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Aggressive behaviour and physiological responses to pheromones are strongly impaired in mice deficient for the olfactory G-protein γ-subunit Gγ8

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Key points

- Pheromones are intraspecies chemical signals that take part in the sexual recognition and choice of appropriate mating partners.
- In the vomeronasal organ (VNO), pheromone responses are probably triggered by two distinct neuronal populations, respectively expressing the heterotrimeric G-proteins Gαi2β2γ2 and Gαoβ2γ8 that, in turn, coexpress with two pheromone receptor families, V1R and V2R.
- We demonstrate that the olfactory-specific G-protein γ8 subunit (Gγ8) plays an important role in pheromone-dependent socio-sexual recognition.
- Deficient mice for Gγ8 show a marked reduction in the pheromone-mediated aggressive behaviour in both females and males that corresponds with a failure to activate V2R targets in the brain. These effects occur in combination with a consistent loss of vomeronasal neurons.
- Thus, Gγ8 is essential for maintenance of the neuronal population of the VNO and for correct transduction of the pheromonal signal.

Abstract Heterotrimeric G-proteins are critical players in the transduction mechanisms underlying odorant and pheromonal signalling. In the vomeronasal organ (VNO) of the adult mouse, two different G-protein complexes have been identified. Gαoβ2γ8 is preferentially expressed in the basal neurons and coexpresses with type-2 vomeronasal pheromone receptors (V2Rs) whereas Gαi2β2γ2 is found in the apical neurons and coexpresses with type-1 vomeronasal pheromone receptors (V1Rs). V2R-expressing neurons project to the posterior accessory olfactory bulb (AOB) whereas neurons expressing V1Rs send their axon to the anterior AOB. Gγ8 is also expressed in developing olfactory neurons where this protein is probably associated with Go. Here, we generated mice with a targeted deletion of the Gγ8 gene and investigated the behavioural effects and the physiological consequences of this mutation. Gγ8$^{-/-}$ mice show a normal development of the main olfactory epithelium; moreover, they do not display major deficits in odour perception. In contrast, the VNO undergoes a slow but remarkable loss of basal neurons starting from the fourth postnatal week, with a 40% reduction of cells at 2 months and 70% at 1 year. This loss is associated with a reduced early-gene expression in the posterior AOB of mice stimulated with pheromones. More interestingly, the Gγ8 deletion specifically leads to a reduced pheromone-mediated aggressiveness in both males and females, all other socio-sexual behaviours remaining unaltered. This study defines a specific role for Gγ8 in maintenance of the

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neuronal population of the VNO and in the mechanisms of pheromonal signalling that involve the aggressive behaviour towards conspecifics.

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Abbreviations AOB, accessory olfactory bulb; BrdU, bromodeoxyuridine; IP_3, inositol 1,4,5-triphosphate; MOE, main olfactory epithelium; PLC, phospholipase-C; TTA, tetracycline transactivator; V1R, type-1 vomeronasal pheromone receptor; V2R, type-2 vomeronasal pheromone receptor; VNO, vomeronasal organ.

Introduction

In addition to conventional odorants, most animals detect pheromones that provide information on the socio-sexual status of conspecifics via the vomeronasal system (Tirindelli *et al.* 2009; Chamero *et al.* 2012).

The vomeronasal organ (VNO) is a tubular structure located at the base of the nasal septum lined by a neuroepithelium (Doving & Trotier, 1998; Jacobson *et al.* 1998).

The organization of the vomeronasal epithelium in apical and basal neurons is characterized by the expression of two heterotrimeric G-proteins, Gi2 and Go (Shinohara *et al.* 1992; Berghard *et al.* 1996; Jia & Halpern, 1996), and two families of pheromone receptors, type-1 and type-2 vomeronasal pheromone receptors (V1Rs and V2Rs; Dulac & Axel, 1995; Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997). The G-protein α-subunit, Gαi2, is expressed in the apical neurons and co-localizes with V1Rs whereas Gαo is expressed in the basal neurons and co-localizes with V2Rs. Recently, a third class of G-protein coupled receptors, formyl peptide receptor-like (FPRs), has been described in the VNO that sense N-formylated peptides (Liberles *et al.* 2009; Riviere *et al.* 2009).

Male rat urine induces inositol 1,4,5-triphosphate (IP_3) production via the activation of Gi2 as well as Go (Wekesa & Anholt, 1997; Krieger *et al.* 1999; Sasaki *et al.* 1999). Interestingly, Gαi2 activation is observed only upon stimulation with the volatile components of rat urine, whereas stimulation of Gαo is elicited by urinary proteins (Krieger *et al.* 1999; Chamero *et al.* 2007).

Mutant mice for both Gαi2 and Gαo show a remarkable reduction in the size of the neuronal layers where these G-proteins were originally expressed (Tanaka *et al.* 1999; Norlin *et al.* 2003; Chamero *et al.* 2011). Furthermore, these mice also display a low level of intermale and maternal aggression (Norlin *et al.* 2003; Chamero *et al.* 2011).

In different tissues, phospholipase-C (PLC) stimulation and IP_3 production appear to be mediated predominantly by the G$\beta\gamma$ complex, in turn released from the α-subunit of Go and Gi (Smrcka, 2008; McIntire, 2009). One G-protein β-subunit (Gβ2) and two γ-subunits (Gγ2 and Gγ8) have been identified in the VNO (Ryba & Tirindelli, 1995; Tirindelli & Ryba, 1996; Runnenburger *et al.* 2002). Gγ2 immunoreactivity is localized to the apical layer of the VNO. Conversely, Gγ8-expressing neurons are preferentially restricted to the basal layer. Interestingly, in contrast to other γ-subunits, Gγ8 appears specific for the olfactory system.

Biochemical studies report that an anti-Gγ2 antibody selectively blocked IP_3 formation, induced by the stimulation of vomeronasal membrane preparations with volatile urinary compounds. In contrast, urinary protein-mediated IP_3 production was inhibited by pre-incubation of the vomeronasal membranes with an antibody against Gγ8 (Runnenburger *et al.* 2002). Thus, both Gγ2 and Gγ8 may control PLC activation through specific and distinct pheromonal stimuli.

To better investigate the role of G-proteins in pheromonal responses, we have generated mice with a homozygous deficiency in the Gγ8 gene. Mice lacking Gγ8 display striking alteration in the vomeronasal epithelium and marked physiological and behavioural changes in response to pheromonal stimulation.

Methods

Generation of Gγ8$^{-/-}$ mice

The Gγ8 locus was targeted by homologous recombination in 129/Sv-derived embryonic stem cells using standard methods. The targeting construct (Fig. 1*A*) was designed to delete the entire coding region of the gene and to replace it with a tetracycline transactivator (TTA) where the starting ATG of TTA exactly replaced that of Gγ8. Approximately 5% of neomycin resistant embryonic stem cell clones were appropriately targeted. One such clone was used to generate chimeric mice by morula-aggregation. For experiments, mice carrying the targeted Gγ8 allele were back-crossed into an FVB/N background for 10 generations to obtain the Gγ8$^{-/-}$ mutants and wild-type littermate controls.

Animals

All tests were conducted on 3-month-old mice unless otherwise indicated.

In experiments implying collection of tissues for immunohistochemistry (fixed) or *in situ* hybridization

(fresh), mice were injected I.P. with a lethal dose of the anaesthetic sodium pentobarbital (30 mg per animal). For immunohistochemistry (fixed tissues), soon after mice ceased breathing, their chest was opened to allow transcardiac perfusion with 4% phosphate-buffered paraformaldehyde.

