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Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination

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To design therapies for demyelinating diseases such as multiple sclerosis, it will be important to understand the mechanisms that control oligodendrocyte progenitor cell (OPC) numbers in the adult central nervous system (CNS). During development, OPC numbers are limited by the supply of platelet-derived growth factor-A (PDGF-A). Here, we examine the role of PDGF-A in regulating OPC numbers in normal and demyelinated adult CNS using transgenic mice that overexpress PDGF-A in astrocytes under the control of the glial fibrillary acidic protein (GFAP) gene promoter (*GFAP-PDGF-A* mice). In adult *GFAP-PDGF-A* mice, there was a marked increase in OPC density, particularly in white matter tracts, indicating that the PDGF-A supply controls OPC numbers in the adult CNS as well as during development. To discover whether increasing PDGF expression increases the number of OPCs following demyelination and whether this enhances the efficiency of remyelination, we induced demyelination in *GFAP-PDGF-A* transgenic mice by intraspinal injection of lysolecithin or dietary administration of cuprizone. In both demyelinating models, OPC density within lesions was significantly increased compared to wild-type mice. However, morphological analysis of lysolecithin lesions did not reveal any difference in the time course or extent of remyelination between *GFAP-PDGF-A* and wild-type mice. We conclude that the availability of OPCs is not rate limiting for remyelination of focal demyelinated lesions in the mouse. Nevertheless, our experiments show that it is possible to increase OPC population density in demyelinated areas by artificially increasing the supply of PDGF.

Introduction

The central nervous system (CNS) has the capacity to remyelinate axons after their myelin sheaths have been destroyed, for example, during human demyelinating diseases such as multiple sclerosis (MS) (Franklin, 2002; Ludwin, 1987). A major source of remyelinating oligodendrocytes is thought to be oligodendrocyte progenitor cells (OPCs), relatively large numbers of which persist in the adult CNS (Levine et al., 2001). OPCs in the normal adult CNS proliferate very slowly. Even so, they are the major proliferating cell population outside the subventricular zone of the lateral ventricles and the hippocampal subgranular zone (Alonso, 2000; Horner et al., 2000). Their proliferation rate is dramatically increased in response to demyelination (Carroll and Jennings, 1994; Keirstead et al., 1998; Levine and Reynolds, 1999; Redwine and Armstrong, 1998; Sim et al., 2002). Presumably, this is due to increased expression of OPC mitogens in and around areas of acute demyelination (Hinks and Franklin, 1999, 2000; Redwine and Armstrong, 1998). This results in local increases in the number of OPCs, some of which subsequently differentiate into remyelinating oligodendrocytes (Gensert and Goldman, 1997; Watanabe et al., 2002).

Remyelination is a common feature of MS lesions in the early stages of disease (Prineas and Connell, 1979; Raine and Wu, 1993). As the disease progresses, however, this inherent capacity for repair becomes exhausted and axons remain persistently demyelinated and vulnerable to atrophy (Lovas et al., 2000; Prineas et al., 1993; Wolswijk, 1998). One strategy that has been suggested for the repair of chronically demyelinated lesions is to enhance or reactivate spontaneous remyelination (Bieber et al., 2002; Cannella et al., 1998; O'Leary et al., 2002; Warrington et al., 2000; Yao et al., 1995). If, for example, the rate of remyelination was dependent on the size of the OPC population, then increasing the number of OPCs by administering appropriate mitogens might be a way to increase remyelination efficiency.

Previous studies have shown that the size of the OPC population during embryonic development is limited by the availability of platelet-derived growth factor A chain homodimers (PDGF-AA) (Calver et al., 1998; Van Heyningen et al., 2001). These studies suggested that OPCs proliferate until the rate at which the population as a whole consumes PDGF (by receptor binding and internalization) balances its rate of supply by neighboring cells. This leads to a steady state in which OPC division almost ceases and cell number remains constant. In the present study, we asked whether the PDGF-A supply determines OPC numbers in the adult

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CNS. We also asked whether PDGF-A influences OPC proliferative response and the remyelination that occurs following CNS demyelination. To this end we followed remyelination of experimentally induced demyelinating lesions in transgenic mice that overexpress PDGF-A under control of the glial fibrillary acidic protein (GFAP) promoter. This promoter is active in a subset of astrocytes in the normal adult CNS and is also upregulated locally in response to lesion induction, thereby targeting overexpression of the PDGF transgene preferentially to sites of demyelination.

Results

PDGF-A regulates OPC numbers in the adult mouse spinal cord

During embryonic development, the size of the OPC population is directly controlled by the availability of PDGF-A (Calver et al., 1998; Van Heyningen et al., 2001). We asked whether the same principle also applies to the adult OPC population using in situ hybridization for PDGF alpha-receptor mRNA (*PDGFRα*) to identify OPCs in cryosections of spinal cords taken from adult mice with different levels of *PDGF-A* expression—wild-type mice and hemizygous *GFAP-PDGF-A* mice which express human PDGF-A (hPDGF-A) under the control of the *GFAP* promoter (Fruttiger et al., 2000; see Experimental methods). In transgenic mice, the density of $PDGFR\alpha^+$ cells in spinal cord sections was increased approximately 4-fold compared to wild-type controls from 66 ± 12 to 255 ± 30 cells/mm^2. The relative change in $PDGFR\alpha^+$ cell density was comparable for white and grey matter, although the density of cells was higher in the grey matter (data not shown). Thus, the number of OPCs in the adult CNS is regulated by the availability of PDGF-A, as it is during development.

PDGF-A augments OPC response to toxin-induced demyelination

Increased expression of PDGF-A is a normal response to demyelination (Hinks and Franklin, 1999; Redwine and Armstrong, 1998) and, given its key role in controlling OPC proliferation during development, is likely to play a role in stimulating OPC proliferation following demyelination. We have asked whether OPC density within demyelinating lesions is increased by overexpressing hPDGF-A in two models of demyelination–remyelination.

