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Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon

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SUMMARY

In the caudal neural tube, oligodendrocyte progenitors (OLPs) originate in the ventral neuroepithelium under the influence of Sonic hedgehog (SHH), then migrate throughout the spinal cord and brainstem before differentiating into myelin-forming cells. We present evidence that oligodendrogenesis in the anterior neural tube follows a similar pattern. We show that OLPs in the embryonic mouse forebrain express platelet-derived growth factor alpha-receptors (PDGFRA), as they do in more caudal regions. They first appear within a region of anterior hypothalamic neuroepithelium that co-expresses mRNA encoding SHH, its receptor PTC1 (PTCH) and the transcription factors OLIG1, OLIG2 and SOX10. *Pdgfra*-positive progenitors later spread through the forebrain into areas where *Shh* is not expressed, including the cerebral cortex. Cyclopamine inhibited OLP development in cultures of mouse basal forebrain, suggesting that hedgehog (HH) signalling is obligatory for oligodendrogenesis in the ventral telencephalon. Moreover, *Pdgfra*-positive progenitors did not appear on schedule in the ventral forebrains of *Nkx2.1* null mice, which lack the telencephalic domain of *Shh* expression. However, OLPs did develop in cultures of *Nkx2.1*$^{-/-}$ basal forebrain and this was blocked by cyclopamine. OLPs also developed in neocortical cultures, even though *Shh* transcripts could not be detected in the embryonic cortex. Here, too, the appearance of OLPs was suppressed by cyclopamine. In keeping with these findings, we detected mRNA encoding SHH and Indian hedgehog (IHH) in both *Nkx2.1*$^{-/-}$ basal forebrain cultures and neocortical cultures. Overall, the data are consistent with the idea that OLPs in the telencephalon, possibly even some of those in the cortex, develop under the influence of SHH in the ventral forebrain.

Key words: Telencephalon, Hedgehog signalling, Oligodendrocyte, Rat, Mouse

INTRODUCTION

Oligodendrocytes in the spinal cord and brainstem are derived from a subset of ventral neuroepithelial cells, under the influence of Sonic hedgehog protein (SHH) from the ventral midline (notochord and floor plate; reviewed by Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). SHH patterns the ventral neuroepithelium by controlling expression of a set of transcription factors including homeodomain proteins NKX2.2 and PAX6, the high mobility group (HMG) protein SOX10 and the basic helix-loop-helix (bHLH) proteins OLIG1 and OLIG2 (Ericson et al., 1997; Kuhlbrodt et al., 1998; Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000). The *Olig* genes and *Sox10* are co-expressed in the oligodendrogenic part of the neuroepithelium a day or two before the appearance of *Pdgfra*-positive oligodendrocyte progenitors (Lu et al., 2000; Zhou et al., 2000). The *Pdgfra*$^{+}$ progenitors then proliferate and migrate away from the ventricular surface into all parts of the spinal cord before differentiating into myelin-forming oligodendrocytes (Pringle and Richardson, 1993; Calver et al., 1998).

The origins of oligodendrocytes at more anterior levels of the neuraxis are less well established. Timsit et al. showed that the myelin proteolipid protein gene *Plp*/*Dm20* is expressed in the ventral neuroepithelium of the embryonic mouse diencephalon from as early as E9 (Timsit et al., 1992); they proposed that this region later goes on to generate oligodendrocytes. Pringle and Richardson (Pringle and Richardson, 1993) described a cluster of *Pdgfra*-positive presumptive oligodendrocyte progenitors (OLPs) in the ventral forebrain of the rat embryo that appeared to proliferate and

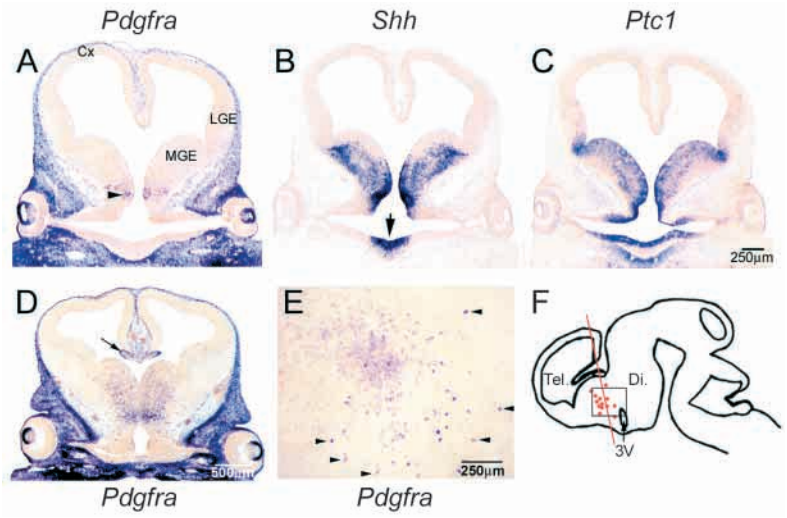

Fig. 1. Expression of *Pdgfra*, *Shh* and *Ptc1* in the embryonic rat forebrain. (A-C), E13.5 anterior forebrain, coronal sections. *Pdgfra* is expressed in the neuroepithelium and adjacent SVZ at the boundary between anterior hypothalamus and MGE (arrowhead in A) within broader domains of *Shh* (B) and *Ptc1* (C) expression. There is an additional domain of *Shh* and *Ptc1* expression in the preoptic recess (arrow in B), but there is no *Pdgfra* expression in this region. (D) Coronal and (E) parasagittal sections of E14.5 anterior forebrain. *Pdgfra* is widely expressed outside the nervous system (D). In the ventral forebrain, *Pdgfra* is strongly expressed in the VZ and SVZ of the anterior hypothalamus, extending dorsally into the MGE (D), but is not expressed in the LGE or cortex at this stage. Strong expression is also observed in the primordia of the choroid plexus (arrow in D). In the SVZ, two categories of *Pdgfra*$^+$ cells are intermingled: closely packed cells that express relatively low levels of *Pdgfra* and smaller cells that are more intensely labelled (arrowheads in E). (F) Diagram to show the plane of section of A-D and the location of the field shown in E. MGE and LGE, medial and lateral ganglionic eminences; Cx, cerebral cortex; Tel, telencephalon; Di, diencephalon; 3V, third ventricle.

