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Conditional knockout of TOG results in CNS hypomyelination

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Abstract

The tumor overexpressed gene (TOG) protein is present in RNA granules that transport myelin basic protein (MBP) mRNA in oligodendrocyte processes to the myelin compartment. Its role was investigated by conditionally knocking it out (KO) in myelinating glia in vivo. TOG KO mice have severe motor deficits that are already apparent at the time of weaning. This phenotype correlates with a paucity of myelin in several CNS regions, the most severe being in the spinal cord. In the TOG KO optic nerve less than 30% of axons are myelinated. The number of oligodendrocytes in the corpus callosum, cerebellum, and cervical spinal cord is normal. In the absence of TOG, the most patent biochemical change is a large reduction in MBP content, yet normal amounts of MBP transcripts are found in the brain of affected animals. MBP transcripts are largely confined to the cell body of the oligodendrocytes in the TOG KO in contrast to the situation in wild type mice where they are found in the processes of the oligodendrocytes and in the myelin compartment. These findings indicate that MBP gene expression involves a post-transcriptional TOG-dependent step. TOG may be necessary for MBP mRNA assembly into translation permissive granules, and/or for transport to preferred sites of translation.

Keywords

mRNA localization; MT-associated protein; myelin basic protein; RNA granule

Introduction

In rodents, CNS myelin formation does not occur in the absence of one of its main component, myelin basic protein (MBP) (Chernoff, 1981; Roach et al., 1985). In contrast to most myelin proteins of the CNS, MBP is synthesized in the myelin compartment itself (Colman et al., 1982). This is accomplished following the assembly of MBP mRNAs in RNA-protein complexes called RNA granules, their microtubule-dependent transport in the oligodendrocyte processes and their translation in myelin (Ainger et al., 1993; Colman et al., 1982). In addition to mRNAs, RNA granules contain microtubule-associated motor proteins, components of the translation machinery and translation regulatory proteins (Barbarese et al., 1995).

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One RNA granule component, the heterogeneous nuclear ribonucleoprotein (hnRNP) A2 binds a sequence located in MBP mRNA termed A2RE (Hoek et al., 1998) that is required for transport of MBP mRNA in oligodendrocyte processes (Ainger et al., 1997). A yeast two-hybrid system analysis using hnRNP A2 as a bait identified the tumor overexpressed gene (TOG) protein (RecName: cytoskeleton associated protein 5 (CKAP5)) as its binding partner (Gao et al., 2008; Kosturko et al., 2005; Shan et al., 2000). Both are found in MBP mRNA granules (Kosturko et al., 2005).

While present at high levels in the adult brain (Charrasse et al., 1996), TOG has been studied mainly in non-neural cells as a microtubule-associated protein that can regulate microtubule dynamics (Al-Bassam and Chang, 2011; Charrasse et al., 1998). It is essential for mitosis but appeared otherwise dispensable for normal cell physiology (Gergely et al., 2003). Our previous data suggest an important role for TOG in the nervous system. Reduced translation of the activity-related cytoskeleton-associated (ARC) protein mRNA was seen in neurons cultured from neuron-specific TOG KO mice (Barbarese et al., 2013). Similarly a decrease was observed in the translation of a MBP-GFP fusion protein in a neuroblastoma cell line after shRNA treatment for TOG (Francone et al., 2007).

TOG protein contains multiple TOG domains that are composed of HEAT repeats (Slep, 2009). Repetitive motifs-containing proteins have been implicated in the assembly of RNA/RNP granules (Wu and Fuxreiter, 2016). This structural feature of TOG suggests that it could lead to the recruitment of mRNAs into translation-permissive granules (Gao et al., 2008). It may furthermore mediate the binding of microtubules to F-actin, a step that is necessary for translation of some mRNAs (Akhmanova & Steinmetz, 2008; Buxbaum et al., 2015). In this regard, the HEAT repeat-containing protein adenomatous polyposis coli protein anchors RNAs in fibroblast protrusions (Mili et al., 2008). Other investigations have shown that TOG can affect a number of cellular functions such as signaling and mRNA regulation and translation although the mechanism by which TOG is implicated in these functions remains to be determined (Fielding et al., 2008; Laursen et al., 2011; Lowery et al., 2010; Skoblov et al., 2013; White et al., 2008).

Here we have characterized the effects of the targeted knockout of TOG in oligodendrocytes in vivo using the cre-lox system to more specifically define the role of TOG in the expression of MBP during the period of myelination in the brain. Our results show that the absence of TOG disrupts the normal pattern of MBP expression which in itself could account for the hypomyelination seen in the CNS. MBP mRNA transport and consequently or independently MBP translation are disrupted in the absence of TOG.

