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Common Developmental Requirement for *Olig* Function Indicates a Motor Neuron/Oligodendrocyte Connection

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Summary

The oligodendrocyte lineage genes *Olig1* and *Olig2* encode related bHLH proteins that are coexpressed in neural progenitors. Targeted disruption of these two genes sheds light on the ontogeny of oligodendroglia and genetic requirements for their development from multipotent CNS progenitors. *Olig2* is required for oligodendrocyte and motor neuron specification in the spinal cord. *Olig1* has roles in development and maturation of oligodendrocytes, evident especially within the brain. Both *Olig* genes contribute to neural pattern formation. Neither *Olig* gene is required for astrocytes. These findings, together with fate mapping analysis of *Olig*-expressing cells, indicate that oligodendrocytes are derived from *Olig*-specified progenitors that give rise also to neurons.

Introduction

During evolution, the appearance of myelinating oligodendrocytes facilitated increased complexity of the vertebrate central nervous system (CNS), in part by enabling saltatory conduction of the nervous impulse. However, the developmental origins of oligodendrocytes and genetic requirements for their specification from multipotent neuroepithelia are poorly understood. Oligodendrocyte precursors emerge from a ventral region of the embryonic spinal cord in which generation of cellular diversity is thought to depend on graded inductive and repressive signals derived from local organizing centers. The secreted glycoprotein, Sonic hedgehog (Shh), produced in ventral midline structures such as the notochord and floorplate, is essential for induction and maintenance of neural cell types including oligodendrocytes (Orentas et al., 1999; Alberta et al., 2001). Downstream targets of Shh include a pair of *Oligodendrocyte lineage genes (Olig1/2)* that encode basic-helix-loop-helix (bHLH) transcription factors (Lu et al. 2000; Zhou et al., 2000).

Transcription factors with bHLH domains have many established and conserved roles during neural development, including determination of neuronal versus glial cell fate (Lee 1997; Kageyama and Nakanishi 1997). *Olig* genes mark oligodendrocyte precursors and their progeny in a restricted domain of the murine ventral spinal cord (Lu et al., 2000; Zhou et al., 2000). The *Olig2* gene has been structurally well conserved from humans down through *Drosophila* and nematode worms. *Olig1* might have arisen more recently via a gene duplication event (see Discussion). Human OLIG1 and OLIG2 proteins are roughly 80% similar to each other within the bHLH domains. Another distinctive feature common to both OLIG proteins is a domain of contiguous serine residues toward the amino terminus. In the human brain, *OLIG1* and *OLIG2* are expressed in mature oligodendrocytes and serve as markers for oligodendroglioma (Lu et al., 2001b; Marie et al., 2001). A third member of the gene family, *Olig3*, is structurally related to *Olig1* and *Olig2*. However, expression of *Olig3* is not confined to the CNS (Takebayashi et al., 2000).

In the vertebrate embryonic neural tube, expression of the *Olig* genes is coordinately regulated by Shh (Lu et al., 2000). *Olig1* and *Olig2* are expressed as early as 8.5 dpc in the murine spinal cord (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000) within a specific region known to give rise both to motor neurons and to oligodendrocyte precursors. In gain-of-function experiments, both *Olig1* and *Olig2* have been shown to be sufficient for formation of oligodendrocytes or early oligodendrocyte progenitor cells (Lu et al., 2001a; Zhou et al., 2001). However, this approach has suggested further biological activities of Olig proteins in neural cell fate acquisition (Mizuguchi et al., 2001; Novitch et al., 2001; Sun et al., 2001; Zhou et al., 2001). For instance, *Olig2* can function as a determinant of motor neuron specification in conjunction with the bHLH factor *Ngn2* and other homeodomain proteins in the ventral neural tube (Mizuguchi et al., 2001; Novitch et al., 2001).

To resolve the developmental requirements for these two genes, we have generated null mutations of *Olig1* and *Olig2*. Collectively, our results demonstrate that *Olig* genes are necessary for fundamental processes of CNS development including formation of oligodendrocytes. However, our data also lead to the surprising conclusion that the two *Olig* genes, though structurally similar and coordinately expressed, encode proteins with distinct biological capabilities. *Olig2*, the "primordial" *Olig* gene, plays especially prominent roles in the developing spinal cord where it is essential for oligodendrocyte and motor neuron specification. *Olig1* is not required for motor neuron development. However, *Olig1* promotes formation and maturation of oligodendrocytes, especially within the brain. Moreover, *Olig1* cooperates with *Olig2* to establish the pMN domain of the embryonic neural tube. A striking feature of the *Olig* null phenotypes is that astrocyte formation proceeds normally in mice that fail to develop oligodendrocytes. This observation, together with fate mapping analysis of *Olig*-expressing cells, challenges the view that oligodendrocytes and astrocytes arise exclusively from a glial-restricted precursor.

Results

Generation of *Olig1* and *Olig2* Null Alleles

We performed single knockouts of *Olig1* and *Olig2* by homologous recombination in embryonic stem (ES) cells.

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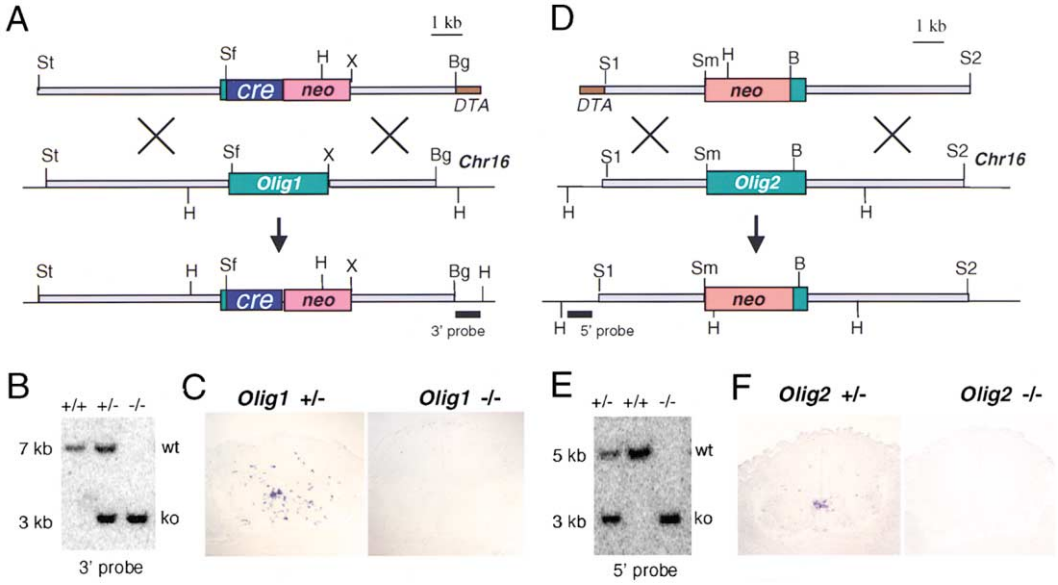

Figure 1. Disruption of *Olig1* and *Olig2* by Gene Targeting

(A) Schematic of the strategy for disruption of *Olig1*. The targeting construct, the *Olig1* locus, and the locus after the predicted homologous recombination event are shown. Abbreviations: *cre*, bacteriophage P1 cre recombinase; *neo*, the *neomycin* positive selection marker cassette; and *DTA,* the diphtheria toxin gene negative selection marker. The thick line below the *Olig1* locus represents the 3′ probe used for Southern analysis of the homologous recombination event.

(B) Southern blot validation of the *Olig1* homologous recombination event. The 3′ *Olig1* probe (above) identifies the targeted locus in homozygous mutant embryos as a 3 kb band following digestion of embryonic DNA with HindIII. The wild-type 7 kb band in heterozygous and wild-type embryos is also seen.

(C) In situ hybridization of *Olig1* heterozygous and homozygous animals for *Olig1* expression. Crosssections of 13.5 dpc spinal cord are shown. Note absence of *Olig1* expression within the null mutant.

(D) Targeting strategy for the *Olig2* locus. The thick line below the *Olig2* locus shows the 5′ probe used for Southern analysis.

