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Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis

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Impaired function/differentiation of progenitor cells might provide an explanation for the limited remyelination observed in the majority of chronic multiple sclerosis lesions. Here, we establish that in the normal adult human CNS, the transcription factors Nkx2.2 and Olig2 are strongly expressed in progenitor cells while mature oligodendrocytes are characterized by low levels of Olig2 or Nkx2.2. *In vitro* studies confirmed the expression of Olig2 in oligodendroglial progenitor cells and mature oligodendrocytes while astrocytes, microglial cells and neurons were negative for Olig2. In early multiple sclerosis lesions, we found Olig2-positive progenitor cells throughout all lesion stages and in periplaque white matter (PPWM). The number of progenitors in PPWM was significantly increased compared with the white matter from controls. In chronic multiple sclerosis lesions progenitor cells were still present, however, in significantly lower numbers than in early multiple sclerosis lesions. A subpopulation of progenitor cells in early multiple sclerosis lesions and PPWM but not in control cases co-expressed NogoA, a marker of mature oligodendrocytes. The co-expression of these two markers suggested that these cells were maturing oligodendrocytes recently recruited from the progenitor pool. In contrast, in chronic multiple sclerosis lesions maturing progenitors were only rarely present. In summary, we provide evidence that a differentiation block of oligodendroglial progenitors is a major determinant of remyelination failure in chronic multiple sclerosis lesions.

Keywords: oligodendroglial progenitors; multiple sclerosis; Olig2; Nkx2.2

Abbreviations: OPCs = oligodendroglial progenitor cells; PPWM = periplaque white matter

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Introduction

Multiple sclerosis is the most frequent demyelinating disease of young adults. Multiple sclerosis lesions are characterized by demyelination, inflammation, axonal loss and reactive gliosis. The myelin maintaining mature oligodendrocytes are a presumed target of the immune mediated demyelinating process in this disease. Oligodendrocyte loss is pronounced in a subset of patients early in the disease process (Brück *et al.*, 1994; Ozawa *et al.*, 1994; Lucchinetti *et al.*, 1999) and may even represent the initial event in lesion formation (Barnett and Prineas, 2004). However, concurrently with these destructive processes, repair mechanisms are initiated in these lesions. Remyelination can be observed in lesions with ongoing demyelinating activity (Prineas *et al.*, 1993; Raine and Wu, 1993). Analyses of chronic multiple sclerosis lesions indicate that although remyelination may be extensive in some of them, it is absent or limited to the plaque border in the majority (Prineas and Connell, 1979; Barkhof *et al.*, 2003; Patrikios *et al.*, 2006; Patani *et al.*, 2007). The underlying cause for this limited remyelination is not known. Possible explanations include a lack of oligodendroglial progenitor cells (OPCs), an impaired differentiation of this cell population and/or disturbed interaction between myelinating cells and axons.

Remyelination of CNS lesions has been shown in animal models to be dependent on the recruitment and

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differentiation of OPCs (Targett et al., 1996; Keirstead and Blakemore, 1997). Increased numbers of OPCs and mature oligodendrocytes have been found in areas of ongoing remyelination in early multiple sclerosis lesions suggesting OPC recruitment and/or differentiation (Raine et al., 1981; Lucchinetti et al., 1999). However, recent publications indicate that the number of OPCs is reduced over time in multiple sclerosis lesions (Chang et al., 2002; Reynolds et al., 2002; Wolswijk 2002; Wilson et al., 2006). In mice repeated episodes of ethidium bromide-induced demyelination with intervening recovery did not affect the extent of remyelination nor the numbers of OPCs (Penderis et al., 2003). In contrast, mice which underwent chronic demyelination over an extended period of time showed progressive depletion of OPCs and lack of remyelination (Mason et al., 2004).

Studies addressing the fate of OPCs in human demyelinating diseases in early versus late disease stages have been limited by the lack of antibodies recognizing these cells in human paraffin embedded tissues as well as the relatively small number of available human tissue specimens from early disease stages. Antibodies frequently used to identify OPCs, such as NG2 and PDGF-receptor (R)α, have until now only successfully been applied to frozen and not human paraffin embedded human tissue sections. Furthermore, NG2 may also label other glial cell populations, such as synantocytes (Butt et al., 2002; Wilson et al., 2006). Additional markers or a combination of markers identifying OPCs in human paraffin embedded tissue sections are therefore desirable.

Olig2 and Nkx2.2 are transcription factors expressed by OPCs and mature oligodendrocytes. Recent studies have revealed the importance of Olig2 for the differentiation of neural progenitor cells into the oligodendroglial lineage (Lignon et al., 2006) while Nkx2.2 regulates the maturation and differentiation of oligodendroglial progenitors (Qi et al., 2001). Overexpression of Olig2 induces differentiation of neural stem cells into mature oligodendrocytes *in vitro* (Copray et al., 2006). Disruption of Olig2 leads to an ablated formation of oligodendrocytes in the spinal cord and lack of NG2 cells (Lu et al., 2002; Lignon et al., 2006). In contrast, loss of Nkx2.2 results in increased numbers of PDGF-Rα-positive oligodendrocytes (Qi et al., 2001). Strong Olig2 or Nkx2.2 signals were observed in OPCs in the spinal cord of adult mice, while mature oligodendrocytes expressed low levels of these two transcription factors (Kitada and Rowitch, 2006).

In this report, we demonstrate that the transcription factors Olig2 and Nkx2.2 identify progenitors in human adult CNS. In early multiple sclerosis lesions, increased numbers of progenitor cells were observed in periplaque white matter (PPWM) compared with white matter from controls. In chronic multiple sclerosis lesions, OPCs were still present, albeit in lower numbers. While about one-third of the progenitor cells in early multiple sclerosis tissue specimens were in the transition to mature oligodendrocytes, only rare individual maturing progenitors were observed in chronic multiple sclerosis lesions—suggesting an impaired ability of OPCs to differentiate over time in multiple sclerosis lesions. Our data provide evidence that a differentiation block of OPCs contributes to limited remyelination observed in chronic multiple sclerosis lesions.