We first created focal areas of demyelination by injecting 1 μl of a 1% solution of lysolecithin into the dorsal funiculus of the spinal cord of hemizygous *GFAP-PDGF-A* mice and wild-type controls (Hall, 1972). The mice received a 2-h pulse of BrdU to label cells in the S-phase just before perfusion-fixation 7 or 14 days later. The lesions were sectioned and subjected to in situ hybridization using DIG-labelled probes for *GFAP* (Fig. 1) or on adjacent sections for *PDGFRα* or *hPDGF-A* (Figs. 2D–I, N–S). Consistent with previous studies on astrocyte response to lysolecithin (Hinks and Franklin, 1999; Woodruff and Franklin, 1999), *GFAP* expression was markedly increased around the edge of lesions identified using the histochemical myelin stain Sudan black (Figs. 1 and 2A–C, K–M). Expression of the *hPDGF-A* transgene was strongly increased in *GFAP-PDGF-A* mice following lysolecithin injection. At 7 days postinjection, expression of *hPDGF-A* was strongest at the lesion edge in a region that corresponded to the increased *GFAP* expression (Fig. 1) but was also detectable in cells within the lesion and, to a lesser extent, in the surrounding grey matter outside the lesion (Fig. 2R). Expression of the transgene within the lesion remained elevated after 14 days, but by this time had subsided within the surrounding tissue (Fig. 2S). Expression of *hPDGF-A* was never observed in wild-type mice, although there was faint background staining within lesions (Figs. 2G–I).

The OPC density in wild-type lesions was increased approximately 15-fold at 7 days postinjection compared to the normal dorsal funiculus (see Figs. 2D, E and 3). The increase in OPC density was less pronounced at 14 days postinjection (see Figs. 2F and 3), reflecting both a downregulation in the level of mitogenic signalling and the onset of OPC differentiation (Hinks and Franklin, 1999). In *GFAP-PDGF-A* mice, the average OPC density across the entire lesions was approximately 8-fold higher at 7 days postlesion than in unlesioned transgenic mice and, in contrast to the wild-type mice, had only marginally decreased (to 7-fold higher) by 14 days (Figs. 2N–P and 3). The OPCs were not

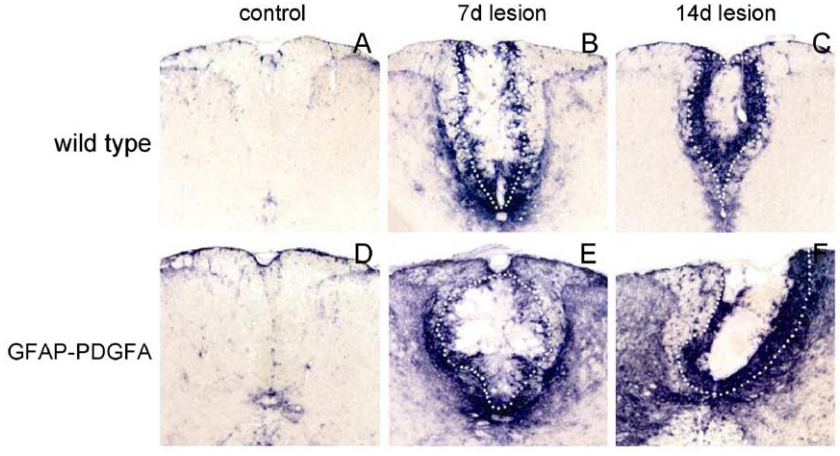

Fig. 1. GFAP expression in control wild-type mice (A) and *GFAP-PDGF-A* mice (D), and after lysolecithin-induced demyelination in the dorsal funiculus of the spinal cord in wild-type (B and C) and *GFAP-PDGF-A* mice (E and F).

