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Neurobiology

Re-Expression of a Developmentally Restricted Potassium Channel in Autoimmune Demyelination

Kv1.4 Is Implicated in Oligodendroglial Proliferation

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Mechanisms of lesion repair in multiple sclerosis are incompletely understood. To some degree, remyelination can occur, associated with an increase of proliferating oligodendroglial cells. Recently, the expression of potassium channels has been implicated in the control of oligodendrocyte precursor cell proliferation *in vitro*. We investigated the expression of Kv1.4 potassium channels in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis, a model of multiple sclerosis. Confocal microscopy revealed expression of Kv1.4 in AN2-positive oligodendrocyte precursor cells and premyelinating oligodendrocytes *in vitro* but neither in mature oligodendrocytes nor in the spinal cords of healthy adult mice. After induction of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis, Kv1.4 immunoreactivity was detected in or around lesions already during disease onset with a peak early and a subsequent decrease in the late phase of the disease. Kv1.4 expression was confined to 2′,3′-cyclic nucleotide 3′-phosphodiesterase-positive oligodendroglial cells, which were actively proliferating and ensheathed naked axons. After a demyelinating episode, the number of Kv1.4 and 2′,3′-cyclic nucleotide 3′-phosphodiesterase double-positive cells was greatly reduced in ciliary neurotrophic factor knockout mice, a model with impaired lesion repair. In summary, the re-expression of an oligodendroglial potassium channel may have a functional implication on oligodendroglial cell cycle progression, thus influencing tissue repair in experimental autoimmune encephalomyelitis and multiple sclerosis. ***(Am J Pathol 2007, 171:589–598; DOI: 10.2353/ajpath.2007.061241)***

Multiple sclerosis (MS) is the most common inflammatory demyelinating disease of the human central nervous system (CNS). Its pathological hallmarks comprise inflammation, gliosis, and, most importantly, axonal damage and demyelination.[1] In MS, lesion repair can take place, although with a high inter- and intraindividual variability.[2] Still, mechanisms of repair are only partially understood, and remyelination of lesions is largely incomplete. In general, less than 40% of new MS lesions remyelinate, giving rise to "shadow plaques."[3,4] To a large extent, inflammatory demyelination is also paralleled by loss of oligodendrocytes (OLs) in the lesions.[5,6] Therefore oligodendroglial repopulation may constitute an important prerequisite for myelin repair. OL precursor cells (OPCs) have been identified in the rodent and human adult CNS.[7] In different experimental models of demyelination, oligodendroglial cells are recruited, proliferate, and can differentiate into mature OLs.[8–12] A substantial number of OPCs are also present around MS lesions.[13,14] However, in chronic MS lesions these cells may be quiescent,[15] or axons are not receptive for remyelination,[16,17] thus limiting sustained myelin repair in the CNS.

Besides previous investigations on glial growth factors,[18,19] recent studies have suggested the re-expression of developmentally regulated oligodendroglial pathways or cytoskeletal proteins during remyelination.[20–22] It is therefore tempting to investigate the role of additional

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molecules implicated in normal oligodendroglial and myelin development during autoimmune demyelination.

Among others, candidates include oligodendroglial ion channels. Potassium channels may play a role in proliferation of different cell types.[23] Inward-rectifying potassium channels are essential for OPC maturation,[24] and the blocking of potassium channels inhibits OPC proliferation.[25] Different studies revealed the importance of the Kv1 family of potassium channels in OPC cell cycle progression *in vitro*. The Kv1.3 and Kv1.4 subunits were implicated in the regulation of OPC proliferation.[26] In particular, the Kv1.4 subunit has been associated with proliferation in pathological states such as spinal cord injury,[27,28] whereas Kv1.3 and 1.5 are expressed in T cells and dendritic cells during autoimmune demyelination.[29,30]

Here, we investigate the expression of Kv1.4 potassium channels in myelin oligodendrocyte glycoprotein peptide 35–55 (MOG 35–55)-induced experimental autoimmune encephalomyelitis, an experimental model mimicking aspects of MS, in particular myelin damage and loss of OLs.[31] Our data reveal a re-expression of this developmentally restricted potassium channel in proliferating premyelinating oligodendroglial cells after autoimmune demyelination and suggest a role for Kv1.4 in oligodendroglial proliferation and remyelination.

Materials and Methods

Cell Culture

Cell culture experiments were performed with primary murine oligodendroglial cells prepared according to established protocols.[32,33] Shaken-off primary OPCs were cultured on poly-L-lysine-coated glass coverslips in SATO medium containing 1% horse serum (Gibco, Eggenstein, Germany), 10 ng/ml platelet-derived growth factor (Sigma, Munich, Germany), and 5 ng/ml basic fibroblast growth factor (Gibco). Reverse transcription-polymerase chain reaction (RT-PCR) experiments were performed by using the OliNeu cell line,[34] either as undifferentiated OPCs or 1 week after differentiation to OLs with 1 mmol/L cAMP (Sigma). OPCs and OLs were stimulated with a supernatant (4%) from concanavalin A-treated Lewis rat spleen cells for 4 or 8 hours, respectively. Alternatively, supernatants of restimulated splenocytes derived from MOG 35–55-immunized C57BL/6 mice were used. In this case, MOG specificity of splenocytes was demonstrated in a [^{3}H]thymidine-based cell proliferation assay (data not shown).

Patch Clamp Recording

Electrophysiological recordings were performed on oligodendroglial progenitors derived from OliNeu and OliNeu-PLP cell lines. Since no specific inhibitors for Kv1.4 are available,[35] we used the lack of sensitivity of Kv1.4 to tetraethylammonium (10 mmol/L) as exclusion criterion. Patch clamp recording and pharmacological blockade were performed as described previously.[36]

Animals

C57BL/6 mice were purchased from Harlan (Borchen, Germany). CNTF$^{-/-}$ mice were generated at Prof. Michael Sendtner's laboratory (Institute of Clinical Neurobiology, Würzburg, Germany) and characterized earlier.[37,38] They were backcrossed onto a C57BL/6 background for at least 10 generations and bred at the in-house animal care facilities at the University of Göttingen. All animal experiments were performed in accordance with the Lower Saxony State regulations for animal welfare.

