The Lim homeobox gene *Lhx2* is required for olfactory sensory neuron identity

Åsa Kolterud[2], Mattias Alenius[1], Leif Carlsson[2] and Staffan Bohm[1,*]

[1]Department of Molecular Biology, Umeå University, Umeå, SE901 87, Sweden
[2]Umea Centre for Molecular Medicine, Umeå University, Umeå, SE901 87, Sweden
*Author for correspondence (e-mail: staffan.bohm@molbiol.umu.se)

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Summary

Progenitor cells in the mouse olfactory epithelium generate over a thousand subpopulations of neurons, each expressing a unique odorant receptor (OR) gene. This event is under the control of spatial cues, since neurons in different epithelial regions are restricted to express region-specific subsets of OR genes. We show that progenitors and neurons express the LIM-homeobox gene *Lhx2* and that neurons in *Lhx2*-null mutant embryos do not diversify into subpopulations expressing different OR genes and other region-restricted genes such as *Nqo1* and *Ncam2*. *Lhx2*$^{-/-}$ embryos have, however, a normal distribution of Mash1-positive and neurogenin 1-positive neuronal progenitors that leave the cell cycle, acquire pan-neuronal traits and form axon bundles. Increased cell death in combination with increased expression of the early differentiation marker *Neurod1*, as well as reduced expression of late differentiation markers (*Gαolf* and *Omp*), suggests that neuronal differentiation in the absence of Lhx2 is primarily inhibited at, or immediate prior to, onset of OR expression. Aberrant regional expression of early and late differentiation markers, taken together with unaltered region-restricted expression of the *Msx1* homeobox gene in the progenitor cell layer of *Lhx2*$^{-/-}$ embryos, shows that Lhx2 function is not required for all aspects of regional specification of progenitors and neurons. Thus, these results indicate that a cell-autonomous function of Lhx2 is required for differentiation of progenitors into a heterogeneous population of individually and regionally specified mature olfactory sensory neurons.

Key words: Lhx2, Neurod1, Olfactory, Gene expression, Odorant receptors, Lim, Homeobox, Neuron, Differentiation, Mouse

Introduction

A major challenge in our understanding of how axonal projection maps are generated is to identify gene regulatory pathways that generate neuronal diversity, both with regard to response properties and axon target selection of individual neurons. A notable example is the highly heterogeneous population of olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) of the nose. Orderly afferent projections of approximately 1000 different subpopulations of mouse OSNs, each expressing a unique odorant receptor (OR) gene, form a projection map decoding olfactory sensory information in the olfactory bulb of the brain (Buck and Axel, 1991; Ressler et al., 1994; Vassar et al., 1994). The scattered distribution of cell bodies of a given OR-specific subpopulation is confined to one of several distinct regions of OE. OSNs expressing different subsets of OR genes thus partition OE into zones (Ressler et al., 1993; Vassar et al., 1993). Some overlap in OR expression between zones divides OE roughly into a dorsomedial, a middle and a ventrolateral zone (Iwema et al., 2004). The zonal organization of OE is also evident from the differential expression of other types of genes, such as the neural cell adhesion molecule 2 (*Ncam2*/*Ocam*/*Rncam*) and NADPH:quinone oxidoreductase 1 (*Nqo1*/DT-diaphorase) (Alenius and Bohm, 1997; Gussing and Bohm, 2004; Yoshihara et al., 1997). Nqo1 catalyzes reduction quinones and is co-expressed with OR genes of the dorsomedial zone (Gussing and Bohm, 2004). Ncam2, which is co-expressed with ORs of the middle and the ventrolateral zones, has been shown to play a role in axonal guidance of OSNs (Alenius and Bohm, 2003).

OE originates from ectodermally derived neurogenic placodes and the appearance of cell layers and zones becomes evident around embryonic day (E) 12.5-13.5 in mouse (Cau et al., 1997; Sullivan et al., 1995). The three major cell layers are: a superficial layer of sustentacular (supporting) cells; a basal cell layer with dividing immediate neuronal progenitors; and an intermediate cell layer containing OSNs. Also progenitor and sustentacular cells differentially express certain genes in a manner that correlates with their zonal position in OE (Miyawaki et al., 1996; Norlin et al., 2001; Tietjen et al., 2003). The significance of zone-specific gene regulation in the progenitor and sustentacular cell layers is not known.

Functional analyses of certain basic helix-loop-helix (bHLH) transcription factors that are expressed in the progenitor cells in all zones have shown that Mash1 (Ascl1 – Mouse Genome Informatics) and neurogenin 1 (Ngn1) are required for the development of OSNs (Cau et al., 2002; Guillemot et al., 1993). Mash1 is required for the survival of OSN progenitors at an early stage in the OSN lineage, whereas the function of Ngn1 appears important in initiating

differentiation, presumably via the bHLH protein Neurod1, which is transiently expressed at the onset of differentiation (Cau et al., 2002). Despite these advances in knowledge of the essential roles played by bHLH in ensuring neurogenesis in OE, transcription factors regulating diversification of OSNs, including the choice of a given OR gene or the formation of OE zones, have not been identified. A candidate gene that is expressed in a Mash1-dependent manner in OE is *Lhx2* (Cau et al., 2002; Tietjen et al., 2003). Lhx2 is a LIM-homeodomain transcription factor that is implied in the specification of several aspects of the neuronal phenotype (Monuki et al., 2001; Porter et al., 1997; Xu et al., 1993). Analyses of Lhx2-deficient mice have shown that Lhx2 is required for the formation of the optic cup that gives rise to the multilayered neural retina (Porter et al., 1997). In addition, lack of Lhx2 function results in agenesis of the hippocampus and profound losses of cortical progenitors and neurons (Monuki et al., 2001; Porter et al., 1997). Other LIM homeobox family members are required for the diversification of neuronal subtypes in the spinal cord (Shirasaki and Pfaff, 2002). A specific role for Lhx2 in the generation of neuronal subtypes has, however, remained elusive. In the present study we have analyzed the function of Lhx2 during neurogenesis in OE. We provide evidence that the expression of Lhx2 in the OSN lineage is required for the generation of OR-and zone-specific subpopulations of OSNs during differentiation.

