TSHZ1-dependent gene regulation is essential for olfactory bulb development and olfaction

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The olfactory bulb (OB) receives odor information from the olfactory epithelium and relays this to the olfactory cortex. Using a mouse model, we found that development and maturation of OB interneurons depends on the zinc finger homeodomain factor teashirt zinc finger family member 1 (TSHZ1). In mice lacking TSHZ1, neuroblasts exhibited a normal tangential migration to the OB; however, upon arrival to the OB, the neuroblasts were distributed aberrantly within the radial dimension, and many immature neuroblasts failed to exit the rostral migratory stream. Conditional deletion of Tshz1 in mice resulted in OB hypoplasia and severe olfactory deficits. We therefore investigated olfaction in human subjects from families with congenital aural atresia that were heterozygous for TSHZ1 loss-of-function mutations. These individuals displayed hyposmia, which is characterized by impaired odor discrimination and reduced olfactory sensitivity. Microarray analysis, in situ hybridization, and ChIP revealed that TSHZ1 bound to and regulated expression of the gene encoding prokineticin receptor 2 (PROKR2), a G protein–coupled receptor essential for OB development. Mutations in PROKR2 lead to Kallmann syndrome, characterized by anosmia and hypogonadotrophic hypogonadism. Our data indicate that TSHZ1 is a key regulator of mammalian OB development and function and controls the expression of molecules involved in human Kallmann syndrome.

Introduction

The olfactory bulb (OB) relays odor information from sensory neurons of the olfactory epithelium to higher brain centers. Interneurons located within the granule cell and glomerular layers mediate the OB’s output through synaptic connections with mitral and tufted cell projection neurons. Remarkably, OB interneurons arise not only during embryonic development, but continue to be generated postnatally by neural stem cells located in regions derived from the dorsolateral ganglionic eminence (dLGE), i.e., the subventricular zone (SVZ) and subependymal zone adjacent to the lateral ventricles and within the rostral migratory stream (RMS). Neuroblasts reach the OB via tangential chain migration within the SVZ/RMS, a process that occurs throughout the entire postnatal life of rodents and during the first year of postnatal life in humans (1–7). Upon arrival in the OB, dLGE-derived interneuron precursors migrate radially within the enlarging bulbs, mature, and integrate into the granule cell layer or the glomerular layer (8).

One molecule expressed in the developing OB and dLGE is teashirt zinc finger family member 1 (TSHZ1) (9). The founding member of the teashirt family, tsh, was first identified in Drosophila as a homeotic gene important for regulating head-trunk specification, with additional functions in patterning of the fly cuticle via modulation of the wingless (Wnt) signaling pathway (10, 11). Interestingly, tsh overexpression in Drosophila imaginal discs resulted in ectopic eye formation, a function previously attributed to the Pax6/PAX6 homolog eyeless, which indicated that tsh lies near the top of the genetic hierarchy controlling organ development in the fly (12). 3 murine homologs of tsh were later identified (Tshz1, Tshz2, and Tshz3), encoding zinc finger proteins containing 3 atypical widely spaced zinc finger motifs, a homeodomain, and 2 classical zinc fingers (13). In the mouse, Tshz1 was found to be essential for correct formation of the middle ear, the axial skeleton, and the soft palate (14), but to our knowledge, no functional analysis in the central nervous system has been published.

Here, we used classical and conditional mutagenesis in mice to investigate the roles of Tshz1 in OB development and function. We found that OB neuroblast differentiation depended on Tshz1, with the majority of interneurons of the granule cell layer and a subpopulation of periglomerular neurons being absent in Tshz1 mutant mice. Furthermore, in these animals, we noted that the radial migration of neuroblasts within the OB was severely impaired, a phenotype accompanied by severe OB hypoplasia. Interestingly, behavioral testing of mutant mice revealed marked olfactory deficits. Our findings with mice prompted us to study human individuals carrying heterozygous mutations in TSHZ1, which were recently described to cause congenital aural atresia (CAA), a congenital malformation of the external auditory canal (15). Intriguingly, we found significant reductions in olfactory sensitivity and discrimination in these patients, whereas...
odor identification was relatively unchanged. Molecular analyses of OBs of Tshz1 mutant mice showed reduced expression of the mRNA encoding for the G protein–coupled prokine-
thin receptor 2 (PROKR2). Previous studies showed that signaling upon binding of the ligand prokineticin 2 (PK2) to PROKR2 plays central roles in neurogenesis and OB development, implicating
this signaling system in neuroblast migration out of the RMS
(16–18). Additionally, mutations in human PROKR2 and PK2 are among those that lead to Kallmann syndrome, which is charac-
terized by anosmia, hypoplastic OBs, and hypogonadism (19, 20).
Based on ChIP analysis of murine OB tissue, we suggest that the role of TSHZ1 in OB development and function may be mediated,
at least in part, through its direct binding to regulatory elements
within Prokr2, thereby promoting the radial migration of neuro-
blasts in the OB. Thus, TSHZ1 is a transcriptional regulator that
impinges on genes involved in Kallmann syndrome and contrib-
tutes to interindividual variation in olfaction in humans.

Results
Tshz1 expression in the olfactory system. We generated polyclonal anti-
bodies against TSHZ1 for immunohistological and biochemical
analyses. TSHZ1 expression was found in a stream of cells extend-
ing from the walls of the lateral ventricle to the OB as well as in
cells scattered throughout the embryonic OB (Figure 1, A and B, and ref. 9). Stronger TSHZ1 expression was observed in the gran-
ule cell layer, where TSHZ1 was coexpressed with the neuronal dif-
f'erentiation marker NeuN (also known as RBFOX3; ref. 21). Weak
expression of TSHZ1 was found postnatally in the RMS of the OB
(RMSo), with stronger expression in the granule cell layer and a
subpopulation of periglomerular neurons (Figure 1, C and D).