Induction and Clinical Evaluation of Active MOG-Experimental Autoimmune Encephalomyelitis (EAE)

For active induction of EAE, mice received s.c. injection at flanks and tail base of 200 μg of MOG 35–55 peptide (Charité, Berlin, Germany) in phosphate-buffered saline emulsified in an equal volume of CFA containing *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) at a final concentration of 1 mg/ml. Two injections of pertussis toxin (400 ng/mouse i.p.; List Biochemicals, Campbell, CA) were given at the time of immunization and 48 hours later. Animals were weighed and scored for clinical signs of disease on a daily basis. For labeling of proliferating cells, 5-bromo-2′-deoxyuridine was injected intraperitoneally (150 mg/kg body weight) in a subset of mice on 3 subsequent days before perfusion.

Tissue Processing

On days 9, 13 or 14, 26, 40, and 56 after immunization, mice were anesthetized with Ketanest (Inresa, Freiburg, Germany) and Rompun (Bayer, Leverkusen, Germany) and transcardially perfused with saline followed by a solution of 4% paraformaldehyde. Spinal cords were carefully removed, postfixed in the same fixative, and then incubated overnight in a solution of 15% sucrose in phosphate-buffered saline. Tissue was snap-frozen in methylbutane (−70°C) and embedded in Tissue-Tek (Sakura, Heppenheim, Germany). Sagittal or transversal serial sections (14 μm) were collected on gelatin-coated slides by using a Reichert cryotome (Leica, Bensheim, Germany).

Immunocytochemistry and Immunohistochemistry

For immunocytochemistry, cells were fixed with 4% paraformaldehyde, then permeabilized in 0.1% Triton X-100 (Sigma), and blocked with 0.2% gelatin in phosphate-buffered saline. For immunohistochemistry, sections were blocked with 0.1% Tween 20 and 1% bovine serum albumin in phosphate-buffered saline (Sigma). Primary antibodies were as follows: mouse anti-Kv1.4 antibody (dilution, 1:25; Alomone); mouse anti-glial fibrillary acidic protein (dilution, 1:3000; Sigma); rat anti-AN2 (dilution, 1:25; kindly provided by K.A. Nave, Max-Planck Institute for Experimental Medicine, Göttingen, Germany); mouse

anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (dilution, 1:100; Sigma); mouse anti-myelin associated glycoprotein antibody (dilution, 1:200; kind gift from C. Stadelmann, Institute of Nephrology, Göttingen, Germany); anti-myelin basic protein (dilution, 1:50; Sigma); rat anti-Mac-3 (dilution, 1:200; BD, Heidelberg, Germany); rat anti-CD3 (dilution, 1:200; Serotec, Wiesbaden, Germany); and anti-Nav1.2 (Scn2a, dilution 1:50; Chemicon, Hofheim, Germany) for damaged axons with an altered expression of sodium channels.[39] Proliferating cells were labeled with a rabbit anti-Ki67 (dilution, 1:50; Dako Cytomation, Hamburg, Germany) and a mouse anti-Ki67 antibody (NCL-Ki67-MM1; Novocastra, Newcastle, UK). 5-Bromo-2′-deoxyuridine staining (anti-5-bromo-2′-deoxyuridine antibody, dilution, 1:100; Bioscience, NatuTec, Frankfurt, Germany) was performed after Kv1.4 staining and pretreatment of slices with 0.2 mol/L glycine and 2 N HCl. Labeling of apoptotic OLs was performed as described.[38]

Specificity of staining was confirmed by omitting the primary antibody. After incubation with the appropriate fluorescence-labeled secondary antibody (Invitrogen, Karlsruhe, Germany) at a dilution of 1:2000, sections were mounted with ProLong containing 4,6-diamidino-2-phenylindole (Invitrogen).

Microscopy and Data Analysis

Cells and sections were analyzed by confocal laser scanning microscopy (Leica) using the appropriate laser and AOBS configurations for Cy3, Alexa Fluor 488, 4,6-diamidino-2-phenylindole, and Cy5. Quantification of CNPase- and Kv1.4-positive cells or proliferating CNPase-positive cells was performed on an average of 10 spinal cord cross sections from at least three different mice per experimental group. Digital images (1024 × 1024 pixels) were taken with a 63× objective. Cells were counted manually by a blinded observer from digital images using the plug-in of the ImageJ cell counter (*http://rsb.info.nih.gov/ij/*). Only cells with a continuous positive staining around the whole cell body were included. Statistical analysis was performed by one-way analysis of variance, Dunnett's test, and Student's *t*-test when appropriate (GraphPad Prism; GraphPad Software, Inc., San Diego, CA).

RT-PCR

RT-PCR was performed using protocols as described[40] and primers (Eurogentec S.A., Herstal, Belgium) with the following sequences: 5′-GGACCTTATTATGGCCCTC-AGT; GTCATTGAATATTGTGTGCCC-3′; and TAMRA-5′-AGCAGATTGCTGAATGACACCTCGGCA-3′-FAM.

Results

Kv1.4 Expression Decreases during OPC Differentiation in Vitro

It is well recognized that proliferation and development of cells of the OL lineage *in vitro* depend on functional potassium channels.[41] We therefore investigated the expression of Kv1.4 during development of murine oligodendroglial cells *in vitro* by immunocytochemistry. First, the signal of Kv1.4 appeared in the soma and processes of AN2-positive oligodendroglial cells (Figure 1A). *In vitro*, some of these AN2-positive cells were already CNPase-positive (Figure 1B). Kv1.4 expression was dramatically reduced in mature, myelin basic protein-positive OLs (Figure 1C, myelin basic protein in red; Kv1.4 in green). Similar results were obtained for O1 and other markers of mature OL (not shown).

To test the functional relevance of Kv channels in oligodendroglial cells, patch clamp recording and pharmacological blockade by tetraethylammonium were performed as described previously.[36] Tetraethylammonium (10 mmol/L) inhibited a majority of the outward currents in different oligodendroglial cell lines. The remaining tetraethylammonium-insensitive current did not show inactivation, indicating that Kv1.4 is present as Kv1 heteromultimers in oligodendroglial cells (data not shown; see Supplemental Figure 1 at *http://ajp.amjpathol.org*), which is well in accordance with previous electrophysiological investigations.[36,42]

In summary, Kv1.4 is down-regulated during the differentiation from OPCs to adult OLs *in vitro*. Consistently, Kv1.4-expressing cells were not present in the adult naive murine spinal cord *in vivo* (Figure 2A).

