# Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice

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## SUMMARY

There is a class of oligodendrocyte progenitors, called O-2A progenitors, that is characterized by expression of platelet-derived growth factor alpha-receptors (PDGFRα). It is not known whether all oligodendrocytes are derived from these PDGFRα-progenitors or whether a subset(s) of oligodendrocytes develops from a different, PDGFRα-negative lineage(s). We investigated the relationship between PDGF and oligodendrogenesis by examining mice that lack either PDGF-A or PDGF-B. *PDGF-A* null mice had many fewer *PDGFRα*-progenitors than either wild-type or *PDGF-B* null mice, demonstrating that proliferation of these cells relies heavily (though not exclusively) on PDGF-AA homodimers. *PDGF-A*-deficient mice also had reduced numbers of oligodendrocytes and a dysmyelinating phenotype (tremor). Not all parts of the central nervous system (CNS) were equally affected in the knockout. For example, there were profound reductions in the numbers of *PDGFRα*-progenitors and oligodendrocytes in the spinal cord and cerebellum, but less severe reductions of both cell types in the medulla. This correlation suggests a close link between *PDGFRα*-progenitors and oligodendrogenesis in most or all parts of the CNS. We also provide evidence that myelin proteolipid protein (*PLP/DM-20*)-positive cells in the late embryonic brainstem are non-dividing cells, presumably immature oligodendrocytes, and not proliferating precursors.

Key words: Central nervous system, Oligodendrocyte, Progenitor cell, PDGF, PDGF receptor, Knockout mouse, PLP/DM-20, MBP, Myelin

## INTRODUCTION

Oligodendrocytes, the myelinating cells of the central nervous system (CNS), are distributed widely throughout the grey and white matter of the mature CNS. Glial progenitor cells that give rise to oligodendrocytes were first identified in cultures of developing rat optic nerve cells (Raff et al., 1983). These progenitors, called O-2A progenitors (Raff et al., 1983), have also been found in several other places including spinal cord, cerebellum and cerebral cortex (reviewed by Pfeiffer et al., 1994). They originate in the ventricular and subventricular zones of the embryo (Pringle and Richardson, 1993; Levison and Goldman, 1993; Ono et al., 1995, 1997; Yu et al., 1994) and subsequently proliferate and migrate throughout the developing CNS (Levison et al., 1993; Noll and Miller, 1993; Leber and Sanes, 1995; Ono et al., 1997; Pringle et al., 1998). In the rodent optic nerve they start to generate myelinating oligodendrocytes around birth, continuing to divide and differentiate into oligodendrocytes during early postnatal life.

O-2A progenitor cell proliferation can be stimulated in vitro by a variety of polypeptide growth factors including PDGF (Noble et al., 1988; Richardson et al., 1988; Raff et al., 1988), basic fibroblast growth factor (bFGF/FGF-2) (Bögler et al., 1990; McKinnon et al., 1990), insulin-like growth factor I (IGF-I) (McMorris and Dubois-Dalcq, 1988), neurotrophin-3 (NT-3) (Barres et al., 1994) and neuregulin/glial growth factor (GGF2) (Canoll et al., 1996). Of these, only PDGF is known to be important for stimulating progenitor cell proliferation in vivo (Calver et al., 1998; this study). Active PDGF is composed of homo- or hetero-dimers of A and B chains, which are related in sequence but encoded by separate genes. In the CNS, PDGF-A is made by many neurons as well as by astrocytes (Richardson et al., 1988; Yeh et al., 1991; Mudhar et al., 1993). PDGF-B is made by capillary endothelial cells (Lindahl et al.,

1997a) and, at lower levels, by many neurons in the postnatal and adult CNS (Sasahara et al., 1991, 1995). PDGF binds to and activates two high-affinity cell-surface receptors, PDGFRα and PDGFRβ, which are also products of separate genes (Heldin and Westermark, 1989). O-2A progenitors express PDGFRα, which can be activated in vitro by all three dimeric isoforms of PDGF (AA, AB, BB) (Heldin and Westermark, 1989; Pringle et al., 1989).

It is not known whether all oligodendrocytes develop from *PDGFRα*+ O-2A progenitors or whether there are other oligodendrocyte lineages that generate different subtypes of oligodendrocytes, or that operate in different parts of the CNS. For example, there are cells in the embryonic mouse brain and spinal cord that express mRNA encoding DM-20, an alternative-splice isoform of the myelin proteolipid protein (PLP) (Timsit et al., 1992, 1995; Ikenaka et al., 1993; Peyron et al., 1997; Spassky et al., 1998). It has been suggested that these cells might represent a distinct class of progenitors that generate oligodendrocytes in some parts of the CNS instead of, or in addition to, O-2A progenitors (Peyron et al., 1997; Spassky et al., 1998).

To assess the contribution made by *PDGFRα*+ O-2A progenitors to myelination throughout the CNS, we investigated the relationship between O-2A progenitors, *PLP/DM-20*-positive cells and oligodendrogenesis in wild-type and *PDGF* knockout mice. Our observations are consistent with the view that oligodendrocytes in all regions of the CNS develop from *PDGFRα*-progenitors and provide no compelling argument for a separate, *PDGFRα*-independent oligodendrocyte lineage.

## MATERIALS AND METHODS

### Knockout mice

*PDGF-A* null mutant mice and wild-type littermates were obtained from heterozygous crosses (lines 29 and 33; bred as 129 Ola/C57 Bl6 hybrids) (Boström et al., 1996). Genotypes were determined by three-primer PCR of tail DNA. The forward primer 5′-CCTTTGGCTCTAGGGTGGAATTTC and the two reverse primers 5′-TGGATGTGGAATGTGTGCGAAG and 5′-ACACGAATGAA-CAGGGATGGG yielded 470 bp wild-type and 368 bp mutant allele products in a 40-cycle reaction (96°C for 30 seconds, 55°C for 30 seconds, 65°C for 3 minutes).

### In situ hybridization

Brains and spinal cords were dissected in ice-cold phosphate-buffered saline (PBS) and fixed overnight in 4% (w/v) paraformaldehyde (PF) in PBS. For in situ hybridization, tissue was infused with 0.5 M sucrose in PBS overnight, then mounted in OCT embedding compound (BDH), frozen on dry ice and sectioned at 10 μm. In situ hybridization was performed as described (Pringle et al., 1996), except that digoxigenin (DIG)-labeled probes were used. The *PDGFRα* probe was transcribed from a 1,637 bp *Eco*RI cDNA fragment encoding most of the extracellular domain of mouse *PDGFRα* cloned into Bluescript KS (a gift from Chiayeng Wang, University of Chicago). The *PDGF-A* probe (from Chiayeng Wang) was a 907 bp full-length mouse cDNA cloned into the *Eco*RI site of pGEM-1 (Promega). The *PLP/DM-20* probe was transcribed from a 747 bp cDNA fragment encompassing the entire mouse DM-20 coding sequence cloned into Bluescript KS (pBS-DM-20; Timsit et al., 1992). RNA polymerases were obtained from Pharmacia and DIG labelling mix from Boehringer. The transcription reaction was run as recommended by Boehringer. RNA hybrids were visualized in situ by immunohistochemistry with an alkaline phosphatase-conjugated anti-DIG antibody (Boehringer kit) according to the manufacturer's

instructions, except that polyvinyl alcohol (PVA; 5% v/v) was included in the final colour reaction to increase sensitivity.

