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

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**Keywords:** G-protein-coupled receptor, mouse, odourant receptor, olfactory epithelium, olfactory bulb, projection, transgene

## 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 TCC AGC TTC AAT-3'. Amplification was carried out using 2U TaqI-Polymerase (Life Technologies, Eggenstein, Germany), 2 mM MgCl<sub>2</sub>, 1 μM of each



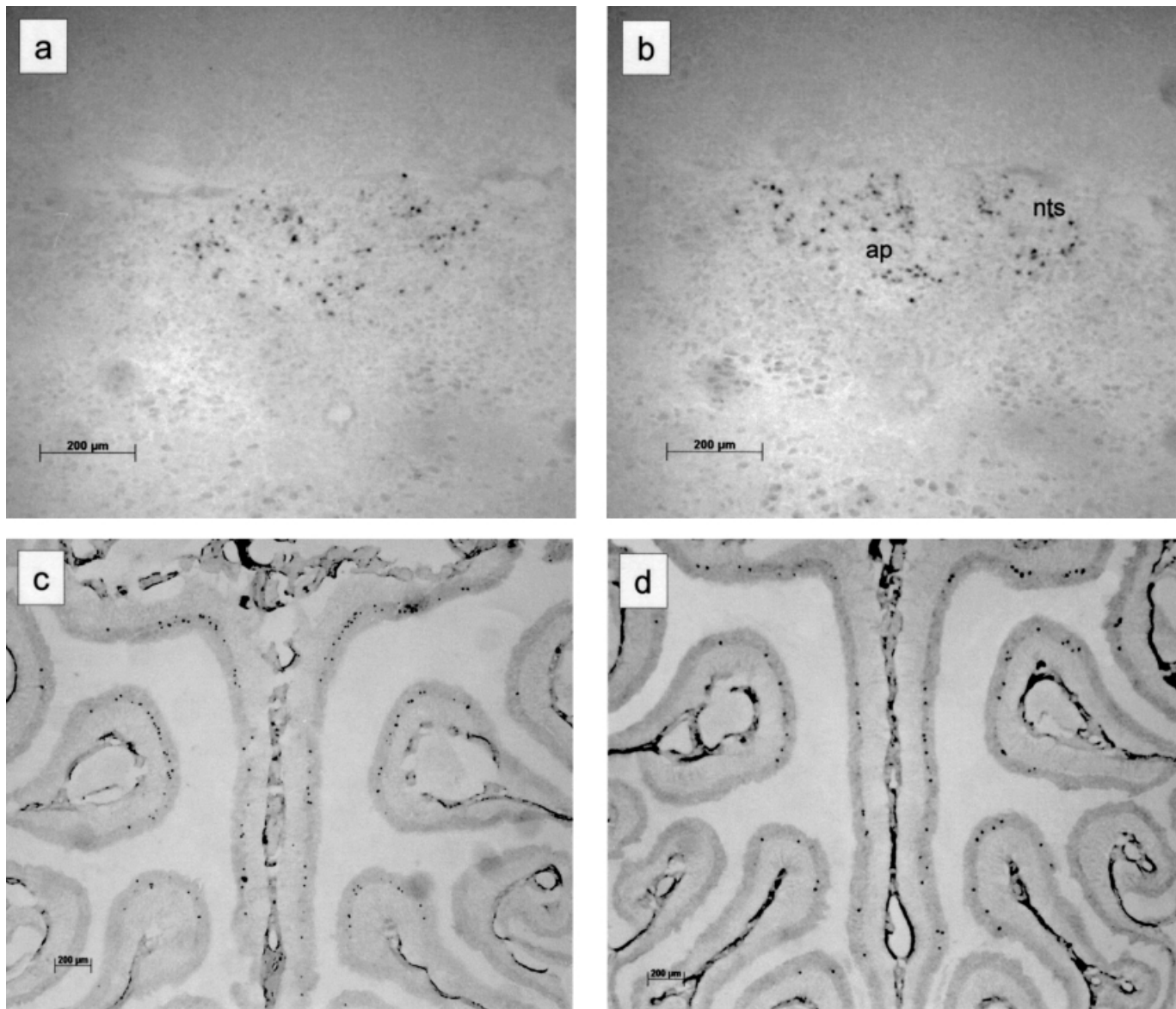


FIG. 2. Digoxigenin-labelled antisense riboprobes of (a and c) *MOL2.3* and (b and d) *MOL8.17* hybridized to adjacent coronal sections through (a and b) the medulla oblongata and (c and d) the mouse nasal cavity. In the medulla oblongata, *MOL2.3*- as well as *MOL8.17*-reactive cells are located in the area postrema (a.p.) and the nucleus tractus solitarius (n.t.s.). With both probes, neurons located in the dorsal zone of the olfactory epithelium are stained (dorsal to the top).

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 mL of *in situ* grade hybridization buffer (Amersham Pharmacia Biotech, Freiburg, Germany) containing 50% deionized formamide. Mice (>6-week-old) were killed by CO<sub>2</sub> 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 Axiophot microscope and the image analysis program Semper6 (Synoptics, Cambridge, UK). At 400× magnification, the vertical

