Ascl1 defines sequentially generated lineage-restricted neuronal and oligodendrocyte precursor cells in the spinal cord

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The neural basic helix-loop-helix transcription factor Ascl1 (previously Mash1) is present in ventricular zone cells in restricted domains throughout the developing nervous system. This study uses genetic fate mapping to define the stage and neural lineages in the developing spinal cord that are derived from Ascl1-expressing cells. We find that Ascl1 is present in progenitors to both neurons and oligodendrocytes, but not astrocytes. Temporal control of the fate-mapping paradigm reveals rapid cell-cycle exit and differentiation of Ascl1-expressing cells. At embryonic day 11, Ascl1 identifies neuronal-restricted precursor cells that become dorsal horn neurons in the superficial laminae. By contrast, at embryonic day 16, Ascl1 identifies oligodendrocyte-restricted precursor cells that distribute throughout the spinal cord. These data demonstrate that sequentially generated Ascl1-expressing progenitors give rise first to dorsal horn interneurons and subsequently to late-born oligodendrocytes. Furthermore, Ascl1-null cells in the spinal cord have a diminished capacity to undergo neuronal differentiation, with a subset of these cells retaining characteristics of immature glial cells.

KEY WORDS: Mash1 (Ascl1), bHLH transcription factor, Spinal cord development, In vivo genetic fate mapping, Mouse

INTRODUCTION

Neurogenesis and gliogenesis in invertebrates and vertebrates is characterized by a series of symmetric and asymmetric divisions that result in the correct number of neurons and glia needed to form a mature nervous system (reviewed by Huttner and Kosodo, 2005; Roegiers and Jan, 2004). In vertebrate organisms, different neural cell types are generated from ventricular zone cells sequentially, with neurogenesis preceding gliogenesis. Recently, elegant time-lapse imaging of cortical neurogenesis in mammals has revealed a model in which mitosis at the ventricular surface is either symmetric, yielding two progenitors, or asymmetric, yielding a progenitor cell and either a neuron or a neuron-restricted precursor (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). The restricted precursor undergoes additional mitoses at non-surface locations, usually symmetrically, yielding two postmitotic neuron daughter cells. By contrast, non-cortical brain regions may have different characteristic division patterns. This is suggested by studies in chick and zebrafish hindbrain that demonstrate that neurons are preferentially derived from symmetric terminal divisions rather than asymmetric divisions (Lyons et al., 2003). Oligodendrocytes and astrocytes are generated from dividing cells in the ventricular zone at later stages. These precise descriptions of neural cell lineage are important for providing a framework to address the molecular control of neurogliogenesis and for understanding the transition of neural stem cells as they differentiate into one of the three major classes of cells in the central nervous system (CNS).

Transcription factors of the Basic helix-loop-helix (bHLH) family are one class of molecules essential in both neurogenesis and gliogenesis from Drosophila to mammals (reviewed by Bertrand et al., 2002; Rowitch, 2004). Here we use recombination-based lineage tracing in vivo to identify the position within the neural lineage marked by the bHLH factor, Ascl1 (previously Mash1). Ascl1 is a vertebrate homolog of the Drosophila proneural genes of the Achaete-scute complex (Johnson et al., 1990). Ascl1 is present within the ventricular zone, in at least some mitotically active cells, in distinct regions along the rostrocaudal and dorsoventral axes of the neural tube (Guillemot et al., 1993; Helms et al., 2005; Ma et al., 1997; Porteus et al., 1994; Torii et al., 1999). Over the past decade, analyses of mice null for Ascl1 have demonstrated its essential role in the generation of specific subsets of neurons in many regions, including the forebrain, hindbrain, autonomic nervous system, olfactory epithelium, retina and spinal cord (Akagi et al., 2004; Blaugrund et al., 1996; Casarosa et al., 1999; Cau et al., 1997; Guillemot et al., 1993; Helms et al., 2005; Hirsch et al., 1998; Pattyn et al., 2004). Roles for Ascl1 in inducing neuronal differentiation and in neuronal specification were revealed by combining results from the mouse mutant with those from overexpression paradigms in cell culture (Farah et al., 2000) or chick neural tube (Nakada et al., 2004). For example, in the chick neural tube, high levels of Ascl1 induce cells to stop cycling, move laterally out of the ventricular zone, and begin expressing both general neuronal markers and neuronal-type-specific markers (Helms et al., 2005; Kriks et al., 2005; Müller et al., 2005; Nakada et al., 2004). Loss of Ascl1 function results in loss or decrease of specific interneuron populations in the mouse spinal cord (Helms et al., 2005; Li et al., 2005). In addition to these specific losses, there is a more general defect as cells stall in the ventricular zone and different aspects of neuronal differentiation become uncoordinated (Casarosa et al., 1999; Helms et al., 2005; Horton et al., 1999; Torii et al., 1999). Together, these studies define Ascl1 as an essential player in vertebrate neuronal differentiation and specification.

