Loss-of-function mutations in sodium channel Na\textsubscript{v1.7} cause anosmia

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The inability to sense odours is known as general anosmia; individuals born with this phenotype are afflicted with congenital general anosmia. Except for some syndromic cases such as Kallmann syndrome, no causative genes for human congenital general anosmia have been identified so far\textsuperscript{1-3}. Nine mammalian genes encoding voltage-gated sodium channel \(\alpha\)-subunits have been cloned and shown to be differentially expressed in the nervous system\textsuperscript{4, 5}. Of these, SCN9A, encoding the tetrodotoxin (TTX)-sensitive sodium channel Na\textsubscript{v1.7}, has received specific attention because of its key role in human pain perception. Individuals carrying loss-of-function mutations in SCN9A are unable to experience pain, and an essential requirement of Na\textsubscript{v1.7} function for nociception in humans has been established\textsuperscript{6-9}. Whether all other sensory modalities are fully preserved in these individuals remained unclear, although an association between congenital inability to experience pain and sense of smell deficits has been suggested\textsuperscript{7}. In this study we examine human patients carrying SCN9A loss-of-function mutations and demonstrate that they fail to sense odours. We establish a mouse model of congenital general anosmia and provide mechanistic insight into the role of Na\textsubscript{v1.7} in olfaction. Together with previous findings\textsuperscript{6-8, 10}, our results establish that loss-of-function mutations in a single gene, SCN9A, cause a general loss of two major senses—nociception and smell—thus providing a mechanistic link between these two sensory modalities.

Requirement for Na\textsubscript{v1.7} in human olfaction

Three individuals with congenital analgesia were ascertained and studied. All three were in their third decade of life and had never experienced acute pain but had no other neurological, cognitive, growth, appearance or health problems. All had suffered from multiple painless fractures and other injuries. Two had given birth painlessly. A living mother of the siblings was found to be heterozygous for the 11-base-pair deletion and the father heterozygous for the nonsense mutation. Therefore the diagnosis of CAIP was substantiated. We next assessed their sense of smell; none complained of having no sense of smell, one had been a cigarette smoker, none had chronic nasal problems. In the first woman smell function was assessed using the University of Pennsylvania Smell Identification Test (UPSIT), a standardized 40-item smell test. The results revealed that she was unable to detect any of the odours (Fig. 1a, black bar). Nine healthy, young individuals served as controls (Fig. 1a, grey bars). In the sibling pair we assessed the parents and their two affected offspring together. All were tested in sequence with cotton wool pads suffused with selected odour stimuli: balsamic vinegar, orange, mint, perfume, water (control) and coffee. Both parents correctly identified all stimuli, including smelling nothing for the water. The siblings detected none of the odours. For the siblings the test was repeated using subjectively unpleasant amounts of balsamic vinegar and perfume: the parents identified the odours correctly and found them unpleasant; the siblings neither identified the odours nor experienced any discomfort.

We proposed that these odour-sensing deficits are caused by loss of Na\textsubscript{v1.7} function in olfactory sensory neurons (OSNs). Indeed, when we investigated expression of Na\textsubscript{v1.7} in normal human olfactory epithelium, we detected messenger RNA for Na\textsubscript{v1.7} and the GTP-binding protein Go\textsubscript{olf}, a prototypical signature of classical OSNs (Fig. 1b). Immunohistochemistry using an antibody specific to Na\textsubscript{v1.7} verified that Na\textsubscript{v1.7} is normally expressed in human OSNs (Fig. 1c, d).
Conditional Na<sub>1.7</sub> null mice

To investigate the mechanisms that underlie the essential role of Na<sub>1.7</sub> in olfactory perception, we first examined Na<sub>1.7</sub> expression in the mouse olfactory system and then used the Cre-loxP system to delete the channel in those cells that express olfactory marker protein (OMP), which includes all classical OSNs<sup>11</sup>. These mice enabled us to examine the mechanisms underlying Na<sub>1.7</sub>-associated anosmia and the behavioural consequences. Consistent with our findings in human OSNs, OSNs from wild-type mice (C57BL/6, referred to as B6) showed Na<sub>1.7</sub> immunoreactivity at their somata (Supplementary Fig. 1). Of greater interest, coronal sections containing main olfactory epithelium (MOE), olfactory nerves and the two olfactory bulbs revealed the most marked Na<sub>1.7</sub> staining in the superficial olfactory nerve layer (ONL, containing axons from OSNs) as well as the glomerular layer (a complex neuropil that includes the presynaptic OSN boutons) of the olfactory bulb (Fig. 2a–c). Higher magnification of individual glomeruli verified co-expression of Na<sub>1.7</sub> with OMP in the glomerular neuropil (Fig. 2b), whereas olfactory bulb projection neurons (the mitral/tufted or M/T cells) and local interneurons did not show Na<sub>1.7</sub> immunoreactivity (Fig. 2a). Thus, Na<sub>1.7</sub> occupies a critical presynaptic location at the first synapse in the olfactory system.

