G protein γ subunit Gγ13 is essential for olfactory function and aggressive behavior in mice

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Most olfactory receptors in vertebrates are G protein-coupled receptors, whose activation by odorants initiates intracellular signaling cascades through heterotrimeric G proteins consisting of α, β, and γ subunits. Abolishment of the α subunits such as Gαolf in the main olfactory epithelium and Gαi2 and Gαo in the vomeronasal organ resulted in anosmia and/or impaired behavioral responses. In this study, we report that a G protein γ subunit, Gγ13, is expressed in a spatiotemporal manner similar to those of Gαolf and Gαi2 in the olfactory system and vomeronasal organ, respectively. In addition, Gγ13 was found in the glomeruli of the main olfactory bulb but was largely absent in the glomeruli of the accessory olfactory bulb. Using the Cre-loxP system, the Gγ13's gene *Gng13* was nullified in the mature olfactory sensory neurons and apical vomeronasal sensory neurons where the Cre recombinase was expressed under the promoter of the *Omp* gene for the olfactory marker protein. Immunohistochemistry indicated much reduced expression of Gγ13 in the apical vomeronasal epithelium of the mutant mice. Behavioral experiments showed that the frequency and duration of aggressive encounters in the male mutant mice were significantly lower than in WT male mice. Taken together, these data suggest that the Gγ13 subunit is a critical signaling component in both the main olfactory epithelium and apical vomeronasal epithelium, and it plays an essential role in odor-triggered social behaviors including male–male aggression. *NeuroReport* 29:1333–1339 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Olfaction is a special sense that enables humans and animals to detect external chemical environment and plays an important role in animals' survival, social interaction and reproduction [1]. Mouse olfactory system is located in the nasal cavity and comprises two major subsystems: (i) the main olfactory system, with its olfactory sensory neurons (OSNs) residing in the main olfactory epithelium (MOE) and their axons projecting to the main olfactory bulb (MOB), and (ii) the accessory olfactory system, with the vomeronasal sensory neurons (VSNs) located in the vomeronasal organ (VNO) and their axons projecting to the accessory olfactory bulb (AOB). Furthermore, the vomeronasal epithelium (VSE) in the VNO is divided into two layers: apical and basal, from which the apical and basal VSNs send their axons to the anterior and posterior parts of the AOB, respectively [2]. Inputs from the neurons located in these three anatomically distinct structures, the MOE, apical vomeronasal epithelium (aVSE), and basal vomeronasal epithelium (bVSE), appear to be integrated in the brain regions to generate behavioral outputs [3]. It is believed that the MOE is more important to the sensation of general odors, whereas the VNO is critical to pheromonal detection that can result in sex identification and intermale aggression [4,5]. The underlying molecular mechanisms, however, are not fully understood.

Sensory neurons in the MOE, aVSE, and bVSE utilize different receptors and G proteins to receive and transduce respective olfactory and pheromonal stimuli. Most OSNs express olfactory receptors and G proteins consisting of Gαolf and Gβ1γ13 [6,7], whereas in the VNO, apical VSNs express type I vomeronasal receptors (V1Rs) along with G protein α subunit Gαi2, and basal VSNs express type II vomeronasal receptors (V2Rs) together with G protein α subunit Gαo, correspondingly [8]. However, less is clear about G protein βγ subunits in the apical and basal VSNs, which are believed to be as important as the Gα subunits in the signal transduction pathways [9]; each of the Gα subunit and Gβγ dimer can activate their respective downstream effector enzymes and ion channels [10].

Recent studies indicate that the G protein subunit Gγ13, encoded by the *Gng13* gene, is a key signaling component in the MOE [7,11,12]. Conditional knockout of the gene in the OMP-Cre: $Gng13^{loxP/loxP}$ mice ($Gng13^{-/-}$) significantly altered the MOE's gene expression profile, diminished its electrical responses to many odorants, and

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impaired the mutant animals' food seeking capabilities [7]. In this study, we further investigated the Gγ13 expression in both MOE and VNO over different developmental stages and characterized the impact of its conditional knockout on gene expression of the VSE and on the aggressive behavior of the mutant mice. Our results suggest that Gγ13 is the Gγ subunit forming a heterotrimeric G protein with Gαi2 in the aVSE.

