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Sonic hedgehog contributes to oligodendrocyte specification in the mammalian forebrain

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SUMMARY

This study addresses the role of Sonic hedgehog (Shh) in promoting the generation of oligodendrocytes in the mouse telencephalon. We show that in the forebrain, expression of the early oligodendrocyte markers *Olig2*, *plp/dm20* and *PDGFRα* corresponds to regions of *Shh* expression. To directly test if Shh can induce the development of oligodendrocytes within the telencephalon, we use retroviral vectors to ectopically express Shh within the mouse embryonic telencephalon. We find that infections with Shh-expressing retrovirus at embryonic day 9.5, result in ectopic *Olig2* and *PDGFRα* expression by mid-embryogenesis. By postnatal day 21, cells expressing ectopic Shh overwhelmingly adopt an oligodendrocyte identity. To determine if the loss of telencephalic Shh correspondingly results in the loss of oligodendrocyte production, we studied *Nkx2.1* mutant mice in which telencephalic expression of Shh is selectively lost. In accordance with Shh playing a role in oligodendrogenesis, within the medial ganglionic eminence of *Nkx2.1* mutants, the early expression of *PDGFRα* is absent and the level of *Olig2* expression is diminished in this region. In addition, in these same mutants, expression of both *Shh* and *plp/dm20* is lost in the hypothalamus. Notably, in the prospective amygdala region where *Shh* expression persists in the *Nkx2.1* mutant, the presence of *plp/dm20* is unperturbed. Further supporting the idea that Shh is required for the in vivo establishment of early oligodendrocyte populations, expression of *PDGFRα* can be partially rescued by virally mediated expression of Shh in the *Nkx2.1* mutant telencephalon. Interestingly, despite the apparent requirement for Shh for oligodendrocyte specification in vivo, all regions of either wild-type or *Nkx2.1* mutant telencephalon are competent to produce oligodendrocytes in vitro. Furthermore, analysis of CNS tissue from *Shh* null animals definitively shows that, in vitro, Shh is not required for the generation of oligodendrocytes. We propose that oligodendrocyte specification is negatively regulated in vivo and that *Shh* generates oligodendrocytes by overcoming this inhibition. Furthermore, it appears that a *Shh*-independent pathway for generating oligodendrocytes exists.

Keywords: Mouse, Shh, Oligodendrocytes, Telencephalon, *Olig1*, *Olig2*, PDGFRα, *Plp*

INTRODUCTION

Oligodendrocytes, through their ability to myelinate axon fascicles, play an essential role in the mature nervous system. Although the function of oligodendrocytes is well understood in the adult nervous system, little is known about the embryonic origins of this cell type. While studies of the development of myelinating cells have been undertaken in the spinal cord (Hall et al., 1996; Noll and Miller, 1993), optic nerve (Barres and Raff, 1994; Shi et al., 1998) and peripheral nervous system (Dong et al., 1999; Jessen and Mirsky, 1997), the origins of oligodendrocytes within the embryonic mouse telencephalon have not been thoroughly addressed.

In the spinal cord, oligodendrocyte precursors are thought to differentiate from bipolar migratory precursor cells that arise from specific regions of the ventral neuroepithelium and then disperse throughout the developing gray matter and populate white matter tracts (reviewed by Miller, 1996; Miller et al., 1999; Richardson et al., 2000; Spassky et al., 2000). Oligodendrocyte precursors in spinal cord express a number of molecular markers including *PDGFRα* (*Pdgfra* – Mouse Genome Informatics; Hall et al., 1996; Pringle and Richardson, 1993), *plp*/*dm20* (*Plp* – Mouse Genome Informatics; Spassky et al., 1998; Timsit et al., 1995), and the recently cloned bHLH transcription factors *Olig1* and *Olig2* (Lu et al., 2000; Zhou et al., 2000). The generation of oligodendrocyte progenitors appears to require Sonic hedgehog (Shh) signaling, which at least partially explains their ventral origin within the spinal cord. In this regard, antibody neutralization of Shh signaling results in a blockade in the emergence of oligodendrocytes and conversely ectopic oligodendrocyte precursors can be induced dorsally by Shh (Orentas et al., 1999; Poncet et al., 1996; Pringle et al., 1996). Furthermore, it has been shown that similar concentrations of Shh are required for the induction of

motoneurons and oligodendrocytes, suggesting that a common motor neuron-oligodendrocyte precursor may give rise to both populations (Orentas et al., 1999; Pringle et al., 1996; Richardson et al., 1997).

In embryonic day (E) 12.5 mouse spinal cord (E14 in rat, E7 in chick), *PDGFRα* expression in a few cells on each side of the ventral portion of the central canal provides one of the earliest markers of oligodendrocyte progenitors. As development proceeds, these cells increase in number and transit away from the midline into the parenchyma of the spinal cord. By E17 in the mouse *PDGFRα*-expressing cells are evenly distributed throughout the spinal cord (Pringle and Richardson, 1993; Pringle et al., 1996). That *PDGFRα*-expressing cells give rise to oligodendrocytes in the spinal cord was shown by immunoselection of these cells followed by their in vitro differentiation into oligodendrocytes (Hall et al., 1996). Furthermore, PDGF-A null mice have fewer *PDGFRα* progenitors and reduced numbers of oligodendrocytes compared with wild-type (wt) mice, suggesting a causal relationship between PDGF signaling and oligodendrogenesis (Fruttiger et al., 1999).

Other groups, based on work done in chick and mouse rhombencephalon, diencephalon and spinal cord, have tracked oligodendrocyte precursor populations by the expression of *Plp* (Perez Villegas et al., 1999; Spassky et al., 1998; Timsit et al., 1995). These transcripts are expressed from E9.5 in the mouse spinal cord, diencephalon, hypothalamus and rhombencephalon (Timsit et al., 1995). Similar to the immunoselection carried out with *PDGFRα*-expressing cells described above, Spassky et al. (1998) have shown that *lacZ*-positive cells selected from a mouse in which the expression of this reporter is directed by a *Plp* promoter also differentiate into oligodendrocytes in vitro. Presently it is not clear whether or not *Plp*- and *PDGFRα*-positive cells represent the same or distinct populations of oligodendrocyte precursors based on the observation that their spatial expression patterns are different (reviewed by Richardson et al., 2000; Spassky et al., 2000). Indeed, it has been long suggested that oligodendrocyte populations may be heterogeneous (Del Rio-Hortega, 1928; Bjartmar et al., 1994; Butt et al., 1994; Anderson et al., 1999a; Anderson et al., 1999b).

The identification of two novel bHLH genes, *Olig1* and *Olig2* represents the first known transcription factors that have been suggested to direct progenitors to assume an oligodendrocyte fate. In support of this notion, these genes mark various populations of developing oligodendrocytes and their expression is lost in *Shh* null mice. Although loss-of-function data are not yet available, these genes at present provide the best candidates for direct regulators of oligodendrocyte fate (Lu et al., 2000; Zhou et al., 2000).

Unlike the spinal cord, where the development of oligodendrocytes is well documented, the origin of oligodendrocytes in the mouse telencephalon is less clear. In accordance with findings in the spinal cord, in vitro work in rat suggests that only ventral telencephalic regions are able to give rise to oligodendrocytes (Birling and Price, 1998). Alternatively, others have suggested that telencephalic oligodendrocytes develop from a number of distinct sources including the ventral diencephalon (Pringle and Richardson, 1993; Richardson et al., 1997), the zona limitans intrathalamica (ZLI) and the entopeduncular area (Spassky et al., 1998). In either case, implied by these studies is the notion that, as in the spinal cord, oligodendrocytes within the telencephalon originate within specific sites and only later migrate to populate dorsal aspects of the brain.

Here, we further address the origin and the molecular specification of oligodendrocytes in the telencephalon, using both gain- and loss-of-function analyses. Specifically, we are interested in whether the mechanism of oligodendrocyte specification in the forebrain is similar to that observed in the spinal cord. We demonstrate that regions of the telencephalon where Shh is expressed are the first to express early oligodendrocyte markers. To see if this is a causal relationship, we have used an in vivo gain-of-function system to misexpress Shh in the forebrain at early times during telencephalic development (Gaiano et al., 1999). We find that Shh induces cells to take an oligodendrocyte fate. To complement this with a loss-of-function analysis, we studied oligodendrocyte development in *Nkx2.1* mutant mice, in which selective aspects of telencephalic *Shh* expression are lost (Sussel et al., 1999). We find that early oligodendrocyte markers fail to appear at normal times in the telencephalon of these mutants in regions where *Shh* expression is absent. In contrast, in the prospective amygdala (i.e. the ventral aspect of the temporal cortex), where *Shh* expression persists in these mutants, oligodendrocyte development appears unaffected based on the maintenance of *Plp* (*PDGFRα* is not expressed in this region). Furthermore, we demonstrate a partial rescue of *PDGFRα* expression in the E14.5 *Nkx2.1* mutant telencephalon using Shh in vivo gain-of-function. This is consistent with the loss of early oligodendrocyte markers in *Nkx2.1* mutants being a result of the absence of Shh. Together, these gain- and loss-of-function results support the hypothesis that in vivo Shh signaling is central to the generation of oligodendrocyte progenitors in the mouse telencephalon. In contrast our in vitro analysis suggests that all regions of the telencephalon, regardless of whether they are derived from wt or *Nkx2.1* mutants, are able to generate oligodendrocytes. Consistent with this, we present evidence that neural tissue from *Shh* null animals can generate oligodendrocytes in vitro. We hypothesize that the generation of oligodendrocytes is under negative regulation in vivo that can be relieved by Shh signaling. We also suggest that there exists a Shh-independent pathway for generating oligodendrocytes.

MATERIALS AND METHODS

Animals, virus preparation and surgery

Swiss Webster (Taconic, Germantown, New York) or CD1 (Charles River, Connecticut) mice used in these studies were maintained according to protocols approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. The day when the sperm plug was detected was considered E0.5. Animal care, preparation for surgery and the use of the ultrasound scanner (UBM scanner) have been described elsewhere (Liu et al., 1998). Virus preparation was performed as previously described (Gaiano et al., 1999). Stocks of CLE (control) and CLES (Shh-expressing virus) were injected at titers of $7\text{-}8\times10^7$ and $1\text{-}3\times10^7$ cfu/ml, respectively. Quantification of cell types in CLE and CLES infected brains were determined by morphology. In each case, approximately 100-200 cells from each of three brains infected with either virus were scored for the presence of neurons, astrocytes and oligodendrocytes.