The experiments comply with the Principles of Animal Care (publication no. 85-23, revised 1985) of the National Institutes of Health and with the current law of the European Union and Italy. The present project was approved by the Ethical Committee of the University of Parma: approval ID: 63/11, 20 July 2011.

Immunohistochemistry

Cryostat-cut sections (20 μm) were treated with 0.5% sodium dodecyl sulphate for 15 min and washed in PBS prior to incubation with the primary antibody.

For immunohistochemistry, sections were blocked in 1% albumin and 0.3% Triton X-100 for 20 min and incubated with the primary antibodies in the same blocking solution for 36 h at 4°C.

Specific immunoreactivity was detected by the biotin–avidin–horseradish peroxidase–diaminobenzidine method (ABC kit) as recommended by the supplier (Vector, Burlingame, CA, USA) or by a secondary antibody conjugated with Alexa-488 or Alexa-546. Images were taken with a digital Nikon camera. All primary antibodies were used at a 1:100 dilution unless otherwise indicated.

Fluorescence images were obtained using a Zeiss fluorescence microscope.

In situ hybridization

Fresh VNOs were included in optical cutting temperature (OCT) compound and frozen in pentane-cooled liquid nitrogen.

Frozen sections were cut at 16 μm and attached to silanized slides. Digoxigenin-labelled cRNA probes and *in situ* hybridization procedures were essentially as described (Schaeren-Wiemers & Gerfin-Moser, 1993) except that sections were treated with 1% Triton X-100 before acetylation. Double label *in situ* hybridization was performed by labelling probes with digoxigenin and biotin. Sections were then developed with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics, Mannheim, Germany) coupled to Fast Red (Perking Elmer, Waltham, MA, USA) followed by treatment with Alexa488-conjugated streptavidin (Life Technolgies Italia, Monza, Italy and Perkin Elmer, Waltham, MA, USA). Finally, sections were analysed with a Zeiss microscope equipped with a CCD camera or with a Zeiss confocal microscope (Carl Zeiss Microscopy, GmbH, Jena, Germany).

Cell counting and area measurements

Mice were anaesthetized with pentobarbital and transcardially perfused as previously described. VNOs or

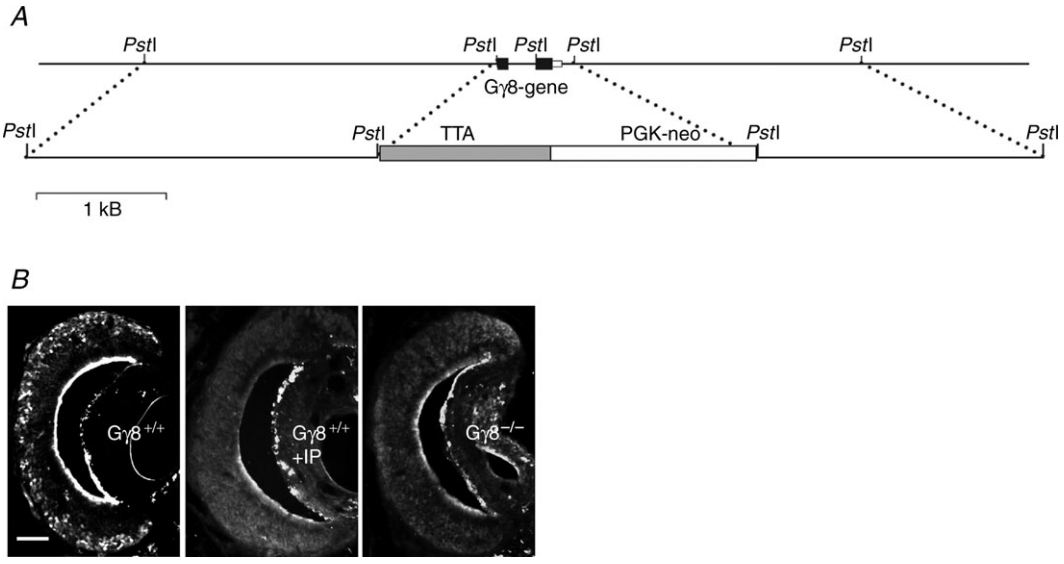

Figure 1. Targeted mutagenesis of the Gγ8 locus
A, the upper diagram shows the two Gγ8 coding exons with open boxes representing 5′ and 3′ non-translated sequences and the filled boxes representing coding sequence; also shown are surrounding *Pst*I sites. The lower diagram represents the targeting construct that consisted of an approximately 2.8 kB 5′ *Pst*I fragment that was fused to the coding region of TTA so that the starting ATG of TTA faithfully replaced the initiation codon of the Gγ8 gene. Also included in the construct was a standard PGK-neomycin cassette (PGK-neo) and an approximately 2.2 kB *Pst*I fragment encoding a region 3′ to the Gγ8 coding sequence. *B*, Gγ8 immunostaining in 3-month-old $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ mice. IP, anti-Gγ8 antibody preincubated with its immunogenic peptide. Scale bar = 100 μm.

accessory olfactory bulb (AOB) were then dissected, post-fixed for 4 h at 4°C and cryo-protected overnight at 4°C in 30% sucrose. Subsequently, tissues were included in OCT embedding solution (CellPath, Newtown, Wales, UK) and frozen in liquid nitrogen-cooled pentane.

Coronal or sagittal sections were collected throughout the entire VNO or AOB, respectively.

For measurement of the VNO area, eight coronal sections of the mid part of the organ were considered for each animal. The area containing the apical and basal neurons was visualized by *in situ* hybridization with antisense probes for Gαi2 and Gαo, respectively, or by immunostaining with anti-V2R antibodies (Silvotti *et al.* 2007). The mean number of positive cells was obtained from the 12 mid sections of the VNO.

For measurements of the AOB area, 10 sagittal sections of the mid part of the region were considered for each animal. The posterior glomerular region of the AOB was distinguished from the anterior AOB by staining sections with an antibody against Gαo. In initial experiments, sections were double stained with an anti-olfactory marker protein (OMP) (at 1:5000) and anti-Gαo antibody to distinguish posterior from anterior AOB. Successively, it was found that the whole glomerular region of the AOB was perfectly detectable by its autofluorescence and consequently the OMP staining was omitted.

For measurements of the main olfactory epithelium (MOE), coronal sections of the mid part of the turbinates and septal region were considered for each animal. Antibodies against OMP (Dako, Carpinteria, CA, USA; 1:5000), Gαolf (Santa Cruz, Santa Cruz, CA, USA; 1:100), type III adenylyl cyclase (Santa Cruz; 1:200), Tmem16b (anoctamine-2) (Sigma, St Louis, MO, USA; 1:100), GAP-43 (Chemicon, Temecula, CA, USA) and acetylated α-tubulin (Sigma; 1:400) were employed after treatment of the sections with 0.5% SDS for 5 min.

For *in situ* hybridization in the olfactory epithelium, a riboprobe was synthesized from the coding region of the mouse OMP cDNA as template.

The NIS-Elements software (Nikon, Tokyo, Japan) was used for performing measurements on microphotographs.

Cell proliferation assay

Mice were injected I.P. with bromodeoxyuridine (BrdU) at 60 mg kg^{-1}.

Twenty-four hours after injection mice were anaesthetized and transcardially perfused and tissues were processed as above described. Coronal sections were collected from the 12 mid sections of the VNO and MOE. To denature DNA, sections were boiled for 20 min in citrate buffer (10 mM, pH 5.5), rinsed in distilled water and then incubated for 2 min in 1 M HCl. Sections were then incubated overnight at 4°C with mouse monoclonal anti-BrdU antibody (G3G4, Developmental Studies Hybridoma Bank, Iowa University, IA, USA) diluted 1:200 in 0.3%Triton-X100 and 1% albumin.