Materials and methods

Subjects

C57BL/6 mice were purchased from the Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). Male mice were housed individually in standard plastic cages whereas females were housed in groups of four per cage with both food and water ad libitum. The mouse rooms were specific pathogens free and maintained at a temperature of 23 ± 2°C and relative humidity of 57 ± 5%, with a 12 h light per day. *Gng13* conditional knockout mice ($Gng13^{-/-}$) were generated by crossing $Gng13^{flox/flox}$ [7] with OMP-Cre mice [13]. The total number of mice used was 40. Male and female mice were used for breeding and for generating embryos as well as for in-situ hybridization and immunohistochemistry experiments. Male mice were also used for male–male aggression experiments. All experiments with mice were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Tissue preparation

Mouse embryos at embryonic day 15 (E15) were dissected out and the heads were fixed in 4% paraformaldehyde (PFA)/PBS for 2 h and cryoprotected in 20% sucrose/PBS overnight at 4°C. Mice at postnasal day 5 (P5) and day 21 (P21) were anesthetized with sodium pentobarbital (40 mg/kg) and perfused transcardially with ice-cold 4% PFA/PBS. Then, the olfactory organs were dissected out, post-fixed for 2 h with 4% PFA/PBS and decalcified in 500 mM EDTA/PBS overnight for young mice or 5 days for older mice at 4°C. The organs were cryoprotected in 20% sucrose/PBS overnight and embedded with OCT compound. Coronal sections (10 μm thick) were cut and stored at −80°C until use.

In-situ hybridization

Digoxigenin (DIG)-labeled RNA probes for Gαolf, Gγ8, and Gγ13 were synthesized using a DIG-labeled probe synthesis kit (11175025910; Roche, Indianapolis, Indiana, USA), and then diluted in the hybridization solution (50% deionized formamide, 10 mM Tris-HCl pH 8.0, 200 μg/ml yeast tRNA, 10% dextran sulfate, 1× Denhardt's solution, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, pH 8.0, dissolved in DEPC-treated sterile water) into a working concentration of 0.1–0.2 μg/ml. In-situ hybridization was performed as previously described [14]. In brief, the tissue sections were incubated with 0.1% H_2O_2/PBS for 30 min to block endogenous peroxidase, and then with the hybridization solution containing the probe at 65°C for 16 h. The sections were then blocked with the blocking solution (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1% goat serum) for 30 min, followed by incubation with alkaline phosphatase-labeled anti-DIG-AP antibody (11093274910, 1 : 500; Roche) overnight at 4°C. Sections were washed with the solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min, and then washed with PBS three times, and finally reacted with BM Purple AP (11442074001; Roche) and observed every 1 h until a color reaction occurred.

Immunofluorescence

The sections were blocked in the blocking buffer (10% normal goat serum, 2% bovine serum albumin in 0.5% Triton X-100/PBS solution) for 1 h, followed by incubation with the primary antibodies. The anti-Gγ13 antibody (produced by the Laboratory of Chemosensory Sciences, College of Life Sciences, Zhejiang University) and anti-Gαi2 antibody (sc-13534; Santa Cruz, Dallas, Texas, USA) were diluted at 1 : 1000 in the blocking buffer and applied to the sections for 16 h at 4°C, which were then incubated with the secondary antibody (AF-488 labeled goat anti-rabbit lgG, A-11034, 1 : 500; Life Technology, Waltham, Massachusetts, USA) for 1 h at room temperature in the dark. Finally, the sections were washed, covered with 0.5% glycerol/PBS and imaged.

