NOTE *Anatomy*

Identification of G Protein α Subunits in the Main Olfactory System and Vomeronasal System of the Japanese Striped Snake, *Elaphe quadrivirgata*

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ABSTRACT. In the olfactory system, G proteins couple to the olfactory receptors, and G proteins expressed in the main olfactory system and vomeronasal system vary according to animal species. In this study, G protein α subunits expressed in the main olfactory system and vomeronasal system of the snake were identified by immunohistochemistry. In the olfactory epithelium, only anti-$G_{\alpha olf/s}$ antibody labeled the cilia of the receptor cells. In the vomeronasal epithelium, only anti-$G_{\alpha o}$ antibody labeled the microvilli of the receptor cells. In the accessory olfactory bulb, anti-$G_{\alpha o}$ antibody stained the whole glomerular layer. These results suggest that the main olfactory system and the vomeronasal system of the snake express $G_{\alpha olf}$ and $G_{\alpha o}$ as G proteins coupling to the olfactory receptors, respectively.
KEY WORDS: electron microscopy, nervous system, reptiles, squamates, western blotting.

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Most tetrapods possess 2 olfactory systems: the main olfactory system (MOS) and the vomeronasal system (VNS). The MOS possesses the olfactory epithelium (OE) in the nasal cavity as the receptor organ and the main olfactory bulb as the primary center, while the VNS possesses the vomeronasal epithelium (VNE) in the vomeronasal organ as the receptor organ and the accessory olfactory bulb as the primary center. In most vertebrates, the olfactory receptors are divided into 3 families; the odorant receptors (OR), vomeronasal receptors 1 (V1R) and vomeronasal receptors 2 (V2R). The OR is coupled to G protein α-olf subunit ($G_{\alpha olf}$) [6], and the V1R and V2R are coupled to G protein α-i2 subunit ($G_{\alpha i2}$) and α-o subunit ($G_{\alpha o}$), respectively [1]. These receptor families and G proteins expressed in the MOS and VNS vary according to animal species [3]. However, there are few reports on G proteins expressed in the MOS and VNS of Squamates (snakes and lizards), although it is well known that they have the most developed VNS among vertebrates [4, 10].

Unlike many other animal species, snakes directly depend on both the MOS and VNS in predatory, defensive and courtship behaviors [8, 11, 13]. The VNS of snakes receives the environmental substances sampled by the tongue-flicking, while the MOS receives the substances through airway [4]. To understand the unique olfactory system of snakes in more detail, it appears to be helpful that G proteins coupling to the olfactory receptors expressed in the MOS and the VNS are identified in snakes. In this study, G proteins expressed in the MOS and VNS of the Japanese striped snake, *Elaphe quadrivirgata*, were identified by immunohistochemistry.

Adult male (n=9) and female (n=7) Japanese striped snakes were used. They were purchased from the Japan Snake Institute (Ota, Japan), where they were kept in near-natural conditions. The study was conducted according to the Guideline for Animal Experiment of Iwate University, and an experimental protocol accepted by the Animal Research Committee of Iwate University.

First of all, we determined the partial sequences of the snake $G_{\alpha olf}$, $G_{\alpha i2}$ and $G_{\alpha o}$ to conform the protein homology of the snake G proteins and mammal G proteins. Total RNA was purified from brain by the use of ISOGEN (Nippon Gene, Tokyo, Japan). The cDNA library synthesized by Omniscript Reverse Transcriptase kit (QIAGEN, Valencia, CA, U.S.A.) was amplified by polymerase chain reaction (PCR) using AmpliTaq Gold PCR Master Mix (Applied Biosystems Inc., Foster City, CA, U.S.A.) and degenerate primers. The degenerate primers used in this study were $G_{\alpha olf}$ sense (5′-GTG ACC ATA GTT TCA GCA ATG-3′), $G_{\alpha olf}$ antisense (5′-TGC ATY CKC TGG ATG ATG TC-3′), $G_{\alpha i2}$ sense (5′-ATG GGC TGY ACS KYS WSN GCC GA-3′), $G_{\alpha i2}$ antisense (5′-GTG TAG ATC TCC TTG GTG TCW TT-3′), $G_{\alpha o}$ sense (5′-AGY ACS ATY GTS AAR CAG ATG-3′) and $G_{\alpha o}$ antisense (5′-CTC AAA RCA GTG RAT CCA YTT CTT-3′). PCR amplifications were performed for 30 sec at 95°C, 30