Immunoreactivity was developed using biotin-labelled anti-mouse IgG antibody, Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) and DAB Enhanced Liquid Substrate System (Sigma).

The mean number of positive cells was obtained by counting immune-positive cells in the 12 mid sections of the VNO.

Apotosis assay

Mice were anaesthetized with pentobarbital and transcardially perfused with a solution of 4% phosphate-buffered paraformaldehyde as described above. Coronal sections of the VNO were considered for this study. Sections were incubated with an antibody against caspase-3. Immunoreactivity was developed using Alexa-488-labelled anti-rabbit IgG antibody.

The mean number of positive cells was obtained by counting immune-positive cells in the 12 mid sections of the VNO.

Physiological responses

Bedding was obtained from individually caged adult mice. For each experiment, soiled bedding from four mice was pooled and divided into six cages. Naïve Gγ8$^{-/-}$ and Gγ8$^{+/+}$ adult females were introduced individually and at the same time to each cage and left undisturbed for 2 h. Female mice were then anaesthetized and transcardially perfused and tissues were processed as described above. Sagittal sections were collected throughout the entire AOB. Then, 10 sections of the mid part of the AOB were considered for each animal. Sections were incubated with a mouse anti-c-Fos antibody (Molecular Probes, Carlsbad, CA, USA) for 48 h at 4°C and immunoreactivity was revealed by using an anti-mouse Alexa-488-conjugated antibody. After taking images and cell counting, the boundaries between anterior and posterior AOB were distinguished by counterstaining sections with an antibody against Gαo (Santa Cruz) followed by an anti-rabbit Alexa-546-conjugated antibody.

Behavioural tests

Resident–intruder. Sexually naïve, resident male Gγ8$^{-/-}$ and Gγ8$^{+/+}$ mice were isolated for 6 days without changing beddings. Testing lasted 10 min and began when a sexually inexperienced adult intruder male was placed in the home cage of the test mouse (resident). Recorded parameters included latency to the first attack, number of

attacks and bites, and cumulative time spent for attacks and for social investigation.

Maternal aggression. Pregnant $G\gamma8^{-/-}$ and $G\gamma8^{+/+}$ female mice were housed singly until the day of testing. On postpartum days 7 and 8 each housed mother was exposed to an intruder male or female for 10 min. Pups were removed from the cage just before testing. Recorded parameters included cumulative attack duration, and number of attacks and bites.

Social recognition. Three-month-old mice were assessed in a social recognition task as previously described (Choleris *et al.* 2003). Both $G\gamma8^{-/-}$ and $G\gamma8^{+/+}$ mice were isolated 24 h before the day of testing. Individual mice were tested five times (tests 1–5) in their home cage, where a cylinder containing a stimulus mouse, namely an unfamiliar conspecific of the same sex and age, was introduced. Each test lasted 5 min, and tests were repeated at 15 min intervals. In tests 1–4, the same stimulus mouse was used, whereas in test 5, a novel stimulus mouse was introduced. The stimulus mouse was enclosed in a small, round wire cage (diameter 10.5 cm, height 11 cm and bars spaced 1 cm apart; Galaxy Cup, Spectrum Diversified Design), which allowed nose contact throughout the bars, but prevented fighting; the experimental subject had been previously habituated to the empty round wire cage that was introduced into the home cage 30 min before the beginning of the test. The stimulus mouse was habituated to stay inside the round wire cage for 5 min just before testing. The response variables recorded were: latency to the first snout contact with the stimulus mouse, total duration of social investigation of the stimulus mouse, and general horizontal and vertical motor activity not related to social investigation.

Cookie finding. Mice were evaluated for their ability to locate a food source (common food pellet) hidden beneath the bedding. Male $G\gamma8^{-/-}$ and $G\gamma8^{+/+}$ mice were left without food overnight. Then, mice were briefly removed from their cage and a food pellet was buried beneath the bedding layer (3 cm). Time from introduction of a mouse into the cage until food pellet retrieval was measured in seconds up to a maximum of 300 s.

Maternal behaviour and pup retrieval. Experiments were performed in bedded home cages of dams. On postnatal day 5, the lactating female was temporarily moved into a clean cage and pups were removed from the nest and distributed in sex-biased groups in the corners of the cage opposite to the nest. The mother was then returned to the nest cage. The total duration of the test was 1 h. Behaviour of the dams was recorded with a digital camera for the experimental times indicated and analysed using the Observer software. Video analyses were done randomly and blindly. The following behaviours shown by each lactating dam were measured: (1) latency to retrieval – time from the first contact with a pup to its retrieval into the nest; (2) in nest – time spent inside the nest; (3) nursing – time spent feeding pups; (4) licking pups – time spent licking or grooming pups; and (5) nest building – time spent building a nest.

Statistical tests

Data were analysed using SPSS statistical software.

Unpaired Student's *t* test was used to measure the significance of differences between two groups, with respect to anatomical measurements and physiological responses. The Mann–Whitney U test, a non-parametric inferential statistic, was used to determine whether two groups showed significant behavioural differences as data were not normally distributed.

Results

Before evaluating the effect of the Gγ8 mutation, we carried out experiments to exclude the possibility that $G\gamma8^{-/-}$ mice showed major neurodevelopmental abnormalities. Body weight of these mice could not be distinguished from that of littermate controls. Moreover, $G\gamma8^{-/-}$ mice are fertile and produce normal litter sizes. $G\gamma8^{-/-}$ mice did not show major locomotor impairments although they displayed somewhat higher levels of horizontal locomotion (walking) but lower rearing and sniffing behaviour. Other aspects of spontaneous maternal behaviour that are reported to be independent of an intact olfactory system, such as the time spent retrieving or licking pups, and building or settling in the nest, appear similar between genotypes. Overall, $G\gamma8^{-/-}$ and control mice did not differ in their capacity for social recognition of individuals of the same sex. When $G\gamma8^{-/-}$ mice were assayed to evaluate olfactory functions, they did not display marked deficits. Mice were tested for three consecutive days for their capacity to recognize a buried piece of food and in none of the trials was there a significant difference between genotypes (Table 1).

We then analysed possible deficits in the MOE of Gγ8-deficient mice as this G-protein subunit is normally expressed in developing olfactory neurons. Overall, our study revealed no gross anatomical alterations in the main olfactory system of $G\gamma8^{-/-}$ mice. Specifically, we measured the thickness of the olfactory epithelium after staining sections with an antibody against OMP (Danciger *et al.* 1989). No significant differences were observed between $G\gamma8^{-/-}$ (23.6 ± 4.6) and control mice

Table 1. Body weight, offspring numbers and overall locomotor activity were not significantly different between the genotypes