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Although not studied as extensively, there are accumulating data that suggest Ascl1 is a player in oligodendrogenesis as well as neurogenesis. Ascl1 was first described in oligodendrocyte progenitor cells isolated from optic nerve (Kondo and Raff, 2000; Wang et al., 2001), and more recently it was shown to overlap with markers of oligodendrocyte precursors in the telencephalon (Gokhan et al., 2005) although its requirement in these cells is not clear. At postnatal stages, Ascl1 functions in the telencephalic subventricular zone (SVZ), the source of replenishing neurons in the olfactory bulb and hypothalamus, although its requirement in these cells is not clear. At postnatal stages, Ascl1-expressing cells in the telencephalic subventricular zone (SVZ), the source of replenishing neurons in the olfactory bulb and hypothalamus, although its requirement in these cells is not clear.

Ascl1 is detected in progenitors common to both neurons and oligodendrocytes, or whether it is present in precursors restricted to either of these two lineages. We developed mouse models expressing Cre or the tamoxifen-inducible Cre recombinase to identify lineages derived from Ascl1-expressing cells at different stages of development. Using cervical spinal cord as a model, we found that during neurogenic periods [embryonic day (E) 9.5-13.5], Ascl1-expressing cells were lineage-restricted neuronal precursors, but later in embryogenesis, during gliogenic periods in the dorsal neural tube (E14.5-early postnatal), Ascl1-expressing cells were lineage-restricted oligodendrocyte precursors. In this paradigm, Ascl1 is not detected in progenitors common to both lineages, as the genetically marked cells rapidly exit the cell cycle, move laterally out of the ventricular zone, and differentiate. Analysis of lineage markers in Ascl1 mutant spinal cords demonstrates a role for Ascl1 in efficient neuronal differentiation; without Ascl1, the cells maintain characteristics of immature oligodendrocytes and astrocytes.

MATERIALS AND METHODS

BAC recombination and generation of transgenic mice

The Roswell Park Bacterial Artificial Chromosome (BAC) library was screened to identify clones that contained the Ascl1 protein coding region. RPCI-428P21 was chosen for further study as it contains a genomic insert of 305 kb with 98 kb 5' and 206 kb 3' of the Ascl1 translation start codon (Fig. 1). Homologous recombination in bacteria (Yang et al., 1997) was used to replace the Ascl1 coding region precisely with coding sequence for EGFP (Clontech) and Cre separated by an internal ribosomal entry site (IRES) (Ascl1-GIC, or CreERTM (Hayashi and McMahon, 2002) with a heterologous 3' cassette from the bovine growth hormone (Ascl1-CreERTM). Transgenic mice were generated by standard procedures (Hogan et al., 1986) using fertilized eggs from B6D2F1 (C57Bl/6 × DBA) crosses. Purified BAC DNA was injected at 0.5-1 μg/μl in 10 mmol/l Tris pH 7.5, 0.1 mmol/l EDTA, 100 mmol/l NaCl. Two lines of each transgene type were examined. Data in Fig. 2 are from Ascl1-GIC (B1A-2), and in Figs 3, 4, 5 and 6 are from Ascl1-CreERTM (B2C-6), while Ascl1-CreERTM (B2C-8) is shown in Fig. S2 in the supplementary material. Transgenic animals were identified by PCR analysis using tail or yolk sac DNA with primers to Cre: GCATGTTAGCTCGACCGGTCG; GCATAACAGTGAACCA-GCATGGCTG.

Ascl1-CreER2 was created by subcloning CreER2 from pCreERT2 (Indra et al., 1999) into the Sort and Nhel site of Nestin Xh5 plasmid (gift from W. Zhong, Yale University). The rat Nestin gene was initially identified to contain four exons spanning three introns (Lendahl et al., 1990) and was recently identified to contain five exons spanning four introns (Wiese et al., 2004). The Xh5 plasmid contains ~5.4 kb upstream of the initiation codon, exons 1-4 and adjacent introns 1-4, and part of the 5' region of exon 5 of rat nestin (see Fig. S1A in the supplementary material). The Xh5 plasmid has been previously published in other mouse models and contains similar elements to Nes/PlacZ3 introns (Beech et al., 2004; Petersen et al., 2002; Zimmerman et al., 1994). The Nestin-CreER2 founder mice were generated by pronuclear injection of Smol digest of Nes-CreER2 into C57Bl/6J fertilized eggs. Five independent lines were generated and the data presented here are from one line (K). A detailed characterization of this transgenic strain will be published elsewhere. A Nestin-CreER2; R26R-stop-lacZ E11.5 embryo induced with tamoxifen at E10.5 illustrates nervous system expression of the transgene along the rostrocaudal axis (see Fig. S1B in the supplementary material). No expression was seen in somites and no expression observed outside of the CNS. A cross section through the neural tube reveals reporter gene expression in the ventricular and mantle zones, and at this stage it is enriched ventrally (see Fig. S1C in the supplementary material).

