Chapter 3

Concluding Remarks and Future Directions

Are LIM genes necessary for the development of correct target specificity of various MEA neuronal populations?

Utilizing genetically encoded and conventional axonal tracers, I have demonstrated that different LIM homeodomain transcription factors mark subpopulations of MEA neurons (or PVT neurons for Lhx9) with distinct projection targets. In particular, I have found that *Lhx6* delineates a pathway for reproductive behavior, comprising the MEApd and BSTpr and their projections to the hypothalamus.

There is considerable evidence from *Drosophila,* chick, and mice to indicate that LIM homeodomain proteins control aspects of motoneuron projection specificity, as well as neurotransmitter identity (Jessell, 2000; Shirasaki and Pfaff, 2002; Thor et al., 1999; Thor and Thomas, 1997). These data raise the question of whether *Lhx6* functions in the development of amygdalar-hypothalamic pathway mediating reproductive behaviors. Our *Lhx6*$^{-/-}$ animals died before projections from the amygdala to the hypothalamus were established, thus requiring conditional mutants to determine whether LHX6 is essential for this pathway.

In order to gain more insight in the functional role of Lhx6 in the specification of the amygdalar-hypothalamic pathway for reproduction, I have generated a construct, which contains cDNA of Lhx6 flanked by LoxP sites and followed by farnesylated green fluorescent protein (fEGFP) (Fig. 1a). This construct will be used to target the *Lhx6* chromosomal locus and to replace its coding region via homologous recombination in embryonic stem cells. In these mice, the expression of CRE recombinase will abrogate the expression of Lhx6 cDNA and instead yield the expression of fEGFP. fEGFP, being

membrane-bound (Zylka et al., 2005), will be used as a genetically encoded axonal tracer to investigate projection phenotypes of the cells without LHX6.

I have also shown that there are two distinct subpopulations of neurons in the MEAa and the PVT that project to the defensive hypothalamic nucleus (VMHdm), and not to the reproductive nucleus (VMHvl). The observation that they are also marked by LIM homeodomain transcription factors, Lhx5 and Lhx9, raised questions of whether the expressions of the LIM genes are accountable for their projection specificity. In order to address this question, I have generated additional targeting constructs in which, Lhx6 cDNA is flanked by loxp sequences and followed by either Lhx5 or Lhx9 cDNA – IRES – fEGFP (Figure 1b and 1c). If the expression of LIM genes is sufficient to shape the amygdalar-hypothalamic projection patterns, then the formally Lhx6-expression neurons will not only fail to project to the reproductive nuclei but will instead project to their defensive counterparts.

I have decided to use Aromatase-Cre as a brain region-specific cre-deleter line. Double staining for β-gal and LHX6 in Aromatase-LacZ reporter mice demonstrated that co-expression is observed only in MEApd and BSTpr, the regions of interest, as early as in embryonic day 14.5 (Figure 2). Every $LHX6^{+}$ neuron in MEApd and BSTpr does not express β-gal. However, since fEGFP will be turned on only in the cells with the Cre, assessing its expression should be able to allow the investigation of projection phenotypes of only those cells without functional LHX6. Moreover, deletion of *Lhx6* in a subpopulation of *Lhx6*-expressing neurons in MEApd and BSTpr may be enough to produce behavioral deficits because of the essential roles Aromatase and possibly the cells expressing Aromatase play in the development of male and female social behaviors (Balthazart, 1989; Luttage, 1979).

Figure 1. Targeting constructs for conditional knock-out of Lhx6.

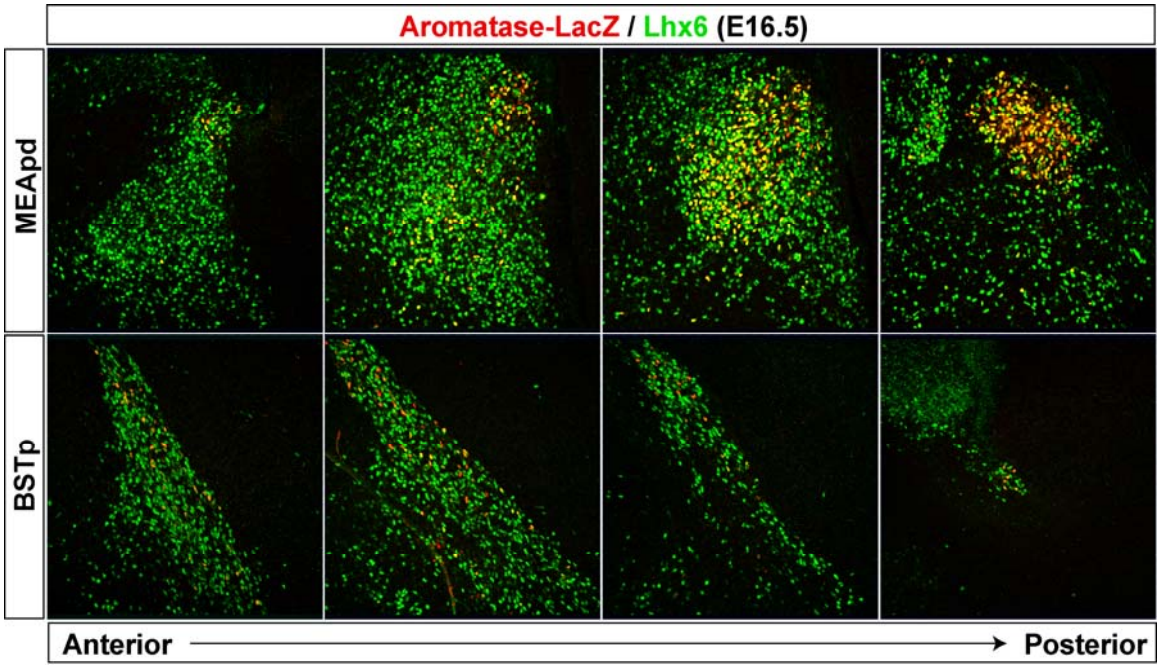

Figure 2. Expression of Aromatase and Lhx6 in MEApd and BST.

Aromatase is (indicated by a reporter gene *LacZ* in Aromatase-LacZ mice) co-expressed in a subset of Lhx6-expressing neurons in the MEApd and BSTpr.

Search of markers for the MEApv neurons that project to either reproductive or defensive nuclei of the hypothalamus

One crucial conclusion that came out of my study is that each nucleus, which has been treated as a single anatomical unit, consists of several distinct populations of neurons with different projection specificities and neurotransmitter phenotypes intermingled together. For example, the MEApv contains at least four different subpopulations of neurons: two glutamatergic populations projecting to either reproductive or defensive nuclei and two populations expressing *Lhx6* or *Lhx9* with unknown projection specificities. Such heterogeneity of neuronal populations thus necessitates dissection of circuits – relating projection specificities to the stimulus selectivity or neurotransmitter phenotypes – at the level of single cells.

The heterogeneity of individual nuclei also requires a novel approach to finding markers for behavioral circuits. A simple search for the genes that are expressed in the MEApv but not in the MEApd failed to find molecular markers for the two glutamatergic neuronal populations in MEApv. The markers for these populations could have been diluted out in the samples prepared from the whole MEApv. Moreover, the initial assumption that they would not be expressed in the MEApd may not be valid, in light of the fact that axonal projection patterns of motoneuron populations are determined not by a single "mastermind gene," but by a combinatorial code of transcription factors (Jessell, 2000; Shirasaki and Pfaff, 2002). Since the MEApd neurons and the neuronal populations of our interest in the MEApv already differ in the expression of one gene, *Lhx6*, they do not necessarily have to show a differential expression for other genes concerned with the projection specificities. A more successful approach, therefore, would require retrogradely-labeling individual populations

from either reproductive or defensive targets, isolating them at a single-cell level, and comparing their gene-expression profiles to each other rather than to that of MEApd.

VMHvl as a potential neural substrate for gating reproductive behavior by threatening stimuli

Animals faced with conflicting cues in their environment must often choose between mutually incompatible behaviors, such as reproduction or defense. Typically, threatening stimuli, such as predators or aggressive conspecifics, will suppress reproductive behavior. However, there is, so far, no clear model to suggest where and how decisions between these competing behaviors might be controlled.

My results indicate that the VMHvl receives convergent inputs, of opposite "signs," from different subpopulations of medial amygdalar neurons that are activated by reproductive or defensive stimuli, respectively. In the simplest model, the inhibitory projections from $Lhx6^{+}$ neurons in the MEApd would release reproductive behavior by inhibiting inhibitory interneurons in the VMHvl. In that case, the glutamatergic projections from the MEApv, which are activated by predator odors, could excite these same interneurons, thereby suppressing output from the VMHvl. Thus, within such a circuit organization, the VMHvl may serve as a neural substrate for gating reproductive behavior by threatening stimuli.

One can test the model using pharmacologic manipulation of glutamatergic synaptic transmission within the VMHvl. According to the model, infusion of a glutamate receptor antagonist would remove the suppression of reproductive behaviors by defensive stimuli. In these animals, thus, cat odor would no longer be able to suppress male courtship vocalization

in response to female urine. However, such a scenario is based on the assumption that the glutamate antagonist has an effect only on the GABAergic inhibitory neurons and not on the glutamatergic excitatory neuron within the VMHvl. A more elegant experiment would be, therefore, to find a marker and genetically silence or kill the neuronal population within the MEApv that are activated by defensive stimuli but that project to the VMHvl.

The role of the main olfactory system in eliciting reproductive behaviors

My study was carried out on the traditionally-held assumption that the accessory olfactory system, which has been shown to be activated by the non-volatile substances, is the major modality detecting the chemosensory cues that elicit reproductive behaviors. However, recent evidence increasingly suggests an important role for the main olfactory system in processing reproductive stimuli (Belluscio et al., 1998; Keverne, 2002; Leypold et al., 2002; Stowers et al., 2002). *TRPC2 -/-* male mice with the genetically-silenced vomeronasal neurons did not lose copulatory behaviors or the courtship vocalization in the presence of females. Data from Pankevich et al. indicated that the rates of mounting and intromission, and the timing of ejaculation were equivalent in sexually naïve, sham-operated males and VNO-removed males (Pankevich et al., 2004), when the mating performance was assessed over extended periods (4 hours) rather than only during the initial encounter with the females (20 minutes) (Wysocki and Lepri, 1991).

The foregoing data suggesting that the reproductive stimuli may be detected by the main olfactory epithelium rather than by the VNO, however, should be taken with caution. Although the VNO ablation impairs sexual behavior of the male mice minimally, the deficits are clearly apparent if the damage is made prior to any adult contact with females (Wysocki

and Lepri, 1991). My preliminary results also indicate that only sexually experienced males, and not native males, display courtship vocalization upon exposure to the volatile compounds of female urine, which are presumably detected only by the main olfactory epithelium. Moreover, it should be noted that Stowers et al. exposed *TRPC2 -/-* males to intact females rather than female-derived odors when they observed the courtship vocalization from these animals. Consequently, one cannot rule out the possibility that learning and experience during interactions with females as well as cues detected by sensory modalities other than olfaction may be capable of overriding the otherwise detrimental effect of the VNO elimination.

In order to formally determine whether the Lhx6-expressing neurons in our circuit are activated by the MOE-derived or VNO-derived reproductive stimuli, I have obtained *TRPC2* and generally anosmic *CNG* (cyclic nucleotide-gated cation channel) (Brunet et al., 1996) mutant animals. Assessing whether female urine is capable of activating *Lhx6*-expressing neurons in these animals should shed some light on the contribution the main and accessory olfactory systems make to evoking reproductive behaviors through the amygdalar-hypothalamic circuit.

How does my model fit with the current model of VNO/AOB function?

Genetically silencing vomeronasal neurons by eliminating *TRPC2* expression results in the male-male mating behavior (Leypold et al., 2002; Stowers et al., 2002). While this phenotype has been interpreted to reflect a defect in gender recognition, it may actually involve a dis-inhibition of mating behavior, as a secondary consequence of an inability to detect aggression-promoting cues. Consistent with this notion, the *TRPC2* mutant mice show

reduced male-male aggression (Stowers et al., 2002) as well as maternal aggression (Leypold et al., 2002). Moreover, it has been shown recently that male mice with VNO removal are still able to reliably discriminate between urinary odors from males and females (Pankevich et al., 2004), arguing against the loss of gender recognition in the *TRPC2* mutants.

My preliminary results show that cat odor can prevent the courtship vocalization displayed by males when exposed to female urine (Fig. 3 and Fig. 4). If the reproductive stimuli prove to be detected by the MOE, then I can test whether or not the cat odor's ability to suppress the courtship vocalization is lost in *TRPC2* mutant males. If so, I can also assess whether the *c-fos* activation in the MEApv in response to cat odor is lost in these mice. These experiments should be able to address whether the circuit identified in my study could explain the suppression of reproductive behaviors by signals that normally promote aggression, in relation to *TRPC2* mutant phenotypes.