	$G\gamma8^{-/-}$	$G\gamma8^{+/+}$	
Weight at weaning (g)	10.4 ± 0.8	12.7 ± 0.5	$F = 0.6$, $P = 0.1$ ($n = 40$)
No. of offspring	7.8 ± 1	8 ± 0.6	$F = 3.9$, $P = 0.9$ ($n = 40$)
Locomotor activity in OF(s):			
Walking (horizontal exploration)	187 ± 7.57	142 ± 7.55	$F = 12.9$, $P < 0.005$ ($n = 24$)
Sniffing	42.5 ± 14.2	62.5 ± 24	$F = 6.13$, $P < 0.05$ ($n = 24$)
Rearing (vertical exploration)	33.2 ± 7.9	52 ± 5	$F = 3.9$, $P = 0.060$ ($n = 24$)
Maternal spontaneous behaviour (s):			
In nest	34.8 ± 7.6	28.2 ± 6.7	$Z = -0.6$, $P = 0.6$ ($n = 10$)
Nest building	9.4 ± 2.8	13.1 ± 3.7	$Z = -0.6$, $P = 0.5$ ($n = 10$)
Nursing	129.9 ± 10.1	105.3 ± 16.1	$Z = -1.7$, $P = 0.1$ ($n = 10$)
Pup licking	6.9 ± 1.6	4 ± 1.5	$Z = -1.4$, $P = 0.2$ ($n = 10$)
Cookie test (s):			
Day 1	201.3 ± 22.9	166.9 ± 28.4	$F = 0.89$, $P = 0.35$ ($n = 24$)
Day 2	138.8 ± 20.2	148.7 ± 21.3	$F = 0.11$, $P = 0.74$ ($n = 24$)
Day 3	41.4 ± 9.8	64.6 ± 16	$F = 1.52$, $P = 0.23$ ($n = 24$)
Social recognition (s):			
Test 1	92.76 ± 6.9	84.87 ± 8.5	
Test 2	69.1 ± 7.9	90.13 ± 5.8	genotype: $F = 0.12$, $P = 0.7$
Test 3	71.7 ± 8.9	64.01 ± 8.1	test: $F = 7.6$, $P < 0.001$
Test 4 (same stimulus mouse)	50.85 ± 7.3	46.74 ± 7.5	G x T: $F = 1.45$, $P = 0.22$
Test 5 (novel stimulus mouse)	83.73 ± 10.5	71.9 ± 8.7	

The test of olfactory function was repeated for three consecutive days. Both genotypes learn to find the cookie faster with repeated trials. No significant differences were observed between genotypes. Overall, social recognition tests were not significant between genotypes. Cookie finding, locomotor activity and maternal spontaneous behaviour were investigated in 3-month-old mice. Values are means ± SD.

(21.6 ± 3.6) in the thickness of the olfactory epithelium taken in the mid-septal, dorsal and ventral regions ($n = 3$, $F = 0.64$, $P = 0.21$; Supplementary Fig. S1A). Moreover, OMP staining was also present in the glomeruli of the olfactory bulb of Gγ8-deficient mice (Fig. S2B). The number of immature olfactory neurons was also measured as Gγ8 is specifically expressed in these cells (Tirindelli & Ryba, 1996). For this, sections were stained with an antibody against GAP-43, a developmental marker that characterizes the immature olfactory neurons that are located in the basal layer of the MOE (Verhaagen *et al.* 1989). Again, no significant differences were observed between $G\gamma8^{-/-}$ and control mice in the number of immature olfactory neurons ($n = 3$, $F = 0.09$, $P = 0.71$; Fig. S1B). Furthermore, mature olfactory neurons of Gγ8-deficient mice appear to normally express the ciliary transduction molecules type III adenylyl cyclase, G-protein α-subunit, Gαolf, Ca^{2+}-activated chloride channel TMEM16b and the ciliary structural protein α-tubulin (Jones & Reed, 1989; Bakalyar & Reed, 1990; Belluscio *et al.* 1998; Rasche *et al.* 2010; Fig. S2A).

BrdU incorporation studies were also performed to reveal possible alterations of the proliferative rate in mutant and control mice. No difference was observed when mice were injected with BrdU 24 h before being killed (Fig. S3).

Overall, although we cannot exclude subtle differences with respect to controls, $G\gamma8^{-/-}$ mice do not appear to show marked deficits of the main olfactory system.

Morphological alterations in the vomeronasal system of Gγ8^{-}deficient mice

Immunohistochemical experiments with anti-V2R antibodies (anti-family A3 and anti-family C) that specifically stain distinct subpopulations of basal neurons were used to probe effects of the Gγ8 knockout on the VNO (Martini *et al.* 2001; Silvotti *et al.* 2007). These experiments indicated that a laminar organization persists in the vomeronasal epithelium of $G\gamma8^{-/-}$ mice (Martini *et al.* 2001). In these mice, A3 and family-C-positive neurons remained localized to the basal region of the VNO just as in control animals. However, importantly, their number was reduced strongly (Fig. 2*A*).

In situ hybridization with cRNA probes encoding the α-subunit of Go and Gi2 (Gαo and Gαi2) confirmed the strong reduction in cell number within the basal neuronal layer of 3-month-old $G\gamma8^{-}/^{-}$ mice (Fig. 2*B*; Fig. S4). In fact, when VNO sections were probed with an antisense cRNA for Gαi2, the absolute area of the apical layer remained unaltered in $G\gamma8^{-}/^{-}$ mice compared with

controls of the same age (26 weeks) ($F = 0.82$, $P = 0.36$ *t* test). Conversely, when VNO sections were probed with an antisense cRNA for Gαo, a 30% reduction of the basal layer was observed in Gγ8^{-}/$^{-}$ mice compared with controls ($F = 4.01$, $P = 0.001$).

The reduction of the basal chemosensory neurons might reflect a deficit that occurs during the early generation of these cells or, alternatively, might reveal an altered balance between adult neurogenesis and apoptotic processes that occurs throughout life. The number of basal neurons in Gγ8^{-}/$^{-}$ mice appeared normal compared with controls at birth ($F = 5.6$, $P = 0.54$) and remained indistinguishable from control mice up to 3 weeks of age ($F = 0.36$, $P = 0.96$). By this developmental stage, in both males and females, the number of basal neurons started to decline to reach a 70% reduction, relative to control mice, at about 1 year of age (52 weeks) (Fig. 3*A*). In Gγ8^{-}/$^{-}$ mice, no difference in the number of A3-positive neurons was observed between sexes at 6 weeks ($F = 3.4$, $P = 0.09$) or 12 weeks ($F = 1.67$, $P = 0.88$) (Fig. 3*B*).

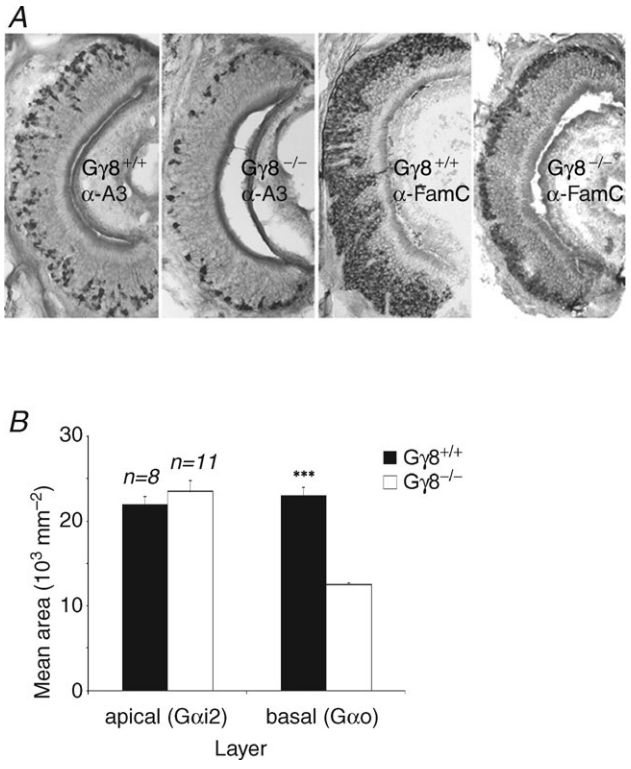

Figure 2. Loss of basal neurons in the VNO of Gγ8^{-}/$^{-}$ mice
A, expression pattern of V2Rs in the VNO of 6-month-old Gγ8^{+}/$^{+}$ ($n = 7$) and Gγ8^{-}/$^{-}$ ($n = 8$) mice. Sections were stained with antibodies against family-C (panC) and subfamily-A3 V2Rs. Scale bar = 100 μm. *B*, areas with Gαi2 and Gαo mRNA expression were measured in VNO sections of 3-month-old Gγ8^{+}/$^{+}$ ($n = 10$) and Gγ8^{-}/$^{-}$ ($n = 10$) mice. Data are means ± SEM. Asterisks indicate significant differences between conditions (***$P = 0.001$).