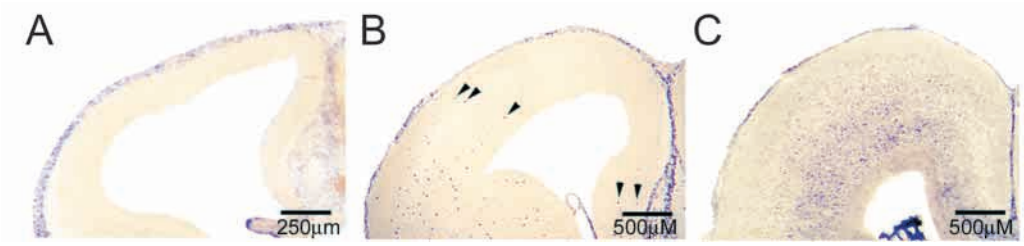

Fig. 2. Scattered *Pdgfra*$^+$ cells spread into the cerebral cortex in lateral-to-medial and ventral-to-dorsal directions. (A-C) Coronal sections through the neocortex at different ages, hybrized in situ with a probe for *Pdgfra*. (A) At E14.5, there are no *Pdgfra*$^+$ cells in the developing cortex. (B) By E17.5, intensely labelled *Pdgfra*$^+$ cells are present within the developing cortical plate and presumptive sub-cortical white matter at the lateral margin of the cortex as well as the medial cortex (arrowheads in B). (C) By E20.5, there are numerous *Pdgfra*$^+$ cells in the cortex, especially in the sub-cortical white matter. At all stages, *Pdgfra* is also expressed by the meninges and skull.

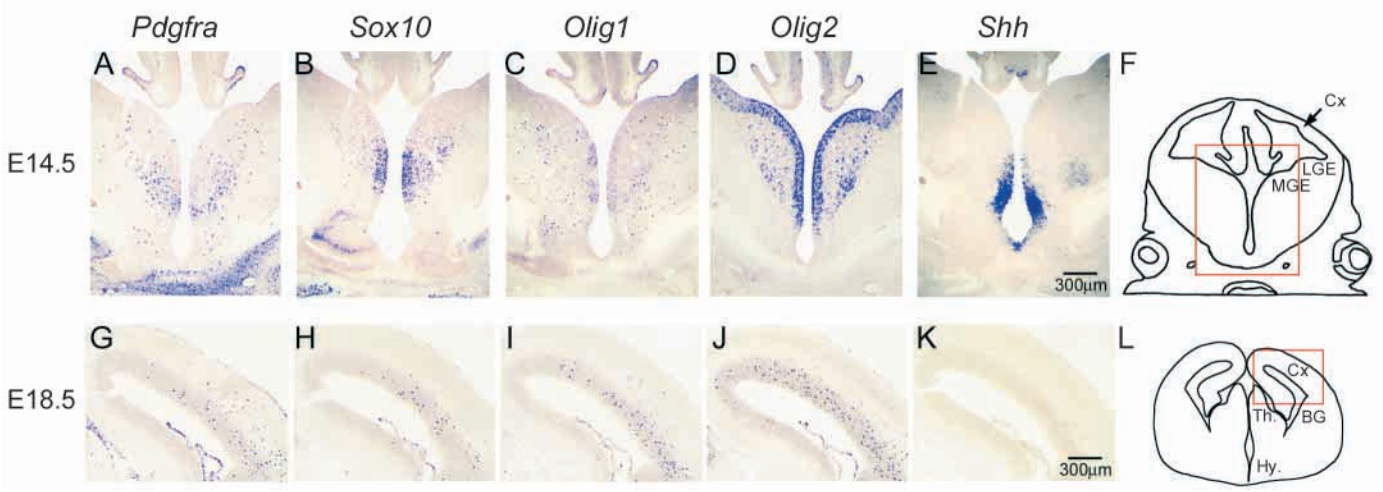

Fig. 3. Overlapping expression of *Pdgfra*, *Sox10*, *Olig1*, *Olig2* and *Shh* in the ventral forebrain and neocortex. (A-E) Serial coronal sections of rat E14.5 forebrain. The four presumptive oligodendrocyte lineage markers show overlapping but non-identical patterns of expression in the neuroepithelium, SVZ and mantle zones of the anterior hypothalamus and MGE. Their expression domains also overlap *Shh* expression in the neuroepithelium (E). The neuroepithelial expression of *Olig2* extends further than the rest, through the MGE and LGE (D). (F) Diagram to indicate the approximate position of sections A-E. (G-K) Serial coronal sections through the rat cortex at E18.5. Cells expressing *Pdgfra*, *Sox10*, *Olig1* and *Olig2* have a similar scattered distribution in the cortex at this stage. *Shh* cannot be detected in the cortex (K). The position of sections G-K is indicated in L. Cx, cortex; Th, thalamus; Hy, hypothalamus; BG, basal ganglia.

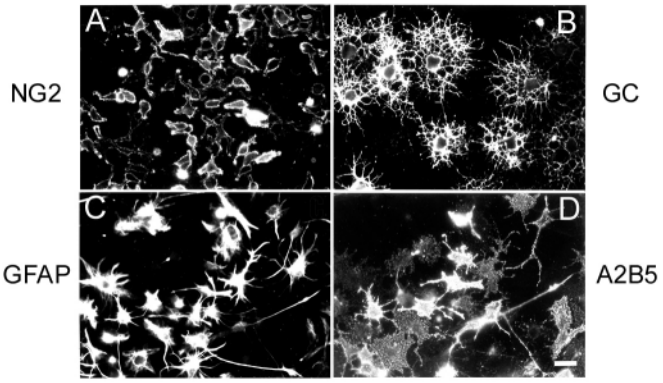

Fig. 4. *Pdgfra*$^+$ cells from embryonic rat brain differentiate in vitro into GC$^+$ oligodendrocytes or (GFAP$^+$, A2B5$^+$) type-2 astrocytes. These cells were immunoselected from whole brain, but similar results were obtained with cells from cortex or basal ganglia. (A) More than 99% of the process-bearing cells were NG2$^+$. These cells were also (PDGFRA$^+$, A2B5$^+$, O4$^-$, GC$^-$) (not shown). (B) After culturing for a further 2-3 days in the presence of 0.5% FCS without added PDGF-AA more than 99% of the cells differentiated into GC$^+$ oligodendrocytes. (C,D) If the immunoselected cells were instead cultured in the presence of 10% FCS, more than 99% of the cells became GFAP$^+$ astrocytes (C), many of which also labelled with A2B5 (D). Scale bar, 50 μm.

