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REGULAR PAPER

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Survival and mitosis of myelinating oligodendrocytes in experimental autoimmune encephalomyelitis: an immunocytochemical study with Rip antibody

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Abstract The fate of myelin-forming oligodendrocytes in the spinal cord of Lewis rats with acute and chronic relapsing experimental autoimmune encephalomyelitis (EAE) was studied using the pre-embedding immunolabelling technique with the Rip monoclonal antibody which specifically labels the cytoplasm of the cell body and processes of the mature oligodendrocyte. Morphologically normal Rip-positive (Rip^+) cells were found in close contact with demyelinated axons at the onset of demyelination and during the course of disease, indicating that oligodendrocytes survive the acute demyelinating insult. Occasional Rip^+ oligodendrocytes were undergoing mitosis at the time of onset of neurological signs. These mitotic oligodendrocytes were present in both the grey and white matter. The majority of the mitotic oligodendrocytes had processes in direct contact with myelin sheaths for considerable lengths, indicating that they were myelinating cells. This study indicates that oligodendrocytes survive the acute demyelinating insult in EAE and that mature myelinating oligodendrocytes are able to undergo mitosis.

Key words Experimental autoimmune encephalomyelitis · Demyelination · Mitosis · Oligodendrocyte · Rip antibody

Introduction

The fate of oligodendrocytes, the myelin-forming cells in the central nervous system (CNS), in inflammatory demyelinating disorders such as multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) is an interesting and controversial issue. Previous studies have reached different conclusions on whether the myelin sheath or the oligodendrocyte is the primary target of the immune response. It is also unclear whether mature myelinating oligodendrocytes are able to proliferate, although it is well established that astrocytes and microglia are able to divide in response to CNS injury.

Although oligodendrocytes have been reported to be present in established demyelinated lesions in EAE [4, 19], it is not known whether these cells represent oligodendrocytes that have survived the acute demyelinating insult or whether they are immigrants from surrounding tissue or newly generated cells. The observed increase in oligodendrocyte numbers in EAE [21, 29] indicates proliferation of cells of the oligodendrocyte lineage. Proliferation of oligodendrocyte progenitors may contribute to this but it is also possible that there is proliferation of mature myelin-forming oligodendrocytes. It has been reported that mature oligodendrocytes can proliferate in demyelinating diseases [1, 8, 28] and after CNS trauma [16, 17]. However, this has been based on the nuclear incorporation of tritiated thymidine which is not specific for mitosis, as it can also occur during DNA repair [35]. Mature oligodendrocytes displaying mitotic figures have not been demonstrated. Furthermore, the definitive identification of oligodendrocytes by standard light and electron microscopy is often difficult because of the morphological similarities between oligodendrocytes and macrophages/microglia [13].

In the present study, we have studied the fate of oligodendrocytes in EAE using the technique of pre-embedding immunolabelling with the Rip monoclonal antibody, which specifically labels the cytoplasm of the cell bodies and processes of myelinating and pro-myelinating oligodendrocytes [3, 5, 7, 10] but does not label oligodendroglial progenitors [30]. Our results indicate that oligodendrocytes survive the acute demyelinating insult in EAE and that mature myelinating oligodendrocytes are able to undergo mitosis.

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Materials and methods

Animals

Male Lewis rats (JC strain), 7–9 weeks old, were obtained from the Central Animal Breeding House of The University of Queensland and from the Animal Services Division of the Australian National University, Canberra, ACT, Australia. They were fed rat and mouse cubes and water ad libitum. The "Principles of laboratory animal care" (NIH publication no. 86-23, revised 1985) were followed, as was the "Australian code of practice for the care and use of animals for scientific purposes" (NHMRC/CSIRO/AAC).

Induction of EAE

Myelin basic protein-induced acute EAE (acute MBP-EAE)

Myelin basic protein (MBP) was prepared from guinea-pig CNS tissue by the method of Deibler et al. [6]. MBP in 0.9% saline was emulsified with an equal volume of incomplete Freund's adjuvant containing 4 mg/ml of added *Mycobacterium butyricum* (Difco, USA). Ten rats were inoculated by the intradermal injection of 0.1 ml of emulsion in the footpad of the right hindfoot. The total dose of MBP was 50 μg/rat. The rats were killed at the onset of neurological signs [9 days post inoculation (DPI)], 2 days after the onset of signs (11 DPI), at the peak of neurological signs (13–14 DPI) and during clinical recovery (15–16 DPI).

Whole spinal cord-induced acute EAE (acute WSC-EAE)

The inoculum was a homogenous mixture of 1 g guinea-pig spinal cord, 1 ml 0.9% saline, 1 ml complete Freund's adjuvant (Difco, USA) and 10 mg *M. tuberculosis* H37RA (Difco, USA). Ten rats were inoculated intradermally with 0.1 ml of inoculum in the footpad of the right hindfoot. The rats were killed 2 days (9 DPI) and 1 day (10 DPI) before the usual time of onset of neurological signs, at the onset of signs (11–12 DPI), 2 days after the onset (13–14 DPI), at the peak of neurological signs (14–15 DPI) and during clinical recovery (16–17 DPI).

