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Immunocytochemical Study of $G_i2\alpha$ and $G_o\alpha$ on the Epithelium Surface of the Rat Vomeronasal Organ

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Abstract

To investigate in detail the distribution of G protein subtypes $G_i2\alpha$ and $G_o\alpha$ along the surface of the vomeronasal epithelium, we used double labeling immunocytochemical methods and electron microscopy. We examined the immunoreactivity of these surface structures with antibodies against $G_i2\alpha$- and $G_o\alpha$. $G_i2\alpha$ and $G_o\alpha$-positive cells were observed at the epithelial surface and were evenly distributed. Electron microscopy revealed that strong immunoreactivities to both antibodies were observed on the microvilli and knob-like surface structures of receptor cells. No immunoreactivity was found on the microvilli or surface membranes of supporting cells. This expression pattern is similar to that reported for putative pheromone receptors. These data confirm that there are two distinct classes of vomeronasal receptor cells expressed at the surface of the epithelium. These two classes of receptors correspond to the same $G_i2\alpha$- and $G_o\alpha$-positive cells distributed in cell body layers of the epithelium and in the axon terminals in the accessory olfactory bulb.

Introduction

The mammalian vomeronasal organ (VNO) is a tubular structure within the nasal septum and contains sensory receptor cells that detect chemical signals, such as conspecific pheromones (Meredith, 1983; Farbman, 1992). In rodents, the VNO plays an important role in sexual and reproductive behaviors (Keverne and de la Riva, 1982; Kaba and Nakanishi, 1995). Vomeronasal receptor cells are bipolar neurons whose apical dendrites reach the epithelial surface and form a knob-like structure covered with microvilli (Rodriguez *et al.*, 1999; Matsuoka *et al.*, 2000). VNO receptor cells express pheromone receptors (Dulac and Axel, 1995; Matsunami and Buck, 1997) and project axons directly to the accessory olfactory bulb (AOB) (Belluscio *et al.*, 1999; Rodriguez *et al.*, 1999). Vomeronasal receptors are classified into two groups: the V1R receptor subtype, which is coexpressed with the G protein $G_i2\alpha$, and the V2R subtype, which is coexpressed with $G_o\alpha$ (Bargmann, 1997; Matsunami and Buck, 1997). *In situ* hybridization studies have shown that receptor cells coexpressing V1R and $G_i2\alpha$ are located in the apical region of the vomeronasal sensory epithelium, while receptor cells coexpressing V2R and $G_o\alpha$ are located in the basal region (Bargmann, 1997; Herrada and Dulac, 1997). In rodents, immunoreactivity for $G_i2\alpha$ is preferentially located in the rostral region of the AOB, while that for $G_o\alpha$ is located in the caudal region (Shinohara *et al.*, 1992; Halpern *et al.*, 1995; Jia and Halpern, 1996). Both G proteins and pheromone receptors are thought to play an essential role in the receptor signaling cascade (Berghard and Buck, 1996). In addition, it has been suggested that the zone-specific expression of G protein subtypes and their mapping to different locations in the AOB may have functional significance for discrimination of pheromones (Inamura *et al.*, 1999; Kumar *et al.*, 1999). Thus, the distribution of the G protein subtypes in the vomeronasal epithelium and their axon projections back to the AOB may play an important role in the transmission and encoding of pheromonal signals.

There have been no detailed studies examining G protein expression patterns at the epithelium surface, even though this is considered to be the region of pheromone–receptor interaction. Recently, it was reported that a putative pheromone receptor is expressed on the microvilli and knob-like surface structures of vomeronasal receptor cells. (Takigami *et al.*, 1999). In the present study, we investigated in detail

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the distribution of G protein subtypes $G_i2\alpha$ and $G_o\alpha$ at the surface of the vomeronasal epithelium using immunocytochemical double labeling methods and electron microscopy.