Materials and methods

Mice

All mice were maintained at the animal facility at Umeå University under pathogen-free conditions. Generation of mice with a targeted *Lhx2* gene has been described elsewhere (Porter et al., 1997), and mice and embryos were genotyped as previously described (Monuki et al., 2001). *Lhx2*$^{+/-}$ mice were maintained on a mixed 129/Sv×C57BL/6 background and were mated to generate embryos for analyses. The morning of the vaginal plug was considered as E0.5. No obvious difference in gene expression patterns could be observed between wild-type or heterozygous E14.5-15.5 embryos and differences in gene expression between E14.5 and E15.5 *Lhx2*$^{-/-}$ embryos were not observed. All animal experiments were approved by the ethical committee at Umeå University.

In-situ hybridization

Embryos were postfixed in 4% paraformaldehyde (PFA), cryoprotected (30% sucrose in PBS), embedded in Tissue-Tek OCT compound and cryosectioned. Cryosections (10 μm) for in-situ hybridization using ^{35}S-labeled probes were pretreated according to Breitschopf et al. (Breitschopf et al., 1992) and hybridization and washing conditions were as described (Sassoon and Rosenthal, 1993). Slides were dehydrated and processed for autoradiography using NTB-emulsion (Kodak), and exposure was for 5-14 days at 4°C. In-situ hybridization using digoxygenin (Dig)-labeled probes was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993), with some modifications. Briefly sections were treated with 5 μg/ml proteinase K (Roche) in PBS for 15 minutes in Fast Red (Roche). Probes specific for Gαolf, Omp and OR genes (M40, K20, L45, A16, and M50) have been described previously (Berghard et al., 1996; Ressler et al., 1993; Sullivan et al., 1995). The *Ncam2*-specific probe corresponded to the extracellular *Ncam2* domain (Alenius and Bohm, 1997). The Lhx2 probe (460-1750 bp, accession no. NM_010710) hybridized to both wild-type and mutated *Lhx2* transcripts (Monuki et al., 2001). Probes corresponded to published sequences of *Nqo1* (94-1626 bp, accession no. NM_008706), *Msx1* (894-1600 bp, accession no. BC016426), and *Alk6* (*Bmpr1b* – Mouse Genome Informatics) (1-1935 bp, accession no. MMALK6A). The *Mash1*-, *Ngn1*- and *Neurod1*-specific probes corresponded to the published sequences of *Mash1* (256-900 bp, accession no. MUSHASH1X), *Ngn1* (75-840 bp, accession no. YO9166), and *Neurod1* (1-1207 bp, accession no. BC018241). Hoechst 33258 (Sigma) was used as nuclear counterstain.

Immunohistochemistry

Embryos were fixed in 4% PFA for 1-2 hours at room temperature and cryoprotected at 4°C for 24 hours. Retrieval of Nquo1, Rncam and Gap43 antigens was achieved by boiling in 10 mM citrate buffer (pH 6.0) for 10 minutes. Sections were then incubated for 1 hour in blocking solution (3% normal donkey serum, 0.3% Triton X-100 in PBS) followed by overnight incubation at 4°C in blocking solution containing affinity-purified anti-Ncam2 (1:100 dilution (Alenius and Bohm, 2003), anti-Gap43 (1:1000 dilution, AB5220, Lot#22010885, Chemicon), anti-caspase-3 antibody (1:1000 dilution, 557035, Lot#M051497, PharMingen), anti-Omp (1:2000 dilution, gift from Dr Margolis), anti-Ncam (1:100 dilution, 556323, clone 12F11, PharMingen), phospho-H3 (1:500 dilution, 06-570, Lot#24019, Upstate biotech), anti-Stathmin/SCG10 antibody (1:1000 dilution (Holmfeldt et al., 2003), and neuronal class III β-tubulin (1:2000 dilution, PRB-435P, Nordic BioSite AB), anti-LH2A [1:500 (Liem et al., 1997)]. Positive immunoreaction was visualized after a 1 hour incubation with Alexa Fluor 546 donkey anti-goat IgG (A-11056) or Alexa Fluor 488 donkey anti-rabbit IgG (A-21206, Molecular Probes) diluted 1:250 in blocking solution. Sections were counterstained with Hoechst. Microphotographs of immunohistochemistry and in-situ hybridization analyses were taken using light, fluorescent or dark-field optics on a Zeiss Axioskop microscope with a Hamamatsu digital CCD camera. Confocal microscopy was performed on a Nikon confocal microscope. Images were processed using Adobe Photoshop 7.0.1. Brightness and contrast adjustments of images were done linearly.

Results

Lack of OR gene expression in *Lhx2*-null embryos

Double Lhx2 and neuronal class III β-tubulin (TubIII) immunohistochemistry analysis of E15.5 mouse embryos showed nuclear Lhx2 in TubIII-positive OSNs (Fig. 1A-C). A fraction of TubIII-negative cells in the basal progenitor cell layer stained positive with the Lhx2 antibody (arrow in 1C). The sustentacular cell layer or in cells located in lamina propria under the OE were negative. OR gene expression was first observed at E11.5 in the developing OE. The number of OR-positive cells increases dramatically thereafter and zonal OR expression is apparent by E13 (Sullivan et al., 1995). Homozygous *Lhx2* knockout mouse (*Lhx2*$^{-/-}$) die in utero after E15.5 (Porter et al., 1997). *Lhx2*$^{-/-}$ and littermate control embryos (*Lhx2*$^{+/-}$ and *Lhx2*$^{+/+}$) at two ages (E14.5 and E15.5) were analyzed in this study. *Lhx2* and OR gene expression was examined by in-situ hybridization (Fig. 1D-J). *Lhx2* mRNA was, in concordance with the result of the immunohistochemical analysis, expressed in both OSNs and a fraction of cells in the basal progenitor cell layer in control embryos (Fig. 1D). The targeted *Lhx2* allele produces a fusion transcript of *Lhx2* exon 1 and the neomycin resistance gene that allows for the identification of cells that have an active *Lhx2* promoter also in *Lhx2*$^{-/-}$ embryos (Monuki et al., 2001). Importantly, we detected a similar cell layer organization and distribution of *Lhx2*-promoter active cells in control and homozygous mutant mice (Fig. 1D,E). The result suggested