Immunostaining with antibodies against NeuN revealed that the
majority of TSHZ1+ cells in the neuronal layers of the OB were
differentiated neurons.

Tshz1 mutation affects the distribution and differentiation of granule cell neurons of the developing OB. We next used targeted mutations to analyze Tshz1 function in murine OB (see Methods and Supple-
mental Figure 1, A–D; supplemental material available online with
this article; doi:10.1172/JCI72466DS1). A cassette encoding GFP was introduced into the Tshz1 locus to serve as a reporter of
gene expression, with concomitant disruption of Tshz1 protein-
coding sequences. In order to circumvent the embryonic lethality of homozygous Tshz1-null mutants, we established mice with a
Tshz1flh allele and crossed these with animals harboring the nestin-
cre transgene (22) to generate nestin-cre;Tshz1GFP/+ mice, with condi-
tional mutation of Tshz1 (referred to herein as coTshz1 mutant mice).
The success of both gene targeting strategies was confirmed
by analysis of GFP and TSHZ1 expression in OBs of control
Tshz1GFP/+ and Tshz1GFP/flh mice and loss of TSHZ1 expression at
the protein and mRNA levels in Tshz1flh-/- and coTshz1 mutant
mice (Supplemental Figure 1, E–H). Macroscopic examination of
the brains of coTshz1 mutants at birth indicated no drastic change
in OB size at this stage; similarly, upon histological examination,
OBs of coTshz1 mutant mice appeared indistinguishable from
those of controls (Figure 2, A and B). Immunostaining of OBs of
control mice with antibodies directed against GFP revealed a ring
of GFP+ cells that were not NeuN+. Scale bars: 1 mm (A); 500 μm (C and D); 200 μm (B); 50 μm (B and D, insets).

Figure 1
Tshz1 expression in developing and postnatal OB. (A) Sagittal sections of P0.5 forebrains showed a stream of cells migrating from the dLGE in a rostral direction toward the OB (arrowheads). Tshz1+ cells (green, Tshz1 mRNA) were located in this stream. (B) Coronal section of embryonic OB revealed cells expressing TSHZ1 protein (green) weakly in the stream (dotted outline), while in the granule cell layer (GCL), TSHZ1 was strongly expressed together with the neuronal differentiation marker NeuN (red). (C and D) In adult OB (P60), TSHZ1 (green) was prominently expressed together with NeuN (red) in neurons of the granule cell layer and with a subpopulation of neurons in the glomerular layer (GL). TSHZ1 was also weakly expressed within migrating cells of the RMS that were not NeuN+. Scale bars: 1 mm (A); 500 μm (C and D); 200 μm (B); 50 μm (B and D, insets).
immunostaining for NeuN, GABA, and tyrosine hydroxylase (TH). Loss of these markers was observed in the outer granule cell layer of coTshz1 as well as homozygous null mutant mice, while GABA and TH expression in the glomerular layer was unchanged (Figure 2, D and E, and Supplemental Figure 1, I and J). We also analyzed the organization of the mitral cell layer by immunostaining for TSHZ2 and detected no changes at P0.5 in homozygous null or coTshz1 mutants (Supplemental Figure 1, E and F). Whereas homozygous Tshz1\textsuperscript{GFP/Δ} and Tshz1\textsuperscript{GFP/GFP} mutants died within 24 hours of birth, displaying aerophagia, coTshz1 mutant mice suckled milk, with 30% surviving to adulthood. However, growth and body weight gain in coTshz1 mutants were severely impaired (Supplemental Figure 2 and Supplemental Methods).

\textit{Tshz1} mutation affects maturation of granule cell neurons of the postnatal OB. We next investigated the role of \textit{Tshz1} in postnatal OB maturation. Macroscopic examination revealed that the OBs of postnatal coTshz1 mutants were substantially smaller than those of controls, while overall brain size was much less affected (Figure 2F), suggestive of aberrant generation and/or migration of neurons that move to the OB. Whereas DAPI staining demonstrated the classical layered architecture of the OB in control mice, coTshz1 mutants displayed a severely disrupted OB: the granule cell and external plexiform layers could not be discerned, the glomerular layer was multilayered, and the RMS\textsubscript{OB} was markedly thickened (Figure 2G). Immunostaining with antibodies against GFP (green, C, D, H, and I), NeuN (red, D and I), or GABA (white, E); or by in situ hybridization with a probe against Gad1 (white, J). Note that C and D as well as H and I show images from the same sections. Arrowheads in C–E denote the outer granule cell layer; arrows in E denote the glomerular layer; arrowhead with asterisk in C denotes the GFP\textsuperscript{+} aggregates observed in coTshz1 mutants; arrows in G denote the RMS\textsubscript{OB}; dotted lines in H and I outline the RMS\textsubscript{OB} border. Scale bars: 3 mm (A); 500 μm (G); 200 μm (B–E and H–J); 50 μm (D, insets).
dcx

A more dispersed zone of
tal sections of control mice revealed a core region of strong
dcx

expression. In situ hybridization with a
Tshz1

cotshz1 mutants (Figure 3A), implying negative autoregulation of
upregulation of
gfp

seen (Figure 3, A and B, and Supplemental Figure 3); however,
alterations in the size of the RMS prior to entering the OB were
also dispersed throughout the granule cell layer in control mice, but
had accumulated in the RMSob in cotshz1 mutants. Insets: DCX+ pro-
cesses (white) were radially oriented in control mice, whereas no clear
orientation was apparent in mutant mice. Scale bars: 1 mm (A and B);
500 μm (D and A, insets); 50 μm (D, insets); 25 μm (C).