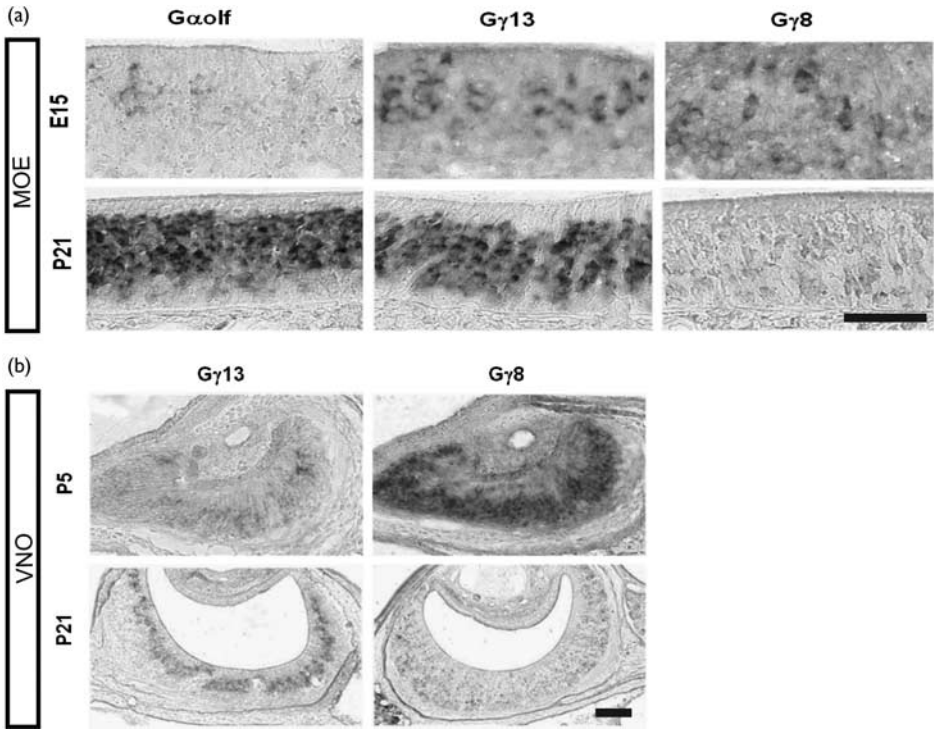
Male–male aggression experiment

WT and $Gng13^{-/-}$ mice were individually caged for 7 days to establish home residency. An age- and body weight-matched castrated C57BL/6 male mouse was introduced into the cage as an intruder. The behavioral responses of the resident mice were video-recorded for 10 min. The castrated mouse was then removed from the cage, swabbed with 50 μl of urine from an intact C57BL/6 male mouse on the back and on the urogenital region, and then reintroduced to the resident's cage for an additional 10 min. The video data were analyzed at 1/5–1/2 speeds by another researcher who was blind to the treatment. Behaviors such as tail rattling, biting, chasing, tumbling/wrestling, kicking and cornering the intruder were designated as aggressive encounters. The number of these encounters and the total time of all encounters during a 10-min session were tallied and averaged for each animal of the same genotypic group.

Statistical analysis

For male–male aggression experiment, the number of aggressive encounters and the total time were expressed as mean ± SEM. Pairwise comparisons of the averaged numbers were analyzed using Student's *t*-tests with PASW Statistics 18.0 software (International Business Machines Corporation, Armonk, New York, USA). *P* value less than 0.05 was the criterion for statistical significance.

Fig. 1



The expression patterns of G protein subunits Gαolf, Gγ13, and Gγ8 in the MOE and VNO at different developmental stages. (a) In-situ hybridization on mouse MOE at E15 and P21. The transcripts for both Gαolf and Gγ13 were detectable at E15 but more abundant at P21, whereas that for Gγ8 seemed to be reversed over the developmental stage. (b) In-situ hybridization on mouse VNO. The transcripts for Gγ13 were present at P5 but more clearly restricted to the apical layer at P21. In contrast, the gene for Gγ8 was highly expressed at P5, but downregulated at P21. Scale bar: 100 μm. E15, embryonic day 15; MOE, main olfactory epithelium; P5, postnasal day 5; P21, postnasal day 21; VNO, vomeronasal organ.

Results

The expression pattern of Gγ13 in the olfactory system

Gγ13 has been shown to be expressed in the adult olfactory system [7,11]. To determine whether it is also expressed at earlier developmental stages, we performed in-situ hybridization on tissue sections of the MOE and VNO at E15, P5, and P21 using the probes for Gαolf, Gγ13, and Gγ8, as the latter is known to be expressed in immature neurons of both MOE and VNO. The results showed that in the MOE, the expression pattern of Gγ13 was in agreement with that of Gαolf, which had a low expression level in few cells at E15 but a high expression level in many more cells at P21. In contrast, the pattern of Gγ8 seemed to be reversed with a relatively high expression level in more cells at E15 but a reduced expression level in fewer cells at P21 (Fig. 1a).