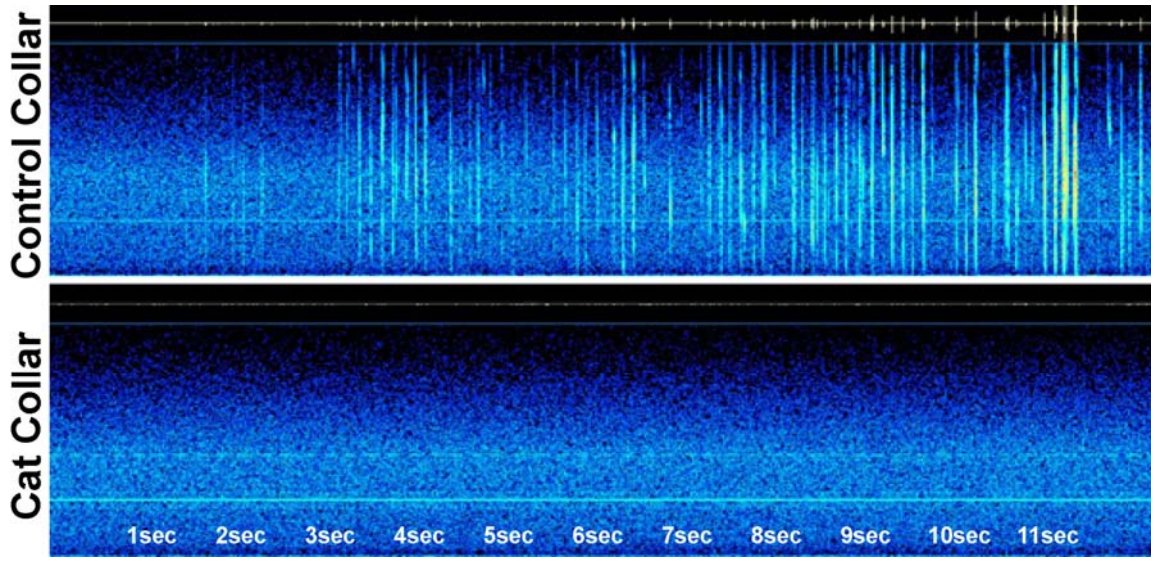

Figure 3. Spectrograms showing a male mouse vocalizing at an ultrasonic range in response to female urine and in the presence or absence of cat odor.

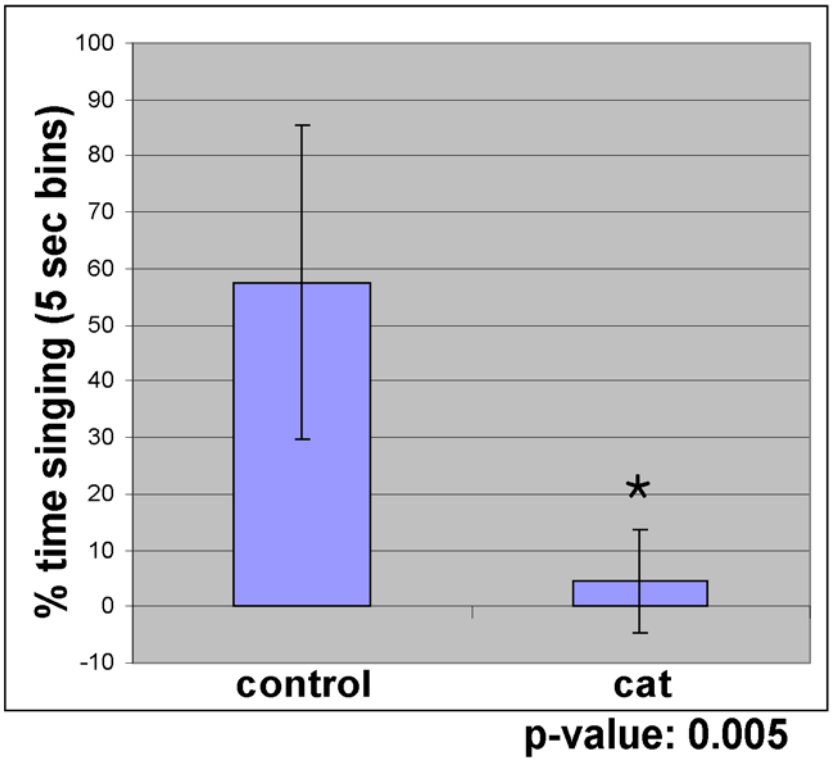

Figure 4. Quantification showing the inhibition of male courtship vocalization in the presence of cat odor.

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Appendix

The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2

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The bHLH Transcription Factor Olig2 Promotes Oligodendrocyte Differentiation in Collaboration with Nkx2.2

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Summary

Olig2, a basic helix-loop-helix (bHLH) transcription factor, is expressed in a restricted domain of the spinal cord ventricular zone that sequentially generates motoneurons and oligodendrocytes. Just prior to oligodendrocyte precursor formation, the domains of *Olig2* and *Nkx2.2* expression switch from being mutually exclusive to overlapping, and *Neurogenins1* and *2* are extinguished within this region. Coexpression of Olig2 with Nkx2.2 in the spinal cord promotes ectopic and precocious oligodendrocyte differentiation. Both proteins function as transcriptional repressors in this assay. This effect is blocked by forced expression of Neurogenin1. By contrast, misexpression of Olig2 alone derepresses *Neurogenins* and promotes motoneuron differentiation. Olig2 therefore functions sequentially in motoneuron and oligodendrocyte fate specification. This dual action is enabled by spatiotemporal changes in the expression domains of other transcription factors with which Olig2 functionally interacts.

Introduction

A fundamental problem in neural development is to understand how multipotential progenitor cells generate the various classes of neurons and glia of the central nervous system (CNS). The production of these different cell types is highly organized in space and in time. Accordingly, two general strategies have evolved to orchestrate the generation of such cellular diversity. In one strategy, different classes of cells are generated from different regions of the ventricular zone at the same time, such as occurs in the ventral spinal cord (Tanabe and Jessell, 1996; Jessell, 2000). In the second strategy, a given region of the ventricular zone sequentially produces different cell types according to a particular schedule, such as occurs in the retina and cortex (Cepko, 1999; Desai and McConnell, 2000). These two strategies are not mutually exclusive. The molecular mechanisms underlying the spatial strategy have been shown to involve patterning processes that convert initially graded morphogen signals into discrete domains of transcription factor gene expression within the neuroepithelium, from which distinct neuronal subtypes arise (Tanabe and Jessell, 1996; Jessell, 2000). Much less is known, however, about the corresponding mechanisms that underlie temporal switches in the generation of particular cell types from a common progenitor domain.

One system in which such a temporal switch occurs is the sequential production of neurons and oligodendrocytes from the ventral spinal cord. Oligodendrocytes, the myelinating glial cell type of the central nervous system, emerge from a discrete domain in the ventral spinal cord, beginning on or about E12.5–E13.5 in the mouse (Miller, 1996). This domain can be visualized by staining for PDGF receptor-α (PDGFRα), a specific marker of oligodendrocyte precursors (Pringle and Richardson, 1993; Hall et al., 1996). At earlier stages of development, this region of the ventricular zone generates one or more neuronal subtypes, including motoneurons and/or interneurons (Jessell, 2000). The molecular mechanisms that control the switch from the production of these neuronal subtypes to oligodendrocytes are not understood.

Studies of the spatial relationship of oligodendrogliogenesis to neurogenesis have been aided by the discovery that the ventricular zone of the ventral spinal cord is subdivided into five distinct progenitor domains, which generate different neuronal subtypes (Briscoe et al., 2000). These progenitor domains are defined by the boundaries of expression of various homeodomain transcription factors, which constitute a combinatorial code for the specification of neuronal progenitor identity (Briscoe et al., 2000). Motoneurons, for example, are generated from the pMN domain. This domain is bounded ventrally by the p3 domain, which is defined by expression of Nkx2.2, and dorsally by the p2 domain, whose ventral boundary is set by Irx3. It has been proposed that oligodendrocytes later emerge from the pMN domain (Richardson et al., 1997; Sun et al., 1998), but recent evidence has been interpreted to suggest that some or all oligodendroglial precursors emerge from the underlying Nkx2.2$^+$ p3 domain (Soula et al., 2001).

Previously, we and others described two related basic helix-loop-helix (bHLH) transcription factors, called Olig1 and Olig2, which delimit the oligodendrocyte precursor domain several days before such precursors can be detected by expression of PDGFRα (Lu et al., 2000; Zhou et al., 2000). Expression of the *Olig* genes persists in migratory oligodendrocyte precursors and in mature oligodendrocytes. Misexpression of Olig2 in chick mesoderm causes ectopic expression of *Sox10*, a marker that is coexpressed with *Olig* genes in oligodendrocyte precursors (Zhou et al., 2000); however, it does not induce later oligodendrocyte markers. In addition, expression of Olig1 in cultured CNS neural precursors increases the number of cells expressing NG2, another oligodendrocyte precursor marker (Lu et al., 2000). These data suggested that *Olig* genes might play an early role in oligodendrocyte precursor specification. Direct evidence of such a role for *Olig* genes in the spinal cord has, however, been lacking.

If the domain of *Olig* gene expression indeed determines the site of origin of oligodendrocytes, then the

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timing of *Olig* gene expression within the spinal cord might determine the time at which such precursors are specified. Paradoxically, however, *Olig2* is also expressed in the pMN domain at earlier stages, when motoneurons are being generated (Takebayashi et al., 2000). Moreover, in the accompanying manuscript, Novitch et al. (2001 [this issue of *Neuron*]) demonstrate that misexpression of *Olig2* promotes motoneuron fate specification. These observations suggest either that *Olig* genes function in motoneuron and not oligodendrocyte fate determination, or alternatively that they play a role in both processes. To address this question, we have isolated a chicken homolog of Olig2, and examined its expression and function in relation to that of other transcription factors involved in neuronal differentiation in the ventral spinal cord (Briscoe et al., 2000).

At E3.0, the time that motoneurons are being generated, the ventral boundary of the *Olig2* expression domain is adjacent to, and nonoverlapping with, that of the underlying Nkx2.2 domain. However, just before oligodendrocyte precursors start to be produced (E6–E7), these two boundaries become overlapping, and numerous Olig2$^+$, Nkx2.2$^+$ cells are seen migrating away from the ventricular zone in a pattern characteristic of oligodendrocyte precursors. Combined misexpression of both *Olig2* and *Nkx2.2*, but not of either gene alone, causes ectopic and precocious oligodendrocyte differentiation in the spinal cord. The normal generation of oligodendrocytes is also preceded by an extinction of the proneural bHLH factors *Ngn1* and *Ngn2* from the Olig2$^+$ domain. Forced expression of *Ngn1* blocks both ectopic oligodendrocyte differentiation induced by coexpression of Olig2+Nkx2.2 as well as normal oligodendrocyte differentiation. Taken together with the results of Novitch et al. (2001), these data suggest that Olig2 plays sequential roles in both motoneuron and oligodendrocyte fate specification. This dual action is enabled by spatio-temporal changes in the expression domains of other transcription factors expressed in the ventral spinal cord, with which *Olig2* functionally interacts.

Results

Identification of a Chick *Olig2* Gene

Previously, two murine *Olig* genes, *Olig1* and *Olig2*, were isolated and characterized (Lu et al., 2000; Zhou et al., 2000). Because the generation of neuronal diversity in the ventral spinal cord has been characterized most extensively in the chick, however (Jessell, 2000), we first sought to isolate chicken orthologs of the *Olig* genes in order to study their expression and function in that system. In situ hybridization to chick embryonic neural tissues, using a mouse *Olig2* cRNA probe, revealed the existence of one or more crosshybridizing species expressed at high levels in E3 and E4 spinal cord (data not shown). Accordingly, we constructed and screened a cDNA library made from dissociated E3 and E3.5 chick neural tubes (see Experimental Procedures). Several full-length chick *Olig2* clones were identified, as well as orthologs of *Olig3* (Takebayashi et al., 2000), but no *Olig1* orthologs were found. The chick and mouse *Olig2* genes have identical bHLH domains and share an overall 75% amino acid sequence identity (Supplemental Figure S1). However, several strings of alanine or glycine repeats present in murine Olig2 are absent in the predicted chick Olig2 protein. Nevertheless, the chick and mouse genes behaved indistinguishably in our functional assays (see below).

Expression of Olig2 in Relation to Other Oligodendrocyte Markers in Embryonic Chick Spinal Cord

We compared the expression profile of chick *Olig2* with that of several other genes that were previously identified as markers of either oligodendrocyte precursors, such as Nkx2.2 (Xu et al., 2000), PDGFRα (Hall et al., 1996), or Sox10 (Zhou et al., 2000), or of differentiated, mature oligodendrocytes, such as myelin basic protein (MBP) or proteolipid protein (PLP/DM20) (Pfeiffer et al., 1993). This analysis was carried out at axial levels just posterior to the forelimb. At E6, the time at which the first oligodendrocyte precursors begin to arise in the spinal cord (Ono et al., 1995), both *Olig2* and *Nkx2.2* are strongly expressed in bilateral ventral foci in the ventricular zone, while the other markers are not yet detectable (Figures 1A–1E, arrows). Beginning on E7, individual *Olig2*- or *Nkx2.2*-positive cells can be seen migrating away from this zone into the gray matter, both laterally and dorsally (Figures 1F and 1G, arrowheads). By E8, many Olig2$^+$ and Nkx2.2$^+$ cells have already moved into the white matter (Figures 1K and 1L, arrowheads). Throughout the stages examined, from E6 to E9.5, the ventral foci of Olig2 and Nkx2.2 expression in the ventricular zone were maintained (Figures 1F, 1G, 1K, 1L, 1P, and 1Q; arrows).

