A novel brain receptor is expressed in a distinct population of olfactory sensory neurons

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Abstract
Three novel G-protein-coupled receptor genes related to the previously described RA1c gene have been isolated from the mouse genome. Expression of these genes has been detected in distinct areas of the brain and also in the olfactory epithelium of the nose. Developmental studies revealed a differential onset of expression: in the brain at embryonic stage 17, in the olfactory system at stage E12. In order to determine which cell type in the olfactory epithelium expresses this unique receptor type, a transgenic approach was employed which allowed a coexpression of histological markers together with the receptor and thus visualization of the appropriate cell population. It was found that the receptor-expressing cells were located very close to the basal membrane of the epithelium; however, the cells extended a dendritic process to the epithelial surface and their axons projected into the main olfactory bulb where they converged onto two or three glomeruli in the dorsal and posterior region of the bulb. Thus, these data provide evidence that this unique type of receptor is expressed in mature olfactory neurons and suggests that it may be involved in the detection of special odour molecules.

Introduction
G-protein-coupled receptors (GPCRs) are integral membrane proteins which mediate signals to the interior of cells via activation of heterotrimeric G-proteins, which subsequently interact with and activate various effector proteins, ultimately resulting in the physiological response. GPCRs are involved in the transduction of a large variety of extracellular signals as diverse as inorganic ions, peptides and lipids or sensory stimuli like photons or odourants. More and more orphan G-protein-coupled receptors have been made available by various cloning procedures such as PCR amplification or systematic sequencing of cDNA libraries (Marchese et al., 1999).

We have recently isolated a cDNA clone (RA1c) from a rat brain library encoding a novel putative GPCR (Raming et al., 1998). Comparison of the deduced amino acid sequence of RA1c shows a low (24–30%) but significant homology to other GPCRs, e.g. to peptide hormone receptors and receptors for adenosine and melatonin. A slightly higher sequence identity was found to olfactory receptors (Buck & Axel, 1991). In situ hybridization studies demonstrated that RA1c was expressed in well-defined areas of the brain stem near the fourth ventricle as well as in the frontal cortex region. In addition, RA1c was expressed in the olfactory epithelium lining the posterior nasal cavity. The cell type that expresses this GPCR, however, could not be identified unequivocally.

The nasal sensory epithelium consists of three principle cell types whose nuclei are generally stratified: sustentacular cells, olfactory receptor neurons and basal cells (Allison, 1953; Graziadei, 1977). The observed zonal organization of RA1c-expressing cells in the epithelium matches the pattern observed with other olfactory receptor (OR) genes (Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994), and thus suggests that it may be expressed in olfactory receptor neurons. However, due to the localization of labelled cells rather deep within the epithelium, expression in, e.g., basal cells cannot be excluded.

To assess the notion that RA1c may be expressed in olfactory sensory neurons, we decided to exploit the characteristic morphological features of the RA1c-expressing cells. Olfactory receptor neurons extend a dendrite to the surface of the epithelium and an unbranched axon into the olfactory bulb. A prerequisite for the analyses therefore was the staining of the entire cell including their processes. For this purpose, a transgenic approach was employed that allows the coordinated translation of the receptor protein along with a marker protein fused to the microtubule-associated protein ‘tau’ (Mombaerts et al., 1996; Wang et al., 1998; Rodriguez et al., 1999). As a first step, we have therefore searched for RA1c-related mouse genes. The isolation of the mouse orthologous gene, designated mouse olfactory-like (MOL)2.3, then allowed generation of a transgenic mouse line and characterization of the cells within the olfactory epithelium expressing this unique receptor.

Materials and methods
PCR analysis of mouse genomic DNA
MOL2.3 was amplified from mouse genomic DNA using the RA1c-specific primer combination 239: 5’-ATG AGC TCC TGC AAC TTC ACT CAC-3’/240: 5’-TCA CGT GTT TCC TGC AGC TTC AAT-3’. Amplification was carried out using 2 U Taq-Polymerase (Life Technologies, Eggenstein, Germany), 2 mM MgCl₂, 1 μM of each

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primer, 200 mM deoxyribonucleotides in 10 mM Tris, pH 8.3 and 50 mM KCl on a Peltier Thermocycler PTC 200 (MJ Research Inc., Waltham, MA, USA). PCR conditions were: 35 cycles of denaturation at 96°C (1 min), annealing at 55°C (1 min) and extension at 72°C (2 min); initial denaturation at 96°C was 5 min and the final extension at 72°C was 10 min.