Material and Methods

Tissue samples

We retrospectively investigated brain tissue from 43 multiple sclerosis patients and five control cases. Biopsies ($n = 33$) had been performed for diagnostic reasons to exclude neoplastic or infectious diseases. Informed consent had been obtained from each patient. None of the study authors was involved in decision-making with respect to biopsy. For 18 patients detailed information was available (sex, age, duration between first symptom of index lesions to biopsy) (Table 1). In addition to the biopsy cases, 11 tissue blocks with 12 lesions from 10 autopsy cases were analysed. The disease duration varied between 4 and 34 years (Table 1). Control tissue specimens were derived from six patients who underwent temporal or occipital lobe surgery due to

Table 1 Sex, disease duration and course of patients

Patient	Sex	Disease duration	Disease course at biopsy/autopsy
Biopsies (months)			
1	F	0.39	CIS
2	F	2.96	CIS
3	M	2.86	RR
4	M	1.94	RR
5	F	0.69	RR
6	M	3.42	CIS
7	F	0.23	RR
8	M	0.59	CIS
9	F	1.25	RR
10	F	1.18	RR
11	M	0.3	CIS
12	F	1.15	RR
13	F	1.91	CIS
14	F	11.74	CIS
15	F	1.55	CIS
16	F	1.41	CIS
17	M	0.2	CIS
18	M	1.02	CIS
Autopsies (years)			
1	M	18	SP
2	M	7	SP
3	F	?	No history
4	M	>4	?
5	F	20	?
6	M	34	SP
7	F	16	SP
8	M	32	SP
9	M	16	SP
10	M	11	SP

CIS = clinically isolated syndrome; RR = relapsing-remitting; SP = secondary progressive; m = male, f = female.

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pharmaco-resistant seizures. The tissue specimens of these control patients revealed unspecific changes such as focal microglial activation and reactive astrogliosis. The study was approved by the Ethics Committee of the University of Göttingen.

In vitro studies

Human foetal oligodendrocyte progenitor culture

Human foetal CNS tissue of 16- to 21-week-old embryos was provided by the Human Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY). The studies were approved by the institutional review boards of McGill University and the Albert Einstein Institute. Human foetal OPCs were isolated as previously described (Miron *et al.*, 2008). Briefly, brain tissue was mechanically diced, incubated with 0.25% trypsin (Invitrogen) and 25 μg/ml DNase I (Roche, Laval, QC) at 37°C and washed through a 132 μm nylon mesh (Industrial Fabrics, Minneapolis, MN). OPCs were isolated by immunomagnetic bead selection according to the manufacturer's instructions using A2B5 IgM antibody purified from hybridoma (neat) and rat anti-mouse IgM antibody (Miltenyi Biotech, Auburn, CA). The A2B5+ cell fraction was resuspended in DMEM-F12 supplemented with 1% penicillin–streptomycin, 1% glutamine (all from Invitrogen, Karlsruhe, Germany), N1 supplement (1X; Sigma), thyroid hormone (T3, 2 ng/ml; Sigma-Aldrich, Munich, Germany), basic fibroblast growth factor (bFGF, 20 ng/ml; Sigma-Aldrich, Munich, Germany) and PDGF (20 ng/ml; Sigma-Aldrich) and plated on lysed cultures of human foetal astrocytes for survival purposes. Cultures were maintained at 37°C for 10 days before fixation. Our previous studies have characterized the purity of these cultures (Miron *et al.*, 2007, 2008).

Human adult mature oligodendrocyte culture

Temporal lobe surgical resections from patients treated for non-tumour related intractable epilepsy were obtained in agreement with the guidelines of the Biomedical Ethics Unit of McGill University. Mature oligodendrocytes were isolated as previously described (Yong and Antel, 1992). Tissue was digested with 0.25% trypsin and 25 μg/ml DNase I, mechanically dissociated through a 132 μm nylon mesh and separated on a linear 30% Percoll density gradient (GE Healthcare, Uppsala, Sweden). Myelin and red blood cells were removed and neural cells were plated in uncoated tissue culture flasks overnight in minimal essential culture medium containing 5% foetal calf serum, 1% penicillin–streptomycin, 1% glutamine, 0.1% glucose (all from Invitrogen, Karlsruhe, Germany). Through two rounds of differential adhesion, less adherent cells (including oligodendrocytes) were harvested and plated in poly-L-lysine coated 16 well glass chamber slides at a density of 10^5 cells per well. Adherent cells (microglia) were plated in uncoated plastic eight well chamber slides at a density of 5×10^4 cells/well. The purity of these cultures has been previously characterized (D'Souza *et al.*, 1996; Wosik *et al.*, 2004).

For isolation of oligodendrocyte progenitor cells from these cultures, the less adherent cells were submitted to immunomagnetic bead separation as described earlier.

Flow cytometry

Ex-vivo human A2B5 + and A2B5− foetal brain cells were washed in staining buffer (PBS with 1% FCS and 0.1% sodium azide), incubated with A2B5 antibody (1 : 100) or isotype IgM control (1 : 10, Dako, Glostrup, Denmark), then washed twice with buffer and incubated with goat anti-mouse secondary antibody conjugated to phycoerythrin (BD Biosciences, Mississauga, ON, Canada). Following fixation in 1% formaldehyde, results were acquired using a FACSCalibur (BD Biosciences) and analysed using FlowJo software (Treestar, Ashland, USA). The positive fraction was typically >90% A2B5 +.

Immunocytochemistry

Cells were fixed with 2% paraformaldehyde and permeabilized with cold methanol for myelin marker stains (MAG, MBP), acetic alcohol for the GFAP stain and 0.25% triton X-100 for the βTubIII, NogoA and Olig2 stains. Cells were blocked with HHG (1nM HEPES, 2% horse serum, 10% goat serum, in Hank's balanced salt solution) and primary antibodies were applied for 1 h at 4°C. Mouse monoclonal primary Abs used include A2B5 (1 : 100), O4 (1: 100; Chemicon International, Temecula, CA), GFAP-Alexa-488 (1 : 100; Molecular Probes, Burlington, Canada), βTubIII (1 : 400; Sigma), myelin-associated glycoprotein MAG (1 : 200; Chemicon International, Temecula, CA), NogoA (1 : 1000; generous gift from Dr Martin Schwab, University of Zurich) and myelin basic protein MBP (1 : 500; Sternberger Monoclonals Inc., Lutherville, MD). Rabbit primary antibodies include NogoA (1 : 100; Chemicon International, Temecula, CA) and Olig2 (1 : 50; IBL, Spring Lake Park, Minnesota). Cultures were incubated with the appropriate secondary antibodies for 30 min at 4°C : goat anti-mouse secondary antibody conjugated to Cy3 (1 : 500; Jackson ImmunoResearch, Westgrove, PA) and goat anti-rabbit conjugated to fluorescein isothiocyanate fluorochrome (FITC) (1 : 100; Cedarlane, Burlington, ON, Canada). Hoechst dye was used to label nuclei (*bis*-benzimide, 1 : 1000; Molecular Probes). Control stainings with isotypes corresponding to primary antibodies showed low non-specific staining. Slides were mounted using Fluoromount-G (Southern Biotech, Birmingham, AL). Epifluorescent microscopy (Leica, Wetzlar, Germany) was used to visualize stains using OpenLab imaging software.