Cre reporter mouse strains R26R-stop-lacZ (Soriano, 1999) and R26R-stop-YFP (Srinivas et al., 2001) were genotyped by PCR using published primers (Soriano, 1999): AAA GTC GCT CTG AGT TGT TAT; GCG AAG AGT TGT TCC TCA ACC; GGA GCC GCG GAA ATG GAT AGT. Ascl1 mutant strain (Guillemot et al., 1993) was genotyped by PCR using: CTCTAGGCAAGAAAC; GCAGCCGATCGCTTCT; CCGAG-ACTCAATAGGACAG. Tamoxifen induction of Cre recombinase was accomplished by interperitoneal injection of pregnant females at 9.5-15.5 days post-coitum (dpc) with 2-3 mg tamoxifen (Sigma, T55648) in sunflower oil per 40 g body weight. Two injections of tamoxifen 6 hours apart were used for experiments shown in Fig. 3.

Immunofluorescence, X-gal staining, and mRNA in situ hybridization

Embryonic day 10.5-13.5 embryos were dissected and processed as previously described for whole mount for GFP or X-gal staining (Gowan et al., 2001), or for cryosectioning for immunofluorescence and mRNA in situ hybridization (Helms et al., 2005). Briefly, for cryosectioning, embryos were fixed in 4%
formaldehyde for 2 hours at 4°C, rinsed well in phosphate buffer, cryoprotected in 30% sucrose, and embedded in OCT. Spinal columns were dissected from embryos older than E15 and fixed in 2% formaldehyde for 16 hours at 4°C, before processing for cryosection as above. Spinal columns were dissected from P14-P30 mice by ventral laminectomy after anesthesia and trans-cardiac perfusion with 4% formaldehyde. Tissue was fixed further for 2 hours at 4°C, vibratome sectioned and X-gal stained, or fixed overnight at 4°C before processing as above for cryosection.

For immunofluorescence, slides were incubated in the appropriate dilution of primary antibody in PBS/1% goat serum/0.1% Triton X-100, followed by either goat anti-rabbit, mouse, or guinea pig IgG conjugated with Alexa Fluor 488, 594 or 647 (Molecular Probes). Primary antibodies used for this study included: rabbit (1:500, Molecular Probes, A6455) or chicken (1:250, Chemicon, AB16901) anti-GFP, mouse anti-Lhx1/5 (1:100, DHSB, 4F2), guinea pig anti-Lmx1b (1:8000, gift from T. Jessell), mouse anti-BrdU (1:25; BD Biosciences, 347580), rabbit anti-β-gal (1:200, Biogenesis, 4600-1505), mouse anti-APC (1:100, Calbiochem, clone CC-1), mouse anti-NeuN (1:1000, Chemicon, MAB377), mouse anti-GFAP (1:400, Sigma-Aldrich, G3893), rabbit anti-Olig2 (1:2000, gift from C. Stiles and R. Lu), guinea pig anti-Sox10 (1:2000, gift M. Wegner), mouse anti-Ki67 (1:100, Novacastra) and guinea pig anti-GLAST (Chemicon, AB1784). The guinea pig anti-Ascl1 antibody was generated using bacterially produced rAscl1 as antigen and used at 1:10,000. For BrdU labeling, pregnant mothers were injected with 200 μg/g of body weight 1 hour before sacrifice. Fluorescence imaging was carried out on a Bio-Rad MRC 1024 confocal microscope. For each experiment multiple sections from at least three different animals were analyzed.

mRNA in situ hybridization was performed essentially as described using a combined protocol (Birren et al., 1993; Ma et al., 1998). A detailed protocol is available upon request. Ascl1 and Cre antisense probes were made from plasmids containing the coding region of each gene.

RESULTS

Transgenic mice expressing GFP and Cre recombinase recapitulate Ascl1 expression

To study the lineages derived from Ascl1-expressing cells, we developed a mouse model that expresses Cre recombinase in these cells. Transgenic mice were generated with a BAC containing the Ascl1 locus that was modified to contain the coding sequence for Cre recombinase in place of the Ascl1 coding sequence. The BAC, RPCI-23 428P21, was chosen for these studies because it contains 100 kb 5' and 200 kb 3' of the Ascl1 start codon (Fig. 1). Thus, this BAC has a high probability of containing all regulatory information for gene expression mimicking endogenous Ascl1. Homologous recombination in bacteria (Yang et al., 1997) was used to replace the Ascl1 coding region with that for GFP and Cre transcribed as a bicistronic message separated by an IRES (Fig. 1) (Helms et al., 2005). This modified BAC was used to generate multiple transgenic mouse lines that expressed the transgene in similar patterns at E11.5. The Ascl1-GIC line used here has been maintained over multiple generations.