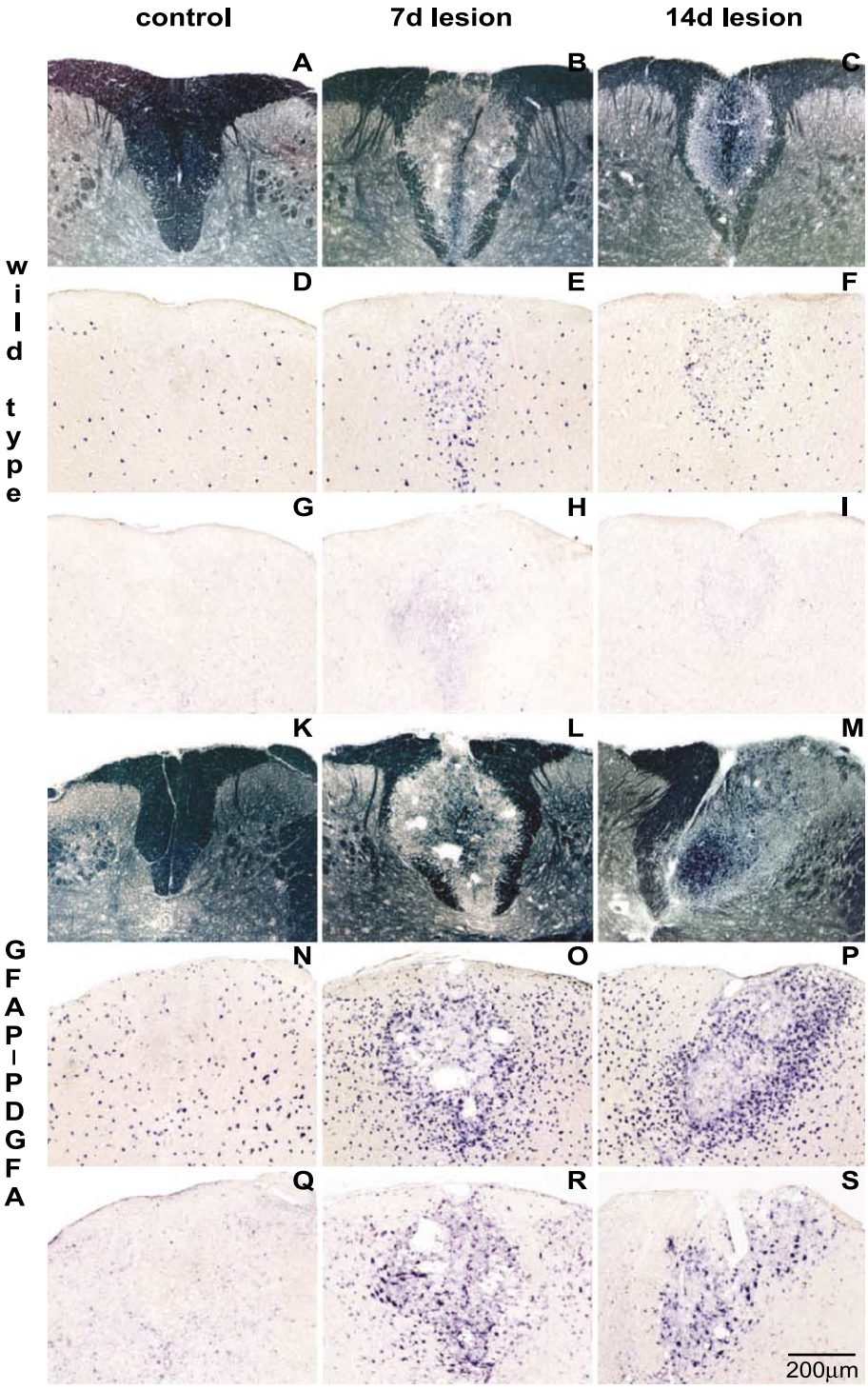

Fig. 2. *PDGFRα*$^{+}$ cells and *hPDGF-A* expression in unlesioned control mice and at 7 and 14 days following lysolecithin-induced demyelination in the dorsal funiculus of the spinal cord of wild-type and *GFAP-PDGF-A* mice. (1) Sudan black-stained sections indicating location of demyelinated area within the dorsal funiculus in wild-type (A–C) and *GFAP-PDGF-A* mice (K–M). (2) *PDGFRα*$^{+}$ cells in wild-type (D–F) and *GFAP-PDGF-A* mice (N–P). (3) *hPDGF-A* expression in wild-type (G–I) and *GFAP-PDGF-A* mice (Q–S).

evenly distributed throughout the lesions but tended to be concentrated at the lesion edge, where they occurred in such high numbers that accurate quantification was difficult and the fold increase for the entire lesion likely to be an underestimate. The concentration of OPCs generally occurred where *hPDGF-A* was most strongly expressed, although there was not always complete correspondence between the two.

Because of the difficulties in accurately quantifying OPC numbers where they occurred at high density at the edges of lysolecithin lesions, we also used an alternative model of toxin-

induced demyelination involving the dietary administration of cuprizone (Blakemore, 1973). At low doses required to avoid high levels of mortality, demyelination is restricted to the corpus callosum (Stidworthy et al., 2003). Although this lesion does not lend itself to accurate assessment of remyelination (Stidworthy et al., 2003), it nevertheless has a more even distribution of reactive astrocytes than lysolecithin lesions allowing a more accurate quantification of OPC numbers (Figs. 1 and 4). Using this model, we observed a similar increase in OPC density in hemizygous *GFAP-PDGF-A* mice and their wild-type littermates. After 4 weeks of cuprizone intoxication, there was evidence of myelin loss within the corpus callosum (Figs. 5B and N), which was accompanied by an increase in the number of *PDGFRα*+ cells in both groups of mice (Figs. 5E, F, Q and R). In *GFAP-PDGF-A* mice, the density of *PDGFRα*$^+$ cells in the demyelinating corpus callosum was nearly four times greater than wild types (Figs. 5 and 6). This correlated with an increase in both the intensity and extent of *hPDGF-A* expression, which extended outside the corpus callosum (see Figs. 5U and V). In contrast to the lysolecithin model, *PDGFRα*$^+$ cells were evenly distributed through the demyelinating region in the cuprizone-treated mice, reflecting spatial differences in *GFAP* mRNA response and hence upregulation of transgene-derived hPDGF-A, between the two models (Figs. 1 and 4).

We used combined in situ hybridization for *PDGFRα* and immunolabelling for BrdU on sections containing lysolecithin-induced lesions to identify OPCs that had been in the S-phase during the 2-h BrdU labelling period. For a given experimental condition, there was no significant difference in the labelling index (LI: percentage of *PDGFRα*$^+$ cells that were also BrdU-labelled) of *GFAP-PDGF-A* mice compared to wild-type mice (Fig. 7). In both groups, the LI was low in normal white matter, consistent with previous reports that the rate of OPC proliferation is low in adult mice (Horner et al., 2000). However, the LI increased substantially in both groups in response to demyelination (Fig. 7). These data show that the OPC density within demyelinating lesions is increased by overexpressing PDGF-A and are consistent with the idea that the proliferative response of OPCs to demyelination is controlled, at least in part, by the supply of PDGF-A.

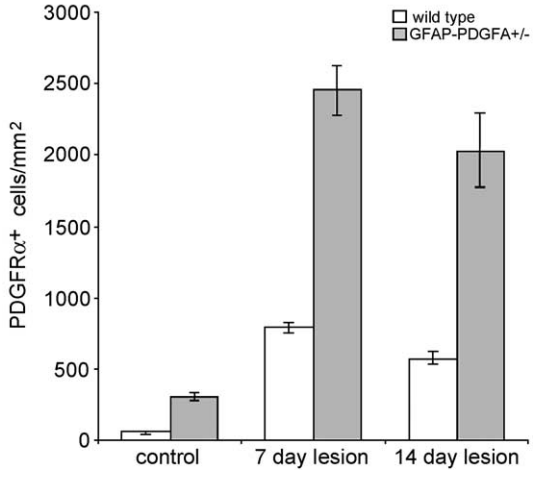

Fig. 3. Comparison of the densities of *PDGFRα*$^+$ cells at 7 and 14 days following lysolecithin-induced demyelination in wild-type and *GFAP-PDGF-A* transgenic mice.

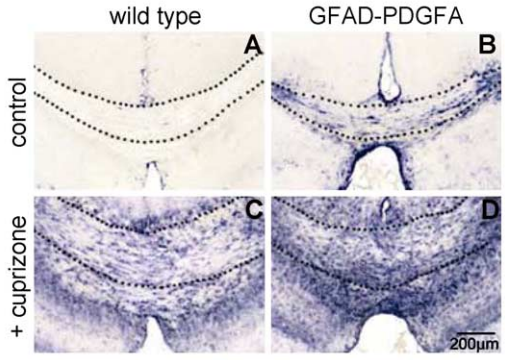

Fig. 4. GFAP expression in the corpus callosum of control wild-type (A) and *GFAP-PDGF-A* mice (B), and after 4 weeks of cuprizone administration in wild-type (C) and *GFAP-PDGF-A* mice (D).