Classification of multiple sclerosis lesions

All lesions fulfilled the generally accepted criteria for the diagnosis of multiple sclerosis (Prineas, 1985; Allen, 1991; Lassmann *et al.*, 1998). Demyelinating activity was classified as described in detail earlier (Brück *et al.*, 1995). In this study, we focused on actively demyelinating, demyelinated and remyelinating lesion areas. Actively demyelinating lesion areas (biopsies: $n = 17$, autopsies: $n = 0$) were located at the plaque border, these areas were partially demyelinated and infiltrated by numerous macrophages containing myelin degradation products within their cytoplasm. Demyelinated plaques were infiltrated by macrophages and T cells, but macrophages did not contain myelin degradation products (biopsies: $n = 5$, autopsies: $n = 10$). In remyelinating plaque areas (biopsies: $n = 11$, autopsies: $n = 3$), thin, irregularly formed myelin sheaths were seen. Remyelinating lesions early during disease course were infiltrated by numerous macrophages and T cells while remyelinating plaques from chronic multiple sclerosis are characterized by a very mild inflammatory reaction. PPWM (biopsies: $n = 15$, autopsies: $n = 12$) showed no signs of demyelination.

Immunohistochemistry

Biopsies were performed in different centres all over Germany and sent to the Department of Neuropathology in Göttingen after completion of routine analyses. Specimens were fixed in

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4% paraformaldehyde and embedded in paraffin. Autopsy cases were derived from a collection of multiple sclerosis autopsies collected at the Montreal Neurological Institute, McGill University, Montreal, Canada. Biopsy and autopsy tissues were cut in 4 µm thick sections that were stained with haematoxylin and eosin, Luxol-fast blue and Bielschowsky's silver impregnation. Immunohistochemical staining was performed with an avidin-biotin technique. After deparaffinization intrinsic peroxidase activity was blocked by incubation with 5% H_2O_2 in PBS for 20 min. Non-specific antibody binding was inhibited with 10% FCS in PBS for 25 min. For biopsies microwave pre-treatment for better antigen retrieval was performed for mouse anti-Nogo-A, rabbit anti-Nogo-A, rabbit anti-Olig2, rat-anti-CD3 and mouse anti-Nkx2.2. Autopsy cases were pre-treated with Triton X (1% for 1 h) prior to incubation with anti-Olig2 and anti-NogoA antibodies. The primary antibodies were rabbit anti-myelin basic protein (1 : 1000) (Boehringer Mannheim, Mannheim, Germany), mouse anti-KiM1P (1 : 5000) (H.-J. Radzun, Department of Pathology, University of Göttingen, Germany), rat anti-CD3 (1 : 200) (Serotec, Oxford, UK), rabbit anti-GFAP (1 : 2000) (Dako, Denmark), mouse anti-GFAP (1 : 50) (Dako, Denmark), mouse anti-NeuN (1 : 100) (Chemicon International, Temecula, CA), rabbit anti-Olig2 (1 : 300) (IBL, Spring Lake Park, Minnesota), mouse anti-Nkx2.2 (1 : 100) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa), rabbit anti-Nogo-A (1 : 750) (Chemicon International, Temecula, CA) and mouse anti-Nogo-A (1 : 15 000) (11c7, a generous gift from M.E. Schwab, Brain Research Institute, University of Zürich and Department of Biology, Swiss Federal Institute of Technology Zürich, Switzerland). Secondary antibodies were anti-mouse, anti-rabbit or anti-rat biotinylated Ig (1 : 200) (Amersham Biosciences, Freiburg, Germany). For double stainings, we combined the rabbit anti-Olig2 or mouse anti-Nkx2.2 antibodies with the mouse or rabbit anti-NogoA antibody, mouse or rabbit anti-GFAP, mouse anti-NeuN or mouse anti-KiM1P. Secondary antibodies were conjugated to either Cy2 or Cy3 (1 : 200) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

Morphometry and statistics

The number of Nogo-A, Olig2 and Nkx2.2-positive cells stained with the corresponding antibodies was determined in at least 10 standardized microscopic fields of 10 000 µm each defined by an ocular morphometric grid. In the text and figures, the mean number of cells/mm^2 ± SEM is given. The numbers of weakly and strongly positive Olig2- and Nkx2.2-positive cells in controls were determined by two independent observers. The intensity of the Olig2 and Nkx2.2 signals in selected control and multiple sclerosis cases was measured using the Image J program confirming that the strongly and weakly Olig2 and Nkx2.2 cells were indeed two distinct cell populations. For statistical analysis, Student *t*-tests were performed. All tests were classified as significant if the *P*-value was <0.05. The GraphPad PRISM™ software was used (Graph Pad Software, Inc., San Diego, CA, USA) for these analyses.