In contast to Olig2 and Nkx2.2, the first PDGFRα-positive cells do not appear in the spinal cord at this axial level until E6–E6.5 (Figures 1C and 1H, arrow). Over the ensuing 2 days, these cells subsequently disperse throughout the spinal cord so that by E9.5, there is a relatively even distribution of PDGFRα^+ cells (Figure 1R). Sox10, which is strongly expressed in murine oligodendrocyte precursors (Zhou et al., 2000), is expressed at high levels only in mature oligodendrocytes in chick (Figure 1S, arrowheads). However, weak expression of Sox10 in the oligodendrocyte precursor domain of the ventricular zone can be detected starting around E7 (Figures 1I and 1N, arrows). Mature oligodendrocyte markers, such as MBP and PLP/DM20, are not detectable until E9.5 (Figure 1T, arrowheads and data not shown). Our observations of Nkx2.2, PDGFRα, and MBP mRNA expression in the chick spinal cord are consistent with those described in previous reports (Xu et al., 2000). The spatio-temporal pattern of *Olig2* expression is consistent with that reported by Novitch et al. (2001). However, the precise timing of the onset of migration of Olig2$^+$ cells is shifted later in development in our study, due to the more caudal levels we have examined and reflecting the strong caudal-to-rostral gradient of oligodendrogliogenesis (Perez Villegas et al., 1999).

A Developmental Switch from Mutually Exclusive to Coincident Expression of Olig2 and Nkx2.2 Correlates with the Emergence of Oligodendrocyte Precursors

Since Olig2 and Nkx2.2 are both expressed in restricted ventricular zone foci, as well as in presumptive migrating

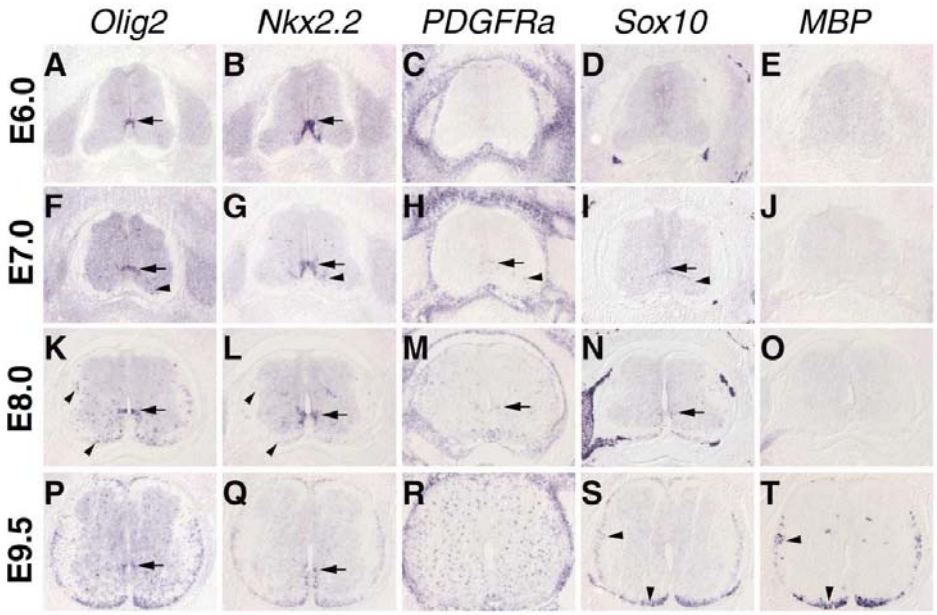

Figure 1. Expression of Olig2 in Relation to that of Oligodendrocyte Markers in Chick Spinal Cord

Nonisotopic in situ hybridizations were performed with the indicated probes at the indicated developmental ages. (A)–(E), (F)–(J), etc. are adjacent serial sections. Arrows indicate the oligodendrocyte precursor domain. Note that migrating oligodendrocyte precursors detected by Olig2, Nkx2.2, or PDGFRα are detected by E7.0 (F, G, and H, arrowheads), while mature oligodendrocyte markers such as MBP and PLP/DM20 are not detected until E9.5 in the white matter (T, arrowheads, and data not shown). Sox10 is first expressed at low levels at E7.0 in the ventricular zone (I and N, arrows) and in some migrating cells (I, arrowhead), but is not expressed at high levels in white matter until E9.5 (S, arrowheads).

oligodendrocyte precursor cells (Figure 1 and Lu et al., 2000; Xu et al., 2000; Zhou et al., 2000; Soula et al., 2001), we sought to compare their expression more directly. To this end, we raised a mouse antibody to chick Olig2 and performed double labeling with a rabbit anti-Nkx2.2 antibody (see Experimental Procedures). From E3 to E4, during the period of neurogenesis, the Olig2 and Nkx2.2 expression domains are adjacent and nonoverlapping (Figures 2A and 2B, open and filled arrowheads). As detailed in the accompanying manuscript (Novitch et al., 2001), the domain of Olig2 expression precisely corresponds to the pMN domain, which overlies the Nkx2.2$^+$ p3 domain. In some cases, a low level of Nkx2.2 expression can be observed to extend dorsally into the pMN domain (B. Novitch, personal communication). Beginning on E5, however, a few cells in the ventricular zone at the pMN/p3 interface are observed to coexpress the two transcription factors at high levels (Figure 2C, arrow). The extent of this coexpression remains largely unchanged at E6 (Figure 2D, arrow), but subsequently expands so that by E7, the majority of Olig2$^+$ cells coexpress Nkx2.2 at levels comparable to that in p3 (Figure 2E, arrow; yellow cells). (There are, however, one or two layers of Olig2$^+$ cells at the dorsal-most aspect of pMN, which do not express detectable levels of Nkx2.2 at all stages examined.)

In addition to this ventricular coexpression, streams of Olig2$^+$, Nkx2.2$^+$ cells appear to delaminate and migrate away from the ventricular foci where Olig2 and Nkx2.2 overlap (Figure 2E, white arrowheads), as well as perhaps from the Nkx2.2$^+$ domain. Nkx2.2$^+$, Olig2$^-$ cells are also observed to migrate from the Nkx2.2-expressing zone (Figure 2E, open arrowheads), beginning on around E5 (Figure 2C, open arrowheads) and continuing until the latest stage examined (E12; data not shown). By E8.0, the number of migrating Olig2$^+$Nkx2.2$^+$ and Nkx2.2$^+$Olig2$^-$ cells has increased dramatically and many of the cells have moved into the ventral white matter (Figure 2F, lower arrowheads). Strikingly, at these stages, no migrating Olig2$^+$Nkx2.2$^-$ cells were observed in either the gray or white matter.

In mouse, Olig2$^+$ cells migrating from the ventricular zone coexpress oligodendrocyte precursor markers such as PDGFRα and Sox10 (Lu et al., 2000; Zhou et al., 2000). The migrating Olig2$^+$Nkx2.2$^+$ and Nkx2.2$^+$Olig2$^-$ cells in chick spinal cord have a similar distribution, and are therefore likely to be oligodendrocyte precursors. Consistent with this, some of the migrating Olig2$^+$ cells are actively dividing (data not shown), and the only proliferating cells in the spinal cord at these late stages are glial precursors (Altman and Bayer, 1984). To confirm this identification, E8.5 chick spinal cords were dissoci-

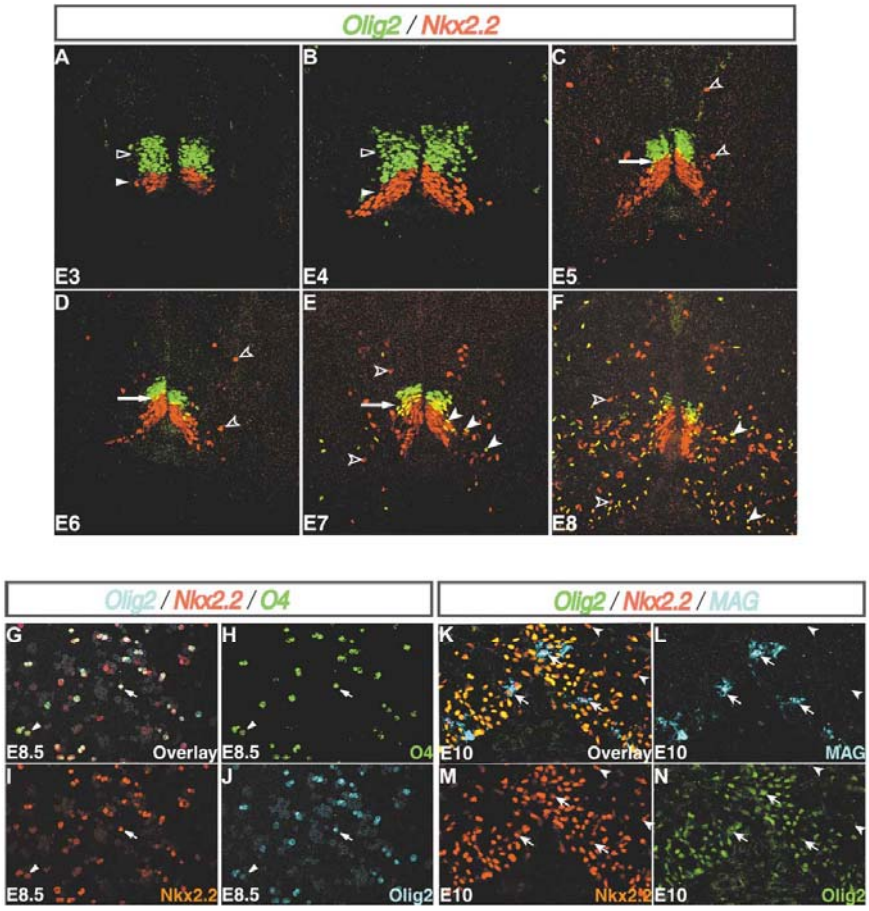

Figure 2. Coexpression of Olig2 and Nkx2.2 in Oligodendrocyte Precursors

(A–F) Double label immunofluorescence confocal microscopy was performed with rabbit anti-Nkx2.2 (red) and mouse anti-Olig2 (green) antibodies at the indicated stages. Double-positive cells (yellow) are first detected at the pMN/p3 boundary at E5.0 (C, arrow). By E7.0, double-positive cells appear to be emigrating in streams from the ventricular zone into the gray matter (E, filled arrowheads), where they later disperse and localize to the white matter (F, lower filled arrowheads). Nkx2.2 single-positive cells begin emigrating at E5.0 (C–F, open arrowheads), but Olig2 single-positive cells are never observed to migrate. (G–J) $Olig2^+$, $Nkx2.2^+$ cells are oligodendrocyte precursors. E8.5 spinal cord was dissociated and triple labeled with antibodies to O4, Olig2, and Nkx2.2. The four panels are from the same field. Most or all (98 ± 1.4%) $Olig2^+$, $Nkx2.2^+$ cells coexpress O4 (arrow). A subset of $Nkx2.2^+$ cells are weak or negative for Olig2, and some of these (20.5 ± 9.3%) coexpress O4 (arrowhead). (K–N) Expression of a mature oligodendrocyte marker by $Olig2^+$, $Nkx2.2^+$ cells in white matter. Triple label immunofluorescence on sections through the ventral region of E10 spinal cord was performed using antibodies to Olig2, Nkx2.2 and myelin-associated glycoprotein (MAG). The four panels are from the same field. Note that all MAG^+ cells are $Olig2^+$, $Nkx2.2^+$ (arrows), while $Nkx2.2^+$, $Olig2^-$ cells are MAG^- (arrowhead). At later stages, virtually all $Nkx2.2^+$ cells are $Olig2^+$ (not shown).

ated and triple-labeled with antibodies to Olig2, Nkx2.2, and the oligodendrocyte precursor cell surface marker O4 (Gard and Pfeiffer, 1990). The results indicated that most (98 ± 1.4%) $Olig2^+$, $Nkx2.2^+$ cells were also $O4^+$ (Figures 2G–2J, arrow). In addition, some (20.5 ± 9.3%) $Nkx2.2^+$ cells that were weak or negative for Olig2 expressed O4 (Figures 2G–2J, arrowhead). Furthermore, at later stages (E10), at least some $Olig2^+Nkx2.2^+$ cells in the white matter coexpress the mature oligodendrocyte marker myelin-associated glycoprotein (MAG) (Philippe et al., 1986) (Figures 2K–2M, arrows). Taken together, these data suggest that from E7.0 onward, both $Olig2^+Nkx2.2^+$ and $Nkx2.2^+Olig2^-$ cells in the ventral spinal cord are likely to be oligodendrocyte precursors. Our results are consistent with recent studies showing that Nkx2.2 is expressed by oligodendrocyte precursors (Xu et al.,