### Immunolabelling

Tissues were embedded in paraffin wax after fixation and serially sectioned at 4 μm. Myelin basic protein (MBP) was visualized with an anti-MBP rabbit serum (provided by David Coleman, Mount Sinai School of Medicine, New York) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, P448). The reaction was developed with diaminobenzidine (DAB). Vimentin was visualized with HRP-conjugated antibodies against vimentin (a gift from Eugenia Wang, Lady Davis Institute, Montreal, Canada), and neurofilament with mouse monoclonal antibodies EPOS (Dako A/S) or anti-NF200 (Novo Castra). The two neurofilament antibodies gave comparable results. Fig. 9 shows the results obtained with the EPOS antibody.

### Combined BrdU immunohistochemistry and in situ hybridization

BrdU (50 μg/g body mass) was injected intraperitoneally into a pregnant female mouse as described before (Calver et al., 1998). After 2 hours the animal was killed and her embryos subjected to in situ hybridization for *PDGFRα* or *PLP/DM-20* as described above. After the final colour reaction, the sections were immersed in PBS for 10 minutes to stop the reaction followed by 70% (v/v) ethanol for 20 minutes at room temperature. The sections were then treated for 30 minutes with 1% Triton X-100 in 6 M HCl, neutralized in 0.1 M Na₂B₄O₇, pH 8.5, and blocked for 30 minutes with 1% Triton X-100, 10% v/v normal goat serum in PBS. The sections were then incubated overnight at 4°C in anti-BrdU (monoclonal Bu209; Magaud et al., 1989) followed by Texas Red-conjugated goat-anti-mouse IgG, and mounted under coverslips for microscopy.

### Histochemistry

Paraffin sections were stained with Hematoxylin and Eosin according to standard protocols. For the detection of nerve cells and myelin, sections were stained using Luxol Fast Blue and Cresyl Violet (Luna, 1968). In some experiments, Sudan Black was used as a myelin stain.

### Cell and myelin quantification

*PDGFRα*-positive and *PLP/DM-20*-positive cells were counted in a dissecting microscope with a graticule eyepiece. In each chosen region of the brain, cell density was estimated in sagittal sections by calculating the average number of cell bodies within three non-overlapping areas (squares of side 0.25 mm) per section. For the spinal cord, we counted all cells within the cross section of the cord and normalized to the area above. For each region of the CNS, we analyzed 4-6 sections from each of two animals of each genotype (from separate litters) and quote the data as means ± s.e.m. MBP-positive myelinated fibres were scored by counting 4-10 randomly selected areas (the size of which varied according to brain region) and normalized to the same area as before (0.25 mm square). One or two sections from one P17 and one P19 animal of each genotype were analyzed and the data expressed as mean ± s.e.m.

Purkinje cells were counted along a distance of 0.25 mm in the Purkinje cell layer, in sagittal sections stained with Hematoxylin and Eosin. Ten separate counts were made from one or two sections from one P17 and one P19 animal of each genotype and the data expressed as mean ± s.e.m. Dorsal hippocampal neurons were similarly counted along a distance of 0.25 mm parallel with the cortical surface in coronal sections.

## RESULTS

### PDGF expression in the normal developing CNS

We analyzed PDGF expression in the developing CNS by in situ hybridization with probes against *PDGF-A* and *PDGF-B*,
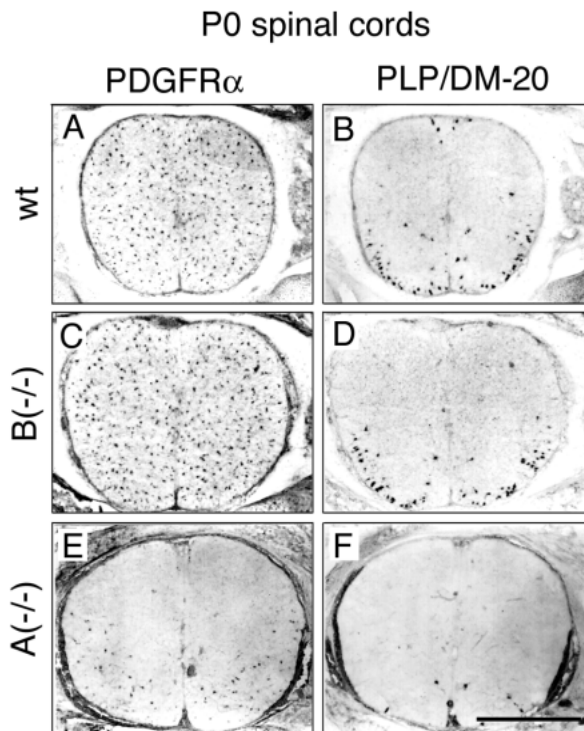
## P0 spinal cords



**Fig. 1.** Oligodendrocyte lineage cells in neonatal mouse spinal cords. Sections of wild-type (A,B), *PDGF-B* null (C,D) or *PDGF-A* null (E,F) cervical spinal cords were subjected to in situ hybridization with probes to *PDGFRα* (A,C,E) or *PLP/DM-20* (B,D,F). Numbers of *PDGFRα*+ O-2A progenitors and *PLP/DM-20*-positive oligodendrocytes appear normal in the *PDGF-B* null cord but are strongly reduced in the *PDGF-A* null cord. Bar, 1 mm.

confirming and extending previous studies. In the spinal cord, *PDGF-A* mRNA was first detected in the floor plate at E11 (Orr-Urtreger and Lonai, 1992), and persisted there until after E12 (data not shown). This is before the appearance of any *PDGFRα*-expressing cells in the ventral cord; *PDGFRα*+ oligodendrocyte precursors first appear around E12.5 in the mouse, at the luminal surface near the floor plate (Pringle et al., 1996; Hardy, 1997; Calver et al., 1998). After E13 *PDGF-A* was expressed by motor neurons and later by neurons in all

parts of the spinal cord (Yeh et al., 1991; Calver et al., 1998). By postnatal day 7 (P7), *PDGF-A* was also detected in numerous small cells, presumably astrocytes, in the developing white matter (Yeh et al., 1991; Mudhar et al., 1993; data not shown). *PDGF-A* was also expressed by many neurons and presumptive astrocytes in the embryonic and postnatal brain (Yeh et al., 1991 and data not shown).

*PDGF-B* was expressed in capillary blood vessels of the embryonic spinal cord and brain (Mudhar et al., 1993; Lindahl et al., 1997a), but could not be detected in either neurons or glia before birth (data not shown). However, after birth *PDGF-B* begins to be expressed at low levels by many cells, presumably neurons, throughout the CNS (Sasahara et al., 1991, 1995; data not shown).

### PDGF-A but not PDGF-B is required for oligodendrogenesis in the developing spinal cord

We analyzed oligodendrocyte development in *PDGF-A* and *PDGF-B* knockout mice, which have been described previously (Lindahl et al., 1997a; Leveén et al., 1994; Boström et al., 1996). Some homozygous *PDGF-A* knockouts die around embryonic day 10 (E10), but others survive and are born outwardly normal though slightly smaller than their wild-type littermates. They die during the first few postnatal weeks. *PDGF-B* knockouts invariably die at birth. They are hemorrhagic because their capillary blood vessels lack pericytes and form rupturing micro-aneurysms (Lindahl et al., 1997a). They also have defective kidney development (Leveén et al., 1994; Lindahl et al., 1998).