(E) Southern blot validation of the *Olig2* homologous recombination event using the 5′ probe following digestion of genomic DNA with HindIII. The targeted locus is identified in homozygous mutant embryos as a 3 kb band by the 5′ probe. The wild-type 5 kb band in heterozygous and wild-type embryos is also shown.

(F) In situ hybridization was used to confirm a null allele of *Olig2*. Crosssections of 13.5 dpc spinal cord are shown. Abbreviations: St, StuI; Sf, SfiI; H, HindIV; Bg, BglII; B, BamHI; X, XbaI; Sm, SmaI; S1, SacI; and S2, SacII.

Our targeting strategy deleted the majority of the *Olig1* or *Olig2* coding region, including the bHLH domain. Additionally, recombination at the *Olig1* locus incorporated knockin of bacteriophage P1 *cre* recombinase. Transmission of both targeted alleles through the germline was confirmed by Southern blotting. Transcripts of *Olig1* and *Olig2* were undetectable by in situ hybridization in the respective homozygous mutant mice (Figure 1). It is technically infeasible to generate *Olig1/2* double-null mice by intercrosses because the two genes are closely linked on mouse chromosome 16. A companion paper to this one describes the phenotype of compound mutant mice generated by an independent targeting approach (Zhou and Anderson, 2002 [this issue of *Cell*]).

Mature animals that were wild-type, heterozygous, and homozygous with respect to the *Olig1* mutation were obtained in Mendelian ratios. However, we failed to generate any pups at weaning age that were homozygous for mutations of *Olig2*. At embryonic and fetal stages as late as 18.5 dpc, *Olig2* null homozygotes were present, indicating that death likely occurs in the neonatal period. Indeed, *Olig2*$^{-/-}$ fetuses lacked spontaneous movement or breathing activity, suggesting a devastating abnormality in neurological function.

Olig Gene Functions Are Essential for Oligodendrocyte Development in the Spinal Cord

In wild-type animals, expression of the early oligodendrocyte markers *PDGFαR* and *Sox10* is detected at 12.5 dpc in the ventral ventricular zone of the neural tube at the forelimb level. The more mature markers *PLP/DM20* and *Myelin basic protein (MBP)* are expressed from 14.5 dpc onward during embryonic development (Figure 2A; Pringle and Richardson, 1993; Qi et al., 2001; Timsit et al., 1995). In *Olig2* null mice, expression of early oligodendrocyte markers is undetectable at any level of the spinal cord, even at the latest collectable date of 18.5 dpc (Figure 2A). The late oligodendrocyte markers likewise are not expressed from 14.5 to 18.5 dpc (Figure 2A).

Does loss of *Olig2* ablate or merely delay the formation of oligodendrocytes in the spinal cord? Following the protocol of Park et al. (2001), we cultured caudal spinal cord explants from E15.5 mouse embryos to the equivalent of P6 in vitro and then immunostained for expression of O4. From wild-type mice, 100% (38/38) of the explants contained O4-positive cells versus none (0/29) of the explants from *Olig2* null mice. Thus, formation of oligodendrocytes evidently is ablated if not severely retarded in the absence of *Olig2* function. In contrast, we note

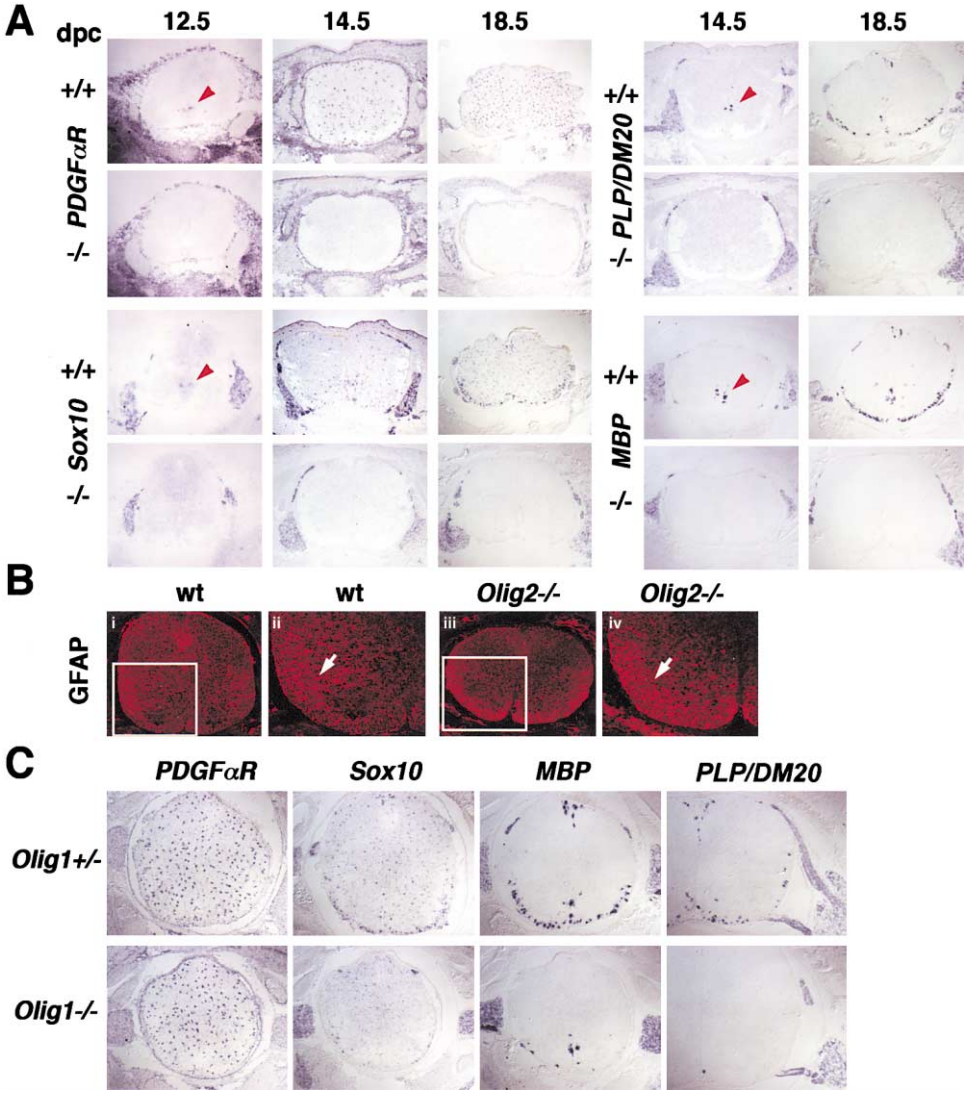

Figure 2. *Olig* Function Is Required for Oligodendrocyte But Not Astrocyte Development in the Murine Spinal Cord

(A) Expression of mRNA transcripts for the oligodendrocyte-associated markers *PDGFαR*, *Sox10*, *PLP/DM20*, and *Myelin basic protein (MBP)* was analyzed in situ on frozen sections of spinal cord taken from wild-type (+/+) or *Olig2* null (−/−) embryos at 12.5 dpc, 14.5 dpc, or 18.5 dpc, as indicated. Note absence of all oligodendrocyte markers in *Olig2* null mutants at all stages tested. The red arrowheads mark the initial expression PDGFαR/*Sox10*-positive *PLP/DM20* and *MB*-positive cells. Note that *PLP/DM20* and *MBP* are also expressed in the peripheral nervous system.

(B) Astrocyte development is normal in the spinal cord of 18.5 dpc (iii and iv) *Olig2* null embryos, compared with (i and ii) wild-type as shown by equivalent expression of Glial Fibrillary Acidic Protein (GFAP) in the ventral spinal cord. The boxed areas in (i) and (iii) are shown at higher power in (ii) and (iv).

(C) Delay of oligodendrocyte maturation in *Olig1* null mice. Expression of the oligodendrocyte-associated markers *PDGFαR*, *Sox10*, *MBP*, and *PLP/DM20* was analyzed in situ on frozen sections of P0 spinal cord taken from heterozygous (+/−) or homozygous null (−/−) *Olig1* animals as indicated. Delay of *Sox10*, *MBP*, and *DM20/PLP* expression in white matter of spinal cord was observed, indicating a role for *Olig1* in oligodendrocyte maturation.

that astrocyte development is apparently unperturbed, because similar levels of GFAP expression are observed in both wild-type and *Olig2* null embryos (Figure 2B).