Materials and Methods

Ethics statement

Use of animals in this study followed the guidelines of the University of Connecticut Health (UConn Health) Institutional Animal Care and Use Committee (IACUC) (Animal Welfare Assurance number A-3124-01) and those of the National Institutes of Health and was approved by IACUC under Protocol # 100686-0416.

Animals

To specifically knock out TOG in myelinating glial cells, C57BL/6 congenic mice with floxed introns 3 and 6 of the TOG gene (Barbarese et al., 2013) were crossed with a Cre driver mouse line that expresses the recombinase in oligodendrocytes and Schwann cells under control of the 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (*Cnp1*) gene (Lappe-Siefke et al., 2003). Wild type mice (C57BL/6J) (JAX 000664) were obtained from the Jackson Laboratories (Bar Harbor, ME). Heterozygous *shi* (+/*shi*) (C3Fe.SWV-Mbpshi/J) (JAX 001428) mice obtained from the same vendor were bred to obtain homozygous *shi* (*shi*/*shi*) mice. All animal use and care protocols were approved by the Institutional Animal Care and Use Committee at UConn Health.

Motor behavior

Male mice were housed 2–3 of the same genotype to a cage on a 12 h light/dark cycle. Tests were carried out during the light cycle in the Scoville Neurobehavioral Suite (UConn Health). General health parameters (body weight, appearance of fur, body posture and gait) were monitored weekly. Animals were habituated to the test room for 1 h before administration of the tests.

Balance, coordination and endurance were measured with a rotarod apparatus as described in Nakajima et al., (2008). Mice were allowed to walk on the rotating rod for 15 sec at constant speed of 4 RPM before acceleration began. Each mouse was subjected to 4 trials (D'Hooge et al. 1999) with an interval of 8 minutes between trials. A trial ended either when the mouse did 2 consecutive loops, indicating loss of balance, or when the 300 s time limit was reached. The average across trials for each genotype was determined.

Stride and gait were visualized by coating the hind paws with gel food coloring to track each paw print as the animal walked on as strip of white paper towards an enclosed dark goal box. Distance between consecutive paw prints was measured manually and analyzed (D'Hooge et al., 1999; Fernagut et al., 2002).

Cell culture

Oligodendrocytes were isolated from mixed glia cultures obtained from cerebral cortices of P0–P2 mice and grown as previously described (Giampetruzzi et al., 2013).

Histochemistry

Fifteen micron thick paraformaldehyde fixed tissue sections were stained for myelin with Black-Gold II (EMD Millipore) according to the manufacturer's protocol (Schmued et al., 2008) and counter stained with cresyl violet.

Immunohistochemistry

Tissue sections and cultured cells were fixed with 4% paraformaldehyde, permeabilized with detergent, incubated successively with blocking reagent, and with primary and secondary antibodies. The following primary antibodies were used: mouse anti-CNP (EMD Millipore, 1:200 dilution), mouse anti-MBP (Covance, 1:2,000 dilution), rabbit anti-OLIG2 (Abcam, 1:1000 dilution) and rabbit anti-TOG antibody (Abcam, 1:400 dilution). Secondary

conjugated antibodies were from Molecular Probes. Nuclei were stained with TO-PRO-3 (1:500) (Invitrogen). Tissue sections and cultured cells were covered with Prolong Gold antifade reagent (Invitrogen). Fluorescent images were acquired by confocal microscopy.

Transmission Electron Microscopy (TEM)

Mice (n = 3) for each genotype were anesthetized and perfused with saline followed by 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2. Optic and sciatic nerves were dissected out and left for 24 hours in the same fixative solution at 4°C. The tissue was embedded in Poly/Bed resins, post-fixed, and stained *en bloc* with 1% aqueous uranyl acetate following standard procedures. Ultrathin sections were observed in a Hitachi H-7650 transmission electron microscope.

For each animal, a series of micrographs was taken of the optic and sciatic nerves from which the percentage of myelinated axons (> 1,000 axons analyzed for each control and TOG KO), axon diameter and g-ratio were determined.