Chronic relapsing EAE

Chronic relapsing EAE was induced as previously described [25]. Five rats were inoculated intradermally in the footpad of the right hindfoot with 0.05 ml of the whole spinal cord inoculum. Commencing on the day of inoculation the rats were given subcutaneous injections of cyclosporin A (Sandoz; 4 mg/kg) on alternate days until and including day 22 DPI. Two of the inoculated rats had a chronic persistent course instead of a chronic relapsing one. The rats were killed at 30 and 37 DPI at which times there was severe inflammation and demyelination in the spinal cord [25]. Two normal rats were studied as controls.

Clinical assessment

The rats were examined daily from 7 DPI and also immediately prior to perfusion for histological and immunocytochemical studies. Tail, hindlimb and forelimb weakness were each graded on a scale of 0 (no weakness) to 4 (complete paralysis) [22].

Immunocytochemistry

Under anaesthesia with ketamine, xylazine and atropine, the rats were perfused via the aorta with 0.9% saline followed by 4% paraformaldehyde/0.05% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). Segments of the thoracic, lumbar and sacral spinal cord and portions of the optic nerve and cerebrum were removed and further fixed in the above fixative for a total of 3–4 h before being transferred to 0.01 M PBS. Sections (50 and 100 μm) were cut on a TPI vibratome. The Rip monoclonal antibody specific for oligodendrocytes [7] was provided by Dr. B. Friedman (Regeneron Pharmaceuticals, Tarrytown, N.Y.). Antibody to glial fibrillary acidic protein (GFAP) expressed by astrocytes was obtained from Dako, USA. Mouse monoclonal antibodies against the αβ T cell receptor (αβTCR; R73) and CD11b/c (the type 3 complement receptor expressed by macrophages and microglia; OX42) were provided by Dr. J. Sedgwick, Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia. For both light and electron microscopic immunocytochemistry, a modified protocol of Hsu's avidin/biotin peroxidase [9] perembedding immunolabelling technique [33] was employed as described previously [21] and briefly as follows. After endogenous peroxidase and non-specific binding sites had been blocked with 1% H_2O_2/0.1% sodium azide solution and with normal goat serum (Dako) (1:50 in PBS), respectively, vibratome sections were incubated with primary antibodies overnight. Antibody dilutions were as follows: Rip, 1:2–1:5; R73 and OX42, 1:25; anti-GFAP, 1:100. Sections were washed with PBS and then incubated overnight with biotinylated anti-species immunoglobulin G (Vector, USA), 1:300, containing 1% normal rat serum. After being washed again with PBS, the sections were incubated with an avidin-biotinylated peroxidase complex (Vector, USA). The sections were washed with PBS and then reacted with 3,3′-diaminobenzidine-tetrahydrochloride (Sigma, USA). Reactions were stopped with PBS, and the sections were then washed with saline, re-fixed with modified Karnovsky's fixative (2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3–7.4) and washed in saline again. The sections were then flat-osmicated and flat-embedded [20]. Semithin sections (1 μm) were examined unstained or counterstained with toluidine blue. Ultrathin sections were examined with a Jeol JEM-1200 EXII electron microscope.

Standard light and electron microscopy

Under anaesthesia, the rats were perfused via the aorta with 0.9% saline followed by modified Karnovsky's fixative. Thin slices of the cervical, thoracic, lumbar and sacral spinal cord were removed for epoxy embedding. The same specimens were also taken from rats perfused for immunolabelling, after the tissue required for immunolabelling had been removed; these specimens were immersed in the modified Karnovsky's fixative for at least 24 h before being processed and embedded in epoxy resin. Semithin sections (0.5 μm) were stained with toluidine blue. Ultrathin sections were stained with lead citrate and examined with the electron microscope.

Quantification of oligodendrocytes

Rip^+ oligodendrocytes in semithin sections of the spinal cord were quantified by light microscopy with a 40 × objective and the assistance of a 1 ×1-mm eyepiece grid. Only those Rip^+ cells with visible nuclei were counted.

Results

Standard histological findings

In rats with acute MBP-EAE or acute WSC-EAE, inflammation was evident in the spinal cord, with numerous inflammatory cells in the meninges, perivascular spaces and throughout the parenchyma. Primary demyelination was present from the time of onset of neurological signs and was most evident in the CNS portions of the dorsal root

