Deletion of Voltage-Gated Calcium Channels in Astrocytes during Demyelination Reduces Brain Inflammation and Promotes Myelin Regeneration in Mice

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To determine whether Cav1.2 voltage-gated Ca^{2+} channels contribute to astrocyte activation, we generated an inducible conditional knock-out mouse in which the Cav1.2 α subunit was deleted in GFAP-positive astrocytes. This astrocytic Cav1.2 knock-out mouse was tested in the cuprizone model of myelin injury and repair which causes astrocyte and microglia activation in the absence of a lymphocytic response. Deletion of Cav1.2 channels in GFAP-positive astrocytes during cuprizone-induced demyelination leads to a significant reduction in the degree of astrocyte and microglia activation and proliferation in mice of either sex. Concomitantly, the production of proinflammatory factors such as $\text{TNF}\alpha$, $\text{IL1}\beta$ and $\text{TGF}\beta 1$ was significantly decreased in the corpus callosum and cortex of Cav1.2 knock-out mice through demyelination. Furthermore, this mild inflammatory environment promotes oligodendrocyte progenitor cells maturation and myelin regeneration across the remyelination phase of the cuprizone model. Similar results were found in animals treated with nimodipine, a Cav1.2 Ca^{2+} channel inhibitor with high affinity to the CNS. Mice of either sex injected with nimodipine during the demyelination stage of the cuprizone treatment displayed a reduced number of reactive astrocytes and showed a faster and more efficient brain remyelination. Together, these results indicate that Cav1.2 Ca^{2+} channels play a crucial role in the induction and proliferation of reactive astrocytes during demyelination; and that attenuation of astrocytic voltage-gated Ca^{2+} influx may be an effective therapy to reduce brain inflammation and promote myelin recovery in demyelinating diseases.

Key words: astrocytes; Cav1.2; myelination; oligodendrocytes; remyelination; voltage-gated Ca^{2+} channels

Significance Statement

Reducing voltage-gated Ca^{2+} influx in astrocytes during brain demyelination significantly attenuates brain inflammation and astrocyte reactivity. Furthermore, these changes promote myelin restoration and oligodendrocyte maturation throughout remyelination.

Introduction

Astrocytes experience substantial morphologic and molecular changes in response to injury and disease, characterized by both a gain and loss of function (Sofroniew and Vinter, 2010). These alterations in astrocytic functions can impact surrounding neural and glial cells, including oligodendrocytes. It is well established that in demyelinated diseases such as multiple sclerosis, the

pathologic environment and the altered functions observed in reactive astrocytes inhibit endogenous oligodendrocyte progenitor cells (OPCs) ability to mature into myelinating oligodendrocytes (Bannerman et al., 2007; Williams et al., 2007). On the contrary, reactive astrocytes can also have beneficial roles in demyelinated diseases. For example, they can recruit microglia and macrophages to the lesion site and promote phagocytosis of myelin debris through the secretion of proinflammatory factors (DeWitt et al., 1998). The dichotomy observed in reactive astrocytes following demyelination makes the design of therapeutic strategies challenging.

Astrocytes exhibit excitability by way of ionic fluxes, and particularly in the form of intracellular Ca^{2+} oscillations. Levels of intracellular Ca^{2+} are critical for numerous homeostatic cellular functions in astrocytes, including migration and proliferation (Gao et al., 2013; Parnis et al., 2013). We have recently reported that voltage-gated Ca^{2+} channels, specifically the L-type Cav1.2 isoform, are centrally involved in triggering astrocyte reactivity

Received July 10, 2019; revised Feb. 28, 2020; accepted Mar. 3, 2020.

Author contributions: N.N.Z., V.T.C., D.A.S.G., and P.M.P. designed research; N.N.Z., V.T.C., D.A.S.G., R.W., and P.M.P. performed research; N.N.Z., V.T.C., D.A.S.G., R.W., and P.M.P. analyzed data; N.N.Z., V.T.C., D.A.S.G., and P.M.P. wrote the paper.

This work was supported by NIH/NINDS Grant 5R01NS078041 and National Multiple Sclerosis Society Grant RG1807-31649.

The authors declare no competing financial interests.

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<https://doi.org/10.1523/JNEUROSCI.1644-19.2020>

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in vitro (Cheli et al., 2016a). Immunocytochemical, Western blot and Ca^{2+} imaging experiments showed a substantial upregulation of Cav1.2 channels in reactive astrocytes. Importantly, Cav1.2 knock-down/out in astrocytes reduce Ca^{2+} influx after plasma membrane depolarization and prevents astrocyte activation and proliferation induced by the endotoxin lipopolysaccharide or by a mechanical injury (Cheli et al., 2016a).

In this work, we have found that Cav1.2 channels play a key role in promoting astrocyte activation in the context of myelin damage. After demyelination, a significant reduction in astrocyte reactivity, microglia activation and production of proinflammatory factors was found in a conditional knock-out mouse in which the Cav1.2 channel was deleted in GFAP-positive astrocytes. Furthermore, Cav1.2 deletion in astrocytes promotes oligodendrocyte maturation and myelin synthesis during the remyelination phase of the cuprizone intoxication. These findings suggest that Cav1.2 channels play a central role in astrocyte activation and propose that attenuation of L-type Ca^{2+} influx in reactive astrocytes may serve of therapeutic value in brain diseases in which astroglialosis plays a detrimental role.

Materials and Methods

Transgenic mice

All animals used in the present study were housed in the University of Buffalo (UB) Division of Laboratory Animal Medicine vivarium. Procedures were approved by UB's Animal Care and Use Committee and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health. The heterozygous *floxed* Cav1.2 mouse was obtained from Murphy Geoffrey (University of Michigan, Ann Arbor; White et al., 2008) and the GFAP CreER^{T2} transgenic line (Jackson Mice 012849) was obtained from The Jackson Laboratory. Experimental animals were generated by crossing the heterozygous *floxed* Cav1.2 line with the hemizygous GFAP CreER^{T2} transgenic line. For ease of reading, conditional knock-out mice (Cav1.2 $^{\text{f/f}}$, GFAP $\text{Cre}^{+/-}$) will be referred to as Cav1.2 $^{\text{KO}}$ and *floxed* controls (Cav1.2 $^{\text{f/f}}$, GFAP $\text{Cre}^{-/-}$) as controls throughout the remainder of the text. For all the experiments presented in this work, mice of either sex were used.

Mice treatments

Postnatal day (P)60 mice were fed pellet chow containing 0.2% cuprizone (CPZ; Teklad-Envigo) for 6 weeks to induce demyelination, followed by a diet of normal pellet chow. In addition, a group of P60 animals were not treated and were maintained on a diet of normal pellet chow. *Cre* activity was induced by intraperitoneal injection of tamoxifen (Sigma-Aldrich). Stock solutions (20 mg/ml) were prepared by dissolving and sonicating tamoxifen in autoclaved corn oil (Sigma-Aldrich). Control and Cav1.2 $^{\text{KO}}$ mice were injected with tamoxifen (100 mg/kg) every other day for 2 weeks for a total of seven injections starting at different time points during the CPZ treatment. Furthermore, P60 C57BL/6J mice were fed pellet chow containing 0.2% CPZ for 6 weeks, followed by a normal diet. Nimodipine or vehicle was applied by subcutaneous injections every other day during the last 3 weeks of the CPZ treatment. Mice were injected with either 10 mg/kg of nimodipine (Sigma-Aldrich) in vehicle solution, which consisted of 5% ethanol, 5% DMSO (Sigma-Aldrich), 40% PEG 400 (Sigma-Aldrich), and normal saline solution or were treated with vehicle solution only. Because nimodipine is very light sensitive, all steps, including stock preparation and administration, were performed protected from light.