Western blotting

Gel electrophoresis and Western blotting were performed as described in Kosturko et al., (2005) using the following antibodies: mouse anti-MBP (Covance, 1:10,000 dilution), mouse anti-myelin oligodendrocyte glycoprotein (MOG) (Millipore, 1:1000 dilution), and mouse anti-proteolipid protein (PLP) (Millipore 1:1,000 dilution), and appropriate horseradish conjugated secondary antibodies (Jackson ImmunoResearch, 1:10,000 dilution). Western blots were visualized using chemiluminescence detection (Pierce). Densitometric analysis was performed using Adobe Photoshop. Optical intensity of bands was integrated after subtracting background; levels of test proteins were normalized to β-actin or nucleoporin protein levels. The levels of proteins from control mice were set at 100%.

qRT-PCR analysis

Total RNA from brain was isolated with the Maxwell 16 Tissue LEV Total RNA Purification kit (Promega) following the manufacturer's protocol. RNA was reverse-transcribed and used for qPCR in the TaqMan Gene Expression Assays (Applied Biosystems). Sense and antisense primers for detection of MBP transcripts are described in Traka et al., (2008). The mRNA copy numbers were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Three brains of each genotype, and two brains of each genotype were used respectively in two independent assays performed in quadruplicate on 45 days old mice.

In situ hybridization (ISH)

Cross sections of the cervical spinal cord and sagittal sections of cerebellum were prepared and ISH was performed as previously described with minor modifications (Ishii et al., 2014) using riboprobes specific for MBP mRNA (Dr. M. Qiu, University of Louisville, KY) and specific for PLP mRNA (Dr. W.B. Macklin, University of Colorado School of Medicine, Aurora, CO). Briefly, after incubation in 1 ug/ml proteinase K at 37°C for 30 min, sections were hybridized overnight at 65°C with digoxigenin-labeled antisense cRNA probe and washed twice in 50% formamide, 2x SSC, and 1% SDS at 65 – 70°C for 15 min each,

followed by two washes in 100mM maleic acid, pH7.5, 150mM NaCl, and 0.1% Tween 20 at room temperature for 30 min each. After blocking in PBS, 0.1% Triton X-100, and 0.2% bovine serum albumin (1 h), sections were incubated overnight in alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000; Roche Diagnostics). Color was developed with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate.

Mitochondria distribution

MitoTracker Red CM-H_2Ros (LifeTechnologies) was added to the culture medium of oligodendrocytes at a concentration of 100nM to label live mitochondria. After incubation at 37°C for 1hr, the cells were rinsed 3X with pre-warmed culture medium without MitoTracker before being visualized. Two independent sets of cultured cells were used for analysis.

Statistical analysis

Comparisons between two groups were performed using unpaired, one- tailed Student's *t*-test. All values are expressed as mean ± SEM. Significance was accepted with $p \leq 0.05$.

Results

Production of the oligodendrocyte-specific TOG conditional knockout (KO)

TOG is ubiquitously expressed and particularly abundant in the nervous system (Charrasse et al., 1996). In order to study its role in mice in vivo, a conditional KO was generated because a full KO is lethal due to the essential role of TOG in mitosis (Barbarese et al., 2013; Gergely et al., 2003). The Cre/loxP-induced conditional knockout system was used to obtain mice in which TOG was knocked out in post-mitotic oligodendrocytes. Mice homozygous for the TOG floxed gene, described in Barbarese et al., (2013), were crossed with CNPcre/+ mice (Lappe-Siefke et al., 2003) to generate CNPcre/+;TOG^{floxed}/+. These were then crossed with +/+;TOG^{floxed}/TOG^{floxed} to obtain CNPcre/+;TOG^{floxed}/TOG^{floxed} (CNP-TOG KO referred to as TOG KO) mice which were present in the expected Mendelian ratio. These conditional TOG KO mice carry only one copy of the CNP gene in which the ORF of CNP has been replaced with that of CRE. This breeding scheme was adopted because the total absence of CNP (CNP^0/CNP^0) in otherwise wild type mice results in demyelination in adulthood. Mice carrying only one copy of the wild type CNP gene are neurologically normal throughout their lifespan (Lappe-Siefke et al., 2003). CNP is expressed in the two myelinating glia of the nervous system oligodendrocytes in the CNS and Schwann cells in the PNS. Fig. 1A shows that TOG is not detectable in the corpus callosum (a region heavily populated by oligodendrocytes) of the TOG KO mice but present in the stratum pyramidale of the hippocampus (a region rich in neuronal cell bodies). TOG is present in the corpus callosum of homozygous *shiverer* mice (Fig. 1B) which were used for comparison because similarly to the TOG KO they fail to produce myelin in the CNS (Chernoff, 1981). Because TOG is also present in neurons (see hippocampal neurons in Fig. 1A) and other brain cell types, it precludes the use of Western blotting for stringent quantification of TOG in oligodendrocytes in the CNP-specific TOG KO brain tissue. However, Western blot was used to show changes in TOG level in the optic nerve where there are no neuronal cell bodies and where cell types are more restricted and

oligodendrocytes are abundant. This analysis shows a 70% decrease of TOG protein in the TOG KO supporting the finding that oligodendrocytes are deficient in TOG in the transgenic mice.