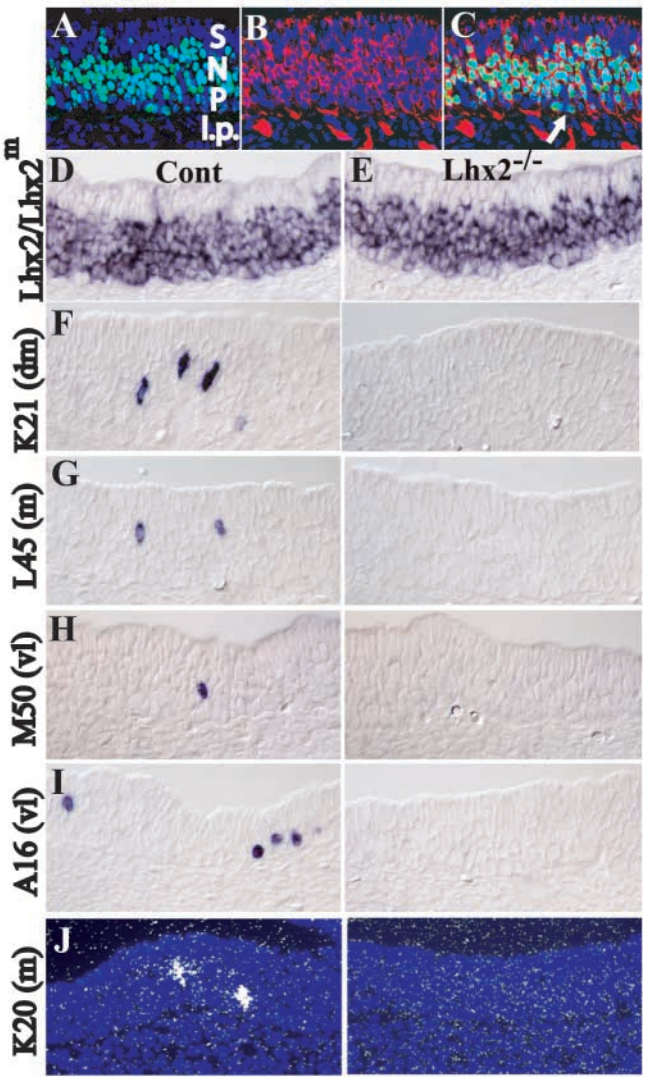


Fig. 1. Unaltered distribution of *Lhx2* expression and lack of OR expression in OE of *Lhx2*$^{-/-}$ embryos. (A-C) Double-immunohistochemical analysis of Lhx2 and TubIII expression in OE of E15.5 mouse embryo is shown. (A) Nuclear Lhx2 immunoreactivity in the neuronal cell layer (N) and in a fraction of cells in the basal progenitor cell layer (P). No Lhx2-positive cells are present in the sustentacular cell layer (S) and the lamina propria (l.p.). (B) TubIII immunoreactivity in neuronal soma and neuronal processes. (C) Images in A and B combined, showing that Lhx2 and TubIII are co-expressed in OSNs. A limited number of TubIII-negative Lhx2-positive cells are present in the progenitor cell layer (arrows in C). (D-I) In-situ hybridization analyses of serial OE sections from control (left panel) and *Lhx2*$^{-/-}$ embryos (right panel) with Dig-labeled cRNA probes that hybridize to *Lhx2* and mutated *Lhx2* transcripts (*Lhx2*^{m}) (D,E), and OR genes (*K21*, *L45*, *M50* and *A16*) (F-I). (J) Autoradiographs of in-situ hybridization analyses with a ^{35}S-labeled cRNA probe specific to the OR gene *K20*. Scattered cells expressing the different OR genes are present in control mice. The zones in which each the OR gene is expressed is indicated, i.e. dorsomedial (dm), the middle (m) or the ventrolateral (vl) zone. In *Lhx2*$^{-/-}$ mice, there are no cells expressing ORs in any epithelial region (representative results are shown).

that Lhx2 is not required for formation of the progenitor and neuronal cell layers of OE. Next we analyzed for the expression of different OR genes that are expressed in different zones, i.e. *K21* (*Olfr145* – Mouse Genome Informatics), *L45*, *M50* (*Olfr6* – Mouse Genome Informatics) and A16 (*Olfr140* – Mouse Genome Informatics). As expected, all four OR probes hybridized to scattered OSNs in sections from control mice (Fig. 1F-I). Interestingly, parallel analyses of *Lhx2*$^{-/-}$ littermates revealed no hybridizing cells (Fig. 1F-I), even though sections throughout the anterior–posterior extent of the nasal cavity were examined. Neither did in-situ hybridization analyses using more sensitive radioactively labeled cRNA probes reveal any OR positive OSNs in mutant embryos (Fig. 1J). These results indicated that the function of *Lhx2* was required for OR expression.

Lack of zone-specific Nqo1 and Ncam2 in *Lhx2*-null embryos

Lack of OR expression in *Lhx2*$^{-/-}$ embryos suggested that the function of *Lhx2* was important specifically for OR expression. However, since mutant mice lacked expression of several ORs that are expressed in different zones, an alternative interpretation was that *Lhx2* is required for zone-specific gene expression in general and not OR gene expression per se. To address this possibility, we analyzed for the expression of other zone-specific markers. *Nqo1* is co-expressed with ORs of the dorsomedial zone, whereas *Ncam2* is co-expressed with ORs of both the middle and the ventrolateral zones (Alenius and Bohm, 2003; Gussing and Bohm, 2004). In-situ hybridization analyses revealed zonal *Nqo1* and *Ncam2* expression in control mice (Fig. 2A, parts B,C and Fig. 2B, parts A,B), whereas no hybridizing cells were observed in the OE of *Lhx2*$^{-/-}$ embryos (Fig. 2A, parts E,F and Fig. 2B, parts C,D). Other cell types that hybridized to Nqo1 and Ncam2 probes were, however, present in both control and *Lhx2*$^{-/-}$ embryos (arrowheads in Fig. 2A, parts B,E,F). Thus, the function of *Lhx2* was required for the development of OSNs that express both ORs and zone-specific genes.