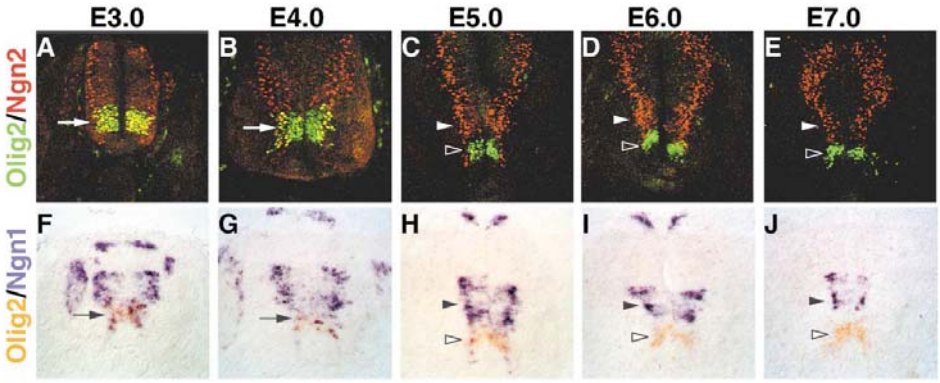

Figure 3. Extinction of Ngns from the Olig2-Expressing Domain Precedes Oligodendrocyte Precursor Formation

(A–E) Double label immunofluorescence microscopy with rabbit anti-Ngn2 (red) and mouse anti-Olig2 (green) antibodies. Arrows in (A) and (B) indicate double-positive (yellow) cells. Note that by E5.0 (C), most Ngn2 expression has already been extinguished from the Olig2$^+$ domain (open arrowhead). (F–J) Double label in situ hybridization with Olig2 and Ngn1 cRNA probes. The domains become well separated by E6.0 (I). Note the gap between the dorsal boundary of Olig2 (J, white arrowhead) and the ventral boundary of Ngn1 (J, black arrowhead) at E7.0.

2000; Soula et al., 2001), but indicate that these precursors are heterogenous with respect to Olig2 coexpression.

Emigration of Olig2$^+$ Oligodendrocyte Precursors from the Ventricular Zone Is Preceded by Extinction of Neurogenin Expression

The observation that Olig2 is expressed in migrating and dividing presumptive oligodendrocyte precursors seems paradoxical, in light of the demonstration that this bHLH factor functions to promote motoneuron differentiation and cell cycle arrest at earlier stages of spinal cord development (Novitch et al., 2001). The induction of motoneuron differentiation by Olig2 in part involves the derepression of *Ngn1* and *Ngn2*, vertebrate proneural genes that activate a program of generic neuronal differentiation and cell cycle withdrawal in uncommitted precursor cells (Ma et al., 1996, 1998; Fode et al., 1998; Farah et al., 2000). We were therefore interested to know whether *Ngn1/2* expression is maintained at later stages when oligodendrocytes are being generated, or rather is extinguished at that time.

Double labeling with antibodies to Ngn2 and Olig2 revealed a strong colocalization of these bHLH proteins in the pMN domain at E3.0–E4.0 (Figures 3A and 3B, arrows; see also Novitch et al., 2001). A similar overlap with Ngn1 in this region was detectable by in situ hybridization (Figures 3F and 3G, arrows), although single cell resolution was more difficult to achieve with the nonfluorescent probes. Between E4.5 and E5.0, however, there was a striking downregulation of Ngn2 within the Olig2$^+$ region of the ventricular zone, although a few Ngn2$^+$ cells persisted laterally to the pMN domain (Figures 3C and 3D, open arrowheads). By E7.0, the stage at which the first Olig2$^+$ oligodendrocyte precursors emigrate from the ventricular zone, Ngn2 expression was no longer detectable either within, or lateral to, the Olig2$^+$ domain of the ventricular zone (Figure 3E, open arrowhead). However, it was still expressed immediately dorsal to this region (Figure 3E, closed arrowhead). A clear separation between the domains of *Ngn1* and *Olig2* mRNA expression was visible by this stage as well (Figure 3J, arrowheads). Thus, the emigration of Olig2$^+$ oligodendrocyte precursors from the ventricular zone is preceded by the extinction of *Ngn1* and *Ngn2* expression within the *Olig2*-expressing domain, as well as by the acquisition of overlap with the Nkx2.2$^+$ domain (Figure 2).

Comisexpression of Olig2 and Nkx2.2 Cell-Autonomously Induces Ectopic and Precocious Oligodendrocyte Differentiation

The coincidence of Olig2 and Nkx2.2 expression within the ventricular zone, together with the observation that migrating Olig2$^+$Nkx2.2$^+$ oligodendrocyte precursors are derived from this domain of overlap, suggested that the combined activity of both transcription factors might be important for the specification and/or differentiation of oligodendroglia. We therefore tested whether comisexpression of these two genes could promote ectopic oligodendrocyte generation. The spinal cord of E2 chick embryos was electroporated in ovo with replication-competent RCASBP(B) retroviral vectors (Morgan and Fekete, 1996) harboring the chick *Olig2* or *Nkx2.2* genes, either singly or in combination. The embryos were harvested 4 days later (E6), at which time endogenous oligodendrocyte precursors are just beginning to appear in the spinal cord, but when overt oligodendrocyte differentiation has not yet occurred (Ono et al., 1995) (Figures 1A–1E).

Coelectroporation of *Olig2* plus *Nkx2.2* yielded ectopic and precocious oligodendrocyte differentiation on the electroporated side of the spinal cord, as assessed by the robust induction of Sox10, PDGFRα, MBP, and PLP/DM20 in embryos harvested at E6 (Figures 4E, 4I, and 4M, arrows; and data not shown). Many of these ectopic oligodendrocytes appeared to be migrating away from the ventricular zone toward the white matter.

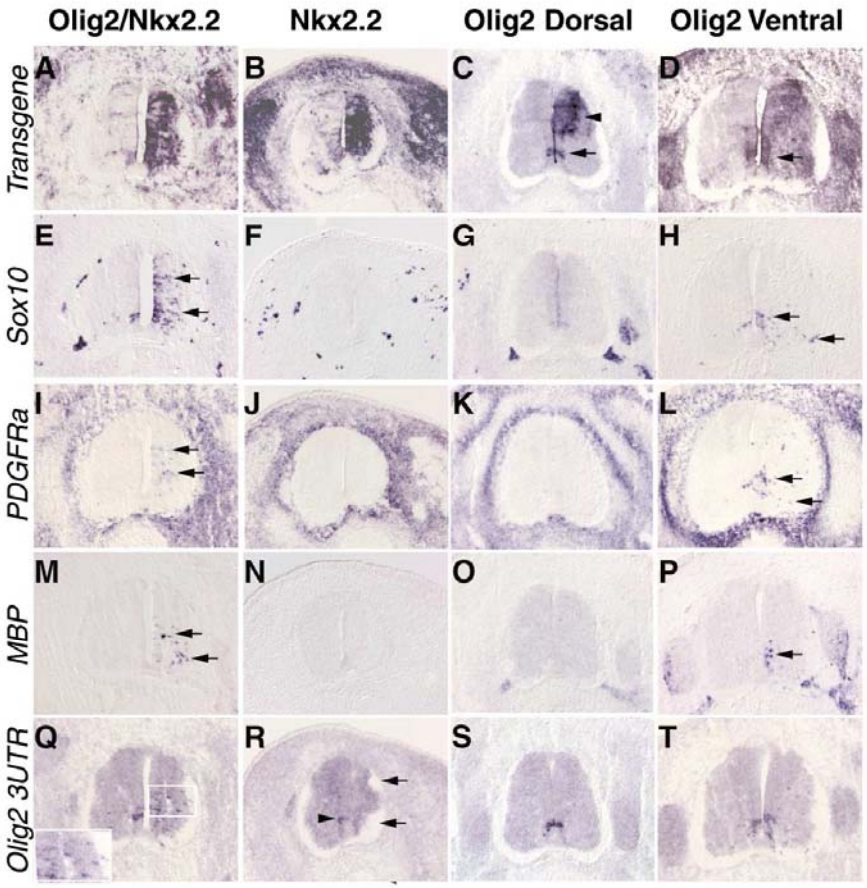

Figure 4. Coelectroporation of Olig2+Nkx2.2 Promotes Ectopic and Precocious Oligodendrocyte Differentiation

E2 embryos were electroporated with the RCASBP(B) plasmids indicated above the columns, harvested 4 days later (E6) and hybridized in situ with the probes indicated to the left of the rows. Note the strong induction of Sox10, PDGFRα, and MBP in embryos coelectroporated with Olig2+Nkx2.2 (E, I, and M, arrows). At this stage (E6), none of these oligodendrocyte markers is normally expressed in the oligodendrocyte precursor region (Figures 2C–2E), thus their induction by Olig2+Nkx2.2 is precocious as well as spatially ectopic. Neither Olig2 alone nor Nkx2.2 alone induces such oligo markers in the dorsal region (F, G, J, K, N, and O). However, electroporation of Olig2 in the ventral spinal cord overlapping the endogenous Nkx2.2$^+$ domain results in premature differentiation of oligodendrocytes in this region (H, L, and P, arrows). Nkx2.2 alone, in contrast, potently represses endogenous *Olig2* expression (R, arrows). However, this repression is overcome by inclusion of exogenous Olig2 (Q, boxed region and inset).

In contrast, neither Olig2 nor Nkx2.2 alone caused such ectopic oligodendrocyte differentiation in the dorsal spinal cord (Figures 4B, 4C, 4F, 4G, 4J, 4K, 4N, and 4O). However, in cases where the *Olig2* transgene was electroporated in the ventral region of the spinal cord (Figure 4D, arrow), oligodendrocytes were observed to differentiate precociously where the transgene overlapped the domain of endogenous *Nkx2.2* expression (Figures 4H, 4L, and 4P, arrows). (The expression of endogenous *Nkx2.2* is not affected by ectopic expression of *Olig2* in the p3 domain [Novitch et al., 2001].) Thus, Olig2 can collaborate with either exogenous or endogenous Nkx2.2 to promote ectopic and/or precocious oligodendrocyte differentiation.

We were not able to determine whether, conversely, exogenous Nkx2.2 can collaborate with endogenous as well as exogenous *Olig2* because electroporation of Nkx2.2 in the pMN domain potently represses endogenous *Olig2* expression (Figure 4R, arrows; see also Novitch et al., 2001). This repression in turn raised the question of how Nkx2.2 and Olig2 can normally be coexpressed in oligodendrocyte precursors (Figure 2). Inter-

estingly, such repression of endogenous *Olig2* was not observed when Olig2+Nkx2.2 were coelectroporated; to the contrary, the combination of these two transcription factors activated ectopic expression of endogenous *Olig2* (Figure 4Q, boxed area and inset). This ectopic expression of the bHLH factor was not activated by forced expression of Olig2 alone (Figure 4S). Taken together, these data suggest that Olig2+Nkx2.2 in combination activate a program of oligodendrocyte differentiation that is not triggered by either gene alone, and that this interaction converts Nkx2.2 from a repressor to a coinducer of *Olig2* expression.

In the accompanying paper, Novitch et al. demonstrate that misexpression of Olig2 alone promotes motoneuron differentiation. This finding raised the possibility that the cells ectopically expressing oligodendrocyte markers in response to combined misexpression of Olig2+Nkx2.2 might coexpress some motoneuron markers, and therefore be phenotypically hybrid cells rather than true oligodendrocytes. To test this, we performed double labeling with motoneuron and oligodendrocyte markers in embryos electroporated with Olig2, in either the absence or presence of Nkx2.2. Consistent with the results of Novitch et al. (2001), a large number of ectopic motoneurons were generated following electroporation of Olig2 alone (Figures 5A and 5D, arrowheads), while Sox10 was not induced. In contrast, both $Sox10^+$ cells and $MNR2^+$ or $LIM3^+$ motoneurons (albeit in smaller numbers) were observed when Olig2 and Nkx2.2 were coelectroporated (Figures 5B and 5E). There was, however, little or no overlap between $Sox10^+$ and $MNR2^+$ cells, or between $Sox10^+$ and $Lim3^+$ cells (Figures 5C and 5F, arrows). Double labeling with the pan-neuronal marker NeuN produced similar results (data not shown). These $NeuN^+$ cells likely include some v3 interneurons as well as motoneurons, as coelectroporation of Olig2+Nkx2.2 yields some cells ectopically expressing Sim1 (not shown), a v3 interneuron marker whose expression is promoted by ectopic *Nkx2.2* expression (Briscoe et al., 1999). Thus, the cells ectopically expressing oligodendrocyte markers in embryos coelectroporated with $Olig2^+$Nkx2.2 are not phenotypic hybrids coexpressing neuronal markers, but rather are glial cells.

