Goofy Coordinates the Acuity of Olfactory Signaling

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The basic scheme of odor perception and signaling from olfactory cilia to the brain is well understood. However, factors that affect olfactory acuity of an animal, the threshold sensitivity to odorants, are less well studied. Using signal sequence trap screening of a mouse olfactory epithelium cDNA library, we identified a novel molecule, Goofy, that is essential for olfactory acuity in mice. Goofy encodes an integral membrane protein with specific expression in the olfactory and vomeronasal sensory neurons and predominant localization to the Golgi compartment. Goofy-deficient mice display aberrant olfactory phenotypes, including the impaired trafficking of adenylyl cyclase III, stunted olfactory cilia, and a higher threshold for physiological and behavioral responses to odorants. In addition, the expression of dominant-negative form of cAMP-dependent protein kinase results in shortening of olfactory cilia, implying a possible mechanistic link between cAMP and ciliogenesis in the olfactory sensory neurons. These results demonstrate that Goofy plays an important role in establishing the acuity of olfactory sensory signaling.

Introduction

The olfactory system integrates sophisticated molecular, cellular, and neural circuit mechanisms for detection and discrimination of a huge variety of odorants in the environment (Axel, 1995; Mori et al., 1999; Mori and Sakano, 2011). Odor molecules inhaled into an animal’s nostrils are detected by the olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) (Buck, 2000). OSNs are bipolar, extending a single axon basally toward the olfactory bulb (OB) and a single dendrite apically toward the epithelial surface. Several long cilia emanate from the dendritic knob into the nasal mucus for efficient reception of odorants (McEwen et al., 2008).

Upon odorant binding to the olfactory cilia, action potentials are generated by the OSN’s signal transduction machinery (Reed, 1992; Touhara and Vosshall, 2009). This signaling process occurs through a sequential activation of a cascade of functional molecules including odorant receptors (ORs), olfactory-specific GTP-binding protein (Golf), adenylyl cyclase III (ACIII), and cyclic nucleotide-gated cation channel, all of which are enriched in the olfactory cilia (Jones and Reed, 1989; Bakalyar and Reed, 1990; Dhallan et al., 1990; Buck and Axel, 1991). Genetic mutations in these signaling molecules lead to olfactory dysfunctions as exemplified by anosmia and hyposmia (McEwen et al., 2008).

Transmembrane and secreted proteins play important roles in a variety of developmental and functional aspects of OSNs. For example, the hierarchical and combinatorial expression of multiple guidance molecules directs convergent projection of OSN axons to target glomeruli in the OB (Mori and Sakano, 2011). In addition, there are other transmembrane and secreted proteins crucial for odor-sensing mechanisms in OE, which facilitate the OR trafficking to plasma membranes (Saito et al., 2004), carry odor molecules in nasal mucus to ORs (Tegoni et al., 2000), and convert odorant information into intracellular second messenger elevation (Gibson and Garbers, 2000). Most of these proteins are characterized by the presence of a signal sequence with a stretch of hydrophobic amino acids at the N terminus.

In this study, we performed a signal sequence trap screening of mouse OE cDNA library and identified a novel transmembrane protein that is specifically expressed in OSNs and vomeronasal sensory neurons (VSNs) and predominantly localized in the Golgi apparatus. Hence, we designated this gene product as Goofy (Golgi protein in olfactory neurons). Genetic ablation of the Goofy gene caused abnormal localization of ACIII, shortening of olfactory cilia, and reduced sensitivity to odorants, indicating a crucial role of Goofy in functioning of the olfactory system.

Materials and Methods

Signal sequence trap screening. Yeast signal sequence trap screening was performed essentially as described previously (Jacobs et al., 1997; Na-
Poly(A) + RNA was prepared from postnatal day (P) 3 mouse OE, reverse-transcribed, and PCR-amplified to generate double-stranded cDNA with EcoRI and XhoI adapters. The double-stranded cDNA was digested with EcoRI and XhoI, size-fractionated by agarose gel electrophoresis (300–800 bp), and ligated unidirectionally into pSUC2T7M13ORI vector (Jacobs et al., 1997) to construct a cDNA library. For screening of cDNAs containing signal peptide sequences, the library was introduced into the invertase-deficient yeast strain YTK12, and selected on Trp plates followed by raffinose-containing plates on which only the transformants harboring cDNA-derived signal peptide-fused invertase gene can grow. Among 686 colonies grown on raffinose plates, cDNA inserts were amplified and sequenced for 258 recombinants, and the presence of a putative signal peptide was confirmed for 215 clones that encoded 49 individual genes.