Primary cultures of cortical astrocytes

Primary cultures of mouse cortical astrocytes were prepared as described by Cheli et al. (2016a). Cerebral hemispheres from 1-d-old pups were dissected under sterile conditions. The tissue samples were mechanically dissociated and plated on poly-D-lysine-coated flasks in DMEM and Ham's F12 (1:1 v/v; Life Technologies), containing 100 $\mu\text{g}/\text{ml}$

gentamycin and supplemented with 4 mg/ml dextrose anhydrous, 3.75 mg/ml HEPES buffer, 2.4 mg/ml sodium bicarbonate, and 10% fetal bovine serum (FBS; Life Technologies). After 24 h the medium was changed and the cells were grown in DMEM/F12 supplemented with insulin (5 $\mu\text{g}/\text{ml}$), human transferrin (50 $\mu\text{g}/\text{ml}$), sodium selenite (30 nM), D-Biotin (10 mM), 0.1% BSA (Sigma-Aldrich), 1% FBS (Omega Scientific), and 1% horse serum (Omega Scientific). After 14 d, oligodendrocytes and microglia were removed from the mixed glial culture by a differential shaking and adhesion procedure and astrocytes were collected from the flasks by trypsinization. The cells were plated on either 24-well glass bottom plates (100 $\times$ 103 cells/well), 60 mm dishes (700 $\times$ 103 cells/dish), or 20 mm micro-well plates (100 $\times$ 103 cells/well) coated with poly-D-lysine (Millipore). Cells were grown for different periods of time or until confluent in defined culture media G5: DMEM/F12 supplemented with hydrocortisone (10 nM), sodium selenite (30 nM), insulin (5 $\mu\text{g}/\text{ml}$), transferrin (50 $\mu\text{g}/\text{ml}$), D-biotin (10 ng/ml), bFGF (5 ng/ml), and EGF (10 ng/ml). To reduce microglial growth, the culture medium was changed every second day. To induce *Cre*-mediated recombination, control and Cav1.2 $^{\text{KO}}$ astrocytes were treated with 4-OH-tamoxifen (1.5 μM) for 3 consecutive days starting 4 d after plating.

Immunocytochemistry

Cells were stained with antibodies against several astrocyte markers following the protocol outlined in Cheli et al. (2016a). Fluorescent images were obtained using a spinning disk confocal microscope (Olympus, IX83-DSU). Quantitative analysis of the results was done counting the antigen-positive and DAPI-positive cells (total number of cells) in 20 randomly selected fields per coverslip, which resulted in counts of >2000 cells. For all experimental conditions, four coverslips per culture were analyzed and data represent pooled results from at least four independent cultures. Cell counting was performed semiautomatically and blind to the genotype of the sample by MetaMorph software (Molecular Devices). The primary antibodies used for immunocytochemistry were against: GFAP (1:1500; Dako; Rabbit) and *Cre* (1:800; Millipore; Mouse).

Immunohistochemistry

All animals were anesthetized with isoflurane and then perfused with 4% of paraformaldehyde in PBS via the left ventricle. The brains were post-fixed overnight in the same fixative solution at 4°C. Coronal brain slices of 50 μm thick were obtained using a vibratome (Leica Biosystems, VT1000-S). Free-floating vibratome sections were incubated in a blocking solution (2% normal goat serum and 1% Triton X-100 in PBS) for 2 h at room temperature and then incubated with the primary antibody overnight at 4°C. Sections were then rinsed in PBS and incubated with Cy3- or Cy5-conjugated secondary antibodies (1:400; Jackson ImmunoResearch) for 2 h at room temperature followed by a counterstain with the nuclear dye DAPI (Life Technologies). After washing, the sections were mounted on to Superfrost Plus slides (Fisher) using coverslips and mounting medium (Aquamount, ThermoFisher Scientific). The primary antibodies used in the present study were against: caspase-3 (1:1000; Cell Signaling Technology; Rabbit), CC1 (1:300; Calbiochem; Mouse), CD68 (1:600; BioLegend; Mouse), *Cre* (1:400; Millipore; Mouse), GFAP (1:1000; Dako; Rabbit), Iba1 (1:800; Wako; Rabbit), Ki67 (1:200; BD Pharmingen; Mouse), MBP (1:1000; BioLegend, Mouse; 1:1000; Covance, Mouse), Olig2 (1:500; Millipore, Rabbit; 1:250; Millipore, Mouse), PLP (1:50; Hybridoma AA3; Rat), Sox2 (1:500; R&D Systems; Mouse), and $\alpha\text{tubulin}$ (1:800; ThermoFisher Scientific). The staining intensity as well as the number of positive cells was assessed in the central area of the corpus callosum, between the midline and below the apex of the cingulum (0.6 mm²) and in the motor and cingulate cortex including M1, M2, Cg1, Cg2 (0.6 mm²; Franklin and Paxinos, 2008, their Fig. 24). The integrated fluorescence intensity was calculated as the product of the area and mean pixel intensity using MetaMorph software (Molecular Devices). For all experiments involving quantification of positive cells and fluorescent intensity in tissue sections, data represent pooled results from at least six brains per experimental group. Ten slices per brain (50 μm each) were used and quantification was performed blind to the genotype of the sample.

Western blot

Protein samples were extracted using lysis buffer as described by Santiago González et al. (2017). Twenty μg of proteins were separated with NuPAGE Novex 4–20% Bis-Tris Protein Gels (Life Technologies) and electro-blotted onto PVDF membranes. Membranes were blocked overnight at 4°C with 5% nonfat milk, 0.1% Tween 20 in PBS. Primary antibodies were diluted with the blocking solution and membranes incubated 3 h at room temperature with agitation. Protein bands were detected by chemiluminescence using the GE Healthcare ECL kit (GE Healthcare) with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and scanned with a C-Digit Bot Scanner (LI-COR). Protein bands were quantified using the Image Studio Software (LI-COR). Data represent pooled results from at least four independent experiments. The primary antibodies used for Western blots were against: Cav1.2 (1:1000; Alomone; Rabbit), CNP (1:1000; Neo-Markers; Mouse), GFAP (1:20 000; Dako; Rabbit), MBP (1:1000; BioLegend; Mouse), PLP (1:3000; Hybridoma AA3; Rat), P84 (1:5000; Genetec; Mouse), and vimentin (1:300; BD Pharmingen; Mouse).

RT-PCR

Total RNA was isolated using Trizol reagent (Life Technologies). RNA content was estimated by measuring the absorbance at 260 nm and the purity was assessed by measuring the ratio of absorbance: 260/280 nm. First strand cDNA was prepared from 1 μg of total RNA using PrimeScript (Takara) and 1 μg of oligo(dT) or random hexamers. The mRNA samples were denatured at 65°C for 5 min. Reverse transcription was performed at 50°C for 60 min and was stopped by heating the samples at 70°C for 15 min. The cDNA was amplified by PCR using specific primers and PCR Platinum Supermix Reagent (Life Technologies). Optimal PCR conditions varied for each set of primers. PCR products were visualized on a SYBR Safe stained agarose gel and the bands digitized using a Gel Doc EZ System (Bio-Rad). Data represent pooled results from at least four independent experiments.

Calcium imaging *in vitro*

Methods were similar to those described previously (Cheli et al., 2016a). Briefly, primary astrocytes were washed in serum and phenol red-free DMEM containing final concentration of 4 μM fura-2 (AM; Life Technologies) plus 0.08% Pluronic F127 (Life Technologies) to load dye into the cells, incubated for 25 min at 37°C, 5% CO_2 , then washed four times in DMEM and stored in DMEM for 10 min before being imaged. Calcium influx and resting Ca^{2+} levels were measured in serum and phenol red-free HBSS containing 1.3 mM Ca^{2+} and 1 mM Mg^{2+} . The fluorescence of fura-2 was excited alternatively at wavelengths of 340 and 380 nm every 2 s by means of a high-speed wavelength-switching device (λ DG4, Sutter Instruments). A spinning disk confocal microscope (Olympus, IX83-DSU) equipped with a CCD camera (Hamamatsu, ORCA-R2) measured the fluorescence. Calcium influx and resting Ca^{2+} levels were measured on individual astrocytes using the image analysis software MetaFluor (Molecular Devices). More than 600 cells for each experimental condition were analyzed and the results from four individual experiments were pooled.