TOG KO has severe motor impairments

TOG KO mice are readily identifiable at the time of weaning by their intention tremor which is absent in mice of all other genotypes generated in the CNPcre/+;TOG^{floxed}/+ by +/+;TOG^{floxed}/TOG^{floxed} cross. PCR genotyping of mice in a blind fashion shows a full correlation between the CNPcre/+;TOG^{floxed}/TOG^{floxed} genotype and the shivering phenotype. Visualization of the paw prints shows that the walking pattern of the TOG KO (Fig. 2A, right lane) is blurred and different than wild type (left lane). The stride length of the TOG KO (3.3 ± 0.8 cm), measured from the paw prints, is shorter than wild type (6.8 ± 0.4 cm) (Fig. 2B). Motor coordination and strength were assessed in a rotarod test. Here too, TOG KO mice display a severe phenotype frequently falling even before the rod is set in motion (Fig. 2C) while wild type mice have a latency of 208 ± 60 s. TOG KO mice develop seizures as they age and become progressively more impaired; they die at about 4 months of age. A similarly reduced lifespan is observed in some dysmyelination mutants (Chernoff, 1981; Duncan et al, 2011).

TOG KO shows CNS dysmyelination/hypomyelination

The early appearance of the motor deficits described above suggests that the TOG KO phenotype could be due to a defect in myelin formation. This was investigated by staining the spinal cord, brain and cerebellum of the control and TOG KO mice with Black-Gold II to visualize myelin (Schmued et al., 2008). Fig. 3A shows the typical staining pattern of the cervical spinal cord in the control mice where myelin appears brownish red with some stained fibers penetrating in the grey matter. In sharp contrast, only faint and regionally limited myelin staining is seen in the TOG KO spinal cord (Fig. 3B). The weakly stained region above the spinal canal (sc) corresponding to the dorsal corticospinal tract is indicated by an arrow in Fig. 3B and magnified in Fig. 3D. The same region of the control is clearly heavily myelinated (Fig. 3C). All TOG KO mice (n = 3) show the identical regional staining of the dorsal corticospinal tract. Section of the mouse spinal cord appearing in Fig. 3B and D has the most intense myelin staining of the corresponding sections in the 2 other mice analyzed. Except for that lightly positive stained region, the rest of the TOG KO spinal cord section shows little above background staining. As an example, the region encompassing white and grey matter that is outlined by a box in Fig. 3B was magnified and compared to the same region in control (box in Fig. 3A). The control (Fig. 3E) shows strong white matter staining with myelinated fibers projecting in the grey matter. The corresponding TOG KO (Fig. 3F) has few isolated red puncta that were not clearly identifiable.

Brain sagittal sections show a similar discrepancy in the myelin staining intensity of the control and TOG KO mice. Fig. 3G shows the strong myelin staining of the corpus callosum (cc) (above the hippocampus (H)) and ventral hippocampal commissure (vhc) in the control. The staining of the corpus callosum (cc) in the TOG KO (Fig. 3H) is apparent but of low intensity. The staining of the ventral hippocampal commissure (vhc) of the TOG KO (Fig. 3H) is less intense than that of the control (Fig. 3G) but appears significant.

The cerebellum of the TOG KO mice (Fig. 3J) has a myelin staining pattern resembling that of control (Fig. 3I) only also less intense. This pattern and intensity was consistent among the 3 TOG KO analyzed. The reduction in myelin appears less severe in the cerebellum than in other CNS regions of the TOG KO. Overall, the level of myelin staining intensity is greatly reduced in the TOG KO CNS. Interestingly, the distribution pattern of myelin remaining in the TOG KO spinal cord, corpus callosum and cerebellum cannot be accounted for by random failure of cre recombination.