Na<sub>1.7</sub> is not the sole Na<sub>+</sub> channel expressed in mouse OSNs. Real-time quantitative polymerase chain reaction with reverse transcription (qRT–PCR) analysis identified Na<sub>1.3</sub> as an additional candidate (Supplementary Fig. 2) and immunohistochemistry verified its expression in OSNs and their axons (Fig. 2d). However, unlike Na<sub>1.7</sub> we did not observe Na<sub>1.3</sub> immunoreactivity in individual glomeruli (Fig. 2d), indicating that Na<sub>1.7</sub> could be the sole Na<sub>+</sub> channel underlying action potential propagation in olfactory glomeruli and OSN nerve terminals.

To create a conditional knockout mouse model, we crossed ‘floxed’ Na<sub>1.7</sub> mice harbouring a loxP-flanked Scn9a gene<sup>12</sup> to homozygous OMP–Cre mice in which the OMP-coding region is replaced by that of Cre recombinase<sup>13</sup>. Further breeding established offspring that were both homozygous for the floxed Scn9a alleles and heterozygous for cre and Omp. In these mice, Cre-mediated Na<sub>1.7</sub> deletion was restricted to OMP-positive cells (henceforward referred to as cNa<sub>1.7</sub>−/− mice). These mice lacked Na<sub>1.7</sub> expression in a tissue-specific manner...
Loss of synaptic transfer in olfactory glomeruli

To define the function of Na\textsubscript{1.7} in OSNs, we prepared MOE tissue slices\textsuperscript{19} and recorded sodium currents in voltage-clamped OSNs. Both Na\textsubscript{1.7}\textsuperscript{+/−} and Na\textsubscript{1.7}\textsuperscript{−/−} OSNs displayed sizeable, TTX-sensitive sodium currents in response to step depolarizations (Fig. 3a, b). On the basis of its biophysical properties, Na\textsubscript{1.7} has been suggested to transduce generator potentials into action potentials in sensory neurons\textsuperscript{6}. However, peak current densities of voltage-activated sodium currents were reduced only moderately, by about 20%, in Na\textsubscript{1.7}\textsuperscript{−/−} OSNs (Fig. 3b). To determine whether Na\textsubscript{1.7}\textsuperscript{−/−} OSNs could still produce odour-evoked action potentials, we used extracellular loose-patch recording from visually identified OSN dendritic knobs\textsuperscript{20} and analysed spike frequency histograms after brief odour exposure (Fig. 3c). There was no obvious difference in odour responsiveness in Na\textsubscript{1.7}\textsuperscript{+/*−} versus Na\textsubscript{1.7}\textsuperscript{−/−} OSNs (Fig. 3c). We obtained similar results when we stimulated the cells with 3-isobutyl-1-methylxanthine (IBMX)\textsuperscript{41}, which raises intracellular cAMP by inhibiting endogenous phosphodiesterase activity (Fig. 3c). Thus, although the initial site of odour-evoked action potential generation in OSNs is unknown, Na\textsubscript{1.7} is not essential for this activity.