Materials and methods

Double labeling with two G-protein antibodies

Adult male Sprague–Dawley rats (>8 weeks old) were used for $G_i2\alpha$ and $G_o\alpha$ double staining immunocytochemical examination. Three animals were deeply anesthetized with pentobarbital and perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The VNOs were removed and postfixed overnight with 4% PFA. They were then cut into 8-μm-thick sections in a cryostat (JUNG GM 3000, Leica), and the sections were mounted on gelatin-coated slides. For $G_i2\alpha$ and $G_o\alpha$ immunocytochemistry, the sections were incubated with an anti-$G_i2\alpha$ antibody (mouse monoclonal IgG, Chemicon, Temecula, CA) and an anti-$G_o\alpha$ antibody (rabbit polyclonal IgG, Medical and Biological Lab., Japan) both diluted at 1:100, for 1 h at 37°C. After washing in phosphate-buffered saline (PBS) for 30 min, the sections were incubated with an anti-mouse Ig biotinylated antibody and anti-rabbit rhodamine diluted at 1:100, for 1 h at 37°C, washed in PBS for 30 min, and incubated with streptavidin labeled with a fluorescent dye. The sections were washed in PBS for 30 min, then coverslipped with a water-based mounting solution (PermaFlour, LIPSHAW), and examined under a confocal laser scan microscope (LSM510, Zeiss).

Immunocytochemical electron microscopy and data analysis

Seven adult male Sprague–Dawley rats (>8 weeks old) were used for electron microscopy observations. Five animals were used to study the normal distribution of the G proteins in the vomeronasal epithelium. In the two remaining animals, degeneration of the receptor cells was induced by a vomeronasal nerve transection (Ichikawa *et al.*, 1998). After six days, the two treated and five intact animals were prepared for immunocytochemical electron microscopy. The animals were deeply anesthetized and perfused with 0.9% saline followed with 4% PFA in 0.1 M phosphate buffer. The VNOs were removed and postfixed overnight with 4% PFA. For each animal, two sets of 1 μm thick samples were cut from the middle region of the VNO. The samples were rinsed with 0.1 M PBS and nonspecific binding components were then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. One set of the VNO samples was incubated with an antibody against $G_i2\alpha$ (rabbit polyclonal IgG, Wako, Japan; diluted 1:500 with BSA–PBS) for ~50 h at 4°C. The other set was incubated with the above-mentioned antibody against $G_o\alpha$ diluted 1:500 with BSA–PBS. After washing in PBS for 10 min, all sections were incubated with a biotinylated goat anti-rabbit IgG (Vector Laboratories) in PBS with 0.5% BSA for 2 h at room temperature. After a second wash in PBS, the sections were incubated with a streptavidin–biotinylated horseradish peroxidase (HRP) complex (Amersham) in PBS for 1 h. The sections were then rinsed again with PBS, washed twice with 175 mM sodium acetate buffer and incubated with 0.004% 3,3′-diaminobenzidine (DAB). Samples were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated and embedded in epoxy resin (Quetol 812). Ultrathin sections of vomeronasal epithelium were prepared and examined under an electron microscope (Hitachi, model H-7500) and photomicrographed. Data analysis consisted of identifying the characteristics of supporting and receptor cells, counting the number of immunopositive receptor cells and calculating the ratio between the number of immunopositive cells and the total number of receptor cells. Measurements of the surface membrane length for all immunopositive receptor cells were obtained using a computer-based image analysis system (Zeiss Videoplan) (Matsuoka *et al.*, 2000). All procedures were approved by the Institutional Animal Care Ethics Committee of Tokyo Metropolitan Institute for Neuroscience.

Results

Double labeling with anti-$G_i2\alpha$ and anti-$G_o\alpha$ antibodies

We double stained the vomeronasal sensory epithelium with antibodies against $G_i2\alpha$ and $G_o\alpha$ to investigate the distribution of these G protein subtypes within the epithelium. In the lower portion of the vomeronasal sensory epithelium, we observed many $G_o\alpha$-positive neurons, whose dendrite processes reached the epithelial surface. In the upper portion of the epithelium, we observed $G_i2\alpha$-positive neurons whose dendrite processes also reached the epithelial surface (Figure 1). In the uppermost region of the $G_i2\alpha$-positive area, we also observed cell bodies of supporting cells, which were not labeled (Figure 1). There appeared to be an overlap of the areas labeled by the two antibodies at the epithelial surface where microvilli extend into the lumen. However, there was no overlap among the dendrite processes reaching the surface of the epithelium. In some regions, there were small groups of cells that stained with the same antibody (Figure 1; arrowheads), however, in most regions there was an alternating distribution of dendrites staining for the two different antibodies (Figure 1; between large arrows). Thus, at the surface of the vomeronasal epithelial, cells exhibiting immunopositivity for the two G protein subtype antibodies appeared to be randomly distributed as shown in Figure 1.

