Expression of bHLH Transcription Factors NSCL1 and NSCL2 in the Mouse Olfactory System

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Abstract

We examined the expression of basic helix–loop–helix transcription factors NSCL1 and NSCL2 in the olfactory epithelium (OE) and the vomeronasal organ (VNO) during development. As detected by in situ hybridization, at embryonic day (E) 10 NSCL1 was weakly expressed in the entire olfactory placodes. From E12 to postnatal day (P) 3, NSCL1 was expressed in olfactory receptor neurons (ORNs) and receptor neurons of the VNO. The expression pattern of NSCL2 was similar to that of NSCL1. By Northern blot analysis, strong expression of NSCL1 was detected in the OE from E12 to P7, but the expression there was low in the adult (P35). NSCL2 mRNA was detected in the E12 and P1 OE, but its level was very low in the P7 and adult OE. The spatial pattern of expression suggests that NSCL1 and NSCL2 contribute to the maturation of ORNs (VNO receptor neurons) or maintenance of their differentiated state. Moreover, the temporal pattern of expression suggests that NSCL1 and NSCL2 may function during development rather than in the adult stage.

Key words: in situ hybridization, NSCL1, NSCL2, olfactory epithelium, vomeronasal organ

Introduction

NSCL, one of the basic helix–loop–helix (bHLH) transcription factors, was identified by screening a 11.5 day embryonic mouse cDNA library with a stem cell leukemia (SCL) probe, also called Hen1 (Brown et al., 1992) or Nhlh1 (Good et al., 1997). The NSCL gene in fact comprises two closely related genes, NSCL1 and NSCL2. The cDNAs for these two genes encode predicted proteins of similar size and structure. The carboxyl-terminal section of the two proteins contains the bHLH motif and differs between them by only three amino acid changes, whereas the amino-terminal portion has diverged (Gobel et al., 1992; Lipkowitz et al., 1992). A previous in situ hybridization study showed that expression of NSCL1 was restricted to neural tissue, e.g. developing brain, dorsal root ganglia and cranial ganglia, and to the nasal epithelium, but was not found in non-neural tissue (Begley et al., 1992). As the in situ hybridization study was done by an autoradiographic technique, the identity of the cell types expressing NSCL1 in the olfactory epithelium (OE) was not clarified. NSCL2 also is expressed in the developing central nervous system and peripheral nervous system (Gobel et al., 1992; Haire and Chiaramello, 1996). However, little is known about its expression in the olfactory tissue.

Unlike other neuronal cells, olfactory receptor neurons (ORNs) continually die and are replaced by their progenitors throughout life. A number of positively and negatively regulating bHLH genes have been found to be expressed in the OE during development as well as during the regeneration process. Mash1 is a determination gene for ORNs since Mash1 null mutant mice fail to produce their progenitors (Cau et al., 1997). The expression of Mash1 was observed in basal progenitor cells in the embryonic OE (Cau et al., 1997) and in globose basal cells (GBCs), which give rise to new ORNs in the postnatal OE (Gordon et al., 1995). Neurogenin (NGN1) was also found to be a determination gene expressed in basal progenitors (Cau et al., 2002). The downstream genes Hes6 and NeuroD promote the differentiation of ORNs (Nibu et al., 1999; Bae et al., 2000). Hes6 was found to be expressed in basal progenitors and in GBCs (Suzuki et al., 2003); and NeuroD was found to be expressed in both GBCs and cells superficial to them (Nibu et al., 1999; Suzuki et al., 2003). Hes1 and Hes5 inhibit differentiation and were expressed in apical and basal progenitors, respectively (Cau et al., 2000). The relation between NSCL and other bHLH transcription factors in the OE is not clear. Moreover, little is yet known about the expression of bHLH transcription factors in the vomeronasal organ (VNO). In the present study, we examined the expression of NSCL1 and NSCL2 in both the OE and VNO of mice.
Materials and methods

Animals
Timed pregnant and postnatal (P1, P3, P7 and P35) ddY mice were obtained from Sankyo Laboratories. All animals were maintained in a heat- and humidity-controlled vivarium on food and water provided ad libitum.

Tissue preparation
To obtain embryos, pregnant females were killed by cervical dislocation and their uteri with fetuses (E10–18) carefully dissected out. Postnatal mice were killed by an overdose of Nembutal given by i.p. injection. The heads were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. The specimens from P3 and P7 mice were decalcified in 10% EDTA in Tris buffer (pH 7.6), and cryo-protected with 25% sucrose, embedded in OCT compound (Tissue-Tek, Miles, Elkhart, IN), and frozen in a spray freezer (Oken, Japan). The tissues were sectioned coronally at a thickness of 8–10 μm, and the sections were collected and placed on silane-coated slides.