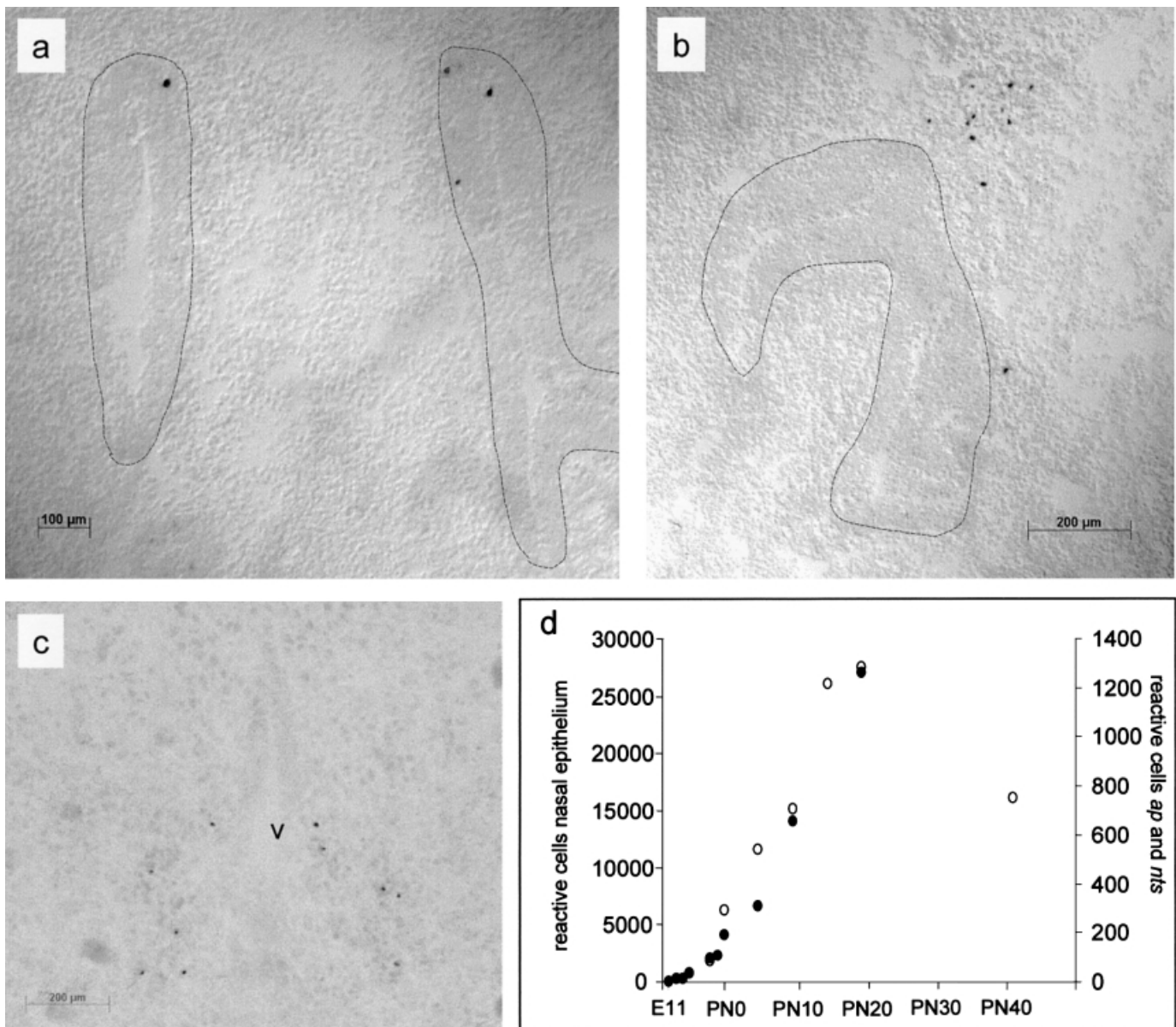


FIG. 3. *MOL2.3*-specific antisense riboprobe (dorsal to the top). The extent of the sensory epithelium is indicated by the dotted lines. (a) In the anterior region of the cavity, a few reactive cells located in the dorsal part of the sensory epithelium are visible. (b) In the posterior region of the cavity, a group of *MOL2.3*-reactive cells located outside the sensory epithelium in the region between the placode and the telencephalic vesicle are detectable. (c) Coronal section through the medulla oblongata of an E17 mouse embryo in the region around the fourth ventricle (v). *In situ* hybridization with a *MOL2.3*-specific probe labels cells surrounding the ventricle (dorsal to the top). (d) Quantitative analysis of *MOL2.3*-reactive cells in the nasal epithelium (filled circles) and in the area postrema (a.p.) and the nucleus tractus solitarius (n.t.s.) (open circles) during development.

