


# Tropomyosin Receptor Kinase B Expressed in Oligodendrocyte Lineage Cells Functions to Promote Myelin Following a Demyelinating Lesion

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## Abstract

The levels of brain-derived neurotrophic factor (BDNF) in the corpus callosum have previously been shown to have a critical impact on oligodendrocyte (OLG) lineage cells during cuprizone-elicited demyelination. In particular, BDNF<sup>+/-</sup> mice exhibit greater losses in myelin protein levels compared to wild-type mice after cuprizone. To investigate whether OLGs may directly mediate these effects of BDNF during a lesion *in vivo*, we used the cuprizone model of demyelination with inducible conditional male knockout mice to specifically delete the high-affinity tropomyosin receptor kinase B (TrkB) receptor from proteolipid protein + OLGs during cuprizone-elicited demyelination and subsequent remyelination. The loss of TrkB during cuprizone-elicited demyelination results in an increased sensitivity to demyelination as demonstrated by greater deficits in myelin protein levels, greater decreases in numbers of mature OLGs, increased numbers of demyelinated axons, and decreased myelin thickness. When mice are removed from cuprizone, they exhibit a delayed recovery in myelin proteins and myelin. Our data indicate that following a demyelinating lesion, TrkB in OLGs positively regulates myelin protein expression, myelin itself, and remyelination.

## Keywords

cuprizone, myelin-associated glycoprotein, myelin basic protein, NEURO repair, oligodendrocyte, remyelination, TrkB

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Oligodendrocytes (OLGs) are critical for the proper functioning of the central nervous system (CNS), with the responsibility of synthesizing myelin sheaths that allow for the rapid conductance of nerve signals and metabolic support (e.g., Waxman and Foster, 1980; Lee et al., 2012; Ronzano et al., 2020). The maintenance, survival, and replenishment of OLGs are often compromised in diseases like multiple sclerosis (e.g., Barnett and Prineas, 2004; Lassmann, 2005). Chronic multiple sclerosis lesions that fail to remyelinate are attributed to the loss of OLGs (Wolswijk, 2000), the inability of OLG progenitors to differentiate into mature OLGs (Kuhlmann et al., 2008), and the failure of premyelinating OLGs to myelinate axons (Chang et al., 2002). Therefore, understanding factors that impact the development and health of OLG lineage cells is important in identifying the regulatory mechanisms necessary to maintain proper OLG function.

A variety of endogenous factors have been found to elicit regulatory effects on OLG growth and maintenance after demyelinating injuries (Huang and Dreyfus, 2016). In particular, we have evaluated the effects of brain-derived neurotrophic factor (BDNF). Following a cuprizone-induced demyelinating lesion, BDNF<sup>+/-</sup> mice exhibit deficits in the proliferation of OLG progenitors compared to wild-type littermates (Tsiperson et al.,

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2015), as well as deficits in myelin proteins during demyelination and remyelination (VonDran et al., 2011). Further, others have reported that an intraventricular infusion of BDNF or a BDNF mimetic for 1 week following a cuprizone-induced demyelinating lesion enhances myelin thickness, while mice with tropomyosin receptor kinase B (TrkB) deleted from 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase)+ cells from birth block this enhanced response to the BDNF mimetic, suggesting that the effects to the BDNF mimetic during recovery is due to a direct mediation by the OLGs (Fletcher et al., 2018).

However, such studies do not resolve whether deficits in myelination observed with the BDNF+/- mice or CNPase TrkB-deleted conditional knockout mice are due to impairments that occurred early in development, thus reducing the ability to withstand a cuprizone lesion as an adult, or are specific to the response to a lesion as adults. Second, although previous results indicate that TrkB receptors are on developing OLGs both in culture and *in vivo* and may mediate BDNF action (Du et al., 2006; Van't Veer et al., 2009; Wong et al., 2013; Fletcher et al., 2018), it is unclear whether TrkB on OLGs mediates response during the entire demyelination process associated with cuprizone ingestion.

To address this lack of specification, our present *in vivo* study investigates whether the deletion of OLG-associated TrkB immediately prior to the start of cuprizone administration impacts the response of OLGs to demyelination. Using *PLP-CreER<sup>T2</sup>-TrkB<sup>fl/fl</sup>* mice, TrkB was conditionally deleted from proteolipid protein (PLP)+ OLG lineage cells in adult mice. PLP primarily is known to be expressed by OLGs but is also expressed by OLG progenitors, particularly during development (Mallon et al., 2002; Harlow et al., 2015), and using *PLP-CreER<sup>T2</sup>* reporter mice has been found to be expressed in mature OLGs as well as neuron-glia antigen 2 (NG2) cells in adult animals (Leone et al., 2003). Adult mice were injected with tamoxifen just prior to cuprizone administration, and the corpus callosum was monitored for progression of demyelination and remyelination. Our studies find that the loss of TrkB receptors on OLG lineage cells negatively influences the numbers of OLGs, levels of myelin proteins, and myelin itself within the lesion site following a cuprizone lesion and suggest that BDNF may directly regulate myelination following demyelination and remyelination.

## Materials and Methods

### Experimental Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at Rutgers University and with National Institutes of Health guidelines.

Mice were maintained at the Rutgers Robert Wood Johnson Animal Facility.

The *PLP-CreER<sup>T2</sup>-TrkB<sup>fl/fl</sup>-Rosa26-lacZ* mouse line was generated by crossing *PLP-CreER<sup>T2</sup>* mice (a gift from Ueli Suter, ETH Zürich, Zurich, Switzerland), *TrkB<sup>fl/fl</sup>* mice (a gift from Luis Parada, UT Southwestern, Dallas, TX, USA), and *Rosa26-lacZ* mice (a gift from Michael Matise, Rutgers Robert Wood Johnson Medical School, Piscataway, NJ, USA). *TrkB<sup>fl/fl</sup>-Rosa26-lacZ* mice lacking Cre expression were used as controls, herein indicated as "Cre- mice," and mice heterozygous for *PLP-CreER<sup>T2</sup>* expression, indicated as "Cre+ mice," were used for tamoxifen-inducible conditional TrkB knockouts.

### Cre Activation and Cuprizone Treatment

Eight- to ten-week-old male mice, regardless of Cre genotype, were injected intraperitoneally twice daily with 1 mg of tamoxifen (Sigma-Aldrich, cat# T5648) for 5 consecutive days.

Upon completion of tamoxifen injections, mice were fed 0.2% cuprizone (Sigma-Aldrich, cat# C9012) milled into mouse feed (Envigo Teklad, TD.01453) or identically processed control feed (Envigo Teklad, TD.00217) continuously for 3 to 6 weeks to induce demyelination. To monitor a remyelination phase, mice were fed cuprizone for 6 weeks, followed by 2 or 4 weeks of control feed (6 + 2 or 6 + 4).