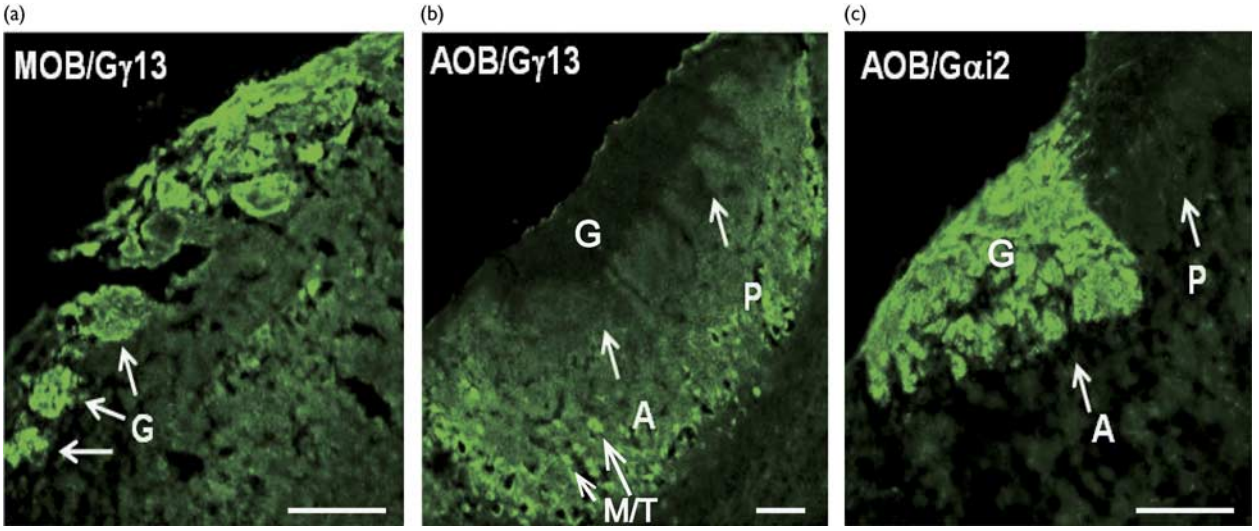
As the rodent VSE reaches maturity later than the MOE, we performed in-situ hybridization on the VSE at a later stage: P5 and P21. Similar to the expression patterns in the MOE, Gγ13 transcripts were barely detectable at P5 but were abundant in the apical neurons at P21, whereas Gγ8 was highly expressed at P5 but was much down-regulated in both aVSE and bVSE at P21 (Fig. 1b).

To determine whether Gγ13 is involved in the path-finding of axonal terminals during their projection from the nasal cavity to the AOB or in the synaptic activity of the axonal terminal in the bulb, we examined its expression in the MOB and AOB. The results showed that Gγ13 was expressed in the glomeruli of the MOB (Fig. 2a). However, it was absent in the glomeruli of both anterior and posterior AOB although some immunostaining signals were detectable in the mitral/tufted cell layer. In contrast, Gαi2 immunoreactivity was present in the anterior but not in the posterior AOB (Fig. 2b and c).

Gng13 knockout reduced its expression in the main olfactory epithelium and vomeronasal organ