Two distinct subsets of basal neurons have been recently described by virtue of their differential expression of specific V2R receptors. These studies revealed that Vmn2r1 and Vmn2r2 (grouped into family-C V2Rs) are expressed in two complementary neuronal subsets of the basal layer that also express specific V2R families and that display evolutionary differences (Silvotti *et al.* 2007, 2011). Therefore, VNO sections of Gγ8^{-}/$^{-}$ mice were stained with antibodies raised against these two family-C V2Rs. Results indicate that the cellular loss was significant in both Vmn1r1- and Vmn2r2-positive neuronal subsets between genotypes ($P = 0.009$ and $P = 0.03$, respectively). However, this loss affects both genotypes equally ($F = 3.72$, $P = 0.55$).

We next used BrdU incorporation to study neural proliferation in the VNO of mutant and control animals; notably, the loss of basal neurons in Gγ8^{-}/$^{-}$ mice does not appear to be associated with a reduction of the proliferative rate ($F = 2.94$, $P = 0.62$; Fig. 4).

To determine whether Gγ8$^{-/-}$ mice displayed an increased rate of programmed cell death, VNOs were assessed for rates of apoptosis using caspase-3 cleavage analysis (Yokoyama *et al.* 2011). Caspase-3 activation is an integral step in programmed cell death pathways and results in protein cleavage, detectable by specific antibodies. Caspase immunoreactivity was used to identify apoptotic cells in Gγ8$^{-/-}$ and control mice. This analysis revealed 15.5 ± 0.64 and 13.8 ± 0.57 ($F = 0.61$, $P = 0.06$) immunoreactive cells for cleaved caspase-3 in Gγ8$^{-/-}$ and control mice, respectively (Fig. 5). Interestingly, we found a higher density of caspase-positive neurons at the boundaries with the non-sensorial epithelium of the VNO. The pattern of cleaved caspase-3 immunoreactivity indicates a difference in dying cells between genotypes, but this did not reach statistical significance ($P = 0.064$).

We then reasoned that the decreased number of chemosensory neurons in Gγ8$^{-/-}$ mice might also reflect changes in the target region for VNO axons, namely the posterior glomerular layer of the AOB. Therefore, we cut sections through the AOB and used anti-Gαo staining to outline the boundary between the anterior glomerular layer that receives projections from the apical neuronal layer of the VNO and the posterior glomerular layer that receives inputs from basal neurons. Anti-Gαo staining of the AOB suggests that, in Gγ8$^{-/-}$ mice, the residual population of basal VNO neurons still project to the posterior glomerular layer. However, the area of this region appeared reduced by 30% in Gγ8$^{-/-}$ mice ($F = 0.49$, $P < 0.005$). Conversely, the anterior AOB did not show changes in morphology or in size ($F = 1.43$, $P = 0.61$; Fig. 6). Thus, the reduction of the posterior glomerular layer of the AOB is consistent with the loss of chemosensory neurons in the VNO of Gγ8$^{-/-}$ mice.

Physiological responses in the VNO of Gγ8-deficient mice

We next tested if the loss of Gγ8 affects pheromone signalling. Expression of the immediate-early gene c-Fos is correlated with sensory activity of the vomeronasal system and is often used as a marker for pheromonal signal input in the AOB. As there is a slow reduction in the number of basal chemosensory neurons with age, all experiments were carried out in 3-month-old mice. Thus, to investigate the responses to pheromonal stimuli in behaving $G\gamma8^{-/-}$ mice at the molecular level, the induction of c-Fos expression was analysed in the anterior and posterior AOB of normally cycling females exposed to male soiled bedding (Guo *et al.* 1997; Halem *et al.* 1999; Inamura & Kashiwayanagi, 2000; Dudley *et al.* 2001). Stimulating control females with soiled bedding of isolated males resulted in an increase in AOB neurons that were immunopositive for c-Fos. As expected, control mice showed a 3- to 4-fold increase in immunopositive glomerular cells of the anterior ($F = 0.24$, $P < 0.005$, t test) and posterior ($F = 2.15$, $P < 0.05$) AOB upon pheromonal exposure when compared with unstimulated animals (Fig. 7; Fig. S5). In $G\gamma8^{-/-}$ mice, c-Fos induction was not observed in the posterior AOB when females were stimulated with male soiled bedding ($F = 6.61$, $P = 0.16$). Conversely, pheromone-mediated c-Fos induction persisted apparently unchanged in the anterior glomerular layer of $G\gamma8^{-/-}$ mice ($F = 2.63$, $P < 0.01$). Notably, the basal level of c-Fos expression (exposure to clean bedding) in the posterior glomerular cell layer was almost identical in $G\gamma8^{-/-}$ and control mice ($F = 0.64$, $P = 0.58$), suggesting that the loss of VNO neurons probably does not affect the number of

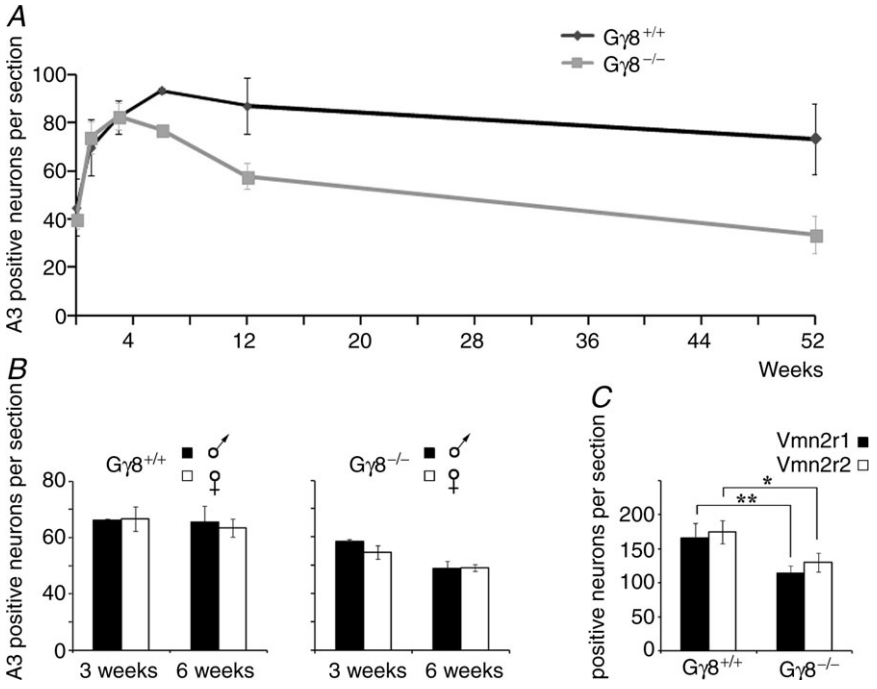

Figure 3. Age- and sex-related loss of VNO basal neurons in $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ mice
A, the number of A3-V2R-positive neurons was measured in $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ male mice at different ages ($n = 6$ for each age). *B*, bar chart showing the number of A3-V2R-positive neurons in both sexes at two different postnatal weeks ($n = 8$). *C*, bar chart showing the number of Vmn2r1- and Vmn2r2-immunopositive neurons in 3-month-old $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ mice ($n = 5$). Data are means ± SD. Asterisks indicate significant differences between conditions (*$P < 0.05$; **$P < 0.01$).