Genotyping of *Nkx2.1* mutant mice

PCR was used to genotype *Nkx2.1* homozygous mice (Kimura et al., 1996). The following primers were used: T/ebp5′ (5′-GGCGAG-CGGCATGAATATGA-3′) and T/ebp3′ (5′-TCTTGTAGCGGTGG-TTCTGGA-3′) that amplify a 250 bp fragment of the Nkx2.1 wt allele; and T/ebp3′ and Neo5′ (5′-TCGCCTTCTATCGCCTTCTT-GACGAG-3′) that amplify an approximately 220 bp from the mutant Nkx2.1 allele. We used the same PCR conditions for both sets of primers: 95°C for 5 minutes; 35 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute; and 72°C for 10 minutes.

Tissue preparation and histology

E9.5 embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, rinsed in PBS, dehydrated in methanol and kept at –20°C until used for whole-mount in situ hybridization. For section in situ hybridization, heads of embryos younger than E16.5 were fixed by immersion in 4% PFA for 2-3 hours at 4°C, and older embryos and P21 animals were transcardially perfused with 4% PFA and their brains were postfixed for 3-4 hours at 4°C. All samples were then cryoprotected in 30% sucrose in PBS, embedded in Histoprep (Fisher Scientific) and frozen. All tissue was sectioned serially at 20 μm and sections were used for histochemical detection of the alkaline phosphatase reporter (PLAP), RNA in situ analysis or immunofluorescence.

In situ hybridization

Whole-mount and section in situ hybridizations were carried out as described by Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993) and Wilkinson and Nieto (Wilkinson and Nieto, 1993) using non-radioactive DIG-labeled probes. The cDNA probes used were Shh (Echelard et al., 1993), Nkx2.1 (Shimamura et al., 1995), PDGFRα (Mercola et al., 1990), Olig2 (Lu et al., 2000) and Plp (Timsit et al., 1992).

Immunohistochemistry

Histochemical detection of PLAP and immunofluorescence were performed as described in Gaiano et al. (Gaiano et al., 1999). Triton X-100 was not used when O4 or O1 were used as the primary antibodies, and a 10 minute ice-cold methanol post-fixation was performed before the blocking step when anti-MBP was the primary antibody. The following antibodies were used for immunofluorescence: mouse anti-CNPase (1:150, Sternenberg Monoclonals), sheep-anti PLAP (1:100, American Research Products), rabbit anti-PLAP (1:100, Accurate Chemical), mouse TuJ1 (IgG2a, 1:200 in sections, 1:1000 in cells, Berkeley Antibody Company), mouse O4 (IgM, 1:10, Chemicon), mouse anti-MBP (SMI99 and SMI94, IgG2b, 1:1000, Sternberger Monoclonals Inc), mouse O1 (IgM supernatant, 1:10, gift from Dr Moses Chao) and mouse anti-GalC (IgG3, 1:10, Chemicon).

Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and were raised in donkeys, except goat anti-mouse IgG2a FITC-coupled secondary, which was purchased from Roche Molecular Biochemicals.

Tissue dissection and culture conditions for matrigel explants

Ventricular zone (VZ) and subventricular zone (SVZ) from medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE) and dorsolateral cortex were dissected from E13.5 wt and Nkx2.1 null embryos, as described in Wichterle et al. (Wichterle et al., 1999). Explants were embedded in matrigel (Collaborative Biomedical Products) and cultured from 24 hours to 12 days in Neurobasal medium (Gibco) supplemented with Pen/Strep (Gibco, 1:100), B27 supplement (Gibco, 1:50) and glutamine (Gibco, 1:100). A parallel set of experiments was run using collagen (Gutherie and Lumsden, 1994) rather than matrigel as the support matrix. In long term cultures, the medium was changed every 3 days. Explants in these experiments were immunostained as described in Gaiano et al. (Gaiano et al., 2000).

Cell dissociation and labeling

VZ and SVZ from MGE, LGE and cortex were dissected from E17.5 wt, Nkx2.1 null embryos and dissociated similarly as described in Gaiano et al. (Gaiano et al., 2000), except that after trituration, cells were washed in DMEM/F12 medium (Gibco) without serum. For Shh null embryos, brain tissue (Chiang et al., 1996) was collected but further regional demarcations could not be discerned. Approximately $0.5\text{-}1.5\times10^5$ cells were plated onto eight-well Lab-Tek II chamber slides coated with poly-D-lysine and cultured for 3 days in DMEM/F12 medium supplemented with B27 (1:100, Gibco) and N2 (1:50, Gibco) supplements, mitoC (1:500, Collaborative Biomedical Products), glutamine (1:100, Gibco) and penicillin-streptomycin (1:100, Gibco). Cells were immunolabeled as described in Gaiano et al. (Gaiano et al., 2000). With the exception of mitoC, all components of the tissue culture media were defined. To rule out the possibility that Shh was contained in the mitoC supplement, Shh standards were used to determine what concentration of Shh could be detected by western blot analysis. While a 22nM Shh solution produced a visible band on a western blot, a 500× mitoC stock solution did not. This suggests that if mitoC contains any Shh it is (at very most) at a concentration fivefold below 0.25 nM (i.e. 22/500 (=0.044 nM)), the minimal Shh concentration that has been demonstrated to be biologically active (Ericson et al., 1997).

RESULTS

Shh, *Nkx2.1* and early oligodendrocyte markers are co-expressed in the forebrain

In the spinal cord, Shh has been shown to be required for oligodendrocyte specification (Orentas et al., 1999; Pringle et al., 1996). Furthermore, the expression of early oligodendrocyte markers, such as *PDGFRα, Plp* and two novel genes *Olig1* and *Olig2* correlates with sites of expression of *Nkx* genes, a family of homeobox-containing transcription factors that can be induced by Shh (Gaiano et al., 1999; Kohtz et al., 1998; Pera and Kessel, 1998; Shimamura and Rubenstein, 1997). However, comparisons of *Shh* and *Nkx* gene expression to the appearance of oligodendrocyte markers have not been made in the mouse telencephalon. To address this issue, we performed in situ hybridizations on E9.5-E18.5 mouse brains and showed that the expression patterns of *Shh* and *Nkx2.1* correlate with the expression patterns of the early oligodendrocyte markers *Olig2* and *PDGFRα* in the telencephalon (*Olig1* is not expressed within the telencephalon at these ages). In addition, regions within the ventral diencephalon show a similar correlation in the expression patterns of *Shh*, *Nkx2.1* and *Plp* within the hypothalamus (Fig. 1).

The expression of both *Shh* and *Nkx2.1* is initiated in the telencephalon between E8.5 and E9 (Shimamura et al., 1995) and precedes the appearance of oligodendrocyte markers. In the E9.5 telencephalon, *Shh* is expressed in the MGE (Fig. 1A), as is *Nkx2.1* (Fig. 1B). By contrast, the oligodendrocyte progenitor marker *Olig2* is only expressed weakly (Fig. 1C) and *PDGFRα* is not detectable at this age (data not shown). By E12.5 in the telencephalon, *Shh* is expressed in the mantle zone of the MGE (N.B. this region has been referred to as the AEP (anterior entopeduncular area), Puelles et al., 2000) and in the preoptic region (Fig. 1E), while *Nkx2.1* is strongly

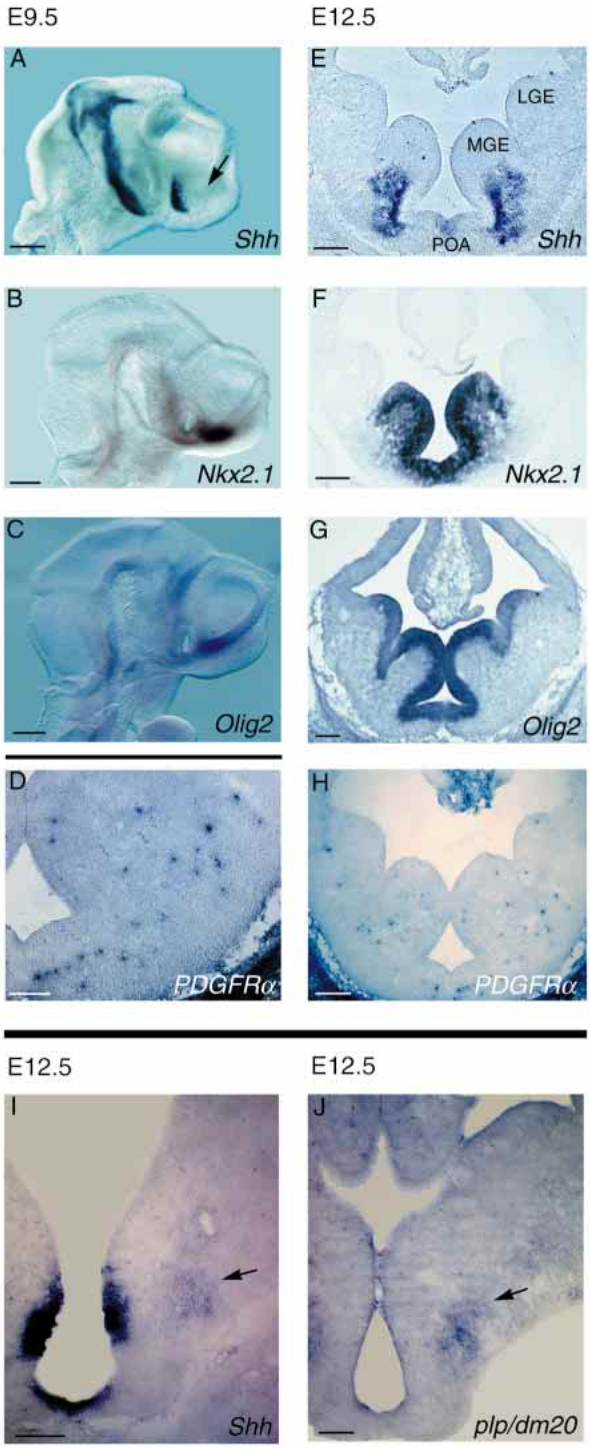

Fig. 1. *Shh*, *Nkx2.1* and early oligodendrocyte markers are co-expressed in the forebrain. (A-C) Whole-mount in situ hybridization of E9.5 heads. Note *Shh* (A) and *Nkx2.1* (B) expression in the ventral telencephalon, in the MGE (arrow in A). The oligodendrocyte precursor marker *Olig2* (C) is also weakly expressed in the same region. (D-H) RNA in situ analysis in coronal sections of the telencephalon at E12.5 showing the ventral telencephalic expression of *Shh* (E), *Nkx2.1* (F) and the oligodendrocyte precursor markers *Olig2* (G) and *PDGFRα* (D,H). (I,J) RNA in situ analysis of *Shh* (I) and *Plp* (J) in the hypothalamus (I,J, arrow) at E12.5. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; POA, preoptic area. Scale bars: 200 μm in A-C,E-H; 100 μm in D,I,J.

expressed throughout the proliferative regions of the MGE and preoptic area, and at lower levels in the mantle zone of the MGE (Fig. 1F). By E12.5, *Olig2* is expressed at high levels in the VZ of the MGE and lower levels in the VZ of the LGE (Fig. 1G) and appears to be too broad to be restricted solely to oligodendrocyte progenitors in the telencephalon. By E12.5, *PDGFRα* is expressed in a subset of cells distributed throughout the MGE in a punctate manner (Fig. 1D,H).