The contribution of *Olig1* function to oligodendrocyte development at spinal cord levels is relatively subtle. Expression of the early oligodendrocyte marker *PDGFαR* is activated on schedule in *Olig1* null mice at 12.5 dpc (data not shown) and is persistent at P0 dpc (Figure 2C). However, expression of the oligodendrocyte progenitor marker, *Sox10*, and the later markers, *MBP* and *DM20/PLP*, are clearly delayed—at least within the white matter of spinal cord (Figure 2C). Although normal oligodendrocytes will eventually form in these animals by P30, the data indicate roles for *Olig1* function in oligodendrocyte maturation. Again, astrocyte formation is unperturbed in *Olig1* null mice (data not shown). Thus, astrocyte development in general does not appear to require *Olig* function or contemporaneous production oligodendro-

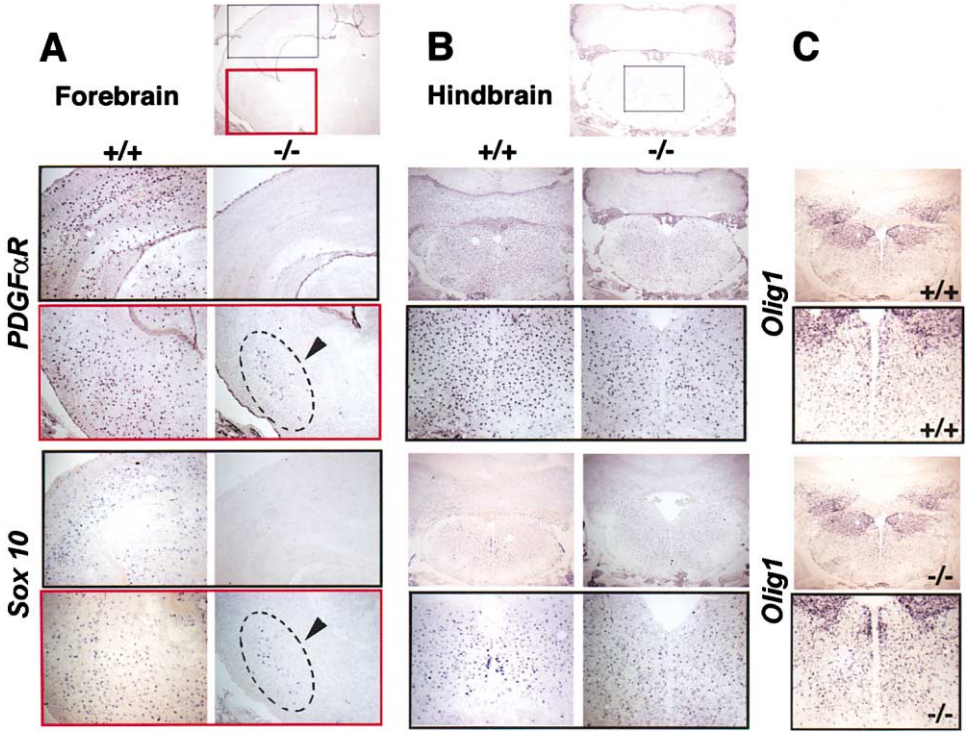

Figure 3. Evidence that *Olig1* Function Compensates for Loss of *Olig2* during Oligodendrocyte Development in the Brain

(A) Presence of oligodendrocyte precursors (OLP) in the forebrain of 18.5 dpc *Olig2*$^{-/-}$ mutants. Expression of *PDGFαR* and *Sox10* was analyzed in situ on frozen sections of forebrain of wild-type (+/+) and *Olig2* null (−/−) mice, as indicated. Black and red borders indicate areas of dorsal and ventral forebrain that correspond (respectively) to the black and red boxes illustrated in the upper schematic. Note presence of a small collection of OLP in the ventral forebrain sections (areas indicated by arrowheads).

(B) Hindbrain images of *Olig2* null mice. Low-power and high-power views are shown. The high-power views (black borders) correspond to the black boxed area in the upper schematic. Note nearly normal numbers of OLP in the hindbrain of *Olig2*$^{-/-}$ mutants.

(C) *Olig1* expression corresponds to area of OLP formation in the hindbrain of *Olig2* null mice. Low-power and high-power (black border) photomicrographs of *Olig1* expression in the hindbrain of wild-type (+/+) and Olig2 null (−/−) mice, as indicated.

cytes. Our results, however, do not rule out a subtle effect on astrocyte subpopulations that might emerge from the *Olig* expression domain.

Olig1 Complements Loss of *Olig2* Function during Oligodendrocyte Development in the Brain

Insights into the functions of *Olig1* within the brain can be drawn by comparing phenotypes of the *Olig2* null mouse shown here with that of the *Olig1/2* double-null mouse described in a companion paper (Zhou and Anderson, 2002 [this issue of *Cell*]). Within the ventral forebrain of the *Olig2* null mice, we observe focal areas of *PDGFαR* and Sox10-expressing oligodendrocyte precursors. Within the hindbrain, early stages of oligodendrocyte development proceed largely unimpaired (Figure 3). These results within the brain are in marked contrast to spinal cord where we observe a total absence of oligodendrocyte precursors in *Olig2*$^{-/-}$ animals. Does the formation of oligodendrocyte progenitors in the brains of Olig2 null mice reflect the activity of *Olig1*? Interestingly, *Olig1* is expressed in the hindbrain at positions where oligodendrocyte precursors are observed in *Olig2* null mice (Figure 3). Moreover, there is a complete failure of oligodendrocyte development in all regions of the brain—including the hindbrain—in mice with compound *Olig1*/*Olig2* null mutations (Zhou and Anderson, 2002 [this issue of *Cell*]). Together, these findings indicate that *Olig1* function is necessary and sufficient for oligodendrocyte development in the absence of *Olig2* function, albeit in a regionally restricted manner (see Discussion).

Olig2 Function Is Required for Somatic Motor Neuron Development in Spinal Cord and Hindbrain

A striking aspect of the *Olig2* mutant phenotype was an absence of spontaneous or elicited movement in newborns, suggesting a neurological abnormality. To establish a possible requirement for *Olig2* function during neuronogenesis, we examined the spinal cord of wild-type and *Olig2* mutant embryos with the postmitotic motor neuron markers *Isl1* and *Isl2* as well as *Hb9*, the earliest known marker of motor neuron development in the mouse (Arber et al., 1999; Pfaff et al., 1996). In wild-type mouse embryos, these markers identify somatic and visceral motor neuron populations of the spinal cord (Figure 4). In dramatic contrast, no *Hb9*- or *Isl2*-positive motor neurons are detected at any stages examined within the spinal cord of *Olig2*$^{-/-}$ embryos (Figure 4). A few *Isl1*-positive cells are observed within the lateral ventral neural tube at 9.5 dpc. However, by 11 dpc all *Isl1*-positive cells are also lost. Within the