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

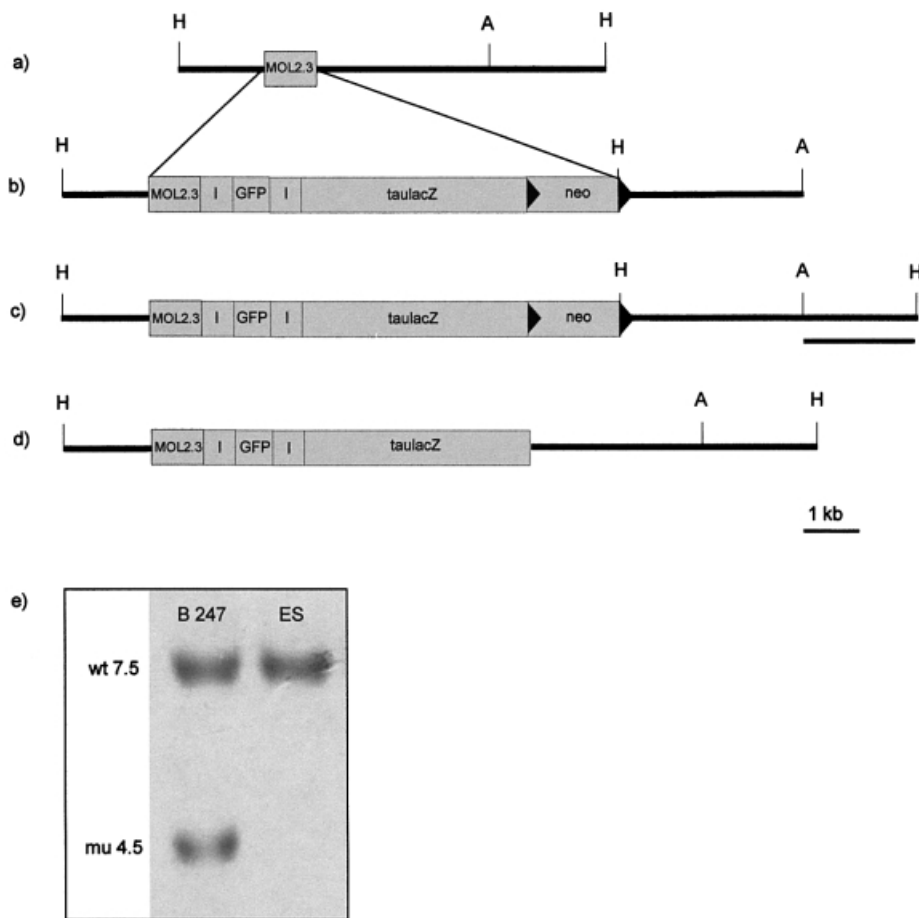


FIG. 4. Targeted mutagenesis of the *MOL2.3* gene. (a) Wild-type *MOL2.3* locus; the coding region is boxed. Restriction sites are indicated for ApaI (A) and HindIII (H). (b) *MOL2.3-IRES-GFP-IRES-tau-lacZ* (*MOL2.3-IGITL*) targeting vector; 'I' represents the *IRES* sequence, 'neo' represents the *neo*-selectable marker LNL flanked by loxP sites (indicated by triangles). (c) *MOL2.3* locus after homologous recombination. The 3' external probe used to detect homologous recombination is indicated as horizontal bar. (d) *MOL2.3-IGITL* mutation after *Cre*-recombination. (e) Southern blot of genomic DNA from targeted clone B247 and wild-type ES cells. DNA was cut with HindIII and hybridized with the 3' external probe. The upper band represents the wild-type allele (7.5 kb); the lower band is the targeted allele (4.5 kb).

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  $\approx 50\%$  sequence identity with *MOL2.3*. All three genes share sequence identity of  $\approx 30\%$  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,  $\approx 200$  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  $\approx 80$  cells were counted in the brain, whereas the number of stained