Because Na\textsubscript{1.7} is expressed in olfactory bulb glomeruli (Fig. 2), we reasoned that it could be required for action potential conduction in OSN terminals. Olfactory glomeruli are delineated spheres of neuropil containing synapses from the OSN axon terminals onto juxtaglomerular interneurons and M/T projection neurons\textsuperscript{22,23}. To examine whether presynaptic activity of Na\textsubscript{1.7} underlies transmitter release in the olfactory glomerulus, we prepared olfactory bulb tissue slices\textsuperscript{24} and combined ONL focal electric stimulation with whole-cell patch-clamp recording from visually identified M/T cells. With the chosen protocol, in control cNa\textsubscript{1.7}\textsuperscript{+/−} mice a single electrical stimulus in the ONL produced a reliable postsynaptic response in M/T cells. Under current clamp, such responses consisted of a prolonged excitation lasting on average for 2.4 ± 0.4 s (Fig. 3d, top; n = 29), with response latencies of 22 ± 4 ms (n = 29). Under voltage clamp, we observed bursts of postsynaptic currents (Fig. 3f; duration, 3.2 ± 0.4 s; n = 26). In stark contrast, in the cNa\textsubscript{1.7}\textsuperscript{−/−} mice such postsynaptic responses were virtually absent in M/T cells, even when the stimulus strength was increased by several-fold (Fig. 3d–f; n = 49). Importantly, M/T cells in these mice still produced normal action potentials when depolarized via current injection through the patch pipette (Fig. 3d, bottom), consistent with the fact that M/T cells lack both OMP and Na\textsubscript{1.7} expression (Fig. 2) and indicating that the effect of deleting Na\textsubscript{1.7} is presynaptic to the M/T cells. The inability of M/T cells to produce synaptic responses to ONL stimulation was not due to a potential deficit in synapse formation because: (1) immunohistochemistry showed normal expression of the vesicular glutamate transporter 2 (vGluT2, which is selectively expressed in OSN axon terminals)\textsuperscript{25,26} (Supplementary Fig. 5); and (2) electron microscopy revealed the existence of normal OSN boutons and synapses in the glomeruli of cNa\textsubscript{1.7}\textsuperscript{−/−} mice (Supplementary Fig. 6). Furthermore, conditional

OSN expression of tetanus toxin light chain, which inhibits synaptic release, does not alter the pattern of axonal targeting in olfactory bulb glomeruli during development\textsuperscript{27}.

Tyrosine hydroxylase (TH) expression in juxtaglomerular neurons of the olfactory bulb, a correlate of axon trans-synaptic activity,
requires olfactory nerve input and odour-stimulated glutamate release by OSN terminals\textsuperscript{28}. Consistent with a loss of OSN synaptic release, TH expression was markedly reduced in cNa\textsubscript{v}1.7\textsuperscript{−/−} mice (Fig. 3h; n = 6). The level of TH downregulation was similar to that observed after odour deprivation by naris occlusion\textsuperscript{29} or after deletion of the Cnga2 cation channel gene\textsuperscript{30}. Thus, we conclude that the presence of Na\textsubscript{v}1.7 in OSN axons is an essential and non-redundant requirement to initiate information transfer from OSN terminals to neurons in the olfactory bulb.

**The absence of odour-guided behaviours**

To further validate these results, we investigated several odour-guided behaviours in B6, cNa\textsubscript{v}1.7\textsuperscript{+/−} and cNa\textsubscript{v}1.7\textsuperscript{−/−} mice. First, we performed an odour preference test\textsuperscript{31} to assess recognition abilities for innate odour qualities (Fig. 4a). Filter papers scented with various cues representing both species-specific and food odours (male and female urine, peanut butter, milk) were presented to the mice and investigation times were analysed. Water was used as a neutral stimulus and 1,8-cineole (eucalyptol), which does not evoke innate attraction, served as the control (n = 7 for each cue and strain, respectively). B6 and cNa\textsubscript{v}1.7\textsuperscript{+/−} mice both showed strong attraction towards conspecific and food odours, whereas cNa\textsubscript{v}1.7\textsuperscript{−/−} mice failed to show any interest in these stimuli.

Second, we explored whether Na\textsubscript{v}1.7 is required for innate avoidance behaviour towards a predator odour, trimethyl-thiazoline (TMT)\textsuperscript{32}, which is normally secreted from the fox anal gland and known to induce aversive behaviour and fear responses in mice. We observed robust avoidance behaviour in both B6 (n = 6) and cNa\textsubscript{v}1.7\textsuperscript{−/−} mice (n = 5) but, notably, cNa\textsubscript{v}1.7\textsuperscript{−/−} mice lacked an innately aversive response in this assay (n = 5; Fig. 4b, c).