Shh (Fig. 1I) and *Nkx2.1* (results not shown) are also expressed at E12.5 in the ventral diencephalon. Examination of *Plp* expression in these regions revealed that this early oligodendrocyte marker shows correlated expression with these genes within parts of the hypothalamus (Fig. 1J).

Shh induces oligodendrocytes in vivo

To assess whether Shh can direct cells to adopt an oligodendrocyte fate in the telencephalon, we misexpressed Shh in the forebrain using a retroviral-based system for gain-of-function gene expression developed in the laboratory (Gaiano et al., 1999). Retroviruses were delivered into the telencephalic vesicles of E9.5 embryos using an ultrasound backscatter microscopy (UBM) system that permits real-time visualization and injection of embryos in utero (Olsson et al., 1997). Virus encoding the complete open-reading frame of human SHH (CLES) and control virus that only contains the reporter gene PLAP (CLE) were injected at a titer of $1\text{-}2\times10^7$ cfu/ml (for the control virus higher titers were also injected, $7\text{-}8\times10^7$). At these titers, embryos (or pups) infected with CLES at E9.5 and killed at E14.5, E18.5, P21 or adult exhibited mild brain malformations, in which the dorsal aspect of the telencephalon was enlarged. In addition, infections in the choroid plexus or its associated mesenchyme resulted in extreme enlargement of this structure (results not shown). By E18.5, the distribution and morphology of histochemically stained PLAP cells in the Shh-infected brains differed from those seen in the control samples. Typically CLES-infected cells were in clusters, whereas CLE infected cells were radially distributed (results not shown). At E14.5, ectopic expression of either *Olig2* (in all cases; Fig. 2A,B) or *PDGFRα* (in five out of nine cases; Fig. 2C,D) could be detected in dorsolateral or dorsomidline regions of CLES-infected brains. Previous studies using a Wnt1 promoter element to misexpress *Shh* in hindbrain and spinal cord have demonstrated that both *Olig2* and *PDGFRα* expression can be ectopically induced in these regions by Shh (Lu et al., 2000; Rowitch et al., 1999).

Since most neurons and glia reach maturity after birth, we also examined animals infected at E9.5 and killed at P21 and as adults, in order to carry out a morphological and immunocytochemical analysis of the mature phenotypes of cells in Shh-infected brains. Sections were stained histochemically to detect the PLAP reporter gene included in the vector and also analyzed by double immunofluorescence for differentiated oligodendrocyte markers.

At P21 in CLES-infected brains there was a preponderance of cells with an oligodendrocyte morphology, with a smaller subset of cells adopting an astrocytic morphology and virtually none resembling neurons (Fig. 2F,G,J). In the brains injected with control virus, we observed a more diverse range of cell morphologies, including neurons, astrocytes and oligodendrocytes (Fig. 2H,I), as has been documented in our

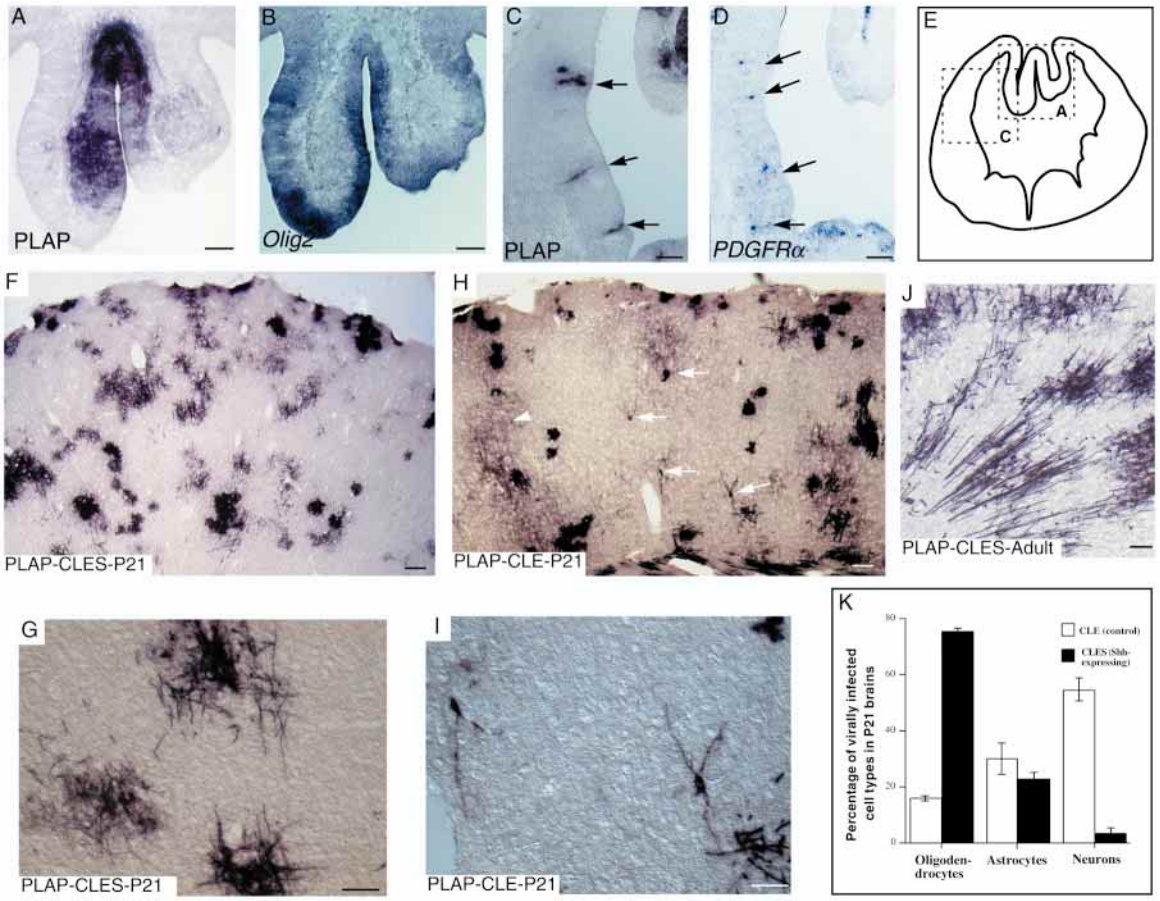

Fig. 2. Shh gain-of-function induces oligodendrocyte formation in the telencephalon. (A-D) Dorsal and lateral virally mediated expression of Shh induces ectopic expression of the oligodendrocyte precursor markers *Olig2* (A,B) and *PDGFRα* (C,D). Embryos were injected at E9.5 and killed at E14.5. Brains were then sectioned coronally. (A,C) Histochemical staining for PLAP revealed the location of CLES-infected cells, dorsal cortex is shown in A, lateral cortex in C. (B,D) In situ hybridization in adjacent sections reveals the ectopic expression of *Olig2* in the dorsal cortex (B), and of *PDGFRα* (D) in the lateral cortex. Arrows in C indicate the clusters of Shh-expressing cells, and in D, the cells ectopically expressing *PDGFRα*. Note that in D, the normal scattered *PDGFRα* expression in the basal telencephalon is also detected. (E) A schematic of the areas shown in A,B (A) and C,D (C). Note that Shh-infected brains are morphologically distorted (Gaiano et al., 1999). (F-J) Morphology of cells infected at E9.5 with CLES (F,G,J) or CLE (H,I) and postnatally identified by histochemical staining for the PLAP reporter gene product. At P21 (F,G) and adulthood (J), CLES-infected cells resemble oligodendrocytes in vivo; while CLE-infected cells (H,I) have more diverse morphologies. Arrows in H indicate neurons, whereas the arrowhead on the left of (H) shows columnar staining characteristic of a large clone of neurons (Gaiano et al., 1999). (J) Oligodendrocytes near the cortical white matter tracts, and (G) oligodendrocytes in the gray matter. (I) Two cells with neuronal morphology, as often seen in CLE-infected samples. (K) A quantitative analysis of the cell types seen in CLE-and CLES-infected brains, as assessed by morphology. Scale bars: 100 μm in A-D,F,H; 50 μm in G,I,J.

previous analysis (Gaiano et al., 1999). Quantification of these results demonstrates that while in control brains neurons comprise the highest percentage of PLAP-positive cells, in CLES-infected brains a large majority of PLAP expressing cells have adopted an oligodendrocyte morphology (Fig. 2K). It is perhaps surprising that Shh, a known inducer of ventral motoneurons and interneurons in spinal cord, fails to induce this cell type in our experiments (Ericson et al., 1997). This may be a result of the timing of our retroviral injections. As shown previously (Orentas et al., 1999), Shh appears to act early in development in spinal cord to induce neurons and later to induce oligodendrocytes.

At this age, in addition to their morphology, oligodendrocytes can be identified by molecular markers, including myelin basic protein (MBP) and 2′,3′ cyclic nucleotide 3′ phosphodiesterase (CNPase), which are both markers of myelinating oligodendrocytes (Bansal et al., 1992; Braun et al., 1988). Shh-infected cells, detected by immunostaining for the PLAP reporter, were found to express MBP (Fig. 3A-C) and CNPase (Fig. 3D-I) when the infected cells were near the white matter or in corticofugal fibers. Interestingly, in the cortex, many of CLES-infected cells were also found in the gray matter and these only rarely expressed mature myelin markers (Miller et al., 1999). These results suggest that while Shh can direct cells to an oligodendrocyte fate, local cues are involved in allowing these cells to adopt a mature oligodendrocyte phenotype. Finally, it is worth noting that based on the pattern of MBP and CNPase staining, we did not observe increased numbers of oligodendrocytes adjacent to Shh-expressing cells in postnatal brains. We however cannot rule out that non-autonomous induction of oligodendrocytes also occurs (see Discussion).