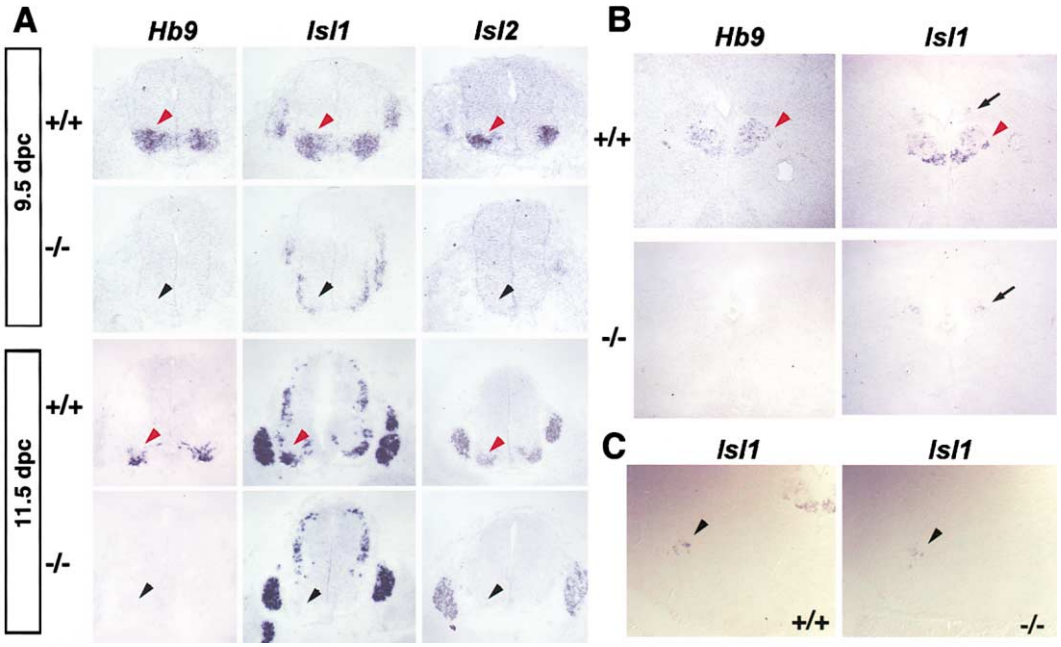

Figure 4. *Olig2* Function Is Required for Development of Somatic Motor Neurons in the Spinal Cord and the Hindbrain

(A) Expression of mRNA transcripts for the motor neuron markers *Hb9*, *Isl1*, and *Isl2* was analyzed in situ on frozen sections of 9.5 dpc and 11.5 dpc spinal cord taken from wild-type (+/+) and *Olig2* null (−/−) embryos, as indicated. Note absence of the motor neurons marked by *Hb9* and *Isl2* in *Olig2*$^{-/-}$ mutants. A transient population of *Isl1*-expressing cells was noted in the ventral neural tube at 9.5 dpc but not at subsequent stages.

(B) Same as above except that tissue shown is the hindbrain taken from 18.5 dpc wild-type (+/+) and *Olig2* null (−/−) animals. Note absence of the somatic motor neurons marked by *Hb9* and *Isl1* in *Olig2*$^{-/-}$ mutants. Preganglion motor neurons marked by *Isl1* (arrows) are unaffected in *Olig2* null mice.

(C) Same as (B) except showing lateral/ventral hindbrain. Here, the *Isl1* marker identifies branchial motor neurons (arrows) that are retained in *Olig2* null mice, providing further evidence that *Olig 2* function is required specifically for development of somatic motor neurons.

hindbrain, *Olig2* null mice are devoid of somatic motor neurons marked by *Hb9* and *Isl1* (Figure 4B). However, preganglion motor neurons and branchial motor neurons marked by *Isl1* are unaffected (Figures 4B and 4C). Visceral motor neurons marked by *Phox2B* are also maintained in the hindbrain of *Olig2* null mice (data not shown). Collectively, our data indicate that *Olig2* is required for development of motor neurons in spinal cord as well as somatic motor neurons in hindbrain. In contrast, V2 interneurons are increased in *Olig2*$^{-/-}$ spinal cords and expanded ventrally into the motor neuron domain (Figure 5A). Antagonism of V2 interneuron development by *Olig2* is further suggested by a reciprocal experiment: ectopic expression of *Olig2* in the chick neural tube inhibits expression of the V2 markers *Chx10* (Figure 5B). No apparent difference is found for the expression of V3 interneurons (marked by *Sim1*). Thus, *Olig2* function is specifically required for development of motor neurons but not V2 or V3 interneurons.

Olig2 Function Is Necessary for Pattern Formation in Ventral Neural Tube

The motor neuron progenitor domain (pMN), which gives rise to motor neurons and oligodendrocytes (Jessell, 2000), is located dorsal to *Nkx2.2*-expressing progenitor cells in a region of low *Pax6* expression (see Figure 5C). In turn, the "p2" domain, which gives rise to V2 interneurons, lies dorsal to the pMN in a region that expresses *Irx3* and high *Pax6* levels. As shown (Figure 5C), in the absence of *Olig2* function we observe normal expression of *Nkx2.2*. The ventral border of the Pax6 low domain expands ventrally to abut the *Nkx2.2*-expressing progenitors in the *Olig2*$^{-/-}$ neural tube, whereas a slight gap between these regions is observed in wild-type mice. In addition, ventral expansion of the p2 progenitor marker, *Irx3*, is also observed in *Olig2* null mice. However, both the *Pax6* low and high domains are detectable. Together, these results suggest that *Olig2* has a role in suppression of *Irx3* expression and that it may also modulate levels of *Pax6* in the pMN.

Olig^{+} Cells in the pMN Domain Are Maintained in the Absence of *Olig2* Function

As shown in Figure 5D, cells that express several markers of pMN progenitor cells (e.g., Nkx6.1, Ngn2) persist in the spinal cord of *Olig2* mutant animals, despite a lack of Hb9-positive motor neuron precursors. These results suggest a failure in motor neuron specification from precursor cells in the *Olig2*$^{-/-}$ mutant spinal cord. To further evaluate the fate of progenitor cells in the pMN region of *Olig2* null mutants, we monitored mRNA transcript expression directly from the loci of *Olig* genes. Previous work has shown that *Olig1* and *Olig2* are coexpressed within cells of the ventral neural tube from 9.5 to 12.5 dpc (Lu et al., 2000; Zhou et al., 2000). However, *Olig2* is expressed consistently at high levels within the

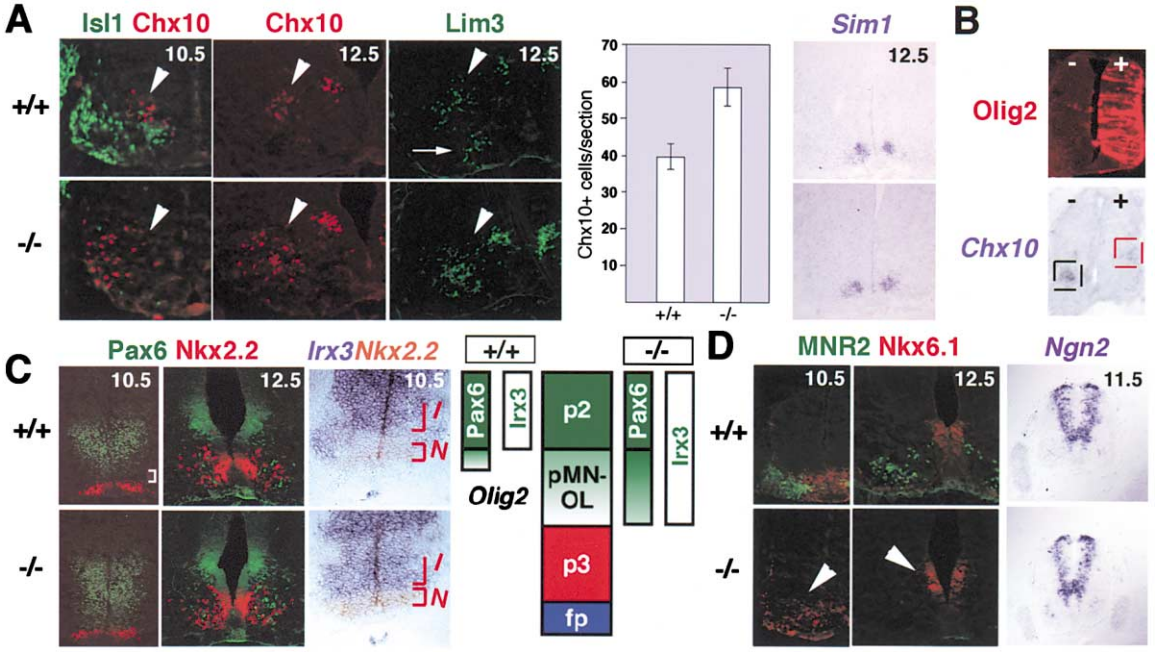

Figure 5. Perturbation of Neural Patterning in Ventral Spinal Cord of Olig2 Null Mice

(A) *Olig2* regulates the number and distribution of V2 interneurons and is not required for V3 interneuron development. Frozen sections of 10.5 and 12.5 dpc spinal cord taken from wild-type (+/+) and *Olig2*$^{-/-}$ (−/−) animals as indicated. V2 interneurons marked by antibodies to Chx10 and to Lim3 (which marks the mouse Lhx3 gene product) were analyzed by immunohistochemistry (red and green, respectively). Chx10^{+} cells were counted on five sections at the forelimb level from four individual mutant and control embryos at E10.5. Increased numbers and ventral expansion of the V2 interneuron population are observed in *Olig2* null mice. Note that Chx10-positive interneurons innervate to the motor neuron domain marked by Isl1/2 and that motor neurons marked by Lim3 (small arrow) are lost in the *Olig2* null embryo. The histogram shows quantitation of Chx10^{+} cells in multiple sections taken from *Olig2*$^{-/-}$ and wild-type embryos. We observed a significant (approximately 45%, $p < 0.001$) increase in V2 interneurons in *Olig2*$^{-/-}$ embryos. Expression of mRNA transcripts for the V3 interneuron marker *Sim1* (assayed by in situ hybridization) is evidently normal in *Olig2* mutants.