Analysis of GFP fluorescence and Cre mRNA in situ in Ascl1-GIC embryos demonstrated that the BAC sequences contain sufficient information to direct expression specifically in Ascl1 domains, including those not detected when smaller genomic sequences were used (Verma-Kurvari et al., 1996). GFP in whole mount was detected in the ventral telencephalon, midbrain and hindbrain, and dorsal neural tube (Fig. 2A). The activity of the Cre recombinase was demonstrated by crossing Ascl1-GIC mice with the Cre-reporter mouse line, R26R-stop-lacZ (Soriano, 1999). Whole mount X-gal stained Ascl1-GIC;R26R-stop-LacZ embryos permanently report the cells or their progeny that have expressed active Cre any time before that stage. At E10.5, there was extensive X-gal staining in midbrain (Fig. 2B). Staining in developing sympathetic neurons was just beginning to be detected at E10.5 but was clearly evident by E11.5 (Fig. 2B,C). E11.5 also revealed expression in the enteric nervous system, diencephalon and hindbrain, with low expression in the dorsal neural tube (Fig. 2C). By E12.5, expression in the dorsal neural tube was clearly detected (Fig. 2D,E). These temporal and spatial expression characteristics reflect expression of endogenous Ascl1 (Guillemot and Joyner, 1993). Cre and Ascl1 detected by mRNA in situ hybridization demonstrates that Cre was present in an Ascl1-specific pattern in the dorsal neural tube at E11.5 (Fig. 2E,F), and in the enteric (Fig. 2G,H) and sympathetic (Fig. 2I) nervous systems. Taken together, these data demonstrate that GFP and Cre in the Ascl1-GIC transgenic mouse strain are expressed in an Ascl1-restricted pattern.

Ascl1-expressing cells produce both neurons and oligodendrocytes

Ascl1 is restricted to the ventricular zone in the neural tube and is known to be required for neuronal differentiation and specification of subsets of neurons in multiple regions of the nervous system.
Ascl1 has also been reported in cells that will become oligodendrocytes (Gokhan et al., 2005; Kondo and Raff, 2000; Parras et al., 2004; Wang et al., 2001). As Ascl1 disappears as cells exit the cell cycle and differentiate, the identity and extent of mature neural cells that comprise the Ascl1 lineage is unknown. To assess the neural cell types derived from the Ascl1 lineage we analyzed lacZ expression in P30 spinal cords of Ascl1-GIC; R26R-stop-lacZ mice. This revealed that the contribution of cells from the Ascl1 lineage was surprisingly broad in the spinal cord and included cells located throughout gray and white matter tissue, and in cells surrounding the central canal (Fig. 2K), a pattern seen at all spinal cord levels.

The location of X-gal stained cells in both the gray and white matter suggested that neurons and glia were labeled. To determine the identity of these cells, P14 spinal cords of Ascl1-GIC; R26R-stop-YFP mice (Srinivas et al., 2001) were analyzed by double-label immunofluorescence. As expected, many YFP + cells in the gray matter co-labeled with the neuronal marker NeuN + (Fig. 2L). In addition, many YFP + cells, particularly in white matter but also in the gray matter, co-labeled with two oligodendrocyte markers, Olig2 + and APC + (Fig. 2M, N). However, no YFP + cells co-labeled with the astrocyte marker GFAP + (Fig. 2O). This contrasts with GFAP co-labeling with YFP from the nestin lineage (data not shown). These results indicate that neurons and oligodendrocytes, but not astrocytes, are derived from Ascl1-expressing cells.

Transgenic mice expressing a tamoxifen-inducible Cre recombinase allow stage-specific fate mapping of the Ascl1-lineage

During neural development, the ventricular zone of the neural tube contains progenitor cells that will give rise to neurons during the period of neurogenesis (~E9-14) and oligodendrocytes during gliogenesis (~E13-postnatal). At E10.5, Ascl1 is present in ventricular zone cells, a subset of which will incorporate BrdU (Helms et al., 2005). Thus, although Ascl1 is best known for its function in neuronal development, the fate-mapping data demonstrate that it is present in cells that will give rise to both neurons and oligodendrocytes. We could either be detecting Ascl1 expression in early neural tube ventricular zone cells that will differentiate to neurons early and then later to oligodendrocytes, or we could be detecting precursors restricted to a neuronal lineage. To distinguish between these two possibilities, we generated additional transgenic mice, Ascl1-CreERTM, that replaced the Ascl1 coding sequence in the BAC transgene with that encoding the tamoxifen-inducible Cre recombinase (CreERTM) (Hayashi and McMahon, 2002) carrying a heterologous 3′ UTR (Fig. 1). Two independent lines were generated that express the CreRTM transgene in a similar pattern but at different levels (compare Fig. 3C and Fig. 4C with Fig. S2 in the supplementary material). The line with the highest expression in the spinal neural tube was used for studies reported here.