RNA probes and in situ hybridization
cDNA fragments of NSCL1, NSCL2, NCAM, NGN1 and Mash1 were cloned by reverse transcription polymerase chain reaction (RT-PCR) using the total RNA extracted from the olfactory mucosa of adult mice and then used for the synthesis of cRNA probes. The sequences of the primers were 5′-ATGATGCTCAACTCCGATACCA-3′ (450–471) and 5′-TCTTCAACCTCTGCGCTA-3′ (1253–1234; Genbank M82874) for NSCL1, 5′-TCCAAAAAACCCCGTCTAT-3′ (1677–1696) and 5′-TAAAATCATCCTCCACGACAA-3′ (2192–2173; Genbank S40532) for NSCL2, 5′-CTACCCCTCACATCTCAACGC-3′ (376–397) and 5′-GACTGGGAAGTCTCTGGCGAT-3′ (1354–1335; Genbank X15049) for NCAM, 5′-TCCAGCTTTCCTACCGAGCA-3′ (70–89) and 5′-GATGAAACAGGGCCTGCG-TG-3′ (704–723; Genbank U6776) for NGN1, and 5′-CTCTGGTTCCTCCTCCCGAG-3′ (44–63) and 5′-GGCTTCCCATTTTGACGTGC-3′ (851–870; Genbank M95603) for Mash1. NSCL2 shows a notable homology within its bHLH motif to NSCL1 (Gobel et al., 1992). To avoid cross-hybridization with NSCL1, we selected a region of NSCL2 that was outside of this shared region as the template for RNA probe. No homology at the nucleotide level was found between the two probes by Blast analysis.

The PCR was carried out for 35 cycles. Each resulting fragment was cloned into HindIII/EcoRI sites of pT7/T3 α18 (Ambion, TX) and sequenced. DIG-UTP-labeled RNA probes were synthesized by use of an RNA transcription kit (Roche Diagnostics, Mannheim).

Sections were immersed in absolute ethanol for 5 min and then washed twice in PBS for 5 min each time. Next, the sections were treated with 2 μg/ml of proteinase K (Takara, Kyoto) at 37°C for 15–20 min, washed in PBS, and refixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min. After having been washed twice in PBS, the sections were air-dried and hybridized. Hybridization was performed at 47°C for 16 h with a digoxigenin (DIG)-labelled RNA probe in a hybridization solution containing 50% formamide, 0.3 M NaCl, 0.02 M Tris–HCl (pH 8.0), 1 mM EDTA, 10% dextran sulphate, 1 × Denhardt’s solution, 1 mg/ml yeast tRNA and 0.02% SDS. Hybridized sections were washed at 47°C in a solution containing 50% formamide and 2 × SSC for 1 h, and thereafter twice in 2 × SSC for 5 min each time. Then, they were treated with 20 μg/ml of RNase (Type II-A, Sigma Chemical Co., St Louis, MO) at 37°C for 30 min, and washed at 47°C in 50% formamide/2 × SSC followed by 50% formamide/1 × SSC for 1 h each. After having been washed three times in PBS, the sections were incubated with 1% blocking reagent (Boeringer Mannheim GmbH, Mannheim) in maleic acid buffer (pH 7.5) for 1 h at room temperature. Subsequently, they were incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG Fab fragments diluted 1:500 in PBS. After three washes in TBS, chromogenic reactions were carried out by using NBT/BCIP (Boeringer).

Northern blot analysis
Total RNA (5–10 μg) from the olfactory mucosa of E12, P1, P7 and P35 mice was separated on a 1% formaldehyde gel, blotted onto a nylon membrane in 2 × SSC for 5 h, and UV crosslinked. Prehybridization was performed at 68°C in hybridization buffer (DIG Easy Hyb, Roche) for 3 h. For the hybridization, 5–15 ng DIG-labeled cRNA probe was added per milliliter of hybridization buffer, and the membrane was incubated overnight at 68°C. For the control, actin DIG-labelled RNA probe (Roche) was used. Blots were washed in 2 × SSC/0.1% SDS for 5 min at RT, and then washed three times in 0.3 × SSC/0.1% SDS for 20 min at 68°C. The membranes were blocked in 1% blocking reagent, then incubated for 30 min with anti-DIG antibody diluted 1:10 000 in maleic buffer, and washed three times in maleic buffer containing 0.3% Tween 20 for 20 min each time. The signal was visualized by chemiluminescence according to the recommendation of the manufacturer (Roche).