**BAC library screen**

A filter library of mouse genomic ES-cell DNA (129Sv) in a BAC vector (Release II; Genome Systems, St. Louis, MI, USA) was screened with 50–100 ng of MOL2.3 DNA labelled with (α-32P)-ATP (New England Nuclear, Boston, MA, USA) by random priming with Klenow enzyme (Life Technologies) according to standard protocols (Sambrook et al., 1989). The probe was hybridized at 65°C in 5 × SSPE (20 × SSPE is 3.6 M NaCl, 200 mM NaH2PO4, 20 mM EDTA, pH 7.4), 5 × Denhardt’s, 0.1% SDS and 10 µg/ml salmon sperm DNA overnight. Washes were performed in 2 × SSC/0.1% SDS at 65°C for 2 × 30 min. Filters were exposed overnight to Fuji X-ray film at −80°C.

**PCR analysis of genomic clones**

Template DNA was prepared by alkaline lysis (Sambrook et al., 1989). For the amplification of OR sequences, 1 ng of BAC DNA was employed as template with primer combination 3.1: 5'-GC(ATGC)-ATGGC(ATGC)TA(C)GA(C)ATGC(T)A-3′ and 7.1: 5'-A-(AG)(ATGC)GC(AT)GA(T)AG(T)GA(A)AC(G)GG(G)AGTT-3′ or P24: 5′-(T)ICA(C)TACCIATGTA-3′ and P28: 5′-(GA)AAIGG(AG)(T)IA(G)CATIGG-3′ and P25: 5′-(CT)IG-(CT)ATGGCITA(C)GA-3′ and P28, respectively. PCR conditions were: 96°C for 45 s, 40°C for 3 min and 72°C with 6-s extension per cycle for 40 cycles, followed by 72°C extension for 10 min. An aliquot of each PCR was analysed by agarose gel electrophoresis on 1.2% gels. PCR products were cloned using the pGEM T-vector system according to the manufacturer’s specifications (Promega, Mannheim, Germany).

**DNA sequencing**

DNA sequencing was carried out using TaqI DNA polymerase and fluorescently labelled deoxy terminator chemistry on an automated DNA sequencer (Model 310; Applied Biosystems, Foster City, CA, USA). Sequencing reactions were assembled and cycle sequencing was carried out according to manufacturer’s protocols using T3, T7 or SP6 primers; custom oligonucleotide primers were used when necessary. All sequences represent double-stranded sequencing or a minimum of two sequences from the same strand. Sequence homologies were determined using the BLAST algorithm. Sequences were assembled and translated using the AutoAssembler program (Applied Biosystems) and aligned using the MegAlign program (DNAStar, Inc., Madison, WI, USA). Phylogenetic analysis in MegAlign was performed by using the clustalV method (Higgins & Sharp, 1989).

**Targeting vectors**

A HindIII fragment containing the coding region of MOL2.3 was subcloned into pBS-SKII (Stratagene, La Jolla, CA, USA). A PacI site was generated by recombinant PCR three nucleotides downstream of the stop codon of the MOL2.3 gene. A PmeI site was generated at the 3′ end of the targeting vectors for linearization of the construct. A PacI fragment containing IRES-GFP-IRES-taulacZ-LoxP-neo-LoxP (IGITIL; IRES, internal ribosome entry site; GFP, green fluorescent protein) was inserted into the PacI restriction site.

**Gene targeting**

The targeting vector was linearized with PmeI and electroporated into R1 cells. Genomic DNA from G418-resistant ES cells was digested with HindIII and analysed by Southern blot hybridization with a 3′ probe external to the targeting vector. Recombinant clones were injected into CD-1 blastocysts, and germline transmission was obtained (clone B247). The neo-selectable marker was subsequently removed by crossing mice heterozygous for the LNL allele to Ella-Cre transgenic mice (Lakso et al., 1996). Intercrosses of loxP-positive mice resulted in loxP-heterozygous and loxP-homozygous mice that were devoid of the Cre transgene. All analyses were performed with mice that did not carry the Cre transgene. Mice were in a mixed (129 × CD-1) background.