Kv1.4 Immunoreactivity Reappears in Adult Mice during Autoimmune Demyelination

Because Kv1.4 is expressed in AN2-positive cells during development, it may be re-expressed in adult mice after myelin or OL damage. We therefore investigated Kv1.4 expression in MOG 35–55 EAE in C57BL/6 mice, a model disease with OL apoptosis and extensive myelin destruction.[38]

Kv1.4 expression was studied by immunohistochemistry at the onset (day 9 p.i.) and the peak of disease (day 13 p.i.) as well as in the early and late phases of MOG-EAE (days 26, 40, and 56 p.i., respectively). At the peak of disease on day 13 p.i., we already detected a strong signal for Kv1.4 in or around lesioned areas on longitudinal frozen sections of lumbar spinal cord of (Figure 2B). Kv1.4 immunoreactivity was strongest in the soma of positive cells but was also present in cell processes. Kv1.4 immunoreactivity followed the clinical severity of EAE with a maximum of Kv1.4 expression at the peak of disease (day 13 p.i.) and a decline of Kv1.4 immunoreactivity during the later phases of MOG-EAE (see also Figures 4 and 5).

To investigate the direct influence of inflammation on oligodendroglial Kv1.4 expression, OPCs or OLs were stimulated with supernatant (4%) from concanavalin A-treated spleen cells containing a mixture of proinflammatory cytokines. In these experiments, Kv1.4 expression was analyzed by RT-PCR. Stimulation for 4 or 8 hours did not lead to an up-regulation of Kv1.4 mRNA in undifferentiated OPCs or partly differentiated OLs, thus indicating that Kv1.4 expression is not mediated by an unspe-

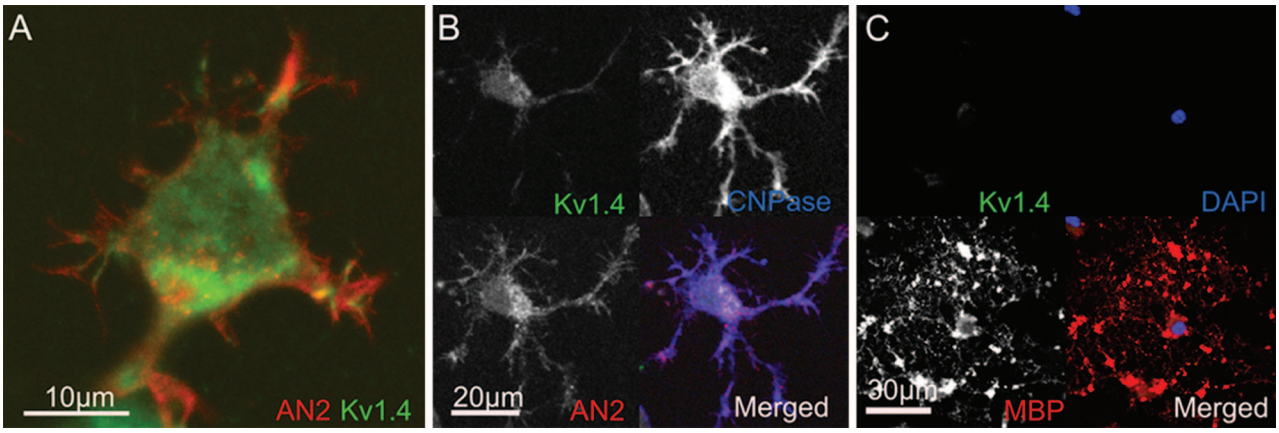

Figure 1. Decrease of Kv1.4 potassium channels during OPC differentiation *in vitro*. **A:** Undifferentiated OPCs are positive for the progenitor marker AN2 (red). These OPCs express high levels of Kv1.4 (green) localized to the soma and short cellular processes. **B:** Two days after induction of morphological differentiation, Kv1.4 is present in cells simultaneously expressing AN2 and CNPase. **C:** Five days later, mature OLs display a complex branched structure expressing markers for mature oligodendrocytes and myelin proteins like myelin basic protein (red). At that time point, Kv1.4 immunoreactivity is reduced to a residual staining (green).

cific stress response (Figure 3). Similar results were obtained after stimulation of OPCs or OLs with splenocyte supernatants from MOG-immunized mice (data not shown; see Supplemental Figure 2 at *http://ajp.amjpathol.org*). In summary, Kv1.4 immunohistochemistry reveals that Kv1.4 is not expressed in healthy spinal cord but reappears during autoimmune demyelination in a process not dependent on proinflammatory cytokines.

Kv1.4 Immunoreactivity during Autoimmune Demyelination Is Confined to CNPase-Positive Oligodendroglial Cells

We next addressed which specific cell types express Kv1.4 during autoimmune demyelination. By morphological criteria, the enlarged cytoplasm of Kv1.4-positive cells was already suggestive of premyelinating oligodendroglial cells.[43] For further clarification, we performed double-label immunohistochemistry for Kv1.4 in combination with specific markers for different resident CNS cells as well as immune cells on day 13 p.i. Cells positive for Kv1.4 were negative for neurofilament, glial fibrillary acidic protein, Mac-3, and CD3. Thus, during MOG-EAE, Kv1.4 is not expressed on neuronal cells, astrocytes, activated microglia, or infiltrating immune cells like T cells or macrophages. However, we found a clear colocalization of Kv1.4 with expression of CNPase, an oligodendroglial enzyme implicated in myelin biosynthesis (Figure 4). All CNPase-positive cell bodies were also Kv1.4-positive. Further staining with other markers of mature OLs revealed that myelin associated glycoprotein-positive but CNPase-negative cells were always Kv1.4-negative (data not shown). Moreover, a very small number of AN2-positive cells were Kv1.4-positive. These cells were also CNPase-positive. In accordance with previous reports,[44] only very few of these CNPase and AN2 double-positive cells were observed.