Figure 3

Impaired radial migration of neuroblasts from the RMSob in the absence of
Tshz1. Shown are sagittal sections of the OB and rostral forebrain
(A and B) or coronal sections of the adult OB (C and D). Tissue was
analyzed by in situ hybridization with probes against Gfp (green, A) or
Dcx (red, B) or with antibodies directed against GFP (green, C) or DCX
(red, C and D). Sagittal sections were also counterstained with DAPI
(blue, A). White dotted line in B denotes the border between the RMSob
and cells that have switched to radial migration; vertical brackets in B
denote sectional planes corresponding to images in C. (C) Note the presence of radially oriented DCX+ processes of migrating neuroblasts
that also stained for GFP in the granule cell layer of control mice. (D) Neuroblasts immunostained for DCX were present in the RMSob
and also dispersed throughout the granule cell layer in control mice, but
had accumulated in the RMSob in cotshz1 mutants. Insets: DCX+ pro-
cesses were interdigitated between the strongly GFP+ neurons in the
granule cell layer of control mice. (E and F) In control mice, DCX+ pro-
cesses extending from the RMSob through the granule cell layer
(Figure 3C). The markedly thickened RMSob of cotshz1 mutants was
characterized by accumulation of DCX+ neuroblasts that strongly
expressed GFP, while radially oriented DCX+ processes outside the
RMSob could not be found (Figure 3, C and D).

Altemations in the number and distribution of OB interneurons
and in the size of the RMSob could reflect changes in proliferation
of neural stem cells or their progeny, or aberrant migration/dif-
ferentiation of neuroblasts within the OB. We therefore injected
BrdU into mice and analyzed the number of BrdU+ cells in the OB 2
hours later or after a chase of 1 or 2 weeks (Figure 4). We chose this
time window based on previous studies in which it was shown that
a newly born neuroblast in the SVZ takes approximately 1 week
to migrate tangentially into the RMSob and begin radial migra-
tion, and a further week to become a differentiated neuron of the
granule cell layer (24). At 2 hours after BrdU injection, we observed
~0.5% proliferating cells in the RMSob in controls, whereas cotshz1
mutants showed an approximately 2.5-fold increase in prolifera-
tion (Figure 4B). At 1 week after injection of control animals, many
BrdU+ cells were found in the RMSob, similar to the density in
cotshz1 mutants (control, 54 ± 4 cells/100-μm quadrant; cotshz1, 38 ± 8 cells/100-μm quadrant; P = NS). The proportion of BrdU+ cells
within the RMSob was unchanged at this time point (Figure 4C), which suggests that tangential migration was not severely
affected in cotshz1 mutants. After 2 weeks of chase, most BrdU+ cells in control animals had exited the RMSob and differentiated
into granule cell neurons, whereas in cotshz1 mutants, consider-
ably more BrdU+ cells remained stuck in the RMSob (Figure 4D).

We next determined the displacement of BrdU+ cells from the ana-
tomical midline of the RMSob at 2 weeks of chase. A significantly
higher proportion of BrdU+ cells were present within 25 μm of the
RMSob midline in cotshz1 mutants (Figure 4, E and F), indica-
tive of impaired radial migration. We also determined the fate of

cotshz1 mutant OBs (Figure 2J). Thus, Tshz1 plays a dual role,
both in the distribution and in the differentiation of granule cell
neurons of the developing and postnatal OB.

Tshz1 mutation affects radial migration of neuroblasts from the RMSob.
We next examined the morphology of the RMS along its route
from the lateral ventricles toward the OB by in situ hybridization
using RNA probes against Gfp and doublecortin (Dcx). No clear
alterations in the size of the RMS prior to entering the OB were
seen (Figure 3, A and B, and Supplemental Figure 3); however,
upregulation of Gfp mRNA was observed within the entire RMS of
cotshz1 mutants (Figure 3A), implying negative autoregulation of
Tshz1 expression. In situ hybridization with a Dcx probe on sagit-
tal sections of control mice revealed a core region of strong Dcx
expression corresponding to the RMSob that was surrounded by
a more dispersed zone of Dcx mRNA+ neuroblasts moving away
into the granule cell layer (Figure 3B). In cotshz1 mutants, disper-
sion of cells away from the RMSob was significantly reduced, with
only the RMSob being clearly apparent. Immunostaining with
antibodies directed against DCX and GFP on coronal sections of
control mice demonstrated the presence of GFP+ DCX+ migrating
neuroblasts in the RMSob, as well as radially oriented DCX+ pro-
cesses extending from the RMSob through the granule cell layer
(Figure 3, C and D, and ref. 23). Such DCX+ processes coexpressed
GFP and were interdigitated between the strongly GFP+ neurons
of the granule cell layer in control mice (i.e., migrating DCX+ neu-
robasts in the granule cell layer belong to the Tshz1 lineage; Fig-
ure 3C). The markedly thickened RMSob of cotshz1 mutants was
characterized by accumulation of DCX+ neuroblasts that strongly
expressed GFP, while radially oriented DCX+ processes outside the
RMSob could not be found (Figure 3, C and D).

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1217
postnatally generated cells in coTshz1 mutants by immunostaining OBs for NeuN and BrdU at the 2-week chase time point and noted a significant increase in the proportion of cells in coTshz1 mutants that had incorporated BrdU, but failed to differentiate into NeuN+ granule cell neurons (Figure 4E and Supplemental Figure 4). To determine whether the increased number of undifferentiated neuroblasts in coTshz1 mutants was accompanied by alterations in cell death, we performed TUNEL staining and found a significantly higher number of apoptotic cells in these animals (Supplemental Figure 5). We conclude that tangential migration of neuroblasts toward the OB proceeds normally in the absence of Tshz1, whereas radial migration of neuroblasts out of the RMSOB into the granule cell layer of the OB is markedly reduced. Furthermore, the differentiation of postnatally generated neuroblasts within the OB of coTshz1 mutants was severely impaired and accompanied by increased programmed cell death.