Inhibited OSN differentiation and increased apoptosis in *Lhx2*$^{-/-}$ embryos

Formally, the phenotype observed might stem from a complete lack of neuronal progenitors, altered commitment of multipotent progenitors or that neuronal differentiation was inhibited at a stage prior to onset of OR and zone-specific gene expression. To address the question of whether *Lhx2* is required for the generation of OSN progenitors, we analyzed for the expression of *Mash1* and *Ngn1*. It has been suggested that the developing OE contains two populations of neuronal progenitors: a Mash1-positive population that divides in the apical part of OE and a population of Mash1- and Ngn1-positive secondary progenitor cells that settle on the basal side of OE, where they continue to divide before differentiating into OSNs (Cau et al., 1997). Expression levels and distribution of Mash1- and Ngn1-positive cells in OE of control and *Lhx2* mutant mice were similar (Fig. 3A,B). Thus, Lhx2 function did not appear to be required for Mash1/Ngn1-dependent neurogenesis. Ngn1 regulates the differentiation-promoting gene *Neurod1*, which is transiently expressed during the onset of OSN differentiation (Cau et al., 2002). Interestingly, *Lhx2* mutant embryos had an increased number of Neurod1-positive

cells (Fig. 3C). *Neurod1* was expressed in the one- to two-cell thick layer of progenitor cells in control mice, whereas Neurod1-positive cells had expanded into the presumptive neuronal cell layer in *Lhx2* mutants (Fig. 3C). Thus, it appeared that lack of *Lhx2* resulted in accumulation of Neurod1-positive progenitor cells and/or immature OSNs. To characterize this phenotype further, we analyzed for expression of OSN-specific genes *Gαolf* and *Omp*, which are expressed at terminal stages of OSN differentiation. The gene for the G-protein alpha subunit Gαolf is turned on at a stage between E13.5-16.5, which is at least 2 days subsequent to the onset of OR expression (Sullivan et al., 1995). *Gαolf* was expressed in neurons of both control and *Lhx2*$^{-/-}$ embryos at E14.5-15.5 (Fig. 3D). This result suggested that the observed phenotype was not a consequence of a developmental delay in the absence of *Lhx2*. The expression level of *Gαolf* was, however, reduced compared with littermate control embryos (Fig. 3D). We next analyzed for the expression of *Omp*, which is a hallmark of mature OSN (Youngentob et al., 2003). Studies of neuronal differentiation in the OE show that ORs are expressed prior to onset of *Omp* expression (Iwema and Schwob, 2003). In-situ hybridization analysis showed that a limited number of neurons in *Lhx2*$^{-/-}$ mice expressed Omp to markedly reduced levels (Fig. 3E). The total number of Omp-positive cells was 133±61 and 13±5 (P<0.001, Student's t-test) per section and nasal cavity in control and *Lhx2*$^{-/-}$ embryos, respectively. The presence of neurons expressing Omp in *Lhx2*$^{-/-}$ mice was confirmed by immunohistochemical analyses (Fig. 3F). Unexpectedly, analyses of sections throughout the nasal cavity revealed that *Omp*-positive and *Gαolf*-positive cells were located selectively in the dorsomedial part of OE (for result and discussion see below). The reduced expression of *Omp* and *Gαolf* suggested that neuronal maturation and/or survival was reduced. To address if reduced expression of *Gαolf* and *Omp* coincided with increased apoptosis, we utilized activated (cleaved) caspase 3 immunohistochemistry analysis. Caspase 3-positive cells were evenly distributed throughout OE (data not shown) and the number of cells with activated caspase 3 in the progenitor and neuronal layers was 5.4±2.1 and 19.4±8.9 (P<0.001, Student's t-test) per OE section in control and *Lhx2*$^{-/-}$ embryos, respectively. The aberrant differentiation of OSNs in *Lhx2*$^{-/-}$ embryos was thus associated with an overall 3.5-fold increased rate of apoptosis. Collectively, these results indicated that a fraction of neurons in the dorsomedial OE acquires an OSN identity in the absence of *Lhx2* but fails to become and/or survive as OR- and zonally specified bona fide OSNs.