### Western Blot

The midline of the corpus callosum overlying the fornix and rostral hippocampus was dissected using a mouse brain matrix, which permits coronal cuts to be made in 1 mm segments along the rostral-caudal axis, and frozen at -80°C. Tissue was lysed, and protein concentrations were quantified using a BCA protein assay kit (Pierce, ref# 23223). Protein was run on 12% Bis-Tris gels (Invitrogen, Ref# NP0341BOX) for myelin basic protein (MBP) and 3% to 8% Tris-Acetate (Invitrogen, Ref# EA0375BOX) gels for myelin-associated glycoprotein (MAG). Protein was transferred to a polyvinylidene fluoride membrane (Millipore, Cat#IPVH00010), blocked in 4% BSA/TBS-T, and incubated with primary antibodies: mouse anti-MBP (Bio-Rad Cat# MCA184S, RRID: AB\_322319) or rabbit anti-MAG (Santa Cruz Biotechnology Cat# SC-15324, RRID:AB\_670104). Corresponding horseradish peroxidase-conjugated secondary anti-mouse (GE Healthcare Cat# NA931, RRID: AB\_772210) or anti-rabbit antibodies (GE Healthcare Cat# NA934, RRID:AB\_772206) were used. Membranes were stripped and reprobed with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Meridian Life Science Cat# H86504M, RRID:

AB\_151542) or anti- $\beta$ -tubulin (Sigma-Aldrich Cat# T4026, RRID:AB\_477577) as loading controls. Bands were visualized with a chemiluminescence system (Perkin Elmer Western Lighting Plus ECL Cat# ORT2655, Cat# ORT2755), and densitometric analysis was performed using Quantity One V 4.2.1 software (Quantity One 1-D Analysis Software, RRID:SCR\_014280).

### Immunohistochemistry

Mice were perfused transcardially with 2% paraformaldehyde in phosphate-buffered saline (PBS). Brains were postfixed for 2 hr in the same fixative, dehydrated in 30% sucrose/PBS for 48 hr, and embedded in optimal cutting temperature (OCT) compound (Tissue Tek, Ref# 4583). Brain tissues were collected at 20  $\mu$ m coronal serial sections.

For TrkB staining, tissue sections were first blocked with 4% donkey serum/3% BSA/PBS/0.1% TritonX-100, incubated with the rabbit anti-TrkB (Santa Cruz Biotechnology Cat# SC-12, RRID:AB\_632557) that recognizes the full length receptor, followed by the cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs Cat# 711-165-152, RRID:AB\_2307443).

For anti-adematous polyposis coli clone CC1 (CC1) staining, the sections were heated at 95°C steam for 10 min in 10 mM sodium citrate buffer (pH 6.0), blocked in 10% donkey serum/PBS/0.3% TritonX-100, and incubated with the mouse anti-CC1 (Millipore Cat# OP80, RRID:AB\_2057371), followed with the AlexaFluor 488 donkey anti-mouse IgG (Jackson ImmunoResearch Labs Cat# 715-545-151, RRID:AB\_2341099).

For cleaved caspase3 staining, tissue sections were heated at 95°C steam for 10 min in 10 mM sodium citrate buffer (pH 6.0), blocked in 20% donkey serum/4% BSA/PBS/0.5% TritonX-100, and incubated with rabbit anti-cleaved caspase3 (Cell Signaling Technology Cat# 9661, RRID:AB\_2341188), followed by the cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs Cat# 711-165-152, RRID:AB\_2307443).

Myelin stain was performed using ferric ammonium sulfate, hematoxylin, and lithium carbonate as described (Jebb and Woolsey, 1977; Hutchins and Weber, 1983).

The staining was visualized using a Leica microscope equipped with a Lumenera Infinity3S camera and Infinity Analyze 6.5 analysis software. Images of 20  $\mu$ m coronal serial sections separated by 80  $\mu$ m were obtained from the midline of corpus callosum overlying the fornix to rostral hippocampus (from bregma .02 to bregma -1.06). For sections imaged at 200 $\times$ , positive cells 235  $\mu$ m on either side of the midline of the corpus callosum were counted. For sections imaged at 400 $\times$ , positive cells 117  $\mu$ m on either side of the midline of the corpus callosum were counted.

**CC1 Analysis.** For total CC1 cell quantification, each experiment contained four mice, each under a different treatment condition: a Cre+ mouse on control feed, a Cre- mouse on control feed, a Cre+ mouse on cuprizone, and a Cre- mouse on cuprizone. Total CC1+ cell numbers were counted from 12 serial sections from each mouse imaged at 200 $\times$ . Within each experiment, sections for each of the four conditions were treated identically and evaluated identically. Within the experiment, the Cre- control-fed mouse cell number was set at 100%, while other group numbers were expressed relative to 100%. For these studies, all mice were injected with tamoxifen and fed with either control feed or cuprizone feed for 5 weeks. The experiment was repeated three times.

**TrkB+, CC1+ Analysis.** For TrkB, CC1+ studies, each experiment contained a control-fed Cre- and Cre+ mouse. Twelve serial sections of the corpus callosum were analyzed at 400 $\times$ . Total numbers of TrkB+, CC1+ as well as total CC1+ cell numbers were counted. A ratio of TrkB+, CC1+/total CC1+ cells was obtained for each animal. The ratio is expressed as a percentage of those in the Cre- samples. For these studies, all mice were injected with tamoxifen at 8 weeks and then fed control feed for 5 weeks or 6 weeks + 4 weeks on control feed.

**Caspase3+, CC1+ Analysis.** For Caspase3+, CC1+ evaluation each experiment contained a cuprizone-fed Cre- and Cre+ mouse. Twelve serial sections were analyzed at 200 $\times$ . Numbers of cells from the 12 sections were summed, and data are presented as above.

Each of these experiments was repeated at least three times.

### Electron Microscopy

Mice were perfused with 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, cat# 01230). Brains were sectioned sagittally in 150  $\mu$ m sections using a Leica vibratome. Sections of the midline of the corpus callosum were postfixed in 1% OsO<sub>4</sub>, stained with uranyl acetate, dehydrated with graded ethanol, and embedded in Poly/Bed812 resin (Polysciences, Inc.). Sections (1  $\mu$ m) were stained with toluidine blue for orientation, and ultrathin sections were cut and visualized using a transmission electron microscope (Philips CM12) at 5,000 $\times$  magnification. For each mouse, images were taken in the corpus callosum immediately caudal to its junction with the fornix. Five images per mouse were assessed in each of three sets of animals per treatment group ( $N=3$ ) to measure g-ratios. Thin sections were evaluated blindly using the imaging program-Image J (ImageJ, RRID:SCR\_003070). A minimal of 150 random axons was

assessed per animal. To count percent of myelinated axons, the entire field ( $14.8\mu\text{m} \times 9.7\mu\text{m}$ ) of the five images was analyzed for myelinated axons and unmyelinated axons, and the data are present as percent of the total number.

### Statistical Analysis

For Western blot analysis, Cre+ and Cre- mice fed cuprizone were compared with their own control-fed mice for the same time points. Differences between Cre- and Cre+ cuprizone-fed mice were then evaluated. Similarly for electron microscopic analysis, Cre+ and Cre- samples were compared to their own controls and to each other when fed cuprizone. Statistical analysis was performed with GraphPad PRISM 8 software (GraphPad Prism, RRID:SCR\_002798), and the data are presented as the mean  $\pm$  SEM. Statistical differences were determined using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. In some cases, the differences between Cre- and Cre+ were determined by paired Student's *t* test. Conditions were considered significant at  $p < .05$ .

For electron microscopy, the scatterplots of g-ratio versus axon diameter were analyzed by GraphPad Prism 8 (GraphPad Prism, RRID:SCR\_002798) for slopes. To follow changes that are more specifically associated with demyelination and remyelination within the myelinated fibers, axons with diameters of  $< 3\mu\text{m}$  (typical of unmyelinated axons) were not analyzed (Mason et al., 2001; Zhou et al., 2012).