Remyelination of lysolecithin-induced lesions is not enhanced by increasing OPC production

We asked whether the increased numbers of OPCs that followed demyelination in *GFAP-PDGF-A* mice affect the rate or extent of subsequent remyelination. This analysis was confined to the lysolecithin model because quantifying changes in the early stages of remyelination of the corpus callosum in the cuprizone model is unreliable (Stidworthy et al., 2003). Lysolecithin lesions were examined at 14 days and 5 weeks after lesion induction. In most (5/7) of the lesions examined in *GFAP-PDGF-A* mice at 14 days there was a striking abundance of small, closely packed cells that distinguished the lesions from those in wild-type mice, which had a much lower cell density (Fig. 8). These cells had a distinctive morphology with a prominent rim of cytoplasm around the nucleus, most likely corresponding to *PDGFRα*$^+$ cells detected by in situ hybridization. In all lesions in which these cells were abundant, remyelination had progressed very little compared to the less cell-rich lesions in wild-type mice. However, while there was a generally negative correlation between the overall cell density in the lesions and the extent of remyelination, a minority (2/7) of the lesions in *GFAP-PDGF-A* mice had a similar cell density as those in wild-type mice, and ranking analysis did not reveal any difference between the extent of remyelination of *GFAP-PDGF-A* mice compared to wild type (Fig. 8). By 5 weeks after lesion induction, remyelination was extensive in all lesions of both *GFAP-PDGF-A* and wild-type mice and no significant differences could be discerned between the two groups. For the most part, remyelination in both groups was by oligodendrocytes, with Schwann cells making only a small contribution.

Discussion

Identifying the factors that control proliferation, migration and differentiation of OPCs during CNS remyelination is essential if we are to understand the regulation of the repair process and devise therapeutic strategies. On the basis of previous studies, it seems likely that PDGF-A is one of the mitogens that contribute to the generation of new OPCs and remyelinating oligodendrocytes in demyelinated lesions. First, PDGF-A is a mitogen for cultured OPCs derived from both newborn and adult rodents (Noble et al.,

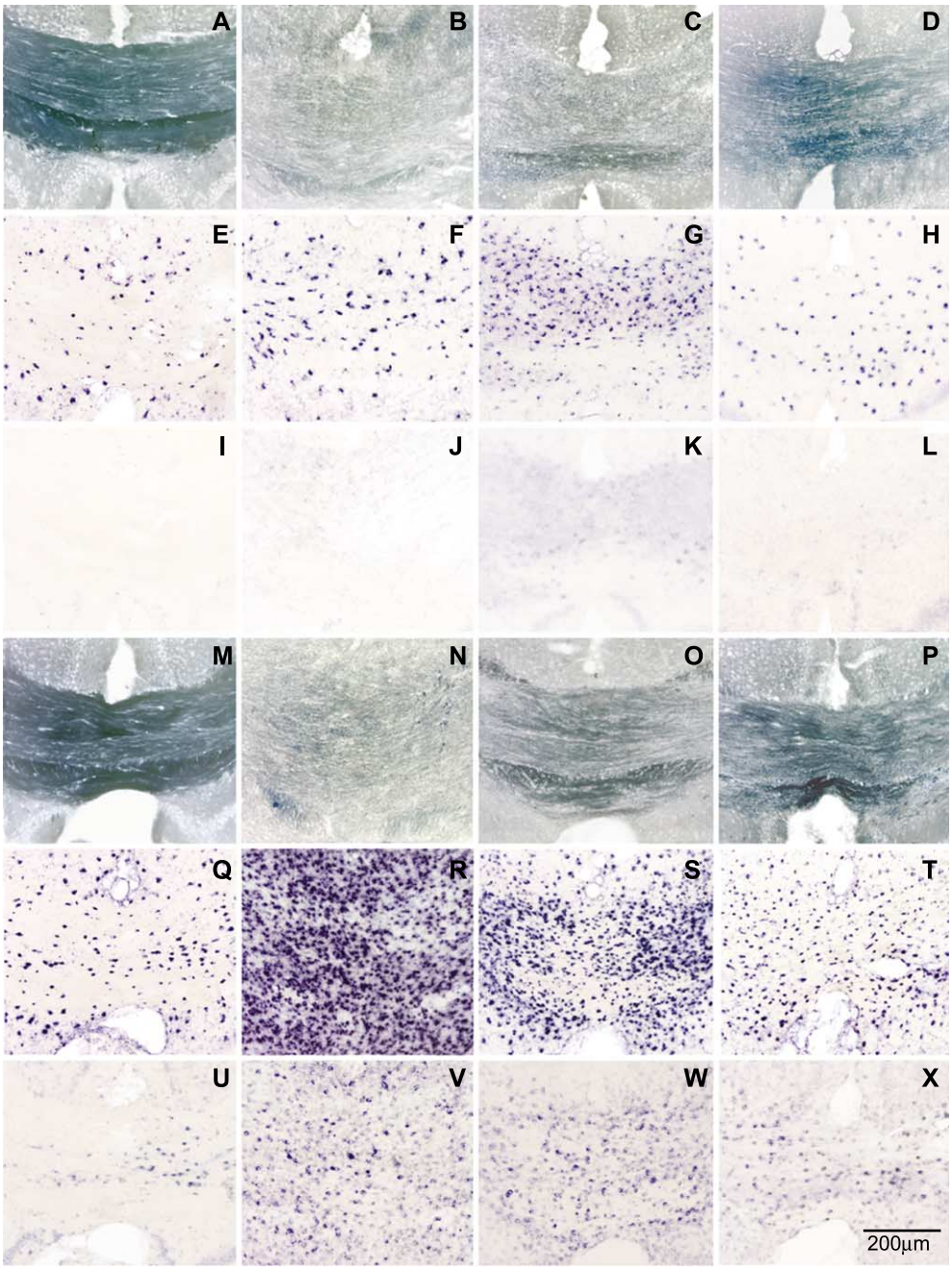

Fig. 5. *PDGFRα*$^{+}$ cells and *hPDGF-A* expression in the corpus callosum of control wild-type (A, E and I) and *GFAP-PDGF-A* mice (M, Q and U); after 4 weeks of exposure to cuprizone in wild-type (B, F and J) and *GFAP-PDGF-A* mice (N, R and V); after 6 weeks of exposure to cuprizone in wild-type (C, G and K) and *GFAP-PDGF-A* mice (O, S and W); with 6 weeks of cuprizone exposure followed by 2 weeks recovery in wild-type (D, H and L) and *GFAP-PDGF-A* mice (P, T and X). (1) Sudan black-stained sections showing the location of the demyelinated–remyelinated area within the corpus callosum in wild-type (A–D) and *GFAP-PDGF-A* mice (M–P). (2) *PDGFRα*$^{+}$ cells in wild-type (E–H) and *GFAP-PDGF-A* mice (Q–T). (3) *hPDGF-A* expression in wild-type (I–L) and *GFAP-PDGF-A* mice (U–X).