In situ hybridization and Northern blot analysis. In situ hybridization and Northern blot analysis were performed as described previously (Yoshihara et al., 1997). One to three mice at individual developmental stages were used for these analyses.

Antibody production. To generate anti-Goofy antibody, a peptide corresponding to the C-terminal 19 aa (RGQRELPSAALSFNFF) of mouse Goofy protein was synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits. This antibody labeled OSNs and VSNs, and its immunoreactivity was completely absent in Goofy-deficient mice, confirming its specificity to Goofy protein.

Figure 1. Discovery of Goofy (#123) by signal sequence trap screening. A, Strategy of the signal sequence trap screening for identification of novel transmembrane and secreted molecules expressed in the OE. B, A summary table for individual 49 cDNA clones obtained by the signal sequence trap screening. C, mRNA expression patterns of six novel genes in P3 OE coronal sections assessed by in situ hybridization analysis. The clone #123 (Goofy) is abundantly and specifically expressed in the OE. NG, Nasal gland. Scale bar: C, 500 μm.
against Olfr6/M50, Olfr17/P2, and rat OR-I7 were generated as described previously (Kaneko-Goto et al., 2008).

**Immunohistochemistry.** Section immunohistochemistry was performed as described previously (Kaneko-Goto et al., 2008). Immunolabeling of olfactory cilia in whole-mount OE preparations was performed as described previously (Strotmann et al., 2004). Primary antibodies used were as follows: rabbit anti-Goofy (1:1000); goat anti-OMP (1:10,000; Wako Pure Chemical Industries); rat anti-NCAM (1:500; Millipore BioScience Reagents); rabbit anti-olfactory cell adhesion molecule (anti-OCAM; 1:1000) (Yoshihara et al., 1997); goat anti-NQO1 (1:500; Abcam); goat anti-neuropilin-1 (1:200; R&D Systems); goat anti-neuropilin-2 (1:200; R&D Systems); goat anti-contactin-4/BIG-2 (1:200; R&D Systems); rabbit anti-Golf (1:1000; Santa Cruz Biotechnology); rabbit anti-cyclic nucleotide-gated channel A2 subunit (anti-CNGA2; 1:200; Alomone Labs); rabbit anti-ACIII (1:1000; Santa Cruz Biotechnology); rabbit anti-Olfr6/M50 (1:2000); guinea pig anti-Olfr2/I7 (1:2000); guinea pig anti-Olfr73/mOR-EG (1:1000); guinea pig anti-Olfr1507/P2 (1:1000); guinea pig anti-rat OR-I7 (1:5000); guinea pig anti-Olfr753/mOR-EG (1:1000); guinea pig anti-Olfr1507/MOR28 (1:1000) (Kaneko-Goto et al., 2008). To label intracellular organelles in OSNs, Organelle Sampler Kits (BD Biosciences) and Alexa488-conjugated secondary antibodies were purchased from the manufacturers’ instructions. Cy3-conjugated and Alexa488-conjugated secondary antibodies were purchased from Jackson ImmunoResearch and Invitrogen, respectively. Immunohistochemical analyses were performed on sections (n = 3–10 mice) and whole-mount OE (n = 3–4 mice) for each antibody.

**Western blot analysis.** Western blot analysis was performed on olfactory cilia fraction (3 μg of protein/lane), OE homogenate (10 μg of protein/lane), and OB homogenate (10 μg of protein/lane) from ≥3 mice of each genotype as described previously (Kaneko-Goto et al., 2008). Olfactory cilia fraction was prepared by a calcium shock method as described previously (Washburn et al., 2002). Primary antibodies used were as follows: rabbit anti-Goofy (1:1000); rabbit anti-ACIII (1:1000; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch. Immunoreactive protein bands were visualized with a chemiluminescence reaction kit (ECL Plus Western Blotting Detection System, GE Healthcare) and an image analysis system (LAS-1000, Fujiﬁlm).