## Results

### Targeting TrkB Deletion From OLGs

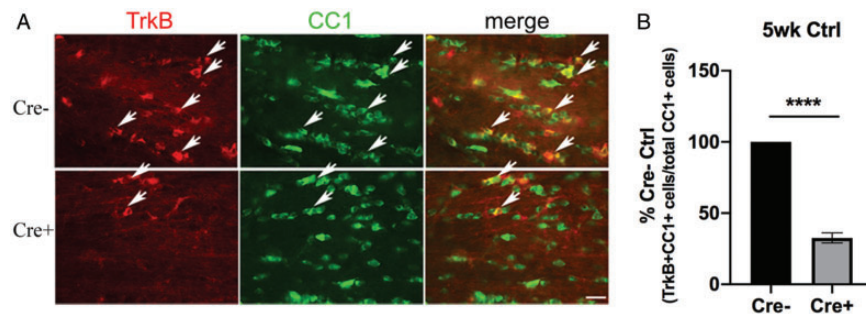
OLGs of the corpus callosum have been found to express the high-affinity BDNF receptor, TrkB, and be responsive to BDNF during development and adulthood

(VonDran et al., 2010, 2011; Wong et al., 2013; Fletcher et al., 2018). Moreover, BDNF+/- mice are more sensitive to myelin protein losses after cuprizone-elicited demyelinating lesions compared to wild-type littermates (VonDran et al., 2011). To investigate whether BDNF may exert a direct effect on OLGs during a demyelinating lesion, we used an inducible knockout mouse model, to specifically delete the TrkB receptor from OLGs, in the cuprizone model of demyelination. *PLP-CreER<sup>T2</sup>-TrkB<sup>fl/fl</sup>-ROSA26-lacZ* mice were generated from mice where tamoxifen-inducible Cre recombinase under the control of the proteolipid protein (PLP) promoter deletes TrkB primarily from postmitotic OLGs, but also from NG2+ cells, following tamoxifen administration in adult mice (Leone et al., 2003).

For our studies, both *PLP-CreER<sup>T2</sup>-TrkB<sup>fl/fl</sup>-ROSA26-lacZ* mice and control Cre recombinase negative *TrkB<sup>fl/fl</sup>-ROSA26-lacZ* mice (herein referred to as Cre+ and Cre- mice, respectively) were injected with tamoxifen. To define the recombination efficiency of our inducible knockout model, and thereby the deletion of TrkB expression from OLGs, we examined the numbers of cells expressing TrkB in the Cre- and Cre+ mice fed control feed for 5 weeks. In agreement with previous work (VonDran et al., 2011), immunohistochemical analysis indicated TrkB colocalization with a subset of CC1+ OLGs in Cre- control-fed mice in the corpus callosum (Figure 1). However, after treatment with tamoxifen, Cre+ mice have a decrease of 67% in TrkB+ CC1+ staining throughout the corpus callosum.

### TrkB Deletion Enhances Losses in Myelin Proteins Following Cuprizone-Induced Lesions

A cuprizone-induced demyelination is characterized by the loss of myelin proteins, death of OLGs, and increases in the OLG progenitor population (Hiremath et al., 1998; Morell et al., 1998; Gao et al., 2000; Mason et al., 2000, 2001; Palumbo et al., 2011). Thus, myelin proteins may



**Figure 1.** TrkB Receptors Are Deleted From OLGs in Cre+ Mice. *PLP-CreERT2-TrkB<sup>fl/fl</sup> Rosa26-lacZ* or control *TrkB<sup>fl/fl</sup> Rosa26-lacZ* mice were injected with tamoxifen. Cre- animals exhibit subsets of CC1+ cells (green) that are TrkB+ (red) (arrows). After tamoxifen, there is 67% reduction of trkB+CC1+/total CC1 cells. Scale bar = 20  $\mu\text{m}$ . \*\*\*\* $p < .0001$ , significantly different from Cre- corpus callosum, analyzed using paired Student's *t* test.  $N = 3$  experiments, each with 2 mice (Cre- and Cre+). Data are presented as mean  $\pm$  SEM. TrkB = tropomyosin receptor kinase B.

provide evidence of myelin deficiencies during demyelination. To define the effects of direct OLG-mediated TrkB signaling, myelin proteins were assessed in the control and lesioned brain following 3, 4, 5, 6, 8, and 10 weeks of control feed, or 3, 4, 5, or 6 weeks of cuprizone, followed by 2 or 4 weeks on control feed. The deletion of TrkB has no effect on MBP expression at any week in animals fed control feed. In the case of MAG, there are no effects of the deletion at 3 or 4 weeks. At 5 weeks, there is a small (12%) significant decrease, but this decrease is gone by 6 weeks, and there is no further difference in MAG levels at 8 or 10 weeks (data not shown). The data suggest that TrkB does not contribute to myelin protein maintenance when mice are fed control feed.

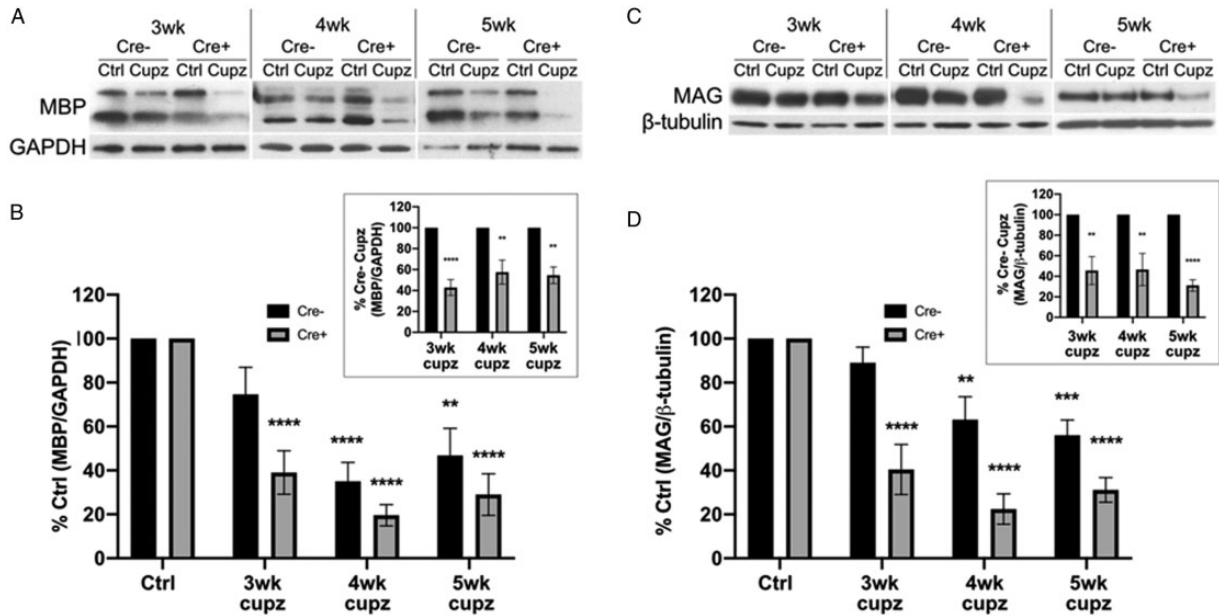
When mice are fed cuprizone, the response to the TrkB deletion becomes evident. Over the course of 5 weeks, cuprizone administration decreases the myelin proteins in both Cre<sup>-</sup> and Cre<sup>+</sup> mice. However, in Cre<sup>-</sup> mice, decreases in myelin proteins are not significantly evident until 4 weeks of cuprizone administration, while decreases in myelin proteins are already evident following 3 weeks of cuprizone in mice with the TrkB deletion (Figure 2). When cuprizone-fed animals

are compared to each other, Cre<sup>+</sup> mice exhibit significant losses in MBP and MAG relative to Cre<sup>-</sup> mice after 3, 4, and 5 weeks (Figure 2 insets). The data suggest that TrkB in OLGs is critical in regulating deficits in myelin protein expression specifically following a demyelinating lesion.