Calcium imaging *in situ*

Calcium imaging acquisitions of cortical astrocytes were performed on living slices at P20 as described elsewhere (Dawitz et al., 2011). Mice were anesthetized with isoflurane, after which brains were rapidly removed and stored in ice-cold slice solution containing the following (in mM): 110 choline chloride, 25 NaHCO_3 , 11.6 sodium ascorbate, 7 MgCl_2 , 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH_2PO_4 , 0.5 CaCl_2 and 10 glucose gassed with 95% O_2 and 5% CO_2 . Coronal slices were cut at 200 μm thickness on a vibratome (Leica VT1000S). Brain slices were kept for 2 h at room temperature in artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 3 KCl, 2.5 MgCl_2 , 1.25 NaH_2PO_4 , 26 NaHCO_3 , 1.6 CaCl_2 , and 10 glucose gassed with 95% O_2 and 5% CO_2 . Intracellular Ca^{2+} measurements were made after loading the tissue with 20 μM of fura-2 (AM) in ACSF for 30 min at 36°C. Then the slices were washed with ACSF for 30 min before being imaged. Calcium influx and resting Ca^{2+} levels were measured in ACSF containing 1.5 mM MgCl_2 .

For cell detection and identification, a z stack using a step size of 1 μm and image $\pm 20 \mu\text{m}$ around the imaged plane of focus was taken. Only on focus astrocytes were selected for the analysis. Four brains per experimental group and six slices per brain were used. Approximately 100 individual cells were evaluated in each slice using the image analysis software MetaFluor (Molecular Devices).

ELISA

Cortical protein samples were collected after 6 weeks of CPZ treatment to measure the levels of IL1 β , IL6, IL10, IFN γ , TNF α , and TGF β 1 using the Mouse Autoimmune Response Multi-Analyte ELISA Array Kits (Qiagen). All ELISA procedures were performed according to the manufacturer's instructions and absorbance was measured at 450 nm with subtraction of absorbance at 570 nm using a microplate reader (Bio-Rad). For all experimental conditions, data represent pooled results from at least six mice.

Electron microscopy

Mouse brains were perfused transcardially with 3% paraformaldehyde and 1% glutaraldehyde. The body of the corpus callosum at the anterior-dorsal level of the hippocampus was dissected and resin embedded. Thin sections were stained with uranyl acetate and lead citrate and photographed with a FEI TecnaiTM F20 transmission electron microscope as previously described (Cheli et al., 2016b). For g-ratio measurements, at least 200 fibers per animal were analyzed. The percentage of myelinated axons was determined in 10 randomly selected fields per sample, which resulted in counts of >1000 axons. The g-ratio and the percentage of myelinated axons were determined semiautomatically and blind to the genotype of the sample using MetaMorph software (Molecular Devices). For all experimental conditions, data represent pooled results from at least four mice.

Magnetic cell sorting of cortical astrocytes

Dissociation of brain cortices was performed as described by Emery and Dugas (2013). Cortical tissue was enzymatically digested for 90 min in a papain buffer that consisted of 1 $\times$ EBSS, MgSO_4 (1 mM), glucose (0.36%), EGTA (2 mM) and NaHCO_3 (26 mM) at 34°C while equilibrated with 95% O_2 /5% CO_2 . Cortical tissue was initially dissociated in low-ovomucoid solution followed by dissociation in a high-ovomucoid solution and centrifuged at $220 \times g$ for 10 min. Tissue samples were resuspended in 1 $\times$ HBSS and then myelin was removed using a Percoll density gradient as described by Orre et al. (2014). Briefly, the cell suspension was overlaid on an isotonic (90%) Percoll layer and centrifuged at $300 \times g$ for 15 min at 4°C. The top layer was discarded, and the Percoll layer containing the cells and the myelin layer were collected and diluted using 1 $\times$ HBSS, followed by centrifugation at $300 \times g$ for 15 min at 4°C. Next, the supernatant containing the myelin was discarded and the pellet was resuspended in cold recovery buffer and centrifuged at $300 \times g$ for 10 min. Cells were magnetically labeled using anti-ACSA-2 (astrocyte cell surface antigen-2) and separated using a LS column according to the manufacturer's instructions (Miltenyi BioTec).

Experimental design and statistical analysis

All datasets were tested for normal distribution using the Kolmogorov-Smirnov test. Single between-group comparisons were made by the unpaired *t* test (Student's *t* test), using a confidence interval of 95%. Multiple comparisons were investigated by one-way ANOVA followed by Bonferroni's multiple-comparison test to detect pairwise between-group differences. For the analysis of g-ratio scatter plots, simple linear regression with a confidence interval of 95% was used. All statistical tests were performed in GraphPad Prism software. A fixed value of $p < 0.05$ for two-tailed test was the criterion for reliable differences between groups. Data are presented as mean $\pm$ SEM. To minimize bias, the quantification of all the experiments described in this work was performed blinded to the sample genotype. Based on previous studies, power calculations and the fact that all comparisons were made between mice with the same genetic background, at least six animals for each genotype were compared for all the morphologic and biochemical endpoints.

Figure 1. Verification of Cav1.2 conditional deletion in cortical astrocytes. **A**, After reaching confluency, Control (*Cre*-negative) and Cav1.2^{KO} astrocytes were treated with LPS (1 $\mu\text{g}/\text{ml}$) for 4 consecutive days and then with 4-OH-tamoxifen for 3 d. **B**, **C**, Quantitative RT-PCR and Western blot analysis of Cav1.2 expression in astrocytes was performed after 3 d of 4-OH-tamoxifen treatment. Representative Western blots for GFAP and vimentin are shown. p84 was used as the internal standard and data from four independent experiments are summarized based on the relative spot intensities and plotted as percentage of controls. Exact *p* values from left to right: 0.0004, 0.0008, 0.0001. **D**, **E**, The expression of GFAP was examined by immunocytochemistry in control and Cav1.2^{KO} astrocytes after 3 d of 4-OH-tamoxifen treatment. High-magnification insets show examples of GFAP/*Cre* double-positive cells. Scale bars top, 80 μm ; bottom, 40 μm . Exact *p* value < 0.0001. **F**, Average Ca^{2+} responses in control and Cav1.2^{KO} astrocytes after depolarization with 75 mM K^{+} ($n = 50$ cells per condition) and representative Ca^{2+} traces from individual control and Cav1.2^{KO} astrocytes. **G**, Bar graph represents the average Ca^{2+} amplitudes calculated from the responding cells expressed as percentage of change in emission intensities following high K^{+} (75 mM) stimulation. Exact *p* value < 0.0001. Values are expressed as mean $\pm$ SEM of at least four independent experiments. ****p* < 0.001 versus control.

Results

Ablation of Cav1.2 channels in GFAP-positive astrocytes attenuates astrogliosis after CPZ-induced demyelination

A conditional knock-out mouse for Cav1.2 channels in astrocytes was generated by crossing the *floxed* mutant Cav1.2 mouse (White et al., 2008) with the GFAP*CreER*^{T2} transgenic line. In the *floxed* mutant Cav1.2, exon 2 of the wild-type Cav1.2 gene (*Cacna1c*) is flanked by *loxP* sites and is eliminated when exposed to *Cre* recombinase. Removal of exon 2 leads to a truncated, non-functional protein. Conditional Cav1.2 deletion in cortical and hippocampal neurons as well as immature oligodendrocytes has been successfully generated in previous experiments (Cheli et al., 2016b; Santiago González et al., 2017). The GFAP*CreER*^{T2} transgenic line expresses a tamoxifen-inducible *Cre* recombinase under the control of the human GFAP promoter (Hirrlinger et al., 2006). Offspring originating from crossbreeding of GFAP*CreER*^{T2} transgenic mice with various *Cre* reporter mice exhibit genomic recombination selectively in astrocytes of almost all brain regions (Hirrlinger et al., 2006). To test

