Published in final edited form as:

Exp Neurol. 2016 September ; 283(Pt A): 330–340. doi:10.1016/j.expneurol.2016.06.033.

A mouse model for testing remyelinating therapies

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Abstract

Used in combination with immunomodulatory therapies, remyelinating therapies are a viable therapeutic approach for treating individuals with multiple sclerosis. Studies of postmortem MS brains identified greater remyelination in demyelinated cerebral cortex than in demyelinated brain white matter and implicated reactive astrocytes as an inhibitor of white matter remyelination. An animal model that recapitulates these phenotypes would benefit the development of remyelination therapeutics. We have used a modified cuprizone protocol that causes a consistent and robust demyelination of mouse white matter and cerebral cortex. Spontaneous remyelination occurred significantly faster in the cerebral cortex than in white matter and reactive astrocytes were more abundant in white matter lesions. Remyelination of white matter and cerebral cortex was therapeutically enhanced by daily injections of thyroid hormone triiodothyronine (T3). In summary, we describe an in vivo demyelination/remyelination paradigm that can be powered to determine efficacy of therapies that enhance white matter and cortical remyelination.

Keywords

myelin; differential remyelination; ultra structure; internodal length; therapeutic; mice

Introduction

Multiple sclerosis (MS) is an inflammatory-mediated demyelinating disease of the central nervous system (CNS). While MS has been historically considered a disease of white matter, it is now clear that cortical and deep gray matter demyelination are significant aspects of MS pathogenesis. Less is known about the dynamics and extent of cortical and deep gray matter

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AUTHOR CONTRIBUTIONS

C.B.B., S.S., and D.E. carried out in vivo experiments; A.R. and E.B. performed 3D-EM experiments; A.R., E.B. and G.K. analyzed 3D-EM datasets. W.M. and R.D. contributed ideas for the experiments. C.B.B., S.M. and B.T. designed the studies, analyzed the data and wrote the paper. All authors edited and approved the paper.

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demyelination because these lesions are not apparent in brain imaging studies of living patients. Estimates of cortical demyelination in postmortem brains, however, have raised the possibility that cortical demyelination may exceed white matter demyelination in many MS patients (Gilmore et al., 2009; Kutzelnigg et al., 2005). More importantly, recent studies indicated that cortical remyelination is much more abundant than white matter remyelination in postmortem brains from MS patients (Chang et al., 2012). Reactive astrocytes are a prominent feature of white matter lesions, but not gray matter lesions in MS brains (Chang et al., 2012). These white matter astrocytes express high levels of the glycosaminoglycan, hyaluronan, which inhibit oligodendrocyte differentiation in vitro and remyelination in rodent white matter (Back et al., 2005; Chang et al., 2012; Sloane et al., 2010).

A number of animal models have been developed to mimic different aspects of MS pathogenesis. The commonly used animal model, experimental autoimmune encephalomyelitis (EAE), has provided proof of principle for many anti-inflammatory MS drugs currently in the market (Steinman and Zamvil, 2006). However, CNS destruction in this autoimmune model includes both myelin and axons, and large areas of demyelinated axons do not occur in predictable locations. This has limited the use of EAE as a reliable animal model to study cellular or molecular mechanisms of CNS remyelination (Ransohoff, 2006; Sriram and Steiner, 2005). A second model, Theiler's virus-mediated demyelination, induces demyelination in predictable areas of spinal cord and has been used to test therapeutics that can enhance spinal cord remyelination (Rodriguez and Lennon, 1990). A third model uses toxin induced demyelination where lysolecithin, ethidium bromide or cuprizone destroys myelin or oligodendrocytes (Blakemore and Franklin, 2008). Focal injections of lysolecithin or ethidium bromide destroy myelin and have been successfully used to test remyelinating therapeutics (Fancy et al., 2009; Huang et al., 2011). This model is technically demanding and restricted to small areas of the CNS around the injection site. In comparison, cuprizone demyelinate both white and gray matter in the brain of C57BL/6J mice (Gudi et al., 2009; Matsushima and Morell, 2001a; Skripuletz et al., 2008). However, because cuprizone does not efficiently destroy new oligodendrocytes, significant spontaneous remyelination occurs during cuprizone-induced demyelination (Koutsoudaki et al., 2010; Mason et al., 2001; Matsushima and Morell, 2001b; Sachs et al., 2014). This results in significant heterogeneity of myelinated axons between and within animals following cuprizone treatment (Schmidt et al., 2013; Stidworthy et al., 2003). The use of different remyelination readouts with variable sensitivity and specificity, including myelin protein immunocytochemistry, histological stains (Luxol fast blue [LFB] and black gold) and/or quantitative Western blots, further complicates comparison of results in different cuprizone studies.

Ultrastructurally, remyelinated internodes are thinner and shorter than normally myelinated internodes. The ratio between axonal circumference and myelin circumference (g-ratio) decreases with normal myelination and is significantly greater following remyelination (Blakemore, 1974). While decreased g-ratios are a reliable indication of remyelination of large diameter axons (Michailov et al., 2004), the use of g-ratio as an indicator of remyelination of small diameter axons in the corpus callosum has been controversial (Franklin and Ffrench-Constant, 2008; Klopfeisch et al., 2008; Mason et al., 2001; Moore et

al., 2014; Poliani et al., 2015; Stidworthy et al., 2003). How internodal lengths correlate with the extent of remyelination of corpus callosum axons has not been established.

The AKT pathway is involved in oligodendrocyte differentiation, as constitutive activation of mTOR in oligodendrocytes resulted in fatal hypermyelination (Flores et al., 2008; Narayanan et al., 2009). This led to the idea that suppression of mTOR activity may inhibit spontaneous remyelination that occurs during cuprizone treatment. Indeed when daily intraperitoneal injections of rapamycin (an mTOR inhibitor) were incorporated in the traditional 6-week cuprizone model, spontaneous remyelination in the corpus callosum was significantly reduced (Sachs et al., 2014). However, detecting therapeutically enhanced remyelination following 6 weeks of cup/rap demyelination is challenging due to the rapid rate of spontaneous remyelination. In the present study, we treated mice with cuprizone plus rapamycin (cup/rap) for 12 weeks. We show here, that 12 weeks of cup/rap demyelination reduced the rate of spontaneous remyelination, recapitulated aspects of demyelination/remyelination in MS brains and can be used to identify therapeutics that enhances remyelination in both white and gray matter.

Material and methods

Animals treatment

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Cleveland Clinic. Six-week old C57/BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used for all experiments. Upon arrival, mice were placed on standard chow for 10–14 days. To induce demyelination, mice were fed a diet containing 0.3% cuprizone (bis-cyclohexanone oxaldihydrazone, Sigma-Aldrich), thoroughly mixed into standard chow and custom-made into pellets by Harlan Teklad (Madison, WI), for 12 weeks *ad libitum*. During this time, mice were also given daily intraperitoneal injection of rapamycin (10 mg/kg body weight), reconstituted fresh in vehicle solution (5% PEG-1000, 5% Tween-80 and 5% ethanol). Cuprizone chow was changed every two days and the weight of mice was monitored weekly. At the end of the 12-week demyelination, mice were either perfused to assess the extent of demyelination, or returned to normal chow minus rapamycin injections to allow for spontaneous remyelination for one to six weeks. Mice were anesthetized with ketamine/xylazine followed by perfusion with 4% paraformaldehyde (PFA) in 0.08M phosphate buffer.