Results

In situ hybridization
The specific signals of NSCL1 and NSCL2 were detected in the E12–15 mouse head. NSCL1 was expressed in the OE, the VNO and the posterior region of the telencephalon (Figure 1A). NSCL2 was expressed in the OE, and the anterior region of the telencephalon (Figure 1B), and in the neuro-epithelium of the developing retina (Figure 1D). Both probes stained also cranial ganglia and dorsal root ganglia (data not shown).
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In the OE, NSCL1 and NSCL2 showed nearly similar expression pattern. At E10, NSCL1 was expressed in the entire olfactory placodes (Figure 2A). Also, NSCL2 was expressed throughout them (Figure 2B). NSCL1 and NSCL2-expressing cell clusters were observed between the olfactory placode and telencephalon (Figure 2A,B). At E12–15, the layer between the basal and middle ones, presumably the ORN layer, was reactive with both probes (Figure 2C,D). The NSCL1-expressing cells were observed also in the lamina propria. The VNO appeared as a tubular structure at E12. A thick sensory epithelium (S-VNO) and a thinner non-sensory epithelium (NS-VNO) could be distinguished. At E15, NSCL1 and NSCL2 were expressed throughout the VNO epithelium except in the basal layer of the S-VNO. In the NS-VNO, a few NSCL1 and NSCL2-expressing cells were observed (Figure 2E,F). After birth, in the OE, globose basal cells (GBCs) and horizontal basal cells (HBCs) differentiated in the basal region. In the VNO, the NS-VNO was replaced by the respiratory epithelium. At P1, in situ signals of NSCL2 were very weak and became undetectable as development proceeded further. The expression of NSCL1 was detected at P3–7; the ORN layer was labeled by the NSCL1 probe (Figure 3A), as were receptor cells in the sensory epithelium of the VNO (Figure 3D). Moreover, in the VNO the expression of NSCL1 was absent in the boundary region between the sensory and the respiratory epithelia (Figure 3D). This expression pattern was similar to that of NCAM (Figure 3B,E), a marker of mature and immature ORNs and receptor cells of the VNO. We used NGN1 and Mash1 as markers of progenitors and GBCs. NGN1-expressing cells were observed in the cell layer above the basal lamina in the OE (Figure 3C), and in the cells at the boundary region between the sensory and the respiratory epithelia in the VNO (Figure 3F). The localization of Mash1-expressing cells was similar to that of NGN 1 (not shown). The signals of NSCL1 mRNA also became weak as the mice continued to grow. Sense controls displayed no reactivity (not shown).

Northern blot analysis

To examine whether NSCL1 and NSCL2 were expressed in the developing and adult OE, we performed Northern analysis. Although high-level expressions of NSCL1 and NSCL2 were observed at E12, these intense expressions were reduced in the postnatal days. The signal of NSCL1 could be observed until P7, but in the P35 OE, very low-intensity signals were detected. The NSCL2 signal was very low in both the P7 and P35 OE (Figure 3).

Discussion

Based on the similarities between NSCL1 and NSCL2 in protein structure, cDNA homology and genomic organiza-
tion, the expression pattern of these genes would be predicted to be similar. This prediction was verified in the olfactory system; but in the developing retina and brain, expression of \textit{NSCL1} and \textit{NSCL2} showed some differences. In the developing retina, \textit{NSCL1} was not expressed, but \textit{NSCL2} was expressed in the inner portion of neuroepithelium adjacent to the vitreous. In the developing chick retina, expression of \textit{cNSCL1} changes during stages: \textit{cNSCL1} is expressed first in developing ganglion cells and then in glial cells. Moreover, between these stages, no cells in the retina expressed \textit{cNSCL1} (Li \textit{et al.}, 1999b). Thus, it is likely that both \textit{NSCL1} and \textit{NSCL2} are expressed in the mouse retina at later stages.

The present study indicates that \textit{NSCL1} and \textit{NSCL2} are exclusively expressed in the ORNs and VNO receptor cell layer, which consists of immature and mature neurons. The area is devoid of progenitors (NGN1- or \textit{Mash1}-expressing). During the embryonic stage, the NS-VNO contains neurons that disappear after birth with the formation of the respiratory epithelium (Tarozzo \textit{et al.}, 1998). \textit{NSCL1}- and \textit{NSCL2}-expressing cells in the NS-VNO may be these neurons. Moreover, the cell clusters expressing \textit{NSCL1} and \textit{NSCL2} in the lamina propria during embryonic stages might be cells migrating from the OE toward the brain, which contain LHRH neurons (cf. Farbman, 1992).