1988; Richardson et al., 1988). Second, levels of PDGF-A expression during development determine the OPC population size in vivo (Calver et al., 1998; Van Heyningen et al., 2001). Third, PDGF expression is increased in response to demyelination with a time course and spatial distribution that is highly suggestive of a functional role in the recruitment of OPCs into areas of demyelination (Hinks and Franklin, 1999, 2000; Redwine and Armstrong, 1998).

We have further investigated the role of PDGF-A in controlling OPC numbers during remyelination by making use of *GFAP-PDGF-A* mice to artificially increase PDGF-A levels within and around demyelinating lesions. We first showed that there is a higher steady-state density of OPCs in the spinal cords of unlesioned adult *GFAP-PDGF-A* mice, demonstrating for the first time that the level of expression of *PDGF-A* is a key factor that controls the number of OPCs in the adult CNS, as it does during develop-

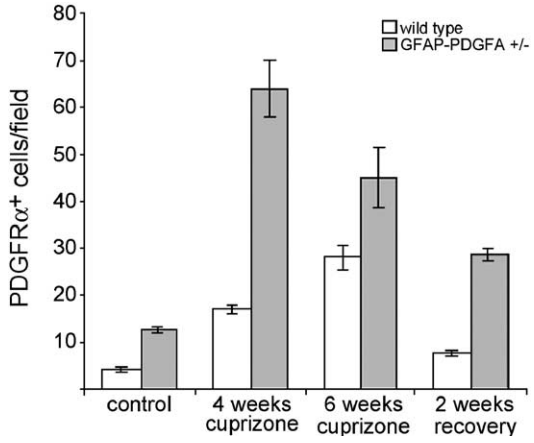

Fig. 6. Comparison of the densities of *PDGFRα*$^+$ cells in the cuprizone model of demyelination–remyelination in wild-type mice and *GFAP-PDGF-A* transgenic mice.

ment (Calver et al., 1998; Van Heyningen et al., 2001). Note that an increased level of *PDGF-A* gene expression, though it leads to an increased amount of *PDGF-A* mRNA and therefore an increased rate of production of PDGF-A protein, does not necessarily lead to an increased concentration or amount of PDGF-A protein at steady state. This is because the PDGF receptor-bearing cells simply proliferate until they consume PDGF (by receptor binding and internalization) as fast as it is produced by neighboring cells. At steady state, the increased rate of PDGF production supports a greater number of cells, but the extracellular PDGF concentration, hence cell cycle time (and BrdU labelling index), is expected to be the same as in wild-type tissue (see Fig. 7; also Calver et al., 1998; Van Heyningen et al., 2001). We have been consistently unable to detect PDGF-A protein in wild-type or transgenic tissue by Western blot presumably because the PDGF protein is normally secreted and turned over rapidly. However, we could easily detect the product of an engineered PDGF-A transgene that carries an endoplasmic reticulum (ER) retention signal at its carboxy-terminus either by immunocytochemistry (Calver et al., 1998) or Western blot (M.F. unpublished data). This ER-retained variant did not increase OPC numbers, demonstrating that secretion of transgene-derived PDGF is required for the OPC phenotype to develop (Calver et al., 1998; Van Heyningen et al., 2001). Moreover, the number of OPCs that developed in *PDGF-A* transgenics with different gene copy numbers was directly proportional to the level of *PDGF-A* mRNA expression—a result that is very difficult to explain other than by altered rates of supply of PDGF protein (Calver et al., 1998; Van Heyningen et al., 2001). Thus, we believe beyond reasonable doubt that the rate of supply of PDGF-A protein is increased in our transgenic mice, even though we have been unable to demonstrate this by direct means.

We visualized OPCs by in situ hybridization for PDGFRα, the cognate receptor for PDGF-A. Some mature or immature neurons can also express PDGFRα. However, because the lesions we analyzed were confined to the white matter, expression in neurons does not alter the interpretation of our experiments. Moreover, although under the conditions of our in situ procedures, we have not seen evidence of this in the postnatal spinal cord. It is possible that *PDGFRα*-positive OPCs might have a greater lineage potential than has generally been realized—it might even correspond to adult stem cells (Belachew et al., 2003; Kondo and Raff, 2000)—but this would not materially affect the conclusions of this study.

We addressed the question of whether PDGF-A is a component of the mitogenic signalling milieu that causes the robust proliferative response of OPCs that normally occurs in response to demyelination (Carroll and Jennings, 1994; Carroll et al., 1998; Cenci di Bello et al., 1999; Keirstead et al., 1998; Levine and Reynolds, 1999; Redwine and Armstrong, 1998; Reynolds and Wilkin, 1993; Sim et al., 2002; Watanabe et al., 2002). Overexpression of PDGF-A in *GFAP-PDGF-A* transgenic mice resulted in a significant increase in OPC density compared to wild-type mice in two models of demyelination–remyelination that have different mechanisms and rates of oligodendrocyte pathology: (1) focal injection of lysolecithin into the white matter of the spinal cord and (2) systemic cuprizone intoxication, which at low doses results in demyelination in the corpus callosum. In the lysolecithin model, we did not find a proportionally greater increase in OPCs in *GFAP-PDGF-A* mice following demyelination. This unpredicted result might reflect the manner in which we express our data, which is likely to underrepresent the scale of the increase in the number of OPCs in *GFAP-PDGF-A* lesions. This is because the increase in *PDGF-A* expression is not uniform across the lesion but is concentrated at the lesion edge where *GFAP* upregulation (hence *hPDGF-A* transgene expression) is highest. Because we averaged the OPC density across the whole area of the lesion, this approach underestimates the real increase in OPC density at the edge, where PDGF-A is most abundant. In addition, OPC density in transgenic mice might have