Further investigation was carried out by TEM imaging of optic nerves of TOG KO and wild type mice. Fig. 4B reveals a paucity of myelinated axons in the KO animals as compared to wild type (Fig. 4A). While over 95% of optic nerve axons are myelinated in the wild type mice, only 29% are in the TOG KO (Fig. 4C). CNP is expressed in the other type of myelinating glia, the Schwann cells, but in contrast, myelination is not affected in the PNS of TOG KO as large (Fig. 4D, left panel) and small caliber (Fig. 4D, right panel) axons of the sciatic nerves have normal appearing myelin sheaths. Sciatic nerves of the TOG KO have only 20% of control MBP level as determined by Western blot but it is documented that even total absence of MBP as in the *shiverer* mouse does not impair myelination of PNS axons (Kirschner & Ganser, 1980).

To determine if the myelin deficit was due to a decrease in oligodendrocytes number, brain sections were stained with anti-OLIG2 and anti-CNP, and positive cells were counted in the corpus callosum of control and TOG KO mice. There is no evidence of a decrease in the number of immature and mature oligodendrocytes in the TOG KO mice when compared to controls (Fig. 4E). The number of TOG KO mature oligodendrocytes positive for the presence PLP mRNA in the cerebellum (Fig. 4F) and spinal cord (Fig. 4G) is also comparable to that of controls. To determine if failure to produce CNS myelin in CNP-TOG KO mice was due to having smaller than normal caliber axons, the number of axons with different diameters were quantified and grouped in a standard bin size of 0.1μm. Overall, axonal calibers are not different between TOG KO (grey bars) and control (black bars) mice (Fig. 4H) indicating that failure to myelinate was independent of axon caliber n the optic nerve.

In optic nerves of TOG KO mice, the axons that are myelinated appear to have thinner myelin compared to controls. This observation was quantified by measuring the g-ratio of the myelinated axons of control and TOG KO mice. The results show that TOG KO mice have a significantly higher g-ratio than control which is indicative of a decrease in myelin thickness (Fig. 4I). Mature nerve fiber populations demonstrate a positive correlation between axon caliber and myelin sheath thickness (Fraher et al., 1989; Hildebrand and Hahn, 1978). While there is a positive linear relationship in control mice (Fig. 4J), there is no significant correlation between axonal diameter and myelin thickness in the TOG KO mice (Fig. 4K).

Myelinated axons of TOG KO mice have all or some portions of the myelin sheath that are compact. MBP is thought to be responsible for this compaction by apposing the cytoplasmic membrane surfaces of myelin where they appear strongly electron dense and form the major dense line (MDL). Two fields of myelinated axons in the control mice are presented in Fig.

5A and B. Segments of compact myelin, delineated by white asterisks in each panel, are magnified in Fig. 5A′ and B′, respectively. MDLs identified by opposing white arrows are well defined. Although in reduced numbers myelinated axons can be found in the TOG KO (Fig. 5C and D) and compacted myelin segments can be seen; those delineated by white asterisks appear at higher magnification in Fig. 5C′ and D′, respectively. MDLs are not as easily discernible in the TOG KO, but are present in the compacted segments. Opposing white arrows identify MDLs in Fig. 5C′ and D′. This feature suggests that the compacted multilayered wrapping of axons in the TOG KO is due to the presence of MBP in some oligodendrocytes.

Morphological assessment of the TOG KO indicates that it has a normal number of oligodendrocytes but a large population of dysmyelinated/hypomyelinated CNS axons. This phenotype could be associated with defects in myelin components as seen in some well characterized CNS myelin mutants.

TOG KO has reduced MBP level

The association of TOG with MBP RNA granules suggests that dysregulation of MBP expression could be implicated in the hypomyelination phenotype observed. A defined level of MBP is necessary for CNS myelination (Popko et al., 1987) and the myelin deficit observed in the TOG KO could be due to the absence or a severely reduced level of MBP in oligodendrocytes. Immunohistochemistry of brain sections with anti-MBP shows a weak signal in the TOG KO (Fig. 6B) in the corpus callosum, a region normally well myelinated, as compared to wild type (Fig. 6A). It also corresponds with the results obtained using Black-Gold II myelin stain showing a strong reduction in staining intensity of the TOG KO corpus callosum (Fig. 3H). The reduction in the level of MBP was quantified by Western blot analysis of brain homogenates which shows that TOG KO has 20% of the normal amount of MBP (Fig. 6C), a level that would be insufficient for normal CNS myelination. The level of MBP in cerebellum homogenates is 40% that of control which might explain why myelin staining intensity seen in that CNS region of the TOG KO (Fig. 3J) is not as dramatically reduced as in the spinal cord or the corpus callosum.