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sec at 55°C and 60 sec at 72°C for 35 cycles. The cDNA fragments ligated into pGEM-T Easy Vector (Promega, Madison, WI, U.S.A.) were transformed into *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA, U.S.A.). Based on the partial nucleotide sequences of $G_{\alpha olf}$ mRNA (851 bp, GeneBank accession number AB733380), $G_{\alpha i2}$ mRNA (965 bp, GeneBank accession number AB733381) and $G_{\alpha o}$ mRNA (507 bp, GeneBank accession number AB733382), the partial peptide sequences of $G_{\alpha olf}$ (281 amino acids relative to estimated full-length 381 amino acids), $G_{\alpha i2}$ (322 amino acids relative to estimated full-length 355 amino acids) and $G_{\alpha o}$ (169 amino acids relative to estimated full-length 354 amino acids) were determined. Although the sequences of $G_{\alpha olf}$, $G_{\alpha i2}$ and $G_{\alpha o}$ determined in this study were partial, it was shown that the snake $G_{\alpha olf}$, $G_{\alpha i2}$ and $G_{\alpha o}$ possess high identities of peptide sequence with each rat G protein α subunit ($G_{\alpha olf}$, 92.9%; $G_{\alpha i2}$, 93.5%; $G_{\alpha o}$, 94.1%).

In immunohistochemistry, polyclonal rabbit anti-$G_{\alpha olf/s}$ (sc-383; Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), monoclonal mouse anti-$G_{\alpha i2}$ (sc-80007; Santa Cruz) and polyclonal rabbit anti-$G_{\alpha o}$ (sc-387; Santa Cruz) antibodies were used as primary antibodies. The peptide sequence of $G_{\alpha olf}$ possesses high identities with that of $G_{\alpha s}$ in mammals, and anti-$G_{\alpha olf/s}$ antibody used in the present study is produced against the common sequence between $G_{\alpha olf}$ and $G_{\alpha s}$. To confirm the specificity of these 3 antibodies, we performed Western blotting. The OE, VNE and brain were homogenized, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes. All steps described below were performed at room temperature unless otherwise indicated. These membranes were incubated with Tris-buffered saline-Tween 20 (TBST) [pH 7.4; 0.05% Tween 20, 25 mM Tris-HCl, 150 mM NaCl] containing 10% EzBlock (ATTO, Tokyo, Japan) and treated with primary antibodies, anti-$G_{\alpha olf/s}$ (1:1,000), anti-$G_{\alpha i2}$ (1: 1,000) or anti-$G_{\alpha o}$ (1: 1,000) antibodies, in TBST for 1 hr. After rinsing in TBST, the membranes were treated with biotinylated secondary antibodies, donkey anti-rabbit IgG (711-066-152; Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) or donkey anti-mouse IgG (715-066-151; Jackson) antibodies, for 30 min and reacted with avidin-biotin complex (ABC) reagent (Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min. Thereafter, the membranes were colorized with 50 mM Tris-HCl containing 0.006% H_2O_2 and 0.02% 3–3′-diaminobenzidine tetrahydrochloride (DAB). Control staining was performed by the use of normal donkey serum to replace primary antibodies. The molecular weight of rat $G_{\alpha olf}$ is 45 kDa, that of rat $G_{\alpha i2}$ is 41 kDa and that of rat $G_{\alpha o}$ is 40 kDa. As the results, anti-$G_{\alpha olf/s}$ antibody detected a protein of approximate 45 kDa in the OE and brain, but not in the VNE (Fig. 1A). Anti-$G_{\alpha i2}$ antibody detected a protein of approximate 40 kDa in the brain, but not in the OE and VNE (Fig. 1B). Anti-$G_{\alpha o}$ antibody detected a protein of approximate 40 kDa in all the tissues examined (Fig. 1C). In the control staining, no specific band was detected (Fig. 1D). These results show that the antibodies used in this study reacted specifically with each snake homolog.