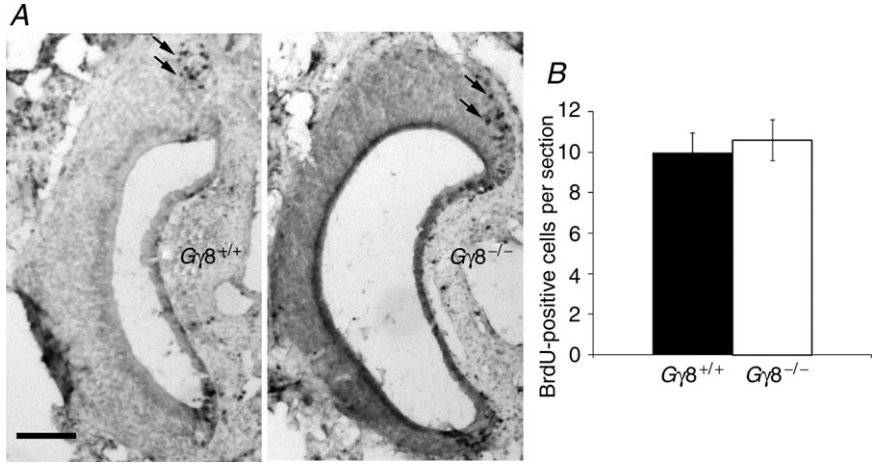

Figure 4. Cell proliferation in the VNO
A, VNO sections of 3-month-old $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ mice immunostained with anti-BrdU. Arrows show the active neuroregenerative region; scale bar = 100 μm. *B*, BrdU-positive neurons quantified in $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ mice ($n = 6$). Data are means ± SD.

glomerular cells (Fig. 7). Loss of pheromone-induced cFos expression in the AOB of Gγ8 mice was also evident in the posterior region of the mitral cell layer ($F = 3.15$, $P < 0.001$). Unfortunately, the c-Fos response to pheromone stimulation in the anterior region of the mitral cell layer was not possible to obtain given the difficulty in defining the precise boundaries of this region. Thus, immediate-early gene induction indicates that there is a major deficit in pheromonal signalling in $G\gamma8^{-/-}$ mice.

Socio-sexual behaviour in Gγ8-deficient mice

VNO removal and genetic ablation of molecular components of its signal transduction pathways are known to influence specific socio-sexual behaviours in mice.

$G\gamma8^{-/-}$ and control mice were then examined by the induced aggression test in males and in lactating females, both these behaviours being dependent on the integrity of the olfactory organs.

A female's aggression serves as a means to protect her litter. Thus, lactating females generally display higher levels of aggression towards male rather than female intruders (Gandelman, 1972; Palanza & Parmigiani, 1994). In our experiments, 3-month-old $G\gamma8^{-/-}$ and wild-type littermate lactating females were confronted in a 10 min test with male and female intruders on post-partum days 7 and 8, respectively (Fig. 8). As expected, control females repeatedly attacked male intruders and generally showed lower aggressive behaviour toward female intruders. In contrast, aggressive behaviour towards male intruders was significantly reduced in $G\gamma8^{-/-}$ lactating females. In fact, $G\gamma8^{-/-}$ dams delivered significantly lower numbers of bites than control lactating females ($Z = -2.5$, $P < 0.05$, Mann–Whitney test) and attacks ($Z = -2.5$, $P < 0.05$) on male intruders. Moreover, $G\gamma8^{-/-}$ dams spent less time attacking male intruders ($Z = -2.7$, $P = 0.005$).

VNO-dependent intermale aggression is a crucial marker of social competitive behaviour that is triggered by compounds present in urine (Maruniak *et al.* 1986; Chamero *et al.* 2007). We used a standard test to assess male aggression whereby a male intruder was introduced into a cage containing a singly housed male resident and the aggressive behaviour of the resident recorded. Results show that 3-month-old $G\gamma8^{-/-}$ male mice displayed an overall reduction of aggressive behaviours towards the same-sex

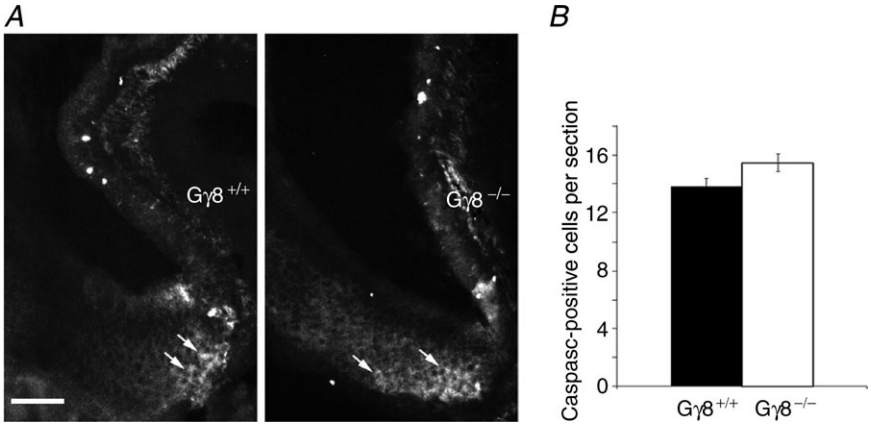

Figure 5. Cell apoptosis in the VNO
A, VNO sections of 3-month-old $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ mice immunostained with anti-caspase-3. Arrows show the apoptotic cells of the neuroregenerative region of the VNO; scale bar = 100 μm. *B*, caspase-positive neurons quantified in $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ mice ($n = 4$). Data are means ± SD.

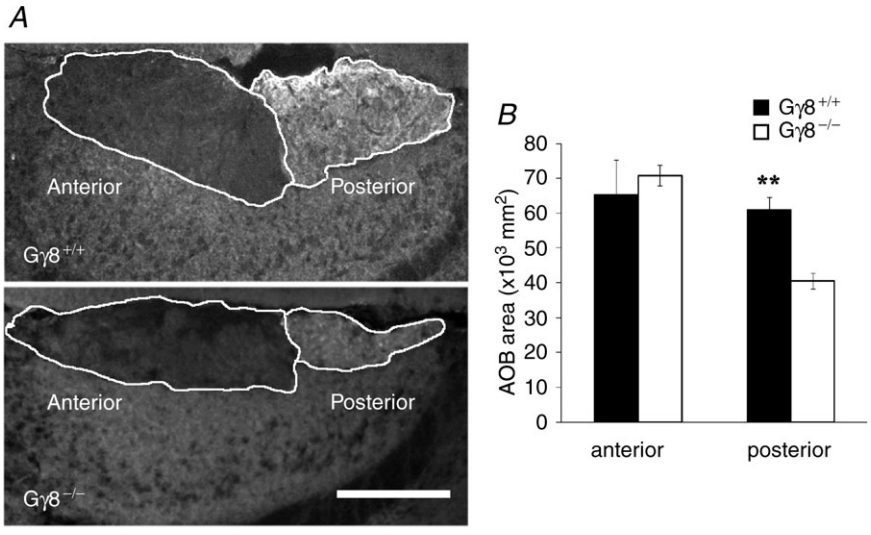

Figure 6. Size reduction of the posterior AOB in $G\gamma8^{-/-}$ mice
A, sagittal sections of the mid part of the AOB. Three-month-old $G\gamma8^{+/+}$ ($n = 11$) and $G\gamma8^{-/-}$ ($n = 12$) mice were stained with anti-Gαo to identify the partition of the two anatomical sub-regions of the AOB. Scale bar = 200 μm. *B*, quantified area of the posterior and anterior AOB in $G\gamma8^{+/+}$ and $G\gamma8^{-/-}$ mice. Data are means ± SD. Asterisks indicate significant differences between conditions (**$P < 0.005$).