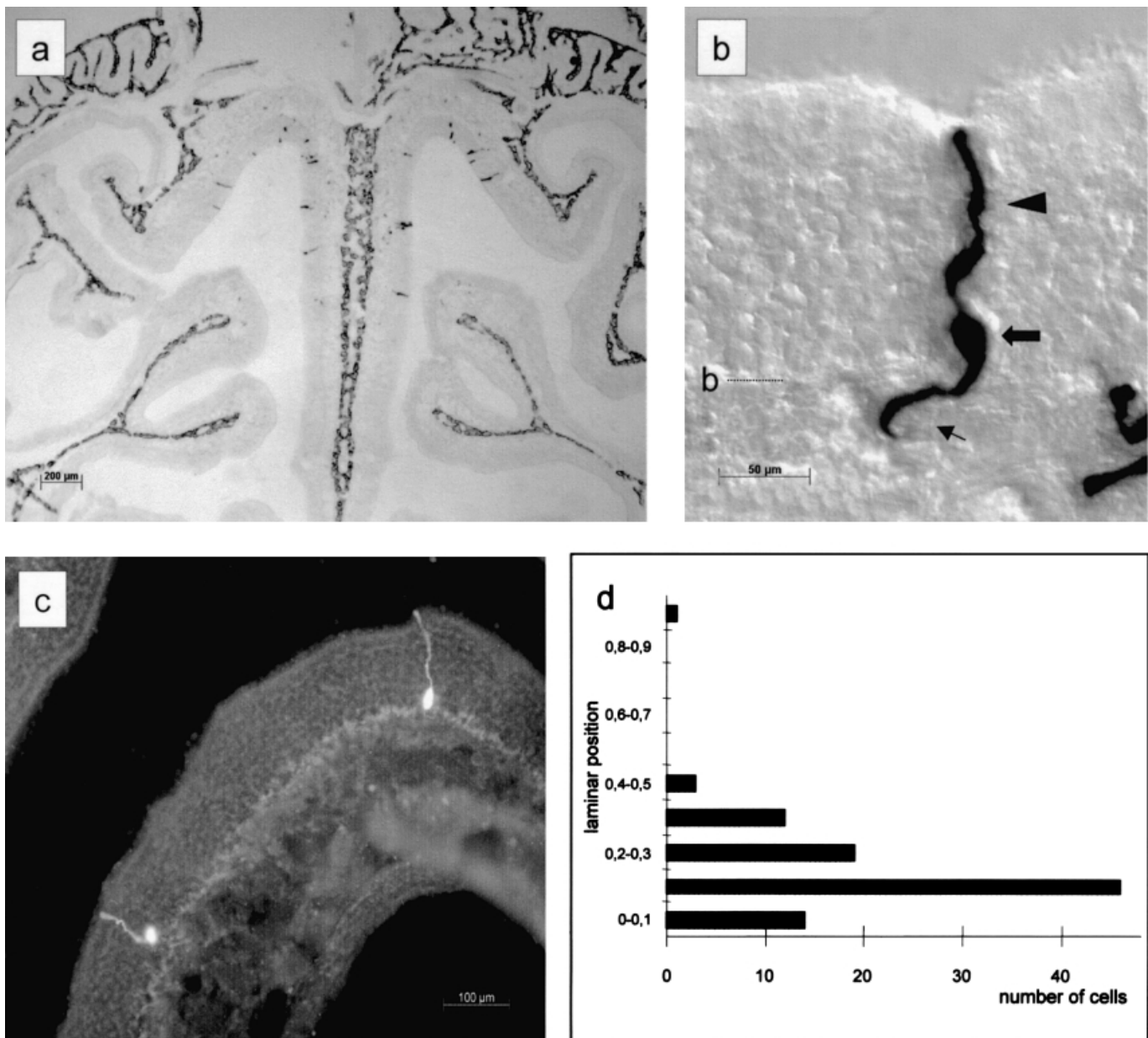


FIG. 5. (a) Coronal section through the nasal cavity from a heterozygous *MOL2.3-IGITL* mouse stained with X-gal. Reactive cells are segregated in the dorsal zone of the epithelium. (b) Cross-section through the olfactory epithelium of a heterozygous *MOL2.3-IGITL* mouse stained with X-gal. At high magnification, cell body (large arrow), dendrite (arrowhead) and axonal process (small arrow) of individual reactive cells are visible. *b*, basal membrane. (c) Cross-section through the olfactory epithelium of a heterozygous *MOL2.3-IGITL* mouse; using a GFP-specific filter set, fluorescent neurons located deep within the olfactory epithelium are visualized. (d) Laminar patterning of *MOL2.3-IGITL* neurons. Graphic representation of the number of reactive cells in different laminae; data were collected from randomly selected and informative sections from four different mice.

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  $\approx 30\,000$  *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 ( $>PN\ 42$ ) 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