For immunohistochemistry, the animals were anesthetized by intraperitoneal injection of pentobarbital and sacrificed by cardiac perfusion with Ringer's solution followed by 4% paraformaldehyde. The OE, VNE and accessory olfactory bulbs were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and cut at 10 μm in thickness. Sections were rinsed in 0.01 M phosphate buffered saline (PBS, pH 7.4), incubated with 5% bovine serum albumin and treated with primary antibodies, anti-$G_{\alpha olf/s}$ (1:500), anti-$G_{\alpha i2}$ (1:100) or anti-$G_{\alpha o}$ (1:200) antibodies, in PBS at 4°C overnight. Several sections were treated with Alexa Flour 594-labeled secondary antibodies (1:100), donkey anti-rabbit IgG (A-11037; Molecular Probes, Eugene, OR, U.S.A.) or donkey anti-mouse IgG (A-11032; Molecular Probes) antibodies, for 1 hr. Other sections were colorized with ABC method in the same manner as the method of Western blotting described above. Control stainings were performed by the preabsorption of anti-$G_{\alpha olf/s}$ and anti-$G_{\alpha o}$ antibodies with each blocking peptide (sc-383P and sc-387P, respectively, Santa Cruz).

In the MOS, anti-$G_{\alpha olf/s}$ antibody labeled the cilia (Fig. 2A, arrowheads), dendrites, cell bodies and axons of the receptor cells in the OE (Fig. 2A). Anti-$G_{\alpha o}$ antibody labeled the dendrites, cell bodies (Fig. 2C) and axons of the olfactory receptor cells in the OE, but did not label the cilia (Fig. 2C, arrowheads). Anti-$G_{\alpha i2}$ antibody did not label the OE (Fig. 2B). In control staining, no specific reaction was observed (Fig. 2D). In the OE of snakes, the receptor cells possess the cilia, and the supporting cells do not possess them as shown by scanning electron microscopy [14]. As only anti-$G_{\alpha olf/s}$ antibody labeled the cilia, we conclude that G protein coupling to the olfactory receptors is only $G_{\alpha olf}$ in the MOS. On the other hand, as it is suggested that $G_{\alpha o}$ is involved in synaptic functions and cell-to-cell contacts in the nervous systems [5], $G_{\alpha o}$ expressed in the MOS appears to play a role in these neuronal activities.

In the VNS, anti-$G_{\alpha o}$ antibody labeled the free border and the cell bodies and axons of the receptor cells in the VNE, although it did not label the protruded ventral wall, which called the mushroom body, covered with the non-sensory epithelium (Fig. 2G). Anti-$G_{\alpha olf/s}$ and anti-$G_{\alpha i2}$ antibodies did not label the VNE (Fig. 2E, F). In addition, anti-$G_{\alpha o}$ antibody stained the whole glomerular layer of accessory olfactory bulb (Fig. 2K), while anti-$G_{\alpha olf/s}$ and anti-$G_{\alpha i2}$ antibodies did not stain them (Fig. 2I, J). In control staining, no specific

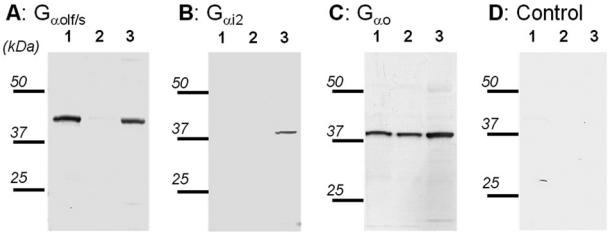

Fig. 1. Western blotting of the Japanese striped snake $G_{\alpha olf}$ (A), $G_{\alpha i2}$ (B), $G_{\alpha o}$(C) and negative control (D). Extracts of the olfactory epithelium (Lane 1), vomeronasal epithelium (Lane 2) and brain (Lane 3) were used.