Tissue processing

The same brain was used to analyze remyelination in cortex, hippocampus and corpus callosum. To obtain corpus callosum tissues for analysis, the brain was placed into a custom-made brain mold containing mm-thick slicing slots for precision slicing. A 1 mm-thick coronal brain slice, located approximately at Bregma 0 to –1 mm, was immediately placed in 2.5% glutaraldehyde and 4% PFA for two additional days. Subsequently, this brain slice was cut in half, and the corpus callosum near the midline (~5×1×1 mm) was processed and embedded for 1 µm-thick sections and 3D-EM analyses. Corpus callosum ultrastructure was similar to that obtained from animals perfused with 2.5% glutaraldehyde and 4% PFA.

Following extraction of the 1 mm-thick slice, the rest of the brain pieces (rostral and caudal) were returned to 4% paraformaldehyde fixative for two days and used for gray matter remyelination analysis. The caudal piece that contains hippocampus was mounted on a Leica freezing microtome and sliced at 30 μ m thickness. Slices were collected and transferred to 24-well plates containing cryostorage solution (0.2M phosphate solution, pH 7.4; 1% polyvinylpyrrolidone; 30% sucrose, 30% ethylene glycol), and examined under dissecting microscope to determine the approximate anteroposterior position. Two 30 μ m-thick brain sections, located at approximately ~Bregma –1.0 to –1.5 mm, were selected for immunohistological staining and analysis for both hippocampus and cortex.

Immunohistochemical staining for free floating slices

Thirty-micron thick brain slices were rinsed in phosphate-buffered saline (PBS), microwaved in 0.01M citric buffer (pH 6.0) for 2 minutes, rinsed in PBS and incubated in 3% hydrogen peroxide and 0.1% Triton-X100 in PBS for 30 minutes. Slices were blocked with 3% normal serum and incubated overnight with primary antibody at 4°C. Slices were then rinsed in PBS with 0.1% Triton X-100 and stained by the avidin-biotin complex (ABC) procedure and diaminobenzidine as described previously (Trapp et al., 1997). After several washes, sections were mounted on glass slides and dried overnight at room temperature. Primary antibodies included: rat anti-proteolipid protein AA3 (1:5000) (Yamamura et al., 1991) and mouse monoclonal Iba1 (1:500, Cleveland Clinic Hybridoma Core), CD44 (1:40, StemCell Technologies) and GFAP (1:5000, DAKO). Hyaluronan was localized with a biotinylated hyaluronan-binding protein (1:50, EMD Millipore).

Quantification of PLP staining in hippocampus and cerebral cortex

PLP-stained sections were scanned in a Mirax high resolution slide scanner equipped with a 20x objective (Zeiss). Each image tile was acquired from 5-focal levels that are 2 μ m apart and projected into one final composite. Images were imported into NIH ImageJ software for analysis. For hippocampus, the ROI was determined by anatomical boundary, excluding borders that are not completely demyelinated following cup/rap treatment (Fig. 1A). For cerebral cortex, the ROI was defined as the region containing piriform cortex lateral to the amygdalar capsule (Fig. 1A). Once ROIs had been outlined, color images were converted into 8-bit grayscale images and thresholded based on intensity. The threshold masks and areas of ROIs were measured to calculate percentage area occupied by PLP staining. The thresholded mask was also pasted over the original image to confirm accuracy of thresholding. Two brain slices were used per mouse, and the results from the hippocampus or cortex were averaged.

Quantification of myelinated axons in corpus callosum

For 1 μ m-thick analysis of myelinated axons, the midline corpus callosum was processed through osmium tetroxide, dehydrated and embedded in epon resin (Electron Microscopy Sciences), and cut into 1 μ m-thick serial sections. Approximately 50–70 sections were collected on 10–12 microscope slides. One from every four slides was stained with toluidine blue and examined under the microscope to determine the slide numbers that are ~0.6 mm lateral to the midline. Two new slides were then selected and stained with 4% paraphenylenediamine (PPD) (Sigma) to visualize myelinated axons. From each slide, two

corpus callosum images were taken from the ROI, which had been validated in our proof-of-concept studies and is approximately ~350 μm rostral to the junction of corpus callosum and fornix (Fig. 1A), using a 63x oil immersion objective (N.A. 1.4). A total of 4 images were obtained from each brain. Images were then imported into ImageJ software for counting. To facilitate counting, a grid of 300 μm^2 was overlaid on top of the images and the number of myelinated axons in each image was counted manually. In the case where there were more than five hundred myelinated axons in one image, myelinated axons in 9 pre-determined boxes were counted and extrapolated for that image. Myelinated axon counts from four images per brain were averaged to obtain a final count for each brain. The counts represent the number of myelinated axons in a 180 μm unit length of longitudinal corpus callosum in the ROI.

3D-EM morphometric analysis

Corpus callosum tissue was stained with OsO_4 -ferricyanide, followed by thiocarbohydrazide, osmium tetroxide, aqueous uranyl acetate, and lead aspartate, dehydrated, and embedded in epon for 3D-EM imaging. Tissue blocks were imaged utilizing a Zeiss Sigma VP scanning electron microscope, fitted with a Gatan 3view2 in-chamber ultra-microtome and a low kV backscatter electron detector. Corpus callosum was imaged in coronal plane at a resolution of 7–10 nm/pixel and slice thicknesses of 60–90 nm at 2.25 kV. Images were imported into ImageJ software for three-dimensional reconstruction. For analysis of internodal length, approximately 15–50 axons, with diameter larger than 0.3 μm , were traced from each block. The distances from the end of the outermost paranodal loops were used to measure internodal length.

For electron microscopy analysis of axon numbers in tissues embedded for PPD staining, 1 μm -thick sections were mounted on coverslips and stained with TEM stains (saturated uranyl acetate in 60% methanol for 10 minutes at 60°C and then lead citrate for 10 minutes at room temperature). Sections were imaged using backscatter electron detector as above. Three fields of corpus callosum were selected at low magnification and then imaged at 8–10 nm/pixel, with beam energy of 2.2 kV which generates an image from ~25 nm-thick slice (interaction volume) beneath the section surface comparable to a thin TEM section. Both myelinated and unmyelinated axons were identified in ImageJ and only axons with diameter larger than 0.3 μm were counted.

Statistical analysis

Samples were analyzed blindly. Parametric or non-parametric statistics were used to assess significant differences. All data sets were first tested for normality using Kolmogorov-Smirnov or Shapiro-Wilk tests. For data with normal distribution, two-tailed Student's *t*-test or one-way ANOVA was used, followed by post hoc testing using Holm-Sidak (or Tukey) method for pair-wise comparison as recommended by SigmaPlot/SigmaStat (SYSTAT, San Jose, CA) or open source software Rkward (KDE.org). Data that did not pass normality test or equal variance test were analyzed using Mann-Whitney Rank Sum Test/U statistics or Kruskal-Wallis ANOVA on Ranks. Outliers were identified based on Chauvenet's criterion and excluded from the analysis. Data were presented as mean $\pm$ standard deviation (SD) or

standard error of the mean (SEM) as indicated. Based upon power analyses, typically 8–15 mice were included in individual experiments.