Results

Expression of Olig2 and Nkx2.2 in adult human CNS tissue

In adult mouse CNS tissues Olig2 and Nkx2.2 are expressed weakly in mature oligodendrocytes and strongly in progenitor cells (Kitada and Rowitch, 2006). To address the question whether Olig2 and Nkx2.2 show a similar expression pattern in adult human CNS tissues, we stained six control biopsies from patients who underwent brain surgery due to pharmaco-resistant seizures with anti-Olig2 and anti-Nkx2.2 antibodies. Olig2 and Nkx2.2-positive cells were found in white matter and cortex (Fig. 1A–D); as expected, significantly higher numbers were found in the white matter (Fig. 1A–D). The majority of cells in the white matter showed a weak nuclear Olig2 (Olig2^{weak} 995 ± 173 cells/mm^2) or Nkx2.2 signal (Nkx2.2^{weak} 968 ± 78 cells/mm^2) (second observer: Olig2^{weak} 924 ± 94 cells/mm^2, Nkx2.2^{weak} 1070 ± 113 cells/mm^2). As expected, the total numbers of weakly labelled Olig2- and Nkx2.2-positive cells in the cortex were lower than in white matter (Olig2^{weak}: 181 ± 24 cells/mm^2; Nkx2.2 weak cells: 228 ± 31 cells/mm^2) (Fig. 1E and F). The distribution pattern of strongly stained Olig2- and Nkx2.2-positive cells showed a similar distribution pattern as described for NG2-positive progenitor cells (Butt *et al*., 2002). In grey and white matter, we found comparable numbers of strongly Olig2- or Nkx2.2-positive cells, respectively (Olig2^{strong} white matter: 46 ± 2 cells/mm^2, Olig2^{strong} cortex: 74 ± 9 cells/mm^2; Nkx2.2^{strong} white matter: 34 ± 7 cells/mm^2, Nkx2.2^{strong}

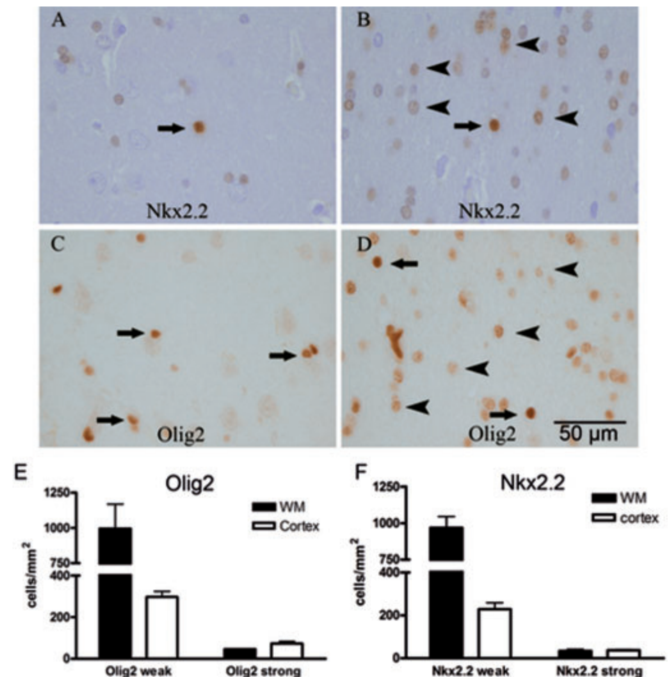

Fig. 1 Expression of Olig2- and Nkx2.2-positive cells in grey and white matter. CNS tissue specimens from controls were stained for Nkx2.2 (**A** and **B**) and Olig2 (**C** and **D**). In grey (**A** and **C**) and white matter (**B** and **D**) weakly (arrowhead) and strongly (arrow) Olig2- and Nkx2.2-positive cells were identified. Quantification revealed that in white matter Olig2^{weak}-positive cells are the dominating cell population while in grey matter similar numbers of Olig2^{strong} and Olig2^{weak} cells are observed (**E**). Nkx2.2^{weak} cells were also predominantly found in white matter (**F**). Scale bars in A–D: 50 µm.

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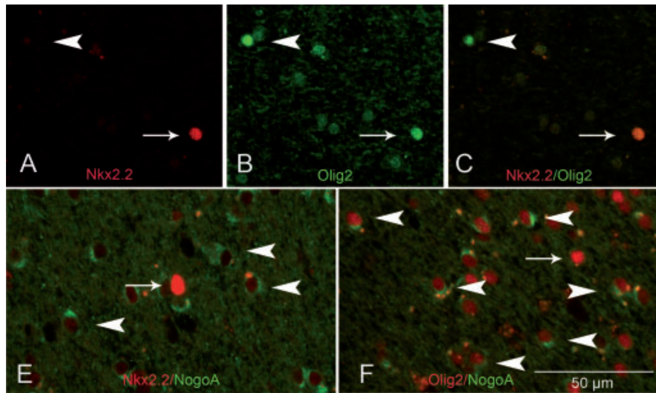

Fig. 2 Olig2^{strong} and Nkx2.2^{strong} cells do not express markers of mature oligodendrocytes. Only a subset of cells expressing high levels of Olig2 also expressed Nkx2.2 [double immuohisto-chemistry for Olig2 (red) and Nkx2.2 (green)] (**A–C**). Cells expressing high levels of Nkx2.2 (arrow) did not co-express NogoA, a marker of mature oligodendrocytes. In contrast, cells expressing low levels of Olig2 also expressed NogoA (arrowhead) [double immunohistochemistry for Nkx2.2 (red) and NogoA (green)] (**E**). Strongly Olig2 expressing progenitors (arrow) were negative for NogoA while mature oligodendrocytes expressed NogoA and low levels of Olig2 (arrowheads) [double immunohistochemistry for Olig2 (red) and NogoA (green)] (**F**). Scale bar in A–E: 50 μm.