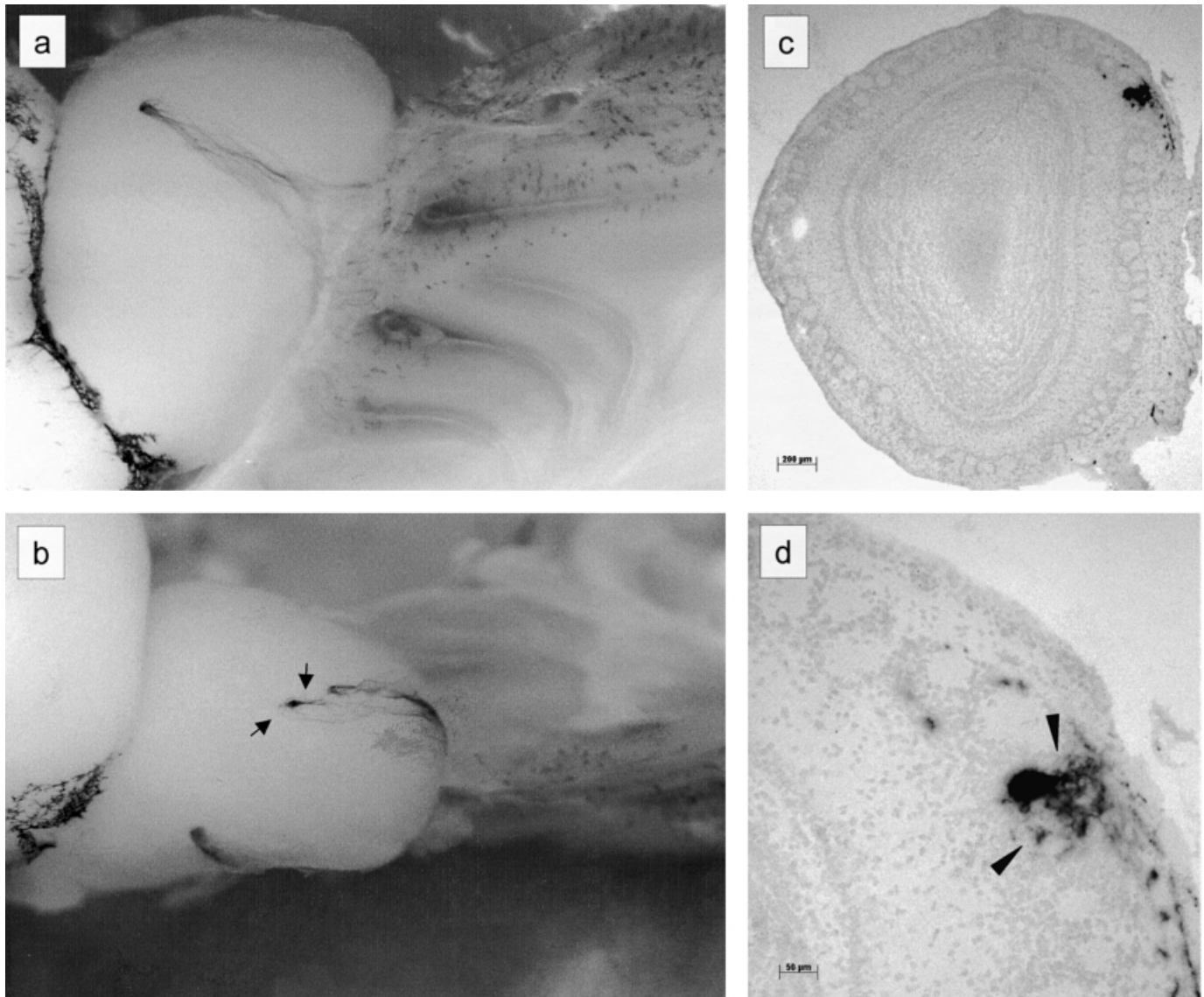


FIG. 6. A heterozygous *MOL2.3-IGITL* mouse, stained with X-gal. (a) Whole-mount view of the left half head; the nasal septum was removed allowing a view of the medial aspect of the turbinates. Blue cells are located in the dorsal region of the turbinates. Their axons converge on the medial aspect in the dorsal and posterior region of the bulb (anterior to the right, dorsal to the top). (b) Whole-mount view of the dorsal aspect of the bulb. Blue axons are visible converging at three distinct positions, one on the medial aspect, two on the dorsolateral aspect (anterior to the right, medial to the bottom). (c) Coronal section through the left olfactory bulb counterstained with neutral red. Stained axons converge in the dorsomedial region of the bulb (dorsal to the top, medial to the right). (d) At higher magnification, a single stained glomerulus is visible.

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 tricistronic 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  $\beta$ -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  $\beta$ -globin locus in mouse, human and chicken (Bulger *et al.*, 1999). It will be interesting to explore whether the  $\beta$ -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 those 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 (Astic *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  $\beta$ -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 (Nef *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 outgrowing 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; Drutel *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-taulacZ; 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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