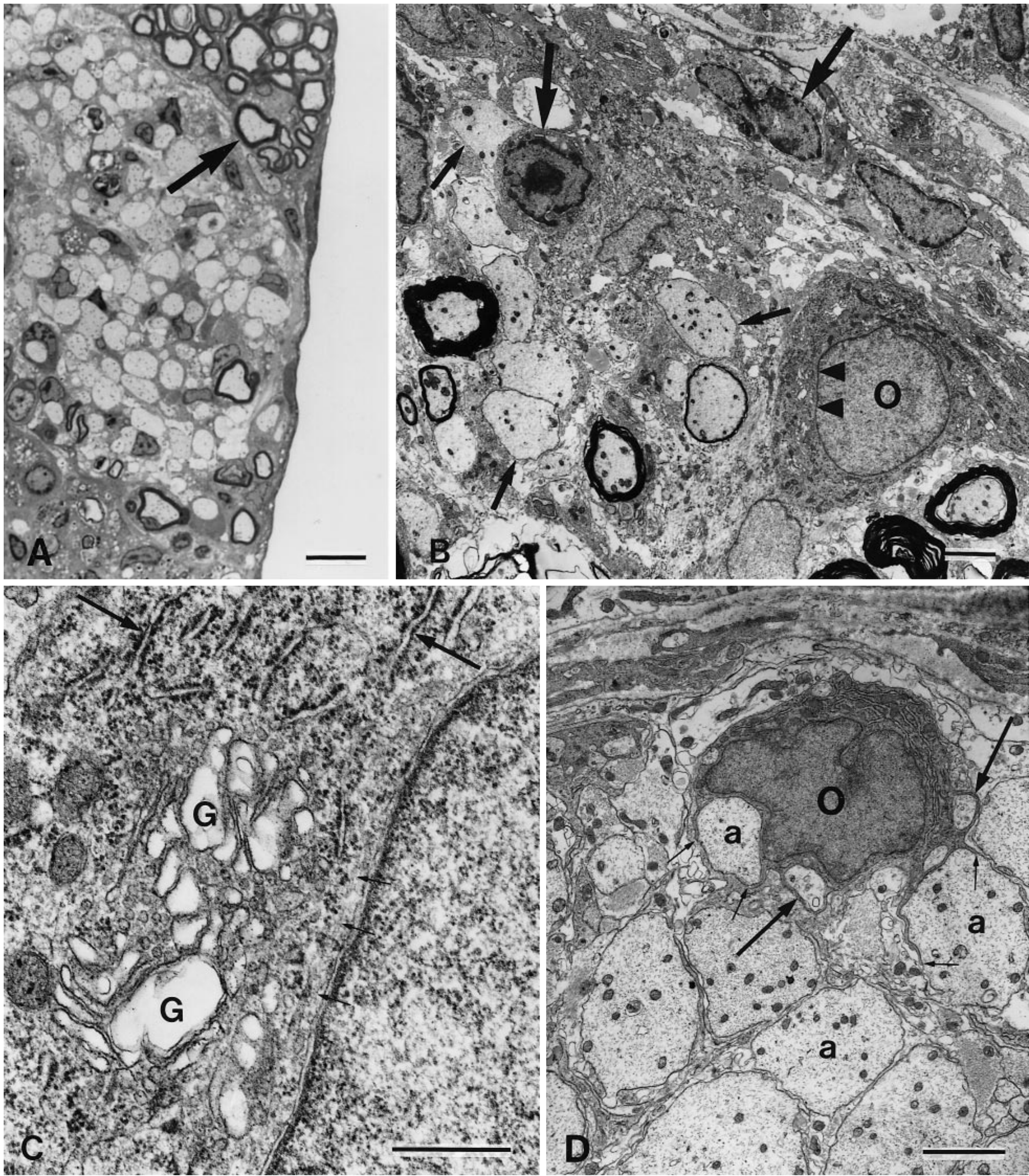
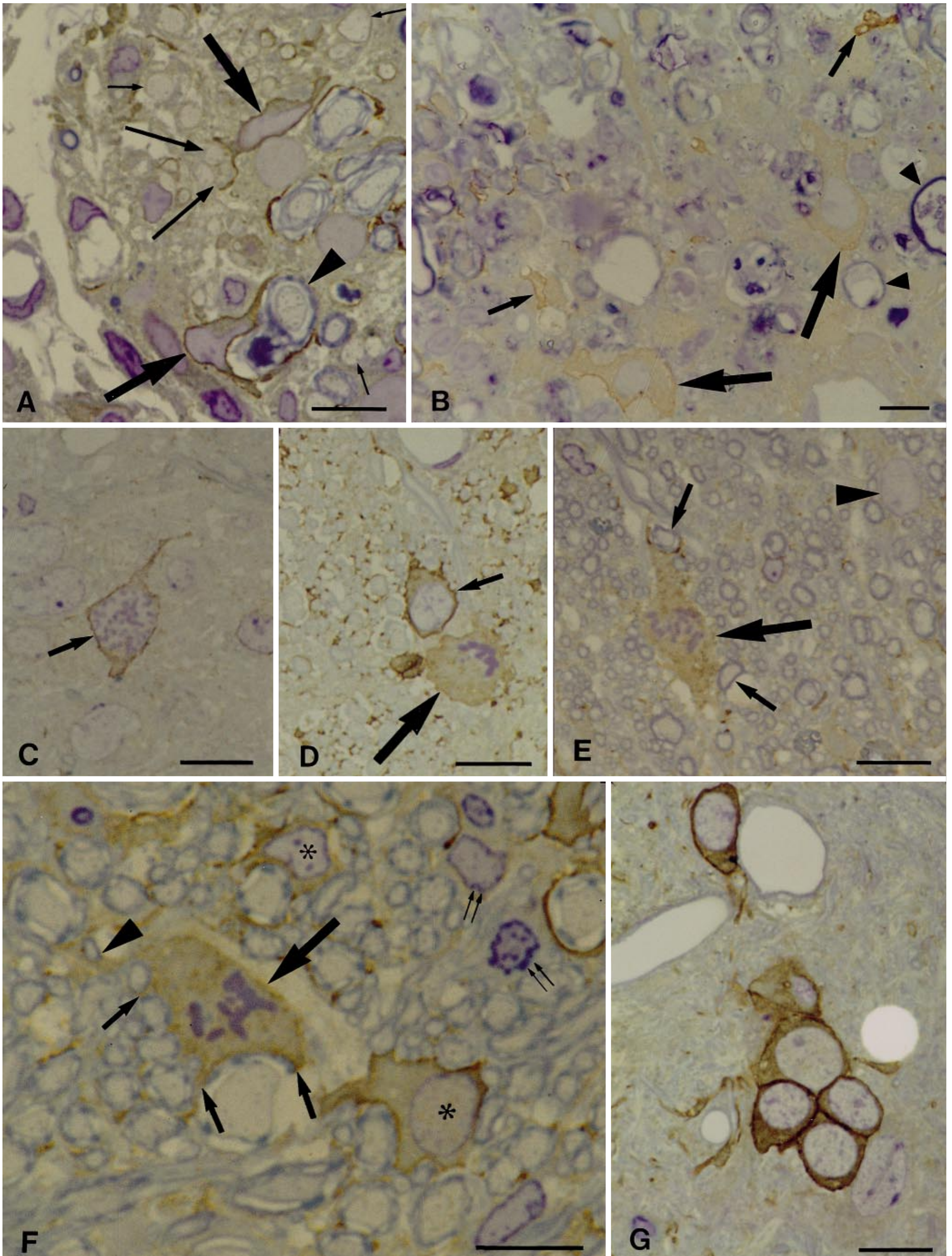