**X-gal staining**

Tissues were immersion-fixed in 2% paraformaldehyde in PBS, pH 7.4, for 60 min at 8°C, incubated in 25% sucrose overnight and frozen in Tissue Freezing Medium (Leica Instruments, Nussloch, Germany). Twelve-micrometre sections were cut on a Leica CM 3000 cryostat (Leica) and stained with X-gal as follows: they were washed with buffer A [100 mM phosphate buffer (pH 7.4), 2 mM MgCl2 and 5 mM EGTA] once for 5 min and once for 25 min at room temperature, followed by two incubations of 5 min at room temperature in buffer B [100 mM phosphate buffer (pH 7.4), 2 mM MgCl2, 0.01% sodium deoxycholate and 0.02% Nonidet P40].

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**Fig. 1.** Deduced amino acid sequences of receptor fragments of the extracellular loop 3 (e3). The predicted positions of transmembrane domains 6 and 7 are indicated by a horizontal line. Amino acid residues conserved in all sequences are shaded in dark grey, those highly conserved in light grey. Additional amino acids in e3 of class I olfactory receptors are indicated by asterisks. Sequences not identified in this study are from: 1Drul et al., 1995; 2Raming et al. (1993); 3Ngai et al. (1993); 4Barth et al. (1997).
blue precipitate was generated by exposure at 37°C to buffer C (buffer B with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/mL of X-gal).

In situ hybridization
Probes were generated from templates using the SP6/T7 in vitro transcription system (Roche Diagnostic, Mannheim, Germany). Linearized vector (2 μg) was transcribed in the presence of 70 nmol digoxigenin-11-uridine-5'-triphosphate. RNA was precipitated with ethanol and resuspended in 20 μL of in situ grade hybridization buffer (Amersham Pharmacia Biotech, Freiburg, Germany) containing 50% deionized formamide. Mice (>6-week-old) were killed by CO₂ asphyxiation and decapitated. The lower jaw and top of the skull were carefully removed using a bone cutter (Fine Science Tools, Heidelberg, Germany). Embryos were obtained from timely mated mice; the time point of vaginal plug was counted as E0. Tissues were embedded in Tissue-Tek and frozen on dry ice. Coronal sections of 10 μm were cut on a cryostat at –24°C, adhered to Superfrost microslides and air-dried for 2 h. For in situ hybridization, tissue sections were covered with 10 μL of hybridization solution containing ~3–5 ng digoxigenin-labelled RNA and coverslipped. Hybridization and posthybridization washes were performed as described earlier (Strotmann et al., 1994).

Quantitative analyses
The relative positions of cell somata within the vertical dimension of the olfactory epithelium were determined as detailed before (Strotmann et al., 1996) using a video camera connected to a Zeiss Axiohot microscope and the image analysis program Semper6 (Synoptics, Cambridge, UK). At 400× magnification, the vertical...
distance of a cell body from the basal membrane (a) was determined. At the same position, the thickness of the cellular layer of the epithelium – the distance from the basal membrane to the nasal lumen (b) – was determined. The relative position of the cell soma was then determined as a divided by b.

Microscopy and photography

Whole mount specimens were photographed using a Wild M8 stereomicroscope (Wild, Heerbrugg, Germany). Sections were analysed with a Zeiss Axiophot microscope. Fluorescence was examined using the appropriate E-GFP filter set.

Results

Identification of RA1c-related GPCRs

Employing RA1c-specific primers 239 and 240 in PCR experiments, a 963-bp DNA fragment (MOL2.3) with 95.2% nucleotide sequence identity to RA1c was amplified from mouse genomic DNA. The deduced amino acid sequence of MOL2.3 is 98.8% identical to RA1c, suggesting that these are orthologous genes. Southern blots using the MOL2.3 coding region as a probe on mouse genomic DNA revealed only one hybridizing band (data not shown), suggesting that it is a single copy gene within the mouse genome. However, due to its
relatedness to the olfactory receptor genes, which are organized in large clusters within the genome (Ben-Arie et al., 1994; Sullivan et al., 1996; Brand-Arpon et al., 1999), this fragment was used to isolate the MOL2.3 genomic locus and assess it for other GPCRs. Using degenerate primers on an isolated BAC clone indeed led to the identification of two additional gene fragments (MOL8.17 and MOL10.8) with 80.6% sequence identity to each other; both share only ≈50% sequence identity with MOL2.3. All three genes share sequence identity of ≈30% with olfactory receptors from mammals as well as from fish, a phenomenon also described for RA1c (Raming et al., 1998). A phylogenetic analysis revealed that all three MOL receptors are related to class I (fish-like) olfactory receptors (Freitag et al., 1995) (data not shown), a notion that is supported by certain motifs, most notably the structural property in extracellular loop 3 (e3) (Fig. 1). The MOL receptors all exhibit an extension of two amino acid residues in e3 which is characteristic for class I olfactory receptors.

