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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

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,

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 (T0 TD 8kEarl1 TplaSDDIG labelling Hybw (2)Ts TfE92at cDN of defi54.9(s medi1 Tm (DMe final)

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

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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lineage defined by reactivity of progenitors with R-mAb prior to O1 galactocerebroside. *J. Neurosci. Res.* **32**, 309-316.