MOG is an oligodendrocyte-specific protein expressed a day or two after MBP. It is reduced in brain homogenate of TOG KO compared to control as is PLP (Fig. 6C) but to a much lesser extent than MBP indicating that the overall program of myelination is not all together compromised in the TOG KO.

It has been reported that the level of MBP protein is commensurate to the level of MBP transcripts when the latter is below 50% of wild type (Shine et al., 1992). In order to determine if the low level of MBP protein could be due to reduced MBP transcription, we performed a quantitative RT-PCR analysis of mRNA isolated from brain homogenates of control and TOG KO mice. The results presented in Fig. 6D show that the level of MBP transcripts in the TOG KO mice is comparable to that of wild type.

MBP transcripts are mislocalized in the TOG KO

The translation of MBP occurs principally in myelin where the bulk of MBP mRNA is found. This was determined by subcellular fractionation and in vitro translation of MBP

mRNA isolated from the myelin fraction (Colman et al., 1982). In situ hybridization (ISH) and immunohistochemistry showed a positive correlation between the localization of MBP transcripts and MBP proteins (Trapp et al., 1987). Since we established that MBP transcripts are present in a normal amount in the TOG KO (Fig. 6D) we sought to determine their intracellular location. ISH was performed on sections from two CNS areas, the cerebellum and the cervical spinal cord. In both areas of the wild type mouse (Fig. 7A, G), MBP transcripts are distributed over the myelinated fibers. At higher magnification, oligodendrocytes cell bodies (red arrows) of the cerebellum show only faint staining for the presence of transcripts (Fig. 7D) as previously reported (Trapp et al., 1987). In contrast to wild type, staining for MBP transcripts is prominent in oligodendrocytes cell bodies (red arrows) of the cerebellum (Fig. 7E) and faint in the regions of axonal fibers in the TOG KO. The same distribution pattern is seen in the TOG KO spinal cord (Fig. 7H, K) when compared to control (Fig. 7G, J). Overall the distribution of MBP transcripts in the TOG KO resembles more closely that of PLP transcripts in the wild type mouse which are shown here (Fig. 7C, F, I, L) than that of MBP mRNA in the wild type mouse. PLP transcripts have been reported to localize to the cell body of oligodendrocytes (red arrows) in the wild type mouse (Verity and Campagnoni, 1988). The similarity in the distribution pattern of MBP mRNA in the TOG KO CNS and PLP mRNA in the wild type CNS indicates that a large proportion of MBP mRNA accumulates in the oligodendrocyte cell body in the TOG KO mouse.

The distribution of mitochondria is unaltered in TOG KO oligodendrocytes

It has previously been established that the transport of MBP mRNA containing granules is microtubule-based and that it can be disrupted by drugs that prevent polymerization (Carson et al., 1997). Excess microtubules also prevents MBP mRNA transport to the myelin compartment and retention of the transcripts in the oligodendrocyte cell body as seen in the *taiep* mutant rat (O, Connor et al., 2000). TOG is a microtubule-associated protein that regulates microtubule dynamics; it has not been linked to the transport of organelles. To test the possibility of a defect in microtubule-based organelles transport in TOG KO, we studied the distribution of mitochondria as a representative organelle of this mode of transport (Chan, 2006) since the transport of mitochondria like that of MBP mRNA utilizes Kif1b (Lyons et al., 2009; Nangaku et al., 1994). We looked for the presence of mitochondria in processes adjoining axons of the optic nerve and their distribution in the processes of oligodendrocytes in culture. A magnification of Fig. 4B is presented in Fig. 8A and shows mitochondria (white arrows) in processes apposing axons (A) in the optic nerves of the TOG KO. When grown in culture, TOG KO oligodendrocytes exhibit a morphology similar to that of wild type. Mitochondria identified with the use of the MitoTracker Red CM-H_2Ros dye are present in the cell body and processes of both wild type and TOG KO cells. Individual mitochondria cannot be distinguished and counted in the cell body but they are distinct and present in the relatively same number (~40) in the sum of the processes of wild type and TOG KO cells. Fig. 8B and C show a wild type and a TOG KO oligodendrocyte in which mitochondria identified with white arrows are found at long distances from the cell body in both cells. The distribution of mitochondria in the processes of TOG KO oligodendrocytes was compared to that of wild type by measuring their distance to the cell body. Fig. 8D shows that mitochondria from TOG KO cell have a distribution similar to that of wild type with a decreasing number at distances greater than 40 μm form the cell body. These data

indicate that retention of MBP transcripts in the cell body is not due to a general failure of microtubule-associated organelles transport. It may instead arise from the failure, in the absence of TOG, to assemble them into transport/translation granules.