Observation by electron microscopy

Electron microscopy made it possible to identify and discriminate between vomeronasal receptor cells and supporting cells along the surface of the epithelium. Receptor cells had low electron density, appeared light and had several centrioles directly under the surface knob. In contrast,

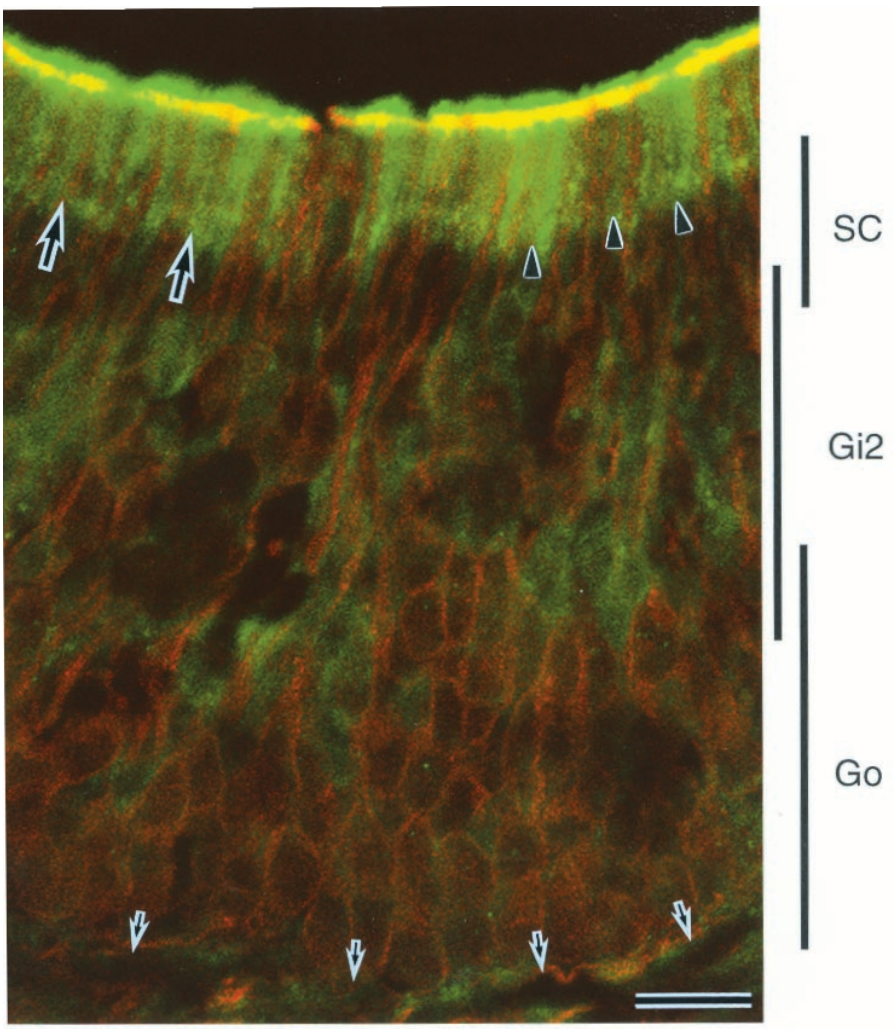

Figure 1 Photomicrograph of the central region of the vomeronasal sensory epithelium. Double staining with $G_i2\alpha$ (green) and $G_o\alpha$ (red) was examined using a confocal laser-scanning microscope. In the lower region of the epithelium (G_o) and extending to the basal lamina (small arrows), many $G_o\alpha$-positive neurons can be seen. In the upper region (G_i2), many $G_i2\alpha$-positive neurons are present. Just above the $G_i2\alpha$-positive region there is a zone of unlabeled supporting cell bodies (SC). However, near the surface, receptor cell dendrite processes are seen, labeled with either $G_i2\alpha$ or $G_o\alpha$. Most cell types are distributed in an alternating fashion (arrows) although there are a few cells of the same type clustered together (arrowheads). There is an overlapping area of $G_i2\alpha$ and $G_o\alpha$ immunoreactivity at the epithelial surface (yellow). Scale bar represents 25 μm.