Cre activity, detected using the R26R-stop-lacZ reporter line, reflects known Ascl1 expression during neurogenesis in the developing spinal cord. Ascl1-CreERTM; R26R-stop-lacZ embryos treated with tamoxifen at E9.5, 10.5, 11.5 and 12.5 were harvested 24 hours later and stained with X-gal (Fig. 3A-D). In a similar way to the Ascl1-GIC (Fig. 2), strong expression in midbrain regions was initiated first in sympathetic neurons, with neural tube just beginning to be detected at E10.5 (Fig. 3A,A′). At E11.5-13.5, X-gal staining reflected Ascl1 expression and was detected in the ventral telencephalon, midbrain and hindbrain, and dorsal neural tube (Fig. 3B-D). By contrast to X-gal staining in Ascl1-GIC, which reported an accumulation of all Ascl1-derived cells, the Ascl1-CreERTM reporter line, indicated neuron labeling at E11.5 and 12.5 in the Ascl1-CreERTM versus the Ascl1-GIC (compare Fig. 2C,D with Fig. 3B,C). Vibratome sections from the whole-mount stained embryos confirmed that Ascl1 lineage cells became sympathetic and ventral interneurons before E10.5, and dorsal horn neurons at E11-13 (Fig. 3A′-D′) (Helms et al., 2005; Li et al., 2005; Lo et al., 1991; Mizuguchi et al., 2006; Wildner et al., 2006). Triple-label immunofluorescence on a cross section of an E10.5 Ascl1-CreERTM; R26R-stop-YFP embryo treated with tamoxifen at E9.0 demonstrated accurate expression of Cre in the Ascl1 lineage in three populations of neurons at this stage (Fig. 3B-D).
The position of the YFP+ cells relative to Lhx1+ and/or Lhx5+ cells and the overlap with Lmx1b identified these cells as dorsal interneurons dI3 and dI5, and a ventral neuronal population (V2), as has been previously reported for Ascl1 lineages (Helms et al., 2005; Li et al., 2005). mRNA in situ hybridization of Cre in Ascl1-CreERTM at E11.5 was restricted to the dorsal neural tube, as expected for Ascl1 expression at this stage (compare Fig. 2E, Fig. 3F with Fig. 3G). Interestingly, the precise distribution of the mRNA accumulation in the mediolateral axis was distinct from endogenous Ascl1, with the mRNA accumulating at the lateral edges of the ventricular zone (Fig. 3G), suggesting a role for the 3' UTR in stabilization of the mRNA (Verma-Kurvari et al., 1998). Overall, within the neural tube, the Cre activity in the Ascl1-CreERTM transgenic line reflects expression recapitulating endogenous Ascl1.

Treatment of Ascl1-CreERTM;R26R-stop-lacZ embryos with tamoxifen at E15.5 and harvest at E16.5 revealed X-gal stained cells and demonstrated that the transgene was still being expressed at this late embryonic stage (Fig. 3H). The presence of Ascl1 in the neural tube at this stage has been reported in a limited number of cells in the dorsal ventricular zone (Cai et al., 2005). Using a newly produced anti-Ascl1 polyclonal antiserum on E16.5 neural tube sections, we detected much more Ascl1 in dorsal and ventral ventricular zone cells, as well as in the gray and white matter, revealing much more extensive expression at this late stage than previously appreciated (Fig. 3I). The specificity of the antisera is demonstrated by the well-documented pattern of labeling in E11.5 neural tube (Fig. 3F), and importantly, by the absence of labeling in an E16.5 Ascl1 null neural tube (Fig. 3J). Thus, endogenous Ascl1 and the transgene-produced Cre are present in late-stage neural tubes when gliogenesis is ongoing.

**Ascl1 expression defines lineage-restricted precursors**

Results from the experiments described above suggest that Ascl1 is present in lineage-restricted precursor cells, both during neurogenesis and later during oligodendrogenesis. To further test this idea, two experiments were performed. One examined the fate of Ascl1-expressing cells from different stages in the mature spinal cord (Fig. 4), and the other examined whether lineage-marked cells could be found in cycling cells in the ventricular zone (Fig. 5).

In the first experiment, R26R-stop-lacZ females crossed to Ascl1-CreERTM males were treated with tamoxifen at 10.5 dpc (embryos undergoing neurogenesis), or 15.5 dpc (embryos undergoing gliogenesis), and the resulting offspring were analyzed for lacZ expression at P30. Activation of Cre at E10.5 resulted in restricted and dense lacZ reporter activity in neurons of the dorsal horn primarily in Lamina I-III, and a few scattered cells in other parts of the gray matter (Fig. 4B). X-gal stained cells were noticeably absent from the spinal cord white matter and central canal (compare Fig. 2K and Fig. 4B). Within the dorsal horn, coexpression of β-gal with NeuN but not APC using double-label immunofluorescence confirmed the identity of these cells as neurons (Fig. 4E,F). By contrast, activating Cre recombinase with tamoxifen at E15.5 resulted in a majority of the X-gal stained cells scattered throughout the white matter at P30 (Fig. 4C). The lineage-traced cells coexpressed the oligodendrocyte marker APC (Fig. 4H) but not the neuronal marker NeuN, even when located in the gray matter (Fig. 4C,G). The lacZ-expressing cells did not coexpress GFAP, indicating that they were not astrocytes (data not shown). Additional time points of tamoxifen treatment confirmed these findings (Fig. 4K-N). Tamoxifen treatment at E9.5 or 12.5 yielded neurons only, while tamoxifen in early postnatal stages yielded oligodendrocytes only. In the absence of tamoxifen, there was no leaky expression of the reporter detected. Thus, these results support the conclusion that Ascl1 is present in oligodendrocyte-restricted precursors in the spinal neural tube by E15.5.