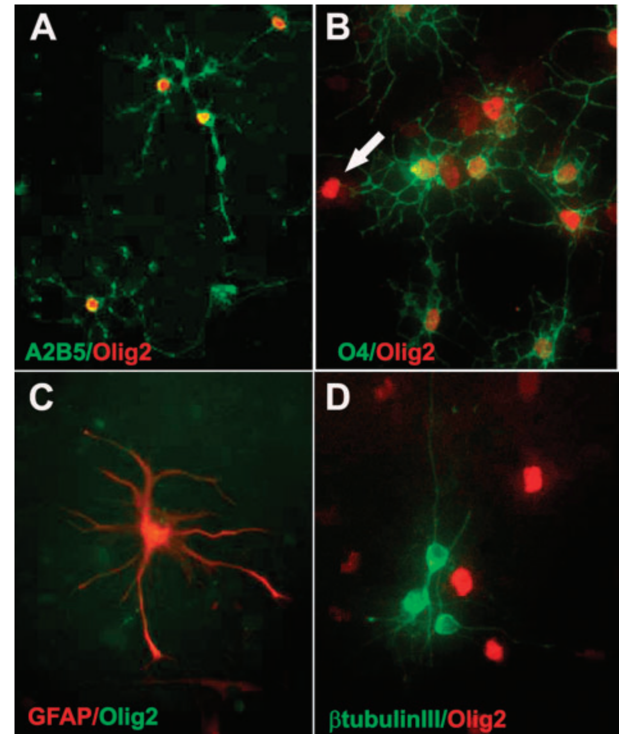

Fig. 3 Foetal oligodendroglial progenitors express Olig2 *in vitro*. Human foetal cells were enriched for A2B5 cells using immunomagnetic bead selection. FACS analysis confirmed the enrichment for A2B5-positive cells. All A2B5 (**A**) and O4-positive cells (**B**) also expressed Olig2. However, there are also Olig2-positive cells, which were negative for O4 (arrow) indicating that these cells are less differentiated oligodendrocyte progenitor cells. GFAP-positive astrocytes (**C**) and βIIITubulin-positive neurons (**D**) which are present in low numbers in foetal cell cultures were negative for Olig2.

cortex: 37 ± 3 cells/mm^2) (second observer Olig2^{strong} white matter: 36 ± 2, Nkx2.2^{strong} white matter: 30 ± 1) (Fig. 1E and F). To clarify whether the same cell populations strongly express Olig2 and Nkx2.2, the appropriate double immunohistochemical staining was conducted. Not all Olig2^{strong} cells also expressed Nkx2.2 strongly (Fig. 2A–C) as expected from our quantitative analysis of the numbers of Nkx2.2^{strong} and Olig2^{strong} cells in white matter and cortex. These findings indicate either the presence of different differentiation stages of progenitors in the human CNS or a heterogeneity among progenitor cells, as suggested for the rodent CNS (Kitada and Rowitch, 2006).

Double immunohistochemistry with anti-Nogo-A, a marker that reliably identifies mature oligodendrocytes in human CNS tissue (Kuhlmann *et al*., 2007), revealed that almost all of the weakly positive Nkx2.2 or Olig2 cells were also Nogo-A positive and were identified as mature oligodendrocytes, therefore (Fig. 2E and F). We also observed a few reactive astrocytes that expressed low levels of Nkx2.2 in the nucleus (data not shown). In contrast, Olig2^{strong} cells were negative for Nogo-A (Fig. 2F) as well as for the astrocytic, neuronal and microglial markers GFAP, NeuN and KiM1P demonstrating that strong nuclear expression of Olig2 identifies selectively progenitor cells in adult human CNS.

Expression of Olig2 in oligodendroglial linage cells *in vitro*

To further confirm that expression of Olig2 is restricted to the oligodendroglial lineage, we studied the expression of Olig2 in cells isolated from human foetal and adult CNS *in vitro*. Foetal CNS derived cells were bead-selected for A2B5 and cultured for 10 days *in vitro*. Flow cytometry (FACS) analysis confirmed that the bead-selected cultures were highly enriched for A2B5-positive cells (data not shown). In these cultures, we found numerous Olig2-positive cells. Co-labelling with two markers of the oligodendroglial lineage (A2B5 and O4) revealed that all A2B5- and O4-positive cells expressed Olig2 (Fig. 3A and B). However, there were also Olig2-positive cells that were negative for O4 (Fig. 3B). NogoA-positive cells were not detected in foetal cell cultures enriched for A2B5-positive cells confirming earlier studies (data not shown) (Ruffini *et al*., 2004). However, we also found single GFAP-positive astrocytes and β-tubulin-III-positive neurons in foetal cell cultures, which did not express Olig2 (Fig. 3C and D). Mature oligodendrocytes that were derived from tissue specimens from adult patients undergoing surgery due to intractable epilepsy expressed GalC, MAG, NogoA as well as Olig2 (Fig. 4A–C). In contrast, microglial cells derived from human adult CNS did not show any Olig2 expression (data not shown).

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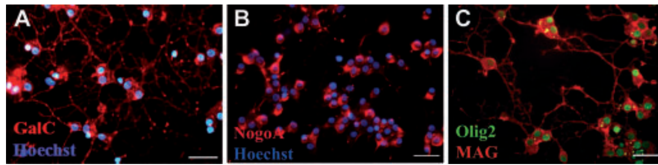

Fig. 4 Mature oligodendrocytes express Olig2 *in vitro*. Mature oligodendrocytes derived from adult human CNS express markers of mature oligodendrocytes, such as GalC (**A**), NogoA (**B**) and MAG (**C**) as well as Olig2 (**C**). Scale bars in A–C: 25 μm.

Progenitor cells in multiple sclerosis

In early multiple sclerosis lesions

Due to the fact that Olig2 recognizes a broader spectrum of progenitor cells than Nkx2.2 in identifying OPCs in adult human CNS, we focussed in the following immunohistochemical studies on Olig2. As in CNS tissue specimens from control cases, we found strongly and weakly labelled Olig2 positive cells in multiple sclerosis lesions (Fig. 5A and B).

We quantified the numbers of Olig2^{strong} cells in active demyelinating and remyelinating lesions as well as in PPWM and compared the results with our findings in white matter from control cases (Fig. 3C). In PPWM from multiple sclerosis patients, a significant increase in the number of Olig2^{strong} cells was observed compared with white matter from control cases (WM 40 ± 1 cells/mm^2, PPWM 133 ± 24 cells/mm^2, $P = 0.024$). In early multiple sclerosis lesions, significantly higher numbers of Olig2^{strong} cells were found in PPWM (133 ± 24 cells/mm^2) compared with active demyelinating (40 ± 10 cells/mm^2, $P = 0.0007$) and demyelinated lesions (44 ± 11 cells/mm^2, $P = 0.048$). To analyse whether the Olig2-positive cells proliferate, we conducted double immunohistochemical stainings for Olig2 and the proliferation marker Ki67 in two multiple sclerosis cases characterized by high numbers of Olig2^{strong} cells. Only rare cells were found to co-express Olig2 and Ki67 (data not shown).

