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Notch1 Signaling Influences V2 Interneuron and Motor Neuron Development in the Spinal Cord

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Abstract
The Notch signaling pathway plays a variety of roles in cell fate decisions during development. Previous studies have shown that reduced Notch signaling results in premature differentiation of neural progenitor cells, while increased Notch activities promote apoptotic death of neural progenitor cells in the developing brain. Whether Notch signaling is involved in the specification of neuronal subtypes is unclear. Here we examine the role of Notch1 in the development of neuronal subtypes in the spinal cord using conditional knockout (cKO) mice lacking *Notch1* specifically in neural progenitor cells. Notch1 inactivation results in accelerated neuronal differentiation in the ventral spinal cord and gradual disappearance of the ventral central canal. These changes are accompanied by reduced expression of *Hes1* and *Hes5* and increased expression of *Mash1* and *Neurogenin 1* and *2.* Using markers (Nkx2.2, Nkx6.1, Olig2, Pax6 and Dbx1) for one or multiple progenitor cell types, we found reductions of all subtypes of progenitor cells in the ventral spinal cord of *Notch1* cKO mice. Similarly, using markers (Islet1/2, Lim3, Sim1, Chox10, En1 and Evx1/2) specific for motor neurons and distinct classes of interneurons, we found increases in the number of V0–2 interneurons in the ventral spinal cord of *Notch1* cKO mice. Specifically, the number of Lim3+/Chox10+ V2 interneurons is markedly increased while the number of Lim3+/Islet+ motor neurons is decreased in the *Notch1* cKO spinal cord, suggesting that V2 interneurons are generated at the expense of motor neurons in the absence of Notch1. These results provide support for a role of Notch1 in neuronal subtype specification in the ventral spinal cord.

Introduction

The developing vertebrate nervous system contains a multitude of distinct neuronal and glial cell types, which are generated from the progenitor cells in the neuroepithelium in a highly coordinated spatial and temporal pattern [Jessell and Sanes, 2000]. Specification of these distinct cell types is controlled by a variety of signaling path-

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ways, including the evolutionarily conserved Notch signaling pathway [Artavanis-Tsakonas et al., 1999; Justice and Jan, 2002]. Upon ligand binding from neighboring cells, Notch receptors are proteolytically cleaved by an ADAM/TACE family metalloprotease [Brou et al., 2000; Mumm et al., 2000] followed by a presenilin-dependent cleavage to release the Notch intracellular domain [De Strooper et al., 1999; Schroeter et al., 1998; Song et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999]. The Notch intracellular domain then translocates into the nucleus and activates transcription of its downstream targets, the *Hes* genes (hairy/enhancer of split), primarily by forming a complex with CSL (CBF1/RBP-Jκ, Suppressor of hairless, Lag-1) [Greenwald, 1998; Kimble and Simpson, 1997; Mumm et al., 2000; Weinmaster, 1998].

During *Drosophila* neural development, Notch signaling plays an essential role in the cell fate decision between neuroblasts and epidermoblasts [Campos-Ortega and Jan, 1991; Simpson, 1997]. Loss of Notch function favors the differentiation of precursor cells into neuroblasts rather than epidermoblasts [Lehmann et al., 1983]. In vertebrate neural development, Notch receptors are primarily expressed in the uncommitted neural progenitor cells within the ventricular zone [Lindsell et al., 1996]. The cell fate decisions mediated by Notch signaling are complex, depending on the spatial and temporal context of the cell. Notch signaling can regulate neuronal differentiation, glial cell type specification, and apoptotic cell death.

The cardinal function of Notch signaling during vertebrate neural development is to maintain neural progenitor identity and to suppress neuronal differentiation [Schuurmans and Guillemot, 2002]. Targeted germ line disruption of *Notch1* or *RBP-Jκ* in mice results in upregulation of proneuronal transcription factors and depletion of neural progenitor cells [Conlon et al., 1995; de la Pompa et al., 1997; Hitoshi et al., 2002; Oka et al., 1995; Swiatek et al., 1994]. Attenuation of Notch signaling in *Presenilin-1 (PS1)* null as well as *Notch1* and *PS1* conditional mutant mice leads to premature neuronal differentiation and subsequently reduced neural progenitor and neuronal populations in the developing brain [Handler et al., 2000; Shen et al., 1997; Wines-Samuelson et al., 2005; Yang et al., 2004]. Furthermore, gain-of-function studies have shown that Notch activation prevents neuronal differentiation [Chambers et al., 2001; Dorsky et al., 1995; Henrique et al., 1997; Hitoshi et al., 2002; Nye et al., 1994; Scheer et al., 2001]. By controlling the timing of neuronal differentiation, Notch signaling may also contribute to the diversification of neuronal populations [Artavanis-Tsakonas et al., 1999].

More recently, Notch signaling has been shown to promote the generation of various glial cell types in a context-dependent manner [Chambers et al., 2001; Gaiano and Fishell, 2002; Lundkvist and Lendahl, 2001; Morrison, 2001; Wang and Barres, 2000]. Notch activation promotes the generation of astroglia in both the peripheral nervous system [Morrison et al., 2000] and the central nervous system (CNS) [Tanigaki et al., 2001]. Notch activation similarly promotes the generation of Müller glia in the retina [Furukawa, 2000; Hojo et al., 2000; Scheer et al., 2001]. In the developing mouse telencephalon, Notch activation has been shown to promote the radial glial lineage [Gaiano et al., 2000], which shares many characteristics with neural progenitor cells [Hartfuss et al., 2001; Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001]. Compound inactivation of the Notch target genes, *Hes1*, *3* and *5,* leads to premature differentiation of radial glia into neurons [Hatakeyama et al., 2004].

An additional role of Notch activation has been discovered to influence cell death in a highly cell type-specific manner. It promotes cell death in the *Drosophila* retina [Cagan and Ready, 1989; Miller and Cagan, 1998; Yu et al., 2002], wing imaginal disk [Milan et al., 2002] and sensory organ [Orgogozo et al., 2002]. In the zebrafish retina, Notch activation plays a similar proapoptotic role [Scheer et al., 2001]. In higher vertebrates, we have recently demonstrated that Notch activation promotes apoptotic cell death in neural progenitor cells of the developing brain through a p53-dependent pathway [Yang et al., 2004]. These observations suggest that during mammalian neural development Notch signaling may play a role in the regulation of apoptotic cell death, contributing to the control of the size and shape of the nervous system [Kuan et al., 2000].

In addition to the role of Notch receptors in the maintenance of neural progenitor population, which may contribute indirectly to the diversification of neuronal populations, they may participate directly in the specification of distinct neuronal subtypes. Studies of Neurogenin1 (Ngn1) and Mash1, expression of which are regulated by Notch target genes, have shown that these basic helix-loop-helix (bHLH) transcription factors are involved in neuronal subtype specification [Akazawa et al., 1995; Gowan et al., 2001; Gradwohl et al., 1996; Guillemot, 1999; Lo et al., 1991; Ma et al., 1996]. Misexpression of *Ngn1* in the chick neural crest directs the cells preferentially towards the sensory neuron fate [Perez et al., 1999]. In contrast, Mash1 is required for the generation of nor-

adrenergic neurons in the sympathetic nervous system and the locus coeruleus [Hirsch et al., 1998; Lo et al., 1998].

To address whether Notch1 plays a role in neuronal subtype specification, we employed a genetic approach to study the effect of Notch1 inactivation in the development of neuronal subtypes in the spinal cord. The spinal cord represents an ideal experimental system for the study of neuronal subtype specification, as it is a well-characterized region of the CNS. Distinct neuronal subtypes emerge in a precise spatial and temporal order from progenitor cells and are topologically positioned along the dorsoventral axis of the neural tube [Briscoe and Ericson, 2001; Jessell, 2000; Lee and Jessell, 1999; Shirasaki and Pfaff, 2002]. The properties of the progenitor cells are defined by gradients of diffusible, inductive signals. For example, in the dorsal spinal cord, bone morphogenetic proteins, which are secreted from the surface ectoderm and the roof plate, control the specification of dorsal cell types such as neural crest cells and dorsal sensory interneurons [Lee and Jessell, 1999]. In the ventral spinal cord, Sonic Hedgehog (Shh) is secreted from the notochord and the floor plate to specify motor neurons and certain classes of ventral interneurons in a concentration-dependent manner [Briscoe and Ericson, 2001; Jessell, 2000].