### OLG-TrkB Deletion Exacerbates Myelin Disruption After Cuprizone

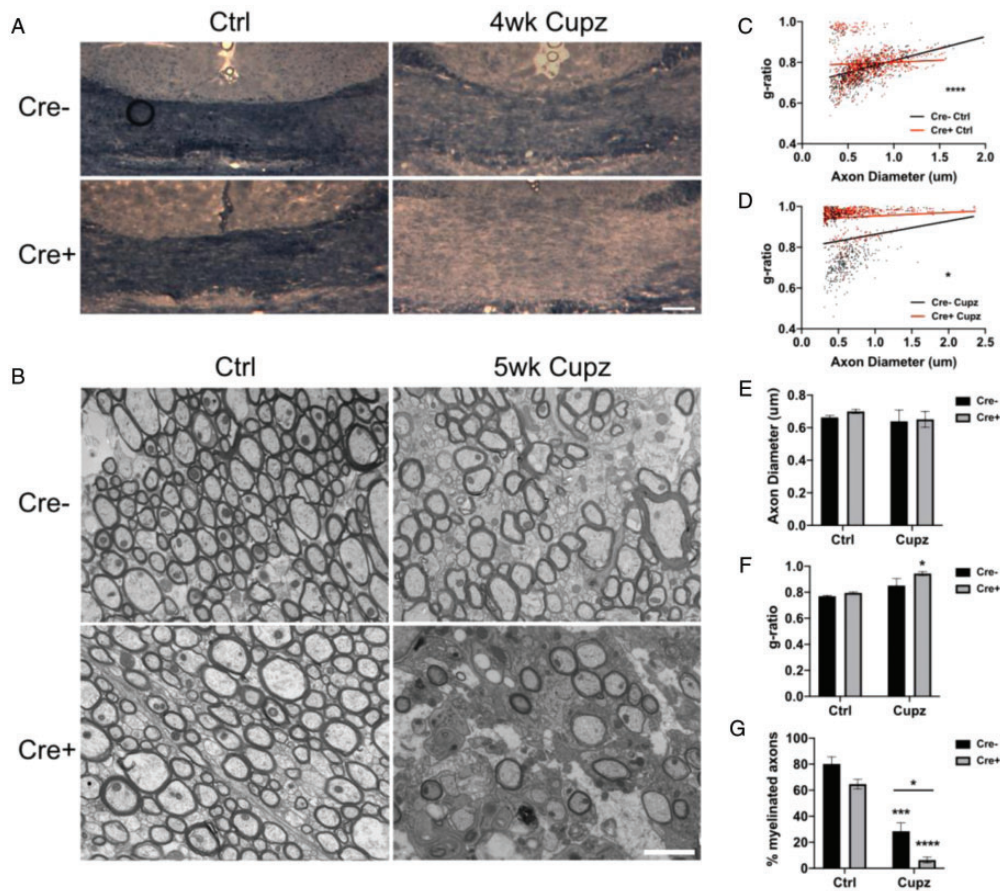
Given the increased loss in myelin protein levels in Cre<sup>+</sup> cuprizone-fed mice compared to their Cre<sup>-</sup> counterparts, myelin integrity was analyzed to further assess the effects of TrkB deletion during demyelination. A myelin stain on coronal sections of the corpus callosum showed lighter myelin stain intensity in the Cre<sup>+</sup> samples following 4 weeks of cuprizone treatment compared to Cre<sup>-</sup> mice (Figure 3A).

To explore the effect of the TrkB deletion on the ultrastructure of myelin, electron micrographs of cross sections of the midline of the corpus callosum were quantitatively assessed by analyzing g-ratios to define myelin thickness (Figure 3B–D, F) and by calculating the percent of myelinated axons (Figure 3B, G) in mice



**Figure 2.** TrkB Deletion From Cre<sup>+</sup> Mice Results in Decreases in Myelin Proteins That Are Greater Than Those Seen in OLG Cre<sup>-</sup> Mice Following a Demyelinating Cuprizone Lesion.

Cre<sup>-</sup> and Cre<sup>+</sup> mice were injected with tamoxifen, and fed control (Ctrl) or cuprizone (cupz) feed for 3 to 5 weeks. (A, B) Western blot and densitometric analysis for both bands of MBP ( $N = 6$  experiments (3 weeks), 8 experiments (4 weeks), and 3 experiments (5 weeks)). (C, D) Western blot and densitometric analysis for MAG ( $N = 4$  experiments (3 weeks), 5 experiments (4 weeks), and 6 experiments (5 weeks)). \*\*\*\* $p < .0001$ , \*\*\* $p < .001$ , \*\* $p < .01$ , significantly different from animals of the same genotype fed control feed analyzed using two-way ANOVA followed by Tukey's multiple comparisons. Inset: \*\*\*\* $p < .0001$ , \*\* $p < .01$ , significantly different from Cre<sup>-</sup> cuprizone-treated animals, analyzed using paired Student's  $t$  test. Data are presented as mean  $\pm$  SEM. In these experiments, each experiment includes four mice (Control-fed Cre<sup>-</sup>/Cre<sup>+</sup> and Cuprizone-fed Cre<sup>-</sup>/Cre<sup>+</sup>). MAG = myelin-associated glycoprotein; MBP = myelin basic protein.



**Figure 3.** Myelin Analysis of the Corpus Callosum After 4 or 5 Weeks of Cuprizone Shows Lighter and More Disrupted Myelin in Cre+ Mice. (A) Myelin staining that uses iron ammonium sulfate, hematoxylin, and lithium carbonate demonstrates the reduction of myelin in TrkB-deleted mice that have received cuprizone (cupz) feed versus control (Ctrl) feed. Scale bar = 30  $\mu$ m. (B) Representative electron microscopic images of sagittal sections of the corpus callosum after 5 weeks of control or cuprizone. Scale bar = 2  $\mu$ m. (C, D) Scatterplot analysis of g-ratios versus axon diameter reveals that there is a significant change in slope in Cre- versus Cre+ mice fed control feed  $***p < .0001$  (C) or cuprizone feed  $*p < .05$  (D), suggesting that small diameter axons are particularly affected. There is no change in axon diameter (E). When Cre+ mice are evaluated, cuprizone-fed mice exhibit a significant increase in average g-ratio in comparison to both Cre- and Cre+ control mice  $*p < .05$  (F). When mice are fed cuprizone, Cre+ mice exhibit a more severe loss in percent myelinated axons than Cre- mice  $*p < .05$ .  $***p < .001$  when Cre- cuprizone mice are compared to Cre- control.  $***p < .0001$  when Cre+ cuprizone mice are compared to Cre- or Cre+ control (G). All analyses use two-way ANOVA followed by Tukey's multiple comparisons.  $N = 3$  independent experiments. Each experiment includes four mice (Control Cre-/Cre+ and Cuprizone-fed Cre-/Cre+). Data are presented as mean  $\pm$  SEM.

fed control or cuprizone feed. In addition, axon diameters were measured to determine if axons are affected by the loss of TrkB from OLGs (Figure 3E).