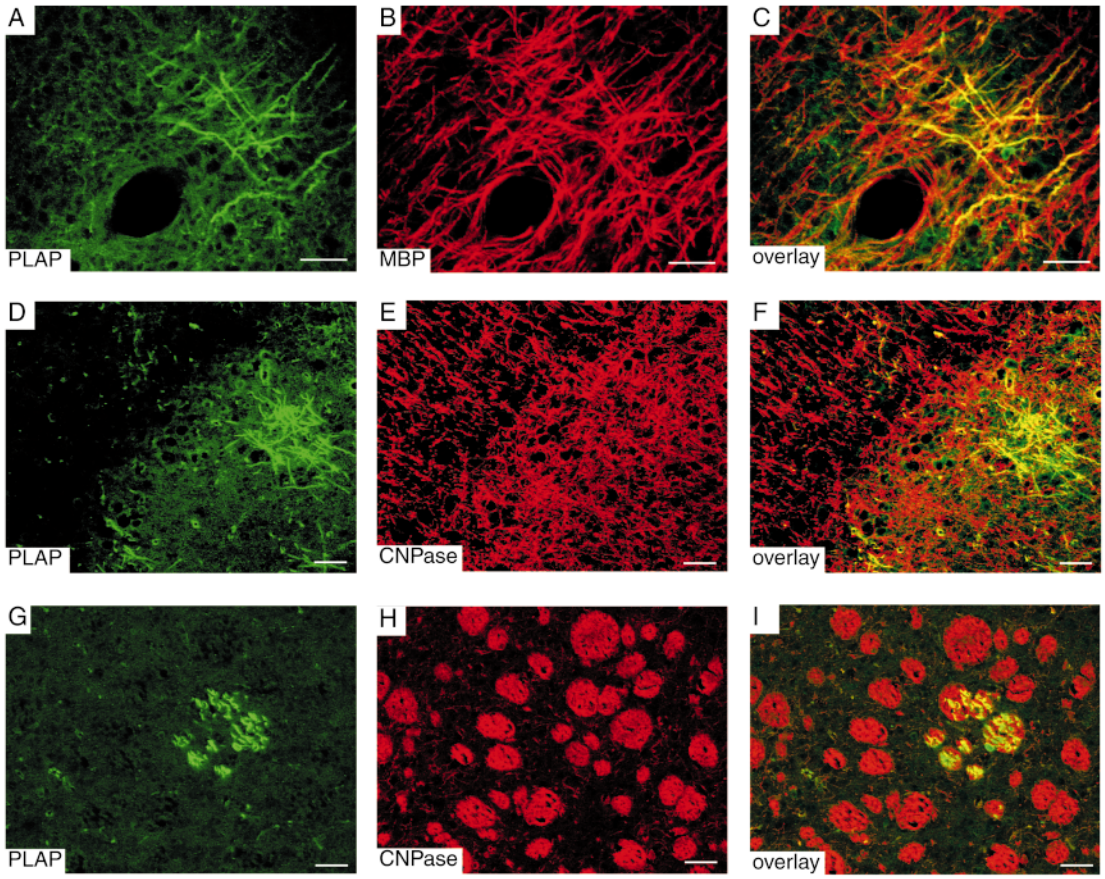

Fig. 3. Shh-infected cells express oligodendrocyte markers. (A-I) Double immunofluorescence performed on E9.5 CLES-infected tissue and analyzed at P21. CLES-infected cells double-immunolabeled for PLAP (A,D,G) and either MBP (B,C) or CNPase (E,F,H,I). (A-F) Two examples of infected cells in the cortex, near the white matter. (G-I) An example in the corticofugal fiber bundles. Scale bars: 50 μm.

Shh and early oligodendrocyte markers are lost in *Nkx2.1* mutants

In *Nkx2.1* mutant mice, *Shh* expression is lost in the MGE (Sussel et al., 1999; Fig. 4A,B). In order to assess whether oligodendrocyte specification in the telencephalon is altered in these mice, we examined the expression of oligodendrocyte markers at different embryonic stages. Consistent with this notion, at E12.5 *PDGFRα* expression is lost. In addition, *Olig2* expression is reduced to LGE levels (data not shown), consistent with previous observations suggesting that the MGE takes on LGE characteristics in *Nkx2.1* mutants (Sussel et al., 1999). At E14.5 in the *Nkx2.1* mutants, these markers show the same alterations in their patterns of expression. Similar to what is seen in the E12.5 telencephalon, E14.5 expression of *Olig2* in wild type is enriched in the MGE versus LGE, while in *Nkx2.1* mutants *Olig2* is uniformly expressed at levels equivalent to that seen in wt LGE (Fig. 4C,D). Furthermore at E14.5, the telencephalic expression of *PDGFRα* is still absent in *Nkx2.1* mutant mice (Fig. 4F,H). In contrast, in E14.5 wt animals, *PDGFRα* expression has expanded both in number and distribution to include the LGE and caudal ganglionic eminence (CGE) (Fig. 4E,G,I,J). Notably, the change in pattern of *PDGFRα* in wt animals is consistent with the suggestion by others that these cells are both proliferative and migratory (Miller et al., 1997).

The loss of early oligodendrocyte markers in *Nkx2.1* mutants is not confined to the telencephalon. Within the ventral diencephalon, Shh expression is also lost in these mutants within the hypothalamus (Fig. 4K,L). In accordance with the notion that Shh expression is required for the expression of early oligodendrocyte markers, the normal expression of *Plp* within this region is also absent (Fig. 4M,N).

Early oligodendrocyte markers are not lost in areas of the *Nkx2.1* mutant forebrain where *Shh* persists

Although a substantial portion of *Shh* transcription is lost in the telencephalon of *Nkx2.1* mutant mice, a subset of regions maintains relative normal patterns of *Shh* expression (Sussel et al., 1999, Fig. 5). Interestingly, these areas also retain the expression of early oligodendrocyte markers. For instance, Shh expression within the ZLI is maintained in *Nkx2.1* mutants, as is the normal expression of *Plp* (Fig. 5). In addition we have identified a previously unreported site of both Shh and *Plp* expression within the prospective amygdaloid region that also remains unaltered in the *Nkx2.1* mutants (Fig. 5), suggesting that this represents an additional area where oligodendrocytes may be generated. The parallels between both the loss or maintenance of Shh and early oligodendrocyte markers in Nkx2.1 mutant mice are striking. Combined with our gain-of-function studies showing that ectopic Shh expression induces

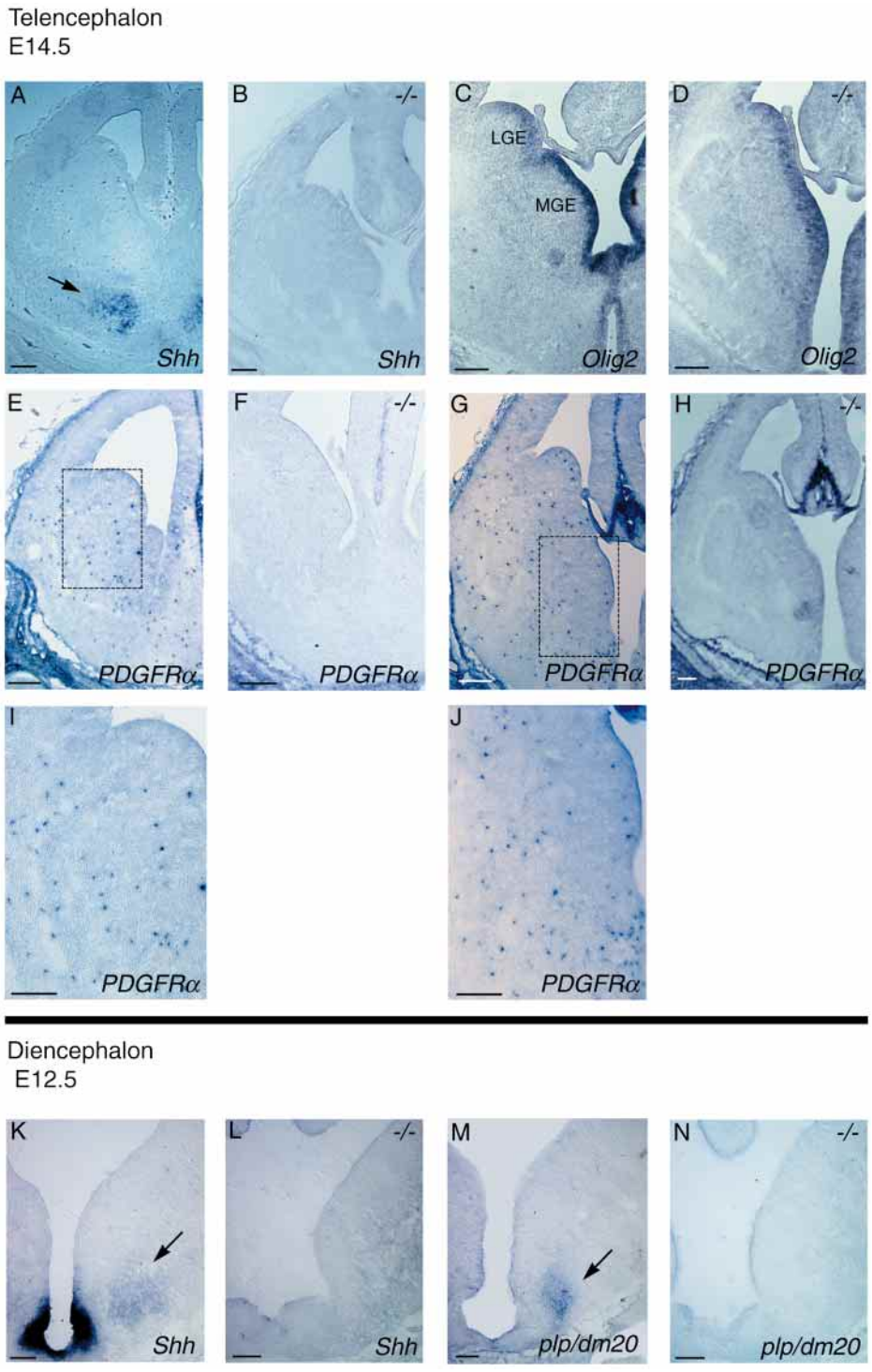

Fig. 4. *Shh* and early oligodendrocyte markers are altered in the *Nkx2.1* mutants. RNA in situ hybridization in coronal sections from control (A,C,E,G,I-K,M) (wt or heterozygotes) and *Nkx2.1* null mutants (B,D,F,H,L,N) (E14.5, A-J; E12.5, K-N). *Shh* is expressed in the telencephalon in ventral pallidal regions (A, arrow) and its expression is lost in the *Nkx2.1* null (B). *Olig2* is normally expressed in the VZ and SVZ of the MGE and in a reduced level in the VZ and SVZ of the LGE (C), and its expression is reduced to LGE levels in the mutant MGE (D). At this age, *PDGFRα*-positive cells are distributed ventrally throughout the MGE, LGE and CGE but not in the cortex (E,G,I,J; I and J are higher power views of the regions shown in E and G, respectively). In the *Nkx2.1* null *PDGFRα* expression is lost (F,H). (K,L) The expression of *Shh* in wt hypothalamus (K, arrow), and its absence in *Nkx2.1* mutant hypothalamus (L). (M,N) Similarly, the expression of *Plp* in wt hypothalamus (M, arrow), and its absence in *Nkx2.1* mutant hypothalamus (N) are shown. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Scale bars: 200 μm in A-H; 100 μm in I-N.

cells to adopt an oligodendrocyte identity, these results collectively suggest that there may be a causal relationship between Shh expression and the appearance of early oligodendrocyte markers.