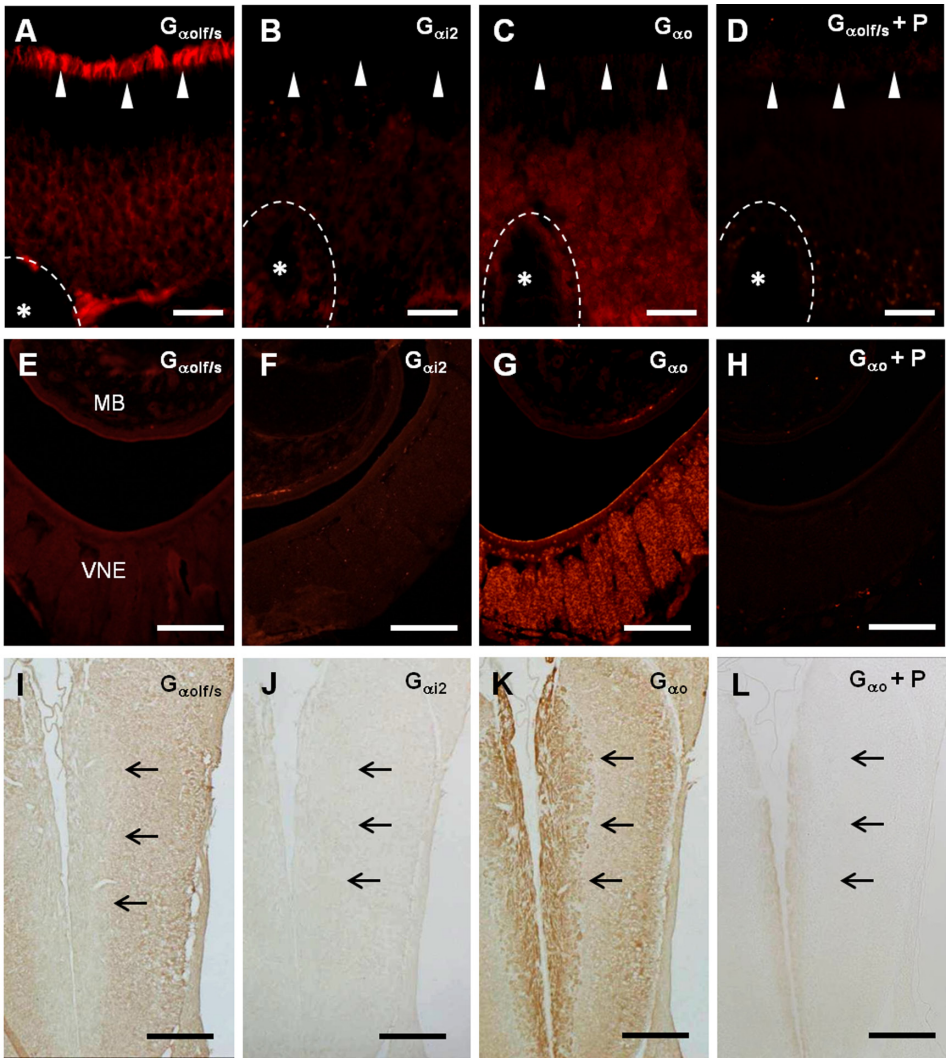

Fig. 2. Immunohistochemistry of $G_{\alpha olf}$ (A, E and I), $G_{\alpha i2}$ (B, F and J), $G_{\alpha o}$ (C, G and K) and control stainings (D, H and L) in the olfactory epithelium (A to D), vomeronasal epithelium (E to H) and accessory olfactory bulb (I to L) of the Japanese striped snake. Arrowheads and asterisks indicate the cilia and the Bowman's glands, respectively, in (A) to (D). Arrows indicate the basal region of the glomerular layer (I) to (L). The left sides of the figures are medial, and the upper, rostral in (I) to (L). VNE, vomeronasal epithelium; MB, mushroom body. Bars=20 μm in (A) to (D), 200 μm in (E) to (H) and 300 μm in (I) to (L).

reaction was observed (Fig. 2H, L). These results indicate that the VNS in the snake expresses only $G_{\alpha o}$. However, as both the receptor cells and supporting cells possess the microvilli as shown by scanning electron microscopy [14], we had to confirm that $G_{\alpha o}$ was expressed in the microvilli of the receptor cells, not supporting cells, to discuss that $G_{\alpha o}$ couples to the olfactory receptors in the VNS.

To detect which structures were positive to anti-$G_{\alpha o}$ antibody, the VNE was examined with transmission electron microscopy (TEM). The animals were anesthetized and sacrificed by cardiac perfusion with Ringer's solution followed by 3% paraformaldehyde and 0.2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4). The VNE was embedded in OCT compound and cut at 25 μm in thickness. Sections were incubated with 2.5% bovine serum albumin at 4°C and treated with anti-$G_{\alpha i2}$ (1:100) or anti-$G_{\alpha o}$ (1:500) antibodies in PBS for 3 days at 4°C. After rinsing in PBS, sections were colorized with ABC method in the same manner as the method of Western blotting described above. Thereafter, sections were postfixed in 1% OsO_4 for 1 hr at 4°C and routinely embedded in epoxy resin. Ultrathin sections of 80 nm in thickness were cut and observed with TEM (H-800; Hitachi, Tokyo, Japan, or JEM-2100; JEOL, Tokyo, Japan). On the free border of the VNE, anti-$G_{\alpha o}$ antibody stained the microvilli of the receptor cells (Fig. 3A, B, arrowheads), but did not stain the microvilli of the supporting cells (Fig.