Within the spinal cord, two different Notch ligands, Delta and Serrate/Jagged, are expressed in complementary subdomains of the ventricular zone [Lindsell et al., 1995; Myat et al., 1996]. Several Notch downstream bHLH proteins are also expressed within discrete domains along the dorsoventral axis of the ventral spinal cord [Gowan et al., 2001; Hatakeyama et al., 2004; Ma et al., 1997; Parras et al., 2002; Scardigli et al., 2001]. Here we show that Notch family members are highly expressed in the ventral spinal cord during early neural development, and inactivation of Notch1 results in a gradual disappearance of the ventral central canal, concomitant with reduced progenitor populations. We further show that V2 interneurons are overproduced at the expense of motor neurons in the absence of Notch1.

Materials and Methods

Generation of Notch1 Conditional Knockout Mice

A modified *Notch1* allele was generated as described in previous reports [Pan et al., 2004; Yang et al., 2004]. Briefly, the targeting vector containing a floxed *PGK-neo* selection cassette and a *loxP* site in the upstream and downstream of the first coding exon was transfected into RW-4 ES cells [Simpson et al., 1997]. The floxed *Notch1* allele *(fN1)* was then generated by transient transfection of a cDNA encoding Cre recombinase. Removal of the floxed *PGK-neo* cassette in ES cells was confirmed by PCR and Southern analyses. ES cells carrying the *fN1* allele were injected into mouse blastocysts to generate chimeric mice, which were then used to generate homozygous *fN1* mice. The *fN1* mouse was then crossed with *Nes-Cre* transgenic mice to generate *Notch1* conditional knockout (*N1* cKO) mice. More than three mice per genotype at each age were used for each experiment.

Preparation of Embryonic Sections

For spinal cord sections, embryos were fixed in 4% paraformaldehyde at 4°C for 40 min (E10.5, 34-somite stage), 1.5 h (E11.5) or 2 h (E12.5 and 13.5), cryoprotected, embedded in OCT and then serially sectioned at 10 μm.

Immunostaining and in situ Hybridization

For immunostaining, sections were blocked with a solution containing 1% BSA, 3% goat serum and 0.1% Triton X-100 for 1 h at room temperature, and incubated with the indicated primary antibodies overnight at 4°C. The following primary antisera or antibodies were used: anti-Chox10 rabbit or guinea pig antiserum (1:1,000, gifts from Drs. R. McInnes and S. Pfaff, respectively), anti-β-tubulin III antibody (TuJ1, 1:500, Covance), anti-Islet1/2 mouse monoclonal antibody (clone 4D5 or clone 2D6, 1:50, Developmental Studies Hybridoma Bank), anti-Nkx2.2 mouse monoclonal antibody (1:20, Developmental Studies Hybridoma Bank), anti-Pax6 mouse monoclonal antibody (1:20, Developmental Studies Hybridoma Bank), anti-Engrailed-1 (En1) mouse monoclonal antibody (1:20, Developmental Studies Hybridoma Bank), anti-Lim3 (Lhx3) mouse monoclonal antibody (1:20, Developmental Studies Hybridoma Bank), anti-Lim3 rabbit polyclonal antibody (1:1,000, Abcam), and anti-Evx1/2 mouse monoclonal antibody (1:20, gift from Dr. T. Jessell). Sections were then washed in PBS and incubated with appropriate Alexa Fluor (488, 568 or 598)-conjugated secondary antibodies (1:300, Molecular Probes) for 1 h at room temperature. In situ hybridization on cryosections was performed as described in Handler et al. [2000].

Quantification of Cell Numbers

Three sections from each embryo were collected 200 μm apart at the forelimb level of the spinal cord, and were stained with appropriate antibodies. For quantification of double-labeled cells at E11.5, an additional 6–8 sections from each embryo were collected 50 μm apart at the forelimb level, and stained with appropriate antibodies. Images were collected on a Zeiss confocal laser scanning microscope and cells were counted on Adobe Photoshop or Scion images. Statistical significance was determined by Student's t test.

Results

Notch Receptors Are Highly Expressed in the Ventral Spinal Cord

It was shown previously that Notch receptors (Notch1–3) are highly expressed in the developing rat CNS during mid-gestation [Lindsell et al., 1996]. Here we used in situ hybridization analysis to determine the expression pat-

tern of *Notch1–3* in the mouse spinal cord. At E11.5, *Notch1–3* transcripts are expressed along the dorsoventral axis in the ventricular zone of the spinal cord, and their expression is higher in the ventral spinal cord (fig. 1A–C). *Notch2* expression is particularly higher in the area surrounding the floor plate (fig. 1B). Notch proteins are also enriched in the floor plate, which is the source of Shh signals [Briscoe and Ericson, 2001; Jessell, 2000; McMahon et al., 2003]. Previous studies have shown that Notch signaling regulates a binary decision within the Xenopus organizer, favoring the floor plate fate at the expense of the notochord [Lopez et al., 2005], and that in the floor plate Notch promotes the expression of *Shh* [Lopez et al., 2003; Paganelli et al., 2001]. These studies suggest that specific Notch receptors may be required for the acquisition of neuronal cell fates within the ventral spinal cord.

To study the function of Notch1 signaling in the spinal cord, we generated an *N1* cKO mouse in neural progenitor cells and neural progenitor cell-derived cells by crossing an *fN1* mouse with *Nestin-Cre* transgenic mice, in which the expression of Cre is under the control of the neural progenitor cell-specific enhancer of the *Nestin* promoter [Tronche et al., 1999; Yang et al., 2004]. Our previous immunohistochemical analysis of *N1* cKO mice using an antibody specific for the intracellular domain of Notch1 revealed the absence of Notch immunoreactivity in the neuroepithelium of *N1* cKO brains at E10.0 [Yang et al., 2004]. To confirm the expression of Cre in the spinal cord, we performed in situ hybridization analysis on sections of *Nestin-Cre* transgenic mice and found that *Cre* transcripts are preferentially expressed in the ventral spinal cord during early neurogenesis, with the highest expression in the area surrounding the floor plate (fig. 1D).

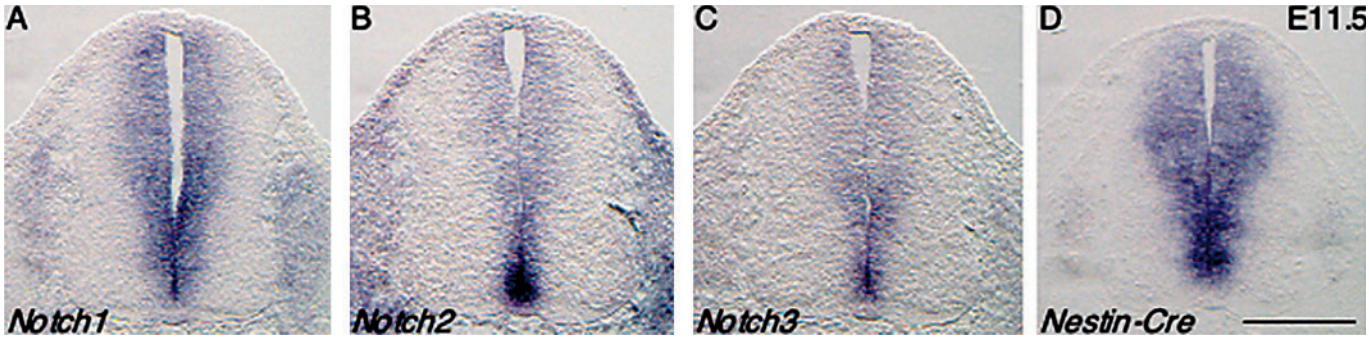
The N1 *cKO Spinal Cord Exhibits a Gradual Fusion of the Ventral Central Canal*