**Transgenic and mutant mice.** Goofy-gapVenus transgenic mice were generated with a standard transgenic method. The 3.0 kb 5’-flanking region of Goofy gene was PCR-ampliﬁed from mouse genomic DNA and inserted into pBStN-gapVenus vector (Mitsui et al., 2011) to generate pGoofy-gapVenus plasmid. Goofy-gapVenus transgene was excised, gel-puriﬁed, and injected into the pronucleus of fertilized eggs that were obtained from crossing C57BL/6 and DBA/2J mice. The manipulated eggs were cultured to the two-cell stage and transferred into oviducts of pseudopregnant foster females (ICR strain). Integration of the transgene was screened by PCR of tail DNA.

**Goofy-deﬁcient mice.** Goofy-deﬁcient mice were generated with a standard gene-targeting method. Two genomic DNA fragments of mouse Goofy gene (2.5 kb Spel-KpnI short arm and 6.7 kb XbaI-EcoRIlong arm) were subcloned into pSLS01 vector (Invitrogen) together with a positive selection marker pgk-neo and a negative selection marker DTA to generate the Goofy-targeting vector. Electroporation into 129/SVEv mouse ES cell line followed by G418 selection resulted in four positive homologous recombinants of 288 clones as determined by PCR screening and Southern blot analysis. Chimeric C57BL/6 males that transmitted the mutant allele were obtained, backcrossed with C57BL/6 mice more than nine times, and used for all the experiments.

**Generation and characterization of Olfr73(mOR-EG)-ires-gapEYFP transgenic mice.** Transgenic mice were described previously (Oka et al., 2006). I7 (WT)-ires-ECFP and I7 (WT)-ires-dnPKA-ires-EYFP transgenic mice (RRBC02931 and RRBC02936) (Imai et al., 2006), generous gifts from Drs. Takeshi Imai and Hitoshi Sakano (The University of Tokyo), were provided by RIKEN Bio Resource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan.

**Results**

**cDNA cloning and structure of Goofy.** To search for membrane-bound or secreted proteins involved in development and/or function of OSNs, we adopted a yeast signal sequence trap method, which enables us to selectively isolate cDNAs encoding proteins with a N-terminal hydrophobic signal peptide. Poly(A)+ RNA was prepared from the OE of P3 mice and a cDNA library was constructed in a signal sequence trap.
plasmid (pSUC2T7M13ORI), which harbors a truncated invertase gene without a signal sequence (Jacobs et al., 1997). When the cDNA library was introduced into the invertase-deficient yeast strain YTK12 (total: 1.5 x 10^6 transformants), only 686 transformants capable of secreting invertase could grow on the glucose-free, raffinose-containing plates, due to in-frame invertaseCould grow on glucose-free raffinose-containing plates.

**Expression of Goofy mRNA**

Northern blot analysis was performed to survey the tissue distribution of Goofy mRNA in adult mouse. Goofy mRNA was detected specifically in the OE, but not in other tissues, such as the brain, thymus, lung, heart, kidney, spleen, liver, small intestine, and testis (Fig. 3A).

**Figure 3.** Expression of Goofy mRNA. A, Tissue distribution of Goofy mRNA in adult mouse assessed by Northern blot analysis. Top, Autoradiogram of a blot probed with 32P-labeled Goofy cDNA. Bottom, Same blot stained with methylene blue. Goofy mRNA is specifically expressed in the olfactory epithelium. B–K, Ontogenic expression of Goofy and OMP mRNAs assessed by in situ hybridization analysis. Coronal sections containing the OE and VNO from E13 (1997; Nakashiba et al., 2000). Of the 258 cDNA-derived signal peptide (Jacobs et al., 1997). When the cDNA library was introduced into the invertase-deficient yeast strain YTK12 (total: 1.5 x 10^6 transformants), only 686 transformants capable of secreting invertase could grow on the glucose-free, raffinose-containing plates, due to in-frame invertaseCould grow on glucose-free raffinose-containing plates.

**Figure 4.** Promoter analysis of Goofy gene. A, Organization of mouse Goofy gene (top). Exons are indicated by boxes with numbers: light blue boxes indicate the open reading frame, and white boxes represent 5'-nontranslated and 3'-nontranslated regions. Red asterisks denote canonical Olf-1-binding sites. Transgene construct (bottom). The Goofy gene's 5'-flanking region (3 kb) was fused to rabbit β-globin intron (gray bar), membrane-targeted Venus cDNA (gapVenus (gV)), and polyadenylation (pA) signals. B, C, Whole-mount fluorescence views of Goofy-gV transgenic mouse at E15.5. Intense gapVenus fluorescence is specifically observed in the olfactory axons projecting from OE to OB even from the outside of embryos. A merged view of fluorescence and visible light is shown in C. D–G, Whole-mount fluorescence views of OE, VNO, OB (E, F), and brain (F, G) from adult Goofy-gV transgenic mouse. Specific and intense fluorescence of gapVenus is detected in the olfactory and vomeronasal axons in all the transgenic mice examined (n = 3). Merged views of fluorescence and visible light are shown in E and G. Cx, Cerebral neocortex; Ob, cerebellum. Scale bars: B, 1 mm; D–G, 2 mm.