In vivo Shh gain-of-function can partially rescue the failure of oligodendrocyte development in *Nkx2.1* mutants

While the loss of oligodendrocyte markers within the MGE of *Nkx2.1* mutants is consistent with a role for Shh in the generation of this cell population, it is possible that this deficit results from actions of Nkx2.1 other than its effect on the induction of Shh in this region (Sussel et al., 1999). To test whether Shh is responsible for the loss of oligodendrocyte markers in this mutant, we examined if in vivo gain-of-function expression of Shh could induce the expression of oligodendrocyte markers within the MGE of *Nkx2.1* mutants. As in the Shh gain-of-function experiments described above, CLES-injected embryos were harvested at E14.5 and PCR genotyping was performed to identify the *Nkx2.1* homozygous embryos. In accordance with the notion that it is the loss of Shh within the MGE that results in failure of early oligodendrocyte markers to appear in *Nkx2.1* mutants, we observed that expression of *PDGFRα* could be induced within the ventral telencephalon of *Nkx2.1* mutants (Fig. 6A,B). Notably the levels of expression were always lower than those seen in similar regions of wt animals and only occurred in 50% of cases. In addition, while we (see above) and others (Lu et al., 2000) have observed that Shh misexpression can induce *Olig2* expression, it was impossible to assess whether Shh could rescue *Olig2* expression within the MGE, given that the expression of *Olig2* was not completely absent in *Nkx2.1* mutants. However, we did note that, as in wt embryos, retrovirally mediated Shh expression did induce *Olig2* (Fig. 6C,D) and *PDGFRα* expression (results not shown)

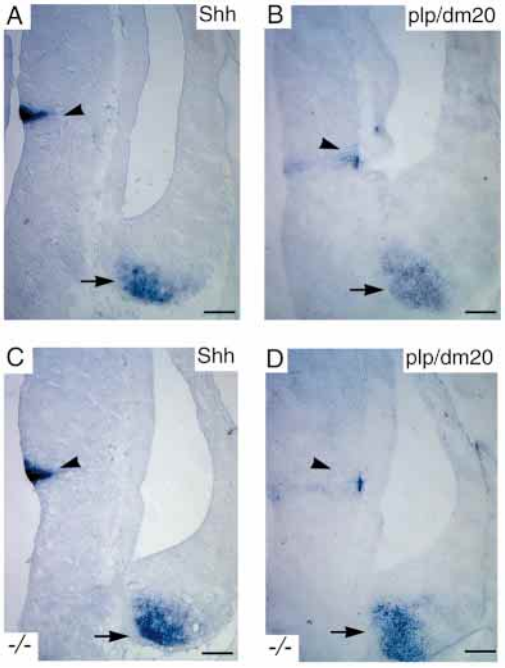

Fig. 5. Early oligodendrocyte markers are not lost in *Nkx2.1* mutants in areas where *Shh* expression persists. In situ hybridization of E12.5 telencephalic coronal sections of wt (A,B) and *Nkx2.1* null embryos (C,D), comparing the pattern of expression of *Shh* (A,C) and *Plp* (B,D). *Shh* (A) and *Plp* (B) are both expressed in the ZLI (arrowheads) and in the amygdaloid region (arrows) and these sites of expression are not affected in *Nkx2.1* nulls (C,D). Scale bar: 50 μm.

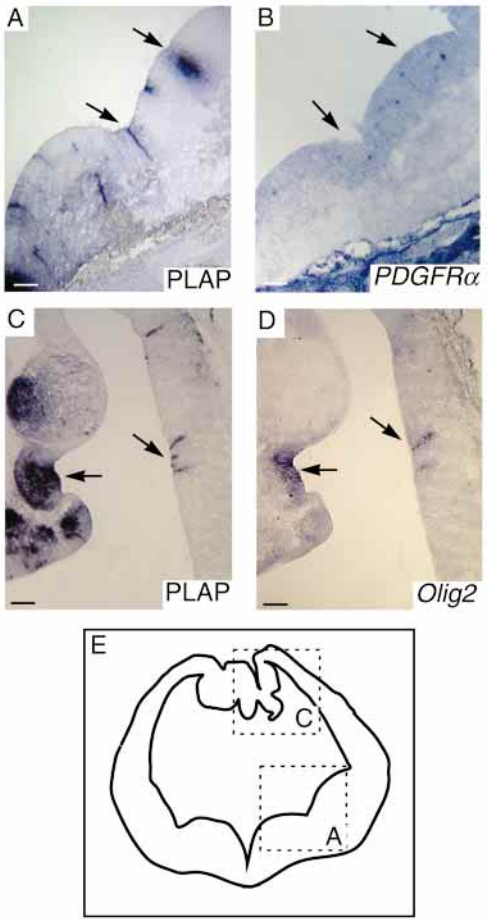

Fig. 6. Ectopic Shh expression in *Nkx2.1* nulls is able to partially rescue the loss of early oligodendrocyte markers. Shh was misexpressed in the telencephalon of E9.5 *Nkx2.1* null embryos and coronal sections of the injected embryos were analyzed at E14.5. Histochemical staining for PLAP shows the viral infection sites (A,C). RNA in situ hybridization was performed in adjacent sections (B,D). Ventral misexpression of Shh in the mutant septum and MGE (A, arrows) is able to partially rescue the ventral telencephalic expression of *PDGFRα* (B, arrows). Ectopic expression of Shh in the cortex (C, arrows) of the mutant embryos is able to induce ectopic *Olig2* expression (D, arrows). (E) Schematic of the regions shown in A,B (A) and C,D (C). Scale bar; 100 μm in A-D.

ectopically within dorsal cortical regions of the *Nkx2.1* mutant.

Shh is not required for the expression of oligodendrocyte markers in vitro

Our analysis of the *Nkx2.1* mutant demonstrates that in areas where *Shh* expression is lost, so is the expression of early oligodendrocyte markers. Surprisingly, by E18.5 the *PDGFRα* expression pattern in *Nkx2.1* null embryos was indistinguishable from wt animals, both of them exhibiting positive cells throughout the telencephalon (results not shown). The fact that *PDGFRα* expression recovers by birth is suggestive of perinatal compensation in oligodendrocyte production. It is, however, unclear whether *PDGFRα* expression is a selective marker of oligodendrocytes during later embryonic development (Oumesmar et al., 1997). Hence, other oligodendrocyte markers must be used to address whether a late embryonic deficit in oligodendrocytes exists in these mutants. While a number of potentially suitable markers exist for premyelinating oligodendrocytes, such as O4 (Bansal et al., 1989; Sommer and Schachner, 1981), O1 and GalC (Bansal and Pfeiffer, 1992; Bansal et al., 1989), we found that these antibodies worked poorly in vivo at perinatal ages within the telencephalon. Further complicating the analysis of the generation of oligodendrocytes in *Nkx2.1* mutants is the fact that these animals die perinatally, likely as a result of a near complete failure in lung development (Kimura et al., 1996). As an alternative approach to examine oligodendrocyte maturation in both dorsal and ventral aspects of *wt* versus *Nkx2.1* mutant telencephalon, we used two complementary culture systems. First, we used an in vitro assay consisting of growing explants in matrigel (Wichterle et al., 1999), which allowed us to examine the ability of different telencephalic regions to generate oligodendrocytes in intact explants where their in vivo milieu is largely maintained. Second, to quantitatively assess oligodendrocyte maturation within different areas of wt versus *Nkx2.1* telencephalon, we used dissociated cultures from various regions of the perinatal telencephalon.

Explants from MGE, LGE and cortex of E13.5 mutant and wt embryos were cultured in matrigel for 1 day (Fig. 7A-D). As previously shown (Wichterle et al., 1999), we observed that MGE cells migrated more than LGE cells (Fig. 7A versus 7C), while cortical cells extended neurites and showed little tendency to migrate (Fig. 7D). We found that *Nkx2.1* mutant MGE cells showed a diminished capacity for migration