intruder compared with wild-type littermates (Fig. 9). More specifically, G$\gamma 8^{-/-}$ mice delivered lower numbers of bites ($Z = -2.8$, $P < 0.005$, Mann–Whitney test) and attacks ($Z = -2$, $P < 0.05$) than controls; latencies to attack the intruders were higher ($Z = -3.13$, $P < 0.001$), whereas duration of attacks ($Z = -2.3$; $P < 0.05$) was significantly lower in G$\gamma 8^{-/-}$ males than in control mice. Finally, G$\gamma 8^{-/-}$ male mice showed a marked increase in social investigation toward the intruder compared with littermates not carrying this mutation ($Z = -3.08$, $P < 0.005$).

Discussion

To address the physiological role of the G$\beta\gamma 8$ complex in pheromonal communication, we generated mice lacking the olfactory specific G-protein γ-subunit, G$\gamma 8$. We demonstrate that deletion of this gene leads to the impairment of specific vomeronasal- rather than olfactory-mediated behaviours such as maternal and intermale aggression, while leaving intact behavioural expressions that do not rely on the VNO functioning. Moreover, the vomeronasal system of mice carrying the $\gamma 8$ subunit deletion produces reduced responses to pheromonal stimulation.

What might be the mechanisms exerted by G$\gamma 8$ to elicit a cellular response in a VNO neuron in order to convey the correct pheromonal information? There is indirect evidence that PLC$\beta 2$ represents the key element in the enzymatic cascade that leads to the opening of the TRPC2 channel (which is ubiquitously expressed in vomeronasal neurons) and the generation of an action potential (Stowers *et al.* 2002; Liman & Dulac, 2007). Unfortunately, PLC$\beta 2$ has only been pharmacologically characterized in the VNO (Inamura *et al.* 1997; Holy *et al.* 2000; Spehr *et al.* 2002); on the other hand, Gαo is

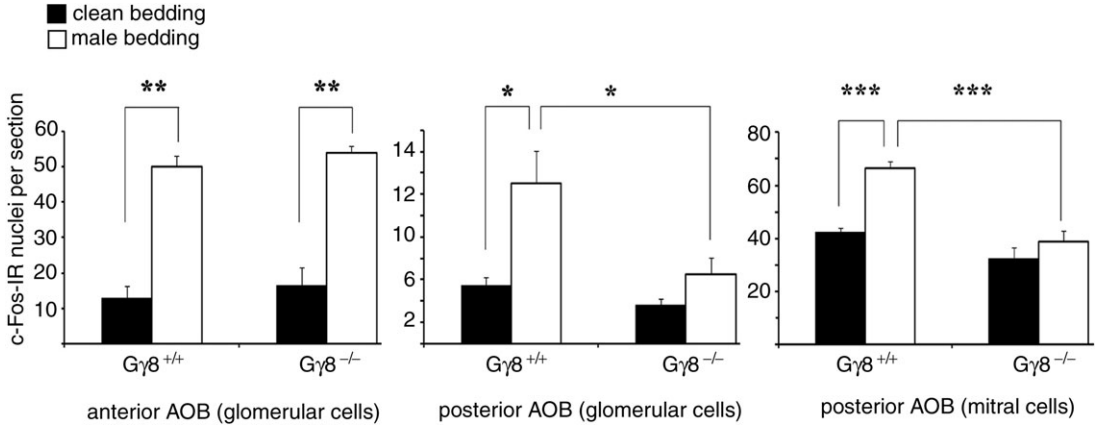

Figure 7. Physiological responses to pheromones are strongly reduced in G$\gamma 8^{-/-}$ mice
c-Fos immunoreactivity in the AOB is reduced in 3-month-old G$\gamma 8^{-/-}$ mice exposed to pheromones. Exposure of females to male bedding increases c-Fos expression in the glomerular and mitral layer of the posterior AOB in G$\gamma 8^{+/+}$ ($n = 15$) but not in G$\gamma 8^{-/-}$ ($n = 14$) mice. Conversely, the pheromonal response in the glomerular layer of the anterior AOB is not affected by the G$\gamma 8$ gene deletion. Asterisks indicate significant differences between conditions (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$).

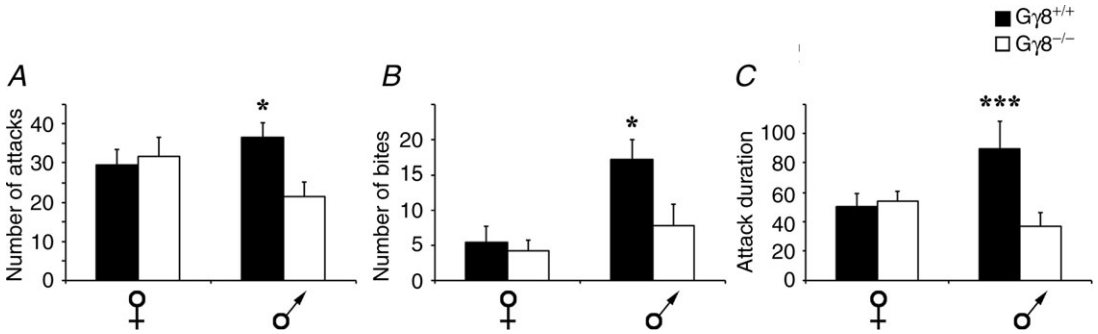

Figure 8. Loss of maternal aggression in G$\gamma 8^{-/-}$ females
Analysis of the number of attacks (*A*) and bites (*B*) and attack duration (*C*) of 3-month-old lactating G$\gamma 8^{+/+}$ ($n = 17$) and G$\gamma 8^{-/-}$ ($n = 20$) females exposed to male or female intruders. In contrast to G$\gamma 8^{-/-}$ females, G$\gamma 8^{+/+}$ females responded aggressively to intruder males. Maternal aggressive behaviour in G$\gamma 8^{-/-}$ females was not significantly different from that observed in G$\gamma 8^{+/+}$ females exposed to female intruders. Asterisks indicate significant differences between conditions (*$P < 0.05$; ***$P < 0.005$).

unlikely to directly couple to PLCβ2 as such interaction has never been described in other systems. Indeed, an antibody against Gαo has been shown to inhibit IP_3 production in stimulated VNO neuron membranes, suggesting that the heterotrimeric Go might be involved in PLCβ2 activity (Krieger *et al.* 1999).