Ontogenic expression of Goofy mRNA was investigated by in situ hybridization analysis in comparison with OMP (olfactory marker protein) mRNA, a well characterized molecule expressed in all mature OSNs and VSNs (Margolis et al., 1991; Halpern et al., 1998). Goofy mRNA was first observed at embryonic day (E) 11.5 in the OE and at E13.5 in the vomeronasal organ (VNO), steadily increased thereafter, reached maximal level at P14, and maintained robust expression level into adulthood (Figs. 1C, 3B–F; data not shown). In contrast, OMP mRNA was first detectable at E15.5 in the OE and postnatally in the VNO (Fig. 3G–K).

These results indicate that the expression of Goofy mRNA is confined to the OE and VNO throughout development and the onset of Goofy expression significantly precedes OMP.
Promoter analysis of Goofy gene

The unique expression of Goofy mRNA prompted us to investigate its transcriptional regulatory mechanism. The mouse Goofy gene consists of five exons and four introns that are compactly arranged within 2.5 kb on the chromosome 7 (Fig. 4A). Intriguingly, the 5′-upstream region of mouse Goofy gene is highly homologous to the previously reported rat DNA sequence 5′.06, which was discovered as one of the potential targets of the olfactory transcription factor Olf-1 (Wang and Reed, 1993; Wang et al., 1993). The mouse Goofy gene contains three canonical Olf-1-binding motifs (YTC-CCYRGGGAR; Fig. 4A, red asterisks) that are commonly present in olfactory neuron-specific genes, such as ORs, Golf, ACIII, CNGA2, and OMP (Wang and Reed, 1993; Wang et al., 1993; Rothman et al., 2005). We constructed a transgene (Goofy-gV) that harbors a 3.0 kb sequence upstream of the transcriptional start site of Goofy gene, rabbit β-globin intron, membrane-targeted yellow fluorescence protein (gapVenus) cDNA, and SV40 polyadenylation signal (Fig. 4A).

Eight independent lines of Goofy-gV transgenic mice were established, among which gapVenus transgene was detected specifically in the OE and VNO of six lines (75%) at different expression levels. Line #3 mice showed the strongest fluorescence in olfactory axons projecting from the OE to the OB, which could be observed in intact E15.5 embryos by epifluorescent stereomicroscopy (Fig. 4B, C). In adults from this line, intense gapVenus fluorescence was detected in the OE, VNO, and OB, but not in other brain regions or in other tissues (Fig. 4D–G). In addition, we observed gapVenus expression in two other olfactory subsystems: the septal organ and Grueneberg ganglion (data not shown).

These results reinforce the OE-specific and VNO-specific expression of Goofy gene. In addition, the 3.0 kb upstream region of mouse Goofy gene can be used as an efficient promoter to drive specific and robust expression of transgenes in all types of sensory neurons in the olfactory system.

Localization of Goofy protein

To address the localization of Goofy protein in the OE, we generated polyclonal antibodies directed against the C-terminal 20 aa peptide of Goofy protein and performed immunohistochemical analysis in comparison with OMP protein, a marker of mature OSNs. On coronal sections of P7 mouse head, both Goofy...
and OMP proteins were present throughout the OE with no zonal preference (Fig. 5A–C). Higher-magnification views revealed that Goofy protein was expressed in both immature and mature OSNs and most abundantly localized to the cell bodies, while OMP protein was present only in mature OSNs and showed cytoplasmic localization with an almost equal level of immunoreactivity in cell bodies, dendrites, and axons (Fig. 5D–F). Goofy protein was not detected in the globose and horizontal basal cells that are located in the most basal region of OE.

