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# 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 embryo murine 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* (*NGN*)*1* 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 cryoprotected 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′-A ATGATGCTCAACTCCGATACCA-3′ (450–471) and 5′-TCCTTCAACCTCTGCCGCTA-3′ (1253–1234; Genbank M82874) for *NSCL1*, 5′-TCCAAAAACACC-CCGTCTAT-3′ (1677–1696) and 5′-TAAAATCATCCCA-CGCACAA-3′ (2192–2173; Genbank S40532) for *NSCL2*, 5′-CTACCCTCACCATCTACAACGC-3′ (376–397) and 5′-GACTGGGAGTCCTGGCCGAT-3′ (1354–1335; Genbank X15049) for *NCAM*, 5′-TCCAGCTTCCTCACCG-ACGA-3′ (70–89) and 5′-GATGAAACAGGGCGTCG-TGT-3′ (704–723; Genbank U6776) for *NGN1*, and 5′-TCTCGTTCTCCCCCGCGACA-3′ (44–63) and 5′-GCCT-CCCCATTTTGACGTCG-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 *Hin*dIII/*Eco*RI 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 in 0.2 N HCl for 20 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 for each. After having been washed three times in PBS, the sections were incubated with 1% blocking reagent (Boeringer Manheim GmbH, Manheim) 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 neuroepithelium of the developing retina (Figure 1D). Both probes stained also cranial ganglia and dorsal root ganglia (data not shown).