Fig. 1 **A** A lesion in the central tissue projection of the dorsal root entry zone of the sacral spinal cord of a rat with acute WSC-EAE, 3 days after the onset of neurological signs. Almost all the fibres in this area are completely demyelinated. A normal PNS portion of the dorsal root (*arrow*) can be seen. **B** An oligodendrocyte (*O*) is present in a subpial demyelinated lesion in the white matter of the sacral spinal cord of a rat with acute WSC-EAE, on the day of onset of neurological signs. Demyelinated axons (*small arrows*) and macrophages (*large arrows*) can be seen. **C** Higher magnification of the *double-arrowed area* of the oligodendrocyte in **B** showing the characteristic morphological features, namely an organelle-rich cytoplasm containing numerous free ribosomes, prominent rough endoplasmic reticulum (*long arrows*), Golgi apparatus (*G*) and microtubules (*short arrows*). **D** An oligodendrocyte (*O*) with processes that completely embrace (*long arrows*) or partially invest (*short arrows*) demyelinated axons (*a*) in a subpial spinal cord lesion at the peak of neurological signs in a rat with acute WSC-EAE. Note the prominent stringy rough endoplasmic reticulum in the oligodendrocyte cytoplasm, a common morphological feature of the remyelinating oligodendrocyte. **A** Semithin 0.5-μm-thick section stained with toluidine blue, **B–D** electron micrographs (*WSC-EAE* whole spinal cord-induced acute experimental autoimmune encephalomyelitis, *PNS* peripheral nervous system). *Bars* **A** 10 μm; **B**, **D** 2 μm; **C** 0.5 μm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entry and ventral root exit zones (Fig. 1A). Cells with the morphological characteristics of oligodendrocytes, namely a well-demarcated soma with microtubules, abundant granular endoplasmic reticulum, numerous free ribosomes and Golgi apparatus [15, 27], were present in inflammatory demyelinated lesions (Fig. 1B, C). At the time of the peak of neurological signs, oligodendrocyte processes in the lesions embraced the demyelinated fibres (Fig. 1D). Mitotic cells were present in the grey and white matter and in the meninges. In rats with chronic relapsing or chronic persistent EAE, there was severe inflammation and demyelination at all levels of the spinal cord, especially the thoracic level.

Immunocytochemical findings

We used the antibody to the αβTCR in acute WSC-EAE to define the time of onset of the earliest inflammatory lesions so that we could determine whether morphological changes occur in Rip^+ oligodendrocytes prior to the onset of demyelination. Two days before the usual onset of neurological signs of acute WSC-EAE, $\alpha\beta TCR^+$ cells were present in the sacral spinal cord, particularly in the central tissue projection of the dorsal root entry zone and in the adjacent meninges, but there was no evidence of CNS demyelination. These cells constituted the majority of the inflammatory cells at this stage of the disease. At the peak of neurological signs $CD11b/c^+$ cells were present in large numbers at all spinal cord levels in the three EAE models. Mitotic $CD11b/c^+$ cells (5 ± 3/sacral spinal cord transverse section) were seen mostly before the peak of neurological signs. $GFAP^+$ cells with hypertrophied processes and cell bodies were present in rats with neurological signs of EAE but there was no evidence of astrocytic damage. Mitotic $GFAP^+$ cells were occasionally noted.