The intracellular localization of Goofy protein in OSNs was examined by double fluorescence labeling with various organelle markers. Goofy protein was colocalized almost perfectly with GM130 (cis-Golgi marker) and WGA (trans-Golgi marker) and partially with BiP (endoplasmic reticulum marker), but not with EEA1 (early endosome marker), Lamp-1 (lysosome marker), and Mcl-1 (mitochondria marker; Fig. 5G–R).

These results indicate that Goofy protein is predominantly localized to the Golgi apparatus in both immature and mature OSNs. A similar Golgi apparatus-specific localization of Goofy protein was also observed in the sensory neurons of the VNO (both apical and basal zones), septal organ, and Grueneberg ganglion, as well as in Goofy cDNA-transfected N2a cells (data not shown).

Abnormal localization of ACIII in Goofy-deficient mice

To elucidate the physiological function of Goofy, we generated Goofy-deficient mice by disrupting the noncoding exons 1 and 2 and a part of exon 3, which encodes the signal peptide of Goofy protein (Fig. 6A,B). The absence of Goofy protein in the mutant mice was validated by Western blot and immunohistochemical analyses (Figs. 6C, 7A, F). Gross anatomy and layer organization of OE and OB in Goofy-deficient mice were not different from those of wild-type mice, as assessed by Nissl staining (Fig. 6D–I).

The olfactory axon wiring from the OE to the OB appeared normal in Goofy-deficient mice, as assessed by both the differential expression patterns of various axon guidance molecules (e.g.,
Goofy

Golf

CNGA2

ACIII

wild-type

Goofy-deficient

A

B

C

D

E

F

G

H

I

J

K

L

M

N

cilia

OE

OB

Figure 7. Abnormal localization of ACIII in Goofy-deficient mice. A–J, Immunofluorescence labeling of OE (A–D, F–I) and OB (E, J) sections with antibodies against Goofy (A, F), Golf (B, G), CNGA2 (C, H), and ACIII (D, E, I, J). In wild-type mice, Golf, CNGA2, and ACIII are highly localized to olfactory cilia, while strong ACIII immunoreactivity is observed also in olfactory axons and glomeruli of mutant mice. K, Immunoblot analysis of ACIII protein in olfactory cilia, whole OE, and OB from three individual mice of each genotype. In Goofy-deficient mice, ACIII is significantly decreased in the cilia (60 ± 7%), slightly decreased in the OE (94 ± 2%), and dramatically increased in the OB (496 ± 41%), compared with wild-type mice (p = 3). L–N, Double immunofluorescence labeling of wild-type OE section with anti-Goofy (L, N) and anti-ACIII (M, N) antibodies. N, A merged image showing colocalization of Goofy (magenta) and ACIII (green) at Golgi apparatus in OSNs. Scale bars: A–D, F–I, 200 μm; E, J, 500 μm; L–N, 20 μm.

Goofy and ACIII from Golgi apparatus to olfactory cilia is impaired in Goofy-deficient mice. In wild-type OE sections with anti-Goofy (epitope), immunofluorescence labeling was observed not only in the olfactory cilia and cell bodies, but also in axons innervating OB glomeruli (Fig. 7 D, E, F, I, J). Western blot analysis also revealed the abnormal localization of ACIII in Goofy-deficient mice with a substantial decrease in the extent of cilial coverage (60 ± 7% of wild-type mice) and a drastic increase in the OB (496 ± 41% of wild-type mice) (Fig. 7 K). A high-magnification image of wild-type OE revealed that Goofy and ACIII proteins are colocalized in Golgi apparatus of OSNs (Fig. 7 I–N). These results suggest that the intracellular trafficking of ACIII from Golgi apparatus to olfactory cilia is impaired in Goofy-deficient mice.