To assess the gross morphology and neuronal populations in the spinal cord of *N1* cKO mice, we collected sections from E10.5 to E15.5, which were stained with DAPI, a nuclear dye, and TuJ1 antibody, a marker for newly generated postmitotic neurons. The spinal cords of *N1* cKO and control mice appear similar at E10.5 (fig. 2A, B), although TuJ1 immunostaining revealed increased size of the neuronal population in the ventral-most region of the *N1* cKO spinal cord, above the floor plate (fig. 2B, arrow). By E11.5, the spinal cord of *N1* cKO mice is readily distinguishable from the control, as the ventral-most portion of the central canal is replaced with TuJ1+ postmitotic neuronal population, leading to a reduced ventricle (fig. 2C, D). On the other hand, the dorsal progenitor cells appear unaffected in the *N1* cKO spinal cord. By E12.5, the reduction of the central canal becomes even more pronounced, with complete disappearance of the ventral half of the central canal (fig. 2E, F). By E15.5, in contrast to the control spinal cord where a central canal extends to the ventral half, the remnant of the *N1* cKO central canal is a tiny opening localized in the dorsal half (data not shown). These results suggest that loss of Notch1 expression causes premature differentiation of progenitor cells in a ventral-to-dorsal direction, leading to the disappearance of the ventral half of the central canal.

Altered Expression of Notch Downstream Genes in the N1 *cKO Spinal Cord*

To determine the molecular consequence of Notch1 inactivation in the spinal cord, we examined the expression of Notch downstream target genes. *Hes1* and *Hes5* are bHLH transcription factors that act as immediate downstream effectors of Notch signaling [Ishibashi et al., 1995; Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999; Tomita et al., 1996]. Decreased expression of *Hes5,* but not of *Hes1,* was reported in *Notch1–/–* and *PS1–/–* mice [de la Pompa et al., 1997; Handler et al., 2000]. We focused our analysis on E10.5 and E11.5, between which time points significant morphological changes take place (fig. 2). In situ hybridization analysis showed that *Hes1* and *Hes5* are expressed in the ventricular zone of the spinal cord in complementary domains: *Hes5* is primarily expressed in two stripes in the ventral domain and the roof plate, while *Hes1* expression is sandwiched between the two *Hes5* stripes in the dorsal spinal cord (fig. 3A–H). Both *Hes1* and *Hes5* are expressed in the floor plate. Thus, *Hes1* and *Hes5* are expressed in distinct and overlapping expression patterns in the developing spinal cord, consistent with a recent report [Hatakeyama et al., 2004; Wu et al., 2003]. Comparison of control and *N1* cKO mice at E10.5 reveals similar *Hes1* expression (fig. 3A, B) but reduced *Hes5* expression in the ventral spinal cord (fig. 3E, F). By E11.5, expression of both transcripts is markedly downregulated throughout the ventricular zone in the *N1* cKO spinal cord (fig. 3C, D, G, H).

The Notch targets Hes1 and Hes5 act as transcription repressors for a subset of proneural bHLH transcription factors including Ngn1–3, Math1 and Mash1 [Akazawa et al., 1995; Gowan et al., 2001; Gradwohl et al., 1996; Guillemot, 1999; Kageyama and Nakanishi, 1997; Lo et al., 1991; Ma et al., 1996]. Within the spinal cord, Ngn1,



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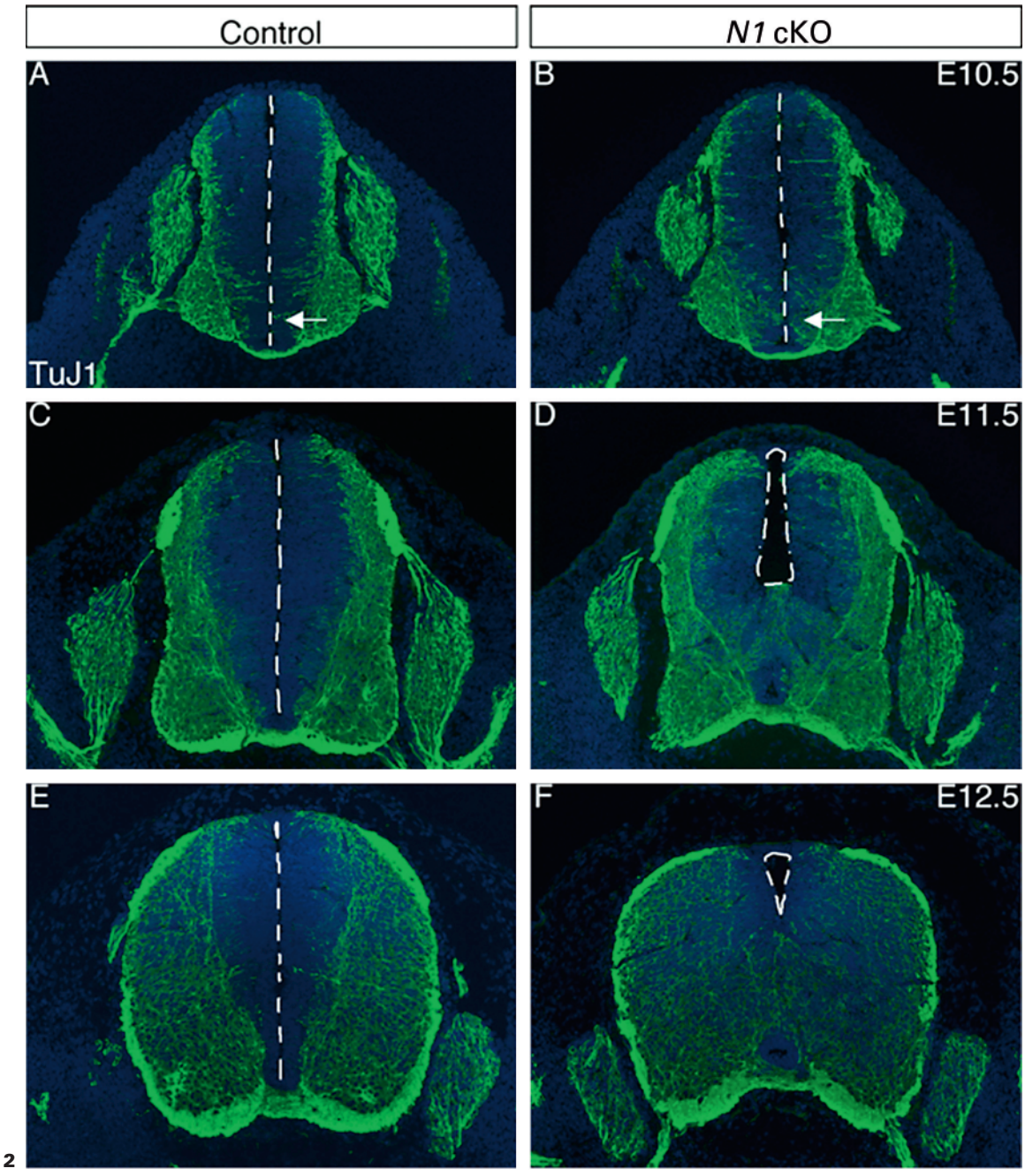