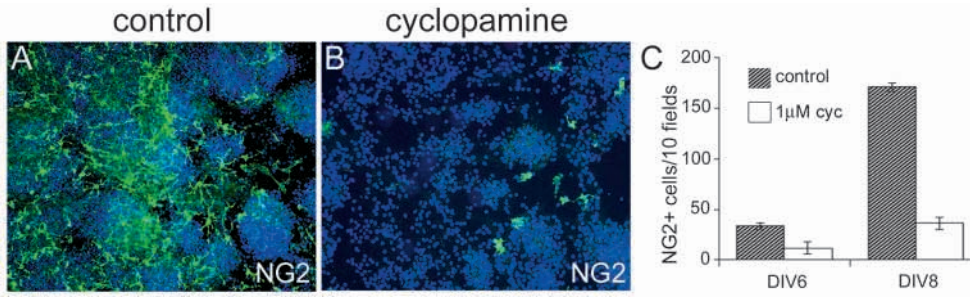

Fig. 5. Hedgehog signalling is required for oligodendrogenesis in the basal forebrain. Dissociated cells of mouse E10.5 ventral forebrain were cultured in defined medium for up to 8DIV in the presence or absence of the HH inhibitor cyclopamine. The cultures were immunolabelled with polyclonal anti-NG2. (A) Numerous NG2$^+$ cells developed after 8DIV in the control. (B) Their development was strongly inhibited by cyclopamine. (C) The experiment was quantified by counting NG2$^+$ cells in more than ten randomly selected fields (×63 microscope objective). Four independent experiments gave similar results, one of which is illustrated (mean ± s.d.).

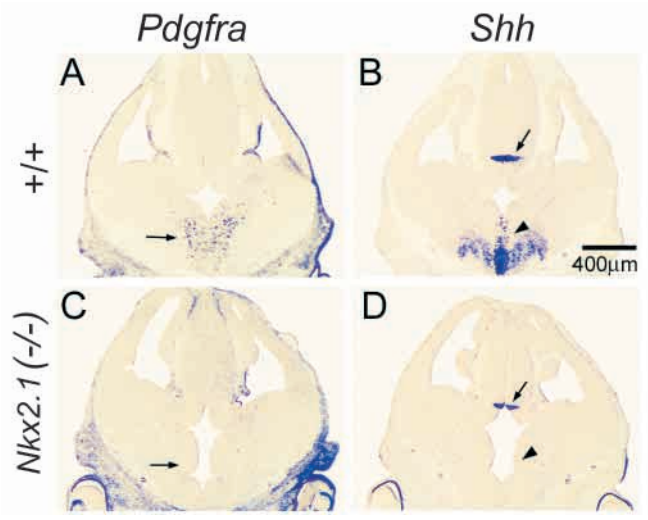

Fig. 6. *Pdgfra*$^+$ OLPs fail to develop in the anterior hypothalamic neuroepithelium of *Nkx2.1* null mice. (A,B) Adjacent coronal sections from the telencephalon of wild-type E12.5 embryos. *Pdgfra*$^+$ OLPs arise in the ventral forebrain (arrow in A) near the region of *Shh* expression (arrowhead in B). *Shh* is also expressed in the diencephalon in the zona limitans intrathalamica (arrow in B) but no *Pdgfra*$^+$ cells are visible there. (C, D) Adjacent coronal sections from the telencephalon of E12.5 *Nkx2.1* null embryos showing a complete absence of *Pdgfra*$^+$ OLPs (arrow in C) and *Shh* expression (arrowhead in D) in the ventral forebrain. In contrast, expression of *Shh* in the zona limitans intrathalamica is maintained in the *Nkx2.1* null (arrow in D).

migrate throughout the developing forebrain. Spassky et al. and Perez-Villegas et al. also provided histological evidence for a ventral source of oligodendrocytes in the rodent and chick forebrains, respectively (Spassky et al., 1998; Perez-Villegas et al., 1999). In keeping with all these studies, it has been reported that precursor cells from E15 rat striatum (ventral telencephalon) have a greater propensity to generate oligodendrocytes than do precursors from the neocortex (dorsal telencephalon), either when cultured in vitro (Birling and Price, 1998) or when transplanted into the eye (Kalman and Tuba, 1998).

It therefore seems likely that there is a region of the ventral forebrain that is specialized for oligodendrogenesis. The experiments reported here further define the location of this site and explore how and when it is established. Our data support the idea that oligodendrogenesis in the rodent telencephalon depends on a localized source of SHH in the ventral forebrain. Even in cultures derived from embryonic neocortex, which does not appear to express SHH or related molecules in situ, generation of oligodendrocyte progenitors was blocked by cyclopamine and appeared to depend on SHH and/or IHH produced in the cultures. Altogether, the available data suggest that at least some oligodendrocytes in the cortex might be derived from progenitor cells that originate in the basal telencephalon – as, for example, the precursors of certain cortical neurons (Parnavelas, 2000).

MATERIALS AND METHODS

Animals

Wild-type rats (Sprague-Dawley) and mice (C57BL/6J) were bred in-house at University College London (UCL). *Nkx2.1* (*Titf1*) knockout

mice were maintained on the 129/Sv or Black/Swiss hybrid background. Midday following appearance of the vaginal plug was designated embryonic day 0.5 (E0.5). Hence, our ages are 0.5 days younger than those of Altman and Bayer (Altman and Bayer, 1995) (e.g. our E13.5 corresponds to their E14). *Nkx2.1* knockout mice (Kimura et al., 1996) and their wild-type littermates were genotyped by polymerase chain reaction (PCR). Primers for amplifying wild-type and mutant alleles were 5′-TCT TGT AGC GGT GGT TCT GGA-3′ and either 5′-GGC GAG CGG CAT GAA TAT GA-3′ (wild-type allele, approx. 250 bp product) or 5′-TCG CCT TCT ATC GCC TTC TTG ACG AG-3′ (null allele, approx. 220 bp product).