supporting cells had high electron density, appeared dark and did not have any centrioles. The microvilli of the receptor cells were thin and tapered, while those of the supporting cells were thicker and more uniform in diameter. The knob-like surface structure of the receptor cells protruded into the lumen while the surface of the supporting cells was flat. Receptor and supporting cells were evenly dispersed along the surface of the vomeronasal epithelium. The pattern of immunoreactivity to antibodies against $G_i2\alpha$ and $G_o\alpha$ along the epithelial surface was identified by examination of photographs taken with the electron microscope. There was no detectable difference in the structural morphology or distribution patterns of the cells that were immunoreactive to the two different G protein subtypes on the surface of the vomeronasal epithelium. In both cases, strong immunoreactivity was observed on the microvilli and surface knob-like structures of receptor cells. Not all receptor cells were immunopositive for any given G protein subtype (Figure 2). None of the supporting cells in the epithelium showed any immunoreactivity towards either of the antibodies. For each of the two G protein subtypes, we calculated the ratio of the number of immunopositive receptor cells to the total number of receptor cells in a given section. There was a slightly higher ratio of $G_i2\alpha$-positive cells, but the difference was not statistically significant. The ratio of immunopositive cells for each G protein antibody was ~50% of all receptor neurons (Table 1). We also measured the surface membrane length (Matsuoka *et al.*,

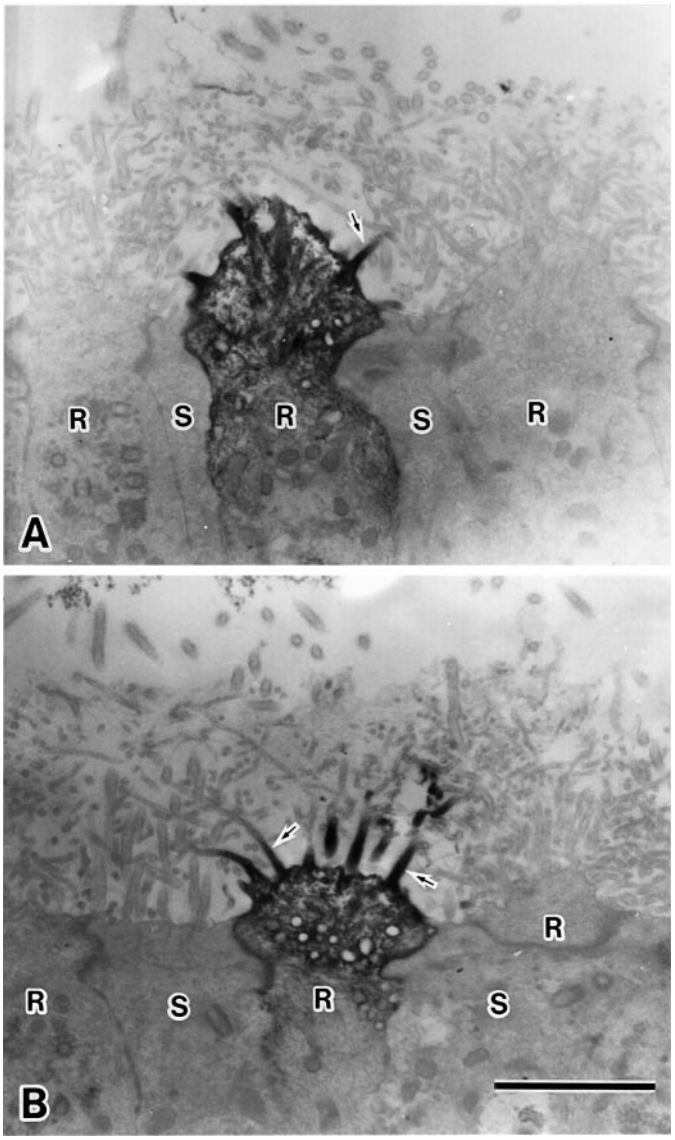

Figure 2 Electron photomicrographs from two sections of the luminal surface of the sensory epithelium stained with antibodies against $G_i2\alpha$ **(A)** and $G_o\alpha$ **(B)**. Immunoreactivity was found on the knob-like structures on the surface and microvilli (arrows) of the receptor cells (R). No immunoreactivity was found on supporting cells (S). Not all receptor cells were immunopositive for $G_i2\alpha$ or $G_o\alpha$ and neighboring receptors cells often did not stain positively for the same antibody. Scale bar represents 2 μm.