If Olig2+Nkx2.2 together promote oligodendrocyte differentiation, why are there any ectopic motoneurons or v3 interneurons in embryos coelectroporated with both transcription factors? In addition to cells ectopically coexpressing Olig2 and Nkx2.2, there are also many cells expressing Olig2 or Nkx2.2 alone in such embryos (Figure 5J, right, green and red arrowheads). This may reflect the fact that some cells incorporate only one of the two plasmids during the initial electroporation. In addition, the RCASBP(B) vectors used to express the transgenes encode replication-competent retroviruses (Morgan and Fekete, 1996). Therefore, following electroporation and expression, infectious virus particles will form and neural tube cells will become secondarily infected within 2–3 days (Morgan and Fekete, 1996). Because the Nkx2.2- and Olig2-expressing viruses are both of the (B) type, they will not coinfect the same cells (Morgan and Fekete, 1996). Thus, cells expressing either *Nkx2.2* or *Olig2*, but not both transgenes, will progressively accumulate at later stages. The existence of such single-positive secondarily infected cells could, therefore, also explain the intermingling of some ectopic motoneurons and v3 interneurons with the ectopic oligodendrocytes, in Olig2+Nkx2.2-electroporated embryos.

The induction of ectopic motor and interneurons in embryos coelectroporated with Olig2+Nkx2.2 raised the possibility that induction of ectopic oligodendrocyte differentiation might not reflect a cell-autonomous collaboration between these two transcription factors, but rather a non-cell-autonomous interaction between the ectopic neurons also generated. To address this question, we deliberately coexpressed Olig2 and Nkx2.2 in nonoverlapping cell populations. To do this, we packaged infectious B-type retroviral particles expressing either Olig2 or Nkx2.2, and coinjected E2 chick embryos with a 1:1 mixture of these particles, then harvested and analyzed them 4 days later (E6). Double labeling with antibodies to Nkx2.2 and Olig2 confirmed that there were many ectopic $Nkx2.2^+$ or $Olig2^+$ cells in such coinfected embryos (Figure 5G, red and green arrowheads) compared to controls (e.g., see Figure 5J, left side), but as expected there were very few double-positive cells (Figure 5G, yellow arrowhead). In such coinfected embryos, no ectopic oligodendrocytes were produced (Figures 5H and 5I), whereas in coelectroporated embryos, many such ectopic cells were seen at this time (Figures 5K and 5L, arrows). As expected, in retrovirally infected embryos where the Olig2 transgene overlapped the endogenous $Nkx2.2^+$ domain (Figure 5G, yellow arrow), premature oligodendrocyte differentiation was observed (Figures 5H and 5I, arrows). These data suggest that Olig2 and Nkx2.2 co-operate in a cell-autonomous manner to promote ectopic oligdodendrocyte differentiation. We cannot exclude, however, that feedback signals from motoneurons (and/or v3 interneurons) may contribute to this process, even if they are not sufficient to promote it.

Neurogenins Interfere with the Promotion of Oligodendrocyte Differentiation by Olig2+Nkx2.2

Recently, Ngn1 has been shown to negatively regulate glial (astrocyte) differentiation, in addition to promoting neuronal differentiation (Sun et al., 2001). The downregulation of Ngns1/2 in the $Olig2^+$ domain prior to the emergence of oligodendrocyte precursors (Figure 3) therefore raised the possibility that these proneural genes might similarly inhibit oligodendrocyte differentiation, and that their downregulation might be required for the specification of these glial cells. Paradoxically, however, misexpression of Olig2 alone promotes ectopic induction of *Ngns*, as part of its activity to induce motoneuron differentiation (Novitch et al., 2001). As Nkx2.2 potently inhibits motoneuron differentiation (Ericson et al., 1997), we reasoned that one essential function of Nkx2.2 in promoting ectopic oligodendrocyte differentiation might be to prevent the induction of *Ngns* by Olig2, thereby suppressing motoneuron differentiation. Consistent with this hypothesis, ectopic expression of Nkx2.2 repressed endogenous *Ngn1* and *2* expression (Figures 6A and 6C, arrow and data not shown). It also prevented the ectopic induction of these proneural genes by Olig2 (Figures 6B and 6D, arrow and data not shown).

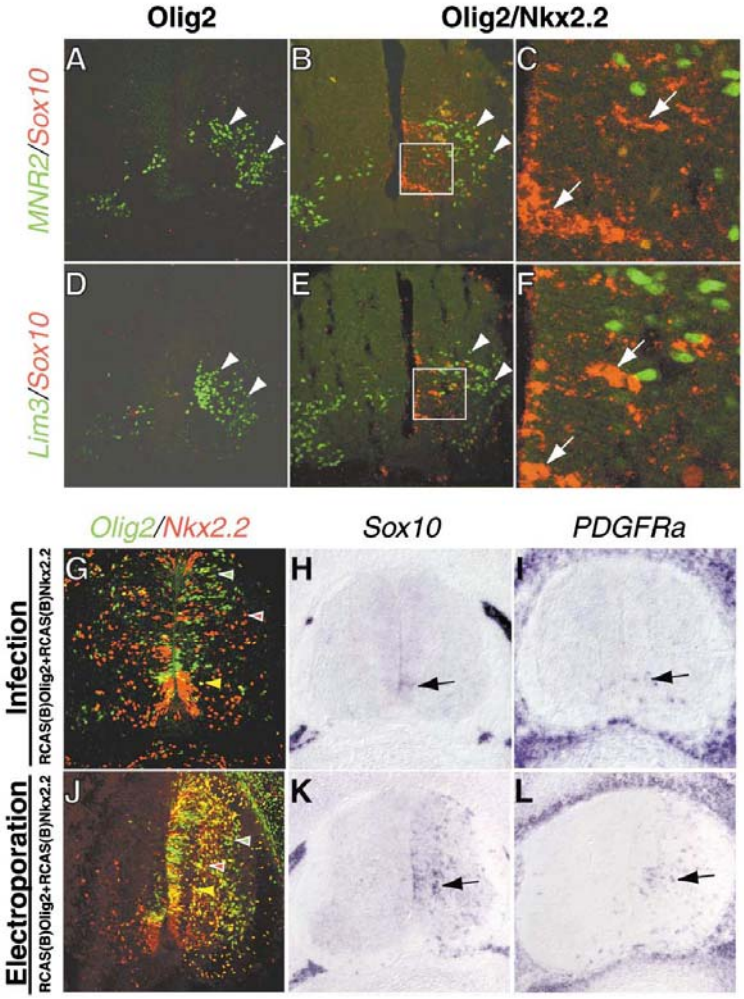

Figure 5. Collaboration between Olig2 and Nkx2.2 Cell-Autonomously Promotes Oligodendrocyte Differentiation at the Expense of Motoneuron Induction

(A–F) Cells expressing the oligo marker Sox10 (C and F, arrows) induced by Olig2+Nkx2.2 do not coexpress the motoneuron markers MNR2 (A and B, green, arrowheads) or Lim3 (D and E, green, arrowheads). The induction of ectopic motoneurons likely reflects cells singly expressing exogenous Olig2 but not Nkx2.2 (Novitch et al., 2001; see panel [J], green arrowhead). (G–L) Promotion of ectopic oligodendrocytes by Olig2 plus Nkx2.2 is cell autonomous. (G–I) Embryos infected with a 1:1 mixture of RCAS(B)-Olig2 and RCAS(B)-Nkx2.2 retroviruses, which do not coinfect the same cells (G, red and green arrowheads), do not produce ectopic oligodendrocyte differentiation (H and I). However, precocious expression of oligo markers is observed where exogenous Olig2 overlaps the endogenous Nkx2.2^{+} domain (G, yellow arrowhead and H and I, arrows). (J–L) Coelectroporation of RCAS(B)-Olig2 and RCAS(B)-Nkx2.2 plasmids results in coexpression of the two transgenes in many cells (J, yellow arrowhead) and induction of ectopic oligodendrocytes (K and L, arrows).

Given that ectopic coexpression of Olig2+Nkx2.2 indeed represses endogenous *Ngns*, we next asked whether such repression is essential for the induction of ectopic oligodendrocytes. To address this question, we triple-electroporated E2 embryos with *Ngn1, Olig2,* and *Nkx2.2* constructs. In the majority (7/10) of such embryos, the induction of ectopic oligodendrocytes by Olig2+Nkx2.2 was potently suppressed (compare Figures 6F and 6H with Figures 6E and 6G, arrow). To determine whether the repression of *Ngns* was also a

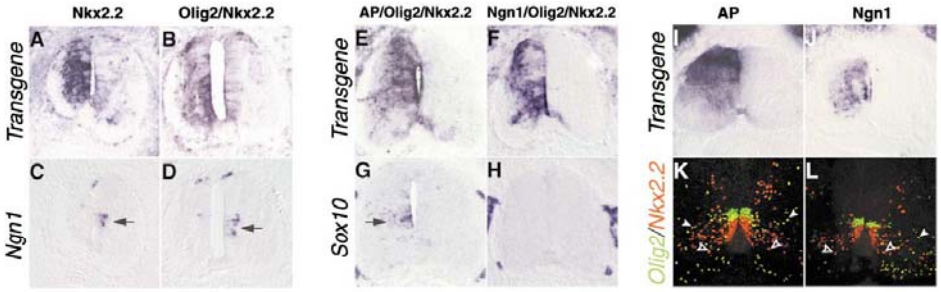

Figure 6. Repression of *Ngns* Is Required for Both Ectopic and Normal Oligodendrocyte Differentiation

(A–D) Nkx2.2 potently represses expression of *Ngn1* (C) and *Ngn2* (not shown), and prevents the induction of ectopic *Ngn1* expression by Olig2 (D and Novitch et al., 2001). (E–H) Inclusion of Ngn1 blocks ectopic oligodendrocyte differentiation induced by misexpression of Olig2+Nkx2.2, as assayed by induction of ectopic Sox10 expression (G, arrow and H) and other oligo markers (not shown). AP, control alkaline phosphatase-expressing plasmid. (I–L) Ngn1 alone blocks emigration of $Olig2^+$, $Nkx2.2^+$ oligodendrocyte precursors from their normal domain. Closed arrowheads in (K) and (L) indicate $Olig2^+$, $Nkx2.2^+$ cells, open arrowheads indicate $Nkx2.2^+$, $Olig2^-$ cells. Note the absence of double-positive cells on the electroporated (left) side of Ngn1-expressing embryos (L). (A)–(J) are in situ hybridizations, (K) and (L) double label immunofluorescence.

prerequisite for the specification of oligodendrocyte precursors in their normal location, we electroporated E2 embryos with the *Ngn1*-expressing vector alone (Figure 6J). In such embryos, most migrating oligodendrocyte precursor cells, identified by coexpression of endogenous Olig2 and Nkx2.2 (Figure 6K, solid arrowheads), were missing on the electroporated side (Figure 6L, left side), as were $Olig2^+Nkx2.2^+$ cells that had settled in the white matter. No such reduction in migrating $Olig2^+$, $Nkx2.2^+$ cells was observed in embryos electroporated with a control alkaline phosphatase (AP)-encoding construct (Figures 6I and 6K, filled arrowheads). Taken together, these data indicate that downregulation of Ngns is an essential prerequisite for both normal oligodendrocyte differentiation, as well as for ectopic oligodendrocyte differentiation elicited by coelectroporation of Olig2+Nkx2.2.

Misexpression of Olig2 in the Absence of Ngns and Nkx2.2 Promotes Development of Oligodendrocyte Precursors

The foregoing data implied that one essential function of Nkx2.2 in collaborating with Olig2 is to repress endogenous Ngns, which otherwise interfere with oligodendrocyte precursor specification. However, during normal development, the clearance of Ngns from the $Olig2^+$ domain of the ventricular zone precedes the overlap with Nkx2.2 in this region (compare Figures 2 and 3). Thus, Nkx2.2 is unlikely to be responsible for repressing Ngns within the $Olig2^+$ domain during normal oligodendrocyte development. This observation raised the question of whether the sole function of Nkx2.2 in collaborating with ectopically expressed Olig2 is to repress the *Ngns* and thereby prevent motoneuron differentiation. If this were the case, then Olig2 should be sufficient to promote ectopic oligodendrocyte differentiation in the absence of Nkx2.2, if the expression or function of *Ngns* could be prevented by other means.

To repress Ngn expression and function, we misexpressed components of the Notch signaling pathway (Artavanis-Tsakonas et al., 1999). Notch signaling is known to be necessary and sufficient to repress expression of *Ngns* in a number of vertebrate systems (Ma et al., 1996, 1998; de la Pompa et al., 1997). Repression of such proneural genes by Notch involves proteolytic cleavage and nuclear translocation of its intracellular domain (ICD), which then interacts with Suppressor of Hairless (Su(H); $RBPJ_k$) to promote expression of transcriptional effectors such as *Hes* genes (reviewed in Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000). In several systems, $Notch^{ICD}$ has been shown to be a constitutively active form of Notch (Struhl et al., 1993; Chitnis et al., 1995). We therefore asked whether repression of *Ngns* by expression of $Notch^{ICD}$ would be sufficient to allow Olig2 to promote ectopic oligodendrocytes in the absence of exogenous Nkx2.2. To ensure that levels of endogenous *Su(H)* were not limiting in this experiment, we coelectroporated a construct expressing that gene as well (Wettstein et al., 1997).