◀ **Fig. 2** **A** Rip^+ oligodendrocytes (*large arrows*) are present within a demyelinated lesion in the central tissue projection of the dorsal root entry zone of the sacral spinal cord. The processes of one of the oligodendrocytes embrace a fibre being demyelinated (*arrowhead*). The processes of another oligodendrocyte are in direct contact with completely demyelinated axons (*long thin arrows*). Other demyelinated axons (*short arrows*) can also be seen. **B** Rip^+ oligodendrocytes (*large arrows*) and oligodendrocyte processes (*small arrows*) are present within a subpial demyelinated plaque in the thoracic spinal cord. Dilated myelin sheaths (*arrowheads*) of degenerating axons can also be seen. **C** A mitotic Rip^+ cell (*arrow*) at early prophase in the grey matter of the sacral spinal cord. **D** A mitotic Rip^+ cell (*large arrow*) and a resting Rip^+ cell (*small arrow*) are present in the white matter of the thoracic spinal cord. **E** A mitotic Rip^+ cell (*large arrow*) in the white matter of the sacral spinal cord. The processes of the oligodendrocyte are in direct contact with myelin sheaths (*small arrows*). A non-labelled astrocyte (*arrowhead*) is also seen. **F** A mitotic Rip^+ cell (*large arrow*) is in direct contact with myelin sheaths (*small arrows*) in the spinal cord. One of the processes of this cell extends out to a myelinated axon (*arrowhead*). Non-mitotic myelin-forming Rip^+ oligodendrocytes (*asterisks*), which are similarly in direct contact with myelin sheaths, can be seen. Inflammatory cells (*double arrows*) are also present. **G** A cluster of five oligodendrocytes in the grey matter of the sacral spinal cord. Three of the cells label more intensely than the other two, suggesting age differences. Sections, 1 µm thick, counterstained with toluidine blue from rats with acute WSC-EAE on the day of onset of neurological signs (**A**, **C–G**), and from a rat with chronic relapsing EAE, 30 DPI (**B**). *Bars* 10 µm

Table 1 Numbers of Rip^+ oligodendrocytes per mm^2 in the sacral spinal cord. Each result was derived from a single transverse section. Sections were taken from one or two vibratome block(s) of the sacral spinal cord from two normal rats, five rats with acute MBP-EAE (11–16 DPI), three rats with acute WSC-EAE (13–15 DPI) and two rats with chronic persistent EAE (30 DPI). In addition, the means ± SD are given. Analysis of variance revealed that there were no significant differences among the groups (*MBP-EAE* myelin basic protein-induced experimental autoimmune encephalomyelitis, *DPI* days post inoculation, *WSC-EAE* whole spinal cord-induced EAE)

	Normal	Acute MBP-EAE	Acute WSC-EAE	Chronic persistent EAE
	164	208	162	178
	175	174	180	160
	168	162	160	172
	151	159	188	169
		172	174	
		169	169	
		178		
		175		
Mean ± SD	165 ± 10	175 ± 15	172 ± 10	170 ± 6

Rip^+ cells were observed at the sites of maximal demyelination, including the severely affected central tissue projection of the dorsal root entry zone, at all stages of acute EAE from the time of initial $\alpha\beta TCR^+$ cell infiltration until after the development of established demyelination. Within these regions they could be seen contacting partially or completely demyelinated fibres (Fig. 2A). In chronic relapsing EAE, Rip^+ cells were commonly observed within recently demyelinated areas containing myelin-laden macrophages (Fig. 2B). There were slight increases in the mean numbers of Rip^+ cells in transverse sections of the sacral spinal cords of rats with EAE compared to normal controls (Table 1), although the differences were not statistically significant. In the two acute EAE models, studied at the onset of neurological signs, occasional (about one cell per two sacral spinal cord transverse sections) Rip^+ cells in the grey matter (Fig. 2C) and the white matter (Fig. 2D–F) showed the characteristic morphological features of mitosis. The majority of these mitotic Rip^+ cells had processes in direct contact with myelin sheaths for considerable lengths, indicating that they were myelinating oligodendrocytes (Fig. 2E, F). These mitotic cells were scattered throughout the cross-sectional area of the spinal cord, and were sometimes located at a distance from the sites of demyelination. Mitotic Rip^+ cells were not observed at later stages of acute EAE or in rats with chronic relapsing EAE. Pairs of Rip^+ cells, but not larger clusters, were found in the spinal cords of the normal rats; however, the number of pairs and larger clusters (up to five cells) (Fig. 2G) of Rip^+ cells was higher in rats with acute EAE, as previously reported [21].