In chronic multiple sclerosis lesions

To address whether progenitor cells are still present in chronic multiple sclerosis lesions, we examined the number of Olig2^{strong} cells in multiple sclerosis lesions derived from 10 patients with a long disease duration (between 4 and 34 years). In all patients Olig2^{strong} cells were found in PPWM (67 ± 13 cells/mm^2) (Fig. 6A). In demyelinated lesion areas, we found significantly lower numbers of strongly Olig2-positive progenitor cells (26 ± 5 cells/mm^2) (Fig. 6B and C). In some demyelinated lesion areas relatively high numbers of Olig2-positive cells were observed despite a complete absence of Nogo-A-positive mature oligodendrocytes. Comparison of the number of Olig2^{strong} cells in early and chronic multiple sclerosis revealed significantly higher numbers of Olig2^{strong} cells in PPWM of early lesions (PPWM early multiple sclerosis 133 ± 24 cells/mm^2, PPWM chronic multiple sclerosis 68 ± 13 cells/mm^2, $P = 0.039$), as well as a trend to a higher number in demyelinated lesions areas (DM early multiple sclerosis 44 ± 11 cells/mm^2, DM chronic multiple sclerosis 26 ± 5 cells/mm^2, $P = 0.058$) in the early disease stage.

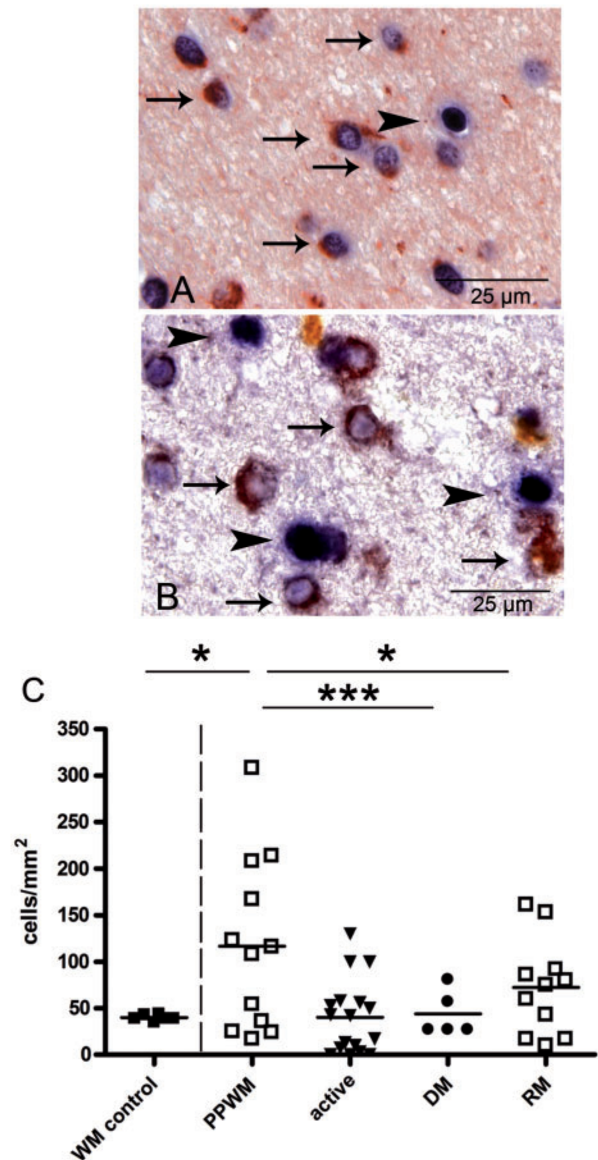

Fig. 5 Progenitor cells in early multiple sclerosis. In white matter from controls without demyelinating diseases, we found progenitors strongly expressing Olig2 (arrowhead) as well as mature oligodendrocytes expressing low levels of Olig2 and NogoA (arrows) [double immunohistochemistry for Olig2 (black) and NogoA (red)] (**A**). In PPWM of multiple sclerosis lesions, we observed increased numbers of Olig2^{strong}-positive progenitors (arrowheads) as well as mature oligodendrocytes expressing NogoA and low levels of Olig2 [double immunohistochemistry for Olig2 (black) and NogoA (red)] (**B**). A quantitative analysis of progenitor cells in early multiple sclerosis revealed significantly increased numbers of Olig2^{strong} cells in PPWM compared with active demyelinating lesions ($P = 0.0007$), demyelinated lesions ($P = 0.048$) and white matter from controls ($P = 0.049$) (**C**). Scale bar in A and B: 25 μm. $^{*}P < 0.05$. $^{***}P < 0.001$.

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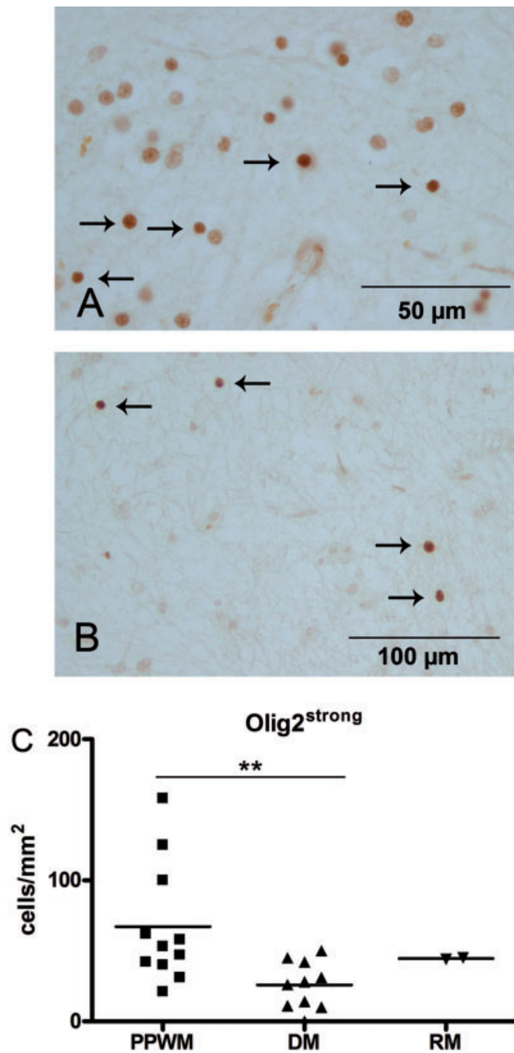

Fig. 6 Progenitor cells in chronic multiple sclerosis lesions. Olig2^{strong} cells were found in PPWM (arrows) (**A**) as well as in demyelinated lesions (arrows) (**B**) [**A** and **B**: immunohistochemistry for Olig2 (brown)]. Highest numbers of Olig2^{strong} cells were found in PPWM in chronic multiple sclerosis lesions (**C**). $^{**}P < 0.01$.