2000) for each immunopositive cell. No significant difference in surface membrane length was found between the two groups of cells (Table 1). Samples taken from the group of animals that underwent vomeronasal nerve transection showed morphological changes characteristic of receptor cell degeneration (Figure 3). The knob-like structures on the receptor cells disappeared and the apical surfaces of these cells appeared to be flat and there were few or no microvilli on the surface of the receptor cells. Immunoreactivity towards antibodies against both $G_i2\alpha$ and $G_o\alpha$, however, was still observed on the surface of degenerated receptor cells.

Table 1 The percentage of receptor cells that were immunoreactive towards antibodies against $G_i2\alpha$ and $G_o\alpha$ and the surface membrane length

G protein subtype	No. of animals	% of immunoreactive cells (mean ± SEM)	Length (μm) (mean ± SEM)
$G_i2\alpha$	5	50.9 ± 6.4	2.84 ± 0.04 (n = 301)
$G_o\alpha$	5	43.8 ± 2.3	2.87 ± 0.06 (n = 301)

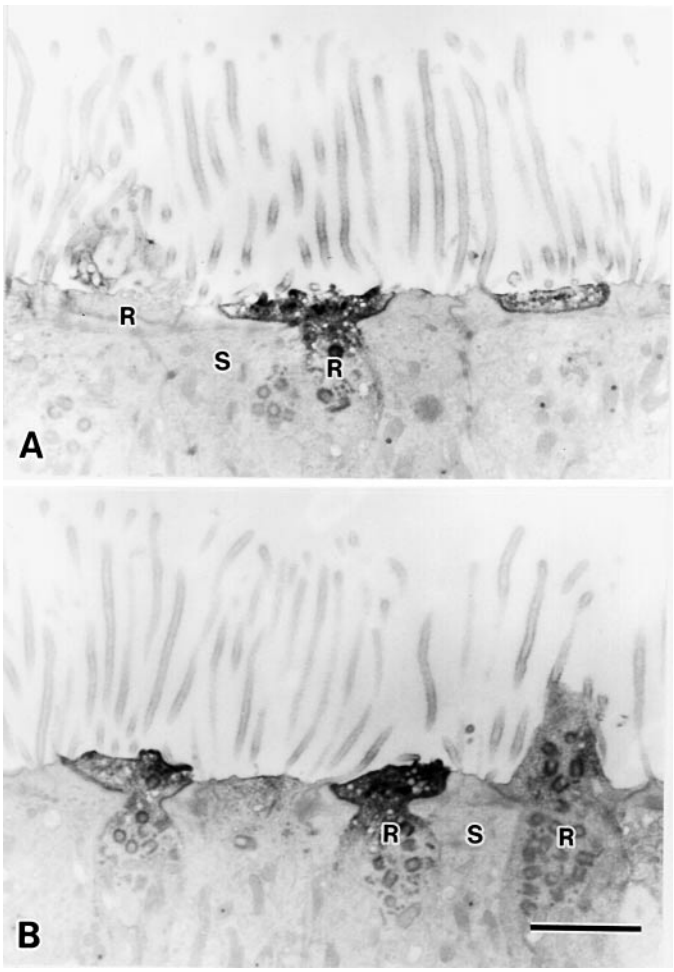

Figure 3 Electron photomicrograph of the surface of the vomeronasal epithelium 6 days after vomeronasal nerve transection. Degenerating receptor cells (R) have few or no microvilli and their surface membranes appear to be flat compared with the knob-like swelling in the controls. Antibody staining for $G_i2\alpha$ **(A)** and $G_o\alpha$ **(B)** is still present in the degenerating receptor cells 6 days after nerve transection. S, supporting cell. Scale bar represents 2 μm.

Discussion

The expression patterns of two G protein subtypes in the sensory neurons of the vomeronasal epithelium have been examined by immunocytochemical and *in situ* hybridization