Expression of $Notch^{ICD}$+Su(H) indeed repressed *Ngn1* on the electroporated side of the neural tube, in either the absence (Figure 7F, arrow) or presence (Figure 7D, arrow) of coelectroporated *Olig2*. Similar results were obtained for *Ngn2* (data not shown). Such repression was by itself insufficient to promote ectopic oligodendrocyte differentiation (Figures 7I, 7L, and 7O; the small number of bilaterally migrating oligodendrocyte precursors in [L] reflects the fact that these particular embryos were analyzed at E6.5 rather than E6.0). In contrast, when embryos were triple electroporated with $Notch^{ICD}$, Su(H) and Olig2, ectopic expression of the oligodendrocyte precursor markers Sox10 and $PDGFR\alpha$ was clearly detected in the dorsal neural tube (Figures 7G and 7J, black arrowheads). Positive cells were observed in both the ventricular zone and in the gray matter, suggesting that migration of such ectopic precursors had occurred. However, expression of mature oligodendrocyte markers, such as MBP, was not detected (Figure 7M).

These data suggested that Olig2 is able, in the absence of Nkx2.2, to promote ectopic oligodendrocyte

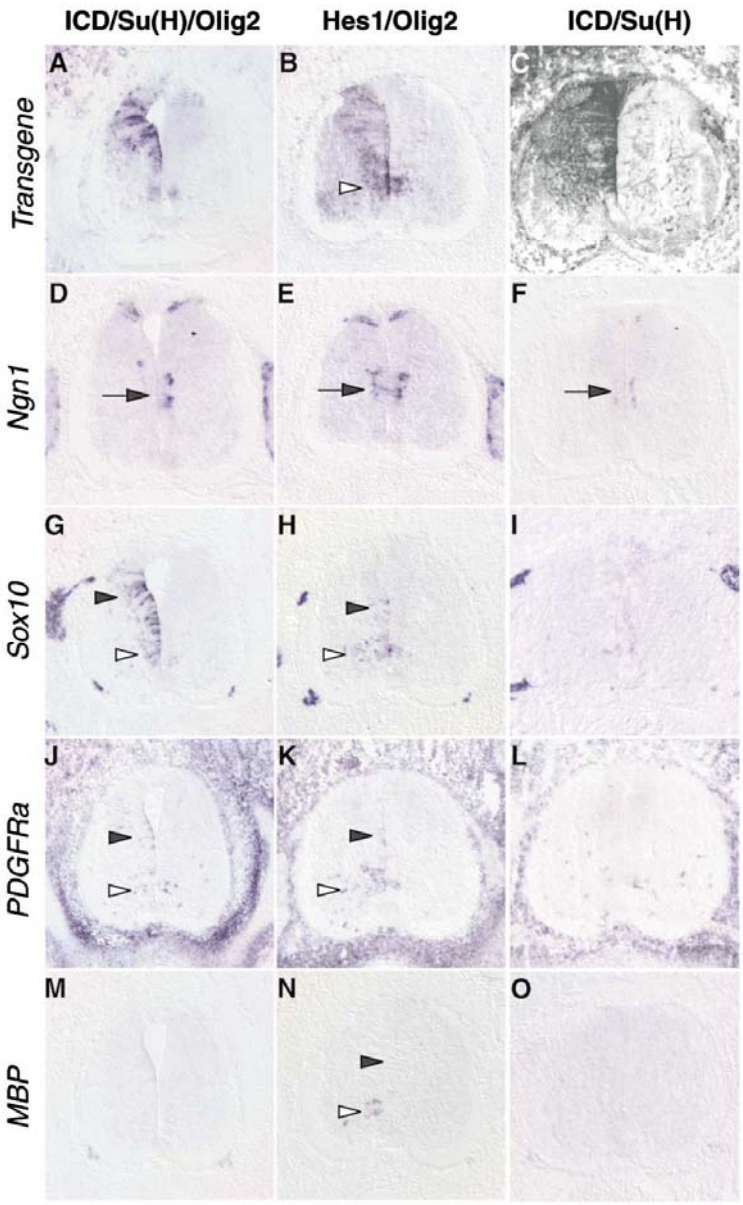

Figure 7. Olig2 Is Sufficient to Induce Ectopic Oligodendrocyte Precursor Markers, but Not MBP$^+$ Oligodendrocytes, in the Absence of Ngns and Nkx2.2

Embryos were electroporated with the mixture of plasmids indicated above the columns, and hybridized with the probes indicated next to the rows. The combination of Notch^{ICD} ("ICD") and Su(H) represses expression of *Ngn1* (F, arrow) and *Ngn2* (not shown), but does not induce ectopic oligo precursor markers (I and L) or MBP (O). In the presence of Olig2, induction of *Ngn1* (D) and *Ngn2* (not shown) is still blocked, and Sox10 (G, closed arrowheads) and PDGFRα (J, closed arrowheads) are ectopically induced. However, no MBP is detected in the dorsal

precursor specification, but not differentiation, if endogenous *Ngns* are repressed. The lack of ectopic MBP$^+$ cells under these circumstances might, therefore, reflect an additional requirement for Nkx2.2 in oligodendrocyte differentiation, independent of its function to repress *Ngns*. Consistent with this, Olig2 alone promoted precocious differentiation of MBP$^+$ cells when expressed in the endogenous Nkx2.2$^+$ p3 domain, where *Ngns* are normally not expressed (Figure 3 and Figure 4P). Alternatively, the lack of ectopic mature oligodendrocyte markers in embryos coelectroporated with Olig2 and Notch^{ICD}+Su(H) might instead reflect an effect of constitutive Notch activation to inhibit terminal differentiation of oligodendrocyte precursors, as described in other studies (Wang et al., 1998).

To try to distinguish these alternatives, we sought a less potent way of mimicking constitutive Notch activation. To this end, we employed Hes1 (Akazawa et al., 1992), a downstream transcriptional effector of Notch that mediates some, but not all, of its biological actions (Ishibashi et al., 1995; Kageyama and Nakanishi, 1997). Consistent with this assumption, electroporation of *Hes1* in either the presence or absence of exogenous *Olig2* produced a detectable, but less pronounced, repression of *Ngn1* (Figure 7E, arrow and data not shown) than was observed using Notch^{ICD}+Su(H) (cf. Figure 7D, arrow). Despite this relatively modest effect on *Ngn* expression, coelectroporation of *Olig2*+*Hes1* also promoted ectopic expression of Sox10 and PDGFRα (Figures 7H and 7K, black arrowheads). Electroporation of *Hes1* alone did not have this effect (not shown). Moreover, in those embryos where *Hes1*+*Olig2* were coelectroporated in the Nkx2.2$^+$ ventral region of the neural tube (Figure 7B, open arrowhead), not only migrating oligodendrocyte precursors (Figures 7H and 7K, open arrowheads), but also MBP$^+$ oligodendrocytes (Figure 7N, open arrowhead) were observed precociously. In contrast, ectopic MBP$^+$ cells were not detected when exogeneous *Olig2* and *Hes1* were coexpressed in more dorsal regions where endogenous *Nkx2.2* is not expressed (Figure 7N, black arrowhead). These data suggest that exogenous Hes1 does not inhibit precocious expression of mature oligodendrocyte markers promoted by exogenous Olig2 plus endogenous Nkx2.2. Taken together, these results suggest that repression of *Ngn* expression by activated Notch (or its downstream effectors) allows specification of ectopic oligodendrocyte precursors by Olig2. Progression of such precursors to MBP$^+$ oligodendrocytes may require additional functions provided by Nkx2.2, as well as abrogation of Notch signaling (Wang et al., 1998).

Olig2 and Nkx2.2 Both Function as Transcriptional Repressors in Promoting Oligodendrocyte Differentiation

Basic helix-loop-helix proteins of the "B" class, such as MyoD, typically function as transcriptional activators (Weintraub et al., 1991). Surprisingly, fusion of Olig2 to the yeast Gal4 DNA binding domain indicated that it acts instead as a repressor (Novitch et al., 2001). Consistent with this observation, a fusion of the Olig2 bHLH domain with the transcriptional repressor domain of *Drosophila* engrailed (Olig2-EnR) mimics the activity of intact Olig2 to promote motoneuron differentiation (Novitch et al., 2001). Conversely, an activator form of Olig2, Olig2-VP16, antagonized motoneuron differentiation (Novitch et al., 2001). These results led us to test whether Olig2-EnR or Olig2-VP16 would phenocopy the activity of intact Olig2 to promote ectopic oligodendrocyte differentiation in combination with Nkx2.2. We found that the Olig2bHLH-EnR fusion phenocopied the intact protein (Figures 8A, 8D, and 8G), while the Olig2-VP16 fusion did not (data not shown). Such an activity was not observed by coelectroporating either a construct containing just the Olig2 bHLH domain, or a Phox2a-EnR fusion, together with Nkx2.2 (data not shown). Thus the effect of Olig2-EnR requires both the EnR domain, and correct DNA binding specificity conferred by the Olig2 bHLH domain. In support of the latter conclusion, a full-length Olig2 construct lacking the basic region was inactive in this assay, confirming that the activity of this protein requires DNA binding.

We also examined whether the Olig2bHLH-EnR fusion could function in the absence of Nkx2.2 to specify oligodendrocyte precursors, using Notch^{ICD}+Su(H) to repress endogenous *Ngns*. Triple electroporation of these constructs indeed promoted formation of ectopic Sox10$^+$ oligodendrocyte precursors (Figure 8F, arrow). These results suggest that Olig2 also acts as a repressor in its Nkx2.2-independent oligodendrocyte precursor-specification function. Importantly, in such triply electroporated embryos, precocious MBP$^+$ oligodendrocytes were still detected in the ventral region where endogenous Nkx2.2 is expressed (Figure 8I, open arrowhead), but not in more dorsal regions (Figure 8I, arrow). These data provide further evidence that the inability of Olig2 alone to promote expression of mature oligodendrocyte markers reflects a requirement for Nkx2.2 in this later differentiation step, and not simply an inhibition of such differentiation by constitutive Notch activation. Nevertheless, we cannot exclude that exogenous Olig2 promoted MBP expression in the Nkx2.2$^+$ domain in only a subset of cells which did not coexpress Notch^{ICD} and/or Su(H).

Previous studies have shown that Nkx2.2 functions as a repressor at earlier stages of neural tube development when it promotes v3 interneuron specification (Muhr et al., 2001). We were therefore curious to know whether Nkx2.2 similarly functions as a repressor in the ectopic oligodendrocyte differentiation assay. When coelectroporated with either the Olig2bHLH-EnR fusion (Figures 8B, 8E, and 8H) or intact Olig2 (not shown), an Nkx2.2 homeodomain-EnR fusion (Nkx2.2EnR; Muhr et al., 2001) also promoted ectopic oligodendrocyte differenti-

domain (M). Similar results were obtained using Hes1 instead of ICD+Su(H) (E, H, K, and N), although the extent of *Ngn* repression was not as pronounced (E, arrow). Note that in the region where exogenous Hes1+Olig2 overlap the ventral domain where endogenous Nkx2.2 is expressed (B, open arrowhead; cf. Figure 1B), not only oligo precursor markers (H and K, open arrowheads), but also MBP (N, open arrowhead) are expressed. This suggests that Hes1 does not block induction of MBP by the combination of exogenous Olig2 plus endogenous Nkx2.2.