Because we found a coexpression of Kv1.4 with CNPase, we investigated the distribution of CNPase during the course of MOG-EAE more closely. Within the

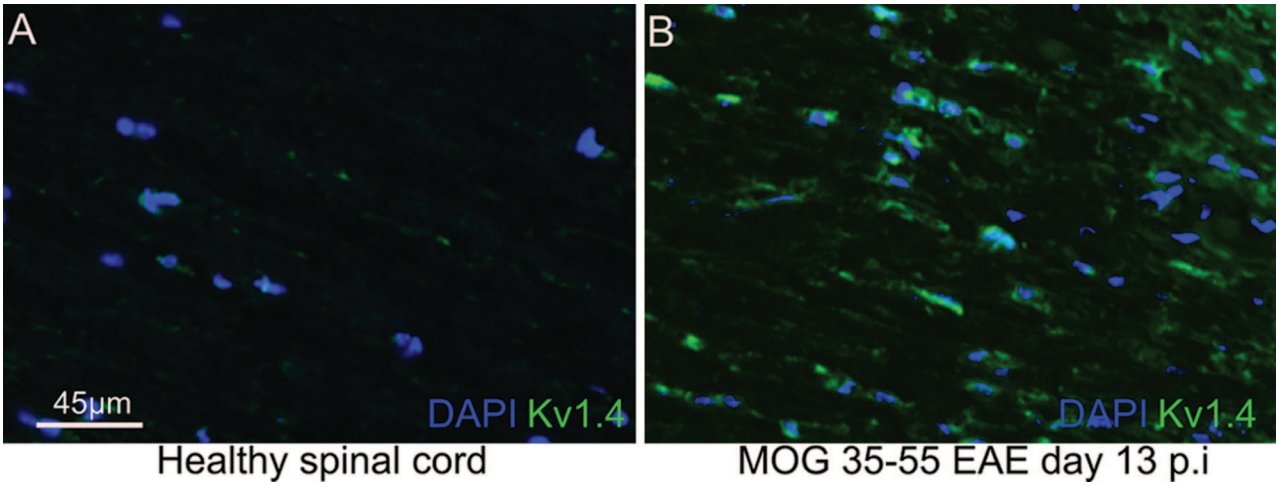

Figure 2. Re-expression of Kv1.4 during autoimmune demyelination. Representative images of longitudinal lumbar spinal cord sections are shown. Nuclei are stained in blue. **A:** No Kv1.4 expression is detected in the spinal cord of a healthy adult control mouse. **B:** During the early phase of MOG-EAE at day 13 p.i., a strong immunoreactivity for Kv1.4 (green) is seen in and around demyelinated lesions.

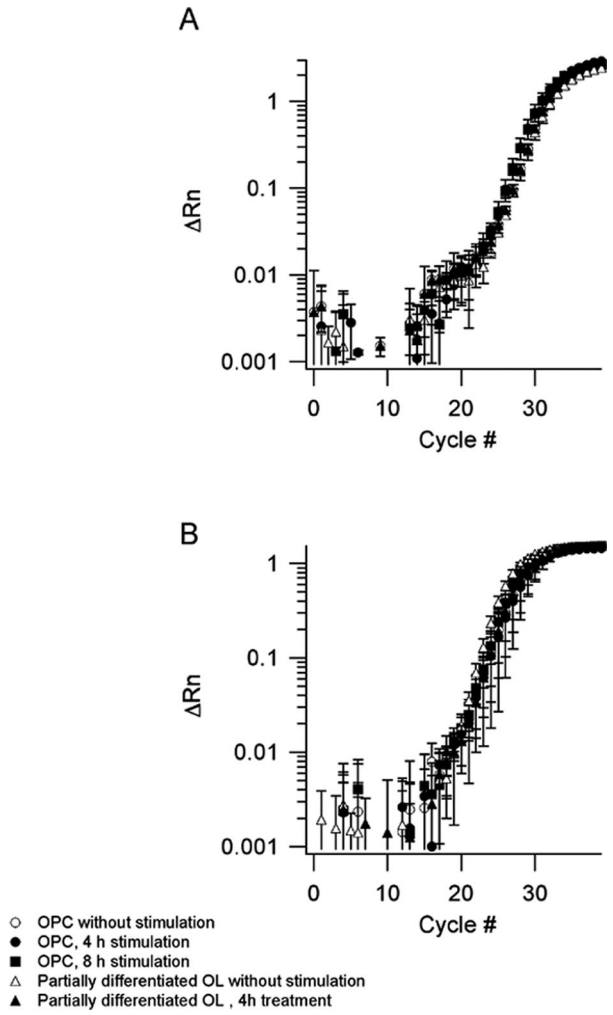

Figure 3. Kv1.4 mRNA expression is not regulated by proinflammatory stimuli. RT-PCR analysis of Kv1.4 expression (**A**) and transferrin receptor expression (**B**) as control. In comparison with naïve OPCs (○), Kv1.4 mRNA is not up-regulated after treatment with 4% supernatant of concanavalin A-stimulated spleen cells for 4 (●) or 8 hours (■). Likewise, there is no difference in Kv1.4 mRNA expression between unstimulated but partly differentiated OPC (△) and partly differentiated OPC after treatment with cytokine supernatant for 4 hours (▲).

plaque and periplaque white matter, CNPase-positive cells were already present at the onset of disease (day 9 p.i.). At that stage, CNPase staining was mainly limited to the cell body (Figure 4A). CNPase immunoreactivity then increased during the progression of the disease. At day 13 p.i., CNPase staining was present in the soma and the processes of cells (Figure 4B). In the later disease stages, numerous thin and irregular myelin sheets labeled positive for CNPase (Figure 4, C and D), whereas CNPase-positive cell bodies were only rarely found.

We next quantified CNPase and Kv1.4 double-positive cells at the different stages of MOG-EAE. Quantification of CNPase- and Kv1.4-positive cells revealed that numbers of CNPase and Kv1.4 double-positive cells peaked at the maximum of disease (day 13 p.i.) and decreased during the later phase of disease (day 26 p.i.). During the very late disease phase (days 40 and 56 p.i.), there were only few CNPase and Kv1.4 double-positive cells found in the spinal cord of EAE-diseased mice (Figure 5). In summary, Kv1.4-positive cells in or around EAE lesions are almost exclusively CNPase-positive oligodendroglial cells.

Expression of CNPase and Kv1.4 in the Early Phase of MOG-EAE Is Associated with Cell Proliferation

After a demyelinating episode, proliferation of oligodendroglial cells may be directly linked with remyelination.[45] Therefore, we asked whether Kv1.4 and CNPase expression might be linked to oligodendroglial proliferation.