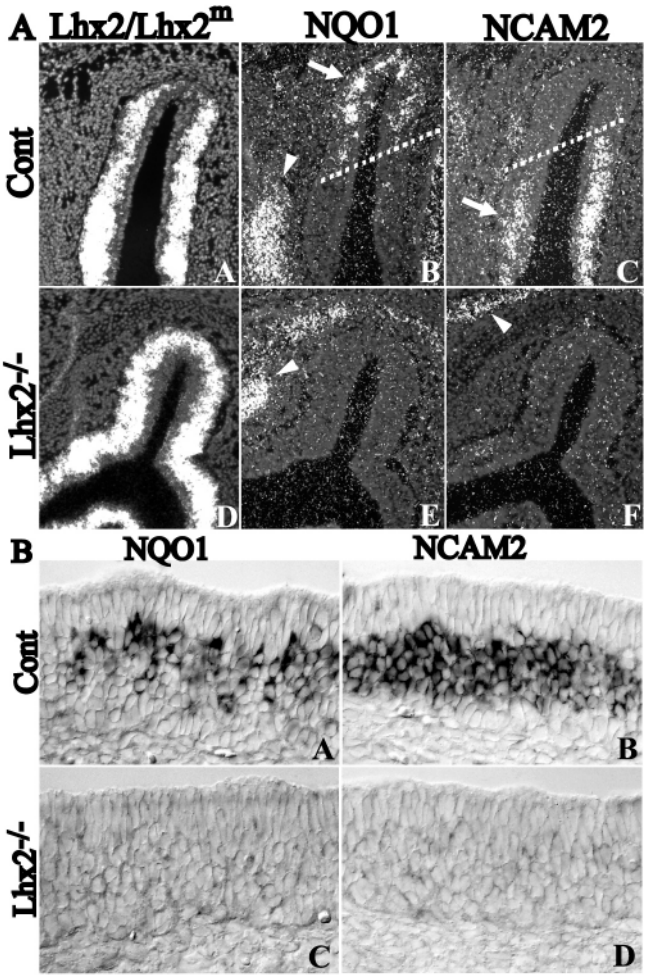


Fig. 2. No expression of the zone-specific genes *Nqo1* and *Ncam2* in OE of *Lhx2*$^{-/-}$ embryos. (A) Autoradiographs of in-situ hybridization analyses of consecutive coronal sections (dorsal up and medial left) of one nasal cavity of control embryos (A-C) and *Lhx2*$^{-/-}$ embryos (D-F). Sections were hybridized with ^{35}S-labeled cRNA probes specific for *Lhx2* and mutated *Lhx2* transcripts (*Lhx2*^{m}) (A,D), *Nqo1* (B,E) and *Ncam2* (C,F). *Lhx2* expression is distributed throughout the OE in both control (A) and *Lhx2*$^{-/-}$ mice (D). *Nqo1* is expressed in OSNs located in the dorsomedial zone in control embryos (arrow in B), whereas no signal is present in the neuronal cell layer of *Lhx2*$^{-/-}$ embryos (E). *Ncam2* is expressed in OSNs located in the middle and the ventrolateral zones in control embryos (arrow in C), whereas no hybridizing signal is present in the neuronal cell layer of *Lhx2*$^{-/-}$ embryos (F). The border between neurons in the dorsomedial zone and the middle zone is indicated (dotted line in B,C). *Nqo1*-specific hybridization to cells in lamina propria (arrowheads in B,E) and *Ncam2*-specific hybridization to cells in the telencephalon (arrowhead in F) are also indicated. (B) High-power magnifications of OE sections hybridized with Dig-labeled cRNA probes specific for *Nqo1* (A,C) and *Ncam2* (B,D) are shown. Both probes hybridize to OSN in control embryos (A,B), whereas no signal over background is evident in sections of *Lhx2*$^{-/-}$ embryos (C,D).

Differentiation is inhibited at, or just prior to, onset of OR expression

Lhx2 deficiency appeared to influence differentiation primarily at a stage that was subsequent to onset of *Neurod1* expression and prior to onset of *Gαolf* and *Omp* expression. To determine if there was an increased number of Neurod1-positive progenitors and/or Neurod1-positive immature neurons in *Lhx2*$^{-/-}$ embryos we analyzed for *Neurod1* expression in relation to dividing cells and onset of early pan-neuronal differentiation markers. Immunohistochemical analyses with antibodies specific for Ncam1, Gap43, Stmn/SCG10 (Stmn2 – Mouse Genome Informatics) and TubIII showed that immature neurons were present throughout OE in both control and mutant embryos (Fig. 4A-F,I,J). Moreover, the staining patterns showed immunoreactivity in axon bundles in the lamina propria and in the OE of *Lhx2*$^{-/-}$ mice (arrows in 4A-F,I,J). Thus, progenitor cells appeared to leave the cell cycle and differentiate to a stage associated with the onset of pan-

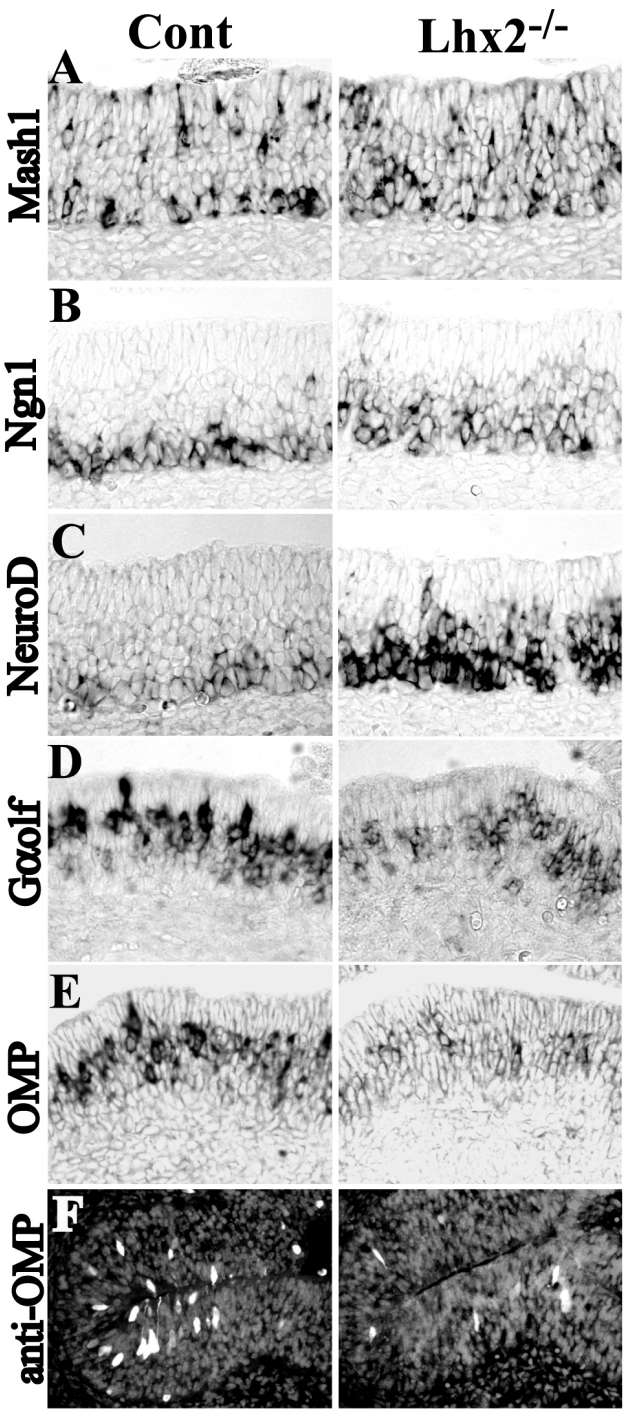


Fig. 3. Unaltered neurogenesis and altered neuronal differentiation in *Lhx2*$^{-/-}$ embryos. In-situ hybridization analyses of OE sections with Dig-labeled cRNA probes specific for *Mash1* (A), *Ngn1* (B), *Neurod1* (C), *Gαolf* (D), and *Omp* (E). Control embryos (left panel) and *Lhx2*$^{-/-}$ embryos (right panel) show an equal distribution and number of apical and basal progenitor cells expressing *Mash1* (A) and basal progenitors expressing *Ngn1* (B). The OE in *Lhx2*$^{-/-}$ embryos contains an increased number of progenitors and/or neurons that express *Neurod1* (C), whereas the expression of OSN-enriched and late differentiation markers *Gαolf* (D) and *Omp* (E) is reduced. (F) Immunohistochemical analyses of the dorsomedial zone (dorsal left and lateral up) with a reduced number of *Omp*-positive cells in OE of *Lhx2*$^{-/-}$ embryos.