(B) Olig2 inhibits production of V2 interneurons. Mouse *Olig2* cDNA was electroporated unilaterally into HH stage 10–12 chick neural tube, and embryos were harvested at stage 21–23 for analysis. Upper and lower images show distribution of Olig2 proteins and expression of *Chx10* mRNA, respectively. Note relative downregulation of *Chx10* on electroporated side (red box). The data is representative of results in 12 electroporated embryos. Similar results were obtained for *Chx10.1* (not shown).

(C) Ventralization of Pax6 and Irx3 neural patterning markers in ventral spinal cord of *Olig2* null mice. Double-labeling with antibodies for Nkx2.2 (red) and Pax6 (green) and double in situ hybridization for Irx3 (purple) and Nkx2.2 (brown) indicates that ventral expansion of Pax6 and Irx3 expression domains in *Olig2* null mice relative to wild-type animals. In *Olig2* null mice, the *Pax6* and *Irx3* domains ventrally abut to the *Nkx2.2* expression domain, which is unchanged relative to wild-type mice (see schematic diagram to right).

(D) Persistence of markers of motor neuron precursor populations within *Olig2* null mutants. Double immunohistochemistry at 10.5 and 12.5 dpc indicates that motor neuron precursor cells (marked by Nkx6.1 in red) but not postmitotic motor neurons (marked by MNR2 in green) are maintained in *Olig2* null mice. The motor neuron precursor domain marked by *Ngn2* (Mizuguchi et al., 2001) is also maintained in *Olig2* null mice, as shown by in situ hybridization at 11.5 dpc.

pMN, whereas *Olig1* is downregulated at 11.5 dpc. In contrast (Figure 6), *Olig1* expression persists and in fact is upregulated at 11.5 dpc in *Olig2* null embryos, relative to wild-type.

Expression of *neomycin (neo)* serves as a proxy marker of the targeted *Olig2* locus itself. *Olig2-neo* expression persists in ventricular zone of the *Olig2* null spinal cord, and the pattern of *Olig2-neo* expression coincides precisely with that of *Olig1*. Indeed, coexpression of *Olig1* and *Olig2-neo* is observed (Figure 6). From 14.5 days onward, the *Olig1*-positive cells appear to migrate outwards from the ventral neural tube of *Olig2* mutants, although the number of cells is much reduced relative to wild-type (see Discussion).

In summary, cells expressing multiple independent markers associated with motor neuron progenitors of pMN persist in *Olig2* null embryos. Despite this, motor neuron precursors fail to develop. The persistent expression of *Olig1* and *Olig2-neo* in the pMN domain of *Olig2* null embryos suggests that *Olig2* function is not essential for survival or growth of pMN progenitor cells. Indeed, TUNEL labeling of wild-type and *Olig2* mutant spinal cords at 9.5–12.5 dpc does not indicate differences in levels of programmed cell death, and similar levels of progenitor cell proliferation are observed (data not shown; Zhou and Anderson, 2002 [this issue of *Cell*]). We conclude that *Olig2* function is required for motor neuron and oligodendrocyte cell fate acquisition in the ventral spinal cord.

Olig1 Is Expressed within Motor Neuron Progenitors

The results presented above indicate that many cells of the pMN-OL domain fail to become specified in *Olig2* mutant embryos. This result is surprising because *Olig1* encodes a structurally related bHLH protein and *Olig1*

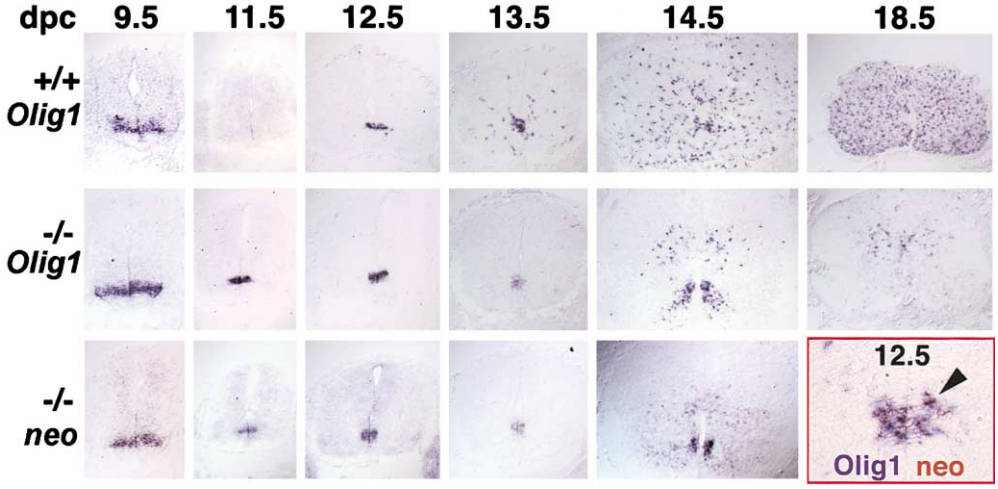

Figure 6. Persistence of *Olig*-Positive Cells in the Absence of *Olig2* Function

(Top and middle rows) Expression of *Olig1* was analyzed in situ on frozen sections of spinal cord taken from wild-type (+/+) or *Olig2* null (−/−) embryos at the indicated developmental stages. Note that at early times (9.5 and 11.5 dpc), *Olig1* expression is actually more robust in *Olig2* null mice. However, at later stages (13.5–18.5 dpc), the migration and/or proliferation of *Olig1*-positive cells is diminished in *Olig2* null mice. (Bottom row) As an independent method of visualizing *Olig*-positive cells in *Olig2* null mice, we monitored expression of the neomycin selectable marker gene within the *Olig2* targeting cassette (see Figure 1). Double in situ hybridization (boxed inset) shows colocalization of *Olig1* and *Olig2-neo* in spinal cord of an *Olig2* null mouse at 12.5 dpc.

expression is strongly maintained in *Olig2* mutants. However, an important caveat is that *Olig1* may not be expressed within the subset of progenitor cells that give rise to motor neurons and/or oligodendrocytes. To determine the fate of cellular progeny from the *Olig* expression domain, we mated *Olig1-cre* heterozygotes to the ROSA26 reporter strain carrying a floxed-STOP allele of *lacZ* (Soriano, 1999). In such matings, cre activity is required to allow synthesis of β-galactosidase proteins (Soriano, 1999). This strategy identifies all cells that express *Olig1* and also the progeny of any cells that have expressed *Olig1* at any time during development. As shown in Figure 7A, β-galactosidase activity is present within the motor neuron domain of F1 bigenic animals and colocalizes with the motor neuron markers *Isl1* and *Hb9* at 10.5 dpc. By 12.5 dpc, early oligodendrocyte markers (*Sox10* and *PDGFαR*) colocalize with expression of β-galactosidase (Figure 7A). These results establish cell-autonomous expression of *Olig1* in progenitors that can give rise to both motor neuron and oligodendrocytes. They further indicate that *Olig1* is insufficient for development of motor neurons in the absence of *Olig2* function.