recombination efficiency, Cav1.2^{KO} (Cav1.2^{f/f}, GFAP*Cre*^{+/−}) and *Cre*-negative control astrocytes (Cav1.2^{f/f}, GFAP*Cre*^{−/−}) were isolated from the cortex of P1–P2 mouse pups. These cells were treated with LPS for 4 d to promote astrocyte activation and then 3 d with 4-OH-tamoxifen to induce *Cre*-mediated recombination (Fig. 1A). RT-PCR and Western blot experiments showed high recombination efficiency and decreased Cav1.2 protein quantities in Cav1.2^{KO} astrocytes (Fig. 1B,C). Suggesting less astrocyte reactivity, Cav1.2^{KO} astrocytes displayed low levels of GFAP and vimentin expression after LPS treatment (Fig. 1C–E). The deletion of Cav1.2 channels was further examined by fura-2 ratio imaging (Fig. 1F,G). In control cells, bath application of a solution containing high K^{+} caused an average Ca^{2+} increase of ~132%. In Cav1.2^{KO} astrocytes, the Ca^{2+} transient induced by high K^{+} was significantly lower (~28%; Fig. 1F,G). Calcium imaging acquisitions of cortical astrocytes were also performed on brain slices from control and Cav1.2^{KO} mice injected with tamoxifen from P2 to P6 (Fig. 2). Cortical astrocytes were labeled with sulforhodamine 101, a specific marker of astroglia in

the neocortex (Nimmerjahn et al., 2004; Fig. 2A,B). Exposure to high K^+ containing medium triggered a significant increase in the intracellular Ca^{2+} concentration in ~85% of cortical astrocytes at P20 (Fig. 2A,B). Suggesting an effective recombination, the intracellular Ca^{2+} changes stimulated by high K^+ in $\text{Cav1.2}^{\text{KO}}$ cortical astrocytes were significantly reduced compare to controls (Fig. 2C,D). Moreover, pharmacological experiments demonstrated that these Ca^{2+} responses were abolished by nifedipine, a Cav1.2 inhibitor (Fig. 2D).

To examine the role of Cav1.2 channels on the induction of reactive astrogliosis in the context of demyelination and remyelination we used the CPZ model of myelin injury and repair (Matsushima and Morell, 2001; Hibbitts et al., 2012). P60 conditional knock-out $\text{Cav1.2}^{\text{KO}}$ animals ($\text{Cav1.2}^{\text{fl/fl}}$, $\text{GFAPCre}^{+/-}$) and Cre -negative control littermates ($\text{Cav1.2}^{\text{fl/fl}}$, $\text{GFAPCre}^{-/-}$) were fed CPZ for 6 weeks to trigger myelin loss. In addition, these mice were compared with animals that were maintained on a normal diet (Untreated). To induce Cre activity in GFAP-positive astrocytes, two different protocols of tamoxifen administration were tested. In Protocol a, control and $\text{Cav1.2}^{\text{KO}}$ mice were injected during the fourth and fifth week of CPZ treatment, which is when inflammation typically commences (Praet et al., 2014). In Protocol b, mice were injected during the last 2 weeks of the CPZ intoxication, which is the peak of astrogliosis in this model (Praet et al., 2014; Fig. 3A). To assess recombination efficiency *in vivo*, $\text{Cav1.2}^{\text{KO}}$ mice were treated with CPZ for 6 weeks and were injected with tamoxifen according to Protocol b. We have performed Cre immunohistochemistry in combination with cell type specific markers. We found that the expression of Cre was restricted to GFAP and $\text{s100}\beta$ -positive cells. The results presented in Figure 3B show high levels of Cre expression in GFAP-positive astrocytes. Approximately, 80% of GFAP-positive astrocytes with morphologic features of reactive astroglia display high levels of Cre expression in the corpus callosum and in the cortex (Figs. 3B). Additionally, we have crossed the hGFAP Cre mouse with the Cre reporter line ROSA26R (Madisen et al., 2010; Jackson Mice 007909). Using the same tamoxifen administration protocol described above, we found recombination exclusively in astrocytes, but not in neurons or oligodendrocytes (data not shown). Verification of Cav1.2 deletion was conducted in pure cortical astrocytes isolated by magnetic cell sorting using ACSA-2 antibodies labeled with micro beads. RT-PCR experiments using primers flanking the exon 2 of the Cav1.2 showed high recombination efficiency and decreased levels of wild-type Cav1.2 mRNAs in Cre -expressing astrocytes (Fig. 3C).

Astrogliosis was initially evaluated in the corpus callosum and cortex using GFAP and $\text{s100}\beta$ as markers of reactive astrocytes. Compared with untreated animals, CPZ treatment induce an important increase in the density of GFAP and $\text{s100}\beta$ -

Figure 2. *In vivo* Cav1.2 conditional ablation in cortical astrocytes. **A, B**, Fura-2 time-lapse series of control astrocytes located in the somatosensory cortex at P20. Arrowheads indicate sulforhodamine 101 (SR-101)-positive astrocytes that were selected for the analysis. An increased fura-2 fluorescence ratio is indicated by warmer colors and time is denoted in minutes at the bottom left. Scale bars: **A**, 120 μm ; **B**, 40 μm . **C**, Average Ca^{2+} responses in control and $\text{Cav1.2}^{\text{KO}}$ astrocytes after depolarization with 50 mM K^+ ($n = 50$ cells per condition). **D**, Bar graph represents the average Ca^{2+} amplitudes calculated from the responding cells expressed as percentage of change in emission intensities following high K^+ (50 mM) stimulation. These experiments were also performed in the presence of nifedipine (5 μM). Exact p values from left to right: 0.0004, 0.6685. Values are expressed as mean $\pm$ SEM. Four animals per experimental group and ~600 cortical astrocytes per mouse were analyzed. *** $p < 0.001$ versus control.

positive cells with the classic morphology of reactive astrocytes in both, control and $\text{Cav1.2}^{\text{KO}}$ mice (Fig. 3D,F). Importantly, the total number of GFAP and $\text{s100}\beta$ -positive astrocytes as well as the level of GFAP and vimentin expression were significant reduced in $\text{Cav1.2}^{\text{KO}}$ brains (Fig. 3D,F,G). These changes were similar in both protocols of tamoxifen injections (Fig. 3F). Next, astrocyte proliferation was stereologically quantified at the end of the CPZ treatment by the coexpression of the mitotic marker Ki67 and GFAP (Fig. 3E,F). Suggesting a decline in the number of mitotic astrocytes, $\text{Cav1.2}^{\text{KO}}$ mouse displayed a significant reduction in the total amount of GFAP/Ki67 double-positive cells in the cortex as well as in the corpus callosum (Fig. 3E,F). We have also quantified microglia activation using markers such as CD68 and Iba1. In agreement with the above presented results and suggesting a milder inflammatory response, reduced CD68 and Iba1 fluorescence intensity was found in $\text{Cav1.2}^{\text{KO}}$ brains (Fig. 4A,B). Again, these changes were equivalent among the two different protocols of tamoxifen administration (Fig. 4B). Furthermore, mRNA and proteins samples were collected from controls and $\text{Cav1.2}^{\text{KO}}$ animals after 6 weeks of CPZ diet to measure the levels of inflammatory cytokines. Quantitative RT-PCR analysis revealed that the mRNAs for the proinflammatory factors $\text{TNF}\alpha$, $\text{IL1}\beta$, and $\text{TGF}\beta 1$ were decreased in the corpus callosum, cortex and cerebellum of $\text{Cav1.2}^{\text{KO}}$ mice (Fig. 4C,D). Similar results were found in pure cortical astrocytes acutely isolated from the cortex of $\text{Cav1.2}^{\text{KO}}$ mice by magnetic cell sorting at the end of the CPZ treatment (Fig. 4C,D). Compare to Cre -negative cells, mRNAs for $\text{TNF}\alpha$, $\text{IL1}\beta$, and $\text{TGF}\beta 1$ were decreased ~50% in $\text{Cav1.2}^{\text{KO}}$ astrocytes (Fig. 4D). In addition,