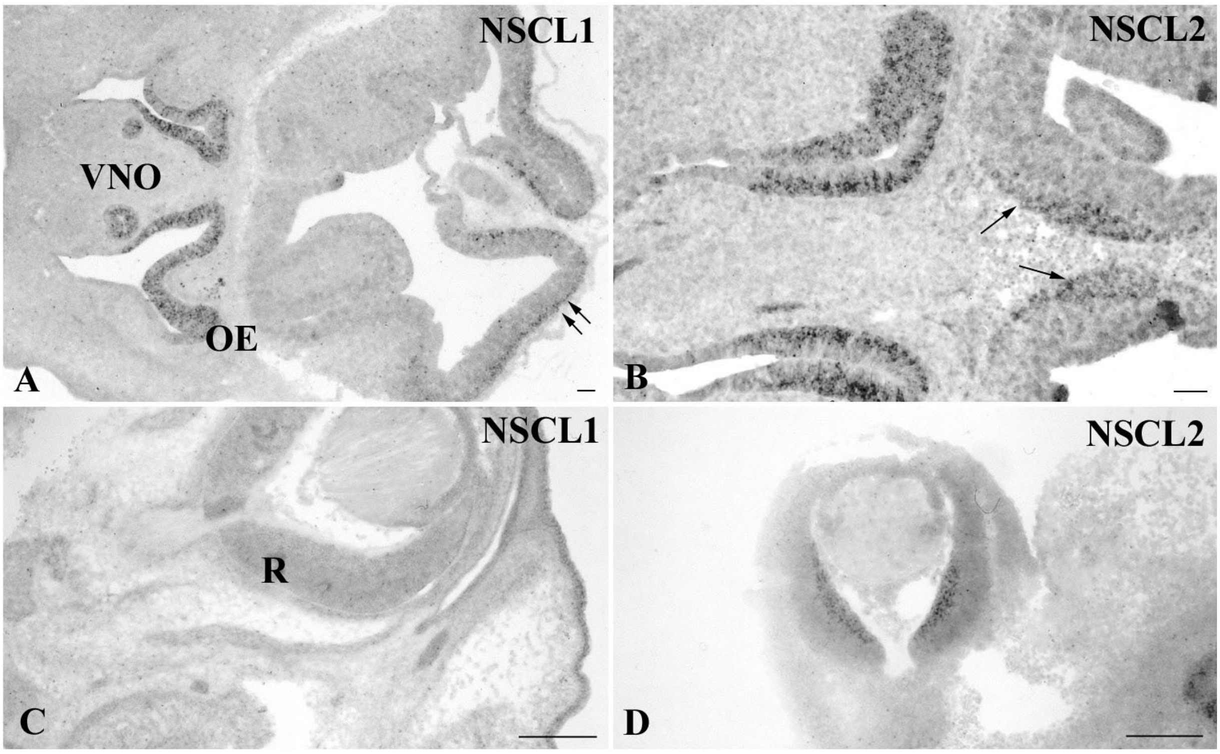


**Figure 1** *In situ* hybridization with RNA probes for NSCL1 **(A, C)** and NSCL2 **(B, D)**, showing distinct expressions. (A) At E12, NSCL1 is expressed in the olfactory epithelium (OE), the vomeronasal organ (VNO), and in the posterior part of the telencephalon (arrows). (B) At E12, NSCL2 is expressed in the OE and in the anterior part of the telencephalon (arrows). (C) No signal for NSCL1 is seen in the neuroepithelium of the retina (R). E15. (D) NSCL2 is expressed in the inner portions of the developing retina, adjacent to the vitreous. E15. Bars 60 µm.