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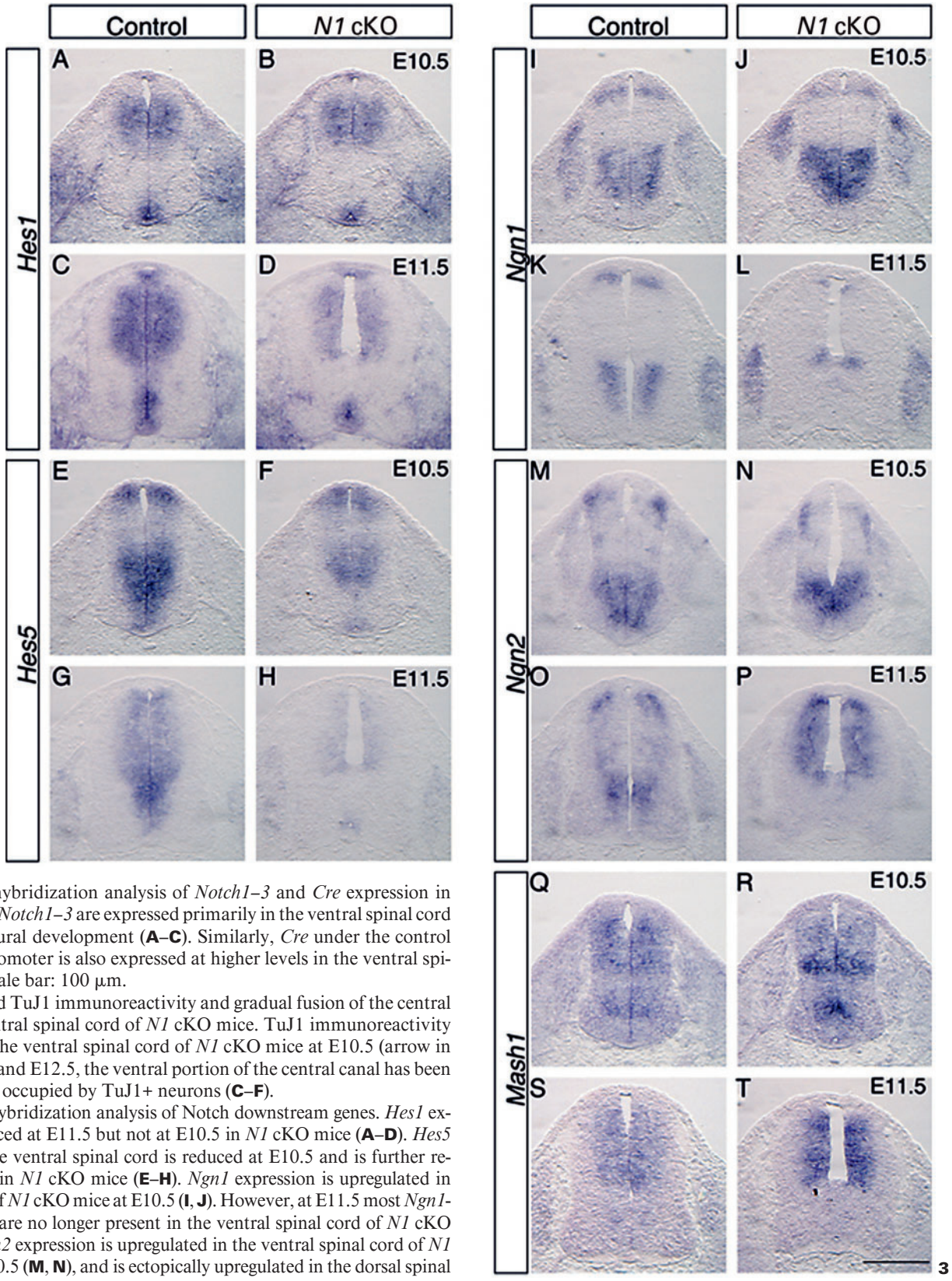


Fig. 1. In situ hybridization analysis of *Notch1–3* and *Cre* expression in the spinal cord. *Notch1–3* are expressed primarily in the ventral spinal cord during early neural development (**A**–**C**). Similarly, *Cre* under the control of the *Nestin* promoter is also expressed at higher levels in the ventral spinal cord (**D**). Scale bar: 100 μm.

Fig. 2. Increased TuJ1 immunoreactivity and gradual fusion of the central canal in the ventral spinal cord of *N1* cKO mice. TuJ1 immunoreactivity is increased in the ventral spinal cord of *N1* cKO mice at E10.5 (arrow in **A**, **B**). At E11.5 and E12.5, the ventral portion of the central canal has been occluded and is occupied by TuJ1+ neurons (**C**–**F**).

Fig. 3. In situ hybridization analysis of Notch downstream genes. *Hes1* expression is reduced at E11.5 but not at E10.5 in *N1* cKO mice (**A**–**D**). *Hes5* expression in the ventral spinal cord is reduced at E10.5 and is further reduced at E11.5 in *N1* cKO mice (**E**–**H**). *Ngn1* expression is upregulated in the spinal cord of *N1* cKO mice at E10.5 (**I**, **J**). However, at E11.5 most *Ngn1*-expressing cells are no longer present in the ventral spinal cord of *N1* cKO mice (**K**, **L**). *Ngn2* expression is upregulated in the ventral spinal cord of *N1* cKO mice at E10.5 (**M**, **N**), and is ectopically upregulated in the dorsal spinal cord of *N1* cKO mice at E11.5 (**O**, **P**). *Mash1* expressing is upregulated in *N1* cKO mice at E10.5 (**Q**, **R**) and E11.5 (**S**, **T**). Scale bar: 100 μm.

Ngn2 and Mash1 have been shown to play a role in the specification of neuronal subtype identity [Gowan et al., 2001; Parras et al., 2002; Scardigli et al., 2001]. We therefore examined the expression of these genes in the spinal cord of *N1* cKO mice. In situ hybridization analysis revealed that expression of *Ngn1, Ngn2* and *Mash1* is upregulated in the *N1* cKO spinal cord at E10.5 (fig. 3I, J, M, N, Q, R). By E11.5, *Ngn1* expression in the ventral spinal cord is largely eliminated, coinciding with the disappearance of neural progenitor cells from the ventricular zone of the ventral spinal cord (fig. 3K, L), while *Ngn2* expression is ectopically upregulated in the remaining dorsal ventricular zone (fig. 3O, P). *Mash1* expression is markedly upregulated in the ventricular zone (fig. 3S, T). These results show that inactivation of Notch1 results in downregulation of its immediate downstream targets Hes1 and Hes 5 and upregulation of bHLH transcription factors in the spinal cord.