We visualized O-2A progenitor cells by in situ hybridization with a *PDGFRα* probe. In the *PDGF-A* knockouts the first *PDGFRα*+ O-2A progenitors appeared at the normal time and place, on E12.5 at the ventricular surface near the floor plate (not shown). However, they failed to increase in number properly in the knockouts so that, at later ages, there was a striking reduction in their number compared to wild-type littermates (compare Fig. 1A,E). At E17 there were less than 5% of the normal number of *PDGFRα*+ cells (Calver et al., 1998), at P0 there were approx. 12% (Fig. 1E; Table 1) and at P9 there were only approx. 3% ($1\pm1$ cells/unit area in the knockout compared to $17\pm3$ in wild type) (Fig. 2D). In contrast, numbers of *PDGFRα*+ progenitors in the spinal cords of *PDGF-B* null mice appeared normal (compare Fig. 1A,C).
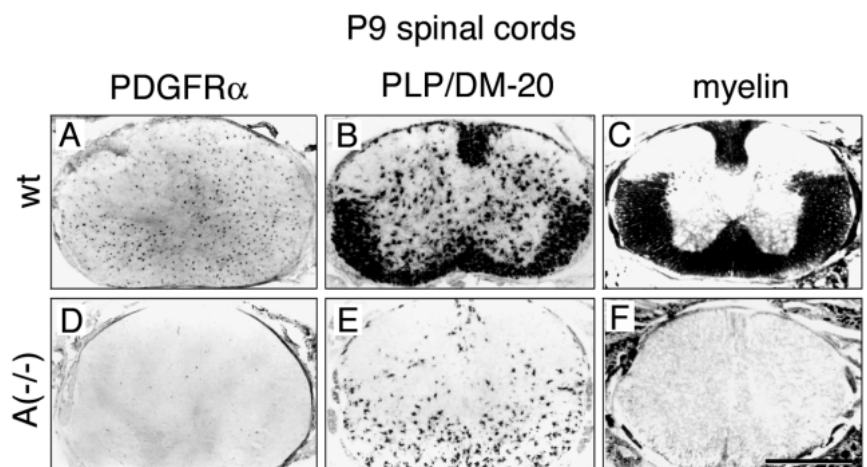
## P9 spinal cords



**Fig. 2.** Oligodendrocyte lineage cells and myelin in P9 spinal cords. Neighbouring sections from the cervical cord of wild-type (A-C) or *PDGF-A* null (D-F) mice were subjected to in situ hybridization with DIG-labelled probes to *PDGFRα* (A,D) or *PLP/DM-20* (B,E), or stained with Sudan Black to visualize myelin (C,F). There is a near-absence of *PDGFRα*+ O-2A progenitors in the knockout cord and severe reductions both in the number of *PLP/DM-20* oligodendrocytes and the amount of myelin. Bar, 1 mm.

**Table 1. Numbers of PDGFRα progenitors, oligodendrocytes and myelin sheaths in wild-type and PDGF-A null mice**

| | PDGFRα+ progenitors in P0 mice | | | Oligodendrocytes in P9 mice | | | Myelin sheaths in P17-P19 mice | | |
|---|---|---|---|---|---|---|---|---|---|
| | wt | −/− | % | wt | −/− | % | wt | −/− | % |
| Brain stem | 37±3 | 16±3 | **42** | 47±5 | 36±4 | **77** | 1013±188 | 513±75 | **51** |
| Cerebral cortex | 14±2 | 3±1 | **20** | 13±3 | 5±2 | **36** | 93±030 | 26±13 | **28** |
| Cerebellum | 24±8 | 4±1 | **15** | 14±4 | 2±1 | **11** | 323±078 | 31±10 | **10** |
| Spinal cord | 19±2 | 2±1 | **12** | 41±3 | 8±2 | **19** | 738±153 | 91±38 | **12** |
| Optic nerve* | 44±4 | 0 | **0** | | | | 423±088 | 5±06 | **2** |

Cells and MBP profiles (means ± s.e.m.) were counted as described in Materials and methods.

Numbers in bold face refer to the *PDGF-A* knockout, expressed as a percentage of wild type.

Those brain regions that displayed the greatest loss of *PDGFRα* progenitors in the knockout also experienced the greatest loss of oligodendrocytes (defined as cells that express high levels of *PLP/DM-20*) and MBP-positive myelin sheaths. Note that a perfect correlation between numbers of *PDGFRα*+ progenitors and oligodendrocytes is not to be expected because their numbers are controlled independently, by mitogens and survival factors, respectively.

*$PDGFR\alpha$ progenitors in the optic nerve were counted midway between chiasm and retina at P4.

We conclude that PDGF-B-containing dimers (BB and AB) are not important for driving progenitor cell proliferation in the embryonic mouse spinal cord.

We visualized oligodendrocytes in the newborn spinal cord by in situ hybridization with a probe for *PLP/DM-20* transcripts (Figs 1, 2). There were rather few oligodendrocytes in the wild-type cord at P0 and these were found mainly in the ventral fibre tracts (Fig. 1B). The number and pattern of *PLP/DM-20*-positive oligodendrocytes was very similar to this in the *PDGF-B* null cords but there were many fewer in the *PDGF-A* null cord (Fig. 1B,D,F). Over the next week or so oligodendrocyte numbers increased in the wild type and the *PDGF-A* knockout, but remained much lower than normal in the knockout (Table 1; compare Fig. 2B,E). The paucity of oligodendrocytes led to a severe lack of myelin, judged by staining sections with Sudan Black (Fig. 2C,F) or immuno-labelling with an antiserum to myelin basic protein (MBP) (not shown).

## PDGF-AA is important for proliferation of *PDGFRα+* progenitors in the embryonic brain

Longitudinal columns of *PDGFRα+* oligodendrocyte progenitors extend all along the ventral ventricular zone of the spinal cord of the E12.5 mouse (E14 rat) into the hindbrain (Pringle and Richardson, 1993). It seems likely that these columns of progenitors generate oligodendrocytes throughout the hindbrain and possibly also the midbrain, as in the spinal cord. We visualized *PDGFRα+* progenitors in the midbrain/hindbrain region of E15 wild-type and *PDGF-A* knockout mice by in situ hybridization as above. In E15 wild-type mice there were many *PDGFRα+* cells scattered through the midbrain and hindbrain. In the knockout hindbrain, these cells were reduced to 42% of normal (9±1 cells/unit area versus 21±2) and 28% of normal in the midbrain (7±1 versus 28±2) (Fig. 3A,B). At this age there were very few *PDGFRα+* cells in the wild-type cerebellum and superior colliculus, and none at all in the *PDGF-A* knockout (Fig. 3A,B). In contrast to the *PDGF-A* knockouts, *PDGF-B* null mice were indistinguishable from wild type (not shown).

## *PLP/DM-20* cells in the E15 hindbrain do not divide and are not affected by loss of PDGF-A

We also visualized *PLP/DM-20*-positive cells in the E15 brain. There were significant numbers of *PLP/DM-20*-positive cells

in the brainstem and cervical spinal cord of wild-type mice (Fig. 3C,E), as reported previously (Timsit et al., 1995; Peyron et al., 1997). Similar numbers of these cells, which were
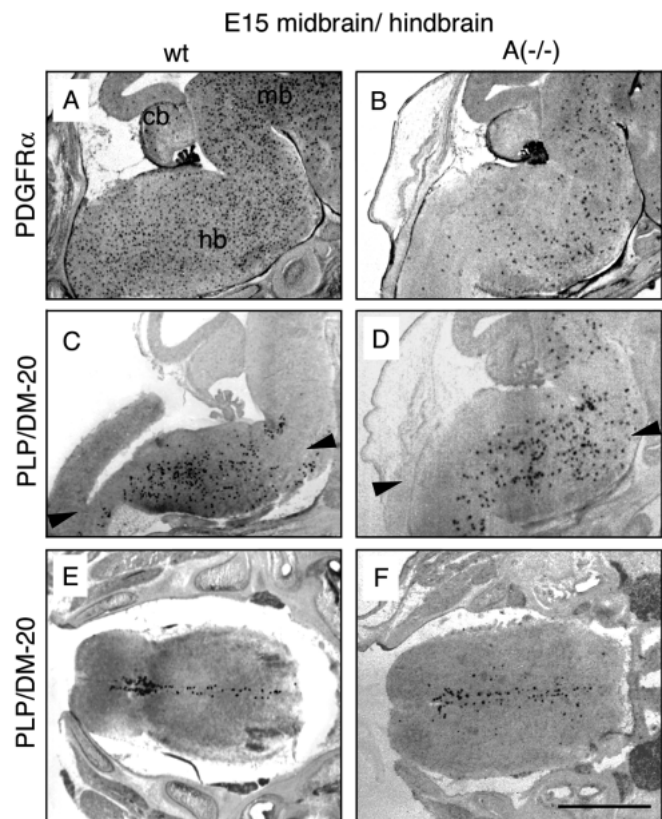