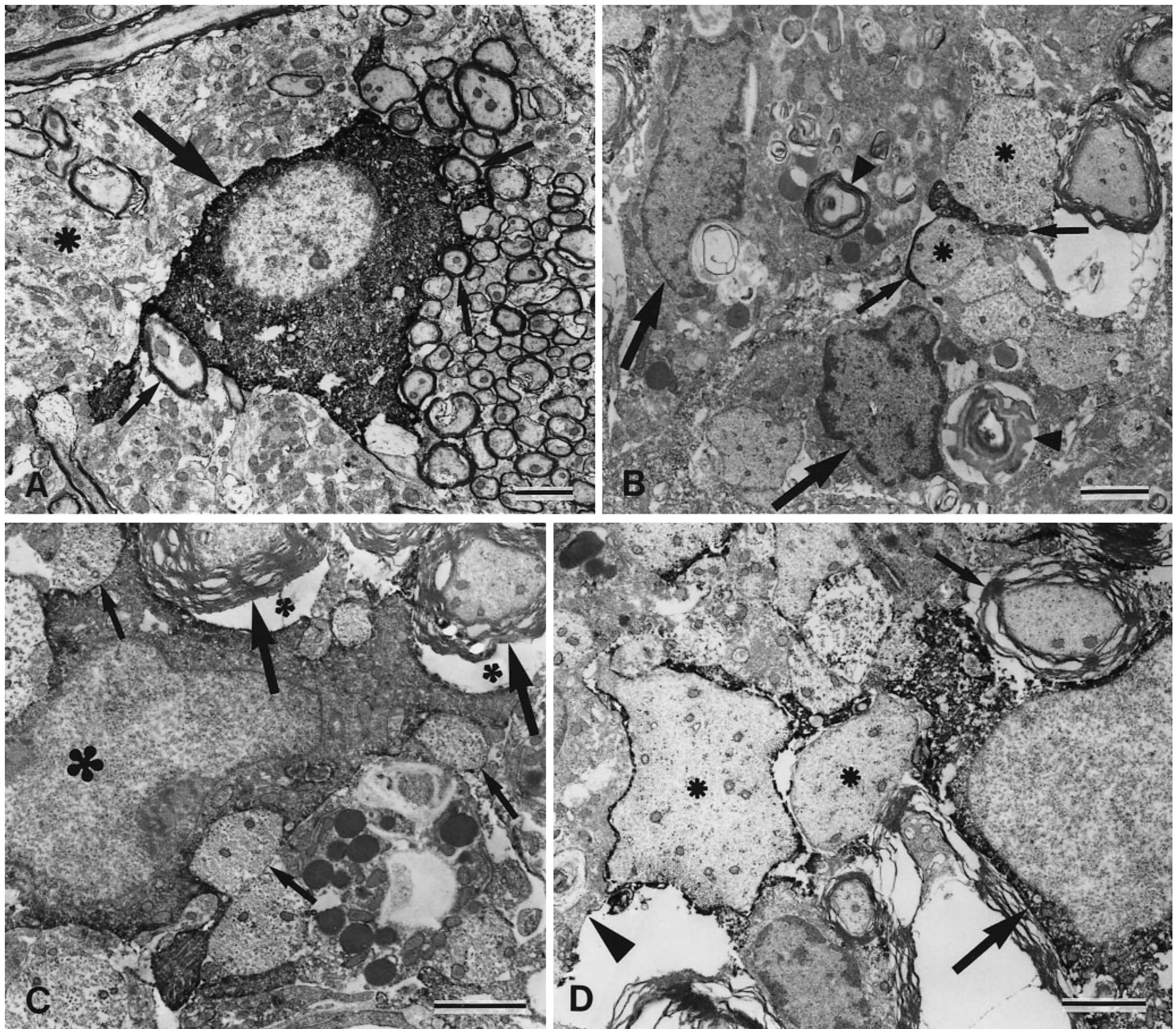

Fig. 3 A–D Electron micrographs of sections labelled with Rip antibody. **A** An oligodendrocyte (*large arrow*) at the junction of normal white and grey matter (*asterisk*). The oligodendrocyte cell body and its processes are in direct contact with many myelin sheaths (*small arrows*), indicating that this is a myelin-forming oligodendrocyte. **B** Labelled oligodendrocyte processes (*small arrows*) are in direct contact with demyelinated axons (*asterisks*) in an active demyelinating lesion. Macrophages (*large arrows*) containing myelin debris (*arrowheads*) are seen nearby. **C** An oligodendrocyte (*large asterisk*) with its labelled cell body and processes in direct contact with both demyelinated axons (*small arrows*) and normal-appearing myelinated fibres (*large arrows*). The gaps (*small asterisks*) between the myelin sheaths and the oligodendrocyte are processing artefacts. **D** An oligodendrocyte (*arrow*) with its processes in direct contact with demyelinated axons (*asterisks*) and a normal-appearing myelinated fibre (*small arrow*). A macrophage (*arrowhead*) containing myelin debris can be seen. All sections are from rats with acute WSC-EAE on the day of onset of neurological signs. *Bars* 2 μm

These clusters were encountered more often in the grey than in the white matter. Mitotic Rip$^+$ or CD11b/c$^+$ cells were not seen in the normal rats.