Figure 3. Reduced astrocyte activation in the Cav1.2^{KO} brain following CPZ treatment. **A**, Control and Cav1.2^{KO} mice were treated with CPZ for 6 weeks and injected with tamoxifen according to Protocols a or b. **B**, Representative coronal sections of the lateral portion of the corpus callosum immunostained for GFAP and Cre. Scale bar, 90 μm. **C**, Quantitative RT-PCR analysis of Cav1.2 expression performed with mRNA from acute isolated cortical astrocytes. **D**, **F**, Representative coronal sections of the corpus callosum (CC) and cortex (CX) immunostained for GFAP and GFAP/Ki67. Brain tissue was collected from untreated and CPZ-treated (CPZ) control and Cav1.2^{KO} mice injected with tamoxifen following Protocol b. Scale bars: **D**, top, 180 μm, bottom, 90 μm; **F**, top, 90 μm; bottom, 45 μm. **E**, The total number of GFAP, s100β and GFAP/Ki67 double-positive cells was quantified in the cortex and in the corpus callosum of untreated and CPZ-treated control and Cav1.2^{KO} mice injected according to Protocols a or b. Exact *p* values from left to right: GFAP CX: <0.0001, <0.0001, <0.0001, <0.0001; GFAP/Ki67 CX: 0.0060, 0.0014, 0.0011, 0.0012; s100β CX: <0.0001, <0.0001, <0.0001, <0.0001; GFAP CC: <0.0001, <0.0001, <0.0001, <0.0001; GFAP/Ki67 CC: 0.0001, 0.0008, 0.0001, 0.0007; s100β CC: <0.0001, <0.0001, <0.0001, <0.0001. Values are expressed as mean ± SEM of six mice per experimental group. ##*p* < 0.01, ###*p* < 0.001 versus untreated; ***p* < 0.01, ****p* < 0.001 versus control. **G**, Representative Western blots for GFAP and vimentin made with brain tissue comprised of the corpus callosum and cortex. p84 was used as the internal standard and data from 4 independent experiments are summarized based on the relative spot intensities and plotted as percentage of untreated. Exact *p* values from left to right: GFAP: <0.0001, 0.0003; vimentin: <0.0001, 0.0008. Values are expressed as mean ± SEM. ##*p* < 0.01 versus untreated; ****p* < 0.001 versus control.

the presence of several proinflammatory cytokines was evaluated by ELISA in cortical tissue samples (Fig. 4E). Except for TGFβ1, the levels of IL1β, IL6, IL10, IFNγ, and TNFα were significantly decreased in the cortex of Cav1.2^{KO} mice after 6 weeks of CPZ treatment (Fig. 4E). Together, these results indicate that specific ablation of Cav1.2 Ca^{2+} channels in GFAP-positive astrocytes during the demyelination phase of the CPZ treatment attenuates astrocyte activation and proliferation, microglia reactivity as well as the production of pro-inflammatory cytokines in the demyelinated mouse brain.

Selective deletion of Cav1.2 channels in reactive astrocytes enhances remyelination of the mouse brain

To study remyelination in Cav1.2^{KO} mice, CPZ was withdrawn and brains were evaluated after 2 and 4 weeks in normal diet (2 and 4 weeks of recovery). Because no significant differences were observed between protocols of tamoxifen administration, all the experiments described in this section were conducted using Protocol b. As shown in Figure 5A and B, the number of GFAP-positive cells were significantly reduced in Cav1.2^{KO} brains after 2 and 4 weeks of recovery. These changes were also reflected in

Figure 4. Attenuated microglia reactivity and reduced production of pro-inflammatory factors in the Cav1.2^{KO} brain. **A**, Representative coronal sections of the corpus callosum and cortex immunostained for CD68 and Iba1. Brain tissue was collected from untreated and CPZ-treated (CPZ) control and Cav1.2^{KO} mice injected with tamoxifen following Protocol b. Scale bar, 180 μm . **B**, The integrated fluorescent intensity of CD68 and Iba1 was measured in the lateral portion of the corpus callosum (CC) and in the cortex (CX) of untreated and CPZ-treated (CPZ) control and Cav1.2^{KO} mice injected with tamoxifen according to Protocol a or b. Exact *p* values from left to right: CD68 CC: <0.0001, <0.0001, <0.0001, <0.0001; CD68 CX: <0.0001, <0.0001, <0.0001, <0.0001; Iba1 CC: <0.0001, <0.0001, <0.0001, 0.0322; Iba1 CX: <0.0001, <0.0001, <0.0001, 0.0002. Values are expressed as mean $\pm$ SEM of six mice per experimental group. ###*p* < 0.001 versus untreated; **p* < 0.05, ****p* < 0.001 versus control. **C**, Quantitative RT-PCR analysis of TNF α , IL1 β and TGF β 1 in tissue samples consisting of the CC, CX, cerebellum (CB), and acutely isolated cortical astrocytes (CX-Ast) from CPZ-treated control and Cav1.2^{KO} mice injected with tamoxifen following Protocol b. **D**, GAPDH was used as the internal standard and data from four independent experiments are summarized based on the relative spot intensities and plotted as percentage of controls. Exact *p* values from left to right: CC/CX: <0.0001, <0.0001, <0.0001; CB: <0.0001, <0.0001, <0.0001; CX-Ast: <0.0001, 0.0001, 0.0001. Values are expressed as mean $\pm$ SEM ****p* < 0.001 versus control. **E**, Cytokine concentrations were measured by ELISA in tissue samples consisting of the CC and CX from CPZ-treated control and Cav1.2^{KO} mice injected with tamoxifen following Protocol b. Results are expressed as pg/mg of proteins. Exact *p* values: ^a0.016; ^b0.012; ^c0.037; ^d0.015; ^e0.0387.

Western blot analysis for GFAP and vimentin (Fig. 5D). Additionally, astrocyte proliferation was substantially reduced during the recovery phase in both, control and Cav1.2^{KO} mice, but no differences between genotypes were found (Fig. 5C). To establish whether Cav1.2 ablation in astrocytes affects the remyelination of the mouse brain, the expression of myelin proteins was evaluated in control and Cav1.2^{KO} animals (Fig. 6). Histologic examination of the corpus callosum and cortex after 6 weeks of CPZ treatment revealed an overall decrease in MBP and PLP immunostaining in both, control and Cav1.2^{KO} mice relative to untreated animals (Fig. 6A,B). These experiments also revealed that Cav1.2^{KO} brains were more sensitive than controls

to the demyelinated outcome of the CPZ intoxication (Fig. 6A, B). In the opposite direction, the production of MBP and PLP was increased in the cortex and corpus callosum of Cav1.2^{KO} brains at both, 2 and 4 weeks of recovery (Fig. 6A–C). In addition, we used electron microscopy to evaluate the degree of remyelination in control and Cav1.2^{KO} mice. 6 weeks of CPZ treatment induced a significant decrease in the proportion of myelinated axons located in the body of the corpus callosum (Fig. 7A,E). In the same line, the g-ratio, a measure of myelin sheath thickness calculated as the ratio of axon diameter to myelinated fiber diameter, was significantly greater in CPZ-treated animals (Fig. 7B,D). In agreement with our previous findings, Cav1.2^{KO}

Figure 5. Gliotic response throughout the recovery period of the CPZ treatment. **A**, Representative coronal sections of the corpus callosum (CC) and cortex (CX) immunostained for GFAP. Tissue was collected from untreated and CPZ-treated control and Cav1.2^{KO} mice at 2 and 4 weeks of recovery (2 or 4W Rec). Scale bar, 180 μm . **B**, **C**, Bar graphs display number of GFAP and GFAP/Ki67 double-positive cells in the lateral CC and in the CX of untreated, control and Cav1.2^{KO} mice at the end of CPZ treatment (CPZ) and after 2 and 4 weeks of recovery (2 or 4W Rec). Exact *p* values from left to right: GFAP CC: <0.0001, <0.0001, <0.0001, 0.0007, <0.0001, 0.0082; GFAP CX: <0.0001, <0.0001, <0.0001, 0.0011, <0.0001, <0.0001; GFAP/Ki67 CC: <0.0001, 0.0007, 0.0066, 0.5727, GFAP/Ki67 CX: 0.0012, 0.0013, 0.6976, 0.8756. Values are expressed as mean $\pm$ SEM of six mice per experimental group. ###*p* < 0.01, ###*p* < 0.001 versus untreated; ***p* < 0.01, ****p* < 0.001 versus control. **D**, Representative Western blots for GFAP and vimentin made with brain tissue comprised of the corpus callosum and cortex. p84 was used as the internal standard and data from four independent experiments are summarized based on the relative spot intensities and plotted as percentage of untreated. Exact *p* values from left to right: GFAP: <0.0001, 0.0003, <0.0001, 0.0001; vimentin: <0.0001, 0.0032, 0.0002, 0.0001. Values are expressed as mean $\pm$ SEM ###*p* < 0.001 versus untreated; ***p* < 0.01, ****p* < 0.001 versus control.

animals displayed lower g-ratios and higher percentage of myelinated axons at 2 and 4 weeks of recovery (Fig. 7A,B,D,E). These data indicate that remyelination is enhanced and thicker myelin sheaths are made during remyelination in the Cav1.2^{KO} corpus callosum. Importantly, no changes in axonal degeneration and/or mean diameter of remyelinated axons were found between genotypes (Fig. 7C).

