. Neurosci. Biobehav. Rev., 3, 301–341.

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