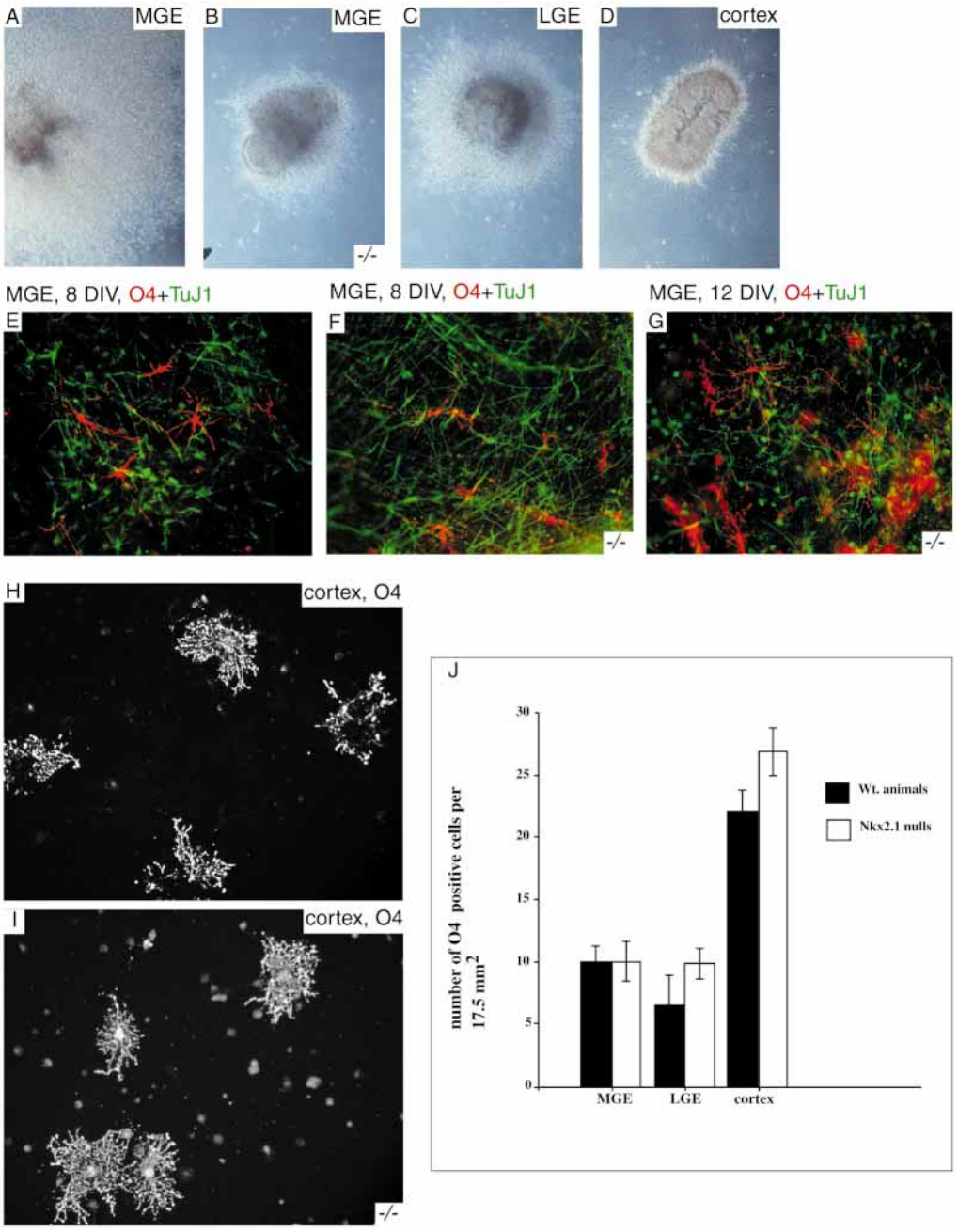

Fig. 7. Oligodendrocytes can develop in vitro from either dorsal or ventral telencephalic regions in wt or *Nkx2.1* mutant animals. (A-D) In vitro explant assay showing that at E13.5 the mutant MGE cells (B) have a migration defect when compared with wt MGE cells (A), behaving more similar to wt LGE cells (C). Cortical cells do not tend to migrate (D). In all cases, explants were cultured in matrigel for one day. (E-G) Both E13.5 wt (E) and *Nkx2.1* mutant (F,G) MGE explants (as well as LGE, and cortical explants, results not shown) express the pre-myelinating marker O4 (red) after longer term culturing (8-12 days). (E,F) wt (E) and *Nkx2.1* (F) MGE explants cultured for 8 days and double-immunostained for O4 (red) and TuJ1 (green). (G) Same as (F) but cultured for 12 days. (H-J) In vitro dissociated cell cultures from E17.5 embryos maintained in culture for 3 days, showing that both normal and mutant MGE, LGE and cortex can generate oligodendrocytes, assessed by O4 immunostaining. (H,I) Typical fields from cortical cultures of wt (H) and null mutants (I). (J) The number of O4-positive cells was counted in 17.5 mm^2 quadrants and plotted for MGE, LGE and cortical cultures of wt and null embryos. This analysis revealed that there is no significant difference in the numbers of O4-positive cells in wt versus *Nkx2.1* null embryos. Results are the mean±s.d. of at least eight different trials.

compared with wt MGE cells and appeared to behave more like the cells in LGE explants (Fig. 7B). This observation is in accordance with recent results (Sussel et al., 1999), which suggested that in *Nkx2.1* nulls the MGE takes on an LGE identity. We next examined the ability of wt versus *Nkx2.1* mutant E13.5 explants to generate oligodendrocytes after 8 or 12 days in vitro. Surprisingly, we found that all explants, regardless of their region of origin or whether they were derived from wt or *Nkx2.1* mutant animals were qualitatively similar in their ability to generate O4-positive cells (Fig. 7E-G; cortical and LGE data not shown). To rule out the possibility that matrigel contains Shh or that factors within matrigel induce the expression of Shh in our explants, we repeated these experiments using instead an inert collagen matrix. These experiments yielded comparable results, suggesting that the findings were not skewed by the presence of matrigel (results not shown).

To evaluate quantitatively the ability of different regions of the *wt* or *Nkx2.1* mutant telencephalon to generate oligodendrocytes, we examined cultures of dissociated cells from these regions. Dissociated cells from the ventricular zone of the MGE, LGE and cortex of E17.5 wt and *Nkx2.1* mutant embryos were cultured for 3 days in serum-free conditions and immunostained for the pre-oligodendrocyte marker O4 and the more mature oligodendrocyte markers O1 and GalC (data not shown). We found that at this age MGE, LGE and cortex of both wt and *Nkx2.1* mutant animals all have the ability to generate oligodendrocytes (Fig.7H-J). These results demonstrate that despite the apparent in vivo requirement for Shh in the generation of oligodendrocytes, forebrain

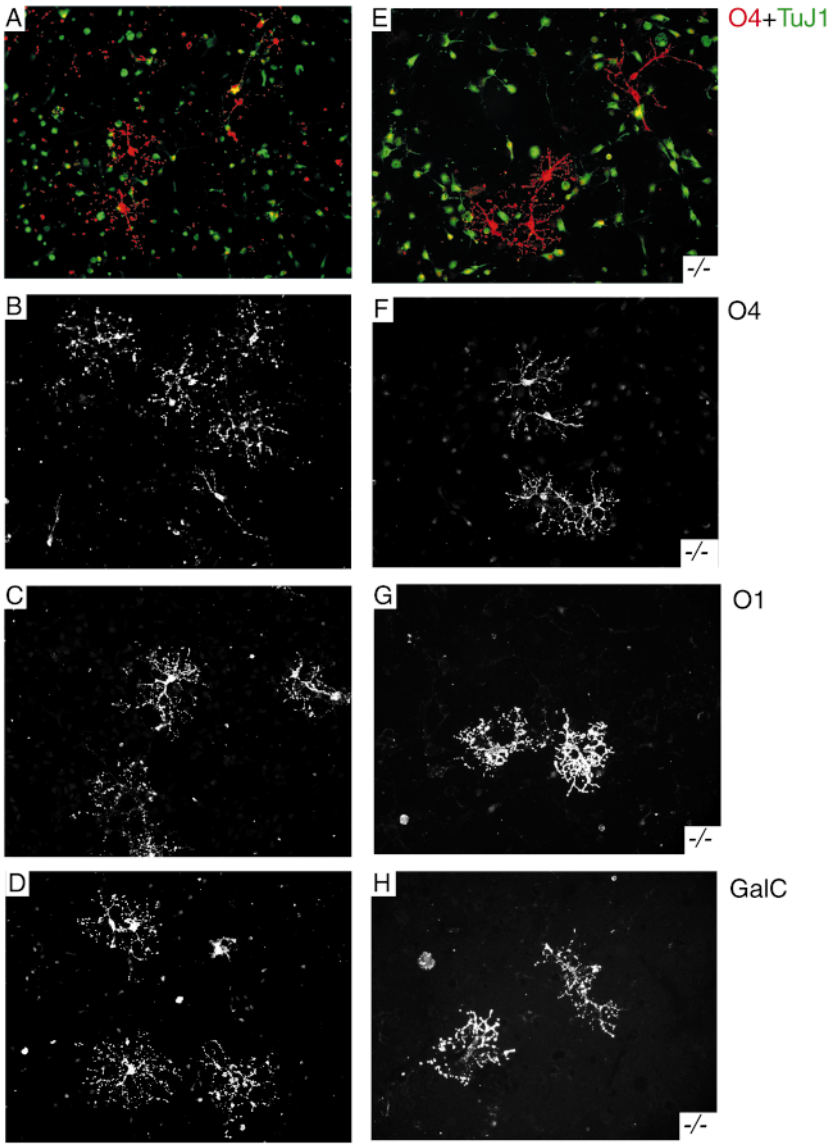

Fig. 8. Shh is not required for the in vitro generation of oligodendrocytes. In vitro dissociated cell cultures from E17.5 wt or *Shh* null embryos maintained in culture for 3 days, showing that both wt (A-D) and Shh mutant (E-H) CNS tissue can generate oligodendrocytes, as assessed by O4, O1 and GalC immunostaining. Typical fields from wt (A-D) or Shh mutant (E-F) cultures stained for O4/TUJ1 (A,E), O4 alone (B,F), O1(C,G) or GalC (D,H).

progenitors retain the capacity to make this cell type in vitro.

To definitively address whether oligodendrocytes can be generated in the absence of Shh in vitro, we repeated the dissociated cells experiment described above using CNS tissue from Shh null animals. Previous analysis of Shh mutant animals showed that these animals fail to express oligodendrocyte progenitor markers in vivo (Lu et al., 2000). Analysis of CNS tissue from Shh null animals shows that neural cells from this mutant can give rise to pre-myelinating oligodendrocytes assessed by O1, O4 and GalC immunoreactivity (Fig. 8). While we have quantified the results of these experiments and compared them with the oligodendrocyte production in wt animals (results not shown), interpretation of whether Shh null tissues are equally competent to make oligodendrocytes is confounded by the generally poor survival of Shh null CNS cells. When plated at equal density, wt cells give rise to about four times the number of surviving cells and twice the number of O4-positive cells compared with cultures consisting of Shh null cells. Regardless of this, these results demonstrate that despite the apparent in vivo requirement for Shh in the generation of oligodendrocytes, forebrain progenitors (in vitro) do not require Shh to make oligodendrocytes.

DISCUSSION

The telencephalic origins of oligodendrocytes

We have investigated whether the mechanism of generating oligodendrocytes within the telencephalon is similar to that used in the spinal cord. We demonstrate that, similar to spinal cord, the appearance of oligodendrocyte precursors in the telencephalon appears to be tightly linked to the expression of *Shh*. Using gain-of-function analysis, we show that Shh is capable of inducing progenitors throughout the telencephalon to adopt an oligodendrocyte phenotype. We also observe that in regions of *Nkx2.1* null mutants where the expression of *Shh* is lost, there is a concomitant loss in the early expression of oligodendrocyte markers *PDGFRα* and *Plp*. Conversely in the prospective amygdaloid region of these mutants, where *Shh* expression is maintained, so is the expression of the early oligodendrocyte marker *Plp*. In accordance with the notion that it is specifically the loss of *Shh* expression in the *Nkx2.1* mutant that results in the absence of oligodendrocyte markers in selective forebrain regions of these mutants, this phenotype can be partially rescued through Shh gain-of-function expression in the affected areas. These results suggest that, in vivo, Shh acts during development in the induction of at least a subpopulation of telencephalic oligodendrocyte precursors. Despite this, we observe that all regions of both the wt and *Nkx2.1* mutant telencephalon have the capacity to generate pre-oligodendrocytes in vitro. Furthermore, neural tissue from *Shh* null mutants is able to give rise to oligodendrocytes. These results argue that even though *Shh* is required in vivo for the generation of oligodendrocyte progenitors, there exists the covert ability for neural progenitors to give rise to oligodendrocytes in vitro.