It is commonly accepted that, upon receptor activation, the Gβγ complex is released from Gα and modulates functions of several effectors (Smrcka, 2008). *In vitro* experiments, in different cellular models, suggest that Gβγ is also capable of directly activating PLCβ2 and adenylyl cyclase II, molecules that are reportedly expressed in VNO neurons (Kuang *et al.* 1996; Chen *et al.* 1997). Thus, the Gβ2γ8 complex, rather than Gαo, might be directly responsible for the production of IP_3, via the activation of PLCβ2. Indeed, antibodies against Gγ8 are effective in dramatically reducing the IP_3 production in VNO membranes stimulated by protein pheromones such as major urinary proteins (Runnenburger *et al.* 2002).

It has been shown that major urinary proteins exert opposing effects on male mice both within species, promoting aggressiveness, and between species, initiating defensive behaviour (Chamero *et al.* 2007; Papes *et al.* 2010). Contextually, here, we found that the Gγ8 deletion reduces male aggressive behaviour. Thus, because intraspecific aggressiveness is thought to be mediated by urinary proteins, Gγ8 is likely to be linked to receptors that recognize these molecules.

In the VNO, deletion of the Gγ8 gene does not interfere with normal development of the organ but does promote a slow but progressive loss of the basal neuronal layer, possibly due to a slowly occurring increase in neuronal death rate rather than to reduced neurogenesis. Accordingly, the glomerular region of the posterior AOB that receives inputs from basal VNO neurons is also proportionally reduced. This observation may suggest that a correct Gγ8 activity is required for the turnover of mature VNO neurons. However, this result also raises the possibility that Gγ8 may be primarily involved in the developmental programme of VNO neurons and that the physiological responses observed in Gγ8-deficient mice reflect the loss of neurons rather than the uncoupling of molecules involved in the pheromone transduction pathway. At present, we cannot rule out both possibilities, although biochemical studies on Gγ8 in the VNO would also seem to support a role in pheromone transduction for this protein (Runnenburger *et al.* 2002). Similarly, we cannot entirely exclude that part of the effects are due to modifications occurring in the main olfactory system, as Gγ8 is indeed expressed in immature olfactory neurons. However, we have been able to detect alterations in the main olfactory system.

The tight correlation between loss of function and neuronal depletion appears to be a general mechanism in the VNO. In fact, mutant mice for Gαo and Gαi2, which are respectively expressed in the apical and basal layer of the VNO, display reduced physiological activity of the VNO (and an altered sexual behaviour) associated with a significant reduction in the size of the neuronal layers of the VNO where these proteins are normally expressed (Tanaka *et al.* 1999; Norlin *et al.* 2003; Chamero *et al.* 2011). Interestingly, although Gγ8 is also expressed (but less abundantly) in the apical layer of the VNO (Ryba & Tirindelli, 1995; Tirindelli & Ryba, 1996), the chemosensory neurons of this layer do not appear to be either anatomically (Figs 2 and 6) or functionally (Fig. 7) affected by the Gγ8 mutation. This might indirectly suggest that the activity of apical neurons in $G\gamma8^{-/-}$ mice is maintained by the expression of other transduction molecules that replace the Gγ8 functions. Noteworthy, apical neurons additionally express Gγ2 (Runnenburger *et al.* 2002), which has similar functional properties to Gγ8 (Ryba & Tirindelli, 1995); thus, it is plausible that, at least in the apical neurons, part of the pheromonal signalling occurs through parallel or overlapping mechanisms.

Centripetal inputs are impaired in $G\gamma8^{-/-}$ mice as pheromonal stimulation greatly affects c-Fos expression in glomerular cells of the posterior AOB, suggesting that the lack of Gγ8 expression may also influence signals directed to central areas of the brain such as the amygdala or

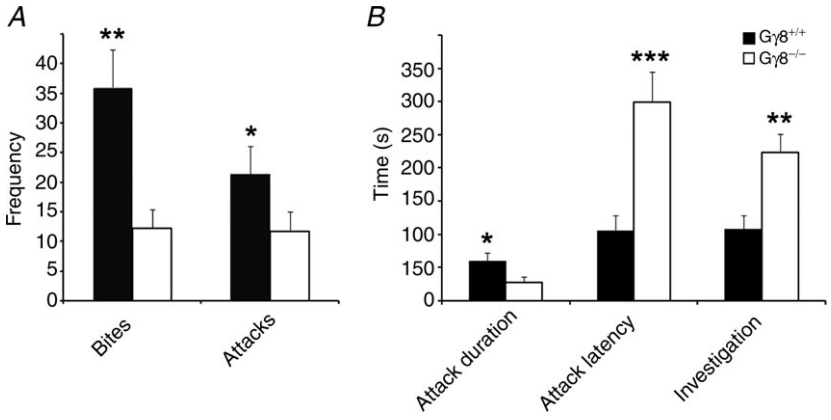

Figure 9. Loss of intermale aggression in $G\gamma8^{-/-}$ mice
Analysis of the number of attacks and bites (*A*) and analysis of attack duration, first attack latency and investigation (*B*) in 3-month-old $G\gamma8^{+/+}$ ($n = 16$) and $G\gamma8^{-/-}$ ($n = 20$) males exposed to male intruders. Asterisks indicate significant differences between conditions (*$P < 0.05$; **$P < 0.005$; ***$P < 0.001$).

hypothalamus. The specific functions of the signal transduction pathways that originate from the two distinct VNO neuronal pathways have not been elucidated. However, it is intriguing but somewhat puzzling why deficits in the signal transduction mechanisms of apical (Gαi2), basal (Gαo, Gγ8) or both neuronal layers (TrpC2) of the VNO all display very similar behavioural alterations (Stowers *et al.* 2002; Norlin *et al.* 2003; Chamero *et al.* 2011). Given that receptors of the apical and basal neurons presumably recognize structurally different pheromonal molecules (Del Punta *et al.* 2002; Leinders-Zufall *et al.* 2009; Haga *et al.* 2010), it is plausible that the presence of two neuronal pathways (apical and basal) may help amplify signals ensuring a higher sensitivity to pheromones. Recently, it has been demonstrated that there is a small group of neurons in the hypothalamus that stimulate aggression and are inhibited by local circuits involving cells that detect sexually related information (Lin *et al.* 2011). It is highly likely that different pheromonal cues are a major input that controls the activation and inhibition of the hypothalamic neurons for driving aggression. Given the similar phenotypes observed in many mutant mice that influence VNO, namely the aggressiveness, it might be suggested that combined input from several distinct pheromone pathways is required to activate hypothalamic aggression neurons. In contrast, other socio-sexual responses, which appear normal in these phenotypes (see Table 1), might be stimulated by any of a number of different pathways, including non-VNO ones. One speculation for this difference would be that it is extremely important at an evolutionary level to maximize the chance of mating but to control aggression to just the very few situations where it is likely to be crucial for an animal to fight to defend territory or its young. Thus, initiating a mating response probably requires just one of many stimuli whereas starting a fight requires a number of different inputs. The clear-cut strong aggression deficit observed in $G\gamma8^{-/-}$ mice emphasizes this view.

Conclusions

Our study demonstrates that the G-protein γ-subunit, Gγ8, is essential for the maintenance of the neuronal population of the VNO and ultimately for correct transmission of the pheromonal signal that is responsible for the aggressive behaviour typically displayed in female and male mice towards intruders.

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Additional information

Competing interests

The authors declare no competing financial interests.

Author contribution

G.M. and S.T. carried out the molecular and cellular studies and analysed the data; V.S. carried out the behavioural studies and analysed the data; P.F. and P.P. participated in conceiving the study, its design and coordination and reviewed the manuscript; A.Z. produced the mutant line of mice; R.T. conceived the study, performed preliminary experiments and wrote the manuscript. All authors read and approved the final manuscript.

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