Our previous study showed that \textit{Hes6} and \textit{NeuroD} were expressed mainly in GBCs and in precursor cells located at the border between the S- and NS-VNO (Suzuki \textit{et al.}, 2003). GBCs or precursor cells in the VNO proliferate and differentiate into immature ORNs or receptor cells of the

**Figure 2** \textit{In situ} hybridization with RNA probes for \textit{NSCL1} (A, C, E) and \textit{NSCL2} (B, D, F) of coronal sections of the olfactory placode (A, B), the OE (C, D) and the VNO (E, F). (A) At E10, NSCL1 is expressed in the olfactory placode (OP). (B) At E10, NSCL2 is expressed in the olfactory placode (OP). The arrows in (A) and (B) indicate cells that are migrating from the OP toward the telencephalon (T). (C) At E15, NSCL1 is expressed in the middle layer of the OE. (D) NSCL2 is expressed in the middle layer of the OE at E15. (E) At E15, NSCL1 expression is seen throughout the S-VNO and NS-VNO. (F) NSCL2 expression is seen in both the NS- and S-VNO. E15. Bars 20 µm.
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During neurogenesis, bHLH genes are sequentially expressed as a result of an activation cascade in which the early genes activate the expression of the late genes. The spatial pattern of expression of NSCL1 and NSCL2 in the VNO and OE suggests that expression of these genes follows that of Hes6 and NeuroD. NSCLs may be activated downstream of Hes6 or NeuroD in the ORN and VNO receptor cell lineage. Because Mash1 and NGN1 are determination genes (Cau et al., 1997, 2002), the Mash1 (NGN1) \( \rightarrow \) NeuroD (Hes6) \( \rightarrow \) NSCL cascade may apply to neurogenesis in the OE and VNO in the mouse. This sequence was confirmed in cranial sensory ganglia where sequential expression of Neurogenin1, NeuroD and NSCL1 was observed in the trigeminal ganglia and otic placode. Also, the expression of a cascade of NGN2, NeuroD and NSCL1 was seen in the nodose, petrosal and geniculate ganglia (Ma et al., 1998).

The function of NSCL1 and NSCL2 in the OE and VNO is not clear, but their spatial pattern of expression suggests that these NSCLs may contribute to maturation and maintenance of differentiated ORNs and receptor cells of the VNO. In fact, the importance of regulated expression of NSCL1 is suggested. Misexpression of cNSCL1 in chick embryos resulted in severe developmental retardations: abnormal brain development (Li et al., 1999a), small eyes with reduced cell proliferation activity, and massive cell death in the neuroepithelium (Li et al., 1999b). Moreover, the temporal pattern of expression showed that NSCL2 plays its role predominantly during early stages of development, whereas NSCL1 may function much longer, even in the adult OE, though its level of expression is low by that time.

Figure 3  In situ hybridization with RNA probes for NSCL1 (A, D), NCAM (B, E) and NGN1 (C, F) of coronal sections of the OE (A–C), and the VNO (D–F). (A) At P3, NSCL1 is expressed in the middle layer. (B) NCAM, a marker of ORNs is expressed in the middle layer (between arrows). P3. (C) NGN1, a marker of progenitor, GBCs is expressed in the cells just above the basal lamina (bl). P3. (D) NSCL1 expression is seen in the basal to middle layer of the s-VNO. The edges of the sensory epithelium are devoid of NSCL1-expressing cells (arrow). P3. (E) NCAM, a marker of receptor cells of the VNO is expressed in the sensory epithelium (SE), not in the respiratory epithelium (RE). The edges of the sensory epithelium are devoid of NCAM-expressing cells (arrow). P3. (F) NGN1 expression (arrows) is seen in the sensory epithelium (SE) near the boundary of the respiratory epithelium (RE). P3. bl basal lamina. Bars 20 \( \mu \)m.

Figure 4  Northern blot analysis of NSCL1 and NSCL2 in the OE obtained from E12, P1, P7, and P35 mice. Total RNA (5 \( \mu \)g for NSCL1, 10 \( \mu \)g for NSCL2 and 5 \( \mu \)g for actin) is loaded onto the gel. Transcripts for NSCL1 (2458 bp in size) are detected in the OEs at E12, P1, P7, and P35, whereas those for NSCL2 (2230 bp in size) are found at E12 and P1, but are at a low level at P7 and P35. Actin controls demonstrate equal loading of the lanes containing total RNA.
References


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