Tissue preparation and in situ hybridization

The heads of embryos were fixed by immersion in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) pH7.5 for 24 hours at 4°C. To aid fixation, the skin and skull were removed from E14.5 and older embryos. Tissue was cryoprotected in 20% (w/v) sucrose in PBS and embedded in OCT (Raymond Lamb). Cryosections (15-20 μm) were collected on Vectabond-coated slides (Vecta labs). Our in situ hybridization protocol was as described before (Sun et al., 1998), except that we omitted proteinase K digestion prior to hybridization. The mouse *Pdgfra* probe was transcribed from a approx. 1.6 kb *Eco*RI cDNA fragment encoding most of the extracellular domain of mouse PDGFRA cloned into pBluescript KS (Mercola et al., 1990); the probe was generated using T7 RNA polymerase (T7pol) from *Hin*dIII-cut plasmid. The rat *Pdgfra* probe was transcribed (T7pol, *Hin*dIII) from an approx. 1.5 kb cDNA encoding most of the extracellular domain of rat PDGFRA cloned into PGEM1 (Pringle et al., 1992). The *Shh* probe was transcribed (T3pol, *Kpn*I) from an approx. 2.6 kb full-length rat cDNA (*Xho*I fragment) in pBluescript SK (from A. McMahon, Harvard Medical School). The *Ptc1* (*Ptch*) probe was transcribed (T7pol, *Hin*dIII) from a 841 bp partial cDNA (*Eco*RI fragment) from the 5′ end of mouse *Ptc1* (Ptch) cloned into pBluescript II KS (also from A. McMahon). The *Sox10* probe was transcribed (T7pol, *Sac*I) from plasmid pZL1/sox10.7.7.1, which contains an approx. 2.3 kb partial rat cDNA (Kuhlbrodt et al., 1998). The *Olig1* probe was transcribed (T7pol, *Hin*dIII) from a plasmid containing 986 bp of a rat cDNA (pBSRGUTR; Lu et al., 2000). The *Olig2* probe was transcribed (T3pol, *Eco*RI) from a plasmid containing an approx. 1 kb mouse cDNA (pRBRAH16; Lu et al., 2000). RNA polymerases were from Promega and the DIG labelling mix from Roche. Hybrids were detected with alkaline phosphatase-conjugated anti-DIG antibodies, using NBT and BCIP (both from Roche) as substrates. To increase sensitivity, 5% (w/v) polyvinyl alcohol was included in the final colour reaction.

Brain cell cultures

Dissociated cell cultures were established from embryonic rat or mouse brain (minus brainstem), neocortex or basal ganglia as previously described for spinal cord cultures (Hall et al., 1996). Brains were dissected in Hepes-buffered minimal essential medium (MEM-H) and meningeal membranes removed. The tissue was transferred to Earle's balanced salt solution without calcium or magnesium (EBSS; Gibco BRL) containing trypsin (0.0125% w/v) and DNaseI (0.005% w/v) and incubated at 37°C in 5% CO_2 for 30 minutes. Cells were dissociated by gentle trituration in the presence of 10% fetal calf serum (FCS), washed by centrifugation and re-suspended in Dulbecco's modified Eagle's medium (DMEM) containing 4% FCS. Cells were plated on poly-D-lysine-coated glass coverslips (3×10^5 cells in 50 μl) and allowed to attach at 37°C. 350 μl of defined medium (Bottenstein and Sato, 1979) was added and incubation continued at 37°C in 5% CO_2.

For explant cultures, fragments of E15.5 rat neocortex or basal ganglia were dissected in MEM-H and cultured in collagen gels (Guthrie and Lumsden, 1994) in defined medium (Bottenstein and Sato, 1979) containing 0.5% FCS.

Immunoselection

A single-cell suspension of E19 rat brain (minus brainstem), neocortex or striatum, prepared as described above, was passed over two bacteriological Petri dishes coated with monoclonal antibody Ran-2 (Bartlett et al., 1981) to remove astrocytes, meningeal cells and macrophages (the latter by non-specific adherence to plastic), then over a dish coated with anti-PDGFRA rabbit serum (#3979; Fretto et al., 1993) as described by Hall et al. (Hall et al., 1996). The PDGFRA-positive ($PDGFRA^+$) cells were removed from the panning dish with trypsin and plated (1000 cells in a 3 μl droplet) on 6 mm diameter poly-D-lysine-coated glass coverslips in defined medium (Bottenstein and Sato, 1979) containing 0.5% or 10% FCS.

Immunocytochemistry

Cells on coverslips were lightly fixed in 4% (w/v) PFA in PBS for 5 minutes at room temperature and washed in PBS. The following primary antibodies were used: anti-PDGFRA rabbit serum, (#3709; Fretto et al., 1993) diluted 1:100 in PBS, monoclonal antibody A2B5 (Eisenbarth et al., 1979), anti-NG2 rabbit serum (Chemicon) or monoclonal anti-NG2 (clone N11.4; Stallcup and Beasley, 1987; Levine and Stallcup, 1987), monoclonal antibody O4 (Sommer and Schachner, 1981; Sommer and Schachner, 1982; Bansal et al., 1992), monoclonal anti-galactocerebroside (GC; Ranscht et al., 1982; Bansal and Pfeiffer, 1992) monoclonal anti-glial fibrillary acidic protein (GFAP; clone GA-5, Sigma). For intracellular antigens the cells were made permeable with 0.1% (v/v) Triton X-100 in PBS. Primary antibody treatments were for 1 hour in a humid chamber at room temperature. Fluorescent secondary antibodies (Perbio Science, UK) were applied for 30 minutes at room temperature. Cells were post-fixed for 5 minutes in 4% (w/v) PFA in PBS and mounted under coverslips in Citifluor (City University, UK).

Cultured explants were fixed with 4% PFA in PBS for 10 minutes at room temperature and washed in PBS. Antibody labelling was at 4°C overnight.

RT-PCR

RNA was prepared from freshly dissociated or cultured cells using Trizol® reagent (Gibco BRL). Reverse-transcription was carried out using the Superscript™ first-strand synthesis system (Gibco BRL). Primers for polymerase chain amplification (PCR) of mouse or rat cDNAs were as follows: *Shh*, 5′-GTG ATG AAC CAG TGG CCT GG-3′ and 5′-GCC GCC ACG GAG TTC TCT GC-3′; *Ihh*, 5′-GGC CAT CTC TGT CAT GAA CC-3′ and 5′-CAG CCA CCT GTC TTG GCA GC-3′; *Dhh*, 5′-GTG CGC AAG CAA CTT GTG CC-3′ and 5′-GAA TCC TGT GCG TGG TGG CC-3′; *Gapdh*, 5′-CCC AGA ACA TCA TCC CTG C-3′ and 5′-GCC ATG AGG TCC ACC ACC C-3′. Typically, we employed a 38 cycle PCR reaction, except for *Gapdh* where the number of cycles was reduced to 30 to avoid saturation.