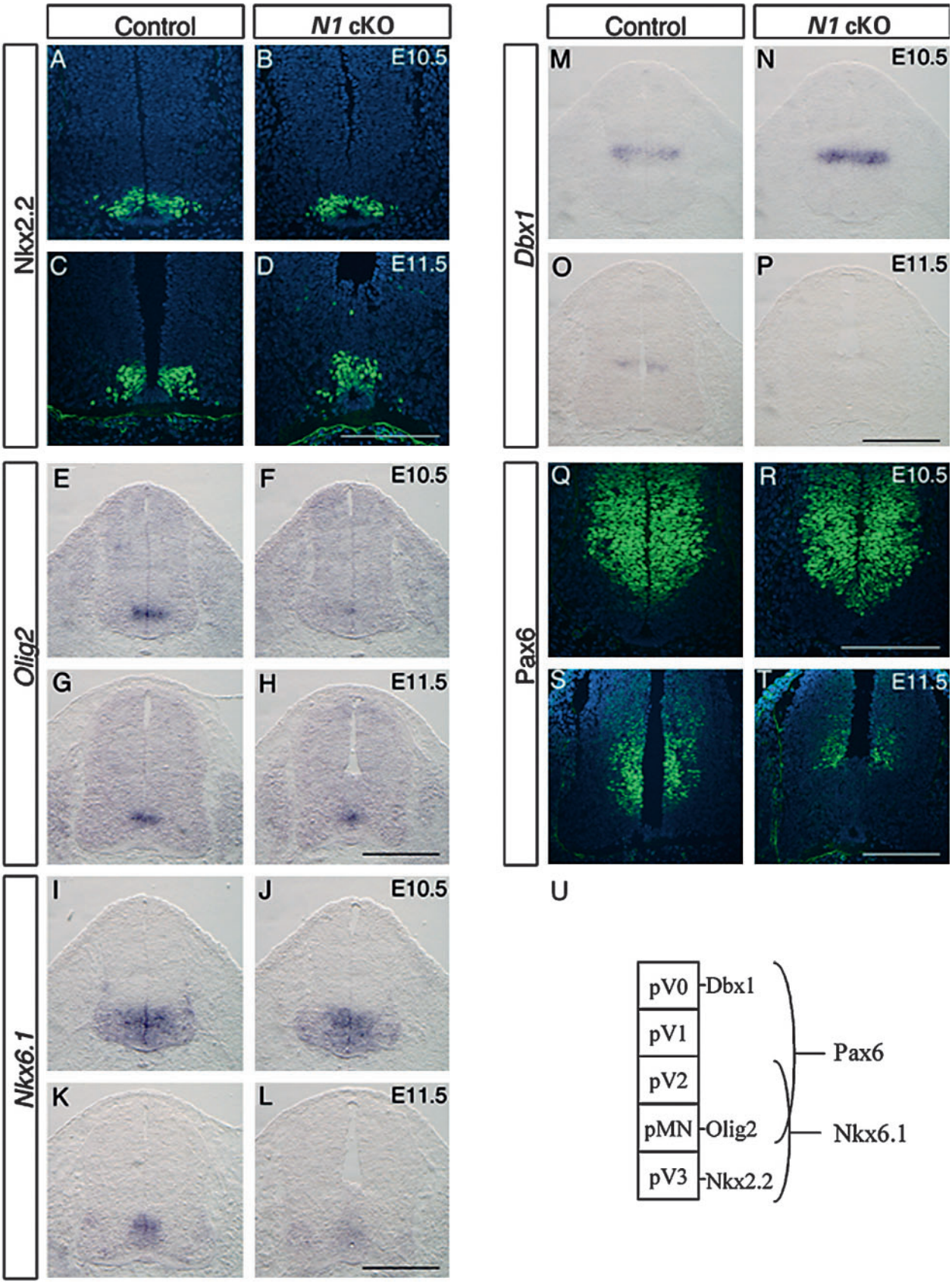
Reduction of All Subtypes of Neural Progenitors in the N1 *cKO Ventral Spinal Cord*

In the ventral spinal cord, Shh secreted from the notochord and the floor plate establishes the patterned expression of homeodomain and bHLH transcription factors. As a result, neural progenitor cells at different dorsoventral positions acquire distinct positional identities [Briscoe et al., 2000; Novitch et al., 2001]. The ventral half of the neural tube is occupied by five progenitor populations that consist of, from ventral to dorsal, pV3, pMN, pV2, pV1 and pV0 (p: progenitor; V: ventral; MN: motor neuron) in the medial ventricular zone (fig. 4U) [Briscoe and Ericson, 2001; Jessell, 2000]. These transcription factors fall into two classes, class I and class II proteins, based on their regulation by Shh [Briscoe et al., 2000]. The class I proteins are constitutively expressed by neural progenitors, and their expression is repressed by Shh. The class II proteins depend on Shh signaling for their neural expression. The selective cross-repressive interactions between class I and class II proteins are necessary to establish and maintain boundaries between distinct ventral progenitor domains [Briscoe and Ericson, 2001; Briscoe et al., 2000; Muhr et al., 2001; Novitch et al., 2001; Vallstedt et al., 2001].

Although inactivation of Notch1 results in premature differentiation of neural progenitor cells in the spinal cord beginning at E10.5 (fig. 2), it is unclear whether all or selective types of progenitor cells are affected in the ventral spinal cord. To address this issue, we performed either in situ hybridization or immunohistochemical analysis on the spinal cord of *N1* cKO and control mice at E10.5 and E11.5 using selective markers of neural progenitors, such as Nkx2.2, Olig2, Nkx6.1 (class II), and Dbx1 and Pax6 (class I), each labeling one or more neural precursor subtypes (fig. 4U). Nkx2.2 is expressed in pV3 cells adjacent to the floor plate, which give rise to V3 interneurons [Briscoe et al., 1999]. Immunostaining using Nkx2.2 antibodies revealed similar numbers of Nkx2.2-expressing pV3 progenitor cells in the ventral spinal cord of *N1* cKO (81 ± 7) and control (83 ± 14, $p = 0.83$) mice at E10.5 (fig. 4A, B). However, at E11.5 there is a small but significant decrease in Nkx2.2+ pV3 progenitor cells in the ventral spinal cord of *N1* cKO mice (84 ± 8), relative to the control (101 ± 5, $p < 0.05$) (fig. 4C, D). *Olig2* is a bHLH transcription factor and is expressed in pMN progenitor cells that give rise to motor neurons and oligodendrocytes [Lu et al., 2002; Novitch et al., 2001; Zhou and Anderson, 2002]. *Olig2* expression is markedly reduced in the *N1* cKO spinal cord at E10.5 (fig. 4E, F) and E11.5 (fig. 4G, H). *Nkx6.1* is another homeodomain gene and its expression demarcates pV3, pMN, and pV2 progenitors [Briscoe et al., 2000; Qiu et al., 1998]. At E10.5, *Nkx6.1* expression is slightly reduced in the ventral spinal cord of *N1* cKO mice (fig. 4I, J). By E11.5, its expression becomes barely detectable in the basal plate (fig. 4K, L).

We next examined the expression of two class I genes, *Dbx1* and *Pax6. Dbx1* expression within the ventral half of the spinal cord gives rise to pV0 progenitors [Pierani et al., 1999]. Surprisingly, *Dbx1* expression in the *N1* cKO spinal cord is increased at E10.5 (fig. 4M, N) but decreased at E11.5 (fig. 4O, P). *Pax6* is another class I homeodomain gene and its expression within the ventral spinal cord overlaps pV0, pV1, pV2, and pMN progenitors [Ericson et al., 1997]. Pax6 immunoreactivity is similar in the spinal cord of *N1* cKO and control mice at E10.5 (fig. 4Q, R). By E11.5, Pax6 immunoreactivity is

Fig. 4. In situ hybridization and immunohistochemical analyses of progenitor markers in the spinal cord. Nkx2.2+ population is not significantly changed in *N1* cKO mice at E10.5 (**A**, **B**). The number of Nkx2.2+ progenitor cells is slightly but significantly reduced in *N1* cKO mice at E11.5 (**C**, **D**). *Olig2* expression is markedly downregulated at E10.5 (**E**, **F**) and E11.5 (**G**, **H**) in the ventral spinal cord of *N1* cKO mice. *Nkx6.1* expression is slightly reduced at E10.5 (**I**, **J**), and more markedly downregulated at E11.5 (**K**, **L**). *Dbx1* expression is upregulated at E10.5 (**M**, **N**), and is downregulated at E11.5 (**O**, **P**) in the ventral spinal cord of *N1* cKO mice. The number of Pax6-expressing progenitor cells is similarly at E10.5 (**Q**, **R**), and is significantly reduced at E11.5 (**S**, **T**) in the ventral *N1* cKO spinal cord. **U** A diagram depicts specific markers and their corresponding progenitor populations. Scale bars: 100 μm.



reduced in the ventral spinal cord of *N1* cKO mice (fig. 4S, T). The reduced expression of all class I and II genes at E11.5 indicates the reduction of all neural progenitor subtypes in the absence of Notch1, although their respective positions are still maintained (fig. 4U). The initial alteration of the expression of individual homeodomain and bHLH factors varies at E10.5: for example, Nkx2.2 expression is unchanged, *Olig2* expression is reduced, and *Dbx1* expression is increased.