Shortened olfactory cilia in Goofy-deficient mice

Next, we compared the OR protein localization in OSNs between wild-type and Goofy-deficient mice. Specific antibodies recognizing four well-characterized OR proteins were used for this analysis: anti-Olfr2 (17), anti-Olfr6 (M50), anti-Olfr17 (P2), and anti-Olfr73 (mOR-EG) (Kaneko-Goto et al., 2008). By whole-mount immunofluorescence labeling of wild-type OE, Olfr2 and Olfr6 proteins were clearly detectable on the olfactory cilia emanating from dendritic knobs of OSNs (Fig. 8 A). In contrast, the length of Olfr2-positive and Olfr6-positive cilia appeared markedly shorter in Goofy-deficient OE (Fig. 8 B). To verify whether the use of OR antibodies is sufficient to assess the cilia length, we used Olfr73-ires-gapEGFP transgenic mice in which membrane-tethered GFP is expressed in Olfr73-expressing OSNs (Oka et al., 2006). Double immunofluorescence labeling of OE sections of Olfr73-ires-gapEGFP mice on Goofy-deficient background revealed that Olfr73 signals in olfactory cilia almost completely overlapped with GFP signals (Fig. 8 C–E). Thus, OR proteins are properly trafficked even to the most distal part of olfactory cilia, validating the appropriateness of using OR antibodies for assessment of the cilia length. When the OE sections of wild-type and Goofy-deficient mice were immunostained with anti-Olfr2, anti-Olfr17, and anti-Olfr73 antibodies, a clear difference was observed in the extent of cilial coverage of individual OSNs on OE surface (Fig. 8 F–K). Quantification of cilia length revealed that the olfactory cilia in Goofy-deficient mice were significantly shorter than those in wild-type mice for all three OR-expressing OSNs examined (Fig. 8 L–N).

In various cell types, the length of primary cilia is regulated through the cAMP signaling pathway. Elevation of intracellular cAMP levels results in the elongation of primary cilia, whereas decreased cAMP leads to cilium shortening (Besscheltnova et al., 2010; Abdul-Majeed et al., 2012). Because the olfactory cilia are classified as a specialized form of the primary cilia (Singla and Reiter, 2006; Berbari et al., 2009), it is conceivable that the cAMP signaling may also control cilia length in the OSNs. To address this possibility, we analyzed two lines of transgenic mice, I7(WT)-ires-ECFP and I7(WT)-ires-dnPKA-ires-EYFP, in which rat OR-17 is expressed in a small population of OSNs with or without the dominant-negative form of cAMP-dependent protein kinase (dnPKA), respectively (Imai et al., 2006). Immunofluorescent labeling of OE sections with rat OR-17-specific antibody revealed that the length of olfactory cilia was greatly shortened in the dnPKA-expressing OSNs, compared with the control OSNs (Fig. 8 O–Q). These results suggest the role of cAMP as a regulator of ciliogenesis in OSNs, implying a possible mechanistic link between the mislocalized ACIII and the shortened olfactory cilia in Goofy-deficient mice.

Reduced olfactory sensitivity in Goofy-deficient mice

The above observation of shortened olfactory cilia led us to examine whether the Goofy deficiency results in impairment of odor-induced physiological and behavioral responses. To evaluate the physiological responses of OSNs, local field potentials (EOG) were recorded from the epithelial surface using intact OE preparations of wild-type and Goofy-deficient mice. The EOG responses to various odorants (eugenol, 2-heptanone, 1-heptanol, safrole, and lilial) were smaller in Goofy-deficient mice than in wild-type mice (Fig. 9 A, B; data not shown). A concentration–response curve for eugenol demonstrates a clear difference of EOG amplitudes between the two genotypes.
(Fig. 9C). Thus, Goofy-deficient OSNs displayed reduced sensitivity to odorants.

Finally, olfactory behaviors were compared between wild-type and Goofy-deficient mice. We used TMT (a component of fox feces odor) as a stimulus odorant, which evokes innate fear responses in rodents (Varnet-Maury et al., 1984; Fendt et al., 2005). TMT exposure induced avoidance and freezing responses in both wild-type and Goofy-deficient mice in a dose-dependent manner, but their sensitivities to TMT were clearly different (Fig. 9D,E). Wild-type mice showed significant responses even at the lowest amount (0.4 μl) of TMT. In contrast, the response threshold to TMT was higher in Goofy-deficient mice, with smaller values of avoidance index and shorter durations of freezing time to the lowest and middle amounts. At the highest amount of TMT, however, there was no difference in the responses between the two genotypes.

Thus, the electrophysiological and behavioral analyses revealed that the olfactory sensitivity of Goofy-deficient mice is significantly lower than that of wild-type mice, suggesting that mice become “dull-nosed” without Goofy function.

Discussion

We discovered a novel membrane protein Goofy that is specifically expressed in OSNs and VSNs and predominantly localized to the Golgi apparatus. The Goofy-deficient mice displayed various olfactory phenotypes, such as abnormal localization of ACIII, shortened olfactory cilia, and reduced electrophysiological and behavioral sensitivities to odorants. These findings demonstrate that Goofy is a crucial molecule for proper function of the olfactory system.