RESULTS

Overlapping expression of *Pdgfra*, *Shh* and *Ptc1* in the ventral forebrain

In the E13.5 rat forebrain, *Pdgfra* was expressed in the ventricular and subventricular zones (VZ and SVZ) in a restricted region spanning the boundary between the anterior hypothalamus (diencephalon) and the medial ganglionic eminence (MGE; telencephalon) (Fig. 1A; Altman and Bayer, 1995). This region has been called the anterior entopeduncular area (AEP; Puelles et al., 2000). The focus of *Pdgfra* lay within broader expression domains of *Shh* and *Ptc1* (Fig. 1B,C). At E14.5, there was a large number of faintly labelled, close-packed $Pdgfra^+$ cells within the SVZ (Fig. 1D,E). Among and

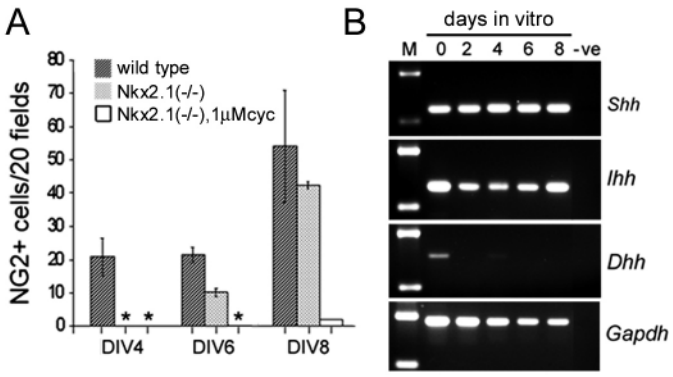

Fig. 7. Hedgehog-dependent development of OLPs in ventral forebrain cultures of wild-type and *Nkx2.1*$^{-/-}$ mice. Dissociated ventral forebrain cells from E13.5 wild-type and *Nkx2.1*$^{-/-}$ mice were cultured in defined medium for up to 8DIV in the presence or absence of the HH inhibitor cyclopamine (cyc, 1 μM). The cultures were immunolabelled with polyclonal anti-NG2 to visualize OLPs and the average number of NG2^{+} cells per 20 fields (×63 microscope objective) was determined for different times in culture. (A) Wild-type cultures contained NG2^{+} process-bearing cells from the outset, whereas *Nkx2.1* null cultures did not develop OLPs until 4-6DIV. The development of OLPs in *Nkx2.1*$^{-/-}$ cultures was strongly inhibited by cyclopamine. Asterisks denote that no NG2^{+} progenitors were detected. (B) Transcripts for SHH and IHH but not DHH could be detected by RT-PCR throughout the culture period. Molecular size markers (M) are 200 bp and 400 bp. The sizes of PCR products were as predicted: *Shh*, 242 bp; *Ihh*, 267 bp; *Dhh*, 311 bp; *Gapdh*, 382 bp.

around these were scattered some smaller, more intensely labelled cells (Fig. 1E, arrowheads). After E14.5, the intensely labelled cells became more numerous and widely distributed, reaching into the SVZ and mantle zones of the MGE and the lateral ganglionic eminence (LGE), while the close-packed *Pdgfra*$^{+}$ cells in the SVZ became less numerous and disappeared around E15.5. By E17 there were small *Pdgfra*$^{+}$ cells scattered more-or-less uniformly throughout the ventral forebrain (not shown). We purified these by immunoselection and showed that they generate oligodendrocytes in vitro (see below).

Lateral-to-medial spread of *Pdgfra*$^{+}$ cells in the cortex suggests immigration from the basal forebrain

Pdgfra^{+} cells began to appear at the cortico-striatal boundary near the lateral tips of the lateral ventricles around E16.5. By E17.5, some were present within the cortical plate and presumptive sub-cortical white matter (Fig. 2B). By E20.5 (just before birth) many *Pdgfra*$^{+}$ cells were present throughout the cortical plate, though they were concentrated in the subcortical white matter (Fig. 2C). They accumulated mainly in a lateral-to-medial direction from the tips of the lateral ventricles but they also appeared to enter the medial cortex in a ventral-to-dorsal direction (Fig. 2B, arrowheads). *Pdgfra* was not detected in the cortical VZ at these ages.

One possible interpretation of these data is that the faint *Pdgfra*$^{+}$ cells in the anterior hypothalamic neuroepithelium give rise to intensely labelled *Pdgfra*$^{+}$ cells, which then migrate away to populate the entire telencephalon. However, we have no direct evidence for migration.

Expression of *Sox10, Olig1* and *Olig2* in the telencephalon

Recent studies have shown that two novel SHH-inducible bHLH genes, *Olig1* and *Olig2*, as well as the HMG protein *Sox10*, are expressed in oligodendrocyte lineage cells in the spinal cord, including the oligodendrogenic part of the ventral VZ where *Pdgfra*$^{+}$ progenitors originate (Kuhlbrodt et al., 1998; Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000). We therefore compared expression of *Sox10*, the *Olig* genes and *Pdgfra* in the rat telencephalon. At E14.5 all of these were expressed in the anterior hypothalamic neuroepithelium and in scattered cells in the adjacent SVZ and mantle zone (Fig. 3A-F). *Olig2* was more widespread in the neuroepithelium than the others, extending through the MGE and LGE.

In the E18.5 cerebral cortex, the expression patterns of *Pdgfra*, *Sox10*, *Olig1* and/or *Olig2* were very similar (Fig. 3G-L). They were all expressed by cells in a scattered distribution from the lateral edges of the cortex towards the midline. We cannot tell whether these cells in serial sections represent one and the same population of cells that express all four genes, or overlapping populations that express different subsets of genes. It is possible, nevertheless, that they represent OLPs in the course of migrating into the cortical plate from the ventral telencephalon. Note that we could not detect *Shh* expression anywhere in the cortex at E18.5 (Fig. 3K) or indeed at any other age between E12.5 and P2 (Fig. 1 and not shown).

PDGFRA^{+} cells in the embryonic rat brain are oligodendrocyte progenitors

To test directly whether PDGFRA^{+} cells in the telencephalon

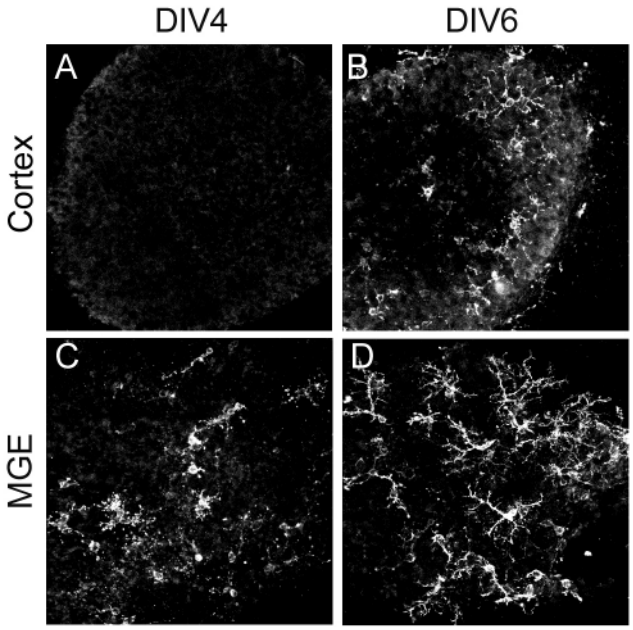

Fig. 8. Rat cortical precursors have latent oligodendrogenic capacity in vitro. Fragments of E15.5 rat cortex or MGE were cultured as explants in collagen gels in basal defined medium for up to 6DIV. The explants were then fixed and immunolabelled with anti-NG2 antibody. (A,B) Cortical explants did not develop NG2^{+} progenitors until 6DIV. (C,D) In contrast, after 4DIV explants of MGE (C) contained process-bearing NG2^{+} cells and these increased in number and complexity by 6DIV (D).