By morphological criteria, CNPase- and Kv1.4-positive oligodendroglial cells were frequently localized in clusters or in doublets with close cell-cell contact (Figure 6, arrow). Because these aggregations are suggestive of proliferation, we performed immunohistochemistry for Ki67 as a marker for proliferating cells. In good correlation with previous studies in MS lesions,[46] CNPase and Ki67 double-positive cells constituted about one-half of all proliferating cells at the beginning and approximately one-third at the later time points of MOG-EAE (Figure 6). A lesser fraction of Ki67-positive cells was preferentially located in the meninges and in clusters around blood vessels, likely representing proliferating immune cells. In the later phase of the disease (day 26 p.i.), fewer CNPase and Ki67 double-positive cells could be found. On days 40 and 56 p.i., proliferating cells were observed only rarely.

Because CNPase-positive cells are positive for proliferation markers and Kv1.4 expression during autoimmune demyelination is confined to CNPase-positive cells, Kv1.4 immunoreactivity should be directly associated with markers of cell proliferation. Indeed, double labeling immunofluorescence confirmed coexpression of Kv1.4 with Ki67 at the maximum of MOG-EAE (Figure 7A). Similar results were obtained by combining anti-Kv1.4 immunofluorescence and 5-bromo-2′-deoxyuridine labeling (Figure 7B). Thus, CNPase and Kv1.4 double-positive cells are actively proliferating in the early phase of MOG-EAE.

Expression of CNPase in the Late Phase of MOG-EAE Is Associated with Remyelination of Naked Axons

Kv1.4 expression is associated with oligodendroglial proliferation and immunoreactivity for CNPase, an enzyme involved in myelin biosynthesis. We were therefore interested to see whether Kv1.4 expression in CNPase-positive cells might relate to tissue repair in MOG-EAE. To that end, we investigated whether CNPase expression is found in the context of remyelination. We performed double-labeled immunohistochemistry for CNPase with staining for injured axons. As a marker for naked axons, we used the sodium channel Nav1.2, which is known to colocalize with axonal APP expression in EAE.[47]

On intact and myelinated axons, Nav1.2 immunoreactivity was confined to the nodes of Ranvier (data not shown). In EAE lesions, immunohistochemistry for Nav1.2 resulted in an extended and diffuse immunostaining along axons (Figure 8, A–D, red). Axons diffusely expressing Nav1.2 (Figure 8A, red) were already visible at

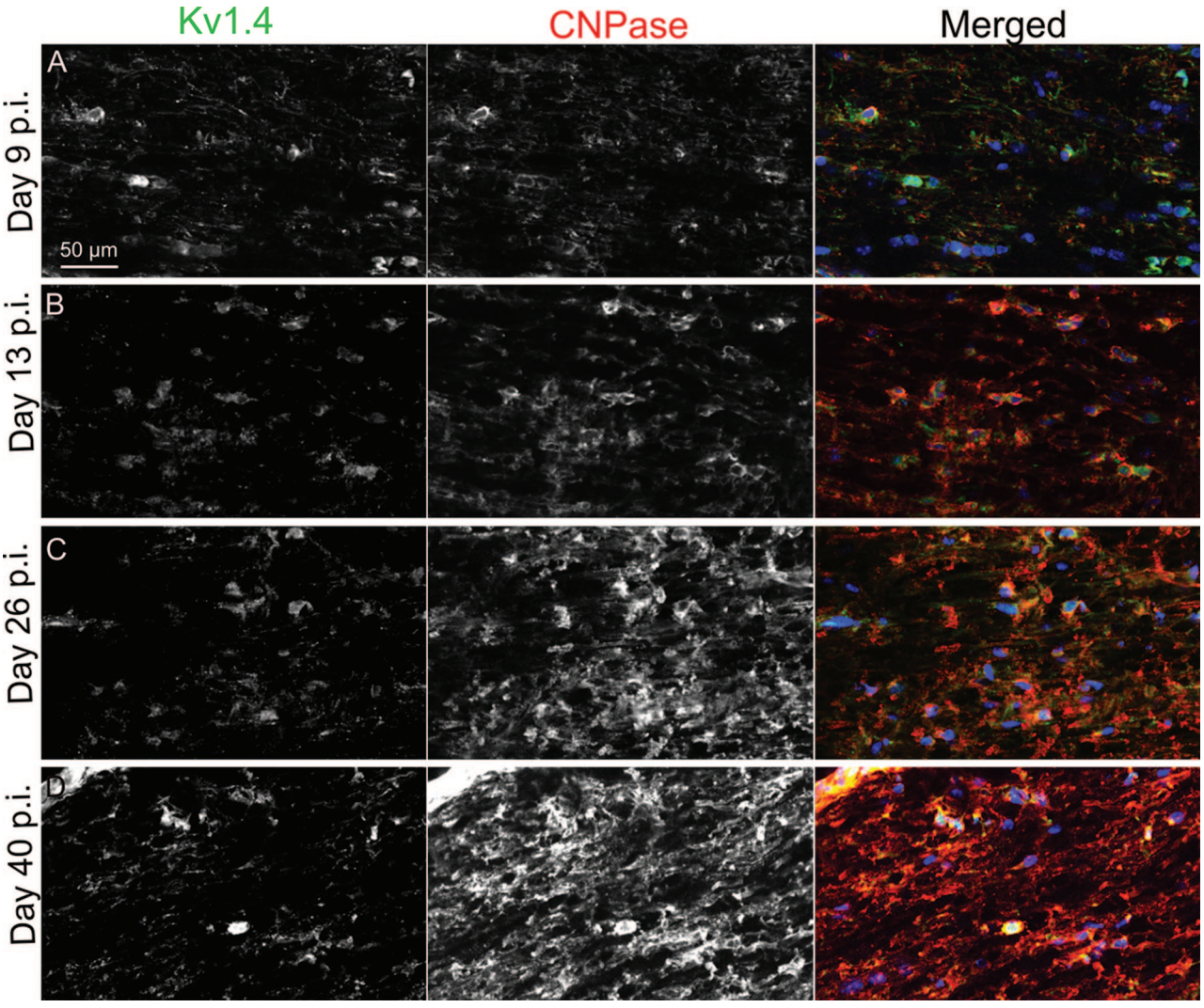

Figure 4. CNPase-positive oligodendroglial cells express Kv1.4 over the course of MOG-EAE. Representative images of longitudinal lumbar spinal cord sections are shown. **A:** Just after disease onset (day 9 p.i.), some cells with a strong signal in the soma for CNPase and Kv1.4 are already present. **B:** The number of CNPase and Kv1.4 double-positive premyelinating oligodendroglial cells increases 13 days p.i. **C** and **D:** The number subsequently decreases in the later phases of the disease (days 26 and 40 p.i.).

the beginning of disease (day 9 p.i.). At that time point, it was already possible to observe some CNPase-positive cells (Figure 8A, green) next to axons with altered expression of Nav1.2. At the maximum of disease (day 13 p.i.), CNPase-positive cells displayed multipolar branched processes but no apparent contact with surrounding axons, which displayed increasing Nav1.2 immunoreactivity (Figure 8B). On day 26 p.i., the CNPase-positive cells contacted, surrounded, and ensheathed Nav1.2-expressing axons (Figure 8C, Nav1.2 staining in red). In the late phase of disease, there were axons with thin and irregular but intact CNPase immunoreactivity (Figure 8D). These axons were Nav1.2-negative.