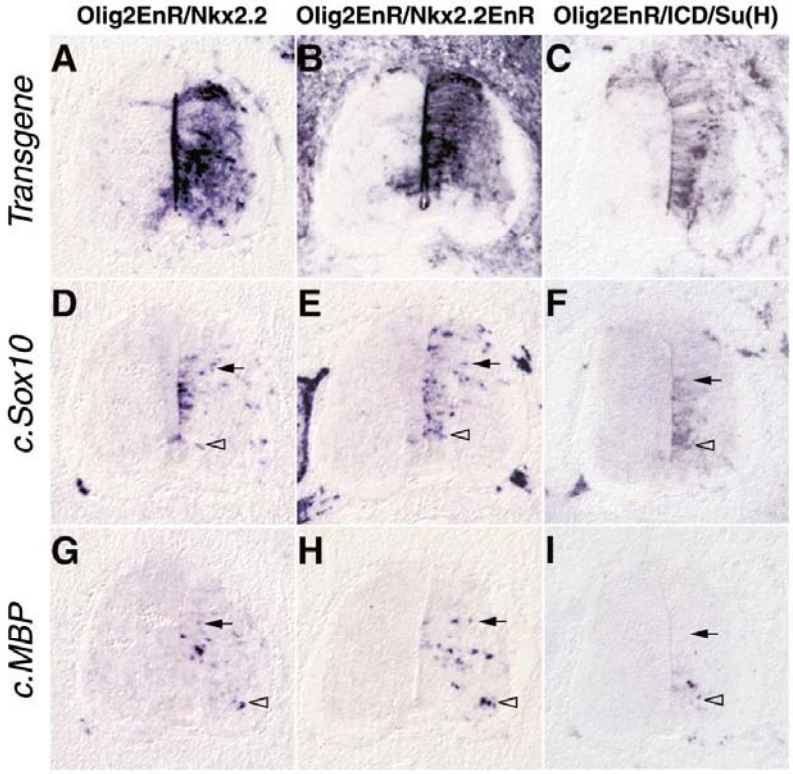

Figure 8. Olig2 and Nkx2.2 Act as Repressors in Promoting Ectopic Oligodendrocyte Differentiation

Coelectroporation of an Olig2bHLH-EnR fusion with either intact Nkx2.2 (A, D, and G) or an Nkx2.2 homeodomain-EnR fusion (Nkx2.2HD-EnR) (B, E, and H) results in ectopic expression of both Sox10 (D and E) and MBP (G and H). Similar results were obtained when EnR fusions of both proteins were coelectroporated (not shown). (C, F, and I) Triple electroporation of Olig2bHLH-EnR+ICD+Su(H) induces ectopic and precocious expression of Sox10 dorsally (F, arrow), but dorsal expression of MBP is not detected (I, arrow). However, precocious MBP expression is detected on the electroporated side in the ventral region where the transgenes overlap the domain of endogenous Nkx2.2 expression. This result again suggests that Nkx2.2 is required for progression to mature oligodendrocytes.

ation. In contrast, the corresponding activator form, Nkx2.2-VP16, did not promote ectopic oligodendrocytes when coelectroporated with full-length Olig2 (data not shown). Thus, EnR fusion proteins phenocopied the activities of both intact Olig2 and Nkx2.2 in this assay. This suggests that both Olig2 and Nkx2.2 function as repressors, rather than as activators, in promoting ectopic oligodendrocyte differentiation.

Olig2-VP16 Inhibits Oligodendrocyte Formation

The finding that Olig2bHLH-EnR phenocopies the activity of intact Olig2 suggested in turn that the corresponding activator form, Olig2bHLH-VP16, might conversely function as a dominant inhibitor of endogenous Olig2 function. Consistent with this notion, the Olig2bHLH-VP16 fusion suppresses motoneuron differentiation (Novitch et al., 2001). Accordingly, we tested whether this Olig2bHLH-VP16 fusion would also inhibit oligodendrocyte precursor formation. Electroporation of this construct caused an approximately 40% reduction in the average number of $Olig2^+$, $Nkx2.2^+$ oligodendrocyte precursors in gray and white matter, in embryos harvested at E8 (Figures 9A, 9B, arrowheads, and 9D). There was also a reduction in the number of $PDGFR\alpha^+$ cells on the electroporated side (Figure 9C, arrowheads). By contrast, the number of $Nkx2.2^+$, $Olig2^-$ progenitors was unaffected (Figure 9D). (Since such single-positive cells constitute only ~25% of the oligodendrocyte precursor population at this stage, their persistence does not mask the reduction of $PDGFR\alpha^+$ cells.)

In addition, among cells in the gray or white matter expressing the Olig2-VP16 transgene, only $8 \pm 2\%$ coexpressed endogenous Olig2. In contrast, cells coexpressing endogenous Olig2 and the Olig2-VP16 transgene could be easily observed in the ventricular zone (Figure 9A, yellow arrow). These data suggest that the reduction in $Olig2^+$ cells in the gray and white matter reflects an inhibition of oligodendrocyte precursor generation or

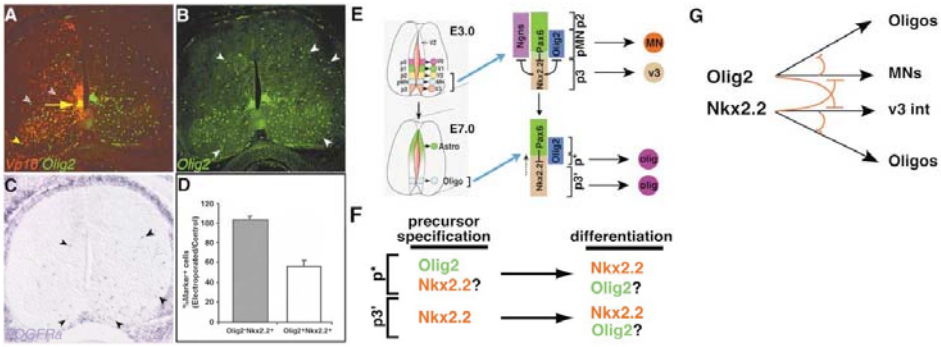

Figure 9. Olig2-VP16 Inhibits Oligodendrocyte Precursor Formation

(A)–(C) show sections from an embryo electroporated with an Olig2-VP16 fusion at E2 and harvested at E8. Migrating oligo precursors are marked by expression of Olig2 (B, arrowheads). Olig2-VP16 causes a reduction in the number of migrating Olig2$^+$ cells (A, green arrowheads and B), as well as of PDGFRα^+ cells (C) on the electroporated (left) side relative to the contralateral control (right) side. Quantification (D) shows an approximately 40% reduction in the number of Olig2$^+$, Nkx2.2$^+$ oligodendrocyte precursors (white bar) on the electroporated versus the contralateral control side. In contrast, the number of Olig2$^-$, Nkx2.2$^+$ cells (shaded bar) is unchanged. Numbers represent the average of six sections from a single experiment; the experiment was performed twice with similar results. (E) Summary of spatio-temporal changes in progenitor domains and their relationship to oligodendrocyte production. Between E3.0 and E7.0, the ventral-most expression domain of Pax6 (light green) disappears, and Nkx2.2 expands dorsally into this region to overlap Olig2. In this way, a new progenitor domain (p*) becomes intercalated dorsally to the erstwhile p3 domain (p3'). (F) Olig2 and Nkx2.2 may act simultaneously and/or sequentially in precursor specification and differentiation. The ventral-most oligodendrocyte precursors that emerge from p3' initially express Nkx2.2 but not Olig2. However, these cells appear to subsequently coexpress Olig2 in white matter. (G) Cooperation of Olig2 and Nkx2.2 in p*-derived oligodendrocyte precursors may involve mutual antagonism of the neuron-promoting activities of each factor. Activation of motoneuron (MN) and v3 interneuron differentiation by Olig2 and Nkx2.2, respectively, is assumed to be mutually exclusive with oligodendrocyte differentiation. Note that this model applies only to p*-derived oligodendrocyte precursors which coexpress Nkx2.2 and Olig2.

survival, and not simply negative regulation of endogenous *Olig2* expression by Olig2-VP16. The inhibition of oligodendrocyte formation obtained with Olig2-VP16 was not observed using just the bHLH domain of Olig2, with the Olig2-EnR fusion, or with a Phox2a-VP16 fusion (not shown). Thus the inhibition reflects transcriptional activation functions conferred by the VP16 domain, and not simply the deletion of the Olig2 domain that it replaces.

That oligo precursor formation was not completely inhibited by the Olig2bHLH-VP16 fusion likely reflects the fact that some ventricular zone cells escaped the initial electroporation. Consistent with this interpretation, most (~66%) of the Olig2$^+$ precursors that did migrate on the electroporated side did not coexpress the Olig2-VP16 transgene (Figure 9A, green arrowheads), although a few did (yellow arrowhead). These latter cells may represent precursors that expressed the Olig2-VP16 fusion only after they had committed to the oligodendrocyte fate, perhaps because they acquired the transgene by secondary infection rather than during the initial electroporation. The increasing proportion of such secondarily infected cells, and our inability to distinguish them from the cells that initially incorporated the transgene by electroporation, precluded an analysis at later stages (E9.5 and older) when mature oligodendrocyte markers such as MBP are first expressed (Figure 1T). Thus these experiments do not address whether Olig2-VP16 inhibits oligodendrocyte maturation. However, they do indicate that this activator form of Olig2 inhibits earlier stages of oligodendrocyte precursor formation or survival.

Discussion

Progenitor cells in the ventricular zone of the ventral spinal cord undergo a developmental transition from generating neurons to producing oligodendrocytes (Sun et al., 1998; Richardson et al., 2000). The evidence presented in this and in the companion paper (Novitch et al., 2001) together indicate that the same bHLH transcription factor, Olig2, plays a determinative and sequential role in both motoneuron and oligodendrocyte fate specification. This dual function is controlled by spatio-temporal changes in the domains of expression of several other transcription factors in relation to the domain of *Olig2* expression. These changes include both the extinction of *Ngn1* and *2* expression within the Olig2$^+$ domain and the acquisition of a partial overlap with the underlying Nkx2.2$^+$ domain. That such changes in expression are functionally important is suggested by the fact that Olig2 can collaborate with Nkx2.2 to promote ectopic oligodendrocyte differentiation, and that this effect is blocked by constitutive expression of Ngn1. In this way, the landscape of gene expression in the spinal cord ventricular zone changes with time, to enable sequential utilization of common transcription factors in the production of different cell types.

Multiple Populations of Oligodendrocyte Precursors

Our double labeling experiments with antibodies to Olig2 and Nkx2.2 suggest the existence of at least two different oligodendrocyte precursor populations, with distinct spatial origins in the chick ventricular zone. One population coexpresses Olig2 and Nkx2.2, and emigrates from

the zone of overlap between these two factors, while the other population expresses Nkx2.2 but not Olig2, and emigrates more ventrally (Figures 9E and 9F). These data are surprising since there was no reason previously to think that spinal cord oligodendrocyte precursors were anything other than a homogenous population.

The observation of oligodendrocyte precursor heterogeneity has several important implications. First, it raises the question of whether it serves to generate different subpopulations of oligodendrocytes with distinct functional properties. Second, it suggests that some oligodendrocyte precursors (i.e., those that are Nkx2.2$^+$ and Olig2$^-$) can be specified in the absence of Olig2. How this occurs is not clear; however, we note that Olig3, which is closely related to Olig2, is coexpressed with Nkx2.2 in the p3 domain (data not shown). Third, it may help to resolve disagreements about whether oligodendrocyte precursors arise from the erstwhile motoneuron progenitor domain (pMN) (Richardson et al., 1997; Sun et al., 1998), or rather the underlying p3 domain (Soula et al., 2001); our discovery raises the possibility that they may arise from both locations (Figure 9E).

Temporal Roles of Olig2 and Nkx2.2 in Oligodendrocyte Fate Determination

Our results demonstrate that exogenous Olig2 and Nkx2.2 can collaborate to promote ectopic oligodendrocyte development. This functional interaction is well correlated with the fact that coexpression of the endogenous proteins in oligodendrocyte precursors coincides with their emergence from the ventricular zone. Nevertheless, it is not completely clear whether this collaboration reflects a simultaneous, or rather sequential, function for the two transcription factors in oligodendrocyte development (Figure 9F). When misexpressed without Nkx2.2 in the context of constitutive Notch signaling, Olig2 is able to ectopically induce early oligodendrocyte precursor markers such as *Sox10* and *PDGFR*α, but not later differentiation markers such as *MBP*. Expression of *MBP* is only detected when *Olig2* is coexpressed with *Nkx2.2* (either exogenous or endogenous). Consistent with these data, misexpression of Olig2 in chick limb bud (which does not express Nkx2.2) induced ectopic expression of *Sox10*, but not of later markers such as MBP (Zhou et al., 2000). In addition, expression of *MBP* is lost in *Nkx2.2*$^{-/-}$ mice, while the number of oligodendrocyte precursors does not appear to be diminished (M. Qiu, personal communication). These observations suggest that Olig2 can on its own induce oligodendrocyte precursor formation under some circumstances, but that Nkx2.2 is required to permit such precursors to progress to later stages of differentiation. Nevertheless, the observation that some oligodendrocyte precursors are initially Nkx2.2$^+$ and Olig2$^-$ raises the possibility that the homeodomain factor may also function in precursor specification in some circumstances (Figure 9F).

Is Olig2, conversely, important only for oligodendrocyte precursor specification, or also for later stages of differentiation? Olig2 expression persists in mature oligodendrocytes (Lu et al., 2000 and this study). In addition, most or all Nkx2.2$^+$, Olig2$^-$ oligodendrocyte precursors appear to subsequently express *Olig2* after they settle in the white matter (Supplemental Figure S2G). Furthermore, at intermediate stages of development, Nkx2.2$^+$ cells in the white matter that are Olig2$^-$ do not yet express MAG, a marker of mature oligodendrocytes (Figures 2K–2N, arrowheads), while all neighboring cells that express MAG are positive for both Nkx2.2 and Olig2 (Figure 2K–2N, arrows). This circumstantial evidence is consistent with the idea that Olig2 may function in oligodendrocyte differentiation as well as in precursor specification. Such a late function may have been obscured in our experiments by the use of constitutive Notch pathway components to examine Olig2 activities in the absence of Nkx2.2 (Wang et al., 1998).