Results

Rapamycin significantly reduces spontaneous remyelination in 12-week cuprizone model

To comprehensively characterize the 12-week cup/rap-mediated demyelination, we examined corpus callosum, cerebral cortex and hippocampus of rap-only (CTL), cup-only and cup/rap treated mice. In 1 μ m-thick epon sections of corpus callosum stained with paraphenylenediamine (PPD) (Fig. 1A, C–E), the number of myelinated axons was $10,315 \pm 976$ within the region of interest (ROI) in CTL mice (Mean $\pm$ SD), $2,841 \pm 608$ in cup-only mice ($\sim 27\%$ of CTL), and 56 ± 49 in cup/rap mice ($\sim 0.5\%$ of CTL) (Fig. 1I). In cerebral cortex and hippocampus, myelin density within perspective ROIs (Fig. 1B) was quantified following immunostaining with PLP antibody. In cerebral cortex, myelin density was $50.1 \pm 2.0\%$ in CTL, $10.5 \pm 1.3\%$ in cup-only mice and $9.8 \pm 0.2\%$ in cup/rap mice (Fig. 1F–H, J). Similarly, hippocampal myelin density was reduced from $47.1 \pm 2.5\%$ in CTL mice to $2.7 \pm 0.7\%$ in cup-only mice, and $2.7 \pm 0.2\%$ in cup/rap mice (Fig. 1K). These results show that rapamycin significantly reduced spontaneous remyelination in the corpus callosum during 12 weeks of cuprizone treatment. Importantly, rapamycin treatment decreased the variation in cortical and hippocampal demyelination, which is critical for powering quantitative remyelination studies.

Astrocytosis and inhibition of myelination in the cup/rap model

To investigate whether astrocytes and microglia are differentially activated in demyelinated white and gray matter following cup/rap treatment, we compared GFAP and Iba1 staining in cup/rap and CTL mice. In corpus callosum, the density of GFAP staining increased ~ 6 fold from $11.2 \pm 1.1\%$ in CTL to $69.6 \pm 3.7\%$ in cup/rap treated mice ($P < 0.01$, t -test) (Fig. 2A–D). In contrast, GFAP staining density increased ~ 2 fold in the cerebral cortex and was unchanged in the hippocampus (Fig. 2A, B, E). Similarly, the density of Iba1 in corpus callosum increased significantly, from $8.2 \pm 2.7\%$ in CTL to $34.7 \pm 2.4\%$ in cup/rap treated mice ($P < 0.01$, t -test) (Fig. 2F–J), while there were no significant changes in cortex or hippocampus (Fig. 2F, G, J). Next we determined whether there was increase in the expression of hyaluronan and CD44, a surface membrane protein participating in binding and organizing hyaluronan (Ponta et al., 2003). Compared with CTL mice, cup/rap mice showed significant increases in immunoreactivities of both hyaluronan and CD44 in corpus callosum (Fig. 2K–T, $P < 0.01$, t -test), while no significant changes occurred in cerebral cortex or hippocampus (Fig. 2O, T). These results show a robust and differential activation of astrocytes and microglia, and increased levels of hyaluronan and hyaluronan-binding membrane protein following 12 weeks of cup/rap demyelination of white matter compared to gray matter, similar to what was described in postmortem MS brains (Back et al., 2005; Chang et al., 2012).

Differential remyelination in gray and white matter of cup/rap model

We next compared white and gray matter remyelination rate following 12 weeks of cup/rap treatment. At 12 week, there were few myelinated axons in corpus callosum of cup/rap mice

($0.5 \pm 0.4\%$ of CTL, Fig. 3A, B, K). As remyelination proceeded, the density of myelinated axons progressively increased from $2.1 \pm 0.47\%$ of CTL at 12+1W to $7.9 \pm 4.22\%$ at 12+3W, and $22.3 \pm 7.8\%$ at 12+6W (Fig. 3C–E, K). Remyelination in cerebral cortex and hippocampus was more robust than in corpus callosum. In cerebral cortex, the density of myelinated axons increased from $33 \pm 19.6\%$ of CTL at 12+1W to $46.2 \pm 13.3\%$ at 12+3W, and $83.2 \pm 8.2\%$ at 12+6W (Fig. 3K). Similarly, the density of myelin in hippocampus increased from $11.6 \pm 4.1\%$ of CTL at 12+1W to $40.3 \pm 10.9\%$ at 12+3W, and $61.2 \pm 8.8\%$ at 12+6W (Fig. 3F–J, K).

To directly compare the rate of remyelination in gray and white matter, we performed linear regression analysis of remyelination in corpus callosum, cerebral cortex and hippocampus, and used parallel line analysis to determine whether slopes of regression lines were significantly different in white and gray matter. Remyelination rate in gray matter (cerebral cortex or hippocampus) was significantly faster than that in white matter (corpus callosum) ($P < 0.01$, parallel line analysis) (Fig. 3L), similar to what was observed in MS lesions (Chang et al., 2012).

To validate the use of quantifying myelinated axons in μm -thick epon sections of corpus callosum, we compared the density of myelinated axons in μm -thick sections and electron micrographs from 12+3W and 12+6W corpus callosi. In μm -thick sections, the density of myelinated axons increased ~ 2.2 fold, from 4.9 ± 1.09 (per $100 \mu\text{m}^2$ corpus callosum, mean $\pm$ SEM) at 12+3W to 11.07 ± 0.99 at 12+6W. In electron micrographs, we quantified the percentage of axons greater than $0.3 \mu\text{m}$ that were myelinated. Myelinated axons increased 1.95 fold, from $22 \pm 2\%$ (mean $\pm$ SEM) at 12+3W to $43 \pm 3\%$ at 12+6W (Fig. 3M). These results validated the use of myelinated axon counts in μm -thick epon sections of corpus callosum.

Three dimensional electron microscopy (3D-EM) study of remyelination in the corpus callosum

To address how myelin ultrastructure changes during remyelination in the cup/rap model, we captured stacks of 300–500 serial EM images per corpus callosum at different remyelination time points and digitally reconstructed the images into 3D stacks (Fig. 4A). The few myelinated fibers present following 12 weeks of cup/rap treatment had average internodal lengths of $57.6 \pm 8.63 \mu\text{m}$ (Mean $\pm$ SEM), much shorter than those in CTL ($106 \pm 5.8 \mu\text{m}$, Fig. 4B), indicating that internodes present at 12W are remyelinated rather than myelinated fibers that survived cup/rap treatment. Following three weeks of remyelination, internodal length increased to $62.2 \pm 4.7 \mu\text{m}$ and was still significantly less than that found in CTL corpus callosum. However at 12+6W, the average internodal length doubled to $103 \pm 3.2 \mu\text{m}$ and was similar to internodal lengths in CTL corpus callosum. To determine how g-ratio changed during remyelination, we plotted myelin thickness and g-ratio against diameter of individual myelinated axons (Fig. 4D). There was a wide spread of myelin thickness for axons of different diameters, including those in corpus callosum of CTL mice. In addition, g-ratio appears to have a complex relationship with axon diameter. For axons smaller than $1 \mu\text{m}$, g-ratio increases as axons get larger. However, for axons larger than $1 \mu\text{m}$, the slope of the g-ratio increase becomes smaller as axonal diameters increase (Fig. 4D). When g-ratios

were compared among remyelination groups, no significant differences in g-ratio between CTL and cup/rap treated groups were found (Fig. 4C, $P>0.05$, ANOVA), indicating that g-ratios are not a reliable correlate of remyelination in the region of corpus callosum examined.