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-

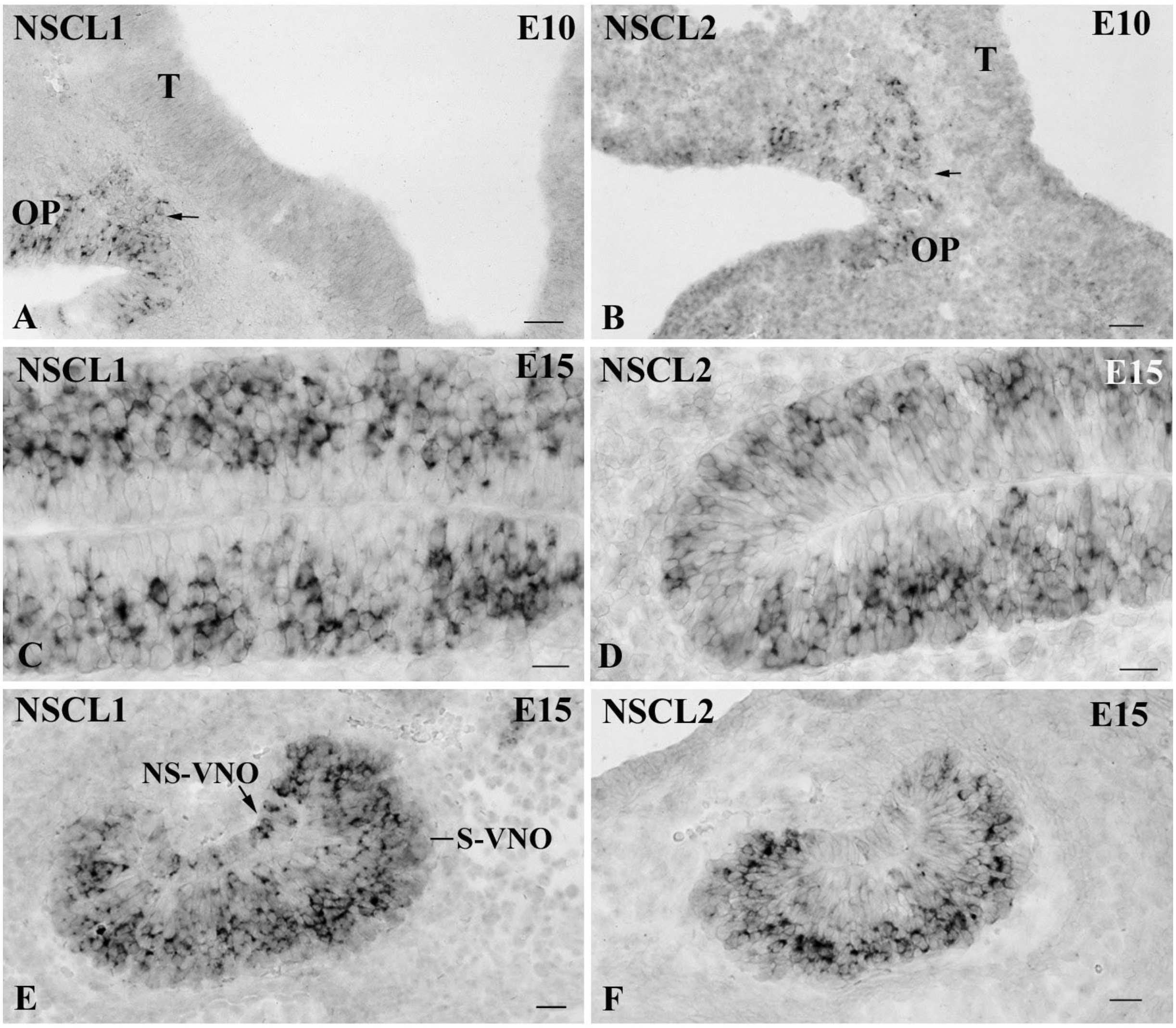


**Figure 2** *In situ* hybridization with RNA probes for NSCL1 **(A, C, E)** and NSCL2 **(B, D, F)** of coronal sections of the olfactory placode (A, B), the OE (C, D) and the VNO (E, F). (A) At E10, NSCL1 is expressed in the olfactory placode (OP). (B) At E10, NSCL2 is expressed in the olfactory placode (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.

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 *NSCL1* and *NSCL2* showed some differences. In the developing retina, *NSCL1* was not expressed, but *NSCL2* was expressed in the inner portion of neuroepithelium adjacent to the vitreous. In the developing chick retina, expression of *cNSCL1* changes during stages: *cNSCL1* is expressed first in developing ganglion cells and then in glial cells. Moreover, between these stages, no cells in the retina expressed *cNSCL1* (Li *et al.*, 1999b). Thus, it is likely that both *NSCL1* and *NSCL2* are expressed in the mouse retina at later stages.

The present study indicates that *NSCL1* and *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 *Mash1*-expressing). During the embryonic stage, the NS-VNO contains neurons that disappear after birth with the formation of the respiratory epithelium (Tarozzo *et al.*, 1998). *NSCL1*- and *NSCL2*-expressing cells in the NS-VNO may be these neurons. Moreover, the cell clusters expressing *NSCL1* and *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 *Hes6* and *NeuroD* were expressed mainly in GBCs and in precursor cells located at the border between the S- and NS-VNO (Suzuki *et al.*, 2003). GBCs or precursor cells in the VNO proliferate and differentiate into immature ORNs or receptor cells of the