neuronal markers and axonal outgrowth. Analyses of serial sections revealed that regions in OE that showed high Neurod1 and TubIII expression correlated (Fig. 4I-L). Confocal microscopy indicated an increased number of cells that co-expressed Neurod1 and TubIII in *Lhx2*$^{-/-}$ embryos (Fig. 4M,N) which is compatible with the suggestion that *Lhx2*$^{-/-}$ embryos have a larger fraction of newly differentiated Neurod1-positive neurons and not proliferating progenitors. To substantiate this suggestion, we analyzed for the mitosis marker phosphorylated histone 3 (phospho-H3). The increased number of Neurod1-positive cells in *Lhx2*$^{-/-}$ embryos did not correlate to an increase in dividing progenitors (Fig. 4G,H,O,P). Thus, these results indicated that the transient differentiation stage, at which Neurod1 and pan-neuronal markers are co-expressed, is prolonged in *Lhx2*$^{-/-}$ embryos. Since there is a lag between onset of pan-neuronal gene expression and onset of OR expression (Iwema and Schwob, 2003), this suggests that neuronal differentiation in *Lhx2*$^{-/-}$ embryos is primarily inhibited at a stage that is normally associated with the onset of OR gene expression.

Lack of *Lhx2* function reveals zone-specific differences in neuronal maturation

Although neurons in *Lhx2*$^{-/-}$ embryos were not zonally specified with regard to OR, *Nqo1* or *Ncam2* expression, we found evidence that *Lhx2* deficiency did not abolish all zonal characteristics of the OE. The ventrolateral region contained a higher fraction of Neurod1-positive cells than did the dorsomedial region (arrowheads in Fig. 4H,L). This regional difference in distribution of Neurod1-positive cells was more apparent in *Lhx2*$^{-/-}$ embryos than controls. Analyses of consecutive sections indicated that the increased expression of *Neurod1* in the ventrolateral part coincided with a high expression of TubIII (arrowheads in Fig. 4J,L). Since phospho-H3 immunoreactive cells were distributed equally throughout the OE (Fig. 4G,H), this result suggested that the ventrolateral region contained a larger fraction of newly differentiated postmitotic neurons compared with the dorsomedial OE region in *Lhx2*$^{-/-}$ embryos. Interestingly, neurons expressing the late differentiation markers (*Gαolf* and *Omp*) were selectively located in the dorsomedial OE region in *Lhx2*$^{-/-}$ embryos (Fig. 5A-D). The *Gαolf*- and *Omp*-positive region appeared to correspond to the dorsomedial zone, namely the dorsal meatus, part of the medial septum, and the tips of the ethmoturbinate projecting into the nasal cavity (Fig. 5B,D and compare with expression of *Nqo1* in Fig. 5E). To confirm the unequal distribution of *Omp* expression, we quantified the number of *Omp*-positive cells that were located on either side of a tentative half circle dividing the OE into two roughly equal parts, one dorsomedial and one ventrolateral part. This analysis revealed that 59.7±14.5% and 95.2±10.9% (P<0.001, Student's t-test) of the *Omp*-positive cells were located in the dorsomedial part in control and *Lhx2*$^{-/-}$ embryos, respectively. Thus, the absence of *Lhx2* expression revealed zone-specific differences in the regulated control of neuronal differentiation.

Intact region-specific expression in sustentacular and progenitor cell layers

One possible scenario was that positional signals determining zone-specific gene expression in OE were absent or altered in *Lhx2*$^{-/-}$ embryos. The type I BMP receptor Alk6 is expressed

in a dorsomedial to ventrolateral gradient by the sustentacular cells, whereas differential expression of the homeobox gene *Msx1* defines a ventrolateral to dorsomedial counter gradient in the progenitor cell layer (Norlin et al., 2001). This spatially limited expression of *Alk6* and *Msx1* was unaltered in *Lhx2*$^{-/-}$ embryos (Fig. 5G-N). Since sustentacular and progenitor cells showed the expected regional differences in gene expression, the result strongly suggested that positional cues are present in OE of *Lhx2*$^{-/-}$ embryos.

Discussion

In this study we show that the function of the LIM homeobox transcription factor Lhx2 is required for the generation of a heterogeneous population of individually and zonally specified OSNs. Lhx2 is apparently not required for the development of zonally specified progenitor cells or supporting cells of OE. Our results thus indicate that *Lhx2* regulates a gene program that is intrinsic to OSN lineage that includes ORs and zone-specific genes. The neurons generated in the absence of *Lhx2* function, and thereby also the functions of ORs and zone-specific genes, express pan-neuronal markers and have axons projecting toward the telencephalic vesicle. Progenitors thus apparently leave the cell cycle and acquire pan-neuronal traits characteristic of postmitotic neurons. We detect an accumulation of neurons that express the differentiation-promoting bHLH transcription factor Neurod1. Since OSNs normally co-express *Neurod1* and pan-neuronal genes only transiently during differentiation (Cau et al., 1997), our results suggest that a majority of neurons in *Lhx2*$^{-/-}$ embryos correspond to immature neurons that have just begun to express pan-neuronal marker genes. Analyses of OR expression in the OE of adult animals indicate that there is a short delay period between the onset of pan-neuronal genes and the start of OR expression (Iwema and Schwob, 2003). Thus our results are compatible with the suggestion that neuronal differentiation in the *Lhx2*$^{-/-}$ embryos does not proceed past this delay period in an appropriate manner. In this regard it is interesting to note that accelerated differentiation as a consequence of OR expression has been suggested to underlie the feedback mechanism in which a given OR protein ensures high levels of expression of its corresponding gene (Lewcock and Reed, 2004). It is therefore conceivable that an *Lhx2*-dependent block in OR expression inhibits neuronal differentiation and/or prolongs the period during which OSN under normal circumstances are competent to select an OR gene for high-