Therapeutic enhancement of remyelination following cup/rap demyelination

T3 increases OPC differentiation *in vitro* (Baas et al., 1997; Jones et al., 2003; Rodríguez-Peña, 1999). Based upon T2-weighted magnetic resonance imaging (Harsan et al., 2008), T3 may also accelerate remyelination in mouse corpus callosum following cuprizone-induced demyelination. To determine whether the 12-week cup/rap model provides a sensitive environment to test for enhancement of remyelination, we injected mice with T3 (3 mg/kg) daily for 3 and 6 weeks following removal of cup/rap. At 12+3W, we found a significant increase in hippocampal (Fig. 5A, $P<0.01$) and cortical remyelination (Fig. 5A, $P<0.01$) in T3-injected mice compared with vehicle-injected mice. However, no significant difference was found in the number of myelinated axons in corpus callosum between T3-injected and vehicle-injected mice (Fig. 5A). We then examined mice that received six weeks of T3. At 12+6W, T3-injected mice showed significantly higher level of remyelination in hippocampus (Fig. 5B, C, D, $P<0.01$), cerebral cortex (Fig. 5B, E, F, $P<0.01$) and corpus callosum (Fig. 5B, G, H, $P<0.01$) compared with vehicle-injected mice. These results establish that the 12-week cup/rap model provides a sensitive platform for testing remyelination therapeutics in both white and gray matter.

Discussion

The cuprizone model offers several advantages for investigating remyelinating therapies as it includes both white and gray matter demyelination. Due to variable cuprizone dosing paradigms and qualitative assessments of remyelination, standardization of the cuprizone remyelination model has not been achieved. The present study describes a cuprizone demyelination paradigm that recapitulates features relevant to remyelination in MS brains, including greater remyelination of gray matter and increased astrogliosis of demyelinated white matter. Most importantly, robust and reproducible quantification of myelinated axons established that the paradigm can detect therapeutic enhancement of remyelination in both white and gray matter. Our studies described a robust pre-clinical *in vivo* model that can reliably test the efficacy of remyelination therapies.

Heterogeneity of demyelination in corpus callosum and inter-animal variability have limited the utility of the cuprizone model (Armstrong et al., 2002; Stidworthy et al., 2003). Factors contributing to the variation include genetics (species, age etc), treatment paradigms and readouts (Gudi et al., 2009; Hiremath et al., 2008; Matsushima and Morell, 2001a; Skripuletz et al., 2008; Taylor et al., 2009). For example, cuprizone treatment for 4, 5, 6, and 7 to 12 weeks has been employed and has resulted in different levels of demyelination/remyelination (Deshmukh et al., 2013; Kang et al., 2012; Moharreh-Khiabani et al., 2010; Olah et al., 2012; Schmidt et al., 2013; Skripuletz et al., 2012; Steelman et al., 2012). Primary readouts used to quantify remyelination also vary and include myelin protein staining (PLP or MBP), histological stains (LFB, black gold, and FluoroMyelin) and

quantitative Western blots. In some reports, histological evaluations are supported by qualitative assessment of myelinated axons in electron micrographs (Deshmukh et al., 2013; Kang et al., 2012; Koutsoudaki et al., 2010; Larsen et al., 2006; Liu et al., 2010; Mi et al., 2011; Moharreh-Khiabani et al., 2010; Olah et al., 2012; Schmidt et al., 2013; Wergeland et al., 2011). These readouts have different sensitivity and specificity. For example, LFB does not reliably stain gray matter myelin (Scripuletz et al., 2008). Lipophilic dyes and myelin protein antibodies do not distinguish between normal myelin and myelin debris, which is commonly found in corpus callosum during demyelination. Our own comparison found that black gold produced false positive and false negative staining in different regions of the brain when compared with PLP staining in adjacent sections (Supplementary Fig. 1). Western blotting can accurately quantify myelin proteins, but requires substantial amounts of tissue and includes myelin debris. In white matter, myelin protein immunostaining has shortcomings, as individual myelinated corpus callosum axons are too small and too close together to be counted. These intensity-based “myelin indexes” have rarely been correlated with myelinated axon numbers in the corpus callosum.

We employed several steps to reduce variability and increase sensitivity of quantifying myelinated axons. First and foremost is the use of rapamycin to inhibit spontaneous regeneration of oligodendrocytes during cuprizone feeding. The utility of this approach was evident following 6 weeks of cuprizone treatment, where significantly greater and more consistent demyelination was noted in cup/rap treated mice (Sachs et al., 2014). The current study extended these earlier observations and focused on 12 weeks of cup/rap treatment, which significantly decreased myelinated corpus callosum axons from $2,841 \pm 608$ in cup-only treated mice to 56 ± 49 in cup/rap treated mice. If both cup-only and cup/rap models were to be used for detection of an increase of 100 myelinated axons, the 12 week cup/rap model would only need 5 mice per group to detect a statistically significant increase in remyelinated axons, while the cup-only model would need 582 mice per group (assuming power of 0.8 and alpha of 0.05 using *t*-test statistics). Besides affecting myelination, rapamycin has also been implicated in regulating other cellular processes, including immune response, metabolism and growth (Janes and Fruman, 2009; Wullschleger et al., 2006). However, rapamycin does not appear to significantly affect activation of microglia and astrocytes, two cell types that are needed for myelin removal (Scripuletz et al., 2012), or PLP expression when brains from mice receiving 12 weeks of rapamycin injection were compared with that of the naïve mice (Supplementary Fig. 2). In addition, rapamycin does not appear to significantly affect adult mice, as these mice behave normally after 12 weeks of daily injections. In this respect, it is interesting to note that long-term (1–2 years) daily feeding of rapamycin to adult mice significantly increases lifespan of both male and female mice (Harrison et al., 2009). In the current studies, the effect of rapamycin during remyelination was minimized because the half-life of rapamycin is only about 6 hr (Comas et al., 2012) and rapamycin was discontinued before drug treatment. The extent of demyelination and rates of remyelination reported here have been consistently found in hundreds of mice used as controls for testing various remyelinating therapies, with different batches of chow and different lots of cuprizone. Furthermore, the mortality rate was less than 5% and the body weight loss in mice receiving cup/rap treatment (Supplementary Fig. 3) is similar to those receiving cuprizone-alone treatment (Sachs et al., 2014). The second

crucial aspect of standardizing this assay was the identification and validation of specific areas of corpus callosum and cerebral cortex that were consistently demyelinated and amenable to drug-induced remyelination, thus reducing tissue heterogeneity. The third aspect is robust detection and quantification methods, including PLP immunostaining to detect myelin in cerebral cortex and hippocampus, and PPD staining to detect myelinated axons in 1 μ m-thick epon sections. In our studies of corpus callosum, quantifying myelinated axons in micron-thick epon sections was a reliable and reproducible readout of remyelination. This conclusion was validated and extended by analysis of the same 1 μ m-thick sections by electron microscopy (Fig. 3M).