As expected, we observed an important reduction in the number of total (Olig2) and mature (CC1) oligodendrocytes in control as well as in Cav1.2^{KO} brains after 6 weeks of CPZ diet (Fig. 8A–C). However, we found a significant expansion in the number of Olig2 and CC1-positive cells in the corpus callosum and cortex of Cav1.2^{KO} mice after 2 and 4 weeks of remyelination (Fig. 8A–C). Suggesting an increase in the rate of OPC proliferation during remyelination, the Cav1.2^{KO} mouse also exhibited a substantial increase in the number of Olig2/Ki67 and Olig2/Sox2 double-positive cells after 6 weeks of CPZ treatment and 2 weeks of recovery (Fig. 8D–F). Finally, to measure apoptotic cell death, the proportion of Olig2/Caspase-3 double-positive cells was quantified after 6 weeks of CPZ treatment and 2 weeks of

recovery (Fig. 8G). CPZ treatment leads to a significant increase in apoptotic Olig2-positive cells in the corpus callosum as well as in the cortex; however, this increase was notably attenuated in the Cav1.2^{KO} brain (Fig. 8G). Together, these results indicate that Cav1.2 deletion in GFAP-positive astrocytes promotes OPC proliferation during myelin recovery and prevents OPC loss by apoptosis throughout demyelination.

Nimodipine significantly attenuates astrogliosis after CPZ-induced demyelination

For these studies, P60 C57BL/6J mice were fed with CPZ for 6 weeks, followed by a diet of normal pellet chow. To pharmacologically block Cav1.2 channels in the CNS, we used the voltage-gated Ca^{2+} channel antagonist nimodipine which has high affinity for the CNS (Levy et al., 1991; Kumar et al., 2012). Nimodipine or vehicle were administered via subcutaneous injections every other day during the last 3 weeks of the CPZ treatment (Fig. 9A). Astrogliosis was apparent in both conditions following CPZ treatment, but we detected a significant reduction in the number of GFAP-positive cells in the corpus

Figure 6. Enhanced myelin proteins synthesis through the remyelination phase of the $\text{Cav1.2}^{\text{KO}}$ brain. **A**, Representative coronal sections of the corpus callosum and cortex immunostained for PLP and MBP. Tissue was collected from untreated, control and $\text{Cav1.2}^{\text{KO}}$ mice at the end of the CPZ treatment (CPZ) and after 4 weeks of recovery (4W Rec). Scale bar, 180 μm . **B**, PLP and MBP fluorescent intensity was quantified in the lateral corpus callosum (CC) and in the cortex (CX). Fluorescent intensity data are presented as percentage of untreated mice. Exact p values from left to right: PLP CC: <0.0001 , <0.0001 , <0.0001 , <0.0001 , <0.0001 , 0.0019 ; PLP CX: <0.0001 , 0.0424 , <0.0001 , <0.0001 , <0.0001 , <0.0001 ; MBP CC: <0.0001 , 0.0027 , <0.0001 , 0.0007 , <0.0001 , 0.0065 ; MBP CX: <0.0001 , 0.3742 , <0.0001 , <0.0001 , <0.0001 , 0.0016 . Values are expressed as mean $\pm$ SEM of six mice per experimental group. ### p < versus untreated; * p < 0.05, ** p < 0.01, *** p < 0.001 versus control. **C**, Representative Western blots for MBP, PLP and CNP made with brain tissue comprised of the corpus callosum and cortex. p84 was used as the internal standard and data from 4 independent experiments are summarized based on the relative spot intensities and plotted as percentage of untreated mice. Exact p values from left to right: MBP: <0.0001 , <0.0001 , <0.0001 , 0.0228 , <0.0001 , 0.0055 ; PLP: <0.0001 , 0.0188 , <0.0001 , 0.0201 , 0.0023 , 0.0002 ; CNP: <0.0001 , 0.0561 , 0.0001 , 0.0322 , 0.1436 , 0.4285 . Values are expressed as mean $\pm$ SEM ## p < 0.01, ### p < 0.001 versus untreated; * p < 0.05, ** p < 0.01, *** p < 0.001 versus control.

callosum of nimodipine-treated mice after 6 weeks of CPZ diet (Fig. 9B,D). These changes were accompanied with an overall decrease in the GFAP fluorescent intensity and with a clear reduction in the number of GFAP/Ki67 double-positive cells (Fig. 9C,D). Along these lines, we found significantly less Iba1 and CD68-positive cells in the corpus callosum of nimodipine-treated mice compared with vehicle-treated animals at the end of the CPZ treatment (Fig. 9E,F). Interestingly, these changes persist after 2 and 4 weeks of recovery. During the remyelination phase of the CPZ model, less GFAP-positive astrocytes as well as

fewer Iba1 and CD68-expressing cells were found in the brain of nimodipine-injected animals (Fig. 9D,F).

To determine whether this milder neuroinflammatory environment affects demyelination and/or remyelination of the mouse brain, immunohistochemical analyses for myelin proteins were performed in nimodipine and vehicle-treated mice. No changes were found at the end of the CPZ treatment; however, compare with vehicle-injected animals, the nimodipine group showed higher levels of MBP and PLP immunofluorescent after 2 and 4 weeks of recovery, particularly in the corpus callosum

Figure 7. Electron microscopy of the Cav1.2^{KO} corpus callosum. **A**, Electron micrographs of axons in the body of the corpus callosum of untreated, control and Cav1.2^{KO} mice at the end of CPZ treatment (CPZ) and after 2 weeks of recovery (2W Rec). Scale bars: top, 8 μm ; bottom, 2 μm . **B**, Scatter plot of g-ratio values of untreated, control and Cav1.2^{KO} mice at the end of the CPZ treatment and after 2 and 4 weeks of recovery (2 or 4W Rec). Four animals per experimental group and ~ 150 fibers per mice were analyzed. The lines represent the regression equation with 95% confidence intervals. CPZ untreated: r^2 : 0.2744; slope: 0.2884; 1/slope: 3.467; F: 134.6; CPZ control: r^2 : 0.4809; slope: 0.2105; 1/slope: 3.442; F: 113.0; CPZ Cav1.2^{KO}: r^2 : 0.4031; slope: 0.2281; 1/slope: 4.385; F: 158.7; 2W Rec control: r^2 : 0.2122; slope: 0.2365; 1/slope: 4.228; F: 120.4; 2W Rec Cav1.2^{KO}: r^2 : 0.5209; slope: 0.2887; 1/slope: 3.463; F: 572.0; 4W Rec control: r^2 : 0.1861; slope: 0.1363; 1/slope: 7.334; F: 115.2; 4W Rec Cav1.2^{KO}: r^2 : 0.2471; slope: 0.1840; 1/slope: 5.436; F: 197.2. **C**, Mean axonal diameter of myelinated axons for the same experimental conditions. **D**, Mean g-ratio values. **E**, Percentage of myelinated axons. Four animals per experimental group and ~ 150 fibers per mice were analyzed. Exact p values from left to right: g-ratios: <0.0001 , <0.0001 , <0.0001 , <0.0001 , <0.0001 , <0.0001 ; percentage of myelinated axons: <0.0001 , 0.0034, <0.0001 , <0.0001 , <0.0001 , <0.0001 . Values are expressed as mean $\pm$ SEM. ### $p < 0.001$ versus untreated; ** $p < 0.01$, *** $p < 0.001$ versus control.

and cortex (Fig. 10A,B). Further observation of the oligodendrocyte population demonstrated that the numbers of CC1-positive cells were significantly increased in nimodipine-treated brains throughout the recovery phase of the CPZ protocol (Fig. 10C,D). Hence, the increased abundance of mature myelinating CC1-positive oligodendrocytes supports our immunohistochemical data showing increased remyelination after nimodipine treatment. Finally, the remyelination of the corpus callosum in nimodipine-treated mice was evaluated by electron microscopy (Fig. 11). In agreement to what we found by immunohistochemistry, nimodipine-injected animals displayed an important decrease in the mean g-ratio of myelinated axons as well as a significant rise

in the percentage of myelinated axons after 2 and 4 weeks of recovery (Fig. 11A–C). Importantly, changes in g-ratios were equal among axons of all sizes (Fig. 11D).