Discussion

Our study reveals that the cell-specific loss of TOG in oligodendrocytes severely reduced myelination in the CNS. The motor deficit of the TOG KO is present at the start of the myelination period and supports the idea that the TOG KO mouse fails to produce myelin (dysmyelination) or sufficient amount of myelin (hypomyelination) rather than loses myelin (demyelination). CNP, the promoter of which drives the expression of the cre recombinase, is expressed in Schwann cells as well but TOG KO mice have normal appearing PNS myelin, a situation not unlike that of the homozygous *shiverer* mice that is attributed to the difference in the amount and in the role of MBP in the CNS and PNS. Regional differences in the level of myelin were found in the TOG KO that cannot be solely attributed to random failure of cre recombination in oligodendrocytes. These differences most likely due to the corresponding differences in the level of MBP can have a variety of causes. Among them is the heterogeneity of oligodendrocytes from various CNS regions that can be traced to their different lineages (Le Bras et al., 2005; Vallsted et al., 2005; Vinet et al., 2010). Similarly to our findings, the study of Walh et al., (2014) reports the extent of myelination impairment the most severe in the spinal cord of mTOR cKO driven by the CNP-cre recombinase compared to other CNS regions. Heterogeneity of the oligodendrocyte population has also been suggested to explain regional differences in the level of myelination found in CNS regions of the *taiep* rat, a mutant with microtubules defect that accumulates MBP mRNA in the oligodendrocyte cell body (O'Connor et al., 2000). The level of cre recombinase driven by the CNP promoter can also vary regionally. For instance, the level of CNP in mouse/rat spinal cord is twice that of the brain (Gravel et al., 1998; Virgili et al., 1990; Vogel et al., 1988). Additionally, the level of TOG, although present in all CNS structures studied here, also varies regionally. As reported for humans and baboons, TOG expression is highest in the cerebellum but very low in the spinal cord (Charrasse et al., 1996). It is not known if the level of TOG in oligodendrocytes in these tissues is representative of the tissue as a whole neither do we know the half-life of TOG in oligodendrocytes in situ. We can only speculate that a combination of low level of recombinase with high level of TOG present in oligodendrocytes before recombination could lead to a degree of myelination in the cerebellum for example that is noticeably higher than that of the spinal cord where CNP/cre recombinase expression is high and TOG level is low. Another factor that could affect the level of myelination is the type of axons present in different CNS regions as well as within a region. The polarity of microtubules in oligodendrocyte processes and the size of axons have been suggested as contributing factors in regional differences observed in myelin levels in hypomyelination the *taiep* mutant (O'Connor et al., 2000). It is possible and may be even likely that these various parameters act together to generate the different patterns of myelin expression seen in the CNS of the TOG KO.

Where myelin is present in TOG KO mice it is thinner than control. Since the size distribution of axon diameters is similar to control in the TOG KO optic nerve we concluded that failure to myelinate lies within the oligodendrocyte as expected from the knockout

strategy that was employed. Thinner CNS myelin has been reported in mice that have a MBP content of less than 50% of wild type (Shine et al., 1992). TOG KO mice have only 20% of the normal MBP brain content. In addition to CNP, TOG KO oligodendrocytes are competent at expressing MOG and PLP suggesting that MBP is the principal myelin component affected by the reduction in TOG and responsible for the observed myelin deficit. Indeed, of the myelin proteins investigated by many groups, MBP is the only one that has been identified as essential for CNS myelin formation (Griffiths et al., 1998; Klugmann et al., 1987; Lappe-Siefke et al., 2003; Pan et al., 2005; Yamamoto et al., 1999; Yin et al., 2006). Our data do not exclude the possibility that other myelin or other oligodendrocytic components contribute to some extent to the myelin deficit of the TOG KO mouse but indicate that the lack of MBP is sufficient to result in CNS dysmyelination.