At the ultra-structural level, we also identified myelin internodal length as a sensitive readout of early remyelination in corpus callosum. Compared to myelin internodes in WT corpus callosum, internodal length was significantly shorter in the few myelinated fibers present at 12W and 12+3W. However, internodal length increased significantly by 12+6W, and was similar to that of the control corpus callosum (Fig. 4B). While g-ratio can be a valid measure for development and repair of large diameter myelinated axons (Franklin and Ffrench-Constant, 2008; Michailov et al., 2004), it was not a valid indicator of remyelination of small diameter axons in the region of corpus callosum analyzed in this study, because g-ratios did not change with remyelination time and were similar in remyelinated and normally myelinated corpus callosum (Fig. 4C).

Previous studies of postmortem leukocortical lesions from individuals with MS implied that remyelination was greater in the cerebral cortex than in subcortical white matter, and that increased sulphated proteoglycans may inhibit white matter remyelination (Chang et al., 2012). Regression line analyses of remyelination rates in our model support this conclusion and establish that cortical and hippocampal remyelination is more robust than corpus callosum remyelination (Fig. 3L). Since demyelination in gray matter is not as complete as that in white matter, an alternative explanation is that faster remyelination occurs because there is less demyelination. However, this seems unlikely because there is no significant difference in the rate of remyelination in cortex and hippocampus ($P>0.05$), despite the fact that the hippocampus is demyelinated to a greater extent than cortex (Fig. 3L). In addition, the rate of remyelination remained relatively constant between 12+1W and 12+6W in all three tissues, despite the different extent of demyelination at 12W. It was also proposed that the decreased rate of white matter remyelination in MS brains was influenced by reactive astrocytes which secrete and organize extracellular matrix proteins that inhibit oligodendrocyte differentiation and myelination (Lau et al., 2013; Ponta et al., 2003; Sherman and Back, 2008). Our studies support this hypothesis and detected significant accumulation of hyaluronan and hyaluronan-binding protein CD44 in the demyelinated corpus callosum, but not in demyelinated cerebral cortex.

In addition to hyaluronan, other extracellular membrane proteins and signaling molecules have also been implicated in remyelination failure of MS. These inhibitors could impact different aspects of the remyelination process, including OPC migration, oligodendrocyte differentiation and myelination. For example, surface protein polysialylated-neural cell adhesion molecule (PSA-NCAM), which is expressed at high levels in chronic MS Lesions (Charles et al., 2002, 2000), may inhibit myelin sheet formation during remyelination.

Signaling molecules, such as jagged1 of the Notch pathway and Tcf4 of the Wnt signaling pathway, are up-regulated in or around MS lesions and may inhibit OPC differentiation and remyelination (Fancy et al., 2009; John et al., 2002). Myelin debris produced during demyelination contains several myelin associated inhibitors and may inhibit OPC differentiation and remyelination (Kotter et al., 2006). Additional factors, such as repulsive guidance protein Semaphorin 3A, was found to be up-regulated in early MS lesions which may inhibit repopulation of OPCs and remyelination (Williams et al., 2007). Another inhibitor of axonal outgrowth, LINGO-1, was found to inhibit oligodendrocyte differentiation and to express in white matter tracks following lesion (Mi et al., 2004). Blocking LINGO-1 function appears to promote CNS remyelination in both slice culture systems and in mouse models (Mi et al., 2009). Due to the large size of white and gray matter lesion area, the cup/rap model is conducive to biochemical analysis and elucidation of factors that inhibit remyelination in white matter.

There are several advantages to having a robust and reproducible rodent model of demyelination/remyelination. Molecular and cellular hypothesis regarding mechanisms responsible for enhanced or restricted remyelination can be tested if the dynamics of the remyelination in the animal model can be manipulated. An important question was whether the cup/rap model has enough power to detect statistical differences in remyelination. We show that T3 significantly increased remyelination in both gray matter and white matter (Fig. 5). Interestingly, the time it takes to produce a significant increase in remyelination is different in gray matter and white matter: three weeks in gray matter versus six weeks in white matter. The slower increase in remyelination in white matter likely reflects a less favorable microenvironment due to accumulation of extracellular inhibitors such as hyaluronan (Fig. 2), rather than different effects of T3 on different tissues, because remyelination steadily increased from 12W to 12+3W and 12+6W in both gray matter and white matter. The ability to detect drug-induced remyelination in both gray and white matter demonstrates the utility of the cup/rap model.

Degeneration of demyelinated axons is a major cause of permanent neurological disability in individuals with MS. Remyelination restores saltatory conduction and provides trophic support that enhances axon survival (Crawford et al., 2009; Duncan et al., 2009; Trapp and Stys, 2009). At present, remyelination is the best documented neuroprotective strategy for individual with MS. Development of future remyelinating strategies will require validation of proof-of-principle in animal models. The data presented here details an experimental paradigm that recapitulate many aspects of demyelination and remyelination in MS brains and provides a reliable platform for testing remyelinating therapeutics. Combining anti-inflammatory and remyelinating therapeutics should decrease permanent neurological disability and possibly restore neurological function in MS patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Caroline Lego, Yongming Jin, Kate Donatto, Doug Chmura, Tasha Elmer, Melissa Maddie for technical assistance to the project. We also thank other members of the Renovo team for help throughout the project. The study was supported by grants from Ohio Third Frontier Program and the NIH R01NS080976 (BDT).