Olig$^+$ Precursor Cells Give Rise to Oligodendrocytes But Not to Astrocytes

The data summarized in Figures 6 and 7A show that *Olig1* and *Olig2* are coordinately expressed within cells that are fated to become motor neurons and oligodendrocytes. Do these *Olig*-positive precursor cells give rise to astrocytes as well? To address this question, we used *Olig1-cre* to conduct further fate mapping experiments in the cortex and optic nerve where development of astrocytes and oligodendrocytes has been extensively characterized (Barres and Barde, 2000). As shown in Figure 7B, β-galactosidase proteins and the astrocyte marker, S100β, appear to segregate from each other in the optic nerve. In addition, β-galactosidase colocalizes with the oligodendrocyte marker protein, CC1, in the optic nerve (Figure 7B), as expected (Lu et al., 2000). Similar observations are made in the brain, where β-galactosidase protein is detected in oligodendrocytes and neurons, but not cells expressing the astrocytic marker S100β (Figure 7B and data not shown). Our findings are unlikely to reflect a lack of reporter gene expression in astrocytes, since β-galactosidase proteins are detected in age-matched optic nerve and cortical astrocytes from ROSA26 mice (data not shown).

Discussion

Do Oligodendrocytes Arise from a Glial-Restricted Progenitor Cell?

During evolution, oligodendrocytes appear coordinately with dorsalization and centralization of the nervous system and are found in all vertebrates higher than the jawless fishes. Astrocytes, the other major type of macroglial cell, have diverse roles in supporting and nurturing the neural cell bodies and in instructing brain capillaries to establish the blood-brain barrier (Kandel, 2000). Previous studies using tissue explants or cultured neural progenitor cells have suggested that oligodendrocytes in CNS arise from a glial-restricted precursor cell (see review by Lee et al., 2000). However, regulation of cell fate acquisition in the vertebrate central nervous system is complex and reliant on both cell-intrinsic factors and position-dependent extracellular cues (Jessell, 2000; McMahon, 2000). Accordingly, developmental options that are unmasked in cell culture/tissue explant studies are not necessarily utilized in developing animals.

In the embryonic spinal cord, several lines of evidence suggest that the development of myelinating oligoden-

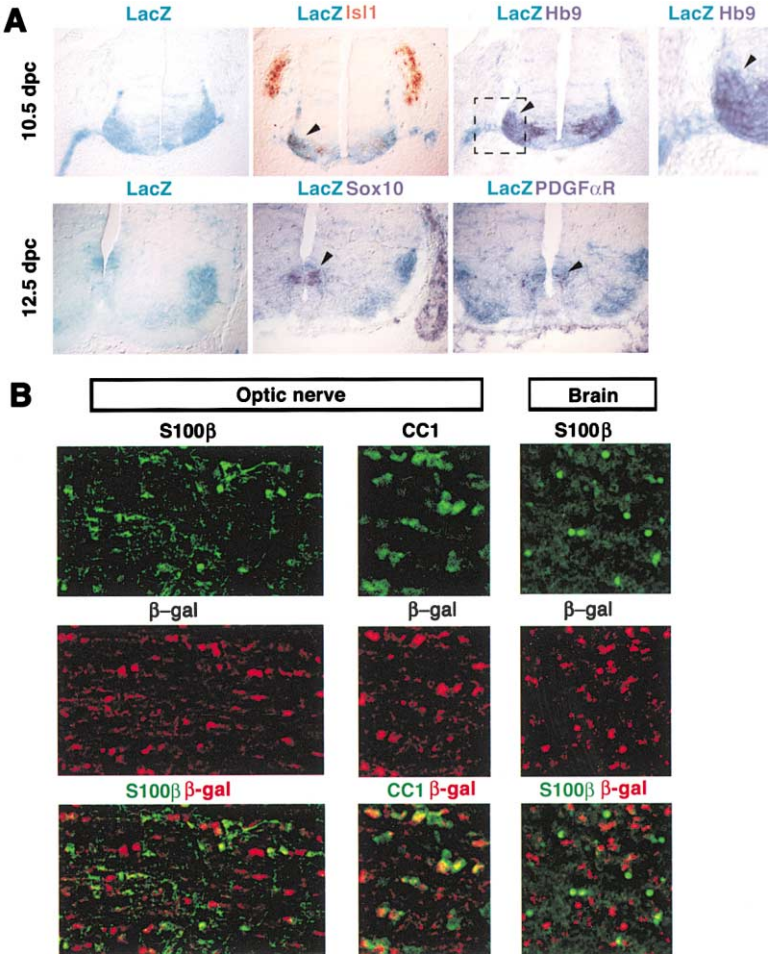

Figure 7. *Olig1* Is Expressed within a Subset of Progenitor Cells that Give Rise to Motor Neurons and Oligodendrocytes But Not Astrocytes

Olig1-cre mice (Figure 1) were intercrossed to a reporter strain of mice containing the STOP-*lacZ* allele at the *ROSA26* locus (Soriano, 1999). In the *cre*/*lacZ*-positive progeny of such intercrosses, β-galactosidase activity is restored in cells that express *cre*. Daughter cells can be followed at later stages of development by incubation with the substrate X-gal or by immunostaining with antibodies to β-galactosidase (β-gal).

(A) Colocalization of β-gal activity with motor neuron markers (*Isl1*, *Hb9*) and oligodendrocyte progenitor markers (*PDGFαR*, *Sox10*), as detected by in situ hybridization. Spinal cord sections were prepared from intercross embryos harvested at 10.5 or 12.5 dpc, as indicated. The boxed inset (right) is an enlarged view to show colocalization of β-gal activity and *Hb9* expression in motor neurons (note projection of axon bundles).

(B) Mutually exclusive expression of β-gal and the astrocyte marker S100β. Frozen sections of optic nerve and of brain from postnatal day 14 double hemizygous (*cre-lacZ*) mice were immunostained for β-gal, the astrocyte marker S100β, or CC1, an oligodendrocyte marker. Note mutually exclusive expression of S100β and β-gal in optic nerve and brain but coexpression of β-gal with CC1 in these representative images.

droglia is more closely linked to motor neurons than to astrocytes (for review see Richardson et al., 2000). For instance, oligodendrocyte progenitor cells arise from a restricted region of the ventral spinal cord where motor neuron fate acquisition is instructed by the organizing signal Shh (see reviews Jessell, 2000; Richardson et al., 2000). In contrast, astrocytes arise from broad and poorly defined areas in both dorsal and ventral regions of the neural tube (Pringle et al., 1998). Moreover, morphological and radiolabeling studies in optic nerve (Skoff, 1990) as well as lineage analysis studies using genetically tagged retroviruses in developing cortex (Luskin et al., 1988; Price and Thurlow, 1988) have generally suggested that oligodendrocytes and astrocytes arise from independent precursor cells in the developing CNS. In a retroviral lineage analysis study of postnatal rat forebrain, occasional clusters of cells that contained both oligodendrocytes and astrocytes were detected, suggesting the possibility of a common glial progenitor cell (Levison and Goldman, 1993). However, even in the postnatal brain, such clusters constituted only a small percentage (~15%) of those examined. The large majority of glial-containing cell clusters comprised only a single glial cell type (Levison and Goldman, 1993).