To demonstrate that the experimental paradigm used here can detect Cre activity in common neural progenitors in the ventricular zone, a novel Nestin-CreERT2 mouse strain (see Materials and methods and Fig. S1 in the supplementary material) that has a tamoxifen-inducible Cre (Feil et al., 1996; Indra et al., 1999) under the control of the promoter and enhancer sequences of the Nestin gene (Lendahl et al., 1990; Zimmerman et al., 1994) was used. Nestin is an intermediate filament protein that is transiently expressed in progenitor cells common to neurons, oligodendrocytes, and astrocytes. Arrowheads indicate individual time points for tamoxifen induction. (B-N) P30 spinal cords from Ascl1-CreERTM;R26R-lacZ (B,C,E-I, K-N) or Nestin-CreERT2;R26R-lacZ (D,I,J) stained for β-gal activity (B-E,D-I) or double-label immunofluorescence for β-gal (green) and NeuN (red) (E,F,I,J) or APC (red) (F,H,I,J). In Ascl1-CreERTM;R26R-lacZ mice, activation of Cre by tamoxifen at E10.5 results in X-gal labeled cells largely restricted to the dorsal horn gray matter (B) in cells that co-label with the neuronal marker NeuN (E, arrows). Activation of Cre at E15.5 results in X-gal labeled cells largely restricted to white matter regions (C) in cells that co-label with the oligodendrocyte marker APC (I, arrows). By contrast, in Nestin-CreERT2;R26R-lacZ, activation of Cre at E10.5 results in X-gal labeled cells in both gray and white matter throughout the dorsoventral axis (D) and cells co-label with both NeuN and APC (J, arrows).
and astrocytes (reviewed by Wiese et al., 2004). By contrast to what was seen with the Ascl1-CreERTM strain, activation of Cre in Nestin-expressing cells at E10.5 and analysis at P30 resulted in X-gal stained cells in both gray and white matter (Fig. 4D). Consistent with the presence of Nestin in neural progenitor cells in the ventricular zone, the lineage-traced cells co-labeled with the neuronal marker NeuN, the oligodendrocyte marker APC or the astrocytic marker GFAP (Fig. 4J and data not shown).

The second experimental paradigm examined early time points after tamoxifen induction to determine if labeled cells could be detected within cycling ventricular zone cells. R26R-stop-YFP females crossed to Ascl1-CreERTM or Nestin-CreERT2 were treated with tamoxifen at 10.5 dpc and embryos were harvested 24 hours later. Using Nestin-CreERT2, YFP+ cells were found scattered throughout the neural tube, present both in mitotically active cells in the ventricular zone, as assessed by the cell cycle protein (Ki67+), and postmitotic cells in the mantle zone (Ki67−) (Fig. 5C,C′). By contrast, using Ascl1-CreERTM, YFP+ cells were found almost exclusively in the mantle zone, and they never co-labeled with Ki67 or incorporated BrdU (Fig. 5A,A′; data not shown). The absence of labeled cells in the ventricular zone was also noted in the Ascl1-CreERTM, R26R-stop-LacZ timecourse (see Fig. 3A−D′). Thus, within the activation and detection limits of these Cre reporter systems, cells expressing Ascl1, and thus, CreERTM, rapidly lose progenitor cell identity. Taken together, these results are consistent with the hypothesis that Ascl1+ cells, at least cells with high levels of Ascl1, do not define progenitors in the ventricular zone common to both neurons and oligodendrocytes but rather define neuronal-restricted precursors during neurogenic periods, and oligodendrocyte-restricted precursors later during gliogenesis.

Ascl1 facilitates restriction of progenitor cells to the neuronal lineage during neurogenesis