**Tshz1 is required for generation of calbindin-positive periglomerular interneurons.** Periglomerular neurons of the glomerular layer of the OB also emerge from the embryonic and postnatal RMSOB (25). They receive synaptic input from olfactory receptor cell axon terminals and form dendrodendritic synapses onto mitral/tufted cell dendrites. Whereas the number of dopaminergic TH+ and calretinin-positive (CR+) periglomerular neurons was relatively unchanged (Figure 5, A–D), a large reduction in the
The number of calbindin-positive (CB+) periglomerular neurons was observed using in situ hybridization or immunostaining (Figure 5, E–G). Furthermore, in coTshz1 mutants, fewer GFP+ cells surrounded the outer parts of the glomeruli (Figure 5G). We also analyzed expression of PAX6, a transcription factor that regulates the generation of a subpopulation of periglomerular neurons (26, 27). In control mice, the expression patterns of PAX6 and Tshz1 (using GFP immunostaining) revealed non-overlapping populations of periglomerular neurons (Supplemental Figure 6A). Interestingly, in coTshz1 mice, we observed an increase in the number of PAX6+ cells surrounding glomeruli, which was accompanied by the presence of ectopic GFP+PAX6+ cells (Supplemental Figure 6), suggestive of functional antagonism between Pax6 and Tshz1 in periglomerular neurons. Thus, we conclude that beyond its role in the development of granule cell neurons, Tshz1 also regulates the migration and/or differentiation of a subpopulation of periglomerular neurons in the postnatal OB, some of which express CB.

Tshz1 mutations lead to poor sense of smell in mice. In order to determine whether the alterations observed in the granule cell and periglomerular layers of the OB affect olfactory function in coTshz1 mutant mice, we carried out behavioral tests. In contrast to control animals, 11 of 13 coTshz1 animals could not find a buried food pellet, whereas food previously soaked in 64 μM vanillin was detected, with latencies approaching those seen in controls (Figure 6A). We next carried out an odor preference test, in which control mice showed a tendency to avoid 2-methyl butyric acid (an unpleasant odor) and a marked preference for peanut butter (a pleasant odor) compared with the time spent investigating a filter paper dosed with water (Figure 6B). coTshz1 mutants were more interested in water than control animals, and no significant differences in preference for 2-methyl butyric acid or peanut butter were observed. Next, in response to 3 consecutive presentations of the same odor, habituation was observed in coTshz1 mutants, but the responses to changes of odor (i.e., dishabituation) were markedly reduced (Figure 6C). In addition, coTshz1 mutants failed to respond to the odor 2,3,5-trimethyl-3-thiazoline (TMT), even when presented at concentrations 100-fold higher than those required to elicit a response in control animals (Supplemental Figure 7A). We therefore conclude that the olfactory function of coTshz1 mutant mice is severely compromised.

TSHZ1 mutations in humans influence sense of smell. Encouraged by these results in mice, we next sought to determine whether TSHZ1 influences olfactory function in humans. Heterozygous mutations in TSHZ1 were recently characterized in families with CAA, including those carrying deletions in 18q22.3 as well as point mutations in TSHZ1 coding sequences (15). Olfactory function was tested in 5 individuals heterozygous for TSHZ1.
mutations (Figure 7A) together with age- and sex-matched controls, using filter pens containing different odors (28). The mutant TSHZ1 alleles harbor point mutations that result in premature stop codons and encode severely truncated mutant proteins with predicted loss of function (Table 1 and ref. 15). Clinical and genetic details of the individuals tested are shown in Tables 1 and 2. Intriguingly, substantial decreases in odor sensitivity and impaired odor discrimination were found in all 5 patients, while odor identification did not differ between the control and the mutant groups (Figure 7, B and C). Thus, haplo-insufficiency of TSHZ1 perturbed olfactory function in humans, which resulted in hyposmia. We noted no significant changes in olfactory function in mice heterozygous for a loss-of-function Tshz1 mutation (Tshz1 Gfp/flox) (Supplemental Figure 7B), which indicates that humans are more sensitive to alterations in TSHZ1 gene dosage than rodents with respect to olfaction.

Expression of Prokr2, which encodes a molecule that regulates OB interneuron migration, depends on Tshz1. In order to better understand the molecular changes occurring downstream of Tshz1 function in the developing and postnatal OB, we used microarray hybridization to profile gene expression in control and Tshz1 mutant mice at the embryonic and postnatal stages (E18.5 and P30, respectively; see Methods). A panel of mRNAs whose expression was altered (Figure 8A) was then analyzed by in situ hybridization on sections of OBs of postnatal cotshz1 mutants (Figure 8, C–F, H–K, and M–P). In addition, probes against Tshz1 or Gfp were used, as well as Reelin, a marker of mitral cells, whose expression was retained in coTshz1 mutants but revealed a somewhat disorganized mitral cell layer (Figure 8, B, G, and L). Several markers of the granule cell layer — including syntabulin (Sybu), sorting nexin 7 (Snx7), Krox20/Egr2, and the immediate early gene Arc — were downregulated in the OB of coTshz1 mutants, in accordance with