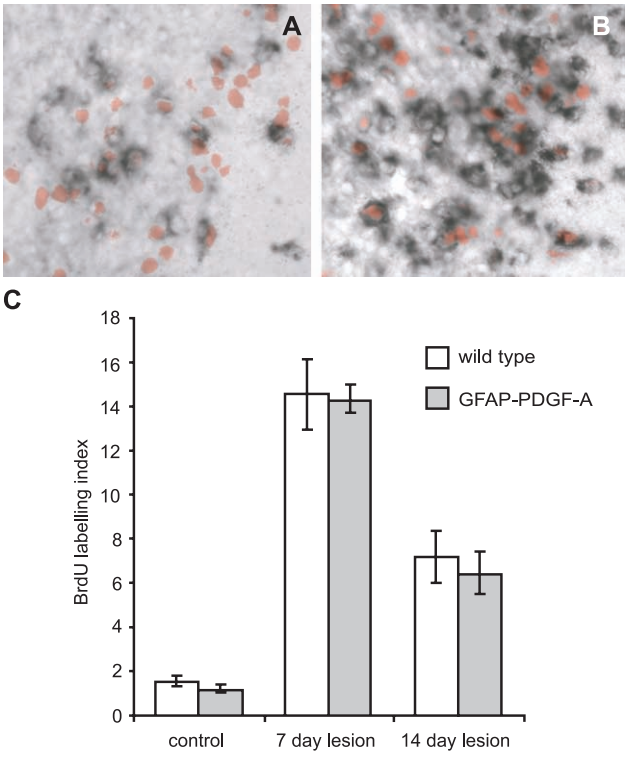

Fig. 7. BrdU$^+$ (red) and *PDGFRα*$^+$ cells in wild-type (A) and *GFAP-PDGF-A* mice (B). Coexpressing cells are identified by superimposing images from sections exposed to BrdU immunocytochemistry and *PDGFRα* in situ hybridization (see Experimental methods). There is no difference in the labelling index (number of BrdU$^+$ cells/number of *PDGFRα*$^+$ cells) between wild-type and *GFAP-PDGF-A* mice following lysolecithin-induced demyelination in the dorsal funiculus of the spinal cord (C).

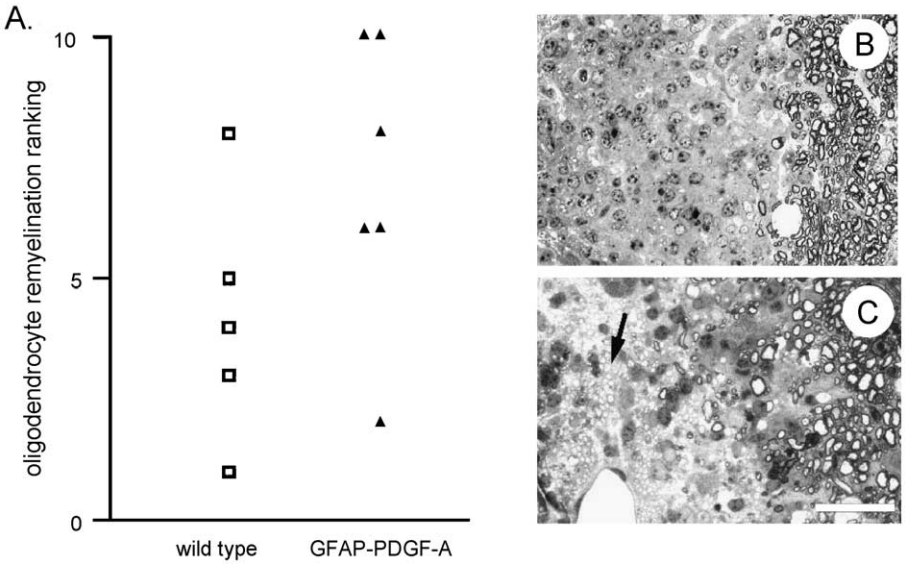

Fig. 8. Remyelination in wild-type and *GFAP-PDGF-A* mice 14 days after lysolecithin-induced demyelination in the dorsal funiculus. (A) Ranking analysis, in which the degree of oligodendrocyte remyelination within the lesion was ranked according to histological criteria (see Experimental methods) with the best remyelination having the lowest rank. There was no significant difference between the two groups according to Mann–Whitney analysis. In contrast to wild-type mice where at 14 days the lesions were characterized by demyelinated axons and thinly remyelinated axons (C), it was often possible to identify areas within the lesion in *GFAP-PDGF-A* mice that were replete with densely packed cells of uniform appearance (B). Within such areas remyelination was rarely observed.

been underestimated due to the technical difficulty of counting closely packed cells. These effects at least partly explain why the increase in OPCs following lysolecithin-induced demyelination seems less in *GFAP-PDGF-A* lesions (8-fold over unlesioned areas) compared to wild-type lesions (15-fold over unlesioned areas).

In the cuprizone model, however, where the transgene is strongly expressed throughout the demyelinating corpus callosum, the OPC density after 4 weeks of cuprizone intoxication is increased to a greater extent in *GFAP-PDGF-A* mice compared to wild-type mice (5- and 4-fold, respectively). Overall, therefore, we believe that our data are consistent with PDGF-A acting as a mitogen for OPCs in demyelinated lesions. Formal proof of this would require conditional knockout of the *PDGF-A* gene in adult mice before lesion induction since germ line *PDGF-A* knockouts die before or shortly after birth (e.g., Fruttiger et al., 1999). The genetic strains necessary for conditional knockout of *PDGF-A* are not yet available. In any case, it is likely that PDGF-A is only one of many mitogenic factors that are active within the acute inflammatory demyelinating environment. FGF-2, for example, is also mitogenic for cultured OPCs (Engel and Wolswijk, 1996; Wolswijk and Noble, 1992) and is expressed following experimental demyelination (Hinks and Franklin, 1999; Messersmith et al., 2000). However, the OPC response to demyelination is not impaired by the absence of FGF-2 (Armstrong et al., 2002), presumably due to compensation by other mitogens, such as PDGF, in a system that is likely to exhibit considerable redundancy.