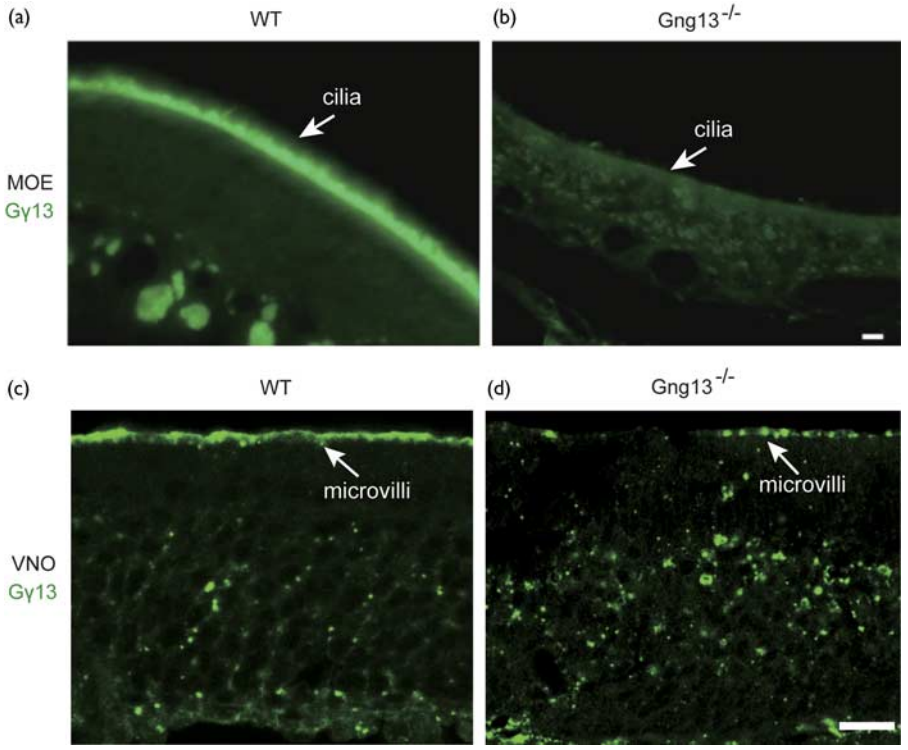
We performed immunostaining to determine whether the Cre recombinases driven by the *Omp* promoter was able to eliminate the expression of Gγ13 in the olfactory system. The results showed that although Gγ13 was abundant in the cilia of WT MOE (Fig. 3a), it was completely absent in the cilia of KO MOE (Fig. 3b). In the VNO, WT microvilli exhibited strong Gγ13 immunostaining signals, indicating the abundance of Gγ13 proteins (Fig. 3c). However, the mutant VNO contained much reduced staining on the microvilli (Fig. 3d).

Fig. 2



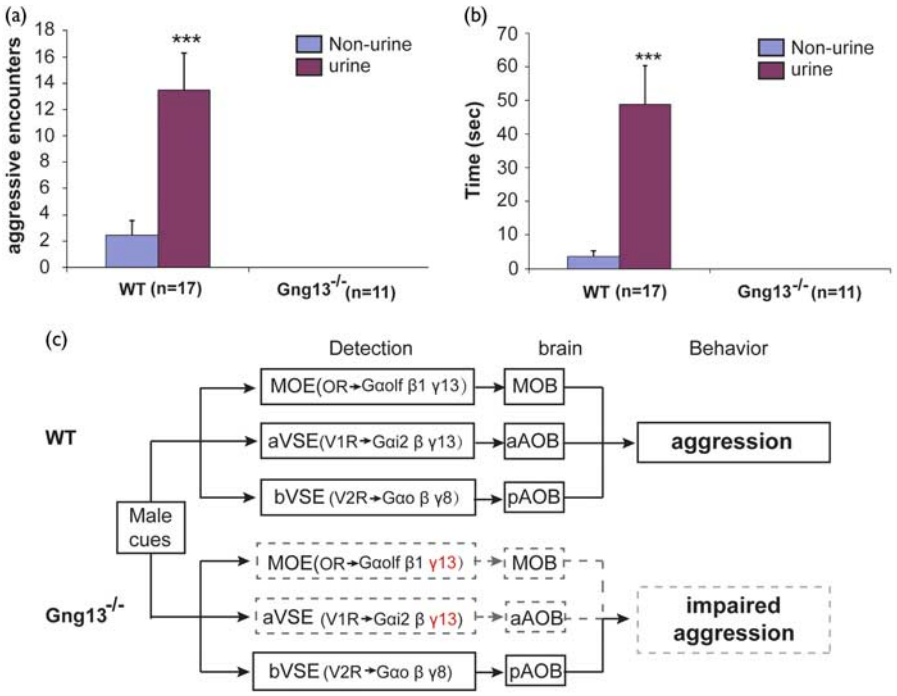
Gγ13 expression in mouse MOB and AOB. Immunostaining with Gγ13 antibody shows the presence of Gγ13 most abundantly in the glomerulus layer (G) of the MOB (a), but absent in the glomerulus layer (G) of both anterior (A) and posterior (P) AOB (b), and present in the mitral and tufted cells (M/T). (c) Immunostaining of mouse AOB sections with Gαi2 antibody shows the staining of the glomeruli (G) in the anterior (A) but not in the posterior (P) part of the AOB. Scale bar: 50 μm. AOB, accessory olfactory bulb; MOB, main olfactory bulb.