Third, we investigated the performance of cNa\textsubscript{v}1.7\textsuperscript{−/−} mice in a habituation–dishabitation assay, which allows for measurement of novel odour investigation, short-term odour learning, and odour discrimination\textsuperscript{32} (Fig. 4d). Mice of both sexes were each presented three distinct stimuli (water, female urine, male urine), each delivered for three successive trials, and investigation time during each trial (3 min) was analysed. Consistent with the results of Fig. 4a, cNa\textsubscript{v}1.7\textsuperscript{−/−} mice (n = 8) failed to show significant odour investigation, habituation, or discrimination abilities when compared with B6 (n = 8) or cNa\textsubscript{v}1.7\textsuperscript{+/−} mice (n = 8) (Fig. 4d; least significant difference (LSD), P < 0.0001).

Last, we examined pup retrieval ability of female mice, a social behaviour that probably depends on a functional main olfactory system (Fig. 4e). Three pups of a litter were removed from the nest, randomly distributed in the cage, and the time to retrieve each pup into the nest was quantified. In contrast to the performance of B6 (n = 12) or cNa\textsubscript{v}1.7\textsuperscript{+/−} mice (n = 6), cNa\textsubscript{v}1.7\textsuperscript{−/−} mice (n = 5) failed to retrieve any of the three pups during a 10-min trial period (Fig. 4e).

**Conclusions and prospects**

Our results establish a critical role of the Na\textsubscript{v}1.7 sodium channel in olfaction. Using conditional Na\textsubscript{v}1.7 null mice, we demonstrate that, in the absence of Na\textsubscript{v}1.7, OSNs are still electrically active and generate odour-evoked action potentials but fail to initiate synaptic signalling to the projection neurons in the olfactory bulb. These results provide evidence that Na\textsubscript{v}1.7 is an essential and non-redundant requirement for action potential propagation in the sections of OSN axons within the olfactory glomerulus. The conditional null mice no longer show a wide range of vital, odour-guided behaviours including innate attraction to food and conspecific odours, odour discrimination and short-term odour learning, innate avoidance towards a predator odour, effective suckling behaviour of newborn pups, and maternal pup retrieval. Within the limits of our anatomical analyses, synapse formation in these mice appears normal, indicating that the behavioural phenotype of the mutant mice is most likely the result of a loss of signalling at the first synapse in the olfactory system. Whether Na\textsubscript{v}1.7 or other sodium channel subunits such as Na\textsubscript{v}1.3 are involved in OSN axon pathfinding and activity-dependent neural map formation\textsuperscript{33} in the mouse olfactory system remains to be seen. Importantly, the phenotype of the mutant mice—the inability to perceive odours—is similar to that observed in human patients with confirmed Na\textsubscript{v}1.7 loss-of-function mutations. Smell tests in three individuals with congenital anosmia establish that they are unable to sense any of the odours. Systematic olfactory testing of patients carrying Na\textsubscript{v}1.7 loss-of-function mutations will be required in the future.

The genetic basis of sensory deficits such as blindness, deafness and pain disorders has been extensively studied in recent years. By comparison, relatively little progress has been made in understanding...
human congenital general anosmia. Mutations in olfactory signal transduction genes such as CNGA2, GNAL and ADCY3 do not seem to be a major cause of human congenital general anosmia. The identification of a sodium channel subunit as a causative gene for an inherited form of general anosmia provides new insight into the molecular pathophysiology of olfaction and should stimulate further research aimed at understanding the genetic basis of the human sense of smell.

**METHODS SUMMARY**

**Human subjects.** All research involving humans was obtained with the informed consent of the patients and performed under protocols approved by the Ethics Committee of the relevant institution. Human nasal mucosa was obtained by biopsy during routine nasal surgery. Further details of the human smell tests can be found in Methods.

**Animals.** The relevant Institutional Animal Care and Use Committee approved all procedures. Experiments used tissue-specific Na\(_{1.7}\)-deficient, C57BL/6J and OMP–GFP mice. See Methods for details.

**PCR analyses.** Human olfactory mucosa samples were examined individually whereas mouse tissue was pooled from four different animals. PCR products were amplified with gene-specific primers and specificity was controlled by sequencing. Primers are specified in Methods.

**Immunohistochemistry and electron microscopy.** These followed previously published procedures as described in Methods.

**Electrophysiology.** We used intact mouse MOE preparations as described previously and electrophysiological experiments are given in Methods.