Discussion

The function of Cav1.2 channels in astrocyte activation

Data presented in this article provide evidence that Cav1.2 Ca^{2+} channels play a key role in promoting astrocyte activation in the context of demyelination. We have found a significant reduction in the inflammation of the Cav1.2^{KO} mouse brain after demyelination marked by a decline in the number of reactive astrocytes,

Figure 8. Increased number of mature oligodendrocytes in the $\text{Cav1.2}^{\text{KO}}$ mouse. **A, D**, Representative coronal sections of the corpus callosum (CC) and cortex (CX) immunostained for CC1, Olig2/Ki67 and Olig2/Sox2. Tissue was collected from untreated, control and $\text{Cav1.2}^{\text{KO}}$ mice after 2 weeks of recovery (2W Rec). Scale bar, 90 μm . **B, C**, The total number of Olig2 and CC1-positive cells was quantified in the lateral portion of the CC and in the CX. Exact p values from left to right: Olig2 CC: <0.0001 , <0.0001 , <0.0001 , <0.0001 , <0.0001 , 0.0157; Olig2 CX: <0.0001 , 0.0002, <0.0001 , <0.0001 , <0.0001 , 0.0004; CC1 CC: <0.0001 , 0.1806, <0.0001 , <0.0001 , <0.0001 , <0.0001 ; CC1 CX: <0.0001 , 0.2527, <0.0001 , <0.0001 , <0.0001 , 0.0073. **E–G**, The total number of Olig2/Ki67 and the percentage of Olig2/Sox2 and Olig2/Caspase3 double-positive cells was quantified in the same brain areas and in the same experimental conditions. Exact p values from left to right: Olig2/Ki67: <0.0001 , 0.0048, <0.0001 , 0.0013, 0.7813, 0.0023, 0.0330, 0.0173; Olig2/Sox2: <0.0001 , <0.0001 , <0.0001 , <0.0001 , <0.0001 , 0.0029, 0.6634, <0.0001 ; Olig2/Caspase3: 0.0144, 0.0323, 0.9792, 0.0784, 0.0039, 0.0094, 0.5897, 0.7812. Values are expressed as mean $\pm$ SEM of six mice per experimental group. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus untreated; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus control.

microglia activation and production of proinflammatory factors. Furthermore, this reduction in astrocyte reactivity persisted throughout the remyelination phase of the CPZ treatment, suggesting that deletion of Cav1.2 channels in astrocytes leads to long-lasting alteration in the demyelinated brain. Significantly, Cav1.2 deletion in GFAP-positive astrocytes during demyelination promotes myelin recovery driven by an increased abundance of mature myelinating oligodendrocytes.

The ability of astrocytes to express functional voltage-gated Ca^{2+} channels *in vivo* has been previously established. For example, Ca^{2+} currents mediated by these channels were detected in astrocytes from the subventricular zone (Young et al., 2010) and the ventrobasal thalamus (Parri et al., 2001; Parri and Crunelli, 2003), and it has been demonstrated that astrocytes in acute

slices show voltage-gated Ca^{2+} oscillations in the ventrobasal thalamus (Komuro and Rakic, 1992, 1998). We confirmed these results in brain tissue slices from young mice. Quiescent astrocytes have a very negative membrane potential and display small voltage deviations due to the high resting K^{+} conductance. However, how the electrical properties of these cells change during neuroinflammation and demyelination in completely unknown. The expression of Cav1.2 Ca^{2+} channels was found increased in reactive astrocytes in several models of brain injury, including mechanical and thermal lesions in the forebrain, hypomyelination in white matter, and ischemia (Westenbroek et al., 1998). We have also found augmented Cav1.2 activity in cortical astrocytes treated with the endotoxin lipopolysaccharide LPS and in a model of astrocyte mechanical injury (Cheli et al.,

Figure 9. Reduced astrocyte reactivity in nimodipine-treated mice. **A**, Nimodipine or vehicle was applied by subcutaneous injections every other day during the last 3 weeks of the CPZ treatment. **B**, **C**, Representative coronal sections of the corpus callosum (CC) and cortex (CX) immunostained for GFAP and GFAP/Ki67. Brain tissue was collected from untreated, and CPZ-treated (CPZ) mice injected with vehicle or nimodipine. Scale bars: **B**, 180 μm ; **C**, top, 90 μm , bottom, 45 μm . **D**, Integrated GFAP fluorescent intensity and number of GFAP and GFAP/Ki67 double-positive cells were measured in the lateral CC. Exact *p* values from left to right: GFAP Int: <0.0001 , <0.0001 , <0.0001 , <0.0001 , <0.0001 , 0.0042; #GFAP: <0.0001 , 0.0035, <0.0001 , <0.0001 , 0.0602, 0.0328; #GFAP/Ki67: <0.0001 , 0.0155, <0.0001 , 0.0179, 0.8831, 0.8741. **E**, Representative coronal sections of the lateral corpus callosum immunostained for Iba1. Brain tissue was collected from vehicle and nimodipine-injected mice at the end of the CPZ treatment. Scale bar, 90 μm . **F**, The integrated fluorescent intensity of Iba1 and CD68 was measured in the lateral portion of the corpus callosum. Fluorescent intensity data are presented as percentage of untreated mice. Exact *p* values from left to right: Iba1: <0.0001 , <0.0001 , <0.0167 , 0.0002, 0.5773, 0.0014; CD68: <0.0001 , <0.0001 , <0.0001 , 0.0001, <0.0001 , 0.0003. Values are expressed as mean $\pm$ SEM of six mice per experimental group. #*p* < 0.05 , ###*p* < 0.001 versus untreated; **p* < 0.05 , ***p* < 0.001 , ****p* < 0.001 versus vehicle.

2016a). Along these lines, *in vitro* and *in vivo* evidence suggest that reactive astrocytes upregulate voltage-gated Na^+ channels and downregulate delayed rectifier K^+ currents (Verkhratsky and Steinhäuser, 2000; Pappalardo et al., 2014). Thus, the increase in Na^+ channels density in combination with a decrease

in delayed rectifier K^+ currents could make these cells excitable. Thus, in a neuroinflammatory environment factors released by demyelinated axons, such as K^+ , glutamate and ATP can potentially depolarize the processes of reactive astrocytes and trigger $\text{Cav}1.2 \text{Ca}^{2+}$ influx.

Figure 10. Improved remyelination of nimodipine-treated brains. **A**, Representative coronal sections of the corpus callosum and cortex immunostained for PLP and MBP from untreated, and CPZ-treated mice injected with vehicle or nimodipine. Brain tissue was collected at the end of the CPZ treatment (CPZ) and after 4 weeks of recovery (4W Rec). Scale bar, 180 μm . **B**, PLP and MBP fluorescent intensity was quantified in the lateral corpus callosum (CC) and in the cortex (CX). Fluorescent intensity data are presented as percentage of untreated mice. Exact p values from left to right: PLP CC: <0.0001, 0.3149, <0.0001, 0.0035, 0.0055, 0.0137; PLP CX: <0.0001, 0.8992, <0.0001, 0.0001, <0.0001, 0.0031; MBP CC: <0.0001, 0.9684, <0.0001, 0.0028, 0.0003, 0.0003; MBP CX: <0.0001, 0.5956, <0.0001, <0.0001, <0.0001, 0.0283. **C**, Representative coronal sections of the corpus callosum and cortex immunostained for CC1 and Olig2. Brain tissue was collected from vehicle and nimodipine-injected mice after 2 weeks of recovery (2W Rec). Scale bar, 90 μm . **D**, The total number of CC1-positive cells was quantified in the lateral portion of the CC and in the CX. Exact p values from left to right: CC1 CC: <0.0001, 0.6577, <0.0001, 0.0019, 0.0054, 0.0463; CC1 CX: <0.0001, 0.2089, <0.0001, 0.0079, 0.0025, 0.0221. Values are expressed as mean $\pm$ SEM of six mice per experimental group. $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus untreated; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus vehicle.