Altered Generation of Neuronal Subtypes in the N1 *cKO Ventral Spinal Cord*

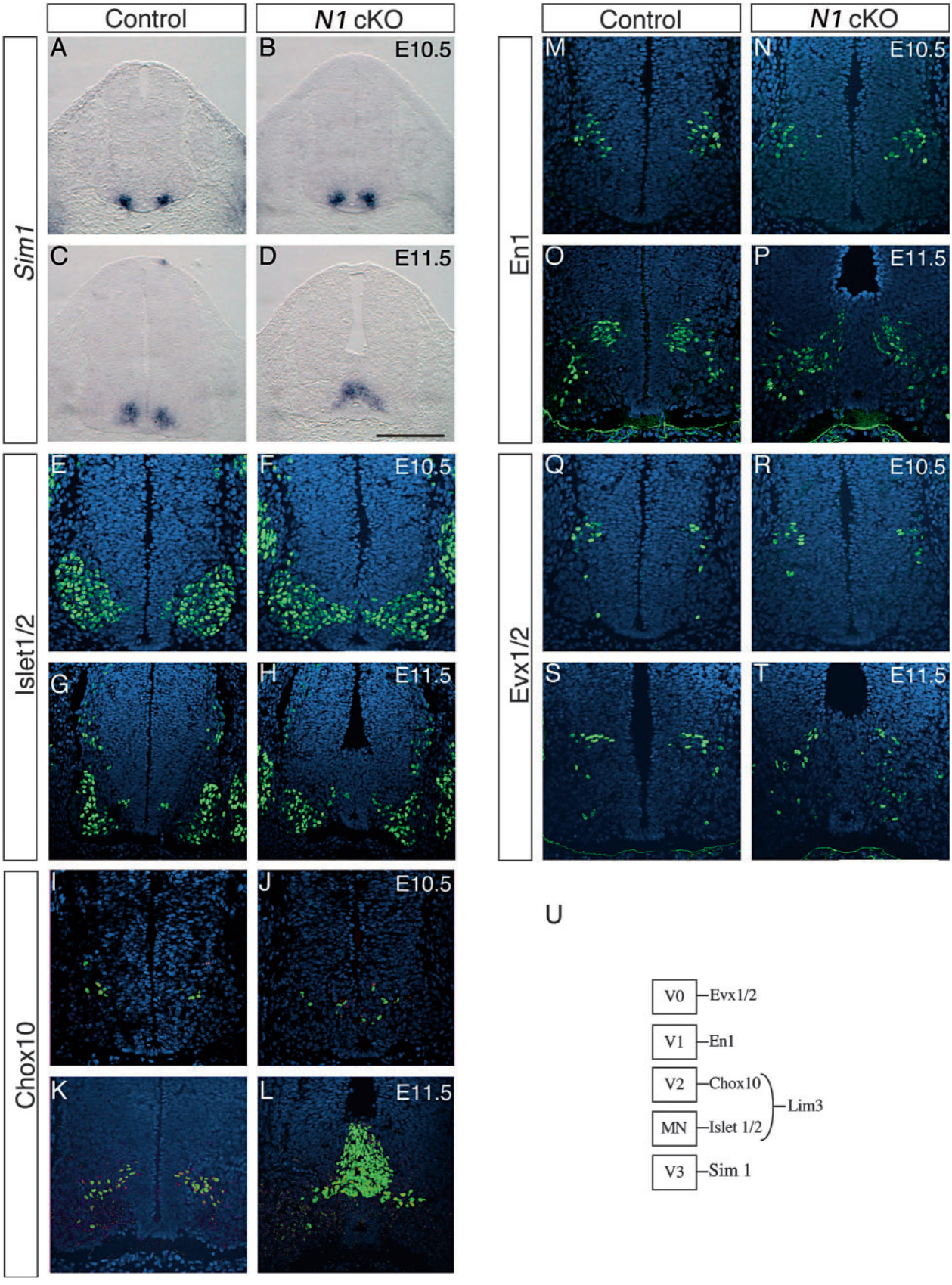
Once established, the expression profile of class I and class II proteins appears to control the fate of neurons by directing the activation of specific downstream determinants that establish the subtype identity of postmitotic neurons [Briscoe et al., 2000; Muhr et al., 2001; Novitch et al., 2001; Pierani et al., 1999, 2001; Zhou and Anderson, 2002]. The ventral half of the spinal cord is occupied by five distinct postmitotic neuronal populations that consist of, from ventral to dorsal, V3, MN, V2, V1, and V0 neurons, which are defined by the expression of Sim1, Islet1/2, Chox10, En1, and Evx1/2, respectively (fig. 5U) [Briscoe and Ericson, 2001; Jessell, 2000]. In situ hybridization analysis revealed that the pattern of *Sim1*-expressing V3 interneurons [Briscoe et al., 1999; Fan et al., 1996] is similar between *N1* cKO and control mice at E10.5, though by E11.5 *Sim1*-expressing V3 interneurons are localized closer to the midline and are fused together (fig. 5A–D). The number of Islet1/2-immunoreactive MNs is similar in the ventral spinal cord of *N1* cKO (283 ± 36) and control (266 ± 31, $p = 0.4$) mice at E10.5 (fig. 5E, F). The Islet1/2+ MN populations are localized closer to the midline in the *N1* cKO spinal cord (fig. 5E, F). Similarly, we did not observe a significant alteration in the number of Islet1/2+ MNs in *N1* cKO (176 ± 12) and control (162 ± 12, $p = 0.19$) mice at E11.5 (fig. 5G, H). At E10.5, the number of Chox10-immunoreactive V2 interneurons is similar in the ventral spinal cord of *N1* cKO (15 ± 8) and control (14 ± 3, $p = 0.8$) mice at E10.5 (fig. 5I, J). By E11.5, immunohistochemical analysis revealed a marked increase in Chox10+ V2 interneurons occupying the midline of the ventral spinal cord of *N1* cKO mice (167 ± 11) compared to the control (54 ± 20, $p < 0.01$) (fig. 5K, L), suggesting that in the absence of Notch1 more Chox10+ V2 interneurons are generated and they fail to migrate laterally to their appropriate domains.

We next examined the populations of En1+ V1 and Evx1/2+ V0 interneurons in the ventral spinal cord [Burrill et al., 1997; Matise and Joyner, 1997; Pierani et al., 1999]. At E10.5, no significant difference was observed in the number of En1+ V1 interneurons between the control (56 ± 13) and *N1* cKO embryos (58 ± 8, $p = 0.73$) (fig. 5M, N). By E11.5, there is a 29% increase in the number of En1+ V1 cells in the *N1* cKO spinal cord (107 ± 8), compared to the control (83 ± 10, $p < 0.01$) (fig. 5O, P). Similarly, at E10.5, we observed no significant difference in the number of Evx1/2+ V0 interneurons between the control (28 ± 7) and *N1* cKO embryos (25 ± 10, $p = 0.46$) (fig. 5Q, R). By E11.5, there is a 22% increase in the number of Evx1/2+ V0 cells in the *N1* cKO embryos (72 ± 8), compared to the control (59 ± 12, $p < 0.05$) (fig. 5S, T).

Homeodomain protein Lim3 defines both V2 interneurons and MNs within the medial division of the median motor column [MMC(m)] [Jessell, 2000; Lee and Pfaff, 2001; Sharma et al., 1998]. Immunostaining at E10.5 revealed that the numbers of Lim3+ cells (green) are not significantly altered in *N1* cKO mice (300 ± 27), relative to the control (262 ± 51, $p = 0.2$) (fig. 6A, B). However, Lim3+ cells are localized more medially in the ventricular zone of the *N1* cKO spinal cord. By E11.5, concomitant with the fusion of the ventral central canal, the midline is occupied by Lim3+ cells, which are markedly increased in *N1* cKO mice (278 ± 32), relative to the control (230 ± 12, $p < 0.01$) (fig. 6G, H).