**Behavioural analyses.** Innate olfactory preference tests and avoidance measures were done as described in Methods.

**IGF-1 assays.** These were done as described in Methods.

**Statistics.** Details of the statistical tests are given in Methods. Unless otherwise stated, results are presented as means ± s.e.m.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Human biopsies. Human nasal mucosa was obtained by biopsy during routine nasal surgery with patients under general anaesthesia. Biopsy specimens were obtained from three individuals and snap-frozen in liquid nitrogen for later processing. All samples were obtained under a protocol approved by the Ethics Committee of the University of Saarland School of Medicine. All biopsy specimens were obtained with the informed consent of the patients.

Human psychophysics. The USPIT was obtained from Sensonics. The test was applied over a period of 25 min. Testing and scoring was done according to standardized operating procedures summarized in the test manual. The reference values have been derived from recorded reference ranges for the USPIT test based on British individuals.

Olfactory mucosa biopsies and PCR analyses. Human surgical material containing olfactory mucosa collected from three different patients was examined individually. RT–PCRs from human samples were performed on a MyCycler (BIO-RAD) with Herculease (Agilent Technologies) following suppliers’ instructions. To amplify human Gαδ1, we used the oligonucleotides TGGAAAAA CTGCAAGCTTGCAG and GGGCCCAACTACAAACATGTGG. Human Na\textsubscript{v}1.7 was amplified by CAGTAAATACGCCGGACGCT and CCTATGCG CTGACGACCAAGGG. PCR conditions were: 95°C for 2 min pre-denaturation, followed by 30 cycles (95°C for 30 s, 60°C for 30 s (Gαδ1) or for 1 min (Na\textsubscript{v}1.7), 72°C for 30 s), followed by a final extension 72°C for 5 min. Mouse tissue was pooled from four different B6 mice (4–8 weeks old). RNA was isolated with the InvitroPREP RNA isolation kit (Analytijena). RNA quality was assessed by gel electrophoresis and photometric measurements. cDNA was synthesized from 0.5 μg of total RNA using the Smart cDNA Synthesis technology (Clontech) and SupercOII reverse transcriptase (Invitrogen). qPCR for different mouse Na\textsubscript{v}s subunits were run on a My-iQ-cycler using iQ SYBRGreen Supermix following the supplier’s instructions (BIO-RAD). We used the following oligonucleotides: Na\textsubscript{v}1.1 (AGCGCTGTAAGATCCTGGGC and TGCCAAC CGCGAAAATAAACG); Na\textsubscript{v}1.2 (TGGGATCTCCACCGCCAGAAATG and TGGGGCAGATTTTTGCGGCA); Na\textsubscript{v}1.3 (AGCTGGCGCTGCGCAAGGT and ATGCGGCACCGCCAAAAATG); Na\textsubscript{v}1.5 (ACACCGAGTGTGAGG GATGATC and GCCTGATTCGTTGGCTCA); Na\textsubscript{v}1.6 (AGGCCAATCTT GACATGTC and CCTGGCTGATCTCTACAGGCCA); Na\textsubscript{v}1.7 (AGGAT GAATTTCAACAGTCGTTCG and GTTCCTGCTTGATCTGCGGCAACAA CA). PCR conditions were: 95°C for 3 min pre-denaturation, followed by 42 cycles 95°C for 30 s, 60°C for 30 s, 72°C for 30 s. Each reaction was performed in three replicates on 96-well plates and analysed with the iQ5 Software (BIO-RAD).

Specificity of all PCR products was confirmed by gel electrophoresis and sequencing.

Mouse. Animal care and experimental procedures were performed in accordance with the guidelines established by the animal welfare committee of the University of Saarland School of Medicine. Mice were kept under a standard light/dark cycle with the guidelines established by the animal welfare committee of the University.

Ultrastructural features of the olfactory sensory axons and their synaptic terminals. Electron microscopy. Following routine processing for electron microscopy, as previously described\textsuperscript{39}, thin 70–100-nm sections were cut on a Reichert Ultracut S and examined on a JEOL 1200 transmission electron microscope. Images were captured at ×12,000, digitized at 1,200 dots per inch (DPI), and examined for ultrastructural features of the olfactory sensory axons and their synaptic terminals.