E15 midbrain/ hindbrain

**Fig. 3.** *PDGFRα+* progenitor cells and *PLP/DM-20*-positive cells in the E15 hindbrain/midbrain region. Neighbouring sagittal (A,B and C,D) and horizontal (E,F) sections of wild-type (A,C,E) and *PDGF-A* null (B,D,F) brains were subjected to in situ hybridization with DIG-labelled probes against *PDGFRα* (A,B) and *PLP/DM-20* (C-F) as before. The number of *PDGFRα+* O-2A progenitors is markedly reduced in the *PDGF-A* knockout whereas *PLP/DM-20*-positive cells occur in similar numbers in wild-type and knockout. The positive cells are located near the midline on either side of the lumen (E,F) as reported by others (Peyron et al., 1997). The approximate planes of section in E and F are indicated by arrowheads in C and D. cb, cerebellum; hb, hindbrain; mb, midbrain. Bar, 1 mm.

restricted to a region close to the midline, were also present in the *PDGF-A* null mice (27±5/unit area versus 24±5 in wild type) (Fig. 3D,F). The *PLP/DM-20* cells might be a separate population of oligodendrocyte precursors, as previously suggested (Timsit et al., 1995; Peyron et al., 1997; Spassky et al., 1998). Alternatively, they might be post-mitotic, pre-myelinating oligodendrocytes that for some reason develop before the main wave of oligodendrogenesis, which starts around birth.

To help distinguish these possibilities we asked whether *PLP/DM-20*-positive cells in the E15 hindbrain were mitotically active, as expected for progenitor cells. We injected BrdU into a pregnant wild-type female, fixed the embryos 2 hours later and double-labelled embryonic brain sections with
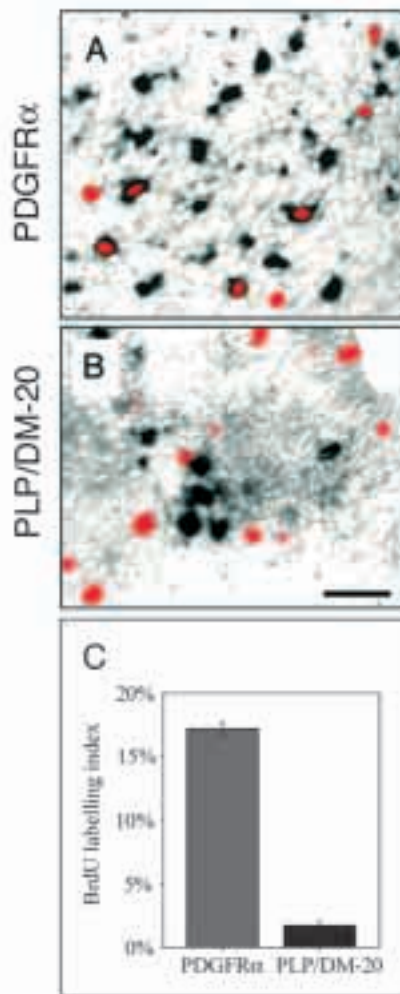
a hybridization probe to *PLP/DM-20* and an antibody against BrdU. We did not find a significant number of double-positive cells (<2% of cells; Fig. 4B,C). However, we found many (*PDGFRα*, BrdU) double-positive cells in parallel control experiments (approx. 17% of cells; Fig. 4A,C). Therefore, it seems likely that the *PLP/DM-20*-positive cells in the E15 brainstem are non-dividing cells, presumably immature oligodendrocytes, not dividing progenitor cells.

## Postnatal oligodendrogenesis in wild-type and PDGF-A deficient brains

At birth, *PDGFRα*[+] progenitors were abundant and fairly evenly distributed through the hindbrain, midbrain and cerebellum of wild-type mice (Fig. 5A). In newborn *PDGF-A* knockout mice, the number of these cells was much reduced in these areas (Fig. 5C and Table 1). However, the brainstem
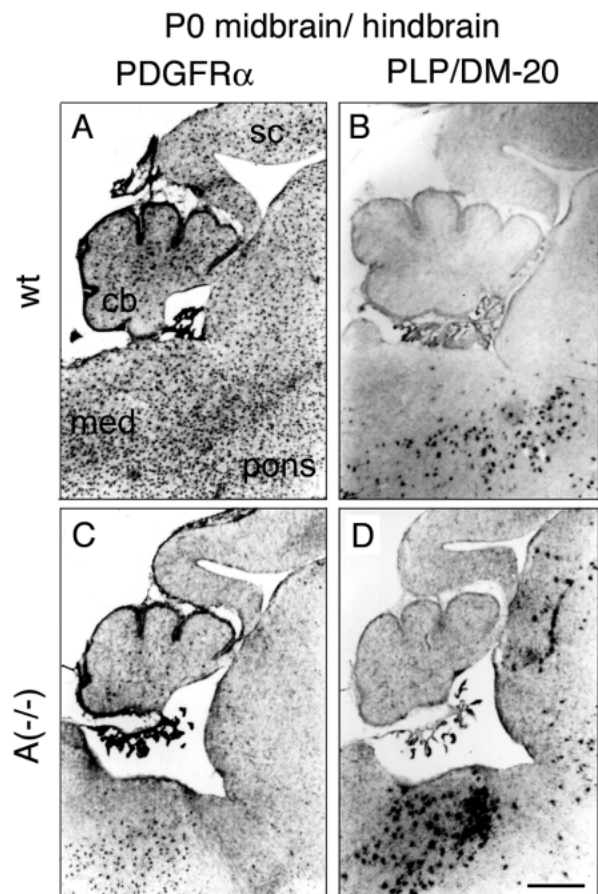


**Fig. 4.** *PLP/DM-20*-positive cells in the E15 brainstem and cervical spinal cord are not proliferating. A pregnant female mouse was injected once with BrdU and the embryos were fixed and processed 2 hours later. Sections through the brain were subjected to BrdU immunohistochemistry combined with DIG in situ hybridization for either *PDGFRα* (A) or *PLP/DM-20* (B). Sections were photographed under bright-field and fluorescence optics and the images digitized, coloured and superimposed by computer. BrdU-positive nuclei are shown in red, DIG-positive cells in black. Double-positive cells were counted and expressed as a percentage of all DIG-positive cells (C). Data are expressed as mean ± s.d. of three independent experiments. Bar, 100 μm.