Since Lim3+ cells give rise to V2 interneurons and a subset of MNs, we next examined the identity of these cells by immunostaining with antibodies specific for Chox10, a V2 interneuron marker. At E10.5, Lim3+/Chox10+ cells (yellow in fig. 6E, F) represent only a small proportion of Lim3+ cells (fig. 6A, B) and the number is

Fig. 5. In situ hybridization and immunohistochemical analyses of V0, V1, MN, and V3 neuronal subtypes in the spinal cord. Sim1 expression, which labels V3 interneurons, is similar in *N1* cKO and control mice at E10.5 (**A**, **B**) and E11.5 (**C**, **D**); although the two lateral populations become fused at E11.5 (**C**, **D**). The number of Islet1/2+ MNs is not significantly altered in *N1* cKO mice at E10.5 (**E**, **F**) and E11.5 (**G**, **H**). The number of Chox10+ V2 interneurons is similar in *N1* cKO and control mice at E10.5 (**I**, **J**), and markedly increased in *N1* cKO mice at E11.5, compared to the control (**K**, **L**). The number of En1+ V1 interneurons is not significantly changed in *N1* cKO mice at E10.5 (**M**, **N**), but it is significantly increased in *N1* cKO mice at E11.5 (**O**, **P**). The number of Evx1/2+ V0 interneurons is not significantly altered in *N1* cKO mice at E10.5 (**Q**, **R**), but it is significantly increased in *N1* cKO mice at E11.5 (**S**, **T**). **U** A diagram depicts specific markers and their corresponding neuronal cell types. Scale bar: 100 μm.



not significantly different between *N1* cKO (12 ± 9) and control (7 ± 1, p = 0.3) mice (fig. 6C, D). By E11.5, almost all Lim3+ cells occupying the midline are Chox10+ V2 interneurons (fig. 6H, J, L). These results demonstrate that V2 interneurons are markedly increased not only in number in *N1* cKO mice (199 ± 12) compared to the control (112 ± 5, p < 0.001), but also in percentage: 78% of the Lim3+ cells are V2 interneurons in *N1* cKO mice, compared to 48% in the control (p < 0.01) (fig. 6K, L). Similar results were obtained using additional sections (6–8 per embryo, 50 μm apart) and embryos (4 per genotype) at E11.5.

To determine whether the increase in the generation of Lim3+/Chox10+ interneurons is at the expense of the generation of motor neurons, we performed double-labeling with antibodies specific for Lim3 and Islet1/2. We found that the number of Lim3+/Islet1/2+ motor neurons (yellow in fig. 6Q, R, W, X) is significantly reduced in *N1* cKO mice (51 ± 8) relative to the control (81 ± 13, p < 0.05) at E11.5 (fig. 6W, X), whereas the number of Lim3+/Islet1/2+ motor neurons is similar in *N1* cKO (201 ± 31) and control (174 ± 29, p = 0.5) mice at E10.5 (fig. 6Q, R). The percentages of Lim3+/Islet1/2+ in total Lim3+ cells are also decreased at E11.5 (*N1* cKO = 17%, control = 35%, p < 0.01), but not at E10.5 (*N1* cKO = 65%, control = 70%, p = 0.7). We repeated the double-labeling experiment at E11.5 using additional sections (6–8 per embryo, 50 μm apart) and embryos (4 per genotype), and similar results were obtained with significantly reduced number and percentage (in total Lim3+ cells) of Lim3+/Islet1/2+ cells in *N1* cKO mice. These results suggest that loss of Notch1 affects the decision between motor neuron and V2 interneuron specification derived from Lim3+ cells.

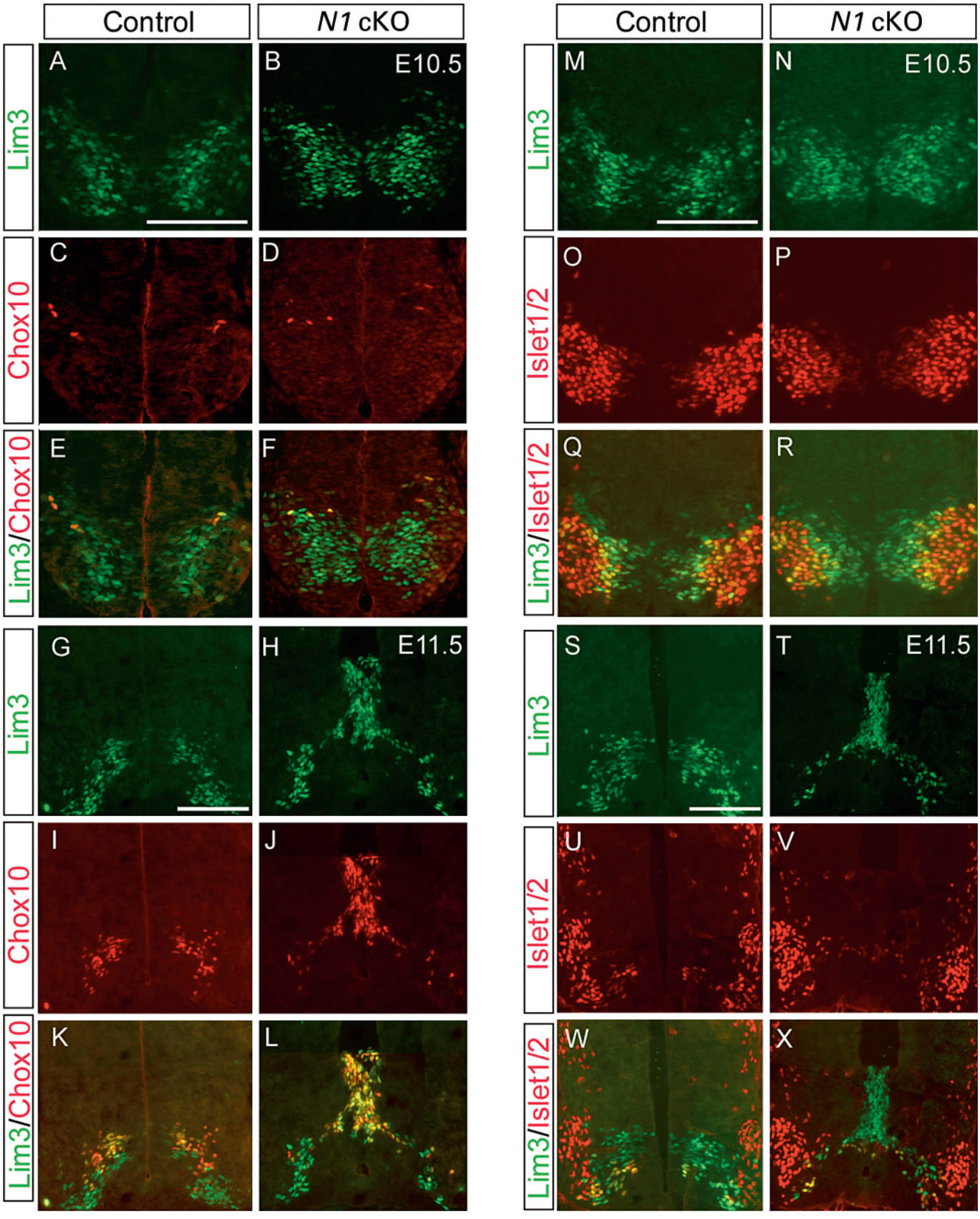
Discussion

Notch Signaling Regulates the Maintenance of All Neural Progenitor Subtypes

Previous studies in a variety of vertebrate systems have established a role for Notch signaling in the cell fate decision between neural progenitor cells and postmitotic neurons [Chambers et al., 2001; Dorsky et al., 1995; Handler et al., 2000; Henrique et al., 1997; Hitoshi et al., 2002; Nye et al., 1994; Scheer et al., 2001; Shen et al., 1997; Wines-Samuelson et al., 2005; Yang et al., 2004]. Reduction of Notch signaling in *PS1* null and conditional mutant mice leads to premature neurogenesis and reduction of progenitor cells in the developing brain [Handler et al., 2000; Hitoshi et al., 2002; Shen et al., 1997; Wines-Samuelson et al., 2005; Yang et al., 2004]. Consistent with these findings, *N1* cKO mice exhibit increased expression of proneural bHLH transcription factors, including *Ngn1, Ngn2* and *Mash1* in the ventral spinal cord, accompanied by the downregulation of Notch immediate targets *Hes5* and *Hes1* (fig. 3). These results are consistent with previous findings showing reduced *Hes5* expression and increased expression of proneural bHLH transcription factors in the brain and spinal cord of *Notch1–/–* and *RBPjk–/–* mice [de la Pompa et al., 1997]. Similarly, *Hes5* expression is reduced in the *PS1–/–* brain [Handler et al., 2000], and is completely abolished in the neural tube of *PS1–/–;PS2–/–* mice [Donoviel et al., 1999]. Interestingly, an in vitro gain-of-function study has shown that *Hes1,* rather than *Hes5,* acts as the major mediator of Notch signaling in gliogenesis [Wu et al., 2003]. The upregulation of these bHLH transcription factors, which are involved in cell cycle exit and promotion of neurogenesis, leads to decreases in neural progenitor cell types and acceleration in neuronal differentiation [Morrow et al., 1999; Farah et al., 2000; Mizuguchi et al., 2001].