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**Figure 6**

Tshz1 mutations in mice result in severe olfactory deficits. (A) Buried food test. The latency of cotshz1 mutant mice to find hidden food pellets substantially increased, although these mice were capable of food detection after soaking pellets in vanillin. Dashed line denotes the maximal 15-minute test period (900 s). (B) Preference test. Time spent sniffing at filter papers containing 1 of 3 odors (water, 2-methyl butyric acid [2-MB], and peanut butter, presented in random order) was determined. Whereas control mice tended to avoid 2-MB and were strongly attracted to peanut butter, no clear responses to aversive or attractive odors were seen in cotshz1 mutants. Gray and black dashed lines denote the investigation time of neutral odor (H2O) of control and cotshz1 mutant mice, respectively. (C) Habituation/dishabituation test. Animals were presented 3 times with different odors on cotton swabs in the order shown, and total time spent sniffing was determined. Inset shows fold change in investigation time in response to the presentation of a new smell (i.e., third test of previous odor compared with first test of next odor). *P < 0.05; **P < 0.01; ***P < 0.001.
CoTshz1 mutants strongly resembled the phenotypes observed in immature cells within the inner layers of the OB, Prokr2 expression was restricted to the inner layers of the OB in control mice at E18.5 (Figure 9A and B). In Tshz1 mutants, Prokr2 expression was strongly downregulated (Figure 9B). The enlarged RMSOB and smaller OBs observed in postnatal coTshz1 mutants strongly resembled the phenotypes observed in mice with mutations in Prokr2 or PK2, and humans with Kallmann Syndrome carrying mutations in PROKR2 or PK2 have hypoplastic OBs (16–20). In postnatal control mice, Prokr2 was expressed in cells both within the RMSOB and in a subpopulation surrounding the RMSOB that was scattered through the granule cell layer (Figure 9C), presumably corresponding to radially migrating neuroblasts. In coTshz1 mutants, Prokr2 expression within the enlarged RMSOB was slightly downregulated; importantly, no cells were seen outside the RMS that still expressed Prokr2. Expression of PK2 was seen in a subpopulation of granule cells within the control OB, but was essentially extinguished in coTshz1 mutant mice (Figure 9D), similar to the loss of expression observed for several other markers of granule cells (Figure 8). Prokr2 expression in cells of the SVZ as well as in neuroblasts migrating tangentially within the RMS was not affected by Tshz1 mutation (Supplemental Figure 8, A–C), which suggests that the function of Tshz1 in regulating Prokr2 expression is confined to the RMSOB, similar to what was seen at embryonic stages. We therefore conclude that in the absence of Tshz1, accumulation of neuroblasts within the RMSOB is accompanied by a major loss of PROKR2-dependent signals.

ChIP reveals association of TSHZ1 with the Prokr2 gene in vivo. We sought to determine whether TSHZ1 physically associates with the Prokr2 locus (Figure 9E) by performing ChIP experiments on murine OB tissue using our anti-TSHZ1 polyclonal antiserum. Western blot analysis of OB lysates demonstrated that the TSHZ1 antiserum used in ChIP recognized a protein species with the size expected for TSHZ1 (Supplemental Figure 8D). We designed primer pairs located within different regions of Prokr2, either covering the promoter region (1.6 kb) or situated within the first intron flanking a region containing sequences conserved between the murine promoters of Sybu, Prokr2, and Tshz1, as well as an element highly conserved across different mammals in the promoter region of Tshz1. No consistent ChIP enrichment was found within the promoter of Tshz1, either covering the promoter region (1.6 kb) or situated within the first intron flanking a region containing sequences conserved between the murine promoters of Sybu, Prokr2, and Tshz1, as well as an element highly conserved across different mammals in the promoter region of Tshz1.

Discussion Characterization of loss-of-function mutations in TSHZ1 in patients with CAA and 18q deletion syndrome previously highlighted the important role of this gene in human craniofacial development (15). Here, we identified hyposmia as another diagnostic criterion of CAA patients with TSHZ1 mutations. Using targeted mutagenesis in mice, we were able to assign crucial key functions to Tshz1 in the development and function of the OB, a structure with essential functions in processing incoming olfactory information.
from the olfactory epithelium and relaying this information to the olfactory cortex. We found that \textit{Tshz1} was important for migration of neuroblasts of the granule cell lineage of the OB as well as for differentiation of granule cells and a subclass of periglomerular interneurons. These functions were in turn required for a normal sense of smell in rodents and humans.

Neurogenesis in the mouse occurs mainly during embryonic to early postnatal stages. However, in the adult rodent brain, there are 2 areas in which new neuroblasts are continually generated: the SVZ and the hippocampal subgranular zone, which migrate to the RMSOB and integrate into the dentate gyrus of the hippocampus, respectively (29, 30). In humans, while hippocampal neurogenesis continues into adulthood (31), generation of neurons in the SVZ that migrate to the OB appears to cease within the first year of postnatal life (7). Using mice, we characterized the expression of \textit{Tshz1} in the postnatal RMS and OB and used conditional mutagenesis to elucidate its function. \textit{Tshz1} expression was weak within the RMS, becoming stronger along its route toward the RMSOB, and could be identified in cells expressing doublecortin, a marker for migrating neuroblasts (23). The strongest site of \textit{Tshz1} expression was that consisting of NeuN + granule cells and a subpopulation of NeuN’ periglomerular neurons (32). In the absence of \textit{Tshz1}, the tangential migration of DCX’ neuroblasts along the RMS was unperturbed, whereas upon arrival in the OB, RMSOB neuroblasts failed to orient their cellular processes toward the periphery of the OB, and did not migrate efficiently in the radial dimension. We found that this related to defects in PROKR2 signaling (see below). In addition, there was a profound failure of differentiation of granule cell neuroblasts in the OB. OB neurogenesis relies on coordination of multiple processes, including cell cycle control in the SVZ; tangential and radial migration of neuroblasts within the RMS and the RMSOB, respectively; and differentiation and formation of synaptic connections in the OB.