Table 1. Development of NG2$^+$ OLPs in forebrain explant cultures

	NG2$^+$ explants after 4DIV		NG2$^+$ explants after 6DIV	
Age	Cortex	MGE	Cortex	MGE
E15.5	0/11	9/9	15/15	15/15
E18.5	12/12	10/10	8/8	9/9

Fragments of rat neocortex or MGE from embryos of the indicated ages were cultured as explants in collagen gels as described in Materials and Methods. After culturing for 4 or 6DIV in basal defined medium the explants were fixed and immunolabelled with monoclonal anti-NG2 proteoglycan to visualize OLPs. The ratio of NG2$^+$ explants/total number of explants are tabulated for one representative experiment at each age. Equivalent results were obtained in at least two repeat experiments and in dissociated cell cultures (not shown). E15.5 cortical explants did not contain any NG2$^+$ cells after overnight culture (not shown) or after 4DIV, but NG2$^+$ cells appeared by 6DIV. In contrast, E18.5 cortical explants did contain NG2$^+$ cells after overnight culture and many more by 4DIV and 6DIV. Explants of E15.5 or E18.5 MGE always contained NG2$^+$ cells. See Fig. 8 for micrographs.

are OLPs, we purified them from E19 rat brains (minus brainstem), neocortex or striatum by immunoselection with antibodies raised in rabbits against PDGFRA (Hall et al., 1996; A. C. Hall, PhD thesis, University of London, 1999; see Materials and Methods). After overnight culture in defined medium containing 0.5% FCS and 10 ng/ml PDGF-AA, which stimulates progenitor cell proliferation and inhibits differentiation (Raff et al., 1988; Richardson et al., 1988; Noble et al., 1988), more than 99% of the immunoselected cells had the morphological and antigenic features of early OLPs: i.e. they were small process-bearing cells that labelled with anti-NG2 proteoglycan (Fig. 4A) in addition to anti-PDGFRA and monoclonal A2B5 (not shown). They did not label with antibody O4 or anti-GC, which identify later stages of the oligodendrocyte lineage, nor with anti-glial fibrillary acidic protein (anti-GFAP; not shown). After overnight culture in the presence of PDGF, the medium was replaced with defined medium lacking PDGF and containing either 0.5% or 10% FCS. After a further 36 hours in the presence of 0.5% FCS, the immunoselected cells developed a more highly branched morphology and many were O4$^+$ (not shown), typical of late oligodendrocyte progenitors (Sommer and Schachner, 1982; Bansal and Pfeiffer, 1992). By 48 hours, almost all (>98%) of the immunoselected cells had differentiated into GC$^+$ oligodendrocytes (Fig. 4B). In the presence of 10% FCS, most of the immunoselected cells differentiated into GFAP$^+$ astrocytes (Fig. 4C), many of which also labelled with A2B5 (Fig. 4D). Therefore, the great majority of PDGFRA$^+$ cells in the late embryonic forebrain, including the neocortex, resemble the oligodendrocyte-type-2 astrocyte (O-2A) progenitors previously characterized in cultures of cells from perinatal rat optic nerve (Raff et al., 1983) or spinal cord (Hall et al., 1996). We refer to these as OLPs. These data are in accord with previous observations in situ showing that PDGFRA is co-expressed with markers for early stages of the oligodendrocyte lineage in several regions of the brain including the cerebral cortex (Ellison and de Vellis, 1994; Nishiyama et al., 1996).

HH signalling is required for oligodendrogenesis in the ventral forebrain

In view of the described parallels between oligodendrogenesis in the spinal cord and forebrain, we asked whether Sonic hedgehog (SHH), which is obligatory for oligodendrocyte lineage specification in the cord, might also be required in the forebrain.

We cultured E10.5 mouse ventral forebrain cells in the presence or absence of the drug cyclopamine, which inhibits HH signalling (Cooper et al., 1998; Incardona et al., 1998; Taipale et al., 2000). The cells were then fixed and OLPs visualized with anti-NG2 antibodies. In the absence of cyclopamine, significant numbers of NG2$^+$ process-bearing progenitor cells appeared between 4 and 6 days in vitro (4-6DIV; Fig. 5). Cyclopamine strongly inhibited the appearance of these cells (Fig. 5), suggesting that in the forebrain, as in more posterior regions of the CNS, oligodendrogenesis is under the control of SHH and/or other related hedgehog molecules.

To further assess the role of SHH in vivo we examined mice with a targeted deletion in the *Nkx2.1* homeobox gene. In these mice, the most anterior region of *Shh* expression (in the basal forebrain) is absent or greatly reduced, while more posterior regions of *Shh* expression remain intact (Fig. 6B,D) (Sussel et al., 1999). At E12.5 numerous OLPs expressing *Pdgfra*, *Sox10*, *Olig1* and *Olig2* were scattered in the anterior hypothalamus and MGE of wild-type mice but were undetectable in *Nkx2.1* mutant littermates (Fig. 6A,C and data not shown). This

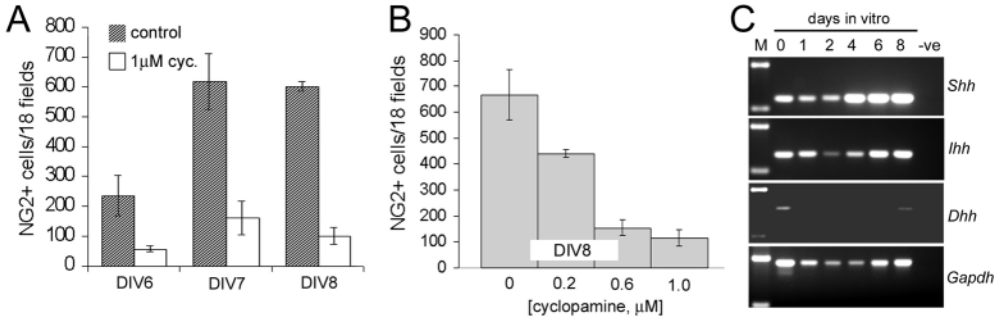

Fig. 9. Hedgehog-dependent development of OLPs in neocortical cultures. (A) Dissociated cells from E15.5 wild-type rat neocortex were cultured in defined medium for up to 8DIV in the presence or absence of cyclopamine (cyc, 1 μM). The cultures were immunolabelled with polyclonal anti-NG2 to visualize OLPs and the number of NG2$^+$ cells per 18 randomly chosen fields (×63 microscope objective) was determined after different times in culture. OLPs did not develop in E15.5 rat cortical cultures until 4-6DIV (see Fig. 8), but after that the number of OLPs increased, peaking after 7DIV. (B) Production of OLPs was inhibited strongly by cyclopamine (A) in a dose-dependent manner. (C) Transcripts encoding SHH and IHH could be detected by RT-PCR as soon as 1 hour after cell dissociation (lane 0), and throughout the culture period up to 8DIV. mRNA coding for DHH was also detected weakly after dissociation and after 8DIV but not at intermediate times. Sizes of PCR products were as predicted (see Fig. 7).