Fig. 3



The *Gng13* KO significantly reduces the expression of Gγ13 in the MOE and VSE. (a, b) Gγ13 is enriched in the cilia of WT MOE (a, arrow) but nearly completely absent in the KO cilia (b, arrow). (c, d) Gγ13 is abundant in the microvillus layer of WT VSE (c, arrow) but much less and punctuated in the KO microvillus layer (d, arrow). Scale bar: 25 μm. MOE, main olfactory epithelium; VSE, vomeronasal epithelium.

Fig. 4



The *Gng13* KO significantly impaired the male–male aggression. (a) Left: comparison of the number of aggressive encounters between WT ($n=17$) and castrated intruder mice (nonurine) to that between WT and castrated intruder mice painted with regular male mouse's urine (urine); right: comparison of the number of aggressive encounters between $Gng13^{-/-}$ ($n=11$) and castrated intruder mice (nonurine) to that between $Gng13^{-/-}$ and castrated intruder mice painted with the urine (urine). (b) Left: comparison of the total duration of aggressive encounters between WT ($n=17$) and castrated intruder mice (nonurine) to that between WT and castrated intruder mice painted with the urine (urine); right: comparison of the total duration of aggressive encounters between $Gng13^{-/-}$ ($n=11$) and castrated intruder mice (nonurine) to that between $Gng13^{-/-}$ and castrated intruder mice painted with the urine (urine). ***$P<0.001$. (c) Schematic model showing the contributions of multiple olfactory circuits to the induction of aggressive behaviors in mice. The male cues are detected by the intact WT MOE as well as both apical and basal VNO. The OSNs in the MOE express olfactory receptors/Gαolf/Gβ1γ13 to detect, transduce, and transmit the cues to the MOB. In parallel, the VSNs in the aVSE and bVSE express V1Rs/Gαi2/Gβγ13 and V2Rs/Gαo/Gβγ8, respectively, and transmit the signals to the aAOB and pAOB. The signals from the three pathways are integrated in the brain, eventually leading to aggressive behavior. In the $Gng13^{-/-}$ mice, however, Gγ13 expression is abolished in the MOE and apical VNO, whereas the basal VNO remains intact. The male cues are detected only by the basal VNO, which is insufficient to trigger any aggressive behavior. AOB, accessory olfactory bulb; aAOB, anterior part of the AOB; aVSE, apical vomeronasal epithelium; bVSE, basal vomeronasal epithelium; MOE, main olfactory epithelium; pAOB, posterior part of the AOB; VNO, vomeronasal organ; VSN, vomeronasal sensory neuron.

Gng13 knockout impaired the aggressive behavior of male mice

To determine whether the *Gng13* KO affects the mutant mice's social interactions with other mice, we performed intermale aggression assays. The results showed that compared with the castrated intruder males, WT male mice had a higher total number of aggression encounters (13.7 ± 2.8 vs. 2.3 ± 1.4, $t=3.642$, $d.f.=32.0$, $P<0.001$) and longer total time of aggressive behaviors (49.5 ± 10.7 vs. 4.8 ± 2.4, $t=4.076$, $d.f.=32.0$, $P<0.001$) against the reintroduced castrated males painted with regular male mice's urine on the back and urogenital region. However, unlike WT mice, the $Gng13^{-/-}$ male mice showed no aggressive behavior when castrated intruder males with or without regular males' urine on the back and urogenital region were reintroduced (Fig. 4a and b), indicating that *Gng13* KO significantly impaired male–male aggression. Otherwise, $Gng13^{-/-}$ mice could run and climb normally without any other apparent behavioral and locomotor dysfunction.

Discussion

Gγ13 is a G protein γ subunit that was first isolated from murine taste bud cells [11]. It has been found in the OSNs in the MOE, interacting with Gαolf, Gβ1 to form the heterotrimeric G protein Gαolf/β1/γ13 and transduce olfactory signals [7,12,15,16]. In this study, we investigated its expression pattern over developmental stages and found that both Gγ13 and Gαolf are expressed in the MOE as early as E5, indicating that the olfactory sensation may function in early embryonic stages (Fig. 1a). In the VNO, which matures later than the MOE, Gγ13 transcripts are also detectable at P5, and much enriched in the apical layer at P21 (Fig. 1b). In contrast, Gγ8 transcripts are more abundant in earlier developmental stages such as E15 and P5 in the MOE and VNO but are reduced at P21 (Fig. 1a and b).