Expression pattern of MOL2.3 and MOL8.17

To determine whether the MOL receptors share the unique expression pattern of RA1c, in situ hybridization experiments with digoxigenin-labelled antisense RNA-probes were performed. Probing sections through the medulla oblongata of the mouse brain revealed MOL2.3-reactive cells in distinct areas close to the fourth ventricle, specifically in the area postrema (a.p.) and the nucleus tractus solitarius (n.t.s.) (Fig. 2a). Hybridization signals were observed neither in other areas of the medulla nor in the neighbouring cerebellum. Probing adjacent sections with a MOL8.17 probe resulted in labelled cells in the same areas (Fig. 2b). The novel MOL receptors thus exhibited the same characteristic distribution pattern within this particular brain region. On cross-sections through the mouse nasal cavity the probes stained a significant number of cells exclusively located in the dorsal zone of the olfactory epithelium (Fig. 2c and d), reminiscent of the results obtained with RA1c in the rat olfactory epithelium (Raming et al., 1998).

Expression of MOL2.3 during development

The moderate relatedness of the MOL receptors to the olfactory receptor gene family and especially their expression in distinct brain regions raises the question about their putative functional role. Analysing the time course of expression in the olfactory epithelium may give some insight, because odourant receptors follow a typical temporal and spatial expression pattern during development (Strotmann et al., 1995; Sullivan et al., 1995). Determining the onset of receptor expression by in situ hybridization revealed the presence of first MOL2.3-positive cells between embryonic days E11 and E12. At E12, ≈200 reactive cells were detectable, specifically located in the dorsal region of the developing nasal cavity (Fig. 3a). Interestingly, the MOL2.3 probe also labelled cells in the mesenchyme located between the olfactory epithelium and the presumptive olfactory bulb (Fig. 3b). In the brain, MOL2.3-reactive cells were not detectable at these embryonic stages (E11/E12). In the medulla oblongata, signals were first detectable at E17 (Fig. 3c). At this stage ≈80 cells were counted in the brain, whereas the number of stained
cells in the olfactory epithelium had already risen to \(\approx 2000\). The almost exponential increase in reactive cells continued during the following pre- and postnatal period (Fig. 3d). At postnatal day (PN) 20 a total number of \(\approx 30000\) MOL2.3-reactive cells were counted in the olfactory epithelium. In the a.p. and n.t.s. the number of cells reached a peak (\(\approx 1200\) cells) at PN20 and then decreased slightly in older animals (\(\geq PN\ 42\)) to \(\approx 750\) cells (Fig. 3d).

**MOL2.3 expression in olfactory neurons**

The observation that MOL receptors are expressed in distinct brain areas as well as in the olfactory epithelium raises the question if the nasal epithelial cells in fact are olfactory neurons and if so, do they display special features? To unequivocally explore the features of cells expressing these special types of GPCRs, a transgenic approach was employed. Following the procedure of Mombaerts et al. (1996), the MOL2.3 gene locus was modified by introducing IGITL (Fig. 4); this design results in specific coexpression of GFP and tau-\(\beta\)-galactosidase along with MOL2.3 from a tricistronic message. Germline transmission was obtained for the targeted allele; subsequently, the neo-selectable marker was removed by Cre-mediated site-specific recombination in mice (Lakso et al., 1996).