situation persisted until E14.5. At E16.5, however, a sparse population of *Pdgfra*$^+$ cells appeared in the telencephalon and by E18.5 there were almost normal numbers of OLPs throughout the cortex and basal ganglia (not shown). We do not know where these cells originate but their scattered distribution outside of the VZ and their delayed appearance raise the possibility that *Pdgfra*$^+$ cells migrate into the telencephalon of *Nkx2.1* null mice from more posterior sites of oligodendrogenesis in the diencephalon or beyond. It is known that OLPs in other regions of the CNS can migrate significant distances during normal embryonic development (Small et al., 1987; Miller et al., 1997; Ono et al., 1997; Pringle et al., 1998).

To avoid the possibility of *Pdgfra*$^+$ progenitors migrating into the *Nkx2.1* null telencephalon from the diencephalon, we dissected basal ganglia from E13.5 *Nkx2.1* null mice – before any *Pdgfra*$^+$ cells could be detected in situ – then dissociated the cells and cultured them in vitro. Unexpectedly, NG2$^+$ progenitors did develop in these cultures; however their appearance was delayed by 4-6DIV relative to wild-type cultures, which contained NG2$^+$ cells from the beginning (Fig. 7A). Nevertheless, by 6DIV there were similar numbers of NG2$^+$ progenitors in wild-type and mutant cultures. Appearance of these cells in both wild-type and *Nkx2.1* null cultures was strongly inhibited by cyclopamine (Fig. 7A), so we tested for the presence of transcripts encoding SHH, Indian hedgehog (IHH) and Desert hedgehog (DHH) in the starting cell populations and after culturing for up to 8DIV. We detected *Shh* and *Ihh* in wild-type (not shown) and *Nkx2.1*$^{-/-}$ basal ganglia within one or two hours of dissociating the cells and throughout the culture period of 2-8DIV (Fig. 7B). *Dhh* was detected only in freshly dissociated cells (Fig. 7B). It appears likely that the appearance of NG2$^+$ progenitors in vitro is related to the presence of cells expressing SHH and/or IHH in the cultures.

Latent oligodendrogenic potential of cortical precursors

HH signalling seems to be required for oligodendrogenesis in basal forebrain cultures (see above). Since we were never able to observe expression of *Shh*, *Ihh* or *Dhh* in the embryonic cortex by in situ hybridization (Figs 1B, 3K and data not shown), this raised the question of whether oligodendrocytes ever normally develop in vivo from indigenous cortical precursors. As already described, the way *Pdgfra*$^+$ progenitors first appear in the ventral forebrain and spread dorsally suggests that OLPs might invade the cortex from germinal zones in the ventral forebrain. We investigated the oligodendrogenic potential of indigenous cortical precursors more directly by culturing explants from E15.5 and E18.5 rat cortex (before and after the apparent influx of *Pdgfra*$^+$ progenitor cells) in defined basal medium. We then fixed and labelled the cells with anti-PDGFRA or anti-NG2 to visualize OLPs or with anti-GC to visualize differentiated oligodendrocytes.

Control explant cultures of E15.5 rat MGE (Fig. 8C, D) or E18.5 neocortex (not shown) contained numerous PDGFRA$^+$ NG2$^+$ cells after overnight incubation and they increased in number during the culture period (Fig. 8D; Table 1 and not shown). GC$^+$ oligodendrocytes also developed in these cultures after 8DIV (not shown). In contrast, no PDGFRA$^+$ or NG2$^+$ progenitor cells were generated from E15.5 cortical explants either overnight or after 4DIV (Fig. 8A; Table 1). However, if E15.5 cortical cells were cultured for a longer period of time – 6DIV or more – many PDGFRA$^+$ NG2$^+$ OLPs did then appear (Fig. 8B and Table 1). Adding cyclopamine to E15.5 cultures strongly inhibited production of OLPs in a dose-dependent manner (Fig. 9A,B), indicating that in long-term cortical cultures SHH or a related molecule (IHH or DHH) was responsible for OLP development. This conclusion was supported by the fact that we could detect mRNA encoding SHH and IHH in cultured cells from E15.5 cortex (Fig. 9C).

DISCUSSION

Oligodendrogenesis in the telencephalon is a ventral, SHH-dependent process

In the spinal cord and brainstem, oligodendrocytes develop from migratory *Pdgfra*$^+$ progenitor cells that are generated in the ventral neuroepithelium under the influence of SHH from the ventral midline (Pringle et al., 1996; Poncet et al., 1996; Orentas and Miller, 1996; Orentas et al., 1999). The appearance of these progenitors is prefigured by expression of the transcription factors OLIG1, OLIG2 and SOX10 in the ventral neuroepithelium (Kuhlbrodt et al., 1998; Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000). The *Pdgfra*$^+$ progenitors subsequently proliferate and migrate throughout the spinal cord and brainstem. Our data reveal that oligodendrogenesis in the forebrain is similar to this in several respects. (1) Specification of OLPs in the forebrain, as in the spinal cord, is dependent on HH expression and activity. (2) Expression of *Sox10*, *Olig1* and *Olig2* overlaps with that of *Pdgfra* in the ventral telencephalon, as in the ventral spinal cord. (3) *Pdgfra*$^+$ OLPs spread laterally and dorsally from their site of first appearance in the ventral telencephalon, just as OLPs spread from their site of origin in the ventral spinal cord. These analogies suggest a common molecular mechanism of oligodendrocyte specification in the telencephalon and more caudal regions of the CNS. Our suggestion that *Pdgfra*$^+$ OLPs in the embryonic cortex arrive there by physical migration from the ventral forebrain is at present a hypothesis that will require direct experimental verification.