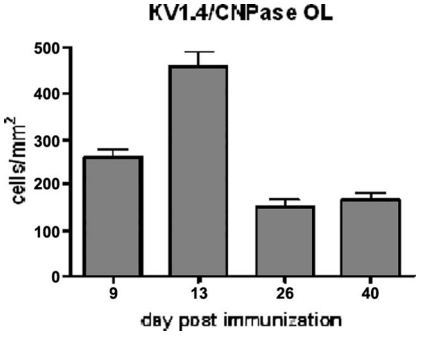

Figure 5. Quantification of Kv1.4- and CNPase-positive cells in the lumbar spinal cord of EAE-diseased mice. Each bar represents the mean density of double-positive cells. CNPase and Kv1.4 double-positive cells are already present at the beginning (day 9 p.i.), peak at the maximum (day 13 p.i.), and subsequently decrease in the later phases of MOG-EAE (days 26 to 40 p.i.).

Despite these morphological indicators for remyelination in the later stages of MOG-EAE, quantification of Nav1.2-positive axons revealed that the number of axons with altered Nav1.2 expression increased over the course of the disease. Although myelinated CNPase-positive axons were present in the late disease phase, a significant number of naked axons with diffuse Nav1.2 expression remained visible (Figure 8D, Nav1.2 in red). In summary, the expression of CNPase is associated with morpholog-

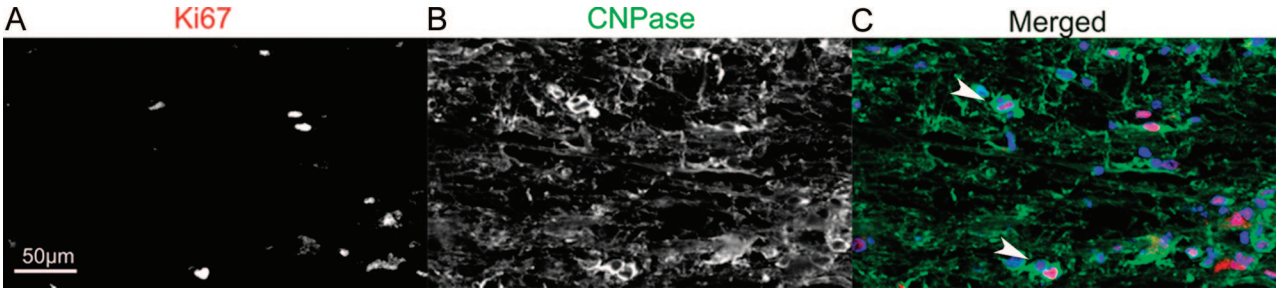

Figure 6. CNPase-positive oligodendroglial cells are actively proliferating. Double staining of MOG-EAE lesions (day 13 p.i.) for CNPase (**B**) and the proliferation marker Ki67 (**A**). **C:** Most of the cells that are Ki67-positive are also immunoreactive for CNPase (double-labeled cells marked with **arrows**).

ical hallmarks of remyelination at the later stages of MOG-EAE, albeit this phase is characterized by persistent redistribution of sodium channels indicating ongoing axonal injury.[40]

Reduced Kv1.4 Expression in a Model of Enhanced Autoimmune Demyelination

Here, we show that expression of the voltage-gated potassium channel Kv1.4 is associated with markers of oligodendroglial cell proliferation and remyelination. To test the functional relevance of this association, we checked for expression of Kv1.4 during autoimmune demyelination in CNTF knockout mice ($CNTF^{-/-}$ mice). Healthy $CNTF^{-/-}$ mice show normal oligodendrocyte numbers.[48] In later stages of MOG-EAE, $CNTF^{-/-}$ mice display an impaired OPC proliferation, increased OL apoptosis, and poor remyelination.[38]

If Kv1.4 is functionally linked to oligodendroglial proliferation, one could expect a reduced expression of this potassium channel early during MOG 35–55 EAE in $CNTF^{-/-}$ mice. Therefore, Kv1.4 immunofluorescence was performed in $CNTF^{-/-}$ and C57BL/6 control mice at the maximum of disease, the time point where the highest number of Kv1.4-positive cells was observed in wild-type mice (Figure 4). At day 14 p.i., Kv1.4 and CNPase double-positive cells were significantly decreased in $CNTF^{-/-}$ mice (Figure 9A). At that time point, the number of apoptotic OLs was very small and not yet increased in $CNTF^{-/-}$ mice (Figure 9B).

In summary, diminished Kv1.4 re-expression in early stages of MOG-EAE in CNTF knockout mice correlates well with impaired recruitment of oligodendroglial cells in these mice. These data further support the notion of a functional relevance of Kv1.4 for oligodendroglial proliferation.