It is known that Ascl1 functions in neuron differentiation and neuron specification in mouse and chick spinal neural tubes (Helms et al., 2005; Nakada et al., 2004; Torii et al., 1999). Here we address the role of Ascl1 in neuron-restricted precursors (∼E11 marked cells) by following their fate in an Ascl1 null background. Ascl1-CreERTM,R26R-stop-YFP;Ascl1+/− or Ascl1-CreERTM,R26R-stop-YFP;Ascl1−/− embryos were treated with tamoxifen at E10.5 and the characteristics of the YFP+ cells were examined at E11.5 and 17.5 (Figs 5, 6). As reported in the previous section, the recombined cells expressing YFP were postmitotic and found lateral to the ventricular zone as soon as they could be detected (Fig. 5A,A′). By contrast, in the Ascl1 mutant, the YFP+ cells aberrantly persisted in the ventricular zone, and some of these cells remained in the cell cycle (Fig. 5B, B′). We next examined whether the fate of these cells was altered in later development. As the Ascl1 null is neonatal lethal (Guillemot et al., 1993), we harvested embryos at E17.5. Normally most YFP+ cells generated from the E10.5 tamoxifen treatment coexpressed NeuN, a neuronal marker (88%; Fig. 6A, graph). Only rarely, if at all, did the YFP+ cells express markers for oligodendrocytes (Sox10 or Olig2; Fig. 6B, E, graph), astrocytes (GFAP+ or Glast+; Fig. 6D, C,D) or mitotically active cells (BrdU+ or Ki67+; Fig. 6F; and data not shown). By contrast, YFP+ cells in the Ascl1 null were less likely to become neurons (54% NeuN+; Fig. 6A′) and more likely to coexpress markers of the other cell identities (Fig. 6B′−F′). The percentage of YFP+ cells coexpressing Olig2 and Glast increased from essentially zero to 16 and 22%, respectively (Fig. 6D′, E′). The Olig2 cells probably represent immature oligodendrocytes, as there was no increase in Sox10 (Fig. 6B′). Likewise, the increase in YFP+ cells expressing Glast probably represents immature astrocytes, as they are largely postmitotic, and thus not progenitor cells (only 1.8 and 0.63% of YFP+ cells were BrdU+ or Ki67+, respectively; Fig. 6F′, graph), and only a small percentage of YFP+ cells labeled with the astrocyte marker GFAP+ (2%; Fig. 6C′). This shift in the fate of cells in the Ascl1 null from mature neurons to cells expressing immature glia markers supports a role for Ascl1 for efficient differentiation of progenitors to a neuronal lineage during neurogenesis.

DISCUSSION

There have been tremendous advances in the past few years using time-lapse imaging to describe the behavior of cells in the vertebrate neural tube as they proliferate and differentiate into neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). These elegant studies have identified radial glia as neuronal progenitor cells, and have characterized division patterns, migration properties and cell-type identity of daughter cells in vivo. The next advances must be in identifying the molecules controlling these processes. It is within this context that we now place the neural differentiation gene Ascl1. Ascl1 is crucial for regulating neural differentiation in multiple regions of the central and peripheral nervous systems. The function for Ascl1 in neuronal differentiation and specification during embryonic stages has been clearly demonstrated (reviewed by Bertrand et al., 2002). Because of the sequential nature of neurogenesis and gliogenesis, and the existence of common progenitor cells for neurons and oligodendrocytes, an independent role for Ascl1 in oligodendrogenesis in neural tube has not been demonstrated. Here we take advantage of the temporal separation of neurogenesis and gliogenesis in the embryonic spinal cord to gain more precision in identifying the stage that Ascl1 is present. We found that Ascl1-expressing cells detected early in the Ascl1-CreERTM transgenic line did not mark common progenitor cells but rather were restricted to neuronal lineages. Later, Ascl1-
Ascl1 defines lineage-restricted precursors

Ascl1 in neuronal lineage-restricted precursor cells

Progenitor cells in the ventricular zone of the vertebrate neural tube undergo a growth phase to increase the population of progenitor cells followed by divisions that begin to generate neurons. This process involves asymmetric divisions that give rise to differentiated cells and a progenitor, and symmetric divisions that give rise to two differentiated cells (Roegiers and Jan, 2004; Wodarz and Huttner, 2003). However, the contribution of these distinct modes of division to different regions of the developing vertebrate nervous system is not clear in many cases, with most studies focusing only on cortical development (Miyata et al., 2004; Noctor et al., 2004; Qian et al., 1998; Qian et al., 2000). In more caudal regions of the CNS such as hindbrain, there is little evidence for asymmetric divisions, but rather a majority of neurons are derived from symmetric divisions yielding two neurons (Lyons et al., 2003). Our data suggest that during neurogenesis Ascl1 is present in cells undergoing their final division, and this division is symmetrical with respect to neural cell fate, with both daughters becoming neurons. This is demonstrated by activation of Cre in Ascl1-CreERTM mice during neurogenesis (E10.5). Analysis of these animals within 24 hours of tamoxifen treatment revealed that the lineage-marked cells were located lateral to the ventricular zone and did not label with Ki67 (Fig. 5), and at P30 the lineage reporter only labeled neurons (Fig. 4). As Ascl1 overlaps with BrdU-incorporating cells in the ventricular zone (Helms et al., 2005), these results are consistent with Ascl1 being present in cells that are undergoing terminal symmetrical divisions to give rise to neurons.