Differentiating oligodendrocyte progenitor cells in multiple sclerosis lesions

In tissue specimens from multiple sclerosis patients, we found a third cell population in addition to mature oligodendrocytes (expressing Nogo-A and low levels of Olig2) and progenitor cells (expressing high levels of Olig2 but no Nogo-A) not observed in control cases: strongly Olig2-positive cells that were also Nogo-A-positive (Olig2^{strong}/Nogo-A) (Fig. 7A).

In early multiple sclerosis tissue specimens, about one-third of Olig2^{strong} cells also expressed Nogo-A. The highest numbers of Olig2^{strong}/NogoA-positive cells were found in PPWM and remyelinating lesions (PPWM 43 ± 11 cells/mm^2, active lesions 19 ± 7 cells/mm^2, demyelinated lesions 17 ± 5, remyelinating lesions 37 ± 10 cells/mm^2) (Fig. 7B).

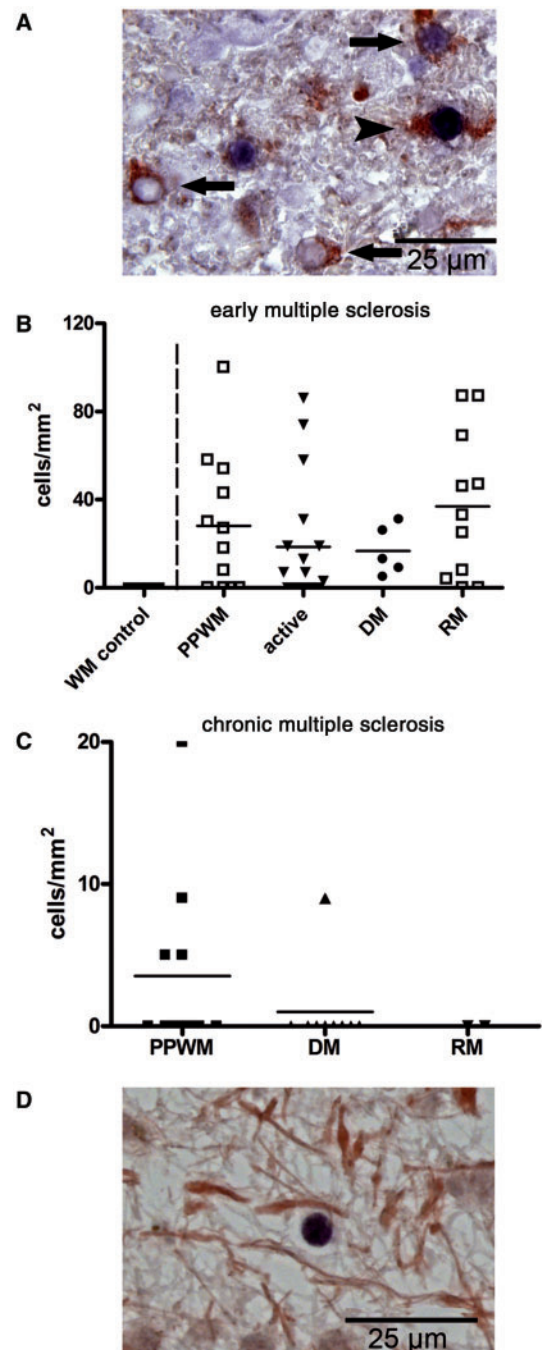

Fig. 7 Maturing OPCs are present in early multiple sclerosis but not in controls. In early multiple sclerosis tissue specimens, we observed a cell population expressing high levels of NogoA and Olig2 (arrowhead) not observed in tissue specimens derived from controls. Mature oligodendrocytes were present as well (arrows) in early multiple sclerosis [double immunohistochemistry for Olig2 (black) and NogoA (red)] (**A**). These maturing progenitor cells were present in PPWM as well as in actively demyelinating, demyelinated and remyelinating lesion areas as revealed by quantitative analysis (**B**). In contrast, in chronic multiple sclerosis Olig2^{strong}/NogoA-positive maturing progenitors were almost completely absent (**C**). In chronic multiple sclerosis lesions Olig2^{strong} cells were found in close proximity to axons [double immunohistochemistry for Olig2 (black) and neurofilament (red)] (**D**). Scale bars in A and D: 25 μm.

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In chronic multiple sclerosis Olig2^{strong}/NogoA-positive maturing progenitor cells were found only occasionally in PPWM or in multiple sclerosis lesions (PPWM: 4 ± 2 cells/mm^2, DM: 1 ± 1 cells/mm^2) as revealed by double-immunohistochemistry (Fig. 7C). To find whether a lack of axons might be responsible for the low numbers of Olig2^{strong}/NogoA-positive cells, we conducted double immunohistochemical stainings for neurofilament and Olig2. Numerous Olig2^{strong} cells were found in close proximity to axons (Fig. 7D).

Discussion

We demonstrate that in adult human CNS, progenitors can be identified by using a combination of antibodies recognizing oligodendroglial transcription factors (Olig2 and Nkx2.2) together with markers of mature oligodendrocytes (NogoA). *In vitro*, Olig2 was detected in OPCs and mature oligodendrocytes, while astrocytes, microglial cells and neurons were negative for Olig2. In early multiple sclerosis lesions, the number of progenitors in PPWM was higher than in white matter from controls. In addition, OPCs undergoing transition to mature oligodendrocytes were observed in early lesions but not in controls or in the majority of chronic multiple sclerosis patients. Our data suggest that a reduction of progenitor cells in combination with a differentiation block contributes to the lack of remyelination in chronic multiple sclerosis lesions.

In control CNS tissue specimens, about 5% of Olig2- and Nkx2.2-positive cells showed strong nuclear immunoreactivity and no co-localization with oligodendroglial, astrocytic, microglial or neuronal markers while the remaining Olig2- and Nkx2.2-positive cells displayed a weak nuclear staining. *In vitro*, we detected Olig2 in OPCs and in mature oligodendrocytes while astrocytes, microglial cells and neurons did not express Olig2. These results demonstrate that Olig2^{strong} and Nkx2.2^{strong} cells in the adult human CNS are progenitor cells as shown for rodent adult CNS (Kitada and Rowitch, 2006). The fact that only a subpopulation of Olig2^{strong} cells also expressed Nkx2.2 at high levels indicates either heterogeneity or the presence of different differentiation stages for progenitor cells in the human CNS as also observed for the rodent CNS (Kitada and Rowitch, 2006). In our study, weakly labelled Olig2- or Nkx2.2-positive cells co-expressed NogoA, a marker for mature oligodendrocytes (Kuhlmann *et al.*, 2007); therefore, we concluded that these cells were mature oligodendrocytes.