One caveat to our conclusion that Olig2 and Nkx2.2 collaborate in oligodendrocyte fate determination is that in rats and mice, Olig2$^+$ oligodendrocyte precursors do not detectably coexpress Nkx2.2 when they first migrate, although some coexpression within the ventricular zone can be detected (Supplemental Figures S2B–S2D). Nevertheless, many Olig2$^+$ cells do eventually coexpress Nkx2.2 after they have settled in the white matter (Supplemental Figure S2F). Thus, the most general implication of our results is that Olig2 and Nkx2.2 collaborate in oligodendrocyte fate determination, but that there may be species and/or lineage differences in the precise time at which these two factors are brought into play.

In addition to the collaboration between Olig2 and Nkx2.2 and the extinction of *Ngn* expression, there may be other time-dependent contextual changes required for oligodendrocyte generation. This is suggested by the observation that the induction of ectopic oligodendrocyte markers by comisexpression of Olig2+Nkx2.2 is detectable at E6, but not at E4. In contrast, ectopic motoneuron differentiation promoted by Olig2 alone is already detectable at E4 (Novitch et al., 2001). Apparently, the dorsal spinal cord is not competent to express oligodendrocyte markers in response to coexpression of Olig2+Nkx2.2 until after E4. This may reflect the fact that neurogenesis continues in the dorsal spinal cord after it has ceased in the motoneuron domain (E5.5–E6), and precludes oligodendrocyte formation. Consistent with this notion, quadruple electroporation of Olig2+Nkx2.2 together with Notch^{ICD}+Su(H) (to inhibit neurogenesis) yielded some ectopic Sox10$^+$ cells as early as E4.5 (data not shown).

Olig2 and Nkx2.2 Act as Repressors in Oligodendrocyte Fate Determination

Olig2-EnR, but not Olig2-VP16, can substitute for wild-type Olig2 in collaborating with Nkx2.2 to promote oligodendrocyte differentiation. These data suggest that Olig2 acts as a repressor in oligodendrocyte, as well as in motoneuron (Novitch et al., 2001), fate determination. What genes does Olig2 repress to promote oligodendrocyte development? As this function involves collaboration with Nkx2.2, and as Nkx2.2 on its own normally promotes v3 interneuron specification, one possibility is that Olig2 represses this interneuron-promoting function of Nkx2.2. Consistent with this idea, induction of ectopic Sim1$^+$ v3 interneurons by Nkx2.2 is inhibited by coelectroporation of *Olig2* (data not shown). If the v3 interneuron-promoting function of Nkx2.2 were incompatible with a concurrent function in gliogenesis, then

antagonism of this neurogenic function by Olig2 might allow Nkx2.2 to promote oligodendrocyte generation (Figure 9G).

Nkx2.2 also appears to function as a repressor in oligodendrocyte differentiation, as it does in v3 interneuron generation (Muhr et al., 2001). One function of this repressor activity may be to antagonize the promotion of motoneuron differentiation by Olig2. Consistent with this, Nkx2.2 strongly inhibits motoneuron differentiation when ectopically expressed in the Olig2$^+$ pMN domain (Briscoe et al., 1999, 2000). Thus, one way of viewing the collaboration between Olig2 and Nkx2.2 in oligodendrocyte differentiation is that each functions to antagonize the other's ability to promote differentiation of a particular neuronal subtype. In this way, oligodendrocytes would emerge when the convergent activity of Olig2 and Nkx2.2 prevents differentiation of either motoneurons or v3 interneurons (Figure 9G). This view does not preclude the possibility that Nkx2.2 plays a dual role as a transcriptional activator (Watada et al., 2000) and repressor in oligodendrocyte differentiation.

An Activator Form of Olig2 Inhibits Oligodendrocyte Development

Our gain-of-function experiments demonstrate that *Olig2* is sufficient to promote ectopic oligodendrocytes when misexpressed together with Nkx2.2, but is this gene required during normal oligodendrocyte development? Since a repressor form of Olig2, Olig2-EnR, phenocopied the function of intact Olig2 to promote ectopic oligodendrocytes in combination with Nkx2.2, we reasoned that the corresponding activator form, Olig2-VP16, might conversely inhibit endogenous oligodendrocyte formation. Consistent with this expectation, the number of oligodendrocyte precursors was reduced by the Olig2-VP16 fusion. The extent of the reduction (~40%) was similar to the extent of reduction of Isl1/2$^+$ motoneurons by Olig2-VP16 (~50%; Novitch et al., 2001).

The inhibition of motoneuron differentiation by Olig2-VP16 raises the possibility that its activity to inhibit oligodendrocyte precursor formation is a secondary, non-cell-autonomous consequence of this earlier effect. Several lines of evidence argue against this possibility. First, the majority (~66%) of oligo precursors that do migrate in Olig2-VP16-electroporated embryos do not coexpress the transgene. Second, only 8 ± 2% of Olig2-VP16$^+$ cells in gray and white matter were oligodendrocyte precursors. Third, oligodendrocytes are generated normally in the absence of motoneurons, in spinal cord explants from *Isl1*$^{-/-}$ embryos (Sun et al., 1998) (in which motoneuron differentiation and survival is prevented; Pfaff et al., 1996). These data suggest that Olig2-VP16 inhibits oligodendrocyte formation cell autonomously. On the assumption (Muhr et al., 2001) that Olig2-VP16 behaves as a dominant-negative (antimorphic) form of Olig2, this inhibition would suggest that endogenous Olig2 (and/or other Olig proteins) is necessary for oligodendrocyte development in vivo. However, we cannot formally exclude that Olig2-VP16 behaves instead as a neomorph that prevents oligodendrocyte formation indirectly, via induction of unknown inhibitory factors. Nevertheless, preliminary analysis of *Olig* knockout mice supports the idea that *Olig* genes are indeed required for oligodendrocyte formation in vivo (Q.Z. and D.J.A., unpublished observations).

Temporal Control of Neural Fate Specification Involves Alterations in Progenitor Domains

At earlier stages of spinal cord development (E3.0–E4.0), motoneurons arise from the pMN domain. This domain is congruent with the zone of *Olig2* expression (Novitch et al., 2001). It is bounded ventrally by the domain of expression of Nkx2.2, which potently represses *Olig2*, and dorsally by Irx3 (Briscoe et al., 2000). Our results indicate that the pMN domain no longer exists at later stages when oligodendrocytes are being generated. Rather, the ventral part of the Olig2$^+$ domain now overlaps the dorsal part of the Nkx2.2$^+$ domain. In this way, a new progenitor domain (Figure 9E, p*) becomes established above p3.

This overlap appears to reflect a dorsal-ward expansion of Nkx2.2 into the Olig2$^+$ domain, rather than vice-versa. At earlier stages, the Olig2$^+$ pMN domain expresses a lower level of Pax6 expression than does the overlying p2 domain (Ericson et al., 1997; Figure 9E, upper, and Supplemental Figures S3A–S3C). However, at later stages (E6–E8), this zone of low Pax6 expression progressively contracts dorsally, and the Nkx2.2 boundary rises until it abuts the more dorsal domain of intermediate-level Pax6 expression (Figure 9E, lower and Supplemental Figures S3D–S3O). Since Pax6 represses Nkx2.2 expression (Briscoe et al., 2000; Muhr et al., 2001), the extinction of Pax6 expression within the ventral part of the Olig2$^+$ domain would permit Nkx2.2 to expand dorsally into this region. Such a change might occur via a temporal increase in *Sonic Hedgehog (Shh)* expression, which positively regulates Nkx2.2 (Briscoe et al., 1999) and negatively regulates Pax6 (Ericson et al., 1997). In this respect, it is interesting that Shh has recently been shown to be important at later stages for oligodendrocyte differentiation (Soula et al., 2001), in addition to its earlier role in neural tube ventralization and motoneuron induction (Ericson et al., 1996).

The observation of overlap between the Olig2$^+$ and Nkx2.2$^+$ domains is surprising, given that Nkx2.2 is a potent repressor of Olig2 at earlier developmental stages (Novitch et al., 2001). Evidently, this repressor activity must be abrogated, at least in the domain where Nkx2.2 and Olig2 are coexpressed. How might this occur? Our data indicate that in the presence of constitutive Olig2, Nkx2.2 is converted from an inhibitor to a coinducer of endogenous *Olig2* expression. Since the overlap between Nkx2.2 and Olig2 occurs by dorsal expansion of *Nkx2.2* expression into the Olig2$^+$ domain, the pre-existing Olig2 protein could conceivably protect *Olig2* from repression by Nkx2.2. Alternatively, other unknown factors might serve to protect *Olig2* from such repression until the positive-feedback loop with Nkx2.2 could be established. Similarly, Olig2 must also lose its erstwhile ability to derepress *Ngn2* (Novitch et al., 2001) in order to promote oligodendrocyte generation. The mechanisms that control these temporal changes in regulatory circuitry will provide interesting topics for further study.

Our results, taken together with those of Novitch et

al. (2001), indicate that the switch from motoneuron to oligodendrocyte generation involves changes in the crossregulatory interactions that position some of the boundaries of transcription factor gene expression in the ventricular zone of the spinal cord (Briscoe et al., 2000). These changes alter the landscape of the progenitor domains established at earlier stages, and permit the same transcription factor to function sequentially in neuronal and glial fate determination. Such dynamic changes in spatial patterning may represent a general mechanism for sequentially generating different neural cell types from a common progenitor domain.

Experimental Procedures

Chick Spinal Cord Library Construction and Isolation of Chick *Olig2* cDNA Clones

Trunk neural tubes of E3 and E3.5 chick embryos were dissociated using a modified enzymatic treatment method based on the protocol of Stemple et al. (Stemple and Anderson, 1992). Total RNA was extracted with TriZol reagent (Gibco), and cDNA synthesis performed with the Superscript Choice System (Gibco) according to the manufacturer's instructions, using an Oligd(T) primer. After ligation into the ZipLox vetor (Gibco), the library was packaged with Gigapack (Stratagene), and amplified. 1.5×10^6 clones of the library were screened with the bHLH region of the mouse *Olig2* gene and 3 full-length cDNA clones corresponding to the chick *Olig2* gene were identified. Sequence alignment was performed with the CLUSTALW program of MacVector.

In Situ Hybridization

Nonradioactive in situ hybridization on frozen sections of chick embryos was performed as previously described (Zhou et al., 2000). The following chick gene probes were used: *Olig2*, *Olig3*, *Nkx2.2* (a gift of Dr. Mengsheng Qiu), *Sox10*, *PDGFRα* (a gift of Dr. Mark Mecola), *MBP*, *PLP/DM20*, *Neurogenin1*, *Neurogenin2*, *Olig2 3′UTR*, and *Olig3 3′UTR*.

Double label in situ hybridization was performed as previously descried (White and Anderson, 1999). The two different probes were labeled with either digoxigenin or fluorescein. The first probe was detected with alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody, developed with Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoly phosphate (BCIP), which yields a purple precipitate. After the NBT/BCIP reaction, the AP-conjugated anti-digoxigenin antibody was inactivated at 85°C, the slides were subsequently incubated with AP-conjugated anti-fluorescin antibody, and detected with 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl terazolium chloride (INT) and BCIP, which yields a reddish brown precipitate. For fluorescent in situ hybridization, the basic protocol is essentially the same except Fast Red was used as the color substrate.

Immunohistochemistry

The peptide APLPAHPGHPASHPAHHPILPPA near the C terminus of the chick Olig2 protein was synthesized and coupled to ovalbumin by ImmunoVison Technologies, Inc. Antibody production was carried out by the Caltech monoclonal facility.

Chick and rat embryos were fixed by immersion in 4% paraformaldehyde from1 hr to overnight at 4°C depending on the age. The following primary antibodies were used: c.Olig2 (1:100, mouse polyclonal), m.Olig2 (1:2000, Rabbit Polyclonal, kind gift of Dr. Takebayashi Hirohide), Nkx2.2 (1:10, hybridoma, Developmental Studies Hybridoma Bank (DSHB)), c.Neurogenin2 (1:1000, Rabbit polyclonal, Dr. Sharon Perez), Lim3 (1:100, hybridoma, DSHB), and MNR2 (1:100, hybridoma, DSHB). Secondary antibodies used were: goat-anti-rabbit-IgG-Alexa 568 and Goat-anti-mouse-IgG-Alexa 488 (Molecular Probes).

Electroporation and Infection of Chick Embryos

All expression constructs were cloned into either the RCASBP(B) or RCASBP(A) (Morgan and Fekete, 1996). The constructs Olig2bHLH-EnR and Olig2bHLH-VP16 are the kind gift of Dr. Ben Novitch. The control virus used in this study is RCASBP(B) encoding alkaline phosphatase. Viral production was performed essentially as described in Perez et al. (1999). Electroporation was carried out with a BTX Electro Squar Porator T820 at HH 13–15 (somites 15–19) (Christ and Ordahl, 1995).

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Accession Numbers

The chick Olig2 sequence reported in this paper has been deposited in GenBank with accession number AF411041.

Note Added in Proof

While our paper was in press, a report appeared showing a requirement for Nkx2.2 in oligodendrocyte differentiation: Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., Rao, M., Sussel, L., Rubenstein, J., and Qiu, M. (2001). Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. Development *128*, 2723–2733.