References

- Armstrong RC, Le TQ, Frost EE, Borke RC, Vana AC. Absence of fibroblast growth factor 2 promotes oligodendroglial repopulation of demyelinated white matter. *J Neurosci*. 2002; 22:8574–8585. [PubMed: 12351731]
- Baas D, Bourbeau D, Sarliève LL, Ittel ME, Dussault JH, Puymirat J. Oligodendrocyte maturation and progenitor cell proliferation are independently regulated by thyroid hormone. *Glia*. 1997; 19:324–332. [PubMed: 9097076]
- Back SA, Tuohy TMF, Chen H, Wallingford N, Craig A, Struve J, Luo NL, Banine F, Liu Y, Chang A, Trapp BD. Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation. *Nat Med*. 2005; 11:966–972. [PubMed: 16086023]
- Blakemore WF. Remyelination of the superior cerebellar peduncle in old mice following demyelination induced by cuprizone. *J Neurol Sci*. 1974; 22:121–126. [PubMed: 4830551]
- Blakemore WF, Franklin RJM. Remyelination in experimental models of toxin-induced demyelination. *Curr Top Microbiol Immunol*. 2008; 318:193–212. [PubMed: 18219819]
- Chang A, Staugaitis SM, Dutta R, Batt CE, Easley KE, Chomyk AM, Yong VW, Fox RJ, Kidd GJ, Trapp BD. Cortical remyelination: a new target for repair therapies in multiple sclerosis. *Ann Neurol*. 2012; 72:918–926. DOI: 10.1002/ana.23693 [PubMed: 23076662]
- Charles P, Hernandez MP, Stankoff B, Aigrot MS, Colin C, Rougon G, Zalc B, Lubetzki C. Negative regulation of central nervous system myelination by polysialylated-neural cell adhesion molecule. *Proc Natl Acad Sci U A*. 2000; 97:7585–90.
- Charles P, Reynolds R, Seilhean D, Rougon G, Aigrot MS, Niezgoda A, Zalc B, Lubetzki C. Re-expression of PSA-NCAM by demyelinated axons: an inhibitor of remyelination in multiple sclerosis? *Brain J Neurol*. 2002; 125:1972–1979.
- Comas M, Toshkov I, Kuropatwinski KK, Chernova OB, Polinsky A, Blagosklonny MV, Gudkov AV, Antoch MP. New nanoformulation of rapamycin Rapatar extends lifespan in homozygous p53^{-/-} mice by delaying carcinogenesis. *Aging*. 2012; 4:715–722. [PubMed: 23117593]
- Crawford DK, Mangiardi M, Xia X, Lopez-Valdes HE, Tiwari-Woodruff SK. Functional recovery of callosal axons following demyelination: a critical window. *Neuroscience*. 2009; 164:1407–1421. [PubMed: 19800949]
- Deshmukh VA, Tardif V, Lyssiotis CA, Green CC, Kerman B, Kim HJ, Padmanabhan K, Swoboda JG, Ahmad I, Kondo T, Gage FH, Theofilopoulos AN, Lawson BR, Schultz PG, Lairson LL. A regenerative approach to the treatment of multiple sclerosis. *Nature*. 2013; 502:327–332. DOI: 10.1038/nature12647 [PubMed: 24107995]
- Duncan ID, Brower A, Kondo Y, Curlee JF, Schultz RD. Extensive remyelination of the CNS leads to functional recovery. *Proc Natl Acad Sci U A*. 2009; 106:6832–6.
- Fancy SP, Baranzini SE, Zhao C, Yuk DI, Irvine KA, Kaing S, Sanai N, Franklin RJ, Rowitch DH. Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS. *Genes Dev*. 2009; 23:1571–85. [PubMed: 19515974]
- Flores AI, Narayanan SP, Morse EN, Shick HE, Yin X, Kidd G, Avila RL, Kirschner DA, Macklin WB. Constitutively active Akt induces enhanced myelination in the CNS. *J Neurosci*. 2008; 28:7174–83. [PubMed: 18614687]
- Franklin RJ, Ffrench-Constant C. Remyelination in the CNS: from biology to therapy. *Nat Rev Neurosci*. 2008; 9:839–55. [PubMed: 18931697]
- Gilmore CP, Donaldson I, Bö L, Owens T, Lowe J, Evangelou N. Regional variations in the extent and pattern of grey matter demyelination in multiple sclerosis: a comparison between the cerebral cortex, cerebellar cortex, deep grey matter nuclei and the spinal cord. *J Neurol Neurosurg Psychiatry*. 2009; 80:182–187. DOI: 10.1136/jnnp.2008.148767 [PubMed: 18829630]

- Gudi V, Moharreggh-Khiabani D, Skripuletz T, Koutsoudaki PN, Kotsiari A, Skuljec J, Trebst C, Stangel M. Regional differences between grey and white matter in cuprizone induced demyelination. *Brain Res.* 2009; 1283:127–138. [PubMed: 19524552]
- Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature.* 2009; 460:392–395. DOI: 10.1038/nature08221 [PubMed: 19587680]
- Harsan LA, Steibel J, Zaremba A, Agin A, Sapin R, Poulet P, Guignard B, Parizel N, Grucker D, Boehm N, Miller RH, Ghandour MS. Recovery from chronic demyelination by thyroid hormone therapy: myelinogenesis induction and assessment by diffusion tensor magnetic resonance imaging. *J Neurosci.* 2008; 28:14189–14201. DOI: 10.1523/JNEUROSCI.4453-08.2008 [PubMed: 19109501]
- Hiremath MM, Chen VS, Suzuki K, Ting JPY, Matsushima GK. MHC class II exacerbates demyelination in vivo independently of T cells. *J Neuroimmunol.* 2008; 203:23–32. DOI: 10.1016/j.jneuroim.2008.06.034 [PubMed: 18805594]
- Huang JK, Jarjour AA, Nait Oumesmar B, Kerninon C, Williams A, Krezel W, Kagechika H, Bauer J, Zhao C, Evercooren AB, Chambon P, Ffrench-Constant C, Franklin RJ. Retinoid X receptor gamma signaling accelerates CNS remyelination. *Nat Neurosci.* 2011; 14:45–53. [PubMed: 21131950]
- Janes MR, Fruman DA. Immune regulation by rapamycin: moving beyond T cells. *Sci Signal.* 2009; 2:pe25. [PubMed: 19383976]
- John GR, Shankar SL, Shafit-Zagardo B, Massimi A, Lee SC, Raine CS, Brosnan CF. Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation. *Nat Med.* 2002; 8:1115–1121. DOI: 10.1038/nm781 [PubMed: 12357247]
- Jones SA, Jolson DM, Cuta KK, Mariash CN, Anderson GW. Triiodothyronine is a survival factor for developing oligodendrocytes. *Mol Cell Endocrinol.* 2003; 199:49–60. [PubMed: 12581879]
- Kang Z, Liu L, Spangler R, Spear C, Wang C, Gulen MF, Veenstra M, Ouyang W, Ransohoff RM, Li X. IL-17-induced Act1-mediated signaling is critical for cuprizone-induced demyelination. *J Neurosci.* 2012; 32:8284–8292. DOI: 10.1523/JNEUROSCI.0841-12.2012 [PubMed: 22699909]
- Klopfleisch S, Merkler D, Schmitz M, Klöppner S, Schedensack M, Jeserich G, Althaus HH, Brück W. Negative impact of statins on oligodendrocytes and myelin formation in vitro and in vivo. *J Neurosci Off J Soc Neurosci.* 2008; 28:13609–13614. DOI: 10.1523/JNEUROSCI.2765-08.2008
- Kotter MR, Li WW, Zhao C, Franklin RJM. Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *J Neurosci Off J Soc Neurosci.* 2006; 26:328–332. DOI: 10.1523/JNEUROSCI.2615-05.2006
- Koutsoudaki PN, Hildebrandt H, Gudi V, Skripuletz T, Skuljec J, Stangel M. Remyelination after cuprizone induced demyelination is accelerated in mice deficient in the polysialic acid synthesizing enzyme St8siaIV. *Neuroscience.* 2010; 171:235–244. [PubMed: 20833231]
- Kutzelnigg A, Lucchinetti CF, Stadelmann C, Brück W, Rauschka H, Bergmann M, Schmidbauer M, Parisi JE, Lassmann H. Cortical demyelination and diffuse white matter injury in multiple sclerosis. *Brain J Neurol.* 2005; 128:2705–2712. DOI: 10.1093/brain/awh641
- Larsen PH, DaSilva AG, Conant K, Yong VW. Myelin Formation during Development of the CNS Is Delayed in Matrix Metalloproteinase-9 and -12 Null Mice. *J Neurosci.* 2006; 26:2207–2214. DOI: 10.1523/JNEUROSCI.1880-05.2006 [PubMed: 16495447]
- Lau LW, Cua R, Keough MB, Haylock-Jacobs S, Yong VW. Pathophysiology of the brain extracellular matrix: a new target for remyelination. *Nat Rev Neurosci.* 2013; 14:722–729. DOI: 10.1038/nrn3550 [PubMed: 23985834]
- Liu LP, Darnall L, Hu T, Choi K, Lane TE, Ransohoff RM. Myelin repair is accelerated by inactivating CXCR2 on nonhematopoietic cells. *J Neurosci.* 2010; 30:9074–9083. [PubMed: 20610741]
- Mason JL, Langaman C, Morell P, Suzuki K, Matsushima GK. Episodic demyelination and subsequent remyelination within the murine central nervous system: changes in axonal calibre. *Neuropathol Appl Neurobiol.* 2001; 27:50–58. [PubMed: 11299002]

- Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol.* 2001a; 11:107–116. [PubMed: 11145196]
- Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol Zurich Switz.* 2001b; 11:107–116.
- Mi S, Lee X, Hu Y, Ji B, Shao Z, Yang W, Huang G, Walus L, Rhodes K, Gong BJ, Miller RH, Pepinsky RB. Death receptor 6 negatively regulates oligodendrocyte survival, maturation and myelination. *Nat Med.* 2011; 17:816–21. [PubMed: 21725297]
- Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB. LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat Neurosci.* 2004; 7:221–228. DOI: 10.1038/nn1188 [PubMed: 14966521]
- Mi S, Miller RH, Tang W, Lee X, Hu B, Wu W, Zhang Y, Shields CB, Miklasz S, Shea D, Mason J, Franklin RJ, Ji B, Shao Z, Chedotal A, Bernard F, Roulois A, Xu J, Jung V, Pepinsky B. Promotion of central nervous system remyelination by induced differentiation of oligodendrocyte precursor cells. *Ann Neurol.* 2009; 65:304–15. [PubMed: 19334062]
- Michailov GV, Sereda MW, Brinkmann BG, Fischer TM, Haug B, Birchmeier C, Role L, Lai C, Schwab MH, Nave KA. Axonal neuregulin-1 regulates myelin sheath thickness. *Science.* 2004; 304:700–703. DOI: 10.1126/science.1095862 [PubMed: 15044753]
- Moharreh-Khiabani D, Blank A, Skripuletz T, Miller E, Kotsiari A, Gudi V, Stangel M. Effects of fumaric acids on cuprizone induced central nervous system de- and remyelination in the mouse. *PLoS One.* 2010; 5:e11769.doi: 10.1371/journal.pone.0011769 [PubMed: 20668697]
- Moore SM, Khalaj AJ, Kumar S, Winchester Z, Yoon J, Yoo T, Martinez-Torres L, Yasui N, Katzenellenbogen JA, Tiwari-Woodruff SK. Multiple functional therapeutic effects of the estrogen receptor β agonist indazole-Cl in a mouse model of multiple sclerosis. *Proc Natl Acad Sci.* 2014; 111:18061–18066. DOI: 10.1073/pnas.1411294111 [PubMed: 25453074]
- Narayanan SP, Flores AI, Wang F, Macklin WB. Akt signals through the mammalian target of rapamycin pathway to regulate CNS myelination. *J Neurosci.* 2009; 29:6860–70. [PubMed: 19474313]
- Olah M, Amor S, Brouwer N, Vinet J, Eggen B, Biber K, Boddeke HWGM. Identification of a microglia phenotype supportive of remyelination. *Glia.* 2012; 60:306–321. DOI: 10.1002/glia.21266 [PubMed: 22072381]
- Poliani PL, Wang Y, Fontana E, Robinette ML, Yamanishi Y, Gilfillan S, Colonna M. TREM2 sustains microglial expansion during aging and response to demyelination. *J Clin Invest.* 2015; doi: 10.1172/JCI77983
- Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol.* 2003; 4:33–45. DOI: 10.1038/nrm1004 [PubMed: 12511867]
- Ransohoff RM. EAE: pitfalls outweigh virtues of screening potential treatments for multiple sclerosis. *Trends Immunol.* 2006; 27:167–168. DOI: 10.1016/j.it.2006.02.007 [PubMed: 16513422]
- Rodriguez M, Lennon VA. Immunoglobulins promote remyelination in the central nervous system. *Ann Neurol.* 1990; 27:12–17. DOI: 10.1002/ana.410270104 [PubMed: 2301922]
- Rodríguez-Peña A. Oligodendrocyte development and thyroid hormone. *J Neurobiol.* 1999; 40:497–512. [PubMed: 10453052]
- Sachs HH, Bercury KK, Popescu DC, Narayanan SP, Macklin WB. A new model of cuprizone-mediated demyelination/remyelination. *ASN Neuro.* 2014; :6.doi: 10.1177/1759091414551955
- Schmidt T, Awad H, Slowik A, Beyer C, Kipp M, Clarner T. Regional heterogeneity of cuprizone-induced demyelination: topographical aspects of the midline of the corpus callosum. *J Mol Neurosci.* 2013; 49:80–88. DOI: 10.1007/s12031-012-9896-0 [PubMed: 23054589]
- Sherman LS, Back SA. A “GAG” reflex prevents repair of the damaged CNS. *Trends Neurosci.* 2008; 31:44–52. DOI: 10.1016/j.tins.2007.11.001 [PubMed: 18063497]
- Skripuletz T, Hackstette D, Bauer K, Gudi V, Pul R, Voss E, Berger K, Kipp M, Baumgärtner W, Stangel M. Astrocytes regulate myelin clearance through recruitment of microglia during cuprizone-induced demyelination. *Brain.* 2012