In PPWM and in remyelinating early multiple sclerosis lesions, we observed a significantly increased number of strongly Olig2-expressing progenitor cells compared with white matter of control patients. This is in line with earlier reports describing the presence of NG2-, O4- or PDGF-R alpha-positive progenitor cells in multiple sclerosis lesions (Chang *et al.*, 2002; Reynolds *et al.*, 2002; Wolswijk, 2002; Wilson *et al.*, 2006). As observed in early multiple sclerosis lesions, increased numbers of progenitor cells can be found in remyelinating animal models. In mice in which demyelination has been induced by toxins, such as ethidium bromide, increased numbers of OPCs expressing high levels of transcription factors, such as Olig2, Nkx2.2 or Olig1 have been described during remyelination (Arnett *et al.*, 2004; Fancy *et al.*, 2004; Watanabe *et al.*, 2004). Our results indicate that in human demyelinating diseases, similar to the findings in remyelinating animal models, OPCs react to a demyelinating stimulus.

The increased numbers of progenitors expressing high levels of Olig2 in early multiple sclerosis lesions may either be caused by the OPC migration, recruitment from a more immature cell population or proliferation. To analyse whether Olig2-positive cells proliferate in early disease stages, we performed double immunohistochemical staining with the proliferation marker Ki67 in two multiple sclerosis cases with high numbers of Olig2^{strong} cells. In our study we rarely observed proliferating Olig2-positive cells. This is in line with an earlier publication demonstrating that the vast majority of proliferating cells in multiple sclerosis lesions are astrocytes and microglial cells (Schönrock *et al.*, 1998) and may indicate a fundamental difference in the proliferating capacity of OPCs in human and mouse CNS (Levine and Reynolds, 1999).

In early multiple sclerosis lesions, we observed significantly lower numbers of Olig2^{strong} cells in actively demyelinating as well demyelinated lesions compared with PPWM suggesting that either increased cell death or impaired migration of progenitors is the cause for low numbers of progenitors in these lesion areas.

In chronic lesions, we found a significantly reduced number of progenitors in PPWM and a trend to lower numbers of progenitors in demyelinated lesion areas compared with tissue specimens derived from patients with early multiple sclerosis confirming earlier studies (Wolswijk, 2000). However, in 8 out of 10 chronic demyelinated lesions Olig2^{strong}-positive progenitors were still present. These results indicate that the limited remyelination found in chronic multiple sclerosis lesions is not simply caused by a lack of progenitors.

In addition to mature oligodendrocytes and progenitors, we found a subpopulation of oligodendroglial lineage cells co-expressing markers of mature oligodendrocytes (Nogo-A) as well as progenitors (high levels of Olig2) suggesting that these cells are either oligodendroglial cells in transition from OPCs to mature oligodendrocytes or mature oligodendrocytes recently recruited from the progenitor pool. This conclusion is supported by findings from a lysophatidylcholine induced demyelination model in which remyelination was associated with the presence of maturing oligodendrocytes expressing high levels of oligodendroglial transcription factors, such as Nkx2.2 (Watanabe *et al.*, 2004). Together these findings suggest progenitors are present in early multiple sclerosis lesions and have the ability to develop into mature oligodendrocytes. In contrast, despite the presence of progenitor cells in chronic multiple

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sclerosis lesions, we observed few oligodendroglial lineage cells in transition to mature oligodendrocytes in chronic multiple sclerosis lesions suggesting a reduction in the differentiation capability of progenitors over time. There are numerous possible explanations for such a differentiation block in chronic multiple sclerosis lesions. One simple explanation could be the lack of contact between progenitors and axons. However, similar to an earlier study (Chang *et al.*, 2002), we found progenitors closely associated with axons indicating that axons are either not receptive to remyelination or that inhibitory factors might prevent it. In recent studies, it has been shown that factors such as Lingo-1 and PSA-NCAM have the capability to prevent remyelination (Charles *et al.*, 2000, 2002; Mi *et al.*, 2005). However, the lack of certain factors such as IGF1, TGF-β1, GGF2 or integrins might also contribute to limited remyelination in multiple sclerosis lesions (Carson *et al.*, 1993; McKinnon *et al.*, 1993; Blaschuk *et al.*, 2000; Mason *et al.*, 2000). In toxin induced demyelinating animal models, depletion of inflammatory cells or lack of pro-inflammatory cytokines (e.g. IL1β, TNF) results in impaired remyelination indicating that the inflammatory infiltrates create a beneficial environment for remyelination (Arnett *et al.*, 2001; Mason *et al.*, 2001; Bieber *et al.*, 2003; Kotter *et al.*, 2005; Chari *et al.*, 2006; Setzu *et al.*, 2006). One major difference between early and chronic multiple sclerosis lesions is the markedly reduced inflammatory infiltrates suggesting that the altered inflammatory response in chronic multiple sclerosis lesions might contribute to the differentiation block of OPCs and limited extent of remyelination. Further studies are needed to identify the mechanisms responsible for the differentiation block of progenitors in multiple sclerosis.

In summary, we show that two types of Olig2- and Nkx2.2-positive cells are present in non-demyelinating CNS. Mature oligodendrocytes show a weak nuclear Olig2 or Nkx2.2 staining signal while progenitors express high levels of Olig2 and/or Nkx2.2. In early multiple sclerosis lesions, an increased number of progenitors and progenitors in transition to mature oligodendrocytes were observed compared with white matter from controls. In chronic multiple sclerosis lesions progenitors are still present but do not mature to oligodendrocytes. Our data support the hypothesis that a differentiation block of progenitors contributes to failed remyelination in multiple sclerosis. The development of neuroprotective treatments preventing the loss of progenitors and promoting the proliferation and differentiation of this cell population might be a promising treatment strategy for multiple sclerosis.

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