These results indicate that Gγ13 is the missing γ partner of Gαi2 in the apical VSNs as it is part of Gαolf/β1/γ13 in the OSNs [7]. In the OSNs, upon activation, Gαolf/β1/

γ13 dissociates into Gαolf and Gβ1γ13 moieties, with the former activating adenylate cyclase III and the latter's effector to be identified. In the apical VSNs, the Gαi2's effector remains unknown [17], but its Gβγ13 moiety is likely to stimulate phospholipase Cβ2, generating the second messengers inositol 1,4,5-triphosphate and diacylglycerol, and diacylglycerol in turn activates transient receptor potential canonical 2 channels [5,18]. Interestingly, Gβγ13 plays a very similar role in taste bud cells, where it acts on the same effector phospholipase Cβ2 as in the apical VSNs [11].

Immunohistochemistry indicates that Gγ13 is also expressed in the glomeruli in the MOB but is absent in the glomeruli of the AOB (Fig. 2a–c). These results indicate that Gγ13 is possibly involved in the signaling activities in the axonal termini of the OSNs including their pathfinding and synaptic activity between the OSNs and their next-order neurons. Therefore, it is also possible that Gγ13 may play a role in axonal projection by the OSNs during the critical period of map formation [19].

In the anterior part of the AOB, it is probably Gαi2, not Gγ13, that contributes to the VSNs' axon terminal pathfinding or synaptic activity between VSNs and their next-order neurons. Instead, Gγ13 is expressed in the mitral/tufted cell layer of the AOB, thus possibly modulating the output of the AOB to other parts of the olfactory cortex [20].

The expression of Gγ13 was completely absent in the cilia of the MOE in the *Gng13*$^{-/-}$ mice (Fig. 3a and b), which is consistent with the previous report [7]. In the KO VNO, Gγ13 was also significantly reduced, but some residual Gγ13 immunostaining signals were visible at the microvillus layer (Fig. 3c and d). The discrepancy in Gγ13 expression between the MOE and VNO is probably attributable to the timing of the Cre expression driven by the *Omp* promoter and the differential developmental stages of the two sensory epithelia. During differentiation and maturation of the progenitor cells into mature OSNs, Gγ13 is expressed earlier than OMP or the knocked-in Cre recombinase, and some Gγ13 proteins may have been synthesized before its floxed gene is excised by Cre [7,20]. As the average lifespan of the OSNs is about 2–3 months, Gγ13 proteins are likely to be used up when the neuron becomes mature, as shown in Fig. 3c. It, however, needs to be determined how long the Gγ13 proteins can last once its gene is deleted. In the VNO, more Gγ13 proteins seemed to be present in the microvilli than in the MOE (Fig. 3b and d). One reason is that mouse VNO is developmentally delayed compared with the MOE, as shown in Fig. 1. Therefore, the VSNs were relatively young and more residual Gγ13 proteins remained.

Gng13 KO significantly reduced both the number and total duration of male–male aggressive encounters over the test sessions (Fig. 4a and b), which is in agreement with our previous studies indicating that the *Gng13* KO in the MOE altered the expression of Gαolf and Gβ1, diminished electrical responses of the MOE to odorants, and impaired odor-guided food-seeking capabilities [7]. Thus, Gγ13 interacts with Gαolf in the MOE but with Gαi2 in the aVSNs and is critical to both odor detection and pheromonal sensation.

Multiple lines of evidence indicate that animals' behavioral outputs may be the integrated responses to the chemosensory inputs by several routes, of which the MOE-MOB, aVSE-anterior part of the AOB, and bVSE-posterior part of the AOB pathways are the chief ones involved in the regulation of the social and sexual behaviors (Fig. 4c). Interruption of the signaling pathways can severely dampen animals' behavioral responses, including food seeking, sex identification and preference, and aggression [21,22]. Previous studies have shown that abolishment of either Gαi2, Gαo or Gγ8 expression in the VNO abrogated the male–male aggression [17,23,24]. Together with our data from this study on Gγ13's expression in the VNO and the impact of its knockout on animals' behavior, we conclude that all the key G protein subunits expressed in the apical and basal VSE are important to male–male aggression, and both VSE layers are required to initiate aggressive behavior. Further studies are warranted to determine the Gγ13's role in other behavioral responses.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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