Both radial migration and neuronal differentiation were affected in the absence of \textit{Tshz1}. This could reflect independent functions of \textit{Tshz1} in these 2 processes or, alternatively, a primary effect of \textit{Tshz1} on radial migration, leading secondarily to defects in differentiation. Interestingly, work on the factor p27Kip1 showed how it independently regulates neuronal differentiation and migration in newborn cortical neurons (33), and studies of basic helix-loop-helix factors showed that they have dual independent functions in migration and neurogenesis (34). shRNA-mediated knockdown of \textit{Dcx} led to accumulation of cells within the RMSOB, but did not affect the capacity of neuroblasts to differentiate into neurons, although changes in dendritic arbors were observed (35). Thus, the lack of granule cell differentiation in \textit{Tshz1} mutants and the migratory deficit that leads to increased numbers of neuroblasts in the RMSOB likely reflect independent functions for the factor in neurogenesis and migration.

It has previously been shown that OB volume positively correlates with olfactory function in children and during aging and is reduced in neuropsychiatric conditions, accompanied by losses in olfactory sensitivity (36, 37). We observed reduced olfactory sensitivity as well as impaired olfactory discrimination in human patients carrying heterozygous mutations in \textit{TSHZ1}. Similarly, co\textit{Tshz1} mutant mice showed significant olfactory impairments as well as displaying smaller OBs, likely as a result of reduced numbers of granule cells and consequent loss of substantial amounts of neuropil. Clinical examinations to determine OB volume,

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### Table 1

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<tr>
<th>Subject</th>
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<th>Sex</th>
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<th>CAA type</th>
<th>Cognitive development</th>
<th>Sensitivity</th>
<th>Olfactory scores</th>
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<td>1.I:1</td>
<td>12.5</td>
<td>Male</td>
<td>Son of 1.I:2</td>
<td>c.723G&gt;A, p.W241X</td>
<td>IIA, bilateral</td>
<td>Normal</td>
<td>Normal</td>
<td>1.25</td>
</tr>
<tr>
<td>Family 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.II:4</td>
<td>44.7</td>
<td>Female</td>
<td>Mother of 2.III:1 and 2.III:2</td>
<td>c.946_947delinsA, p.P316TfsX16</td>
<td>IIA, bilateral</td>
<td>Normal</td>
<td>Normal</td>
<td>5.5</td>
</tr>
<tr>
<td>2.III:2</td>
<td>13.9</td>
<td>Female</td>
<td>Daughter of 2.II:4A</td>
<td>c.946_947delinsA, p.P316TfsX16</td>
<td>IIA, bilateral</td>
<td>Normal</td>
<td>Normal</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Families 1 and 2 correspond to families 3 and 4, respectively, as previously described by Feenstra et al. (15). Both the c.723G>A and c.946_947delinsA mutations lead to major truncations in the mutant TSHZ1 proteins (after 241 and 316 amino acids, respectively; wild-type TSHZ1 contains 1,032 amino acids), with predicted loss of function due to the loss of the majority of the protein sequence. \*Twin.

### Table 2

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>CAA type</th>
<th>Cognitive development</th>
<th>Sensitivity</th>
<th>Olfactory scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>43.0</td>
<td>Female</td>
<td>Normal</td>
<td>Normal</td>
<td>9.25</td>
<td>14</td>
</tr>
<tr>
<td>Control 2</td>
<td>13.4</td>
<td>Male</td>
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<td>Normal</td>
<td>6.75</td>
<td>14</td>
</tr>
<tr>
<td>Control 3</td>
<td>43.5</td>
<td>Female</td>
<td>Normal</td>
<td>Normal</td>
<td>8.75</td>
<td>13</td>
</tr>
<tr>
<td>Control 4</td>
<td>13.5</td>
<td>Female</td>
<td>Normal</td>
<td>Normal</td>
<td>8.5</td>
<td>15</td>
</tr>
<tr>
<td>Control 5</td>
<td>13.6</td>
<td>Female</td>
<td>Normal</td>
<td>Normal</td>
<td>7.5</td>
<td>13</td>
</tr>
</tbody>
</table>

Control subjects were not related to each other and did not have \textit{TSHZ1} mutations.
factor) lead to clear clinical phenotypes (40), whereas heterozygous Nkx2.1+/– mice develop normally, and phenotypes that parallel those seen in humans are observed only in homozygote animals (41).

We showed here that one major function of Tshz1 in the OB of mice is to maintain the expression of Prokr2. Prokineticin signaling is involved in several biological processes, including nociception, circadian rhythm, and neurogenesis (17, 42, 43). PK2 and its receptor, PROKR2, are essential factors for normal OB development (16, 17). Both PK2 and Prokr2 mutant mice displayed an accumulation of immature neuroblasts in the RMSOB, implicating PROKR2/PK2 signaling in the final steps of immature neuron migration in the OB, such as the dissociation of tangentially migrating neuroblast chains in the RMS OB that precedes their switch to radial migration (17, 18). We observed changes in Prokr2 and PK2 expression in the OBs of coTshz1 mutant mice, and propose that TSHZ1 plays a direct role in regulation of Prokr2 in radially migrating neuroblasts. In contrast, the strong reduction in PK2 expression is likely a secondary consequence of the loss of granule cells in TSHZ1+/– mice (14) or altered sense of smell (present study). We interpret this as showing that humans are more sensitive to alterations in TSHZ1 gene dosage than rodents. Such differences between humans and mouse models have been previously noted; for example, heterozygous mutations in Nkx2.1 (encoding a transcription factor) lead to clear clinical phenotypes (40), whereas heterozygous Nkx2.1+/– mice develop normally, and phenotypes that parallel those seen in humans are observed only in homozygote animals (41).

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Including magnetic resonance imaging, could therefore be of diagnostic relevance in patients with bilateral CAA type 2a who also present with olfactory deficits. Based on our findings in mice, we suggest that the marked deficits in olfactory function resulting from mutation in TSHZ1 in humans could be attributable to defects in the granule cell lineage, such as reduced numbers or impaired maturation of these neurons. The importance of granule cells in OB function has been previously shown in studies in mice that demonstrated olfactory impairments when the activity of granule cells had been silenced (38) or in mutant strains with reduced numbers of granule cells (39).