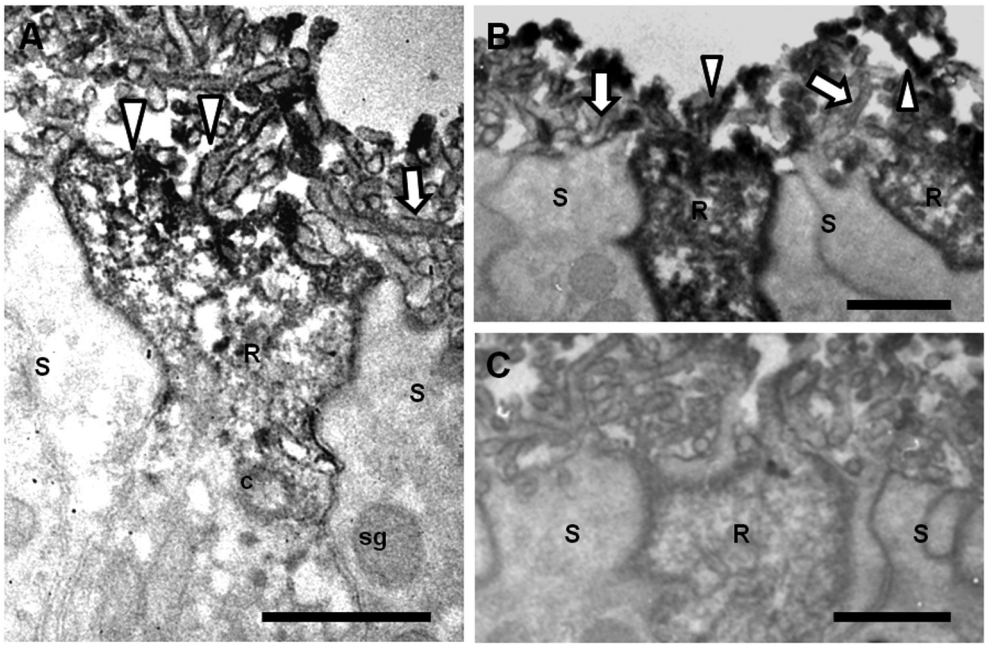

Fig. 3. Transmission electron microscopic immunohistochemistry of $G_{\alpha o}$ (A and B) and $G_{\alpha i2}$ (C) in the vomeronasal epithelium of the Japanese striped snake. Arrowheads indicate the microvilli of the receptor cells (R) with centriol (c), and arrows indicate the microvilli of the supporting cells (S) with the secretory granules (sg) in (A) and (B). Bars=1 μm.

3A, B, arrows). In contrast, anti-$G_{\alpha i2}$ antibody did not stain the microvilli of both the receptor cells and supporting cells at all (Fig. 3C). Considering all results in the present study together, it is strongly indicated that G protein coupling to the olfactory receptors is only $G_{\alpha o}$ in the VNS.

Luo *et al*. reported by immunohistochemistry that $G_{\alpha i}$ and $G_{\alpha o}$ are expressed on the free border of the VNE in the garter snake belonging to the same family as the Japanese striped snake [7]. According to peptide sequence relatedness, $G_{\alpha o}$ belongs to $G_{\alpha i/o}$ family same as $G_{\alpha i2}$, and all members in $G_{\alpha i}$ family possess approximate 70% identities and 80% similarities [5]. In addition, the peptide immunogen for anti-$G_{\alpha i}$ antibody (NNLKECGLY-NH_2) used by Luo *et al*. [7] is corresponding to the sequence of human $G_{\alpha i3}$ and shows 78% identities with both human $G_{\alpha i2}$ and $G_{\alpha o}$ [5]. According to these reports, we consider that $G_{\alpha i}$ expression shown by Luo *et al*. [7] appears to be equal to $G_{\alpha o}$ expression shown by the present immunohistochemistry in the VNE and does not compete to the present results.

We demonstrated that the MOS and VNS of the snake mainly express $G_{\alpha olf}$ and $G_{\alpha o}$ as G proteins coupling to the olfactory receptors, respectively. In the olfactory system, it is suggested that $G_{\alpha olf}$ and $G_{\alpha o}$ couple to the OR and V2R, respectively [1, 6]. The OR shows high affinities for small airborne substances [12, 15], and the V2R shows high affinity for large water-soluble substances [2, 9]. Considering these reports and the present results together, it is suggested that the MOS and the VNS of snakes receive small airborne substances and large water-soluble substances, respectively, although the MOS and VNS of snakes mediate the similar behaviors [8, 11, 13]. As the V2R-$G_{\alpha o}$ shows high affinity for nonvolatile water-soluble substances, only the V2R-$G_{\alpha o}$ expression in the VNS appears to have a disadvantage for terrestrial species. However, as snakes appear to be able to sample efficiently nonvolatile water-soluble substances by their tongue-flicking, it may be a practical advantage that snakes recognize the objects, such as their prey, through nonvolatile water-soluble substances received by the VNS, even after small airborne substances received by the MOS disappear. Phylogenetically, as amphibians possess the V2R-$G_{\alpha o}$ but not the V1R-$G_{\alpha i2}$ in the VNS and many mammals possess the V1R-$G_{\alpha i2}$ in the VNS, the V1R appears to have begun expression in the VNS during the evolution from amphibians to mammals [3]. Therefore, the exclusive expression of $G_{\alpha o}$ in the snake VNS shown by the present study indicates that snakes evolved in the different way from mammals before the onset of the V1R-$G_{\alpha i2}$ expression in the VNS.

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