Using electron microscopy Rip$^+$ myelinating oligodendrocytes and their processes could be seen in direct contact with myelin sheaths in normal regions of the spinal cord (Fig. 3A). It was estimated that more than 95% of the single Rip$^+$ cells in the normal spinal cord, including the perineuronal satellite cells, were in close contact with at least one myelinated fibre, albeit often very small. In the early lesions of acute EAE, Rip$^+$ cells were found in direct contact with demyelinated axons (Fig. 3B–D). Some Rip$^+$ cells directly contacted both demyelinated and normal myelinated fibres (Fig. 3C, D). The majority of the mitotic cells were CD11b/c$^+$ and had the typical round or oval shape and rugged processes of macrophages/microglia (Fig. 4A), in contrast to the smooth outline of the less frequent mitotic Rip$^+$ cells (Fig. 4B–D). Some of the mitotic Rip$^+$ cells in the grey matter had no demonstrable contact

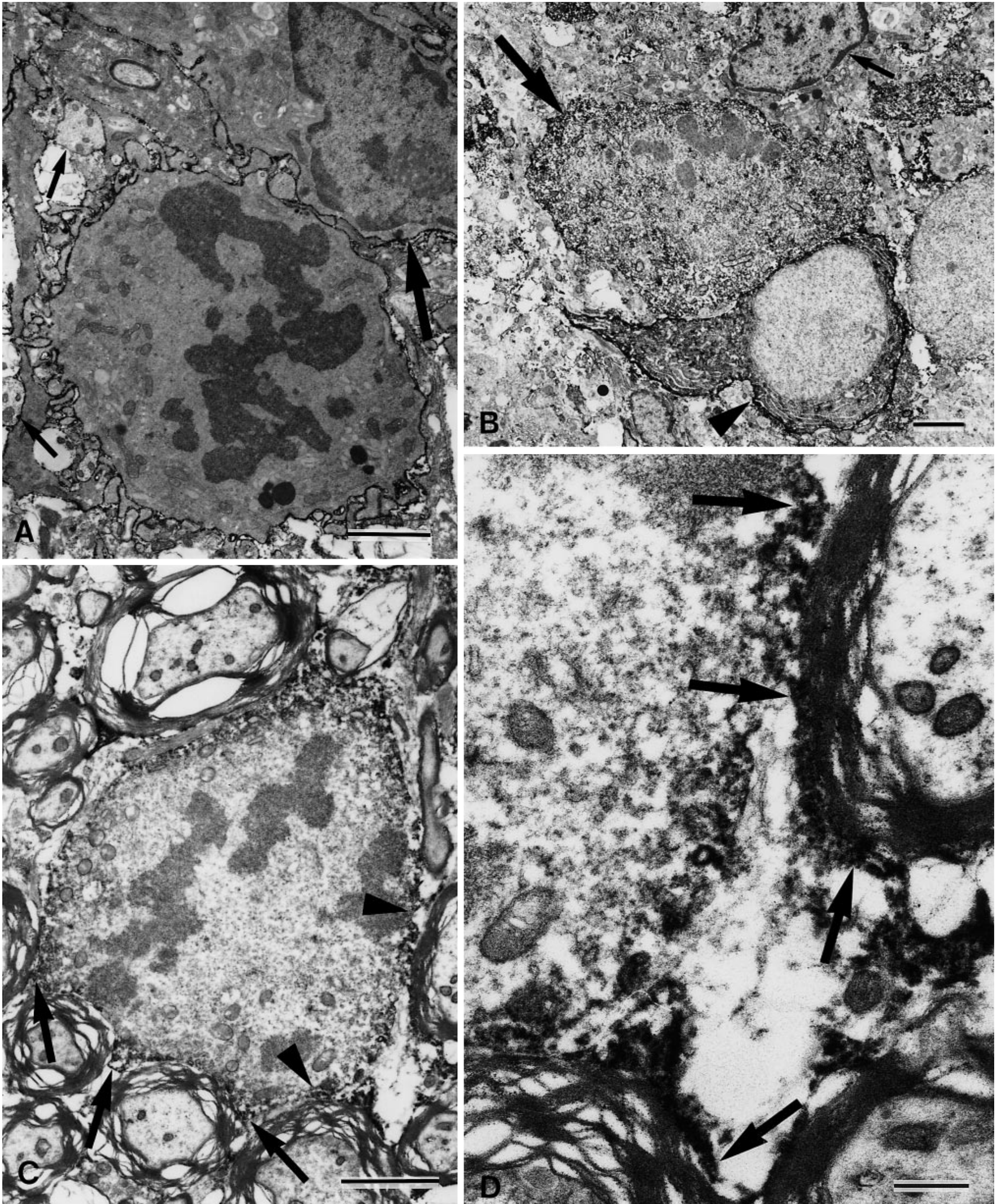

Fig. 4 **A** A mitotic CD11b/c$^+$ cell with rugged processes is present in the spinal cord. A resting CD11b/c$^+$ macrophage or microglial cell (*large arrow*) and demyelinated axons (*small arrows*) can also be seen. **B** A mitotic Rip$^+$ cell (*large arrow*) at early anaphase lies adjacent to a resting Rip$^+$ cell (*arrowhead*) in the grey matter of the spinal cord. The mitotic cell is less intensely labelled than the resting cell. A non-labelled mononuclear leukocyte (*small arrow*) is also seen. **C** A mitotic Rip$^+$ myelin-forming oligodendrocyte with its cell body and processes in direct contact with normal myelin sheaths (*arrows*) in the spinal cord white matter. **D** Higher magnification of the region in **C** indicated by *arrowheads* showing Rip$^+$ oligodendrocyte processes (*arrows*) in direct contact with myelin sheaths for considerable lengths. **A** Rat with acute myelin basic protein-induced EAE; **B–D** rats with acute WSC-EAE on the day of onset of neurological signs. *Bars* **A–C** 2 µm; **D** 0.5 µm