Consistent with reduced progenitor cells in the developing brain of *PS1* null and conditional mutant mice, in the present study we found that Notch1 inactivation in the spinal cord also causes premature neuronal differentiation and reduction of progenitor cells (fig. 2). Using markers specific for one or multiple subtypes of neural progenitor populations, we found reductions of all progenitor populations in the *N1* cKO ventral spinal cord by E11.5 (fig. 4). In contrast to the more marked decreases of Olig2 and Nkx6.1 expression, reductions of the Nkx2.2+ progenitor population are more subtle in *N1* cKO mice, with normal numbers of Nkx2.2+ progenitor

Fig. 6. Increased Lim3+/Chox10+ V2 interneurons and decreased Lim3+/Islet1/2+ motor neurons in the *N1* cKO spinal cord. At E10.5, the number of Lim3+ neurons (green), which include both V2 interneurons and motor neurons, is similar in *N1* cKO and control mice (**A**, **B**, **M**, **N**). At E11.5, the number of Lim3+ neurons is markedly increased in *N1* cKO mice, compared to the control (**G**, **H**, **S**, **T**). Lim3+ neurons cluster along the midline in *N1* cKO mice (**H**, **T**). The number of Lim3+/Chox10+ V2 interneurons (yellow) is not significantly changed in the *N1* cKO spinal cord at E10.5 (**E**, **F**), but it is significantly increased in *N1* cKO mice at E11.5 (**K**, **L**). The number of Lim3+/Islet1/2+ motor neurons (yellow) is also not significantly altered in *N1* cKO mice at E10.5 (**Q**, **R**), but is significantly decreased at E11.5 (**W**, **X**). Scale bars: 100 μm.



cells at E10.5 and a small reduction in Nkx2.2+ progenitor cells at E11.5 (fig. 4). It is noteworthy that the Nkx2.2+ domain is immediately adjacent to the floor plate, where Notch2 is highly expressed. It is possible that the existing Notch2 activity may compensate for the lack of Notch1 signaling in maintaining the Nkx2.2+ population. Another interesting observation is the expression of *Dbx1* is upregulated transiently at E10.5 (fig. 4). It is unclear how the loss of Notch1 activity may transiently increase *Dbx1* expression; nevertheless, this transient upregulation of Dbx1 at E10.5 is likely to have contributed to the subsequent increase in the Evx1/2+ V0 interneuron subpopulation at E11.5 in *N1* cKO mice compared to the control (fig. 5S, T), consistent with the critical role of Dbx1 in V0 neuronal fate decision demonstrated in Dbx1 mutant mice which fail to generate V0 interneurons [Pierani et al., 2001].

Further analysis demonstrated that postmitotic neuronal populations are increased in size, revealed by the premature appearance of TuJ1 immunoreactivity in the ventricular zone of the ventral spinal cord of *N1* cKO mice (fig. 2). It has been shown that distinct postmitotic neuronal populations in the ventral spinal cord are derived from medially localized progenitor cells [Briscoe and Ericson, 2001; Jessell, 2000; Lee and Pfaff, 2001]. Consistent with the reduction in progenitor cells, postmitotic neuronal populations (V0, V1, V2) are increased (fig. 5, 6). Together, these observations show that as in the developing brain, Notch1 controls the timing of neuronal differentiation and the size of neural progenitor populations in the spinal cord.

Loss of Notch1 signaling causes a remarkable morphological change in the ventral spinal cord, in which the lateral walls of the central canal gradually fused together initiating from the floor plate area (fig. 2). This could be explained by the gradual loss of neural progenitor cells normally lining the ventricles and the premature emergence of postmitotic neurons forming interconnections between the two lateral populations. In support of this interpretation, we found that Chox10+ V2 and Sim1+ V3 interneurons in *N1* cKO mice are localized more medially, compared to their usual lateral positions in the control, and eventually become fused together at the midline (fig. 5, 6).

Notch1 Signaling Influences Neuronal Subtype Specification in the Ventral Spinal Cord

One of the fundamental issues in the field of developmental neuroscience is to understand the mechanisms that control the identity of distinct classes of neurons located at defined positions within the nervous system [Briscoe and Ericson, 2001; Jessell, 2000; Lee and Pfaff, 2001]. It was unclear how the specification of neuronal subtypes is integrated with the regulation of neurogenesis, which is largely controlled by Notch signaling [Jessell, 2000], and whether Notch1 plays an additional role in the regulation of neuronal subtype specification. In the present study, we observed that the Lim3 (Lhx3) neuronal subtype is affected by Notch inactivation. Lim3 and the closely related factor Lhx4 are expressed by V2 interneurons and MNs within the medial division of the MMC(m) [Jessell, 2000; Lee and Pfaff, 2001; Sharma et al., 1998], but only MNs express Islet1 [Ericson et al., 1992]. Mice lacking Lim3 and the redundant factor Lhx4 failed to generate V2 interneurons and the proper types of MNs [Sharma et al., 1998]. On the other hand, ectopic expression of Lim3 triggered V2 interneuron formation, whereas the combination of Lim3 and Islet1 led to the ectopic upregulation of an MN marker [Tanabe et al., 1998]. In addition to the overproduction of Lim3+ cells, we found that these cells divert from their normal program of differentiation: instead of generating slightly more Chox10+ V2 interneurons (48%) than Islet1/2+ MNs (35%) at E11.5, in the absence of Notch1, the majority of Lim3+ cells become Chox10+ V2 interneurons (78%), at the expense of Islet1/2+ MMC(m) motor neurons (17%) (fig. 6). These observations suggest that Notch1 functions at two different stages in neuronal subtype specification: first, its presence in progenitors normally restricts the size of the Lim3+ population; second, within the Lim3+ population, Notch1 signaling favors the generation of motor neurons.

It remains unclear how loss of Notch1 signaling and altered regulation of proneural bHLH transcription factors rapidly lead to changes in neuronal subtype specification within the Lim3+ population. Biochemically, it was demonstrated that a 2NLI:2Lim3 tetramer, formed between Lim3 and nuclear LIM interactor NLI (Ldb1, CLIM2), is involved in V2 interneuron generation, whereas 2NLI:2Islet1:2Lim3 hexamers are found to drive MN differentiation [Thaler et al., 2002]. The competitive interactions of Lim3 lead to the formation of hexamers at the expense of tetramers in MNs, thereby serving as a switching mechanism for regulating its cell type-specific functions [Thaler et al., 2002].

Whether Notch regulation of Lim3+ neurons is cell-autonomous awaits further investigation. Future studies are also necessary to confirm whether Notch signaling regulates additional neuronal subtype specification during vertebrate neural development. In the immune sys-

tem, analysis of Notch mutant mice suggests that Notch signaling is involved in B and T cell lineage determination [Pui et al., 1999; Radtke et al., 1999] as well as CD4/CD8 [Chang et al., 2000; Deftos et al., 2000; Izon et al., 2001; Robey et al., 1996; Wolfer et al., 2001] and αβ/γδ [Washburn et al., 1997] T cell lineage determination. Chimera analysis of Notch1-deficient cells and employment of other Cre transgenic mice that target specific subtypes of progenitor cells may address this issue.

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