Rate of remyelination following toxin-induced demyelination is not influenced by increasing OPC density

In general, most toxin-based models of experimental demyelination in rodents undergo complete remyelination, albeit with varying efficiency. This is in contrast to MS in humans where the initial capacity for remyelination is eventually exhausted and chronic demyelination sets in. For this reason, attempts to find ways to enhance remyelination in these models have relied on altering the rate of remyelination—a more subtle alteration than the objective in naturally occurring disease, which is to enhance the final extent of remyelination (Pavelko et al., 1998). One approach that has elicited interest is to use growth factors that are normally involved in myelination as therapeutic agents to stimulate remyelination in clinical disease. We have looked at the effect of increasing PDGF-A expression and OPC number on remyelination of lysolecithin lesions in older adult mice (22–35 weeks), where the time course of repair is slower than in younger adults (Shields et al., 1999; Sim et al., 2002). At the survival times chosen, our results do not show any difference in the rate of remyelination in hemizygous *GFAP-PDGF-A* mice compared to wild types. Indeed, in areas of transgenic lesions that were highly cellular (due, at least in part, to increased OPC density), remyelination tended to be less advanced than wild types, where the degree of cellularity was less, suggesting that prolonged exposure to a mitogen-rich environment may even delay remyelination by extending the recruitment phase of repair. In general, our results indicate that the provision of OPCs is not rate limiting for remyelination under our experimental conditions. This was perhaps predictable because, even in lesioned wild-type mice, more OPCs are produced than are required to generate enough oligodendrocytes to repair small lysolecithin-induced areas of demyelination. In an earlier study, we demonstrated that as animals age there is an impairment of OPC recruitment and differentiation during remyelination, and speculated that both might contribute to the age-related decrease in the rate of remyelination (Sim et al., 2002). The results of the experiments described here indicate that OPC recruitment is less of a bottle-

neck than the delay in differentiation. This conclusion is at odds with a recent report suggesting that exogenous delivery of PDGF-AA protein to lysolecithin lesions created in the corpus callosum of young rats has a beneficial effect on remyelination (Allamargot et al., 2001). We find this result difficult to reconcile with our own observations but may reflect differences in the OPC dynamics following lysolecithin injection into the corpus callosum compared with other white matter regions within the CNS. Alternatively, there might be some inherent disadvantage to starting with the higher basal OPC density present in the unlesioned *GFAP-PDGF-A* transgenics.

Does PDGF have a role in promoting remyelination in multiple sclerosis (MS)? While our results provide no evidence that PDGF-A can enhance the rate or extent of remyelination in mice, this might simply reflect the small size of demyelinated lesions in mouse models compared to human MS patients. Even in rodent models in which OPCs are killed within the area of demyelination, repopulation of the lesion with OPCs might not be a rate-limiting step (Sim et al., 2002). However, the combination of large lesions and the extent of OPC loss found in MS might present greater demands on OPC recruitment (Blakemore et al., 2002; Franklin, 2002). It is conceivable, therefore, that despite our negative results in mice, exogenous PDGF might be beneficial in MS by aiding recruitment of OPCs through its mitogenic and or migration-enhancing activities (Armstrong et al., 1990; Milner et al., 1997). The presence of OPCs does not by itself guarantee successful remyelination unless they are able to differentiate into myelin-forming oligodendrocytes. Several recent studies suggest that this might be a problem because long-term demyelinated lesions can be found that contain OPCs and premyelinating oligodendrocytes that are apparently unable to differentiate further (Chang et al., 2000, 2002; Wolswijk, 1998). Such lesions are unlikely to benefit from OPC mitogens but might conceivably respond to agents that stimulate differentiation or that block endogenous inhibitors of differentiation (Charles et al., 2002; John et al., 2002). So far it has proved difficult to stimulate OPC differentiation in animal models (O'Leary et al., 2002). Another potential difficulty is that while PDGF is mitogenic for rodent OPCs, the same might not be true of human OPCs (Armstrong et al., 1992; Scolding et al., 1995). Nevertheless, our data provide clear evidence that OPC numbers can be manipulated during remyelination by artificially increasing levels of mitogenic growth factor, establishing an important principle relevant to remyelination-enhancing therapies.

Experimental methods

Transgenic mice

The *GFAP-PDGF-A* transgenic mice have been described before (Fruttiger et al., 2000). The transgene encodes the "short" alternative-splice isoform of human PDGF-A (hPDGF-A) with a Myc tag attached to its carboxy terminus under the control of the *GFAP* promoter (Fruttiger et al., 2000). Hemizygous *GFAP-PDGF-A* mice and their wild-type littermates, bred on a C57Bl6J/cba background, were genotyped by Southern blot analysis of tail DNA as described previously (Fruttiger et al., 2000). Homozygous *GFAP-PDGF-A* mice exhibited scoliosis (deformity of the spinal cord) and died within the first few postnatal weeks.

Surgical procedure and injection of lysolecithin for spinal cord lesions

Adult wild-type and transgenic mice (aged 22–35 weeks) were anaesthetized with a mixture of halothane and oxygen. The surface of the spinal cord was exposed by dissecting through the inter-arcuate ligament joining adjacent vertebrae in the caudal thoracic region (T11–T12). The underlying dura was incised close to the midline, and a small hole was made in the pia to allow the intraspinal injection of 1 μl of 1% (w/v) solution of lysolecithin (lysophosphatidyl choline, Sigma) in sterile PBS. The injection was made approximately 0.5 mm beneath the pial surface using a Hamilton syringe secured in a micromanipulator with a glass micropipette attached to its tip. Before suturing the skin wound, the point of injection was marked by a suture placed in the epaxial musculature. The mice were allowed to recover for 1–5 weeks before perfusion and tissue processing for in situ hybridization or morphological analysis (see below).

Demyelination of the corpus callosum by cuprizone intoxication

The method used for the induction of demyelination by feeding cuprizone has been previously described (Morell et al., 2000). Hemizygous *GFAP-PDGF-A* mice and wild-type littermates (aged 8–9 weeks) were placed on a diet of milled chow containing 0.2% (w/w) cuprizone (*bis*-cyclohexanone oxaldihydrazone, Sigma) for 4–6 weeks before perfusion and tissue processing for in situ hybridization (see below). Additional animals were placed on the cuprizone diet for 6 weeks and then allowed to recover on a normal diet for 2 weeks before perfusion. Age-matched control animals were maintained on normal laboratory chow.