- Skripuletz T, Lindner M, Kotsiari A, Garde N, Fokuhl J, Linsmeier F, Trebst C, Stangel M. Cortical demyelination is prominent in the murine cuprizone model and is strain-dependent. *Am J Pathol.* 2008; 172:1053–1061. [PubMed: 18349131]
- Sloane JA, Batt C, Ma Y, Harris ZM, Trapp B, Vartanian T. Hyaluronan blocks oligodendrocyte progenitor maturation and remyelination through TLR2. *Proc Natl Acad Sci U S A.* 2010; 107:11555–11560. DOI: 10.1073/pnas.1006496107 [PubMed: 20534434]
- Sriram S, Steiner I. Experimental allergic encephalomyelitis: a misleading model of multiple sclerosis. *Ann Neurol.* 2005; 58:939–945. DOI: 10.1002/ana.20743 [PubMed: 16315280]
- Steelman AJ, Thompson JP, Li J. Demyelination and remyelination in anatomically distinct regions of the corpus callosum following cuprizone intoxication. *Neurosci Res.* 2012; 72:32–42. DOI: 10.1016/j.neures.2011.10.002 [PubMed: 22015947]
- Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Ann Neurol.* 2006; 60:12–21. DOI: 10.1002/ana.20913 [PubMed: 16802293]
- Stidworthy MF, Genoud S, Suter U, Mantei N, Franklin RJM. Quantifying the Early Stages of Remyelination Following Cuprizone-induced Demyelination. *Brain Pathol.* 2003; 13:329–339. [PubMed: 12946022]
- Taylor LC, Gilmore W, Matsushima GK. SJL mice exposed to cuprizone intoxication reveal strain and gender pattern differences in demyelination. *Brain Pathol.* 2009; 19:467–479. DOI: 10.1111/j.1750-3639.2008.00230.x [PubMed: 19016742]
- Trapp BD, Nishiyama A, Cheng D, Macklin W. Differentiation and death of premyelinating oligodendrocytes in developing rodent brain. *J Cell Biol.* 1997; 137:459–68. [PubMed: 9128255]
- Trapp BD, Stys PK. Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis. *Lancet Neurol.* 2009; 8:280–291. [PubMed: 19233038]
- Wergeland S, Torkildsen Ø, Myhr KM, Aksnes L, Mørk SJ, Bø L. Dietary vitamin D3 supplements reduce demyelination in the cuprizone model. *PLoS One.* 2011; 6:e26262.doi: 10.1371/journal.pone.0026262 [PubMed: 22028844]
- Williams A, Piaton G, Aigrot MS, Belhadi A, Théaudin M, Petermann F, Thomas JL, Zalc B, Lubetzki C. Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis? *Brain J Neurol.* 2007; 130:2554–2565. DOI: 10.1093/brain/awm202
- Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell.* 2006; 124:471–484. DOI: 10.1016/j.cell.2006.01.016 [PubMed: 16469695]
- Yamamura T, Konola JT, Wekerle H, Lees MB. Monoclonal antibodies against myelin proteolipid protein: identification and characterization of two major determinants. *J Neurochem.* 1991; 57:1671–1680. [PubMed: 1717653]

Highlights

- A modified mouse cuprizone model that displays consistent demyelination, differential astrogliosis and differential remyelination in white and gray matter.
- Ultra-structural analysis reveals internodal length as a better readout of remyelination than g-ratio.
- The paradigm can be powered to determine efficacy of remyelinating drugs.

Figure 1.

Rapamycin increases cuprizone-induced demyelination. (A) Schematic of a sagittal brain section showing the region of interest (ROI) in corpus callosum (cc). Corpus callosum is in black and fornix in gray. (B) Schematic of a coronal brain section showing the ROIs for hippocampus (hip) and cortex (cx). (C–E) PPD staining of 1 μm sections detects myelinated axons in corpus callosum. (F–H) PLP immunostaining of 30 μm sections detects myelin in cerebral cortex. The ROI is indicated with red dotted lines. (I–K) Quantification of myelinated axons in corpus callosum, and PLP staining in cerebral cortex and hippocampus. Mice were injected with Rap-only (CTL), fed 0.3% cuprizone only (Cup only) or cuprizone plus daily rapamycin injection (Cup/Rap) for 12 weeks. Error bar: standard deviation. Error bar: standard deviation. Scale: 5 μm (C–E), 100 μm (F–H).

Figure 2.

GFAP, Iba1, hyaluronan and CD44 expression is increased in corpus callosum following 12 weeks of cup/rap treatment. (A, B) Increased density of GFAP staining in corpus callosum of cup/rap-treated brains compared with CTL. (C, D) High magnification images of GFAP staining in corpus callosum. (E) Quantification of area occupied by GFAP staining in corpus callosum, hippocampus and cortex. **, $P < 0.01$, Student's *t*-test. (F, G) Increased Iba1 staining in corpus callosum of cup/rap-treated brains compared with CTL. (H, I) High magnification images of Iba1 staining in corpus callosum. (J) Quantification of area occupied by Iba1 staining. (K–N) Increased immunoreactivity of hyaluronan in corpus callosum, but not in cerebral cortex or hippocampus following cup/rap treatment. (O) Quantification of hyaluronan in different tissues. (P–T) Significant increase in CD44

expression in corpus callosum following cup/rap treatment. Error bar, standard deviation.
Scale: 500 μm (A, B, F, G, K, L, P, Q); 200 μm (C, D, H, I, M, N, R, S).

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Figure 3.

Different rates of remyelination in white and gray matter following cup/rap treatment. (A–E) Changes in the number of myelinated axons in corpus callosum following 12 weeks of demyelination (12W), plus 1 week (12+1W), 3 weeks (12+3W), and 6 weeks (12+6W) of remyelination. (F–J) Changes of PLP staining in hippocampus following demyelination and remyelination. (K) Quantification of remyelinated axons in corpus callosum, and myelin density in cerebral cortex and hippocampus. Note that by 6 weeks of remyelination (12+6W), myelin density in cerebral cortex and hippocampus reached 70 – 80% of CTL while the number of myelinated axons in corpus callosum reached only ~22% of CTL. (L) The rate of remyelination in corpus callosum is significantly slower than that in cortex and hippocampus. For each tissue type (cortex, hippo and corpus callosum), a regression line was generated based on remyelination data of 12W, 12+1W, 12+3W and 12+6W. The slopes of regression line were compared to determine whether one line is significantly different from the other lines using parallel line analysis. (M) Increases in the density of remyelinated axons correlate well with increases in percentage of remyelinated axons in electron micrographs.. The same tissue blocks were used to count myelinated axons in PPD-stained 1 μm -thick sections and in electron micrographs. Note the similar increases (~2 fold) in the density of myelinated axons in μm -thick sections and percentage of myelinated axons in electron microscope from 12+3W to 12+6W. Error bar: standard deviation (K, L); standard error of the mean (M). Scale: 5 μm (A–E); 100 μm (F–J).

Figure 4.

Internodal length is a sensitive ultra-structural readout of early remyelination in corpus callosum. (A) Changes in myelinated axons following demyelination and remyelination in reconstructed 3D-EM image stacks. (B) Quantification of internodal length during early remyelination. Note the significantly shorter internodal length at 12W and 12+3W, but not at 12+6W. (C) Quantification of g-ratios during remyelination. There were no significant differences in g-ratios in the four groups. G-ratios from the same group were averaged. The total numbers of axons included in g-ratio measurement were: 273 for CTL; 280 for 12W; 154 for 12+3W and 890 for 12+6W. **, $P < 0.01$. (D) Scatter plots of myelin thickness, g-ratio and axon diameter. Each dot represents one myelinated axon. Error bar: standard deviation of the mean (B, C).

Figure 5.

Therapeutic enhancement of remyelination by thyroid hormone T3. (A) Three weeks of daily T3 injection increased remyelination in cortex and hippocampus, but not in corpus callosum. (B) Six weeks of daily T3 injections increased remyelination in cortex, hippocampus and corpus callosum. (C–F) PLP immunostaining of hippocampus and cortex of mice injected for 6 weeks with vehicle (CTL) or T3. (G–H) PPD staining of corpus callosum myelinated axons following 6 weeks of T3 injection. CC, corpus callosum. **, $P < 0.01$; ns, not statistically significant. Error bar: standard deviation. Scale, 100 μm (C–F), 5 μm (G–H).