We observe a common requirement for *Olig2* function within cells that give rise to motor neurons and oligodendrocytes but not astrocytes. The dual genetic requirement for *Olig2* does not, in itself, indicate a lineage relationship between motor neurons and oligodendrocytes. However, lineage relationships are revealed through fate mapping experiments of the kind depicted in Figure 7 with *Olig1*. Data shown here (Figure 6) and elsewhere (Zhou et al., 2000) demonstrate that *Olig1* and *Olig2* are expressed in the same cells. Thus, *Olig1* can be used as a surrogate marker of *Olig2*-expressing cells. These fate-mapping experiments demonstrate that *Olig1* is expressed in cells that give rise to both oligodendrocytes and motor neurons in the developing spinal cord. In summary, our genetic analysis of *Olig* gene functions and our fate mapping of *Olig*-expressing progeny cells are incompatible with the view that oligodendrocytes arise from a glial-restricted precursor cell in the developing CNS. Alternative models can be put forward that account for these observations. For example, distinct unipotential $Olig^+$ pMN progenitors for either motor neurons or oligodendrocytes could be envisaged. However, we favor the simplest model: oligodendrocytes arise from a progenitor cell that also gives rise to motor neurons in the developing spinal cord (Figure 8A). Further work will be required to determine whether oligodendrocytes in the brain may also stem from a neuron-oligodendrocyte precursor. In this regard it is interesting to note that a common signal, Sonic hedgehog, is necessary for oligodendrocyte develop-

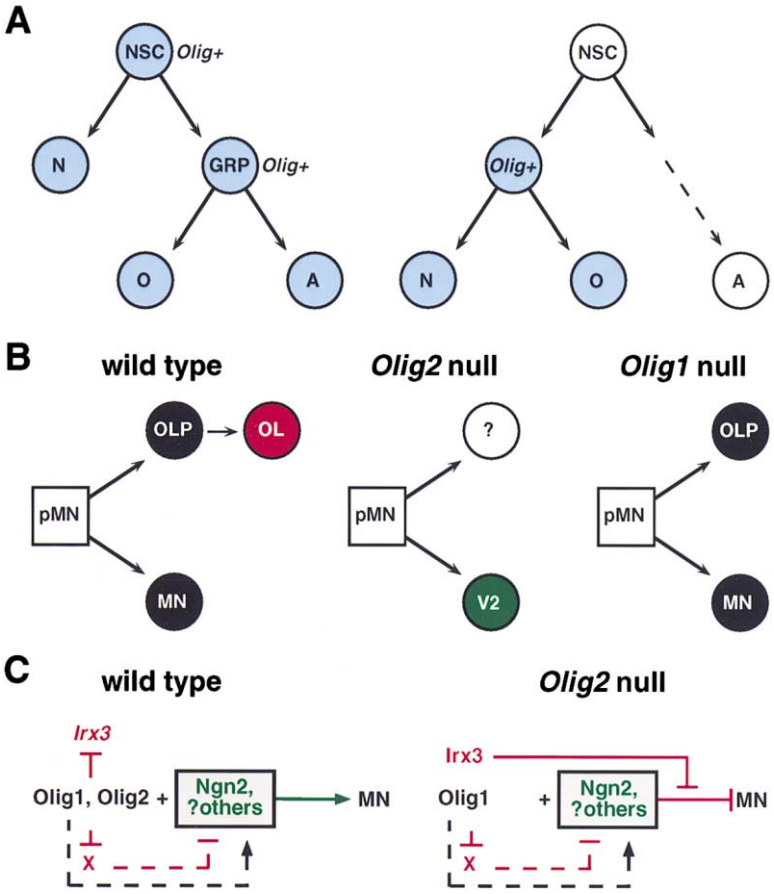

Figure 8. Analyses of *Olig1* and *Olig2* Function in the Developing CNS

(A) Analysis of *Olig* function and fate mapping indicates a neuron-oligodendrocyte lineage connection and challenges notion of a glial-restricted precursor (GRP).

(Left) Scheme for the generally held view of oligodendrocyte development via a GRP. Our studies indicate *Olig* function and expression in the progenitor for neurons (N) and oligodendrocytes (O), i.e., a multipotent neural stem cell (NSC). If so, then *Olig-cre* fate mapping analysis would have labeled all cell types (indicated by blue color), including astrocytes (A). However, β-gal protein/activity was not observed in astrocytes.

(Right) The simplest model to account for our observations is that *Olig* expression identifies a restricted neuron-oligodendrocyte (N-O) progenitor, and that *Olig* expression is excluded from astrocyte progenitors. While alternative models can be proposed to account for the experimental findings, our results preclude *Olig* expression at a GRP stage.

(B) Summary of cell types present in the P0 spinal cord of wild-type and *Olig* null mice.

(Left) Wild-type spinal cord contains mature motor neurons (MN), oligodendrocyte precursors (OLP), and mature oligodendrocytes (OL).

(Middle) In contrast, only V2 interneurons (V2) and unidentified, *Olig*-expressing cells (?) are found in *Olig2* nulls.

(Right) In *Olig1* null animals, we observe a delay in oligodendrocyte maturation.

(C) Functions of Olig proteins in the regulation of pMN cell fate. Proposed functions of *Olig1* and *Olig2* in regulation of pMN gene expression.

(Left) In the wild-type neural tube, *Olig2* antagonizes *Irx3* expression, and Olig proteins may activate/maintain expression of *Ngn2* (black dashed line), possibly via repression of a factor (X) that itself represses *Ngn* (red dashed line) (Novitch et al., 2001). *Olig2* may then interact with *Ngn2* to promote expression of MN target genes (green arrow) (Mizuguchi et al., 2001).

(Right) In *Olig2* mutants, *Irx3* expression invades the pMN. Although *Olig1* is sufficient to activate/maintain expression of *Ngn2* and other pMN markers, there is a failure of MN differentiation. This might be explained by antagonism of MN development by *Irx3* (Briscoe et al., 2000). Alternatively, this could reflect a particular requirement for *Olig2* function as part of a transcriptional complex that promotes expression of essential target genes for MN development.

ment in both the brain and spinal cord (Alberta et al., 2001; Spassky et al. 2001; Nery et al., 2001; Orentas et al., 1999; Tekki-Kessaris et al., 2001). Thus, it is possible that analogous mechanisms underlie oligodendrocyte development throughout the CNS. In keeping with this, our data indicate that astrocytes and oligodendrocytes of the brain and optic nerve arise from distinct progenitor populations.

A Single bHLH Transcription Factor, *Olig2*, Is Required for Specification of All Motor Neurons

Essential functions of *Olig* genes in formation of motor neurons and oligodendrocytes are summarized in Figure 8B. As indicated, *Olig2* is required for specification of all somatic motor neurons and visceral motor neurons in the spinal cord. The persistence of pMN progenitor cells in the spinal cord of *Olig2* null mice indicates that Olig2 proteins are required to establish a state of competence essential for motor neuron development. In principle, Olig2 protein could cooperate with other pMN transcription factors (e.g., Ngn2) to promote neuron formation. Alternatively, it has been proposed that Olig proteins may antagonize a repressor of motor neuron development (Figure 8C; Mizuguchi et al., 2001; Novitch et al., 2001). In any case, our results establish essential but restricted roles for a bHLH transcription factor in motor neuron subtype specification. This is in distinction to the major conserved role of bHLH proteins as general proneural factors (Kageyama and Nakanishi, 1997; Lee, 1997). Finally, it bears mention that we observe a transient population of *Isl1*$^{+}$, *Hb9*$^{-}$ cells in the ventral neural tube of *Olig2* null embryos at 9.5 dpc. This result suggests the possibility that there is an *Olig*-independent mechanism for transient expression of Isl1 within cells of the ventral neural tube.

Olig1 and *Olig2* Have Distinct Roles in the Regulation of the pMN Domain

Although the majority of pMN progenitors fail to become specified in *Olig2* mutants, we observe an approximate 45% increase in the population of V2 interneurons in *Olig2* null mutants. Moreover, an 80% increase in V2 interneurons was observed in *Olig1/2* compound mutants (Zhou and Anderson, 2002 [this issue of *Cell*]). We

observed also that ectopic expression of *Olig2* suppressed expression of two markers of V2 interneurons in the chick ventral neural tube. Together, these results suggest that *Olig2* is a powerful antagonist of V2 interneurons during normal development. Novitch et al. (2001) recently reported that *Olig2* misexpression leads to ectopic V2 neuron induction in dorsal regions of the chick neural tube. However, their data appear consistent with ours with respect to suppression of endogenous V2 interneuron development. Dissimilar levels of Olig2 protein overexpression achieved in the experiments of Novitch et al. (2001) could account for the particular observation of ectopic V2 interneuron induction by *Olig2*.