Figure 5a shows a cross-section through the nasal cavity of a MOL2.3-IGITL mouse stained with X-gal. Reactive cells were segregated within the dorsal region of the olfactory epithelium.
comparable to the wild-type cells. Higher magnification (Fig. 5b) revealed that the stained cells show the characteristic morphology of mature olfactory neurons. Cell bodies are located within the cellular layer of the epithelium; they send a dendritic process to the surface ending in a slight thickening, the olfactory knob. An axonal process is visible extending through the basal membrane into the lamina propria. The use of the transgenic message also allowed visualization of such cells by their GFP fluorescence (Fig. 5c). It is noteworthy that all these stained cell bodies are located in a rather basal layer of the epithelium. Determining the position of a large number of MOL2.3-IGITL cells within the epithelium (n = 93) revealed that they exhibit a very pronounced laminar patterning. The majority of cells are located in a relative position around 0.15 (Fig. 5d), a position which is deep within the olfactory epithelium; neurons expressing, e.g., receptors of the mOR37 subfamily are found in a higher range between 0.4 and 1.0 (Strotmann et al. 2000). The typical morphology of X-gal-positive cells and their characteristic laminar distribution within the epithelium indicate that MOL2.3 is indeed expressed in olfactory sensory neurons.

**Projection pattern of MOL2.3-IGITL-expressing neurons**

Expression of β-galactosidase as a fusion protein with the microtubule-binding protein tau allowed also visualization of the axons of MOL2.3-IGITL neurons and to follow their projection into the olfactory bulb. Figure 6a shows a whole-mount view of the medial aspect of an X-gal-stained nasal cavity. A subpopulation of cells segregated within the dorsal zone of the nasal cavity is stained intensely blue. Their axons are visible as they emerge from the nasal
cavity and pass through the cribriform plate into the olfactory bulb. They course over the surface into the dorsomedial/posterior region of the bulb where they converge at a distinct position. A view of the top of the olfactory bulb (Fig. 6b) reveals two other positions where MOL2.3-IGITL cells converge; these are located in the dorsolateral region of the bulb, slightly anterior to the medial point of convergence. This pattern of one medial and two dorsolateral points of convergence was observed in three out of five MOL2.3-IGITL mice that were analysed. In the two other animals, one medial and one dorsolateral point of convergence were observed; their positions were constant in all these animals. In cross-sections through the olfactory bulb of MOL2.3-IGITL mice it became evident that blue axons terminated in discrete structures which, by counterstaining, were identified as individual glomeruli (Fig. 6c and d). Axons were approaching this target from several directions (see, e.g., arrows in Fig. 6b). Counterstaining of sections revealed that they did so by passing through the surrounding layer of periglomerular cells (arrowheads in Fig. 6d). Serial sections revealed that neighbouring glomeruli did not receive input from these axons (data not shown).

Discussion

This study characterizes mouse GPCR genes which display moderate sequence homology to the olfactory receptor genes and are expressed in the olfactory epithelium as well as in particular regions of the brain. Here, evidence is presented indicating that expression in the nasal epithelium occurs specifically in olfactory sensory neurons; the axons of these neurons project into the main olfactory bulb where they converge onto a small number of glomeruli located in the medial and lateral region of the olfactory bulb.

The genes identified are phylogenetically related to class I or ‘fish-like’ olfactory receptors (Freitag et al., 1995), a fact that is reflected also by a typical structural motif, an extended extracellular loop 3. Interestingly, both criteria are also fulfilled by recently identified putative olfactory receptors encoded by genes which are clustered around the β-globin locus in mouse, human and chicken (Bulger et al., 1999). It will be interesting to explore whether the β-globin-associated OR-like genes are also expressed in other tissues, in particular in the brain.

Our data provide the ultimate proof that the MOL receptors are expressed in olfactory sensory neurons. Coexpression of histological markers with one MOL receptor (MOL2.3) visualized cells in the olfactory epithelium that exhibit the characteristic morphological feature of mature olfactory receptor neurons, including a dendritic process extending to the luminal surface and an axonal process extending into the lamina propria. The vast majority of MOL2.3-IGITL-expressing cells were located in basal layers of the olfactory epithelium, rather close to the basal membrane. These cells displayed a pronounced laminar patterning which has previously been described for a few other olfactory neuron populations (Strotmann et al., 1996).