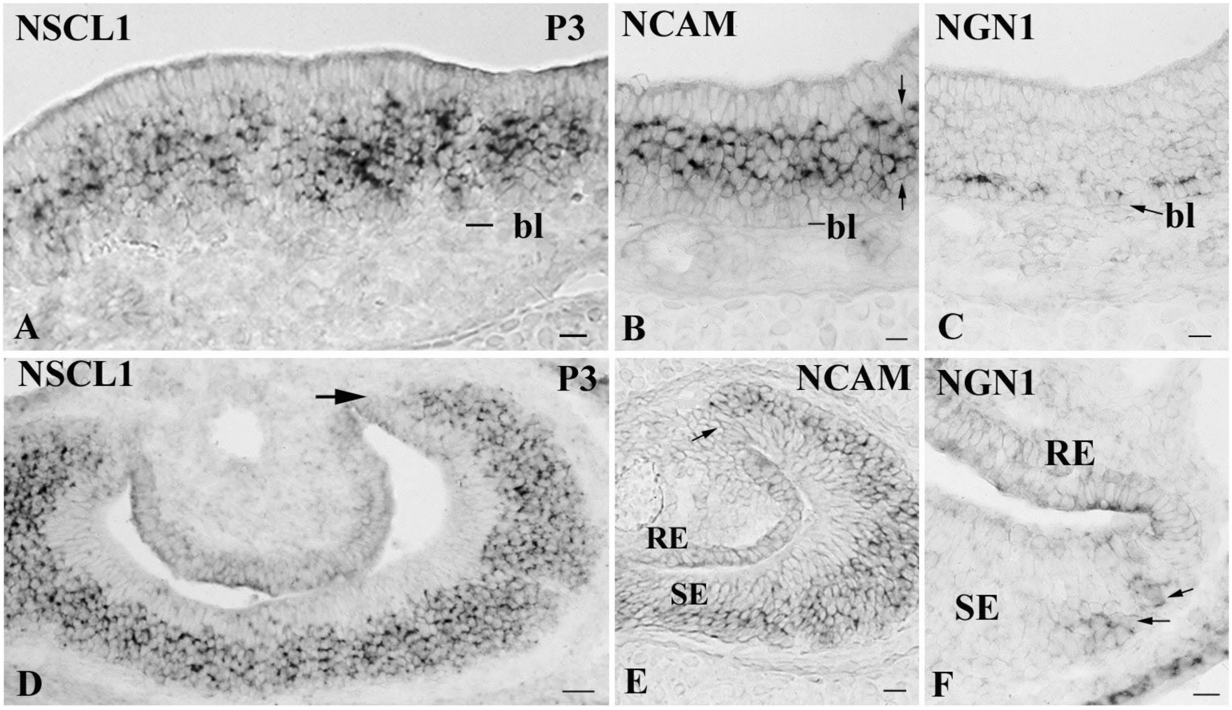


**Figure 3** *In situ* hybridization with RNA probes for NSCL1 **(A, D)**, NCAM **(B, E)** and NGN1 **(C, F)** of coronal sections of the OE (A–C), and the VNO (D–F). (A) At P3, NSCL1is expressed in the middle layer. (B) NCAM, a marker of ORNs is expressed in the middle layer (between arrows). P3. (C) 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 μm.

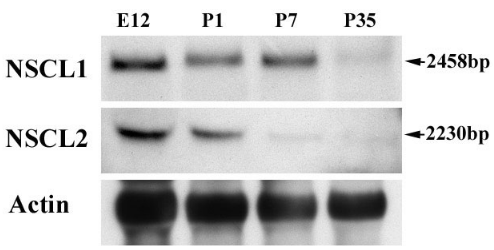


**Figure 4** Northern blot analysis of NSCL1 and NSCL2 in the OE obtained from E12, P1, P7, and P35 mice. Total RNA (5 μg for NSCL1,10 μg for NSCL2 and 5 μg for actin) is loaded onto the gel. Transcripts for NSCL1 (2458 bp in size) are detected in the OEs at E12, P1, P7, and P35, whereas those for NSCL2 (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.

VNO. 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 the 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*) → *NeuroD* (*Hes6*) → *NSCL* cascade may apply to neurogenesis in the OE and the 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 the VNO is not clear, but their spatial pattern of expression suggests that these *NSCL*s 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.

## References


**Bae**, **S.-K.**, **Bessho, Y., Hojo, M.** and **Kageyama, R.** (2000) *The bHLH gene* Hes6, *an inhibitor of* Hes1, *promotes neuronal differentiation*. Development, 127, 2933–2943.

**Begley**, **C.G.**, **Lipkowitz, S., Gobel, V., Mahon, K.A., Bertness, V., Green, A.R., Gough, N.M.** and **Kirch, I.R.** (1992) *Molecular characterization of* NSCL, *a gene encoding a helix–loop–helix protein expressed in the developing nervous system.* Proc. Natl Acad. Sci. USA, 89, 38–42.

**Brown**, **L.**, **Espinosa III, R., Le Beau, M.M., Siciliano, M.J.** and **Baer, R.** (1992) Hen1 *and* Hen2*: a subgroup of basic helix–loop–helix genes that are coexpressed in a human neuroblastoma.* Proc. Natl Acad. Sci. USA, 89, 8492–8496.