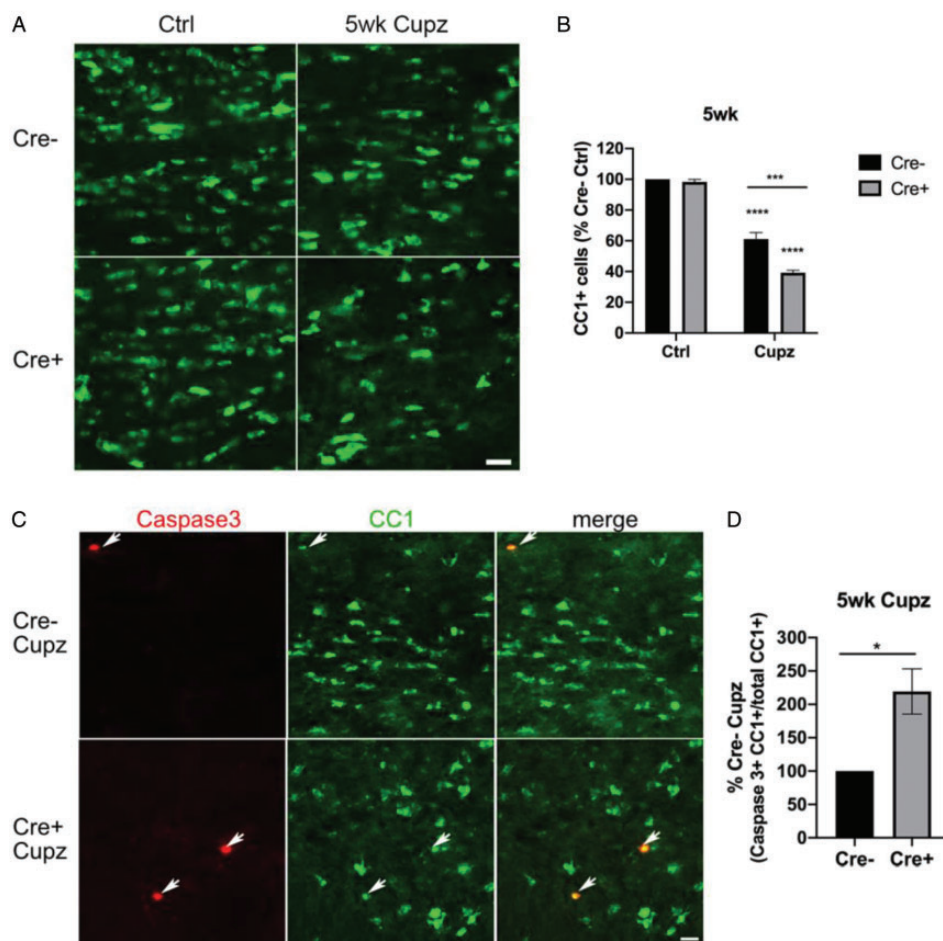
After 5 weeks, there is no change in average axonal diameter in either Cre- or Cre+ mice fed control or cuprizone feed (Figure 3E). However, changes are apparent when myelin is evaluated. In mice fed control feed, a scatterplot analysis comparing the g-ratio of individual axons to their respective axon diameters reveals a change in slope in Cre+ mice that reflects an increase in g-ratio, particularly with respect to the smallest axons (Figure 3C). The overall average g-ratio between control-fed Cre- and Cre+ mice are not different (Figure 3F). When evaluating cuprizone-fed

mice, there is also a significant difference between Cre- and Cre+ mice, with the slope of Cre+ mice suggestive of a loss in myelin thickness in axons of all sizes (Figure 3D). This is reflected by an increase in average g-ratio for the Cre+ cuprizone mice (Figure 3F). Most dramatically, in cuprizone-fed mice, there is a decrease in percent of myelinated axons in comparison to mice fed control feed and a further decrease in myelinated axons when TrkB is deleted (Figure 3G). The data indicate that deletion of OLG TrkB causes myelin deficits in cuprizone mice consistent with the decreases in myelin protein and suggest that TrkB on OLGs directly affects disruption of myelin that occurs upon demyelination.

### Loss of *TrkB* Reduces Numbers of Mature OLGs During Demyelination

Previous results indicated that in response to cuprizone, BDNF-deficient mice exhibit greater myelin protein losses compared to wild-type mice while maintaining comparable numbers of CC1+ OLGs, suggesting that decreases in myelin proteins are due to decreases per cell (VonDrän et al., 2011). To determine if similar results occur when *TrkB* is deleted from PLP+ cells, numbers of CC1+ OLGs were counted after 5 weeks of cuprizone treatment. In mice fed control feed, no differences are noted in CC1+ cells whether or not *TrkB* is deleted. However, in mice

subjected to a cuprizone lesion, deletion of *TrkB* from OLGs in *Cre*+ mice significantly increases the loss of CC1+ OLGs in the corpus callosum compared to *Cre*- mice (Figure 4A, B). To address whether this exacerbated loss is due to cell death, we evaluated numbers of caspase3+CC1+ cells compared to total CC1+ cells under cuprizone feed conditions. A significant increase in numbers of caspase3+CC1+ cells relative to total CC1+ cells is evident after cuprizone feed (Figure 4C, D). Therefore, a global reduction of BDNF (such as is seen in *BDNF*+/- mice) does not affect numbers of OLGs in cuprizone-treated mice, while deletion of *TrkB* from OLGs does. These data suggest that either residual levels of BDNF in



**Figure 4.** Cuprizone-Induced Loss of CC1+ Oligodendrocytes Is More Severe When *TrkB* Is Deleted and Apoptosis in CC1+ Cells Is Increased. (A) Representative immunofluorescence staining of CC1+ cells after 5 weeks of control or cuprizone feed. (B) Number of CC1+ cells is expressed as percent of *Cre*- controls at 5 weeks. \*\*\**Cre*- and *Cre*+ mice fed cuprizone are significantly different from each other,  $p < .001$ . \*\*\**Cre*- or *Cre*+ cuprizone-fed mice are significantly different from control-fed mice,  $p < .0001$ . Analysis used two-way ANOVA followed by Tukey's multiple comparisons.  $N = 3$  experiments. Each experiment includes four mice (Control +/- *Cre* and Cuprizone +/- *Cre*). (C) Representative immunofluorescence staining of Caspase3 and CC1 after 5 weeks of cuprizone. (D) Number of Caspase3+CC1+/total CC1 cells expressed as percentage of *Cre*- cuprizone.  $N = 4$  experiments. Each experiment includes two mice (Cuprizone +/- *Cre*). \*Significantly different from *Cre*- cuprizone-treated animals, analyzed using paired Student's *t* test,  $p < .05$ . Scale bar = 20  $\mu$ m. Arrows = caspase3+ CC1+ cells. Data are presented as mean  $\pm$  SEM.

BDNF<sup>+/-</sup> mice may be sufficient to maintain OLG cell number during cuprizone lesions, or that other compensatory mechanisms exist. Furthermore, loss of TrkB specifically from OLGs is more detrimental a response than a general reduction of BDNF levels.