A number of models could potentially explain our findings. First, the generation of oligodendrocytes in vivo could normally be under negative regulation, and both in vivo Shh signaling or in vitro culturing overcomes this negative regulation, allowing oligodendrocytes to be generated. This hypothesis suggests that CNS tissue from Shh null animals

should be able to generate oligodendrocytes in vitro. Our experiments confirm this prediction (Fig. 8). An alternative but not mutually exclusive hypothesis is that oligodendrocytes may be generated by a number of independent molecular pathways, only one of which requires Shh signaling.

At present, data exist to support both proposed mechanisms. The possible restricted origin of oligodendrocytes to ventral aspects of the nervous system argues for the generation of this cell type being under strict control, consistent with the negative regulator hypothesis (Wada et al., 2000). On the other hand, the observation that different populations of oligodendrocyte precursors apparently express distinct sets of markers and show a variety of morphologies argues that multiple molecular pathways for the generation of this cell type may exist (Del Rio-Hortega, 1928; Bjartmar et al., 1994; Butt et al., 1994; Spassky et al., 1998; Anderson et al., 1999a; Anderson et al., 1999b; present results). Regardless of this, the observation that Shh can direct cells to adopt an oligodendrocyte fate both in spinal cord (Orentas et al., 1999; Pringle et al., 1996) and forebrain (the present results) supports the notion that the Shh pathway plays a fundamental role during oligodendrogenesis.

Molecular mechanisms of Shh induction of oligodendrocytes

While our data show that, as in spinal cord, Shh can specify oligodendrocytes within the telencephalon, the molecular mechanism by which this is accomplished is at present unclear. What is largely known about the function of Shh in determining cell identity has been gleaned from examination of its role in spinal cord (Briscoe et al., 1999; Ericson et al., 1997; Roelink et al., 1995). In this region it has become increasingly apparent that a combinatorial code of transcription factors acts to specify different neuronal cell types. Shh, through its ability to induce and repress a variety of these molecules in a concentration-dependent manner, appears to tightly orchestrate the spatial appearance of specific combinations of gene expression resulting in the establishment of specific neuronal subtypes (Ericson et al., 1995; Roelink et al., 1995; Briscoe et al., 2000). Within the spinal cord, some of the transcription factors in this combinatorial code are Lim-domain genes, *Pax6, Dbx1, Dbx2, Nkx2.2* and *Nkx6.1* (Tsuchida et al., 1994; Bang and Goulding, 1996; Briscoe et al., 1999; Briscoe et al., 2000).

Lineal analysis in spinal cord has suggested that oligodendrocytes do not result from a lineage distinct from those that give rise to neurons (Leber and Sanes, 1995), suggesting that a specific constellation of transcription factor expression may confer an oligodendrocyte identity on ventral neural precursors. Many of the factors that have been implicated as contributing to this process in the spinal cord are either expressed within the telencephalon or have closely related homologs that are. At present, both Nkx genes as well as the recently identified *Olig1* and *Olig2* genes are candidates for being central in a combinatorial code that acts in the specification of oligodendrocytes. In addition, the signaling molecule PDGF has been implicated through knockout analysis, which suggests that PDGF signaling is essential for the generation of most oligodendrocytes (Fruttiger et al., 1999). Beyond these candidates, our molecular understanding of how oligodendrocytes are generated remains rudimentary. Identification of other genes that play a role in this process and loss-of-function analysis of known candidates such as *Olig1* and *Olig2* will no doubt significantly clarify the underlying mechanisms. In addition, the advent of conditional Shh nulls, will allow for the contributions of specific spatial/temporal expression of Shh in the generation of oligodendrocytes to be elucidated.

Does Shh act cell autonomously in the induction of oligodendrocytes?

It is interesting that based on the postnatal pattern of MBP and CNPase staining in our gain-of-function Shh experiments we only observe cell-autonomous induction of oligodendrocytes. Specifically, we do not detect increased numbers of oligodendrocytes adjacent to Shh-expressing cells in postnatal brains. There is presently no evidence, aside from concentration effects, that Shh acts differently in cells producing this signaling molecule versus those passively receiving Shh signaling. Furthermore, it is not clear that oligodendrocyte progenitors normally express Shh. Nonetheless, the suggestion that Shh may have distinct cell autonomous effects is consistent with the fact that regions expressing early oligodendrocyte markers closely match with areas of Shh expression. Alternatively, the propensity of cells infected with Shh-expressing retroviruses to adopt an oligodendrocyte identity may be the result of autocrine effects of Shh. Our present data do not distinguish between Shh-inducing oligodendrocytes through autocrine effects versus acting through another as yet undefined cell-autonomous mechanism.

If our observation that Shh-expressing cells become oligodendrocytes is in fact mediated through an autocrine mechanism, one would predict that cells adjacent to those expressing Shh would be similarly induced to become oligodendrocytes through a paracrine mechanism. Why then do we fail to observe paracrine induction of oligodendrocytes adjacent to retrovirally infected Shh-expressing cells? Numerous factors could obscure the non-autonomous induction of oligodendrocytes. Oligodendrocyte progenitors are known to be highly motile, therefore, between the time of injection and analysis, Shh-expressing cells may migrate away from those cells induced through a paracrine mechanism to become oligodendrocytes. Furthermore, given the high levels of endogenous MBP and CNPase expression, it is easy to understand why the paracrine induction of oligodendrocytes was undetectable in our experiments.

The prospective amygdaloid region is novel source of oligodendrocytes

Aside from considerations of the molecular mechanisms that control oligodendrocytes, it is likely that the progenitors for this cell type within the brain arise from a number of discrete sources. Previous analyses have proposed that the ventral diencephalon, entopeduncular nucleus, ZLI and MGE are all sources of oligodendrocyte precursors (Perez Villegas et al., 1999; Pringle and Richardson, 1993; Spassky et al., 1998). Here, we identify an additional Shh-expressing region within the prospective amygdaloid area of the telencephalon where oligodendrocyte precursors are produced. Previous investigators have suggested that *PDGFRα*- and *Plp*-expressing cells represent distinct populations of oligodendrocyte precursors (Richardson et al., 2000; Spassky et al., 2000). Interestingly,

among the areas of the forebrain where early oligodendrocytes are generated, the amygdaloid region and the ZLI, both of which express *Plp*, are unaffected in the *Nkx2.1* mutant, whereas the expression of *PDGFRα* in the MGE is lost. Whether these different regional sources of oligodendrocytes reflect diversity in oligodendrocyte populations within the mature telencephalon or rather reveal that different molecular pathways underlie the generation of oligodendrocytes in different areas is at present unclear.

Similarities in the development and migration of oligodendrocytes and interneurons

Aside from the diverse sources from which oligodendrocytes arise, one of the most puzzling aspects surrounding the generation of oligodendrocytes, is why a cell type required ubiquitously should be restricted at all as to its sites of origin. In this regard, it has become clear that oligodendrocytes are not unique. Recent analysis has revealed that telencephalic interneurons are generated within the basal aspects of the telencephalon and only later disperse throughout the entire telencephalon (Anderson et al., 1997; Anderson et al., 1999a; Anderson et al., 1999b). Furthermore, this population is diminished by 40% in *Nkx2.1* mutant animals (Sussel et al., 1999).

The dependence of telencephalic interneurons and oligodendrocytes on *Nkx2.1* gene function, coupled with their similar regional induction and their coordinated tangential dispersion warrants further investigation. As noted above, in spinal cord, motoneurons and oligodendrocytes have been shown to be lineally related. As there are no motoneurons within the forebrain, it is interesting to speculate whether oligodendrocytes share a common lineage with other neuronal populations. If so, interneurons provide an attractive candidate for being clonally related to oligodendrocytes.

Future directions

It is now apparent that *Shh* lies at the top of the hierarchy of genes that control the generation of oligodendrocytes in the spinal cord. Here we present evidence to suggest *Shh* plays a similar role in oligodendrocyte development in the telencephalon. Nonetheless, in vitro analysis has demonstrated that dorsal tissues in the absence of Shh are capable of eventually generating this cell type, albeit more slowly (Sussman et al., 2000). In accordance with this, we present evidence that all regions of the telencephalon of wt or *Nkx2.1* mutant animals can generate oligodendrocytes in vitro. Furthermore, although oligodendrocyte progenitors apparently fail to appear in Shh null animals, CNS tissue from these mutants can generate pre-oligodendrocytes in vitro (Fig. 8). Together these findings raise numerous questions. Does Shh initiate the generation of all oligodendrocytes? Do oligodendrocytes represent a single homogenous population? What, if any, later cues are required for oligodendrocytes to attain their mature differentiated phenotypes? While answers to these issues will no doubt be forthcoming, they will require better markers for oligodendrocytes through their various stages of development; and conditional null analysis to overcome the perinatal lethality in animals lacking genes implicated in the generation of oligodendrocytes, such as *Shh* and *Nkx2.1*.

In conclusion, we suggest that, at least in the telencephalon, while Shh initiates the generation of oligodendrocytes in vivo, this cell type can be specified in vitro in its absence. This suggests that the normal function of Shh in vivo may be to overcome a constitutive inhibition to oligodendrocyte production. Perhaps, in the absence of a negative regulator for oligodendrocyte production Shh is not needed to specify this cell type. Alternatively, there may exist a completely separate Shh-independent mechanism for generating oligodendrocytes. Finally, whether in the telencephalon Shh has an additional positive regulatory role in the generation of oligodendrocytes as seen in the spinal cord remains to be determined.

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REFERENCES

Anderson, S. A., Eisenstat, D. D., Shi, L. and Rubenstein, J. L. (1997). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science* **278**, 474-476.

Anderson, E. S., Bjartmar, C., Westermark, G. and Hildebrand, C. (1999a). Molecular heterogeneity of oligodendrocytes in chicken white matter *Glia* **27**, 15-21.

Anderson, S., Mione, M., Yun, K. and Rubenstein, J. L. (1999b). Differential origins of neocortical projection and local circuit neurons: role of Dlx genes in neocortical interneuronogenesis. *Cereb. Cortex* **9**, 646-654.

Bang, A. G. and Goulding, M. D. (1996). Regulation of vertebrate neural cell fate by transcription factors. *Curr. Opin. Neurobiol.* **6**, 25-32.

Bansal, R., Warrington, A. E., Gard, A. L., Ranscht, B. and Pfeiffer, S. E. (1989). Multiple and novel specificities of monoclonal antibodies O1, O4, and R- mAb used in the analysis of oligodendrocyte development. *J. Neurosci. Res.* **24**, 548-557.

Bansal, R. and Pfeiffer, S. E. (1992). Novel stage in the oligodendrocyte lineage defined by reactivity of progenitors with R-mAb prior to O1 anti-galactocerebroside. *J. Neurosci. Res.* **32**, 309-316.