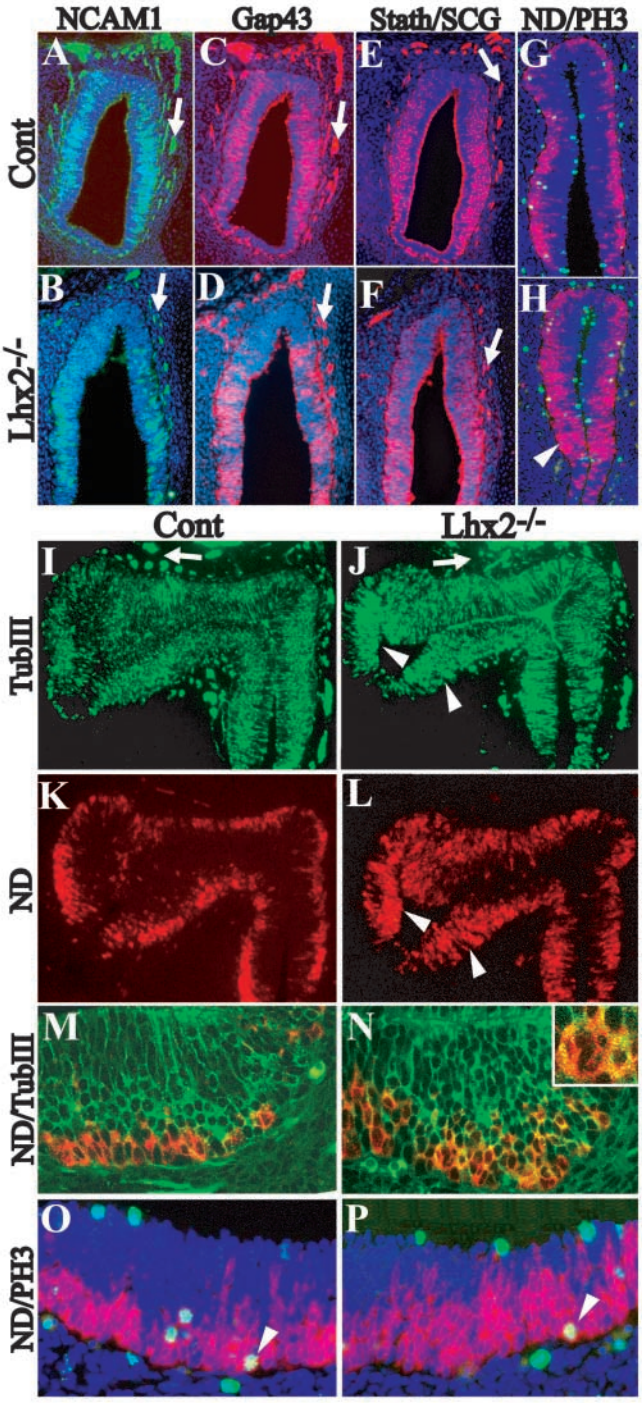
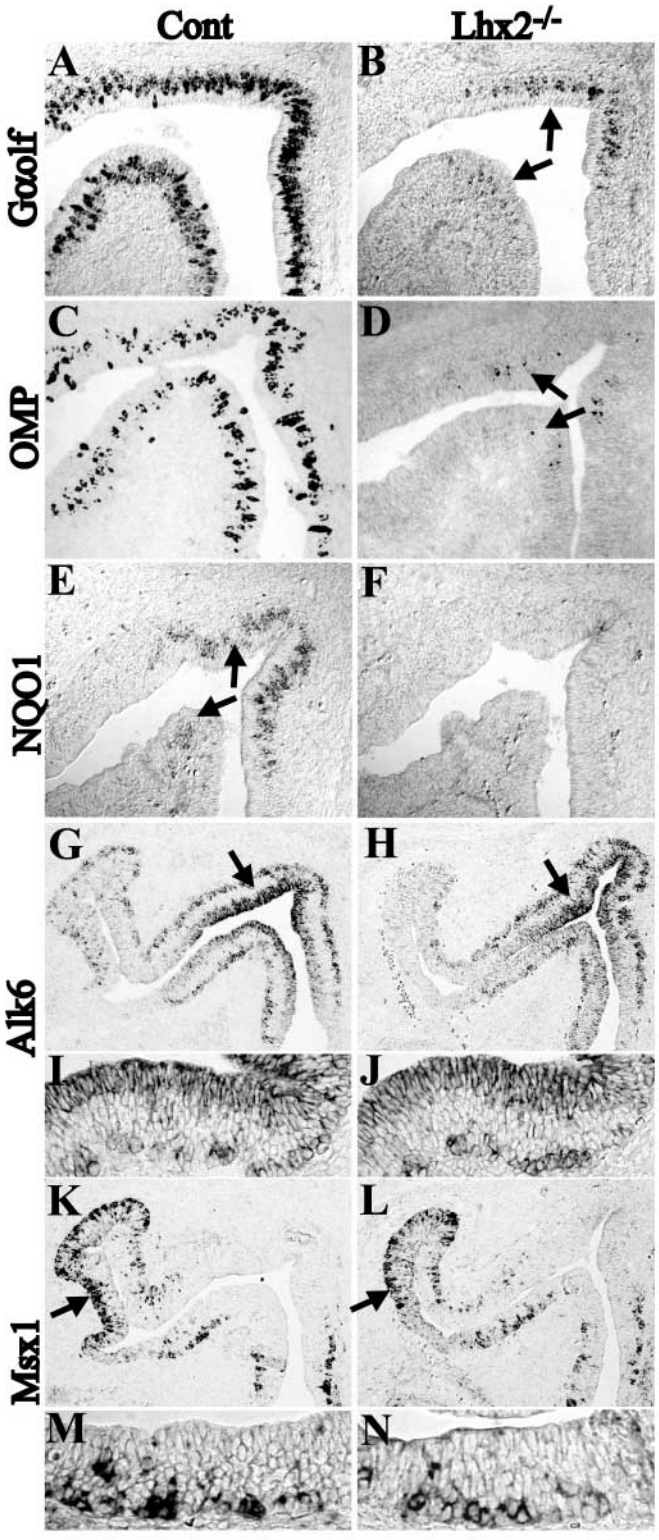