Discussion

In the present study, we show that the potassium channel Kv1.4 is re-expressed in and around demyelinated lesions in the murine CNS. Kv1.4 expression is not found in healthy adult spinal cord or in normal appearing white matter. The re-expression of developmentally regulated molecules in oligodendroglial cells in demyelinating diseases of the CNS gains increasing attention, as shown for the cytoskeleton regulating molecule stathmin or oligodendroglial Notch signaling.[20,22] Depending on the model investigated, this re-expression of developmentally regulated pathways may have distinct functional implications.[21,49]

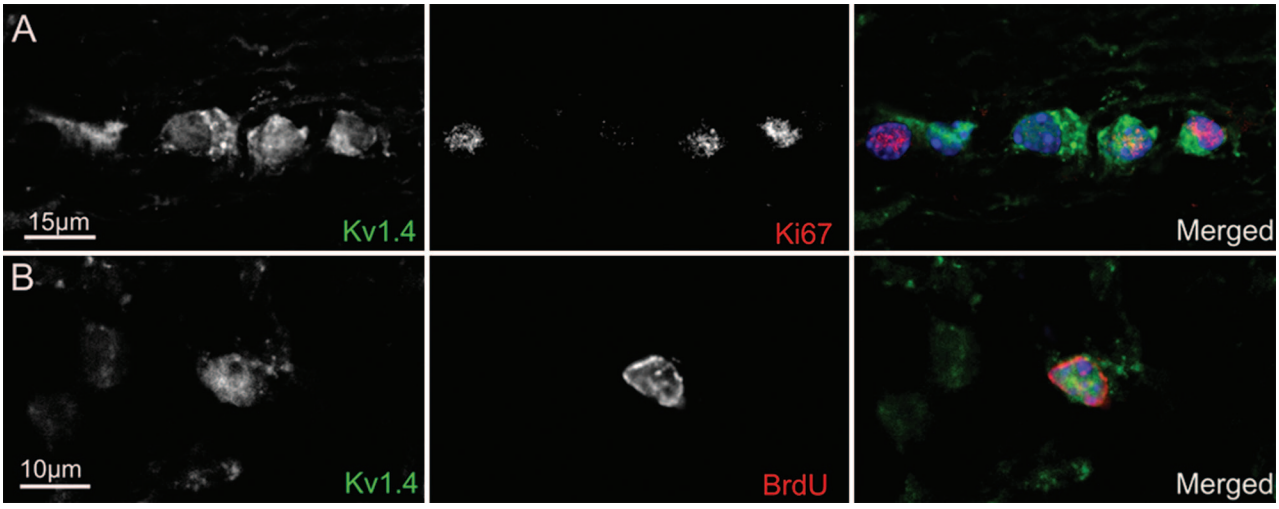

Figure 7. Kv1.4-expressing cells in MOG-EAE lesions are actively proliferating (day 13 p.i.). Representative images from lumbar spinal cord are shown. **A:** High-resolution imaging after immunohistochemistry for Kv1.4 and Ki67 reveals colocalization of both markers (see Merged). **B:** Similar results are obtained after *in vivo* labeling of proliferating cells with 5-bromo-2′-deoxyuridine. Kv1.4-expressing cells are also 5-bromo-2′-deoxyuridine-positive (see Merged).

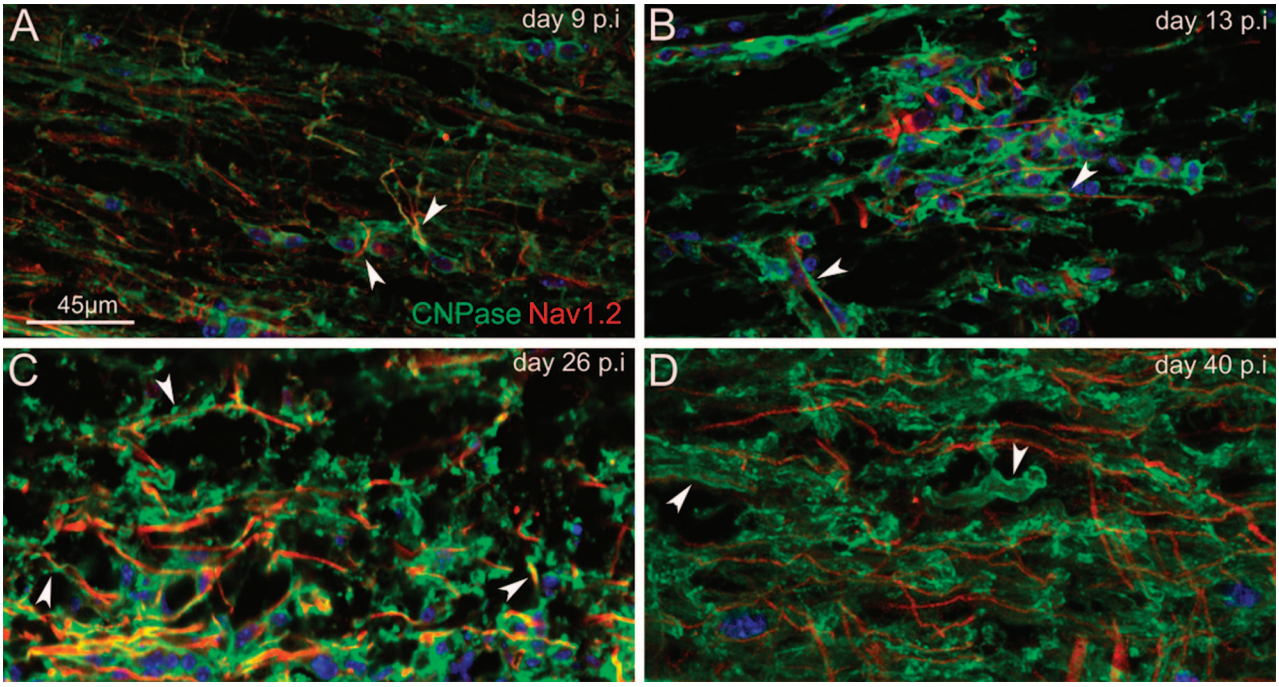

Figure 8. CNPase-positive oligodendroglial cells are associated with remyelination of naked axons expressing Nav1.2 sodium channels (**A–D**, red). **A:** At the onset of the disease (day 9 p.i), CNPase-expressing oligodendroglial cells (green, **arrowheads**) start to appear in the lesion. **B:** At the maximum of disease (day 13 p.i) contact Nav1.2-positive naked axons appear (**arrowheads**). **C:** In the later phase of MOG-EAE, CNPase-positive oligodendroglial cells surround and ensheathed demyelinated axons (**arrowheads**). **D:** Finally (day 40 p.i), irregular and thin myelin sheaths suggestive of remyelination can be seen (**arrowheads**). Representative sections from lumbar spinal cord are shown. **D:** Projection of 24 optical sections.