**Fig. 5.** Oligodendrocyte lineage cells in the newborn hindbrain/midbrain region. Neighbouring sagittal sections of wild-type (A,B) or *PDGF-A* null (C,D) brains were subjected to in situ hybridization with probes against *PDGFRα* (A,C) or *PLP/DM-20* (B,D) as before. There are many fewer *PDGFRα*[+] O-2A progenitors than normal in the hindbrain/midbrain region of the knockout (compare A and C). Note, however, the relatively large population of these cells in the medulla of the mutant. The number of *PLP/DM-20*-positive cells was not dramatically altered in the mutant at this age (compare B and D). Note that the apparent distribution of *PLP/DM-20* cells depends critically on the plane of section, due to their restricted distribution (see Fig. 3). cb, cerebellum; sc, superior colliculus; med, medulla. Bar, 500 μm.

(medulla) was relatively less affected than other regions (Fig. 5C; Table 1). It appears, therefore, that PDGF-AA helps determine O-2A progenitor cell number in all parts of the CNS, but is relatively less important in the medulla than elsewhere.

We visualized *PLP/DM-20*-positive oligodendrocytes in brains of neonatal wild-type and *PDGF-A*-null mice by in situ hybridization. We did not detect strongly labelled *PLP/DM-20* cells anywhere in the newborn brain except for the brainstem, where there were similar numbers of scattered *PLP/DM-20*-positive cells in wild-type and null mutant mice (7±1 and 8±2 cells/unit area respectively) (Fig. 5B,D). In the mutant, there were a few much more weakly labelled cells scattered in the midbrain and hypothalamus, which did not appear to be present in the wild type. In addition we detected localized regions of weak, diffuse labelling in both wild type and mutant, for example in the ventromedial nucleus of the hypothalamus (not shown). We presume that this and some other faintly labelled

## P9 cerebellum



**Fig. 6.** Oligodendrocyte lineage cells in P9 cerebellum and brainstem. Sagittal sections of wild-type (A,B) or *PDGF-A* null (C,D) brains were hybridized with probes to *PDGFRα* (A,C) or *PLP/DM-20* (B,D) as before. There are significant numbers of *PDGFRα*⁺ O-2A progenitors in the wild-type cerebellum and brainstem (A), whereas there are practically none in the *PDGF-A* knockout (C). This is typical of most regions of the P9 brain and spinal cord (e.g. see Fig. 2D). In addition, there are greatly reduced numbers of *PLP/DM-20*-positive oligodendrocytes in the foliar white matter of the knockout cerebellum (compare B and D), although there are near-normal numbers in the brainstem (see Table 1). Bar, 1 mm.

regions represent specific sub-populations of neurons; they are clearly different in character from the scattered, strongly labelled oligodendrocytes in the neonatal brainstem. We did not examine *PLP/DM-20*-positive cells in the ventricular zones of the E9-E12.5 brain, as described by Timsit et al. (1992), because we did not look early enough in this study.

*PDGFRα*⁺ progenitors were still quite plentiful in the brains of P9 wild-type mice (Fig. 6A). At this and later ages there were greatly reduced numbers of *PDGFRα*⁺ progenitors in all parts of the *PDGF-A* knockout brain. For example, we counted 4% of the normal number of these cells in the hindbrain (1±1 cell/unit area versus 25±3) and essentially none in the cerebellum (Fig. 6C). *PLP/DM-20*-positive oligodendrocytes were also generally reduced in number in the P9 knockout, especially in some regions such as the distal parts of the cerebellar white matter and cerebral cortex (Fig 6B,D and Table 1). However, numbers of oligodendrocytes in the brainstem were only slightly reduced (Table 1).
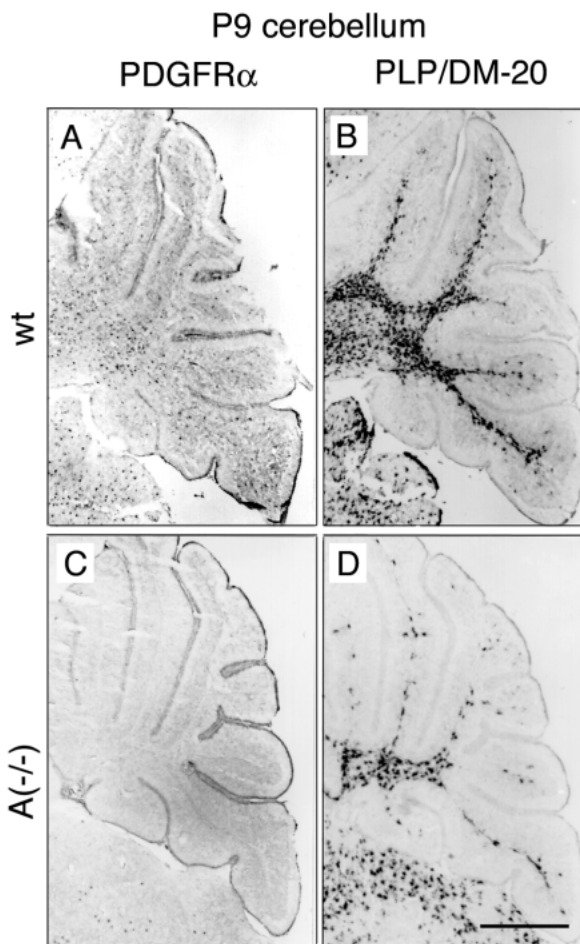
### Hypomyelination in PDGF-A deficient mice

In our colony of *PDGF-A* knockout mice, most of the postnatal animals die before 2 weeks of age. Rarely, some survive for as long as 6 weeks. At birth, *PDGF-A* null animals are slightly smaller than their wild-type littermates but their external morphology appears normal. However, they fail to thrive after birth and become increasingly runted compared to their siblings. They are generally lethargic, but the cause of this is unclear because they have multiple developmental defects outside the nervous system, several of which have been described (Lindahl et al., 1997b).

Knockout mice that survived beyond 3 weeks of age developed tremor in the hindlegs and tail, suggesting dysmyelination, although hindlimb paralysis was not observed even at 6 weeks. We examined the brains from 6 such mice. The optic nerves, optic tracts and other superficial fibre tracts were translucent in the knockouts compared to wild-type or heterozygous littermates, in which these structures were distinctly white (not shown). Also, superficial fibre tracts of the brainstem were less white in the *PDGF-A* knockouts. On dissection, a marked reduction in white matter was also apparent in the cerebellum and corpus callosum. Overall, our macroscopic examinations of *PDGF-A* knockout brains indicated dysmyelination in all parts of the brain, most dramatically in the optic nerves and optic tracts and in the cerebellum.

To examine myelin deficiency in more detail we serially sectioned brains from one P17 and one P19 *PDGF-A* knockout mouse together with control littermates and labelled with an antiserum against myelin basic protein (MBP) (Fig. 7). We compared numbers of MBP-labelled axons in cross-sections of fibre tracts in wild-type and knockout mice. The most dramatic loss of myelin in the knockout mice was in the optic nerves and optic tracts, cerebellar white matter and thoracic spinal cord (Figs 2C,F, 7A-D, 9E,D; Table 1). The corpus callosum and striatum, cerebral cortex and brainstem showed more moderate reductions (Fig. 7E-J; Table 1). In cortex and brainstem the reduction was most marked in the superficial layers (left sides of Fig. 7G-J).