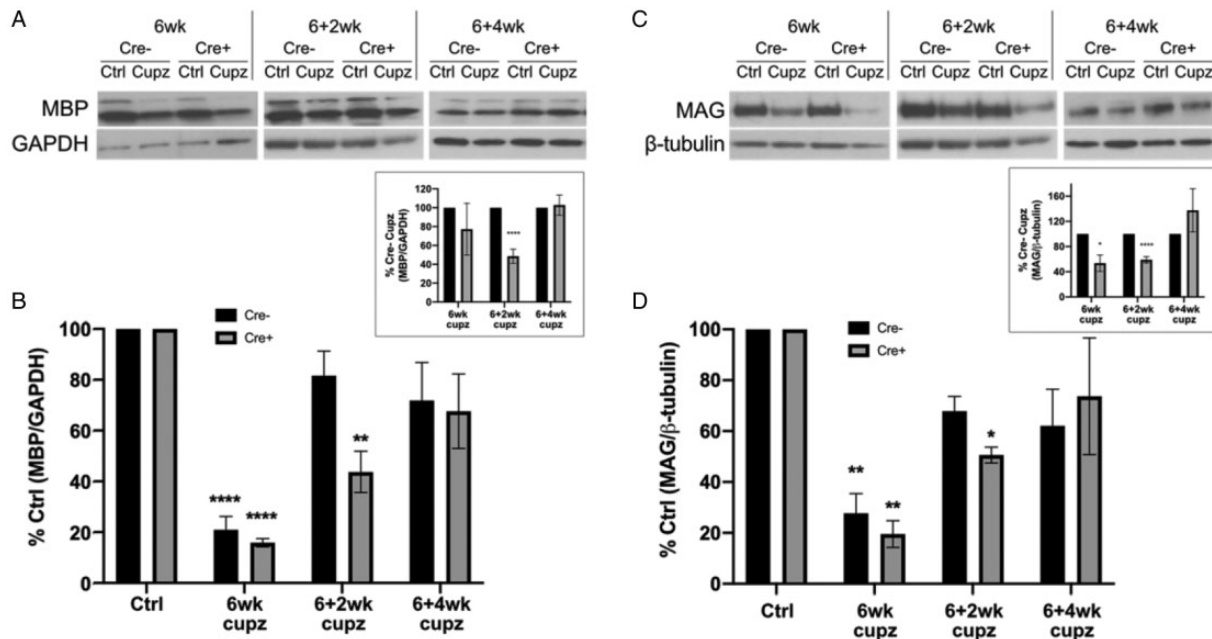
### TrkB Deletion Leads to a Slower Recovery in Myelin Proteins During Remyelination

The studies thus far have focused on effects of TrkB in mitigating the demyelinating effects of cuprizone. To investigate the effects of the OLG TrkB deletion on remyelination, mice were fed cuprizone for 6 weeks and then returned to control feed for an additional 2 or 4 weeks (6+2 or 6+4) before being sacrificed. After 6 weeks of cuprizone, differences in levels of myelin protein between Cre<sup>-</sup> and Cre<sup>+</sup> mice have largely disappeared (Figure 5). However, when taken off cuprizone, Cre<sup>+</sup> mice exhibit a slower recovery of myelin proteins than do the Cre<sup>-</sup> mice. Relative to the 6-week timepoint, MBP and MAG levels increase during the second week of recovery (6+2); however, Cre<sup>+</sup> mice continue to have significant decreases compared to control-fed Cre<sup>+</sup> mice (Figure 5B, D) and cuprizone-fed Cre<sup>-</sup> mice (Figure 5 insets). On the other hand, by the fourth week of recovery (6+4), MBP and MAG levels in the Cre<sup>+</sup> mice reach levels comparable to

the Cre<sup>-</sup> mice and to control-fed mice, indicative of a slowed but eventual recovery (Figure 5B, D).

To determine if differences in myelin proteins at 6+2 weeks are indicative of differences in myelin, g-ratios and percent of myelinated axons were evaluated (Figure 6A–C, E, F). Neither control nor cuprizone-fed mice exhibit differences during the second week of recovery in their axon diameters (Figure 6D) or their g-ratios (Figure 6B, C, E), as indicated by slopes of scatterplots. When examining the percent of myelinated axons, there are no significant differences between control groups or when Cre<sup>-</sup> control and Cre<sup>-</sup> cuprizone mice are compared. However, Cre<sup>+</sup> mice fed cuprizone do exhibit a deficit in their second week of recovery, with a significant decrease in percent myelinated axons when compared to Cre<sup>-</sup> control-fed mice and a trend toward a decrease ( $p = .06$ ) compared to Cre<sup>+</sup> mice control-fed (Figure 6F). The loss of TrkB impacts the numbers of myelinated axons.

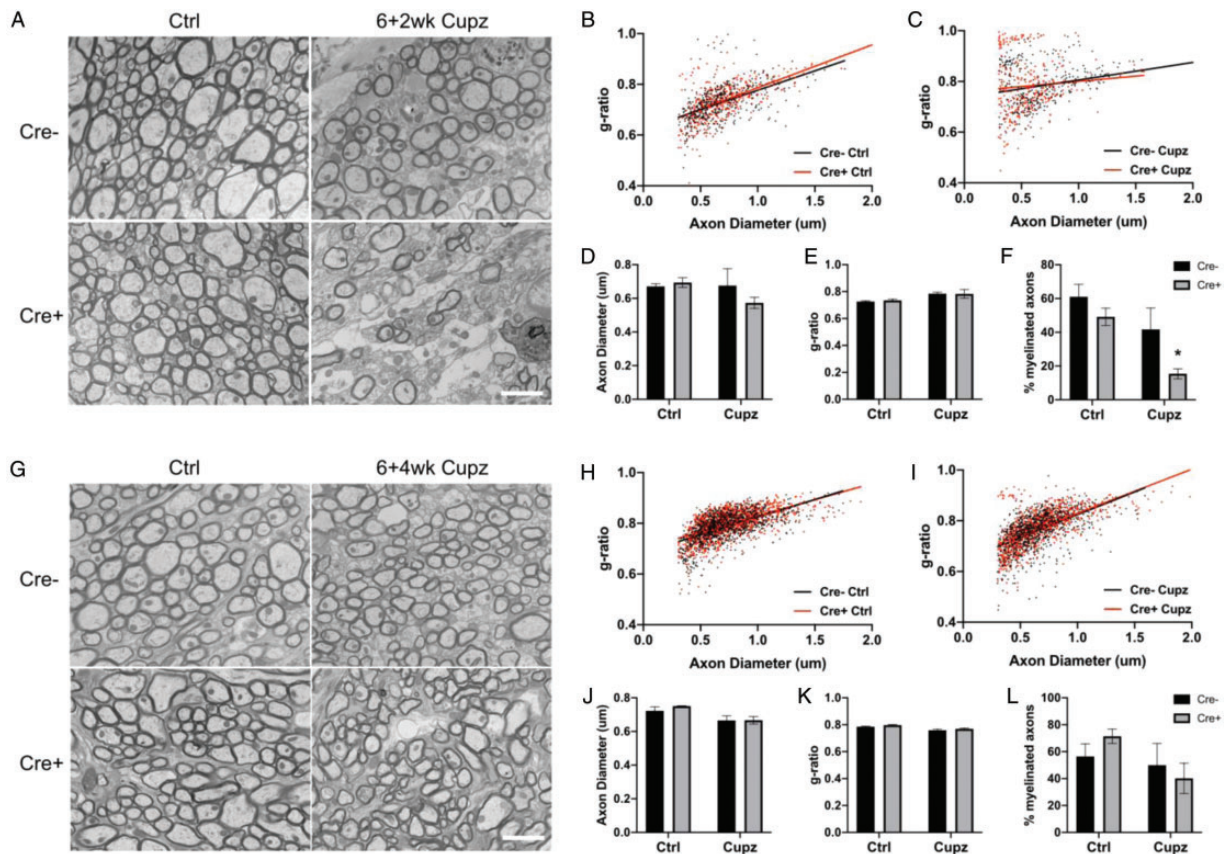
When mice are evaluated 4 weeks after being removed from cuprizone, deficits noted at 6+2 weeks have disappeared. No differences are evident in the percent of myelinated axons in Cre<sup>+</sup> cuprizone mice compared to any control-fed group (Figure 6L). Moreover, no decreases in axon diameter or g-ratio are evident at 4 weeks when Cre<sup>-</sup> and Cre<sup>+</sup> mice are compared (Figure 6H–L).