Bansal, R., Stefansson, K. and Pfeiffer, S. E. (1992). Proligodendroblast antigen (POA), a developmental antigen expressed by A007/O4-positive oligodendrocyte progenitors prior to the appearance of sulfatide and galactocerebroside. *J. Neurochem.* **58**, 2221-2229.

Barres, B. A. and Raff, M. C. (1994). Control of oligodendrocyte number in the developing rat optic nerve. *Neuron* **12**, 935-942.

Birling, M. C. and Price, J. (1998). A study of the potential of the embryonic rat telencephalon to generate oligodendrocytes. *Dev. Biol.* **193**, 100-113.

Bjartmar, C, Hildebrand, C. and Loinder, K. (1994). Morphological heterogeneity of rat oligodendrocytes: electron microscopic studies on serial sections. *Glia* **11**, 235-244.

Braun, P. E., Sandillon, F., Edwards, A., Matthieu, J. M. and Privat, A. (1988). Immunocytochemical localization by electron microscopy of 2'3'-cyclic nucleotide 3'-phosphodiesterase in developing oligodendrocytes of normal and mutant brain. *J. Neurosci.* **8**, 3057-3066.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. and Ericson, J. (1999). Homeobox gene Nkx2.2 and

specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627.

Briscoe, J., Pierani, A., D., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.

Butt, A. M., Colquhoun, K., Tutton, M. and Berry, M. (1994). Three-dimensional morphology of astrocytes and oligodendrocytes in the intact mouse optic nerve. *J. Neurocytol.* **23**, 469-485.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic Hedgehog* gene function. *Nature* **383,** 407-413.

Del Rio-Hortega, P. (1928).Tercera aportación al conocimiento morfológico e interpretación funcional de la oligodendroglia. *Soc. Espan. Hist. Nat.* **14**, 40-122.

Dong, Z., Sinanan, A., Parkinson, D., Parmantier, E., Mirsky, R. and Jessen, K. R. (1999). Schwann cell development in embryonic mouse nerves. *J. Neurosci. Res.* **56**, 334-348.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Ericson, J., Muhr, J., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog: a common signal for ventral patterning along the rostrocaudal axis of the neural tube. *Int. J. Dev. Biol.* **39**, 809-816.

Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. and Jessell, T. M. (1997). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 451-466.

Fruttiger, M., Karlsson, L., Hall, A. C., Abramsson, A., Calver, A. R., Bostrom, H., Willetts, K., Bertold, C. H., Heath, J. K., Betsholtz, C. et al. (1999). Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. *Development* **126**, 457-467.

Gaiano, N., Kohtz, J. D., Turnbull, D. H. and Fishell, G. (1999). A method for rapid gain-of-function studies in the mouse embryonic nervous system. *Nat. Neurosci.* **2**, 812-819.

Gaiano, N., Nye, J. S. and Fishell, G. (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* **26**, 395-404.

Guthrie, S. and Lumsden, A. (1994). Collagen gel coculture of neural tissue. neuroprotocols. *Methods Neurosci.* **4,** 116-120.

Hall, A., Giese, N. A. and Richardson, W. D. (1996). Spinal cord oligodendrocytes develop from ventrally derived progenitor cells that express PDGF alpha-receptors. *Development* **122**, 4085-4094.

Jessen, K. R. and Mirsky, R. (1997). Embryonic Schwann cell development: the biology of Schwann cell precursors and early Schwann cells. *J. Anat.* **191**, 501-505.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzalez, F. J. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **10**, 60-69.

Kohtz, J. D., Baker, D. P., Corte, G. and Fishell, G. (1998). Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog. *Development* **125**, 5079-5089.

Leber, S. M. and Sanes, J. R. (1995). Migratory paths of neurons and glia in the embryonic chick spinal cord. *J. Neurosci.* **15**, 1236-1248.

Liu, A., Joyner, A. L. and Turnbull, D. H. (1998). Alteration of limb and brain patterning in early mouse embryos by ultrasound-guided injection of Shh-expressing cells. *Mech. Dev.* **75**, 107-115.

Lu, Q. R., Yuk, D., Alberta, J. A., Zhu, Z., Pawlitzky, I., Chan, J., McMahon, A. P., Stiles, C. D. and Rowitch, D. H. (2000). Sonic hedgehog-regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. *Neuron* **25**, 317-329.

Mercola, M., Wang, C. Y., Kelly, J., Brownlee, C., Jackson-Grusby, L., Stiles, C. and Bowen-Pope, D. (1990). Selective expression of PDGF A and its receptor during early mouse embryogenesis. *Dev. Biol.* **138**, 114-122.

Miller, R. H. (1996). Oligodendrocyte origins. *Trends Neurosci.* **19**, 92-96.

Miller, R. H., Payne, J., Milner, L., Zhang, H. and Orentas, D. M. (1997). Spinal cord oligodendrocytes develop from a limited number of migratory highly proliferative precursors. *J. Neurosci. Res.* **50**, 157-168.

Miller, R. H., Hayes, J. E., Dyer, K. L. and Sussman, C. R. (1999). Mechanisms of oligodendrocyte commitment in the vertebrate CNS. *Int. J. Dev. Neurosci.* **17**, 753-763.

Noll, E. and Miller, R. H. (1993). Oligodendrocyte precursors originate at the ventral ventricular zone dorsal to the ventral midline region in the embryonic rat spinal cord. *Development* **118**, 563-573.

Olsson, M., Campbell, K. and Turnbull, D. H. (1997). Specification of mouse telencephalic and mid-hindbrain progenitors following heterotopic ultrasound-guided embryonic transplantation. *Neuron* **19**, 761-772.

Orentas, D. M., Hayes, J. E., Dyer, K. L. and Miller, R. H. (1999). Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors. *Development* **126**, 2419-2429.

Oumesmar, B. N., Vignais, L. and Baron-Van Evercooren, A. (1997). Developmental expression of platelet-derived growth factor alpha- receptor in neurons and glial cells of the mouse CNS. *J. Neurosci.* **17**, 125-139.

Pera, E. M. and Kessel, M. (1998). Demarcation of ventral territories by the homeobox gene NKX2.1 during early chick development. *Dev. Genes Evol.* **208**, 168-171.

Perez Villegas, E. M., Olivier, C., Spassky, N., Poncet, C., Cochard, P., Zalc, B., Thomas, J. L. and Martinez, S. (1999). Early specification of oligodendrocytes in the chick embryonic brain. *Dev. Biol.* **216**, 98-113.

Poncet, C., Soula, C., Trousse, F., Kan, P., Hirsinger, E., Pourquie, O., Duprat, A. M. and Cochard, P. (1996). Induction of oligodendrocyte progenitors in the trunk neural tube by ventralizing signals: effects of notochord and floor plate grafts, and of sonic hedgehog. *Mech. Dev.* **60**, 13-32.

Pringle, N. P. and Richardson, W. D. (1993). A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. *Development* **117**, 525-533.

Pringle, N. P., Yu, W. P., Guthrie, S., Roelink, H., Lumsden, A., Peterson, A. C. and Richardson, W. D. (1996). Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog. *Dev. Biol.* **177**, 30-42.

Puelles, L., Kuwana, E., Puelles, E., Bulfone, A., Shimamura, K., Keleher, J, Smiga, S. and Rubenstein, J.L.R. (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon traced by the expression of the genes, *Dlx-2, Emx-1, Nkx2.1, Pax6,* and *Tbr-1*. *J. Comp. Neurol.* **424**, 409–438.

Richardson, W. D., Pringle, N. P., Yu, W. P. and Hall, A. C. (1997). Origins of spinal cord oligodendrocytes: possible developmental and evolutionary relationships with motor neurons. *Dev. Neurosci.* **19**, 58-68.

Richardson, W. D., Smith, H. K., Sun, T., Pringle, N. P., Hall, A. and Woodruff, R. (2000). Oligodendrocyte lineage and the motor neuron connection. *Glia* **29**, 136-142.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Rowitch, D. H., S-Jaques, B., Lee, S. M., Flax, J. D., Snyder, E. Y. and McMahon, A. P. (1999). Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. *J. Neurosci.* **19**, 8954-8965.

Schaeren-Wiemers, N. and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* **100**, 431-440.

Shi, J., Marinovich, A. and Barres, B. A. (1998). Purification and characterization of adult oligodendrocyte precursor cells from the rat optic nerve. *J. Neurosci.* **18**, 4627-4636.

Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.

Shimamura, K. and Rubenstein, J. L. (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124**, 2709-2718.

Sommer, I. and Schachner, M. (1981). Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev. Biol.* **83**, 311-327.

Spassky, N., Goujet-Zalc, C., Parmantier, E., Olivier, C., Martinez, S., Ivanova, A., Ikenaka, K., Macklin, W., Cerruti, I., Zalc, B. et al. (1998). Multiple restricted origin of oligodendrocytes. *J. Neurosci.* **18**, 8331-8343.

Spassky, N., Olivier, C., Perez-Villegas, E., Goujet-Zalc, C., Martinez, S., Thomas, J. and Zalc, B. (2000). Single or multiple oligodendroglial lineages: a controversy. *Glia* **29**, 143-148.

Sussel, L., Marin, O., Kimura, S. and Rubenstein, J. L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126**, 3359-3370.

Sussman, C. R., Dyer, K. L., Marchionni, M. and Miller, R. H. (2000). Local control of oligodendrocyte development in isolated dorsal mouse spinal cord. *J. Neurosci. Res.* **59**, 413-420.

Timsit, S. G., Bally-Cuif, L., Colman, D. R. and Zalc, B. (1992). DM-20

mRNA is expressed during the embryonic development of the nervous system of the mouse. *J. Neurochem.* **58**, 1172-1175.

Timsit, S., Martinez, S., Allinquant, B., Peyron, F., Puelles, L. and Zalc, B. (1995). Oligodendrocytes originate in a restricted zone of the embryonic ventral neural tube defined by DM-20 mRNA expression. *J. Neurosci.* **15**, 1012-1024.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79,** 957-970.

Wada, T., Kagawa, T., Ivanova, A., Zalc, B., Shiraskai, R., Murakami, F., Iemura, S-i., Ueno, N. and Ikenaka, K. (2000). Dorsal spinal cord inhibits oligodendrocyte development. *Dev. Biol.* **227,** 42-55.

Wichterle, H., Garcia-Verdugo, J. M., Herrera, D. G. and Alvarez-Buylla, A. (1999). Young neurons from medial ganglionic eminence disperse in adult and embryonic brain. *Nat. Neurosci.* **2**, 461-466.

Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361-373.

Zhou, Q., Wang, S. and Anderson, D. J. (2000). Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. *Neuron* **25**, 331-343.