We examined the optic nerve in more detail. In wild-type mice, there was a high density of MBP-positive myelin sheaths in the optic chiasm and all along the nerve to the eye. In P17
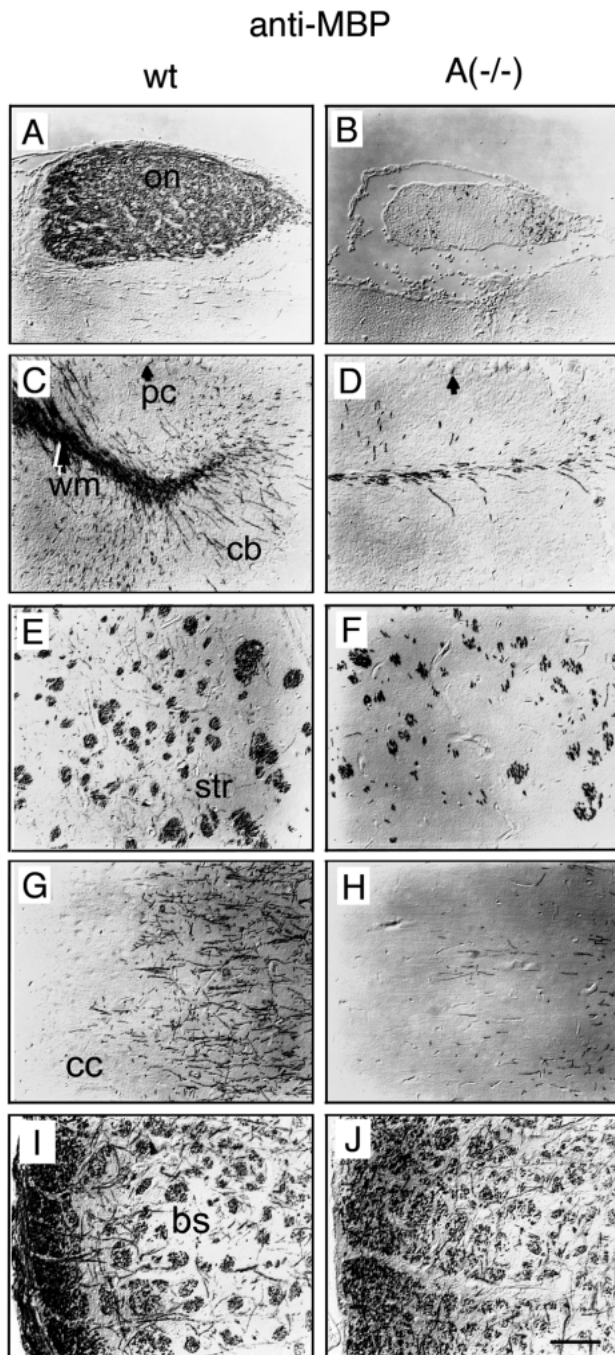
## anti-MBP

wt                    A(-/-)



**Fig. 7.** Reduced density of MBP-positive myelinated fibres in *PDGF-A* null brains. Wild-type (A,C,E,G,I) and *PDGF-A* null (B,D,F,H,J) P19 brain tissue was sectioned, immunolabelled for MBP and photographed under Nomarski optics. The reduction of the number of myelinated nerve profiles in the knockout optic nerve (A,B) is dramatic (>95%, see Table 1), and the nerve is also thinner than normal. The density of myelinated fibres in the cerebellum (C,D) is also greatly reduced (approx. 90%; Table 1), both in the white matter (wm) and in the region between white matter and Purkinje cells (arrows). The striatum (E,F), cerebral cortex (G,H) and brainstem (I,J) also had reduced numbers of myelin profiles (Table 1), particularly in more superficial regions (left sides of H,J). on, optic nerve; cb, cerebellum; wm, white matter; pc, Purkinje cells; str, striatum; cc, cerebral cortex; bs, brainstem. Bar, 50 µm.

and P19 knockout animals the density of myelin was much lower than normal at the chiasmal end of the nerve and declined further from there towards the retinal end, where there was no myelin whatsoever (Fig. 8; Table 1). In addition to the lack of myelin there was a loss of cell bodies; in Luxol Fast Blue/Cresyl Violet-stained sections there were many large, faintly stained cells in the wild type that were almost completely missing from the knockout (Fig. 8). These cells presumably correspond to oligodendrocytes and their precursors (the 'large, pale glioblasts' described by Vaughn et al., 1969). However, there were similar numbers of cells with small, dark nuclei, most likely astrocytes, in the wild-type and knockout optic nerves (Fig. 8). We also examined optic nerves by electron microscopy (Fig. 9). The loss of myelin sheaths in the knockout was striking, although where they were present (e.g. in the optic chiasm) their ultrastructure appeared normal. Oligodendrocytes (large pale cells lacking intermediate filaments and with prominent rough endoplasmic reticulum) were absent, but there were similar numbers of astrocytes (small dark cells with intermediate filament bundles) in wild type and knockout (Fig. 9).

### Neurons are not obviously affected by loss of PDGF-A

In view of reports (Vignais et al., 1995; Nait-Oumesmar et al., 1997) that *PDGFR*α is expressed by many neurons in the developing CNS in addition to O-2A progenitors, we looked for defects in development of neurons and other cell types in *PDGF-A* knockout brains. Staining with Luxol Fast Blue/Cresyl Violet confirmed the dramatic loss of myelin in the P19 knockout cerebellum, but failed to reveal any obvious changes to the overall morphology or disposition of cell bodies in the Purkinje and granule cell layers (Fig. 10A,B). This conclusion was upheld following staining with Haematoxylin and Eosin (Fig. 10C,D).

We immunolabelled brain sections with antibodies against neurofilament to reveal neuronal cell bodies and axons. There were no gross differences in the patterns of labelling between wild-type and *PDGF-A* null mice (Fig. 10G,H). We also immunolabelled sections with antibodies against vimentin. In the cerebellum, anti-vimentin labelled Bergman glia and other cells, presumably astrocytes, in the granule cell layer and in the white matter; there were no discernible differences between wild type and *PDGF-A* null mice (Fig. 10I,J).

Cerebellar Purkinje cells and hippocampal neurons were reported to be strongly labelled for *PDGFR*α (Vignais et al., 1995; Nait-Oumesmar et al., 1997), suggesting that PDGF might be important for the development or maintenance of these cells. Therefore, we counted Purkinje cells in sections of cerebellum from P17 and P19 *PDGF-A* null mice and their wild-type littermates. There was little or no difference between wild type and mutant (Table 2). We also counted neurons in the dorsal hippocampus. Again, there was no significant difference between wild type and mutant (Table 2).

### DISCUSSION

A large body of evidence indicates that PDGF is important for normal development of the oligodendrocyte lineage (e.g. Noble et al., 1988; Richardson et al., 1988; Raff et al., 1988; Armstrong et al., 1991; Barres et al., 1992; Hall et al., 1996;
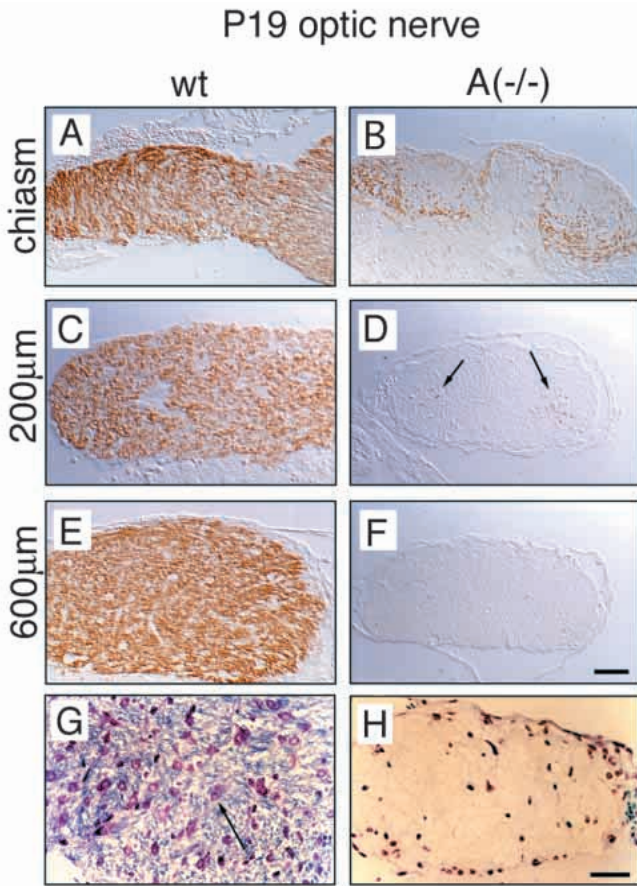
## P19 optic nerve