Northern blot, in situ hybridization, and immunohistochemical analyses revealed that Goofy mRNA and its translation product are expressed by sensory neurons of the olfactory system in a highly restricted manner. This rigorous regulation of Goofy gene transcription was verified by generating transgenic mice in which a fluorescent reporter protein was identified by generating transgenic mice in which a fluorescent reporter protein was targeted to the conserved cis-acting regulatory elements commonly present upstream of ORs, OMP, Golf, ACIII, and CNGA2 as well as two functionally uncharacterized genes 50.06 and 50.11 (Wang and Reed, 1993; Wang et al., 1993). Although the following studies reported that the 50.11 gene encodes an olfactory signal-modulating protein, SLP3/SRO, belonging to the stomatin family (Kobayakawa et al., 2002; Goldstein et al., 2003), the functional role of 50.06 gene product remained to be elucidated. Interestingly, the reported nucleotide sequence (1.0 kb) in the 5‘-upstream region of rat 50.06 gene (Wang et al., 1993) is highly homologous to that of mouse Goofy gene with 86% identity. This significant sequence

Figure 8. Shortened olfactory cilia in Goofy-deficient mice and dnPKA-expressing transgenic mice. A, B, Whole-mount OE preparations from wild-type (A) and Goofy-deficient (B) mice immunolabeled with anti-Olfr2 (I7, magenta) and anti-Olfr6 (M50, green) antibodies. The length of both Olfr2-expressing and Olfr6-expressing olfactory cilia in Goofy-deficient mice is markedly shorter than that in wild-type mice. C–E, Double immunofluorescence labeling of an OE section from Olfr73-ires-gapEGFP transgenic mouse on Goofy-deficient background with anti-Olfr73 (C, E) and anti-GFP (D, E) antibodies. E, A merged image showing perfect overlapping of Olfr73 (magenta) with GFP (green) in the olfactory cilia. F–H, Immunofluorescence labeling of OE sections from wild-type (F–H) and Goofy-deficient (I–K) mice with antibodies against Olfr2 (C, I), Olfr17 (P2; G, J), and Olfr73 (mOR-EG; H, K). L–N, Cumulative frequency plot for length of olfactory cilia expressing Olfr2 (L), Olfr17 (M), and Olfr73 (N) in wild-type (blue) and Goofy-deficient (red) mice. Thin broken lines represent the results from individual mice (n = 3 mice for each genotype; n = 259–805 OSNs for each mouse), while bold lines represent pooled data (n = 1196–1815 OSNs). Insets, Averages of cilia length. For all the three ORs, the length of olfactory cilia in Goofy-deficient mice is significantly shorter than that in wild-type mice. O, Cumulative frequency plot for length of rat 17-expressing olfactory cilia in I7-ires-ECFP (light blue) and I7-ires-dnPKA-ires-EYP (green) transgenic mice. Thin broken lines represent the results from individual mice (n = 3 mice for each genotype; n = 292–693 OSNs for each mouse), while bold lines represent pooled data (n = 1538 for 17-ires-ECFP; n = 1229 for 17-ires-dnPKA-ires-EYP). Insets, Averages of cilia length. P, Q, Immunofluorescence labeling of coronal sections of OE from I7-ires-ECFP (P) and I7-ires-dnPKA-ires-EYP (Q) transgenic mice with anti-rat17-specific antibody. **p < 0.01 (2-tailed test). Scale bars: A, B, 20 μm; C–K, P, Q, 50 μm.
Figure 9. Reduced olfactory sensitivity in Goofy-deficient mice. A–C, Electrophysiological properties of OSNs assessed by measurement of odor-evoked EOG responses (A, B). Representative EOG traces to eugenol, 2-heptanone, and 1-heptanol in wild-type (A) and Goofy-deficient (B) mice. C, Relationship between applied eugenol concentrations and EOG responses in wild-type (blue) and Goofy-deficient (red) mice (n = 12 for each genotype). *p < 0.05 (2-tailed t-test). D, E, Behavioral responses of wild-type (blue) and Goofy-deficient (red) mice to an aversive odor TMT stimulation (n = 8–10 for each genotype and each TMT concentration). Freezing time (D) and avoidance index (E) were measured for the two genotypes at different amounts of TMT. *p < 0.05 (Mann–Whitney U test).

Homology indicates that the 50.06 gene encodes a rat ortholog of Goofy and that Olf-1 plays a crucial role in regulating the restricted expression of Goofy/50.06 in rodent olfactory neurons.