Fig. 4. The neuronal cell layer in OE of *Lhx2*$^{-/-}$ embryos largely contains newly differentiated neurons that have acquired pan-neuronal traits and form axon bundles. Coronal sections (dorsal up and medial left) of one nasal cavity from control embryos (A,C,E,G,I,K,M,O) and *Lhx2*$^{-/-}$ embryos (B,D,F,H,J,L,N,P). (A-F) Immunohistochemical analyses for expression of immature neuronal markers is shown. *Ncam1* (A,B), *Gap43* (c-d), and *Stathmin*/*SCG10* (E,F) are expressed in OE and axon bundles of the lamina propria (arrows) in both control and *Lhx2*$^{-/-}$ embryos. (G,H) Double phospho-H3 (PH3; in green) immunohistochemistry and *Neurod1* (ND; in red) in-situ hybridization analyses showing that the increased expression of *Neurod1*, primarily apparent in the ventral OE (arrowhead in H), does not correlate with the number and distribution of mitotic cells. (I-L) Analyses of consecutive OE sections, showing that cells immunoreactive using an anti-TubIII antibody (I,J) and cells that express *Neurod1* transcripts (K,L) co-localize predominantly in ventrolateral regions of OE (arrowheads in J,L). Expression of TubIII in axon bundles of the lamina propria is indicated (arrows in I,J). (M,N) High magnification confocal images of double TubIII immunohistochemical (in green) and *Neurod*1 in-situ hybridization (in red) analyses showing an increased number of cells in *Lhx2*$^{-/-}$ embryos that co-express *Neurod1* and TubIII (yellow signal; insert in N). (O,P) High magnification confocal images of double phospho-H3 immunohistochemical (in green) and *Neurod1* in-situ hybridization (in red) analyses showing that OE in *Lhx2*$^{-/-}$ embryos does not contain an increased number of progenitors that co-express phospho-H3 and *Neurod1* (in yellow; arrowheads).

level expression. Even though the data does not allow us to determine if *Lhx2* exerts its primary effect in progenitor cells or OSNs, it is interesting to note that Lhx2 has been identified by yeast one-hybrid screening as a protein that binds to conserved sequence motifs in the promoter regions of a defined subfamily of OR genes (Hoppe et al., 2003). Previous studies have demonstrated that promoter proximal regions of certain OR genes are sufficient to fully recapitulate proper expression in transgenic mice (Lewcock and Reed, 2004; Vassalli et al., 2002). Moreover, the feedback control mechanism of OR gene choice was shown to require a defined *cis*-acting locus control region with capacity to enhance transcription of OR genes belonging to different zonal sets (Lewcock and Reed, 2004). Similarly, we find that Lhx2 function appears required for expression of OR genes belonging to different zonal sets, which is compatible with the possibility that *Lhx2* regulates transcription by binding to conserved DNA motifs in zonally expressed genes.



The OE in *Lhx2*$^{-/-}$ embryos shows an aberrant zonal distribution of neurons that express early (*Neurod1* and pan-neuronal) and late (*Gαolf* and *Omp*) differentiation markers, respectively. This result suggests that neuronal differentiation and/or survival is under the influence of zonal factors and thus, that *Lhx2* is not required for all aspects of zonal specification of cells in OE. Evidence for the existence of zone-specific cues in the absence of *Lhx2* is that the region-specific expression of *Alk6* in sustentacular cells and *Msx1* in progenitors is unperturbed in mutant embryos. Several other genes have been found to be expressed in progenitors in middle and ventrolateral zones exclusively (Tietjen et al., 2003). Interestingly, the expression of these genes in progenitors is either dependent or independent of *Mash1* function. Since multipotent progenitors are present in OE it is tempting to speculate that a *Mash1*- and *Lhx2*-independent mechanism specify sustentacular cells to differentially express genes such as *Alk6* in a zone-specific manner. Rbtn1 (Lmo1 – Mouse Genome Informatics), which belongs to the LIM only family, is among the transcription factors that were identified to be zonally expressed in a Mash1-dependent manner. LIM only factors interact with both LIM homeobox and bHLH proteins and can regulate *Neurod1* expression (Bao et al., 2000; Jurata et al., 1996). Thus, the zone-specific differentiation phenotype may be a consequence of lack of a zone-specific signaling protein in OSNs and/or absence of *Lhx2* function in progenitors. In either case it is likely that *Lhx2* deficiency accentuates a zone-specific difference in OSN maturation that is not as apparent in the OE of control animals. Evidence for regional differences under normal conditions has come from

Fig. 5. Abnormal region-specific OSN differentiation and normal region-specific gene expression in sustentacular and progenitor cells. In-situ hybridization analyses of serial coronal (dorsal up and medial left) sections of OE from control (A,C,E,G,I,K,M) and *Lhx2*$^{-/-}$ embryos (B,D,F,H,I,L,N) using Dig-labeled cRNA probes specific for *Gαolf* (A,B), *Omp* (C,D), *Nqo1* (E,F), *Alk6* (G-J) and *Msx1* (K-N). (A-F) *Gαolf*- and *Omp*-positive cells are present in all OE zones in control embryos (A,C) but show a restricted dorsomedial distribution in OE of *Lhx2*$^{-/-}$ embryos (arrows in B,D). The distribution of *Gαolf*- and *Omp*-positive cells in *Lhx2*$^{-/-}$ embryos is similar to the distribution of the dorsomedial-specific marker *Nqo1* in control embryos (arrows in E). No *Nqo1* expression over background is detected in *Lhx2*$^{-/-}$ embryos (F). (G-N) The gradient of *Alk6* expression (G,H; high dorsomedial and low ventrolateral) and the reverse gradient of *Msx1* expression (K,L) are present in both control and *Lhx2*$^{-/-}$ embryos. High-power magnifications of *Alk6* expression in the sustentacular cell layer (I,J) and *Msx1* expression in the basal progenitor cell layer (M,N) is shown. Thus, cells expressing *Alk6* and *Msx1* show a similar distribution in OE cell layers in control and *Lhx2*$^{-/-}$ mice.

the finding that the regenerative capacity of OSNs in an adult animal differs between OE zones (Konzelmann et al., 1998). Moreover, the anterior OE, with a high proportion of the dorsomedial zone, appears to contain more OSNs that co-express early (*Gap43*) and late (*Omp*) differentiation markers compared with middle and ventrolateral zones (Iwema and Schwob, 2003).

Collectively, the results presented show a role for *Lhx2* in supplying a fundamental function in the regulated control of neuronal diversity within the OSN lineage. Further studies of *Lhx2* function in the olfactory system will help clarify the precise role of *Lhx2* in the gene regulatory mechanism, whereby an individual OSN selects a given response profile and acquires topographic precision in axon targeting.

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Note added in proof

During the revision of this manuscript, similar results were reported (Hirota and Mombaerts, 2004).

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