During myelination and demyelination, Kv1.4 is particularly expressed on oligodendroglial cells. Kv1.4 expression is not found on immune cells, although the expression of the related channels Kv1.3 and Kv1.5 may be involved in T-cell activation and dendritic cell function in EAE and MS.[29,30,50,51]

During oligodendroglial development *in vitro*, there is a clear coexpression of Kv1.4 with AN2 in cells, which at a certain stage are also CNPase-positive. During MOG-EAE *in vivo*, Kv1.4 is only found on very few AN2-positive cells, which are also CNPase-positive. AN2-positive oligodendroglial cells exist around EAE lesions *in vivo*,[11,38] yet are mostly CNPase-negative. On the one hand, the quantitative differences in AN2 and CNPase double-positive cells between the *in vitro* and the *in vivo* situation may be explained by the fact that simultaneous expression of both markers reflects a brief transition phase from OPCs to OLs. The highly synchronized OL development *in vitro* may thus facilitate the depiction of these only shortly existing double-positive cells. On the other hand, AN2 may label a distinct, relatively quiescent OPC population in the adult brain with different characteristics from AN2-positive OPCs during development or after activation.[44]

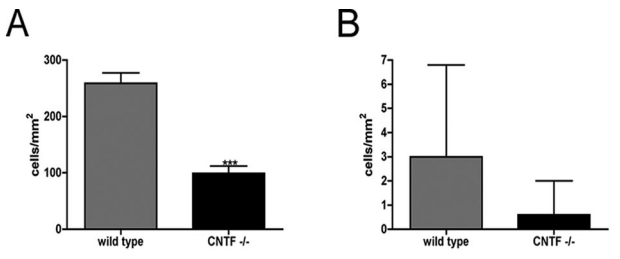

Figure 9. Investigation of Kv1.4 in CNTF$^{-/-}$ mice. **A:** Quantification of Kv1.4 and CNPase double-positive cells during MOG-EAE in CNTF$^{-/-}$ mice at the maximum of disease (day 14 p.i.). CNTF$^{-/-}$ mice display a significantly lower number of double-positive cells (***$P < 0.001$). **B:** Quantification of apoptotic CNPase-positive oligodendroglial cells at the maximum of disease. At that time point, numbers of apoptotic OLs are not increased in CNTF$^{-/-}$ mice.

After demyelination in MOG 35–55 EAE, Kv1.4 expression is in particular confined to cells positive for the oligodendroglial marker CNPase, an enzyme involved in myelin biosynthesis. *In vitro*, the expression of different voltage-gated Kv potassium channels has already been described in cells of the OL lineage, among them Kv1.4.[42] In the present study, Kv1.4-positive, CNPase-positive cells peak at the maximum of MOG 35–55 EAE. In acute MS lesions, CNPase-expressing oligodendroglial cells are known to appear in large numbers after acute myelin breakdown and before new myelin formation.[52] At later disease stages of MOG 35–55 EAE, these cells are able to contact naked axons and to build thin and irregular, newly formed myelin around damaged axons, which express an altered pattern of sodium channels as recently found in EAE and MS lesions.[17,47] However, in chronically demyelinated lesions, this process remains largely incomplete, perhaps because axons (re)-express inhibitory molecules like PSA-NCAM,[53] thus being unreceptive for remyelination.[13,17]

The morphological evidence for remyelination as well as the coexpression of Kv1.4 with CNPase suggests that Kv1.4-positive cells are exclusively premyelinating oligodendroglial cells actively involved in lesion repair. These data are in line with recent evidence for the presence of premyelinating oligodendroglial cells throughout the

adult mammalian CNS. These cells also occur in or around MS lesions[13] and may contribute to lesion repair. To date, it is still unclear whether only cells derived from OPCs can give rise to new myelin or if this process may also occur by dedifferentiation of already existing OLs in the lesions.[54] Correspondingly, our data allow no definite conclusion as to whether Kv1.4 CNPase double-positive cells are derived from OPCs or from pre-existing OLs.

Furthermore, we present morphological and immunohistochemical evidence that CNPase- and Kv1.4-positive cells in the spinal cord are actively proliferating. Immature CNPase-positive oligodendroglial cells have been shown earlier to proliferate actively in acute and chronic MS lesions.[46] In good agreement with these results, an *in vitro* study overexpressing different Kv channels does not report an impact on OL differentiation, but on OPC proliferation: Kv1.3 and Kv1.4 overexpression increases OPC cell proliferation, whereas Kv1.6 overexpression inhibits cell cycle progression.[28] Moreover, Edwards et al[27] describe the up-regulation of Kv1.4 in proliferating oligodendroglial cells after chronic spinal cord injury *in vivo*. However, further data on the *in vivo* expression of Kv1.4 in cells of the OL lineage in the adult CNS are limited. In particular, the role of glial potassium channels for demyelination and remyelination of the CNS *in vivo* has not been investigated so far. Here, we describe the potassium channel Kv1.4 as a new marker for proliferating premyelinating oligodendroglial cells in autoimmune demyelination.

Finally, we found a significantly reduced expression of Kv1.4-positive proliferating oligodendroglial cells in the spinal cords of EAE-diseased CNTF knockout mice. $CNTF^{-/-}$ mice are characterized not only by a reduced expression of Kv1.4 early but also in particular by impaired remyelination late during MOG-EAE.[38] Although not establishing a direct link, it is thus tempting to speculate that expression of Kv1.4 in oligodendroglial cells may also have functional implications. A lack of proliferating oligodendroglial cells can contribute to accumulation of incompletely repaired lesions and finally indirectly add to clinical deterioration later during the disease.[54,55]

In vitro studies already revealed that potassium channels may play a role in entering or keeping oligodendroglial cells in the cell cycle.[23] Our own *in vitro* experiments showed that oligodendroglial Kv1.4 expression cannot be augmented or re-established by proinflammatory cytokines. Although the local milieu in the CNS may differ, these data strongly argue against re-expression of Kv1.4 as part of an unspecific oligodendroglial stress reaction. Thus we propose that Kv1.4 is not only a marker of but rather is functionally involved in proliferation of oligodendroglial cells.

The identification of factors that control the proliferation of oligodendroglial cells is a pivotal step in the creation of new repair strategies for demyelinated lesions in the CNS. In particular, modulating the activity of Kv1.4 channels may present an interesting mechanism for enhanced lesion repair by sustained oligodendroglial proliferation. So far, specific inhibitors for Kv1.4 are not available.[35] Because unspecific potassium channel blockers largely interfere with the immune response,[50,51] they cannot be sensibly used to study the significance of Kv1.4 for remyelination. More studies, eg, in genetically engineered mice, are needed to address further the functional role of Kv1.4 during autoimmune demyelination.

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