Another remarkable feature of Goofy expression is its early appearance in both developmental and differentiation aspects in sharp contrast to the later onset of OMP expression. Ontogenic analysis revealed that Goofy mRNA is detectable as early as at E11.5 in the OE and at E13.5 in the VNO, much earlier than the appearance of OMP. In the neonates and adults, Goofy protein is present in both the immature and mature OSNs residing in the deep and superficial layers of OE, respectively, but not in the globose or horizontal basal cells, the progenitors of OSNs and sustentacular cells (Schwoob, 2002). Thus, the Goofy expression starts at a postmitotic, immature stage during the differentiation process of OSNs.

For the OSN-specific transcript expression, the transcriptional regulatory region of OMP gene has been widely used so far (Mombaerts et al., 1996; Walters et al., 1996; Yoshihara et al., 1999; Bozza et al., 2004; Nguyen et al., 2007). Although the OMP gene promoter/enhancer has proved powerful in many cases, it can drive transgene expression only in the mature OSNs. In contrast, the present study demonstrates that the 3 kb fragment upstream of mouse Goofy gene can efficiently induce transgene expression in both the immature and mature OSNs from developmental stages earlier than the expression onset of OMP and other olfactory signaling molecules. Thus, the Goofy gene promoter/enhancer will become a useful and convenient tool for various studies on differentiation, maturation, and function of OSNs.

Immunohistochemical analysis with various organelle markers revealed that Goofy protein is predominantly localized to the Golgi apparatus of OSNs. The Golgi apparatus is an intracellular central organelle with a well established role as a processing and sorting station in the transport of secretory and transmembrane cargo proteins to their final destination (Wilson et al., 2011). By surveying the expression patterns of various functional molecules in OSNs, we found the abnormal localization of ACIII, an enzyme playing a pivotal dual role in odor-induced signal transduction cascade (Bakalyar and Reed, 1990) and olfactory axon targeting to OB (Imai et al., 2006). ACIII is a transmembrane protein normally highly enriched in olfactory cilia (Bakalyar and Reed, 1990). In Goofy-deficient mice, however, ACIII was also abundantly detected in olfactory axons and glomeruli. Thus, it is likely that Goofy associates with ACIII in Golgi apparatus of OSNs, regulating either its post-translational modification or intracellular trafficking. Further biochemical and proteomics analyses are now in progress to gain mechanistic insight into the interaction between Goofy and ACIII as well as to search for other functional molecules that associate with Goofy.

In addition to this molecular phenotype of ACIII mislocalization, Goofy-deficient mice displayed morphological abnormality of OSNs: the shortening of olfactory cilia. How does Goofy protein in the Golgi apparatus affect the morphology of olfactory cilia? We observed that the length of olfactory cilia is drastically shortened by the transgenic expression of dnPKA, suggesting a crucial role of cAMP signaling in ciliogenesis of OSNs. A similar regulatory mechanism of olfactory cilia architecture by cyclic nucleotide was reported in C. elegans, where cGMP signaling plays an important role (Mukhopadhyay et al., 2008). Taking into consideration all of these findings, we speculate that the abnormal localization of ACIII in Goofy-deficient mice might cause insufficiency of cAMP production in dendrites, resulting in the impaired elongation of olfactory cilia. Consequently, Goofy-deficient mice may display reduced sensitivity to odorants in both physiological and behavioral analyses, rendering themselves goofy-nosed.

The cAMP second messenger system appears to play three important roles in OSN functioning: odor-induced neuronal excitation (Bakalyar and Reed, 1991; Schild and Restrepo, 1998), olfactory axon guidance (Imai et al., 2006; Col et al., 2007), and ciliogenesis (this study). In Goofy-deficient mice, the odor-induced responses were smaller and the olfactory cilia were shorter than in wild-type mice, whereas the olfactory axon projection to target glomeruli appeared normal. It is likely that the degree of odor-induced activation and the length of olfactory cilia are attributable to the absolute cAMP level in individual OSNs in a cell-autonomous manner. In contrast, the olfactory axon innervation may be determined by the relative difference in cAMP-regulated expression levels of axon guidance molecules among distinct OR-expressing OSNs (Mori and Sakano, 2011). Therefore, it is conceivable that the olfactory axons can precisely innervate target glomeruli in appropriate OB regions in Goofy-
References
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