Electrophysiology. Whole-cell patch-clamp recordings from individual OSNs were obtained in acute MOE tissue slices of P1–P5 mice\textsuperscript{43}. The anterior aspect of the head containing olfactory epithelium and bulb was embedded in agarose (4%), placed in oxygenated, ice-cold extracellular solution (95% O\textsubscript{2}, 5% CO\textsubscript{2}) containing: 120 mM NaCl, 25 mM NaHCO\textsubscript{3}, 5 mM KCl, 5 mM Mg\textsubscript{2}+ (N-[2hydroxyethyl]-2-aminoethanesulfonic acid), 1 mM MgSO\textsubscript{4}, 1 mM CaCl\textsubscript{2}, 10 mM glucose, osmolality adjusted to 300 mOsm, pH 7.3. Coronal slices (250 μm) were cut on a vibratome (Microm HM 650 V), transferred to a recording chamber and kept under continuous flow (2 mln l−1) of oxygenated solution or remained on ice in oxygenated solution until needed (for up to 4 h). Experiments were performed at room temperature. The CaCl\textsubscript{2}-based extracellular solution contained: 140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 10 mM HEPES, pH 7.4, 300 mOsm. IBMX was prepared in 10 mM stock solution containing 5% dimethylsulfoxide (DMSO) (v/v). For M/T cell recordings, brains were rapidly dissected in ice-cold oxygenated (95% O\textsubscript{2},5% CO\textsubscript{2}) solution containing: 125 mM NaCl, 26.2 mM NaHCO\textsubscript{3}, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 2.5 mM KCl, 3.3 mM MgSO\textsubscript{4}, 0.5 mM CaCl\textsubscript{2}, 70 mM sucrose, pH 7.3, 300 mOsm. Horizontal olfactory bulb slices (300 μm) were cut in this solution. Until use, slices were transferred to oxygenated modified artificial cerebrospinal fluid (ACSF, 95% O\textsubscript{2}, 5% CO\textsubscript{2}) containing: 25 mM NaHCO\textsubscript{3}, 2.5 mM KCl, 1.25 mM NaH\textsubscript{2}PO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, 25 mM CaCl\textsubscript{2}, and 25 mM glucose. Recording pipettes had resistances of 4–7 MΩ. M/T cells were identified by size and location of their somata and filled with Lucifer Yellow during patch recording. The intracellular solution contained: 140 mM KCl, 1 mM EGTA, 10 mM HEPES, 0.5 mM GTP Na-salt, 2 mM ATP Mg-salt, pH 7.1, 290 mOsm. To assess OSN firing properties under non-invasive conditions, we used extracellular loose-patch recording from OSN knobs\textsuperscript{43}. In this case, the septal epithelium of juvenile (P5–P5) or adult mice was dissected and transferred to a recording chamber. Patch pipettes (9–12 MΩ) were filled with a HEPES-based extracellular solution containing: 140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 10 mM HEPES, pH 7.4, 300 mOsm. IBMX was prepared in 10 mM stock solution containing 5% dimethylsulfoxide (DMSO) (v/v). For M/T cell recordings, brains were rapidly dissected in ice-cold oxygenated (95% O\textsubscript{2},5% CO\textsubscript{2}) solution containing: 125 mM NaCl, 26.2 mM NaHCO\textsubscript{3}, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 2.5 mM KCl, 3.3 mM Mg\textsubscript{2}+, 0.5 mM CaCl\textsubscript{2}, 70 mM sucrose, pH 7.3, 300 mOsm. Horizontal olfactory bulb slices (300 μm) were cut in this solution. Until use, slices were transferred to oxygenated modified artificial cerebrospinal fluid (ACSF, 95% O\textsubscript{2}, 5% CO\textsubscript{2}) containing: 25 mM NaHCO\textsubscript{3}, 2.5 mM KCl, 1.25 mM NaH\textsubscript{2}PO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, 25 mM CaCl\textsubscript{2}, and 25 mM glucose. Recording pipettes had resistances of 4–7 MΩ. M/T cells were identified by size and location of their somata and filled with Lucifer Yellow during patch recording. The intracellular solution contained: 140 mM KCl, 1 mM EGTA, 10 mM HEPES, 1 mM ATP Na-salt, 0.5 mM GTP Mg-salt, 0.1 mM Lucifer Yellow; pH 7.1, 290 mOsm. M/T cells were held at −55 to −60 mV. Input and series resistances were 200–300 MΩ and 15–20 MΩ, respectively. After establishing a whole-cell recording, the ONL was stimulated using a glass electrode (1–1.5 MΩ) filled with HEPES-buffered extracellular solution connected to an electrical stimulator (single stimulus: 20 ms, 40 V, 260–400 μA). The stimulus pipette was placed rostrally to the recorded cell in the ONL. If a given M/T cell showed no postsynaptic response, the position of the stimulus pipette was changed until OSN axon bundles were found that caused M/T cell responses. Ionic currents were analysed using PulseFit 8.54 (HEKA) and IGOR Pro software (WaveMetrics)\textsuperscript{39}. OSNs with leak currents >20 pA and M/T cells with leak currents >100 pA (all measured at −70 mV) were excluded from analysis. Cell capacitance (C\textsubscript{m}) was monitored using the automated function of the EPC-9 amplifier. A stable C\textsubscript{m} value was considered an important criterion for the quality of the experiment. Spike analysis was done off-line using IGOR Pro software with custom-written macros. Chemicals were purchased from Sigma unless otherwise stated. Drugs used in the electrophysiological experiments were prepared as stock solutions in DMSO or distilled water and diluted to the final concentration in HEPES-based extracellular solution. NaCl, MgCl\textsubscript{2}, glucose and CaCl\textsubscript{2} were from Merck. IBMX (100 μM) and cineole (100 μM) were diluted in a HEPES-buffered extracellular solution (pH 7.3) and locally ejected using multibarrel stimulation pipettes.