**Figure 5.** TrkB Deletion in OLGs Results in a Delay in MBP and MAG Expression During Remyelination. Mice are fed cuprizone (cupz) for 6 weeks followed by a switch to control feed (Ctrl) for 2 or 4 weeks. (A, B) Western blot and densitometric analysis for MBP ( $N = 4$  experiments (6 weeks) and 5 experiments (6+2 weeks and 6+4 weeks)). (C, D) Western blot and densitometric analysis for MAG ( $N = 5$  experiments for each time point). \*\*\*\* $p < .0001$ , \*\* $p < .01$ , \* $p < .05$ , significantly different from animals of the same genotype fed control feed, analyzed by two-way ANOVA followed by Tukey's multiple comparisons. Inset: \*\*\*\* $p < .0001$ , \* $p < .05$ , significantly different from Cre<sup>-</sup> cuprizone-treated animals, analyzed by paired Student's  $t$  test. Data are presented as mean  $\pm$  SEM. Each experiment includes four mice (Control  $\pm$  Cre and Cuprizone  $\pm$  Cre).

MAG = myelin-associated glycoprotein; MBP = myelin basic protein.





**Figure 6.** Myelin Analysis of the Corpus Callosum Indicates That TrkB cKO Brain Remyelination Is Delayed (6 + 2 weeks) But Recovers After 4 Weeks (6 + 4 weeks). (A and G) EM representative images of 6 weeks of control (Ctrl) or cuprizone (cupz) followed by 2 (A) or 4 (G) weeks of control feed. At 6 + 2 weeks of recovery, in both control- (B) and cuprizone- (C) fed mice, analysis of g-ratio versus axon diameter indicates no changes in slope for Cre<sup>-</sup> and Cre<sup>+</sup> mice. Similarly, there are no differences in axon diameter (D) or g-ratios (E). On the other hand, significant differences are seen in Cre<sup>+</sup> mice treated with cuprizone when percent myelinated axons is determined (F), analyzed using two-way ANOVA followed by Tukey's multiple comparisons. \* $p < .05$ , significantly different from control-fed Cre<sup>-</sup> mice, and there is a trend ( $p = .06$ ) when compared to Cre<sup>+</sup> control-fed mice. Data are presented as mean  $\pm$  SEM;  $N = 3$  independent experiments. Scatterplot analysis of g-ratios versus axon diameters in mice fed control (H) or cuprizone (I) feed for 6 + 4 weeks. Linear regression analysis reveals that at 6 + 4 weeks, there is no difference in slope. Average axon diameter (J), g-ratios (K), and the percent of myelinated axons (L) are similar when Cre<sup>+</sup> mice are compared to Cre<sup>-</sup> mice fed control or cuprizone feed. Analysis is by two-way ANOVA followed by Tukey's multiple comparisons.  $N = 3$  experiments. Each experiment includes four mice (Control +/- Cre and Cuprizone +/- Cre). Data are presented as mean  $\pm$  SEM.

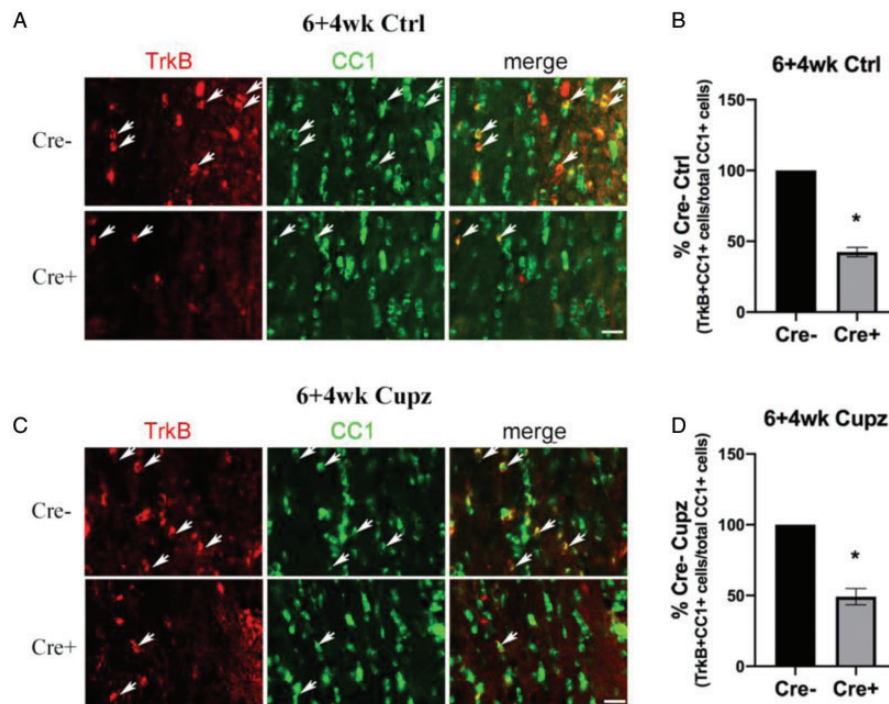
### Equal Reductions in TrkB Expression Persist at 6 + 4 Weeks in Control and Cuprizone-Fed Cre<sup>+</sup> Mice

One possibility for the recovery of myelin and myelin proteins at 6 + 4 weeks could be the OLG lineage cells that escape recombination upon tamoxifen treatment and still express TrkB compensate for the loss of TrkB-deleted CC1<sup>+</sup> cells. To examine this possibility, we evaluated the relative number of TrkB+CC1<sup>+</sup> cells in Cre<sup>-</sup> and Cre<sup>+</sup> mice at 6 + 4 weeks of control or cuprizone feed. In both cases, comparable decreases were observed between Cre<sup>-</sup> and Cre<sup>+</sup> mice. Cre<sup>+</sup> mice fed control feed exhibit a 58% decrease in numbers of TrkB+CC1<sup>+</sup> cells, while Cre<sup>+</sup> mice fed cuprizone feed exhibit a 51%

decrease in numbers of TrkB+CC1<sup>+</sup> cells (Figure 7). These data suggest that decreases in TrkB remain and other compensatory mechanisms occur to allow for eventual recovery. Future experiments will be necessary to explore this possibility.

## Discussion

Our studies demonstrate that TrkB expression in OLGs is important in response to a demyelinating lesion. With the deletion of TrkB from OLGs, Cre<sup>+</sup> mice show increased sensitivity to a cuprizone-elicited lesion in the corpus callosum compared to Cre<sup>-</sup> mice. Cre<sup>+</sup> mice have



**Figure 7.** TrkB Receptors Are Deleted From OLGs in Cre+ Mice after Removal from Cuprizone for 4 weeks. Cre+ or Cre- mice were injected with tamoxifen. In both control- (A, B) and cuprizone- (C, D) fed mice, Cre- animals exhibit subsets of CC1+ cells (green) that are TrkB+(red) (arrows). After tamoxifen, there is a 58% reduction of TrkB+CC1+/total CC1 cells in control mice and a 51% reduction in cuprizone mice. Scale bar = 20  $\mu$ m. \*Significantly different from Cre- corpus callosum, analyzed using paired Student's *t* test. \**p* < .05. *N* = 3 experiments. Each experiment includes four mice (Control +/- Cre and Cuprizone +/- Cre). Data are presented as mean  $\pm$  SEM. TrkB = tropomyosin receptor kinase B.

exacerbated losses in myelin protein levels, increased myelin disruption characterized by fewer numbers of myelinated axons, and decreased myelin thickness. Moreover, there is a delayed recovery in myelinated axons and myelin proteins that occurs once cuprizone is removed.