Cav1.2 channel activation may promote reactive astrocytosis directly by inducing biochemical changes in quiescent astroglia. For example, Ca^{2+} entry through Cav1.2 channels have been shown to increase GFAP expression, phosphorylation, and assembly, essential steps to induce morphologic transformations in reactive cells (Harrison and Mobley, 1991; Nakamura et al., 1992). Furthermore, L-type Ca^{2+} channels are capable of transmitting signals to the nucleus which influence gene transcription

(Bading et al., 1993). Calcium influx through Cav1.2 activates transcription factors such as CREB, MEF, and NFAT (Sheng et al., 1990; Graef et al., 1999; Mao et al., 1999), that lead to the expression of genes such as c-fos and BDNF (Morgan and Curran, 1986; Zafra et al., 1990; Murphy et al., 1991). Two mechanisms link Cav1.2 channels to the activation of transcription factors. Calcium entering through the channels can diffuse to the nucleus and activate nuclear Ca^{2+} -dependent enzymes, such

Figure 11. Electron microscopy of nimodipine-treated corpus callosum. **A**, Electron micrographs of axons in the body of the corpus callosum from CPZ-treated mice injected with vehicle or nimodipine. Brain tissue was collected after 2 and 4 weeks of recovery (2 or 4W Rec). Scale bar = 8 μm top; 2 μm bottom. **B**, Percentage of myelinated axons. **C**, Mean g-ratio values. Four animals per experimental group and ~150 fibers per mice were analyzed. Exact p values from left to right: g-ratios: <0.0001 , <0.0001 , <0.0001 , <0.0001 ; percentage of myelinated axons: <0.0001 , <0.0001 , <0.0001 , <0.0001 . Values are expressed as mean $\pm$ SEM. ### p < 0.001 versus untreated; *** p < 0.001 versus vehicle. **D**, Scatter plot of g-ratio values of vehicle and nimodipine-treated mice after 2 and 4 weeks of recovery. Four animals per experimental group and ~150 fibers per mice were analyzed. The lines represent the regression equation with 95% confidence intervals. 2W Rec vehicle: r^2 : 0.6105; slope: 0.1792; 1/slope: 5.581; F: 846.4; 2W Rec nimodipine: r^2 : 0.5723; slope: 0.2261; 1/slope: 4.424; F: 978.2; 4W Rec vehicle: r^2 : 0.6666; slope: 0.2175; 1/slope: 4.598; F: 899.6; 4W Rec nimodipine: r^2 : 0.5838; slope: 0.2082; 1/slope: 4.802; F: 676.0.

as CaMKIV, that regulate the activity of transcription factors and co-regulators (Hardingham et al., 2001). In addition, Ca^{2+} influx through Cav1.2 channels can interact with Ca^{2+} -dependent signaling proteins located near the channel, and this can activate the MAPK signaling pathway which can send this localized signal to the nucleus (Dolmetsch et al., 2001). Thus, future experiments are needed to study how the activity of Cav1.2 channels modulate the expression of genes

associated with astrocyte reactivity under different pathologic environments.

Voltage-gated calcium channels in reactive astrocytes, a possible therapeutic target to reduce brain inflammation and promote remyelination

Reactive astrocytes undergo substantial changes that can be either inhibitory or permissive to demyelinating diseases. These

changes ultimately contribute to an overall extracellular environment that affects the regeneration of the myelin sheath by influencing the ability of OPCs to migrate into a demyelinated lesion and differentiate into mature, myelin producing oligodendrocytes (Wheeler and Fuss, 2016). Although deletion of the Cav1.2 channels in GFAP astrocytes did not prevent myelin damage during the CPZ treatment, attenuation of Cav1.2 mediated Ca^{2+} entry did have beneficial implications on remyelination. For example, the Cav1.2^{KO} corpus callosum showed an increase in the percentage of myelinated fibers and a reduction in the mean g-ratio of myelinated axons during the recovery phases of CPZ intoxication. The enhanced remyelination of the Cav1.2^{KO} brain was accompanied by a significant increase in the number of myelinating oligodendrocytes and in the density of proliferating OPCs. This expansion in the OPC pool is a necessary and vital prerequisite for effective remyelination of the brain. Together, these findings suggest that reducing astrocyte and microglia activation during demyelination is beneficial for myelin regeneration and for the development of new OPCs in the adult brain.

In mature neurons, Cav1.2 channels are located postsynaptically at somatodendritic locations and they are involved in synaptic modulation as well as in the propagation of dendritic Ca^{2+} spikes (Obermair et al., 2004). Inhibition of Cav1.2 channels in the brain at therapeutic doses in humans cause measurable changes in neuronal plasticity but no obvious side effects (Wankerl et al., 2010; Ortner and Striessnig, 2016). Voltage-gated Ca^{2+} channel blockers have been used successfully to treat absence seizures, and are emerging as potential therapeutic avenues for pathologies such as Parkinson's disease, addiction and anxiety (Zamponi, 2016). However, recent findings suggest that Cav1.2 channel inhibitors are also effective in the treatment of neuroinflammatory diseases. For example, new studies have reported that the Cav1.2 channel blockers nimodipine and verapamil exert their neuroprotective effects through anti-inflammatory properties (Michelucci et al., 2009; Liu et al., 2011). Interestingly, nimodipine and verapamil are very efficient in preventing microglial activation (Hashioka et al., 2012) and the anti-inflammatory effect of nimodipine has been indicated to down-regulate TNF α and IL1 β expression in the hippocampus and IL1 β expression in microglia (Sanz et al., 2012). Although these studies suggest that Cav1.2 channel blockers might have beneficial effects in multiple sclerosis, the role of voltage-gated Ca^{2+} channel inhibitors in inflammatory demyelinating events has not been established. Cav1.2 channel blockers-mediated effects in the CNS have been claimed to result mainly from the modulation of neuronal activity and microglia survival (Li et al., 2009; Schampel et al., 2017). However, microglial cells do not express functional Cav1.2 channels (Stebbing et al., 2015; Schampel et al., 2017), thus we believe that the anti-inflammatory effects of these drugs are mainly mediated by their effect on astrocytic Ca^{2+} channels.

The Cav1.2 antagonist nimodipine has been found to be beneficial in many CNS disorders, including stroke, brain injury, cerebral ischemia, epilepsy, dementia, and age-related degenerative diseases (Scriabine et al., 1989; Taya et al., 2000; Horn et al., 2001; Li et al., 2009). In this work, we have shown that animals injected with nimodipine during the demyelination stage of the CPZ treatment displayed a reduce number of astrocyte and microglial cells activation and proliferation as well as a faster and more efficient brain remyelination. Cav1.2 channels are not present in mature oligodendrocytes (Paez et al., 2010; Cheli et al., 2015) but they are expressed by OPCs and they are essential for their maturation (Cheli et al., 2016b; Santiago González et al.,

2017). Thus, to prevent negative effects on OPC development, nimodipine was administered at the peak of astrocyte activation and not through the remyelination phase, in which OPC maturation and remyelination happens. Together, our data suggest that blocking Cav1.2 channels in the brain of demyelinated animals significantly promote myelin restoration and oligodendrocyte maturation, and in combination with the results obtained in the astrocytic Cav1.2^{KO} mouse, indicate that these effects are consequence of a reduce population of reactive astrocytes. Because patients treated with brain-permeant dihydropyridines do not experience adverse CNS effects, understanding how these Ca^{2+} channel blockers influence astrocyte reactivity in the context of brain inflammation and demyelination may have therapeutic value.

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