studies (Shinohara *et al.*, 1992; Halpern *et al.*, 1995; Jia and Halpern, 1996; Bargmann, 1997; Herrada and Dulac, 1997). *In situ* hybridization studies have shown that rat receptor cell bodies expressing $G_i2\alpha$ or $G_o\alpha$ are localized in different layers within the vomeronasal epithelium (Matsunami and Buck, 1997). In addition, immunoreactivity to antibodies against $G_i2\alpha$ or $G_o\alpha$ in the glomerular layer of the AOB is separate, being found in the rostral and caudal regions, respectively (Shinohara *et al.*, 1992). However, some overlap in the distribution of immunoreactivity towards the two antibodies cannot be excluded completely. We double stained the vomeronasal sensory epithelium with antibodies against $G_i2\alpha$ and $G_o\alpha$ and obtained results consistent with those of previous reports. We found that all neurons in the vomeronasal epithelium expressed either $G_i2\alpha$ or $G_o\alpha$. In areas adjacent to the vomeronasal lumen, apical dendrites of the receptor cells reached the epithelial surface and were covered with microvilli, which extended into the lumen in all directions. Many microvilli of adjacent neurons are entwined and overlapping within the lumen (Matsuoka *et al.*, 2000). It is presumed that antibody staining of microvilli at the epithelial surface reflects this overlapping. Hence, overlapping of microvilli at the surface does not rule out the possibility that individual vomeronasal receptor cell expresses only one G protein. Using immunocytochemical methods and electron microscopy, we performed a quantitative analysis of receptor cells at the surface of the vomeronasal epithelium. We found that, for each of the two G protein subtypes, ~50% of the receptor cells were immunoreactive. The ratio for $G_i2\alpha$-positive cells (50.9%) was slightly higher than for $G_o\alpha$-positive cells (43.8%); however, this difference was not statistically significant (Table 1). Methods to detect other G-protein subtypes are not presently available in the vomeronasal epithelium. The results of the present study are the first to demonstrate that $G_i2\alpha$- and $G_o\alpha$-positive cells are present in about the same ratio, half of all neurons, and are distributed evenly along the surface of the vomeronasal epithelium. These results are consistent with previous findings that classify receptors into two large groups in the cell body layers of the epithelium and among the axon terminals projecting to the AOB.

Previous studies used light microscopy to investigate expression patterns of G proteins in the vomeronasal system and were limited to examination of the cell body layers of the epithelium and axon terminals in the AOB (Shinohara *et al.*, 1992; Takigami *et al.*, 2000). In the present study, electron microscopy allowed us to examine the expression of G protein subtypes along the surface of the vomeronasal epithelium. Electron microscopy revealed fine structures such as microvilli not seen by light microscopy. Samples from the two animals that underwent vomeronasal nerve transection contained immunopositive receptor cells that degenerated rapidly. No changes were observed in the supporting cells. These results are consistent with those of a previous report (Matsuoka *et al.*, 2000) and provide additional evidence that the G protein subtypes are found in receptor cells. It has been suggested that G protein expression in vomeronasal receptor cells is coupled to pheromone receptors. Recently, immunoreactivity to a polyclonal antibody for the putative pheromone receptor V1R type VN6 was observed on the microvilli and knob-like structures of vomeronasal receptor cells. Not all vomeronasal receptor cells were immunoreactive towards the pheromone receptor antibody, suggesting that the V1R type pheromone receptor is not present in all receptor cells (Takigami *et al.*, 1999). We attempted to demonstrate a correlation between the expression patterns of the G proteins and the putative pheromone receptors on the surface of the vomeronasal epithelium by examining in detail the surface knobs and microvilli of receptor cells in the present study. Immunoreactivity towards the anti-$G_i2\alpha$ antibody was observed on microvilli and knob-like structures on the surface of receptor cells. This correlation suggests that the putative pheromone receptor VN6 could be coupled to $G_i2\alpha$. It is possible that the coexpression of the G proteins and pheromone receptors represents functional coupling of the two molecules in the pheromonal transmission cascade. Additional studies using morphological, physiological or molecular strategies may be helpful in confirming this possibility.

We attempted to quantify the immunoreactivity of vomeronasal receptor cells to antibodies against G protein using electron microscopy. In previous studies of receptor cell body layers and glomerular layers in the AOB, quantification of G protein distribution in individual cells was not practical. However, by electron microscopic observations of the epithelial surface it is relatively easy to identify and count immunopositive cells. No morphological differences were detected between the receptor cells immunopositive for the two G protein subtypes. In addition, measurements of the surface membrane length revealed no significant difference between the two classes of immunopositive cells (Table 1). Thus, at the vomeronasal epithelial surface, it was not possible to distinguish, by morphological characteristics alone, between receptor cells expressing $G_i2\alpha$ and $G_o\alpha$, that is, V1R-type receptor cells and V2R-type receptor cells. Electron immunocytochemical methods used in the present study may be useful in the future to identify V1R-type and V2R-type vomeronasal receptor cells.

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