In contrast to the CAA and olfactory deficits seen in human TSHZ1+/– patients, heterozygous Tshz1+/– mice do not display middle-ear defects (14) or altered sense of smell (present study). We interpret this as showing that humans are more sensitive to alterations in TSHZ1 gene dosage than rodents. Such differences between humans and mouse models have been previously noted; for example, heterozygous mutations in Nkx2.1 (encoding a transcription factor) lead to clear clinical phenotypes (40), whereas heterozygous Nkx2.1+/– mice develop normally, and phenotypes that parallel those seen in humans are observed only in homozygote animals (41).
TSHZ1 levels in order to migrate out of the RMS OB, probably via maintenance of Prokr2 expression. The high levels of TSHZ1 seen within granule cells, and the failure to generate these neurons in the absence of the factor, would favor a model whereby maintenance of TSHZ1 expression is also required for neuronal differentiation once neuroblasts have migrated out of the RMS OB and reached the granule cell layer.

Matsumoto et al. showed abnormal development not just of the OB, but also of the gonads and associated reproductive tracts, in mice lacking Prokr2 (16). Furthermore, recent studies showed that human mutations in PROKR2 or PK2 result in Kallmann syndrome, characterized not only by defective OB development and anosmia, but also by hypogonadism (19, 20, 44, 45). This phenotype relates to a role of prokineticin signaling in the development of hypothalamic gonadotrophin-releasing hormone (GnRH) neurons, which originate in the olfactory placode and migrate to the hypothalamus. We failed to obtain litters from coTshz1 mutant female and male mice, which suggests that fertility and/or sexual behavior are affected in these animals. Although initiation of Prokr2 expression in tangentially migrating neuroblasts of the SVZ and along the RMS prior to the OB was unaffected in coTshz1 mutant mice, maintenance of Prokr2 expression in the RMS OB was impaired, and Prokr2-expressing cells outside the RMS OB were absent in these animals. In order to address a direct role of TSHZ1 in regulation of Prokr2 expression, we conducted ChIP experiments on OB tissue. Although such experiments rely on detecting TSHZ1-Prokr2 complexes that may be restricted to the small proportion of OB tissue containing radially migrating neuroblasts, we found significant enrichment of the first intron of Prokr2 in the ChIP immunoprecipitate isolated with anti-TSHZ1 antibodies. Future studies will address the importance of this interaction in regulation of Prokr2 expression.

We used a Gfp cassette in the Tshz1 locus to mark mutant cells and serve as a reporter of transcriptional activity. We observed upregulation of GFP protein and mRNA in coTshz1 mutant mice within the RMS and RMS OB, which suggests that TSHZ1 acts as an autorepressor at these sites. TSHZ1 levels might have to be tightly controlled in the RMS and RMS OB to prevent precocious radial migration out of the RMS into other brain regions. Upon reaching the OB, radially migrating neuroblasts could require increased TSHZ1 levels in order to migrate out of the RMS OB, probably via maintenance of Prokr2 expression. The high levels of TSHZ1 seen within granule cells, and the failure to generate these neurons in the absence of the factor, would favor a model whereby maintenance of TSHZ1 expression is also required for neuronal differentiation once neuroblasts have migrated out of the RMS OB and reached the granule cell layer.

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Whether nestin-cre targets GnRH neurons sufficiently early in their development remains unclear; a proper functional assessment of the role of Tshz1 in GnRH neuron development will require future work using more suitable cre drivers. However, our data showing the importance of TSHZ1 in regulating expression in vivo work using more suitable drivers. However, our data showing the role of TSHZ1 in regulating expression in vivo will require future work using more suitable drivers.

Methods

Mice. See Supplemental Methods for detailed information about generation of mice carrying the Tshz1<sup>fl/fl</sup> and Tshz1<sup>cre</sup> alleles.

In situ hybridization, histology, and immunohistochemistry. Single and double in situ hybridization and immunohistochemical analyses were performed as previously described (47). See Supplemental Methods for details.

Pulse-chase experiments with BrdU. BrdU was injected into postnatal coTshz1 mutants and control littermates at a dosage of 50 μg/g body weight. Brains were harvested 2 hours, 1 week, or 2 weeks after pulsing with BrdU, then processed for preparation of frozen sections. After staining for primary antigens, sections were postfixed in 4% PFA/phosphate buffer for 15 minutes at room temperature. DNA was then denatured by incubation in 2.4 M HCl for 10–15 minutes at 57°C. After extensive washing, immunohistochemistry with anti-BrdU antibodies was performed (see Supplemental Methods).

Microarray analysis. Microarray analysis was initially performed on OB tissue from E18.5 Tshz1<sup>Δ^27/27</sup> (control) and Tshz1<sup>Δ^27/27;A</sup> embryos prior to establishment of a coTshz1 mutant colony (nestin-cre;Tshz1<sup> Δ^27/27;A</sup> condi-
tional mutant). We found the phenotype in the OB of Tshz1<sup>Δ^27/27</sup> and coTshz1 mutants at E18.5 to be identical (Supplemental Figure 1). Subsequently, OB tissue from P30 nestin-cre;Tshz1<sup>Δ^27/27;A</sup> (control) and coTshz1 animals was isolated. OB tissue samples were homogenized in TRIzol, and total RNA extraction was performed according to the manufacturer’s instructions. 1.125 μg cRNA from each sample (n = 8 biological replicates per genotype) was hybridized to Illumina MouseRef-8 version 2.0 arrays, after labeling using the Illumina TotalPrep RNA amplification kit. Arrays were quantile normalized on probe level without background correction using Illumina Genome Studio software version 2011.1, with gene expression module version 1.9.0. Subsequently, data were log-transformed after an offset addition of +16. Probes failing to display a detection P value less than 0.05 in all samples were discarded before performing statistical comparisons. Probes and samples were analyzed for significant differences in expression according to genotype using an ANOVA algorithm, followed by a false discovery rate (FDR) multiple-testing correction (48). ANOVA was performed for adult and embryo samples separately by controlling for the “slide” cofactor. Probes with FDR 0.1 were selected as differentially expressed (n = 865 probes [P30], 92 probes [E18.5]). After selection of candidates for verification by in situ hybridization or immunohistochemistry, standardized mean values of 37 differentially expressed probes (representing 31 genes) were clustered using hierarchical average linkage clustering. Microarray data were deposited in GEO (accession no. GSE51761).