Olig2 function is required to repress expression *Irx3* in the pMN. However, maintenance of the Pax6 low domain in the *Olig2*$^{-/-}$ neural tube coupled with persistent expression of *Nkx6.1*, *Ngn2*, *Olig1*, and *Olig2-neo* rules out a complete pMN to p2 conversion in the absence of *Olig2*. In contrast, a complete pMN to p2 conversion does take place in *Olig1/2* compound mutants (Zhou and Anderson, 2002 [this issue of *Cell*]). It follows that *Olig1* can partially compensate for *Olig2* function to maintain the expression of several markers of the pMN. *Olig2* has previously been proposed to derepress expression of *Ngn2* (Novitch et al., 2001), and Ngn2^{+} progenitors are not detected in compound *Olig1/2* mutants. Thus, *Olig1* is able to activate and/or maintain *Ngn2* expression in the absence of *Olig2* function. However, it is clearly unable to prevent ectopic expression of *Irx3* in the pMN, which might explain the block MN formation observed in *Olig2* mutants (Figure 8C; Briscoe et al., 2000). Together, these results demonstrate essential roles for *Olig* genes in neural pattern formation and maintenance of the pMN.

Findings from gain-of-function studies (Mizuguchi et al., 2001, Novitch et al. 2001) indicate Irx3 is a repressor of *Olig2* expression. However, we observed that *Olig1* and *Olig2-neo* continue to be expressed in the pMN of *Olig2*$^{-/-}$ embryos, and appropriate expression of a *GFP* reporter gene was similarly maintained in compound mutant *Olig1/2* null animals that lack a defined pMN (Zhou and Anderson, 2002 [this issue of *Cell*]). One possibility is that a *cis*-acting DNA binding site for Irx3 is deleted in the targeted *Olig2* loci. Alternatively, repressive effects of Irx3 could be due to superphysiologic protein levels in the overexpression studies.

Olig Function Is Required for Oligodendrocyte Precursor Development in the Absence of Somatic Motor Neurons

It has been proposed in a "sequential model" (reviewed in Hardy, 1997) that inductive signals from somatic motor neurons are required for establishment of oligodendrocyte precursors. Our results, however, show that oligodendrocyte precursors are readily detected in the hindbrain of *Olig2* null animals despite a lack of somatic motor neuron precursors. Visceral motor neurons remain in the hindbrain of *Olig2* null mice. However, oligodendrocytes fail to form in the hindbrain of *Olig1/2* compound mutant mice despite the presence of these visceral motor neurons (Zhou and Anderson, 2002 [this issue of *Cell*]). Further, explant studies of spinal cord tissue from *Isl1* null mice indicate that oligodendrocytes can form in the absence of mature motor neurons (Sun et al., 1998). Thus, the validity of the sequential model is doubtful. These findings suggest that *Olig* function is required for oligodendrocyte specification per se, independent of its roles in motor neuron specification.

Unique Biological Functions of *Olig1*

The *Olig2* gene has been structurally well conserved from humans down through *Drosophila* and nematode worms. In mammals, *Olig1* and *Olig2* colocalize to within 40 kb of each other (on human chromosome 21 and on mouse chromosome 16). However, exhaustive efforts have so far failed to detect an *Olig1* ortholog in chicken (D. Anderson, personal communication) and zebrafish (unpublished observations). Collectively these observations suggest that *Olig2* is the primordial *Olig* gene and that *Olig1* was derived from *Olig2* via gene duplication during evolution. However, oligodendrocytes are found in all vertebrates excepting jawless fishes, so the appearance of *Olig1* cannot explain the innovation of myelinating cells in the CNS.

What are the biological functions of *Olig1*? Despite the dramatic differences in phenotype of *Olig1* and *Olig2* mutant animals, it appears that the encoded proteins have some similar activities at the molecular level. Gain-of-function studies show that *Olig1* can promote the formation of oligodendrocyte progenitor cells and mature oligodendrocytes in culture and in developing mice (Lu et al., 2000, 2001a). Moreover, *Olig1* function is necessary and sufficient for oligodendrocyte precursor development in the brain of *Olig2* null mice. In addition, it is clear that *Olig1* activity overlaps with *Olig2* in the maintenance of the pMN-OL domain of the ventral neural tube. On the other hand, our data indicate that *Olig1* is insufficient for the formation of motor neurons or oligodendrocytes in the embryonic spinal cord in the absence of *Olig2*.

A final important consideration is the potential roles of *Olig* factors in human disease. Further insights into Olig functions may facilitate therapeutic approaches to dysmyelinating diseases such as multiple sclerosis as well as spinal cord injury. In addition, human *OLIG1* and *OLIG2* are localized to a region of chromosome 21 associated with severe mental retardation in Down syndrome. This raises the possibility that neurological aspects of Down syndrome might result from an increased dosage of OLIG proteins and consequent abnormalities in cell fate specification within neural progenitors.

Experimental Procedures

Construction of *Olig1* and *Olig2* Targeting Vectors

To construct the Olig1 targeting vector, a 12 kb StuI-BglII genomic fragment was cloned into pKO-915 (Stratagene) vector carrying the DTA gene. In a homologous recombination event, the *Cre-neo* cassette thus replaces a 2.0 kb SfiI-XbaI fragment containing the *Olig1* coding region. Note that *Cre* protein expression is in-frame fused to the *Olig1* coding region immediately after the translation start site. To construct the Olig2 targeting vector, a 10 kb SacI-SacII genomic fragment was cloned into the pKO-915 vector. In a homologous recombination event, a 1.0 kb SmaI-BamHI fragment containing the majority of the *Olig2* coding region including the bHLH domain is thus replaced by the *neo* cassette.

Generation of Mutant ES Cell Lines, Chimeric Mice, and Animal Strains

Both targeting vectors were linearized with NotI and electroporated into mouse J1 ES cells. G418-resistant cells were selected. Genomic DNA from drug-resistant cells was digested with HindIII and analyzed by Southern hybridization using a 0.5 kb 3′ BglII-HindIII fragment as a probe for *Olig1*, or a 0.5 kb or HindIII-SacI fragment as a 5′ probe for *Olig2*. Chimeric mice and F1 heterozygotes were then generated from two *Olig1* and *Olig2* mutant ES cell lines. Transmission of the target allele through the germline was confirmed by Southern blotting. Mutant mice were maintained on C57BL/6J (Jackson Laboratory) hybrid background and analyzed. The ROSA26 conditional reporter line (Soriano, 1999) was kindly supplied by Dr. Philippe Soriano (Hutchinson Cancer Research Center, Seattle).

RNA In Situ Hybridization

RNA in situ hybridization was carried out as described in Lu et al. (2000). The probes used were the following: *Olig1*, *Olig2*, *PDGFαR*, and *PLP*/*DM20* (Lu et al., 2000); *MBP* and *Irx3* (gifts from Mengsheng Qiu of University of Louisville); *Ngn1* and *Ngn2* (gifts from Qiufu Ma of the Dana-Farber Cancer Institute); and Hb9 (a gift from John Rubenstien of the University of California, San Francisco).

Whole-Mount X-Gal Staining and Immunohistology

Mouse embryos at E10.5–E12.5 that were heterozygous for both *Olig1-cre* and the ROSA26 conditional *lacZ* reporter allele were fixed with 4% paraformaldehyde for 30 min and stained with X-gal as described (Franco et al., 2001). X-gal-stained embryos were embedded in OCT and then sectioned at 15 μm. Spinal cord sections were subjected to in situ hybridization with *Isl1* and *Hb9* cRNA probes. Immunostaining methods with tissue sections such as mouse brain and optic nerves were described previously (Lu et al., 2000). Confocal analysis was carried out on a Zeiss microscope. The authors thank Dr. Connie Cepko (Harvard Medical School) for Chx10 rabbit polyclonal antibody, Dr. Mensheng Qui (University of Louisville) for Nkx6.1 polyclonal antibody, and Dr. Thomas Jessell (Columbia University) for Nkx2.2 polyclonal antibody. Rabbit polyclonal anti-β-galactosidase and monoclonal anti-S100 β were purchased from ICN and Sigma, respectively. Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to Alex488 (Molecular Probe) and Cy3 (Jackson Immunoresearch) were used for double-labeling experiments. The monoclonal antibodies against Pax6, MNR2 (considered to label Hb9-positive cells; Scardigli et al., 2001), Isl1, and Lim3 (marking the mouse Lhx3 gene product) were developed by Dr. Thomas Jessell and obtained from the Developmental Studies Hybridoma Bank established under the auspices of the National Institutes of Health and Child Development and maintained by the University of Iowa.

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