Behavioural tests. The innate olfactory preference test followed previously described procedures\textsuperscript{44}. Briefly, mice were habituated to the test conditions before experiment started. Mice were placed in a cotton imbibed with water and then transferred to a new cage. This habituation was repeated three to four times for each animal. Soon after habituation, mice were transferred to the test cage, and a filter paper scented with a test odorant was introduced. Investigation times of the filter paper during the 3-min test period was recorded and quantified. Odour
stimuli were freshly collected male and female B6 mouse urine (5 µl), peanut butter (10% w/v, 15 µl), milk powder (10% w/v, 15 µl), water (15 µl) and cineole (100 µM, 15 µl).

For the innate olfactory avoidance test, following habituation (see innate preference test), a filter paper scented with 5 µl TMT (7.6 mM) was placed in one corner of the test cage. Mouse behaviour was recorded for 30 min. The test cage was subdivided into three equally sized areas. Time spent in area 1 of the cage (farthest distance from the TMT source) was evaluated as avoidance, whereas time spent in area 2 (consisting of the TMT source) was evaluated as attraction. Animal movements were tracked with SwisTrack (Swarm Intelligent Systems Group, Swiss Federal Institute of Technology).

For the olfactory habituation–dishabituation assay, following habituation (see innate preference test) mice were exposed for 3 min to distilled water (15 µl). This procedure was repeated three times with 1-min intervals, followed by a three-time presentation of female urine (5 µl) and a three-time presentation of male urine (5 µl). Investigation times during the 3-min test periods were measured.

For the pup retrieval test, lactating mice were habituated to the experiment for several minutes. Experiments were performed in the bedded home cages of the dams. Three pups (1–3-days old) were removed from the nest and randomly distributed in the cage. The latency for pup retrieval back into the nest was measured. If a dam had not completed retrieval within 10 min the test was terminated, resulting in a latency of 600 s.

Experiments were performed in empty standard cages (38 × 19 × 12 cm) and test substances were applied on filter paper (~1 × 3 cm). Mouse behaviour was recorded with a digital camera (Sony) for the experimental times indicated. Statistical video analyses were done randomly and blindly. Peanut butter (Barney’s Best) and milk powder (Bio-Anfangsmilch, Hipp) were diluted to 10% (w/v) in water.

**IGF-1 assays.** IGF-1 levels were measured by sandwich ELISA (ALPCO Diagnostics). IGF-1 was dissociated from the binding proteins by diluting samples with an acidic buffer. The analytical sensitivity of the assay was 0.029 ng ml^{-1}. Inter and intra-assay variability was below 7%. Experiments used plasma of 4–5-weeks-old mice (n = 4, each genotype).

**Statistics.** Data were analysed using NCSS 2004 statistical software (NCSS). The Student’s t-test (two-tailed) was used for measuring the significance of difference between two distributions. Multiple groups were compared using a one-way or two-way analysis of variance (ANOVA) with Fisher’s LSD as a post hoc comparison. Unless otherwise stated, results are presented as means ± s.e.m.