**Fig. 8.** Loss of MBP-positive nerve fibres and oligodendrocytes in *PDGF-A* knockout optic nerve. Wild-type (A,C,E,G) and *PDGF-A* null (B,D,F,H) sections of P19 optic nerve were compared with MBP immunolabelling (A-F), or Luxol Fast Blue/Cresyl Violet (G,H) to visualize cells (purple/violet) and myelin (blue). MBP-positive fibres are present but less numerous in the optic chiasm of null mutants compared to wild type. With increasing distance from the chiasm towards the eye, MBP-positive fibres gradually disappear in the knockout optic nerve (compare D,F). There is an abundance of blue fibres (myelin) and presumptive oligodendrocyte lineage cells (large lightly stained cells, arrow), in the wild-type optic nerve (G), which are almost completely absent from the *PDGF-A* knockout nerve (B). The remaining small cells with dark nuclei are presumably astrocytes. Bars, 30 μm.



**Fig. 9.** Ultrastructure of the *PDGF-A* null optic nerve. Electron micrograph showing corresponding regions of the optic nerve in a wild-type (A) and *PDGF-A* knockout (B) mouse at 28 days of age. Note the lack of myelinated fibres in the *PDGF-A* null tissue (B). The cell body in A was judged to be an oligodendrocyte since its cytoplasm lacked bundles of intermediate filaments but contained a prominent rough endoplasmic reticulum. These cells were abundant in wild-type nerves, but were absent from the knockouts. The cell body in B was judged to be an astrocyte because of the presence of dense bundles of intermediate filaments. Similar numbers of the latter cells were found in wild-type and knockout nerves. Bar, 5 μm.

Calver et al., 1998). Our studies of PDGF knockout mice described here provide striking, direct evidence that PDGF is important for oligodendrocyte development in vivo. However, some O-2A progenitor cells do accumulate in the absence of embryonic PDGF-A, particularly in the brainstem. It is possible that other growth factors normally co-operate with PDGF-A and exert a limited effect in its absence. It is also possible that maternal PDGF-AA might cross the placenta and partially complement the embryonic PDGF-A deficiency, although why this would preferentially affect the brainstem is not obvious.

In the *PDGF-A* knockouts, a reduction in the number of PDGFRα+ progenitor cells present at birth was followed by a parallel reduction in the number of oligodendrocytes and the amount of myelin that form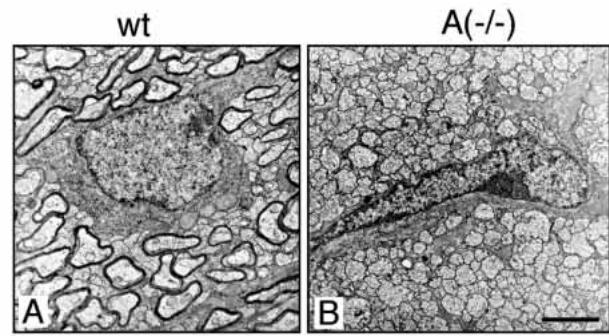ed postnatally. Those regions that experienced the greatest loss of PDGFRα+ progenitors in the embryo – spinal cord, optic nerve and cerebellum – were also those regions where myelin loss was most severe. Where the loss of PDGFRα+ precursors was less pronounced, in the brainstem and parts of the cerebral cortex, for example, there was a more modest loss of myelin internodes. This correlation suggests a link between PDGFRα+ progenitors and myelinating oligodendrocytes and is compatible with the idea that oligodendrocytes in all regions of the CNS develop from a single class of PDGFRα+ progenitors. Note that one would not necessarily expect a perfect correlation between progenitors and oligodendrocytes since their numbers are controlled separately, progenitors by mitogens (mainly PDGF; this study) and oligodendrocytes by survival factors (Barres et al., 1992; Calver et al., 1998; this study).

There is a population of PDGFRα-negative, *PLP/DM-20*-positive cells in the late embryonic brainstem and cervical spinal cord (Timsit et al., 1995; Peyron et al., 1997; this study). The location of these cells – close to the ventricular surface – led to the suggestion that the *PLP/DM-20*-positive cells and the PDGFRα+ cells are distinct types of oligodendrocyte progenitors that belong to independent cell lineages (Timsit et al., 1995; Peyron et al., 1997; Spassky et al., 1998). To test whether the *PLP/DM-20*-positive cells are dividing, as expected for a progenitor cell population, we labelled E15

**Table 2. Numbers of neurons in *PDGF-A* null and normal brains**

|  | Purkinje cells | | Hippocampal neurons | |
|---|---|---|---|---|
|  | wt | -/- | wt | -/- |
| P17 | 17.3±1.6 | 18.2±2.1 | 93±16 | 93±8 |
| P19 | 17.5±2.6 | 20.5±2.1 | 104±9 | 98±7 |

Neuronal populations (means ± s.e.m.) were estimated as described in Materials and methods. There were no significant differences between the wild type and *PDGF-A* knockout.
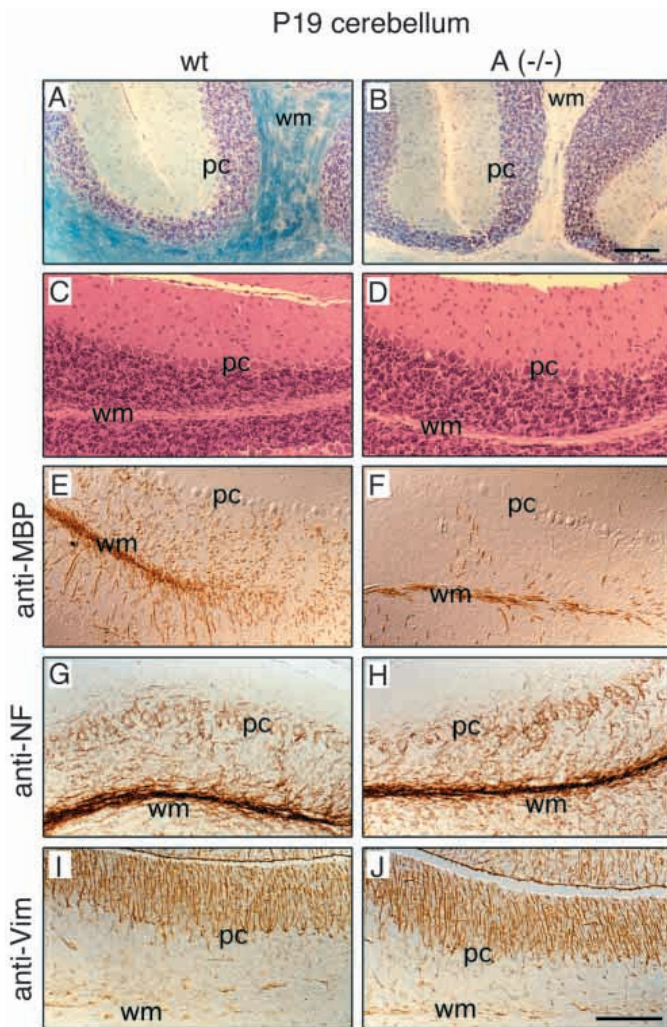
## P19 cerebellum