Tissue preparation and in situ hybridization

Mice were perfused via the left ventricle with 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) pH 7.4 under terminal anesthesia. Spinal cords were removed from mice that had received an intraspinal injection of lysolecithin, having first marked the point of injection with Indian ink. The spinal cords of age-matched controls were processed in the same way. The brains were removed from mice that had been treated with cuprizone and from age-matched controls. Tissue was postfixed overnight in 4% PFA at 4°C, cryoprotected at 4°C in 20% (w/v) sucrose in PBS for 24 or 48 h (spinal cord or brain, respectively) and embedded in OCT (Raymond Lamb). Spinal cords were bisected at the point of injection and the two halves of the lesion were embedded side by side. Cryosections (20 μm) were collected onto Vectabond-coated slides (Vecta laboratories). Our in situ hybridization protocol was as described before (Sun et al., 1998), except that we omitted proteinase K digestion before hybridization. The mouse *PDGFRα* and *PDGF-A* probes have been described by Van Heyningen et al. (2001) (the *PDGF-A* probe is 687 bp, not 68 bp, as stated there). The mouse *GFAP* probe was a gift from James Cohen (King's College London). RNA polymerases were from Promega and the digoxigenin (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 (BDH) was included in the final color reaction.

Combined BrdU immunolabelling and in situ hybridization

Mice were injected intraperitoneally with BrdU (10 mg/ml in PBS) at a dose rate of 50 μg/g body weight 2 h before perfusion fixation. Following in situ hybridization for *PDGFRα*, the color reaction was stopped by washing in PBS for 10 min at room temperature (RT). The sections were fixed in 70% (v/v) ethanol for 30 min at −20°C and then permeabilized by treatment for 15 min at room temperature with 1% (v/v) Triton X-100 in PBS. Treatment of sections with 6 M HCl and 0.1 M $Na_2B_4O_7$ (Sun et al., 1998) was omitted, as these steps were found to be unnecessary. The sections were then incubated overnight at 4°C with anti-BrdU antibody (monoclonal BU209; Magaud et al., 1989), washed in PBS and then incubated for 1 h at room temperature with a rhodamine-conjugated goat anti-mouse secondary antibody. Sections were mounted in Citifluor (City University, London) for microscopy. Labelled cells were counted on a fluorescence microscope using a 40× objective and the labelling index (LI) (the fraction of *PDGFRα*$^+$ cells that was also BrdU positive) was calculated. To calculate LI within spinal cord lysolecithin lesions, a minimum of nine fields per lesion, which were taken from at least three sections and contained a minimum of 300 *PDGFRα*+ cells, were counted. For controls, where the *PDGFRα*$^+$ cells were more sparsely distributed, at least 300 *PDGFRα*$^+$ cells in the white matter were counted per animal. In situ hybridization images were captured using a video camera under bright field and BrdU-labelling under fluorescence illumination. Images were superimposed using Adobe software.

Quantification of PDFGRα$^+$ *cells*

Numbers of *PDGFRα*$^+$ cells were counted in transverse sections taken through the spinal cords of unlesioned hemizygous *GFAP-PDGF-A* mice and their wild-type littermates using image analysis software (Simple PCI). Previous studies had confirmed that data obtained using this method were closely similar to those obtained from manual counts. Spinal cord sections were examined using a low power objective (2.5×) and a digital image was captured using a video camera. The cross-sectional area of the section in the captured image was delineated by tracing around its edge. *PDGFRα*$^+$ cells within the area of interest were highlighted using a threshold tool and their spatial density calculated. Measurements were taken from five sections per animal and the mean was calculated. When the density of cells was too high to mark individual cells with the threshold tool, *PDGFRα*$^+$ cells were counted manually with the aid of an eyepiece graticule. For lysolecithin lesions, the mean density of *PDGFRα*$^+$ cells per lesion cross-section was calculated since the cells were unevenly distributed within lesions. The total number of *PDGFRα*$^+$ cells per lesion cross-section was determined by counting adjacent, non-overlapping fields under a 20× objective. The lesion cross-sectional area was measured using a captured image including the whole lesion cross-section. The boundary between the demyelinated lesion and normal white matter was identified by examining adjacent sections stained with Sudan black (see below). In control mice, the density of *PDGFRα*$^+$ cells in the dorsal funiculus (where the lesions were located) was calculated. Five sections per animal were analyzed and the mean density of *PDGFRα*$^+$ cells per lesion cross-section was calculated for each. In contrast to lysolecithin lesions in the spinal cord, *PDGFRα*$^+$ cells were evenly distributed within areas of demyelination–remyelination in the corpus callosum following cuprizone intoxication. In this instance, relative changes in the density of *PDGFRα*$^+$ cells within the corpus callosum were determined by counting the number of cells per field using the 63× objective. Two fields, adjacent to the midline, were counted per section and five sections were counted from each animal. For all experiments, three or four animals were analyzed per group and the results were expressed as the mean ± standard error of the mean (SEM).

Sudan black histochemistry

The histochemical stain Sudan black was used to stain myelin in cryosections. Sections were fixed for 1 min in 70% ethanol before immersion in Sudan black for 1 h. Excess stain was removed by washing in 70% ethanol before mounting sections in Citifluor.

Tissue preparation for morphological analysis of spinal cord lesions

Mice were perfused with 4% gluteraldehyde in phosphate buffer (pH 7.4) via the left ventricle under terminal anesthesia. The spinal cords were removed and postfixed in 4% gluteraldehyde for at least 5 h, after which they were cut into eight 1-mm coronal blocks around the site of injection. The tissue blocks were washed with phosphate buffer, postfixed with osmium tetroxide, dehydrated through increasing concentrations of ethanol and embedded in TAAB resin. Semi-thin (1 μm) sections were cut onto plain glass slides and stained with alkaline toluidine blue.

Morphological analysis of remyelination

Sections were cut from each lesion-containing block, stained with toluidine blue and examined by light microscopy. To establish whether the extent of oligodendrocyte remyelination in lysolecithin lesions differed at 14 days between the wild-type and *GFAP-PDGF-A* mice a ranking analysis was performed. In this method, the highest rank was given to the animal exhibiting the highest proportion of oligodendrocyte remyelination. If it was not possible to differentiate two animals using this method, then they were given the same rank. In this method, no attempt was made to assign a value to the proportion of remyelination, but simply to establish how a section ranks relative to others. Statistical comparisons were made using a nonparametric test (Mann–Whitney test, 5% confidence interval).

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