We demonstrate that Ascl1 is required for efficient neuronal differentiation. This supports conclusions from previous studies (Casarosa et al., 1999; Horton et al., 1999; Torii et al., 1999), but here we follow the fate of the mutant cell into the late-stage embryo. Ascl1 mutant cells identified at E10.5 were less likely to become neurons (30% reduction) than if Ascl1 was present. The neurons that did develop may have used an alternative neural bHLH factor such as Neurog1, Neurog2 and Neurod4 (previously Ngn1, Ngn2 and Math3, respectively) and if so the specification of neuronal type may be altered, as suggested by previous studies (Helms et al., 2005; Kriks et al., 2005; Mizuguchi et al., 2006; Nakada et al., 2004; Wildner et al., 2006). In addition to the reduction in the percentage of YFP+ cells that became neurons, in the Ascl1 null some cells were maintained in an aberrant progenitor state – as defined by BrdU incorporation and expression of Ki67, and expression of Olig2 in the absence of Sox10 – or aberrantly became astrocytic as defined by Glast and GFAP expression (Fig. 6). The simplest interpretation of these data is that the mutant cells have a diminished capacity to differentiate properly. However, this interpretation is complicated by the fact that the loss of Ascl1 results in an increase in expression from the Ascl1 locus due to negative feedback (Horton et al., 1999; Meredith and Johnson, 2000). Thus, in the Ascl1 null, we may be detecting upregulation of Cre in cells not normally expressing Ascl1.

The specific pathways controlled by Ascl1 continue to be an open question. The best-characterized downstream effect of Ascl1 activity is the increase in expression of the Notch ligands Delta1 and Delta3, which should activate Notch signaling in adjacent cells and suppress differentiation (reviewed by Yoon and Gaiano, 2005). What other changes in gene expression are controlled by Ascl1 to induce the cell to differentiate? The transcriptional targets for Ascl1 may be the same in both neurogenesis and oligodendrogenesis; however, it is also possible that the downstream targets change during these two processes based on the context-dependent co-factors.
Ascl1 in oligodendrocyte lineage-restricted precursor cells

Our results demonstrate Ascl1-expressing cells in the spinal neural tube give rise to oligodendrocytes throughout the spinal cord dorsally and ventrally, with an enrichment in the dorsal funiculus. Furthermore, the Ascl1-derived oligodendrocytes begin appearing after E14.5, suggesting that they may represent the late-developing oligodendrocytes originating from the dorsal neural tube rather than the early oligodendrocytes generated as early as E12.5 from more ventral regions (reviewed by Cai et al., 2005; Fogarty et al., 2005; Richardson et al., 2006; Vallstedt et al., 2005). Expression of Pax7 and Ascl1 in a subset of the Olig2 cells at E14.5 suggested that the dorsal oligodendrocytes originate from dp3, dp4 and dp5 (Cai et al., 2005), progenitor domains defined by Ascl1 (Gross et al., 2002; Helms et al., 2005; Müller et al., 2002). We used a newly generated antibody to demonstrate that at E16.5 Ascl1 was not restricted to dorsal domains but rather was detected throughout the ventricular zone and in scattered cells of the gray and white matter. This suggests that the oligodendrocytes derived from these cells are late formed but are not necessarily restricted to a dorsal origin. However, genetic fate mapping of another gene, Mx3, which is restricted to the dorsal neural tube, resulted in oligodendrocytes concentrated in the dorsal funiculus (M. Fogarty, Thesis, University of London, 2005), similar to that seen here for Ascl1. This contrasts with the distribution of oligodendrocytes derived from nestin-expressing cells from ~E11 (Fig. 4C,D), illustrating the distinct temporal and spatial origins of these populations.

The function of Ascl1 in the oligodendrocyte lineage in the spinal cord has not been addressed in the Ascl1 mutant. Discerning the phenotype in the oligodendrocyte population is complicated by the disruption of the progenitor domain at earlier stages (Figs 5, 6). However, a role for Ascl1 in the development of the oligodendrocyte lineage in the postnatal brain has recently been examined (Parras et al., 2004). The SVZ in the postnatal forebrain is the source for the rostral migratory stream (RMS), which supplies the olfactory bulb with neurons and oligodendrocytes (Marshall et al., 2003; Pencea and Luskin, 2003). Recently, it was demonstrated using a short-term lineage-tracing method that Ascl1 in the SVZ and RMS identifies the transit amplifying cells that give rise to these cells (Parras et al., 2004). Furthermore, in the Ascl1 mutant, neuropeptide spheres derived from this region are altered in their potential; they have a decreased ability to generate both the neurons and oligodendrocytes, whereas there is an increase in generation of astrocytes (Parras et al., 2004). The precise function of Ascl1 in the oligodendrocyte lineage in embryonic and postnatal stages remains to be determined. Understanding the full repertoire of Ascl1 function in nervous system development will require dissection of these functions in a conditional Ascl1 knockout paradigm.

The results presented here place Ascl1 distinctly in cells that are transitioning from cycling progenitor or stem cells to progenitors with limited potential for cell division and restrictions on the cell types formed. Importantly, oligodendrocytes derived from the Ascl1 lineage may be more extensive than previously appreciated. Indeed, Ascl1 cannot be thought of as simply a neuronal differentiation factor, but rather as a more general differentiation factor, and the cell type that arises depends on the stage at which Ascl1 is expressed.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/2/285/DC1

References
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