Mouse behavioral tests. To determine whether coTshz1 mutant mice had an impaired sense of smell, we performed several olfactory tests: (a) the buried food test, which measures the ability of mice to locate familiar food hidden underneath bedding (49); (b) the preference test, designed to identify abilities to sense attractive or aversive odors (50, 51); (c) the habituation/dishabituation test, to determine an animal’s ability to habituate to different odors; and (d) the threshold test, using different concentrations of the odor TMT (51). See Supplemental Methods for details.

Olfactory testing of human subjects. Olfactory testing was performed in 5 individuals heterozygous for loss-of-function TSHZ1 mutations, previously described by Feenstra et al. (15), and 5 controls matched for age and sex. Testing was performed using the Sniffin’ Sticks test kit (Burghart Instruments; ref. 28), which is divided into 3 subtests. Odor sensitivity was tested using a 3-alternative, forced-choice task and a staircase paradigm using triplets of odor sticks, 1 with n-butanol and 2 blanks for each dilution step. Odor discrimination was tested using a 3-alternative forced-choice task and 16 triplets of odor sticks, with 2 containing the same odor and 1 containing a different odor. Odor identification was tested using a 4-alternative, forced-choice task with a list of 4 descriptions for each of 16 odor sticks with different odors. To determine whether the results of the control group differed significantly from those of the mutant group, unpaired t test was used. The hyposmic scores on the identification test in both control and mutant groups can be explained by the age of the participants: 6 of the individuals tested were children and were not familiar with all odors. All tests were performed by a single trained examiner.

ChIP. Chromatin was extracted from adult mouse OBs, followed by immunoprecipitation using anti-TSHZ1 antisera or serum before immunization (preimmune serum) as a negative control. ChIP was performed as previously described (52), with minor changes. Briefly, OBs of control and mutant mice were dissociated and cross-linked with 1% formaldehyde for 15 minutes at room temperature. Chromatin was released using SDS lysis buffer during 25 cycles of sonication (30 s on, 30 s off), and the size of DNA in the chromatin fragments was checked by gel electrophoresis (Supplemental Figure 8E). Chromatin samples were then split, and DNA-protein complexes were immunoprecipitated with either anti-TSHZ1 antiserum or preimmune serum using protein A–coupled beads. After RNase and protease treatment of chromatin, reverse cross-linking was performed, followed by DNA purification using phenol-chloroform extraction and ethanol precipitation. Conventional PCR was used to test for ChIP enrichment using primers amplifying different segments covering a region 1.5 kb upstream of exon 1 and in intron 1. We showed results for 2 sets of primers: promoter (5’-CACACTCAAACACTCTGGCACACTTATG-3’ and 5’-CGGAGCTCTGAGACCCCTAATTCGAT-3’; 154 bp), which served as a negative control for binding in the promoter sequence, and intron 1 (5’-TTCAAGGGCATTCTCAGAACCGGACTA-3’ and 5’-ACTGCTCCTGGCGCAGAAGGGTAT-3’; 236 bp), which served to detect a putative binding site of TSHZ1 in the first intron of Prokr2. Quantitative PCR using the same primers and a SybrGreen-based kit was used to examine ChIP enrichment across a total of 5 independent tissue samples, each consisting of a pool of OBs from 4 control animals. IP enrichment was calculated as the ratio of amplified product after precipitation with anti-TSHZ1 antiserum relative to preimmune serum.

Western blot. Dissected OBs of adult mice were homogenized in lysis buffer (10 mM Tris, pH 8; 1 mM EDTA; 0.5 mM EGTA; 1% Triton-X; 0.1% SDS; 0.1% Na deoxycholate; 1× Complete Protease Inhibitor Cocktail, Roche) using a Dounce homogenizer and 5× 1-minute sonication. 30 μg protein lysate was resolved using a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Whatman) and blocked with 5% skimmed milk powder (Merck) in PBS with 0.1% Tween20 (PBST) for 1 hour at room temperature. Primary guinea pig anti-TSHZ1 antisera (1:10,000; see above) was incubated in blocking solution for 2 hours at room temperature. Blots were washed 3 times for 10 minutes with PBST, then incubated with HRP-conjugated antibody (1:3,000 goat anti–guinea pig; Jackson Immunoresearch). A protein band corresponding to the expected size of TSHZ1 (115 KDa) was observed in Western blotting of control OB tissue, but was absent in coTshz1 mutant animals.

Statistics. Statistical significance was assessed using unpaired, 2-tailed Student’s t test. Results (mean ± SEM) were obtained using GraphPad Prism 5. P values less than 0.05 were considered significant.

Study approval. All procedures with patients were approved by, and performed in accordance with the ethical standards of, the Radboud University Nijmegen Medical Centre ethical committee. Informed consent
was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki. All animal experiments described herein were approved in accordance with German animal welfare regulations (LaGeSo, Berlin, Germany).

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