with myelin sheaths (Fig. 4B) but the majority of mitotic Rip+ cells had processes in direct contact with myelin sheaths for considerable lengths (Fig. 4C, D), indicating that they were myelin-forming oligodendrocytes. Mitotic Rip+ cells usually labelled less intensely than resting Rip+ cells (Fig. 4B). There was no evidence of oligodendrocyte apoptosis or necrosis in any of the three EAE models. We did not find any evidence of dying-back gliopathy as found in other conditions [18, 31].

Discussion

In the present study we have used pre-embedding immunolabelling with the Rip monoclonal antibody, which specifically labels the cytoplasm of mature oligodendrocytes but not oligodendroglial progenitors [30], to study the fate of oligodendrocytes in the CNS in EAE. We have shown that oligodendrocytes survive the acute demyelinating insult in EAE and that mature myelinating oligodendrocytes are able to undergo mitosis. Using the Rip antibody we did not identify any apoptotic oligodendrocytes which, on the basis of standard light and electron microscopy, we have previously suggested might occur in the CNS in EAE in addition to apoptotic mononuclear leukocytes [26].

Oligodendrocytes were observed at the sites of maximal demyelination, including the severely affected central tissue projection of the dorsal root entry zone, at all stages of the disease from the time of the initial αβ T cell infiltration until after the development of established demyelination. The presence of morphologically normal oligodendrocytes in direct contact with fibres in the process of being demyelinated indicates that these cells are surviving myelinating oligodendrocytes rather than newly generated or recruited oligodendrocytes. The presence of surviving oligodendrocytes and the absence of oligodendrocyte apoptosis or necrosis indicate that myelin, not the oligodendrocyte, is the primary target of the immune attack in EAE. The direct contact between individual normal oligodendrocytes and both myelinated and demyelinated fibres (Fig. 3C, D) further supports this conclusion. Nevertheless, it remains possible that a sublethal injury to oligodendrocytes might contribute to the demyelinating process.

Oligodendrocytes isolated from the adult rat are able to undergo mitosis in vitro [34] and mitotic myelin-forming oligodendrocytes have been identified by standard light microscopy in the normal developing mouse CNS [32]. However, it has been unclear whether mature myelin-forming oligodendrocytes are able to proliferate in the adult CNS in vivo. Some studies have reported the incorporation of tritiated thymidine into the nuclei of mature oligodendrocytes [1, 8, 16, 17, 28] as evidence of oligodendrocyte proliferation, but the nuclear incorporation of tritiated thymidine is not specific for mitosis, as it can also occur during DNA repair [35]. The percentages of incorporating cells were very high in some studies [8, 28] despite the absence of mitotic figures.

To our knowledge the present study is the first to describe immunocytochemically identified myelinating oligodendrocytes displaying mitotic features in situ, in the adult CNS. Pre-embedding immunolabelling with the Rip antibody, which specifically labels the cytoplasm of both the oligodendrocyte cell body and its processes [3, 7], revealed that the processes of mitotic oligodendrocytes were in direct contact with myelin sheaths for considerable lengths. Nevertheless, mitosis of mature oligodendrocytes was an infrequently observed event. This is consistent with the statistically insignificant increase in the total number of oligodendrocytes in spinal cord sections. Our finding of mitotic Rip+ oligodendrocytes is in contrast to the results of a recent study which found that Rip+ oligodendrocytes do not incorporate bromodeoxyuridine in rats with CNS demyelination induced after spinal cord irradiation and which concluded that mature oligodendrocytes do not undergo mitosis [11]. The mitosis of myelinating oligodendrocytes is likely to contribute to the formation of the oligodendrocyte clusters observed in the present and a previous study [21]. It may also provide one source of the oligodendrocytes that remyelinate the CNS during clinical recovery from EAE [12, 14, 23–25].

Although the factors inducing the mitosis of mature oligodendrocytes in EAE are as yet undetermined, the occurrence of mitotic oligodendrocytes at some distance from the sites of demyelination and at the onset of neurological signs suggests that soluble factors released by CNS parenchymal cells or inflammatory cells might be responsible. For example, the proliferation of oligodendrocytes could be triggered by T cell-derived cytokines such as tumour necrosis factor, which stimulates the proliferation of many cell types, or interleukin-2, which stimulates oligodendrocyte proliferation [2]. Although the mechanism of demyelination in multiple sclerosis may differ from that in EAE, our finding of survival and mitosis of myelinating oligodendrocytes in EAE may boost the search for factors that facilitate remyelination by increasing the number of myelinating oligodendrocytes.

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