Oligodendrogenesis in the ventral telencephalon appears to be dependent on locally produced SHH from ventral neuroepithelial cells. The neuroepithelial expression domain of *Pdgfra* in the anterior hypothalamus is nested within broader expression domains of *Shh* and its receptor *Ptc1*, indicating that the forerunners of *Pdgfra*$^+$ progenitors are normally exposed to and responsive to SHH. Oligodendrogenesis in cultures of rat ventral forebrain cells was blocked by cyclopamine, an inhibitor of HH signalling pathways. Moreover, *Pdgfra*$^+$ OLPs did not develop in the VZ or SVZ of the ventral forebrain of *Nkx2.1* null mutant mice, which lack the telencephalic domain of *Shh* expression. Further evidence for the involvement of SHH comes from in vivo gain-of-function experiments in which SHH was ectopically expressed in the telencephalic neuroepithelium of mouse embryos using a retrovirus vector (Nery et al., 2001). SHH-expressing cells developed into OLPs and later into oligodendrocytes (Nery et al., 2001). These cells would not normally express SHH persistently but, nevertheless, this experiment provides a striking demonstration of the OLP-inducing activity of SHH in vivo.

Pdgfra$^+$ progenitors did appear belatedly in the telencephalon of *Nkx2.1* null mice. However, they first appeared in small numbers scattered outside the germinal zones, not tightly packed within the VZ and SVZ as in wild-type mice, suggesting that *Pdgfra*$^+$ cells might migrate into the *Nkx2.1* null forebrain from more caudal regions where *Shh* expression is unaffected in the mutant. OLPs also appeared in cultures derived from *Nkx2.1* null ventral forebrain, where there was no possibility of immigration from other brain regions. However, their production was suppressed by cyclopamine and furthermore we could detect expression of *Shh* and *Ihh* in the cultures, so HH signalling was presumably responsible for OLP induction in vitro. Whether the *Shh* and *Ihh* expression we detected reflects aberrant up-regulation of these molecules following cell dissociation, or normal expression that is undetectable in situ, we do not know. In any case, it is not necessary to invoke a SHH-independent pathway of oligodendrogenesis to explain the appearance of OLPs in the *Nkx2.1* null telencephalon, as suggested recently by Nery et al. (Nery et al., 2001). Indeed, expression of IHH in culture might explain the observation that some OLPs developed in cultures derived from *Shh* knockout brain (Nery et al., 2001). This could be tested by culturing *Shh* null brain cells in the presence of cyclopamine. It remains possible that there is a SHH-independent (or HH-independent) route(s) to oligodendrocyte development but this requires further investigation.

Do cortical oligodendrocytes originate in the ventral telencephalon?

A possibility raised by this work is that some cortical oligodendrocytes might develop, not from endogenous cortical precursors, but rather from OLPs that migrate from the ventral forebrain. At present we have no direct evidence for migration. Nevertheless, *Pdgfra*$^+$ OLPs in the telencephalon appear similar to those in optic nerve or spinal cord, which are known to migrate relatively long distances during development (Small et al., 1987; Miller et al., 1997; Ono et al., 1997; Pringle et al., 1998) or following transplantation into dysmyelinating or demyelinating hosts (e.g. Warrington et al., 1993; Vignais et al., 1993). Moreover, there is a delay in appearance of *Pdgfra*$^+$ progenitors in the cortex of *Nkx2.1* mutant embryos, which are primarily defective in ventral structures (Sussel et al., 1999), consistent with the idea that at least the early-appearing cortical OLPs are derived from the ventral forebrain. There is ample precedent for migration of progenitor cells into the neocortex from ventral telencephalon. For example, the progenitors of many GABAergic non-pyramidal neurons migrate into the developing cortex from the MGE or LGE (Anderson et al., 1997; Lavdas et al., 1999; reviewed by Parnavelas, 2000).

Latent oligodendrogenic potential of cortical precursors

We found that E15.5 rat cortical cells did not generate oligodendrocyte lineage cells in short term (4DIV) cultures, in contrast to cells from E15.5 ventral forebrain or E18.5 cortex. This confirms the finding of Birling and Price (Birling and Price, 1998) that E15 rat cortical cells have a reduced oligodendrogenic capacity in vitro compared to either E15 striatal cells or E18 cortical cells. It also tallies with the experiments of Kalman and Tuba (Kalman and Tuba, 1998) who showed that fragments of E18 rat cortex, but not E15 cortex, generate oligodendrocytes when transplanted into the eye of a new-born rat. Thus, the oligodendrogenic capacity of rat neocortex increases markedly between around E15 and E18, consistent with and as predicted by the in situ hybridization data, which show *Pdgfra*$^+$ progenitors apparently migrating into the cortex after E16.5 (Figs 3, 4).

Nevertheless, E15.5 rat cortical cells did generate OLPs when cultured for long periods of time (≥6DIV), demonstrating that rat cortical precursors have latent oligodendrogenic potential. This is analogous to the recent report that E14 rat dorsal spinal cord cells can generate oligodendrocytes in long-term, though not short-term cultures (Sussman et al., 2000). Several previous studies have demonstrated that early cortical cells from rodents can generate oligodendrocytes in vitro (e.g. Williams et al., 1991; Davis and Temple, 1994).

The appearance of OLPs in long-term cultures of E15.5 rat neocortex was puzzling, since we could not detect expression of *Shh*, *Ihh* or *Dhh* in the embryonic cortex by in situ hybridization. However, production of oligodendrocytes in vitro was inhibited by cyclopamine and, in addition, mRNAs coding for SHH and IHH could be detected in the cultures. Perhaps HH expression is normally repressed in the cortex in vivo but under our culture conditions the inhibitory signals are rendered ineffective by dilution or otherwise. Note that *Shh* is clearly expressed in certain neurons in the rat cortex from about a week after birth (Traiffort et al., 1999 and our unpublished results). Whether this late-onset expression contributes to postnatal oligodendrogenesis is not known.

Is *Olig2* oligodendrocyte lineage-specific?

We found that *Olig2* is expressed more widely than *Olig1*, *Sox10* or *Pdgfra* in both the neuroepithelium and the surrounding mantle zone (not shown). Whether the *Olig1*$^-$ *Olig2*$^+$ cells are OLPs is an open question, since it is by no means clear that the *Olig* genes and particularly *Olig2* are restricted to the oligodendrocyte lineage(s). It is known, for example, that *Olig2* is expressed by olfactory neurons and their precursors in the olfactory epithelium (Takebayashi et al., 2000). It is possible that the *Olig1*$^-$ *Olig2*$^+$ cells in the telencephalon might be related to the pluripotent neuroglial precursors that Goldman and colleagues identified in the germinal zones surrounding the lateral ventricles after birth (Levison and Goldman, 1993; Levison and Goldman, 1997).

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