**Cau**, **E.**, **Gradwohl, G., Fode, C.** and **Guillemot, F.** (1997) Mash1 *activates a cascade of bHLH regulators in olfactory neuron progenitors.* Development, 124, 1611–1621.

**Cau**, **E.**, **Gradwohl, G., Casarosa, S., Kageyama, R.** and **Guillemot, F.** (2000) Hes *genes regulate sequential stages of neurogenesis in the olfactory epithelium.* Development, 127, 2323–2332.

**Cau**, **E.**, **Casarosa, S.** and **Guillemot, F.** (2002) Mash1 *and* Ngn1 *control distinct steps of determination and differentiation in the olfactory sensory neuron lineage.* Development, 129, 1871–1880.

**Farbman**, **A.I.** (1992) Cell Biology of Olfaction. Cambridge University Press, New York.

**Gobel**, **V.**, **Lipkowitz, S., Kozak, C.A.** and **Kirsch, I.R.** (1992) NSCL-2*: a basic domain helix–loop–helix gene expressed in early neurogenesis.* Cell Growth Differ., 3, 143–148.

**Good**, **D.J.**, **Porter, F.D., Mahon, K.A., Parlow, A.F., Westphal, H.** and **Kirsch I.R.** (1997) *Hypogonadism and obesity in mice with a targeted deletion of the* Nh1h2 *gene*. Nature Genet., 15, 397–401.

**Gordon**, **M.K.**, **Mumm, J.S., Davis, R.A., Holcomb, J.D.** and **Calof, A.L.** (1995) *Dynamics of* MASH1 *expression* in vitro *and* in vivo *suggest a non-stem cell site of* MASH1 *action in the olfactory receptor neuron lineage*. Mol. Cell. Neurosci., 6, 363–379.

**Haire**, **M.F.** and **Chiaramello, A.** (1996) *Transient expression of the basic helix–loop–helix protein NSCL-2 in the mouse cerebellum during postnatal development*. Mol. Brain Res., 36, 174–178.

**Li**, **C.-M.**, **Yan, R.-T.** and **Wang, S.-Z.** (1999a) *Misexpression of a bHLH gene*, cNSCL1, *results in abnormal brain development.* Dev. Dyn., 215, 238–247.

**Li**, **C.-M.**, **Yan, R.-T.** and **Wang, S.-Z.** (1999b) *Misexpression of* cNSCL1 *disrupts retinal development.* Mol. Cell. Neurosci., 14, 17–27.

**Lipkowitz**, **S.**, **Gobel, V., Varterasian, M.L., Nakahara, K., Tchorz, K.** and **Kirsch, I.R.** (1992) *Comparative structural characterization of the human* NSCL-1 *and* NSCL-2 *genes.* J. Biol. Chem., 267, 21065–21071.

**Ma**, **Q.**, **Chen, Z., Barrantes, I., Pompa, J.L.** and **Anderson, D.J.** (1998) *Neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia*. Neuron, 20, 469–482.

**Nibu**, **K.**, **Li, G., Zhang, X., Rawson, N.E., Restrepo, D., Kaga, K., Lowry, L.D., Keane, W.** and **Rothstein, J. L.** (1999) *Olfactory neuron-specific expression of* NeuroD *in mouse and human nasal mucosa.* Cell Tissue Res., 298, 405–414.

**Suzuki**, **Y.**, **Mizoguchi, I., Nishiyama, H., Takeda, M.** and **Obara, N.** (2003) *Expression of* Hes6 *and* NeuroD *in the olfactory epithelium, vomeronasal organ and non-sensory patches*. Chem. Senses 28, 197–205.

**Tarozzo**, **G.**, **Cappello, P., De Andrea, M., Walters, E., Margolis, F.L., Oestreicher, B.** and **Fasolo, A.** (1998) *Prenatal differentiation of mouse vomeronasal neurons*. Eur. J. Neurosci., 10, 392–396.


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