The regulation of OLG lineage cell development by BDNF signaling has been well established by previous studies. In cultured basal forebrain OLG progenitors, BDNF increases DNA synthesis and increases myelin protein expression through TrkB (Du et al., 2006; Van't Veer et al., 2009). *In vivo*, the basal forebrain of BDNF+/- mice exhibits reduced numbers of NG2+ progenitors and reduced levels of myelin proteins compared to wild-type littermates throughout development and as adults (VonDran et al., 2010). The conditional knockout of TrkB from the CNS during embryonic development of the neocortex results in hypomyelination, with reduced MBP expression and myelin thickness, and fewer myelinated axons (Medina et al., 2004). The deletion of TrkB specifically from OLGs also shows a hypomyelinating phenotype during development with normal myelination occurring in adults (Wong et al., 2013).

The impact of BDNF on OLGs is similarly reflected during lesions and injuries. Following a demyelinating cuprizone lesion, BDNF+/- mice exhibit blunted

increases in NG2 levels, decreases in proliferation of OLG progenitors, as well as greater deficits in myelin proteins in the corpus callosum compared to wild-type mice (VonDran et al., 2011; Tsperson et al., 2015). During a spinal cord injury, intrathecal delivery of BDNF upregulates MBP expression and decreases apoptosis of OLGs (Ikeda et al., 2002; Koda et al., 2002), while BDNF expressing fibroblast grafts enhance the proliferation and myelination of OLGs (McTigue et al., 1998). More recent work suggests that these effects may be due to actions of TrkB on OLGs as deletion of the receptor from CNPase cells throughout life results in a reduction of effects of a BDNF mimetic on the remyelination process that is occurring 1 week following removal from cuprizone (Fletcher et al., 2018). Our work extends these studies to evaluate direct effects of the TrkB deletion specifically during the cuprizone lesion by use of an inducible conditional knockout model of TrkB from PLP+ cells. Moreover, it examines both the demyelination as well as a prolonged remyelination process.

Interestingly, results in which BDNF+/- mice are exposed to cuprizone as described (VonDran et al., 2011) differ in some respects from what we observe in the present work. When BDNF+/- mice are examined,

no effects on numbers of mature OLGs are apparent during demyelination, whereas conditional knockout mice with induced OLG TrkB-specific deletions prior to cuprizone exhibit a reduced number of mature OLGs during cuprizone administration. In addition, we found that BDNF<sup>+/-</sup> mice do not recover levels of myelin protein after cuprizone removal (VonDran et al., 2011), while effects of the TrkB deletion are not permanent, and recovery does occur although it was delayed.

Why do these differences occur? With respect to the difference in loss of OLGs, one possibility is that the reduced level of BDNF expression in the heterogeneous mice may be sufficient to maintain the survival of OLGs. Alternatively, neurotrophin 4 (NT4) that also interacts with TrkB (Barbacid, 1994), but presumably is not affected in the BDNF-deficient mouse, may be sufficient to maintain survival of OLGs. On the other hand, it may be that when TrkB is deleted, both BDNF and NT4 would be impacted in their ability to influence OLG lineage cells, resulting in a more severe phenotype than would be present when there is a 50% decrease in BDNF. The role of NT4 in conjunction with BDNF on myelination following a demyelinating lesion has yet to be explored.

In addition, why do Cre<sup>+</sup> cuprizone mice recover from myelin loss at 4 weeks? One possibility for this recovery may be due to the use of tamoxifen-inducible Cre recombination (Leone et al., 2003) where some OLG TrkB<sup>+</sup> cells that escaped recombination could ultimately compensate for the loss of PLP-TrkB<sup>+</sup> cells. This possibility can be tested in the future by injection of tamoxifen at 6 weeks of cuprizone and analyzing increases in OLGs when cuprizone is removed. However, based on our data thus far, this does not appear to be the case because we see approximately the same reduction in TrkB<sup>+</sup>CC1<sup>+</sup> cells at 6+4 weeks of recovery (Figure 7) as at 5 weeks of cuprizone treatment (Figure 1) when the deletion of TrkB results in deficits in OLG function. Another possibility is that during the remyelination process, factors are stimulated to be produced that do not depend on TrkB, resulting in the remyelination of demyelinated axons. OLGs are known to be a heterogeneous population responsive to a number of trophins and factors (Barres et al., 1993, 1994; Scarisbrick et al., 2000; Du et al., 2003; Dimou and Simons, 2017). Not only are there regional differences within the CNS, there is heterogeneity that also exists between OLGs within the same area (Ornelas et al., 2016). For example, across the basal forebrain, corpus callosum, and spinal cord, subpopulations of OLGs express TrkB, with other OLGs expressing TrkA, and TrkC (VonDran et al., 2010, 2011; Coulibaly et al., 2014). Moreover, factors such as insulin-like growth factor, fibroblast growth factor, and neuregulin-1 type III are known to have effects on differentiation of OLG lineage cells that may also

contribute to recovery following a lesion (Huang and Dreyfus, 2016). To fully understand how the diverse arrays of growth factors impact remyelination, it is important to explore how these factors function in concert with effects of BDNF and should be the focus of future studies.

Another question to consider is why remyelination is affected at all. PLP has been presumed to be expressed only in maturing OLGs, and it can be argued that oligodendrocyte progenitor cells (OPCs) that would be responsible for the remyelination would repair the lesion as normal. It is possible, however, that mature OLGs communicate with OPCs and OLG death influences the ability of the OPCs to mature and myelinate denuded axons. For example, it is known that OLGs produce BDNF (Dai et al., 2003) and that OPCs express TrkB and respond to BDNF (Van't Veer et al., 2009; VonDran et al., 2010; Tshiperson et al., 2015). On the other hand it may be important to consider which cells exhibit the TrkB deletion. Although PLP is generally considered a mature OLG marker, PLP gene expression is known to be present in NG2 cells in the developing brain (Mallon et al., 2002), and OPC-associated PLP protein may influence migration of OPCs in response to excitatory stimulation (Harlow et al., 2015). Our mouse is derived from mice in which recombination occurs in mature OLGs as well as NG2<sup>+</sup> cells (Leone et al., 2003). An intriguing possibility is that deletion of TrkB from PLP cells also impacts OPCs of the cuprizone lesion and delays their maturation into mature OLGs. The role that the TrkB deletion plays through NG2<sup>+</sup> cells during remyelination, as well as the demyelination process is unexplored and a fertile ground for exploration in the future.

In sum, we have found that TrkB on OLGs is critical for the response of OLGs to a demyelination lesion with the loss of this receptor impacting demyelinating and remyelinating cells. The data are consistent with the possibility that BDNF-TrkB signaling influences the ability of OLG lineage cells to withstand and recover from a demyelinating lesion and that at least some of the BDNF effects are directly on OLGs. Moreover, it suggests that multiple signaling pathways may be stimulated to collaborate to enhance remyelination in the absence of TrkB signaling.

## Summary

The current study uses inducible PLP+OLG TrkB-deleted mice and reports that TrkB affects OLGs during and following cuprizone treatment. It suggests that BDNF plays a critical direct role in the reaction of OLGs to demyelination.


## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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