The use of the axonal marker tau-lacZ allowed visualization of the axons of MOL2.3-IGITL-expressing neurons and clearly showed that they project into the main olfactory bulb, the first relay station of olfactory information processing in the brain. The axons of MOL2.3-IGITL neurons converge onto glomeruli located in the medial and the lateral aspect of each bulb. This projection pattern is characteristic for receptor cells located in the same epithelial zone expressing the same olfactory receptor gene (Mombaerts et al., 1996; Wang et al., 1998). The glomeruli targeted by MOL2.3-IGITL-expressing neurons are located in the dorsal region of the bulb; the zone-to-zone topography of projection which is typical for populations distributed throughout broad zones of the olfactory epithelium (Astice et al., 1987; Wang et al., 1998) is thus also maintained for the MOL2.3-IGITL neurons. Together, these data suggest that the newly discovered GPCRs indeed encode odourant receptors; their ligand(s), however, remains elusive. The relatedness to ‘fish-like’ receptors and their expression in distinct brain regions, however, suggest that the MOL receptors may interact with very special, possibly nonvolatile, molecules. The finding that the MOL receptors are expressed by cells located in the a.p., which is one of the circumventricular organs (CVOs) located at the blood–brain barrier, suggests that they are involved in communication between the circulation and the CNS. It has been well documented that blood-borne peptides, such as angiotensin, vasopressin or endothelin, influence brain function by acting on the CVOs (Ferguson & Bains, 1996). Future studies using the MOL-GFP transgenic mice may allow identification of the ligands for the MOL receptors, e.g. by screening extracts obtained from appropriate biological fluids.

The genes identified in this study are expressed in the dorsal zone of the nasal epithelium and this is the case also for those putative OR genes flanking the mouse β-globin gene cluster (Bulger et al., 1999), suggesting that this region may be dedicated to the detection of special odourous molecules. This notion may be corroborated by the endowment of this zone with a unique set of bio-transformation enzymes including drug-metabolizing enzymes and metallothioneins (Miyawaki et al., 1996). It has been proposed that this area of the nose may be exposed to the highest concentration of inhaled chemicals (Miyawaki et al., 1996), which may be a prerequisite for the detection of nonvolatile molecules. An additional unique feature is the absence of particular marker proteins, such as the olfactory specific adhesion molecule OCAM, which is restricted to the medial and lateral regions of the epithelium (Yoshihara et al., 1997). Thus, the dorsal zone of the olfactory epithelium may have specialized functions compared to the other zones of the nasal epithelium.

Expression of MOL2.3 has been detected not only in the sensory neurons of the olfactory system, but also in a population of cells located between the placode and the telencephalic vesicle. During the critical developmental period, when massive axon outgrowth from the epithelium towards the presumptive bulb occurs, cells located in that region express MOL2.3 receptors; this has also been described for a few other OR genes (Net et al., 1996; Saito et al., 1998). This observation supports the speculation that these OR-expressing cells may play a role as ‘guide-posts’ determining pathways for out-growing sensory axons. This view implies that olfactory receptors may not only be involved in odour detection but could also serve as guidance molecules, e.g. by homophilic interaction via extracellular domains of the receptor protein (Singer et al., 1995). Recent research in fact suggests that olfactory receptor proteins may play an important role in targeting olfactory axons to the bulb (Wang et al., 1998; Rodriguez et al., 1999).

Meanwhile several studies have demonstrated that OR genes are expressed in tissues other than the olfactory epithelium (Nef et al., 1992; Drulet et al., 1995). The widespread expression in different tissues has led to a revival of the ‘area code hypothesis’ (Dreyer, 1998) proposing that these receptors may be involved in cell–cell recognition and in cell positioning during development. In this context, expression of the MOL receptors in certain brain regions is of particular interest. The molecular mechanisms underlying the formation of the precise topological maps of neurons in distinct layers and nuclei of the brain are largely unknown. The genetic approach may allow study of the connectivity of brain neurons expressing MOL receptors and thus provide further insight into this topic.
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Abbreviations
a.p., area postrema; CVOs, circumventricular organs; E, embryonic day; e3, extracellular loop 3; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; IGITL, IRES-GFP-IRES-tauLucZ; IRES, internal ribosome entry site; MOL, mouse olfactory-like; n.t.s., nucleus tractus solitarius; OR, olfactory receptor; PN, postnatal day.

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