**Fig. 10.** Morphology and cellular composition of *PDGF-A* null cerebellum. Sagittal sections from cerebellar folia were stained with Luxol Fast Blue/Cresyl Violet (A,B) or with Hematoxylin and Eosin (C,D). Immunohistochemistry was performed with anti-MBP (E,F), anti-neurofilament (G,H) or anti-vimentin (I,J). There is a dramatic loss of myelin and MBP immunoreactivity in the foliar white matter (wm) in *PDGF-A* null mice (compare A,B and E,F*)*. However, overall morphology appears normal, and there are no obvious differences in the distribution of Purkinje cells (pc) or granule cells (I,J). Bars, 100 μm.

embryos with a short pulse of BrdU. We found that few if any (<2%) of the *PLP/DM-20*-positive cells incorporated BrdU, whereas a significant proportion (approx. 20%) of *PDGFRα*⁺ progenitors incorporated label. Hardy and Friedrich (1996) previously found that *PLP/DM-20*-positive cells in the embryonic mouse spinal cord did not incorporate BrdU either. Therefore, we suggest that the *PLP/DM-20*-positive cells in the E15 spinal cord and brain are not proliferating progenitor cells but post-mitotic oligodendrocytes that are derived from *PDGFRα*⁺ O-2A progenitors. This interpretation is consistent with the observation that the embryonic *PLP/DM-20*-positive cells co-express other markers of differentiated oligodendrocytes such as MBP and CNP (Peyron et al., 1997; data not shown). The *PLP/DM-20* cells might be a specialized

class of non-myelinating oligodendrocytes, of unknown function, or they might be nascent, pre-myelinating oligodendrocytes that are awaiting signals to mature into fully fledged myelinating cells later in development.

Note that there are *PLP/DM-20*-positive cells present in the brain as early as E9 (Timsit et al., 1992; Ikenaka et al., 1993; Peyron et al., 1997; Spassky et al., 1998), much earlier than the embryos we examined in this study and long before the first appearance of *PDGFRα*⁺ O-2A progenitors around E12-E13. At E9, the *PLP/DM-20*-positive cells are clustered in the ventricular zones (VZ) of the ventral diencephalon and elsewhere (Timsit et al., 1992; Peyron et al., 1997; Spassky et al., 1998). These weakly labelled cells clustered in the VZ are clearly different in nature from the scattered, strongly *PLP/DM-20*-positive cells in the late embryonic spinal cord and brainstem, discussed above. The early *PLP/DM-20*-positive cells in the VZ are undoubtedly neuroepithelial precursors but it remains to be determined what types of cells they generate during later development. It is possible that they are already dedicated to the production of oligodendrocytes from as early as E9. It is perhaps more likely that they are pluripotent precursors whose later progeny might include oligodendrocytes as well as neurons and/or astrocytes. In either case, we imagine that they must downregulate *PLP/DM-20* while upregulating *PDGFRα* as they proliferate and migrate away from the VZ, then strongly upregulate *PLP/DM-20* once again as they leave the cell cycle and differentiate into oligodendrocytes.

Setting aside these arguments, are there any grounds for thinking that there might be distinct lineages and/or subclasses of oligodendrocytes? Different morphological subtypes of oligodendrocytes have been described (del Rio Hortega, 1942; Hildebrand et al., 1993; Butt et al., 1994, 1997). For example, oligodendrocytes that ensheath large-diameter axons have large cell bodies and elaborate only one or a few short myelin internodes, while those that ensheath small-diameter axons have smaller cell bodies and generate many longer internodes. There are also reported biochemical differences between large and small oligodendrocytes in vivo (e.g. Butt et al., 1995). In addition, two putative morphological variants of oligodendrocytes were recently described in cultures of rat spinal cord cells (Fanarraga and Milward, 1997). It is not known whether these variations reflect heterogeneous cell lineages, or developmental plasticity within a single lineage. However, there is evidence that O-2A lineage cells from the optic nerve are indeed plastic and are capable of myelinating a wide range of axonal diameters in vivo (Fanarraga et al., 1998). Plasticity is also indicated by the fact that a single oligodendrocyte can simultaneously myelinate axons belonging to different functional subclasses of neurons (Belichenko and Celio, 1997). At present, therefore, the case for different oligodendrocyte lineages appears weak.

It has been reported (Vignais et al., 1995; Nait-Oumesmar et al., 1997) that many post-mitotic neurons in the developing and adult brain express PDGFRα. We ourselves have not observed reproducible labelling of neurons by in situ hybridization for *PDGFRα*. Other workers have also reported finding PDGFRα immunoreactivity and/or mRNA in O-2A progenitors but not neurons (Nishiyama et al., 1996; Ellison et al., 1996). We do not know the reason for this discrepancy. Setting aside the question of *PDGFRα* expression by post-

mitotic neurons, there is evidence that it is expressed by some neuronal precursors in the embryonic VZ (Pringle and Richardson, 1993; Williams et al., 1997). We therefore looked for morphological abnormalities that might reflect defects in neuronal development, by serially sectioning brains of postnatal *PDGF-A* knockout mice. We did not detect any abnormalities. We also labelled neurons with antibodies against neurofilament but again found no abnormalities. Finally we counted numbers of cerebellar Purkinje neurons and hippocampal neurons, which were reported to express *PDGFRα* strongly (Vignais et al., 1995; Nait-Oumesmar et al., 1997), but found no significant differences in the numbers of these cells. Therefore, while we do not rule out subtle alterations to neuronal development or phenotype, or the loss of specific neuronal subpopulations, it seems that loss of PDGF-A does not have a major effect on neuronogenesis.

In general, it seems that regions that are more distant from the periventricular germinal zones, such as optic nerve, cerebellum and superficial cerebral cortex, are also those regions that showed the most severe loss of myelin in the *PDGF-A* knockout mice. This was well illustrated in the optic nerve, where there was a gradation of myelin in the knockout, declining from the optic chiasm toward the retina. Therefore, loss of PDGF-A might inhibit long-range migration of O-2A progenitors as well as inhibiting their proliferation. An analogous situation has been described for some other types of progenitor cells that are PDGF-dependent in vivo (for review see Lindahl and Betsholtz, 1998). One example relates to lung development. Alveolar smooth muscle cells (SMC) are situated in lung alveolar septa and are the most distally located SMC associated with the respiratory tract. All respiratory tract SMC seem to originate from a population of PDGFRα-positive mesenchymal progenitors (Lindahl et al., 1997b) but it is only the alveolar SMC that are lost in *PDGF-A* knockout lungs (Boström et al., 1996). The developing respiratory epithelium expresses PDGF-A, which probably drives proliferation of the alveolar SMC progenitors and possibly also their migration to the developing alveolar saccules. Angiogenesis provides another example. During this process, PDGF-B is expressed by the sprouting vascular endothelial cells and attracts PDGFRβ-positive vascular SMC progenitors, which migrate from pre-existing blood vessels along the newly forming capillaries. These SMC progenitors subsequently form pericytes that surround and reinforce the new vessels. Proliferation and migration of the SMC progenitors is impaired in *PDGF-B* knockout mice, which consequently lack pericytes and have leaky blood vessels (Lindahl et al., 1997a). A third example is kidney development. Here, PDGF-B from endothelial cells stimulates proliferation and migration of PDGFRβ-positive SMC progenitors that subsequently give rise to mesangial cells at the distal tips of the kidney glomeruli; these cells are missing in *PDGF-B* knockout mice (Lindahl et al., 1998). Thus, while PDGFs are critical for the development of diverse cell types originating from different germ layers, its principal effects on these cells might be similar.

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