The low level of MBP protein comes from the failure to efficiently translate MBP mRNA that is found at normal level in the TOG KO but largely confined to the cell body. TOG may normally regulate MBP post-transcriptional expression by affecting microtubule dynamics and organelles transport thus preferentially locating MBP transcripts in translation-competent areas of the cell, and/or by promoting RNA granule assembly thus recruiting components of the translation machinery and promoting high efficiency translation. Our data indicate that the distribution of mitochondria in the oligodendrocyte processes which depends on a functional microtubule-based transport mechanism similar to that of RNA granules is not affected in the TOG KO. This suggests that while changes in microtubule dynamics may occur in the absence of TOG they do not account for the retention of MBP transcripts in the cell body. MBP transcripts could be poorly translated because they are not localized near axons that provide positive translation signals. Enhanced MBP expression has been observed when oligodendrocytes are co-cultured with neurons or treated with neuron-conditioned medium (Bologa et al., 1986; Coelho et al., 2009; Flores et al., 2008; Kramer-Albers and White, 2011; Laursen et al., 2011; Wake et al., 2011; White et al., 2008). However, MBP protein still is synthesized when oligodendrocytes are cultured in the absence of neurons and even when its mRNA is transfected in non-glial cells (Francone et al., 2007). The presence of MBP protein has also been observed in the oligodendrocyte cell body in vivo prior to myelin formation (Sternberger et al., 1978) and in zebrafish proximal glia processes when mRNA transport is deficient (Lyons et al., 2009). More recently, it has been reported that the downregulation of Tau, another microtubule-associated protein, results in a decreased localization of MBP mRNAs to the oligodendrocyte processes without affecting MBP translation *per se* (Seiberlich et al., 2015). So while proximity to axons enhances MBP translation it appears not to be the single determinant of translational output. Furthermore, MBP protein accumulates in the cell body indicating that MBP synthesized in the cell body is not subjected to extensive degradation.

TOG may be necessary for RNA granule assembly thus for recruiting components of the translation machinery and promoting high efficiency translation (Barbarese et al., 1995). The spatial confinement of MBP mRNA in granules would allow for rapid recycling and reloading of ribosomes onto mRNAs (Buchan et al., 2014). TOG could participate in the assembly of MBP mRNA and other components into granules because it has scaffolding properties and may be in complexes with several other proteins (Slep, 2010). It has also been proposed that granule assembly sequesters the mRNAs from translation interferences

(Buchan et al., 2014). These considerations suggest that failure to assemble MBP mRNA into granules could be the major determinant in the lack of MBP translation.

Release of translation inhibitory factors associated with MBP mRNAs may also require TOG independently of the location of the mRNAs. We have previously reported that hnRNP E1 in association with hnRNP A2 reduces MBP mRNA translation (Kosturko et al., 2006). This partial inhibition is released with the phosphorylation of hnRNP A2 by Fyn kinase. This step may necessitate TOG which has been implicated in Fyn kinase activation and also in integrin signaling (Fielding et al., 2008; Laursen et al., 2011; Lowery et al., 2010; Skoblov et al., 2013; White et al., 2008). It is likely that other translation inhibitors such as sncRNA715 are associated with MBP mRNA but the mechanism of their release is unknown and may or may not implicate TOG (Bauer et al., 2012).

By regulating the post-transcriptional expression of MBP, TOG has a critical role in the process of myelination. Report that MBP is absent in chronic inactive MS white matter lesions despite the presence of a normal amount of MBP transcripts suggests a similar importance of components and pathways associated with the post-transcriptional regulation of MBP expression in the process of remyelination (Bauer et al., 2012; Michel et al., 2015). Identifying them offers the potential that they can be modulated by drugs or small molecules to restore myelination.

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Main Points

TOG is required for the expression of myelin basic protein (MBP).

MBP transcripts level is not affected by the absence of TOG but in contrast to control they remain in the oligodendrocyte cell body.

MBP regulates CNS myelin level.

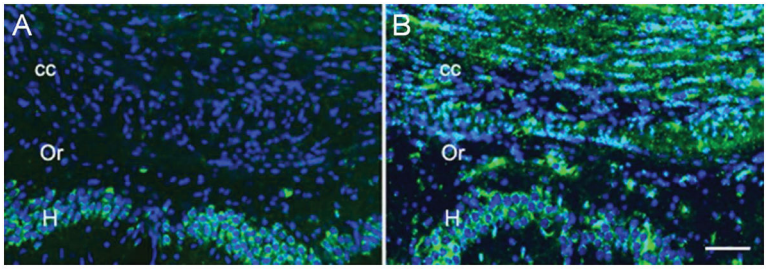

Figure 1. TOG is reduced or absent in oligodendrocytes of the CNP-conditional TOG KO mouse

(A) 45 days old TOG conditional KO (TOG KO) mouse brain coronal section and (B) *shiverer* mouse brain coronal section immunostained with anti-TOG (green). Cell nuclei were visualized with TO-PRO-3 (blue). Abbreviations: corpus callosum: cc; oriens layer: or; hippocampus: H. Scale bar = 50 μm.

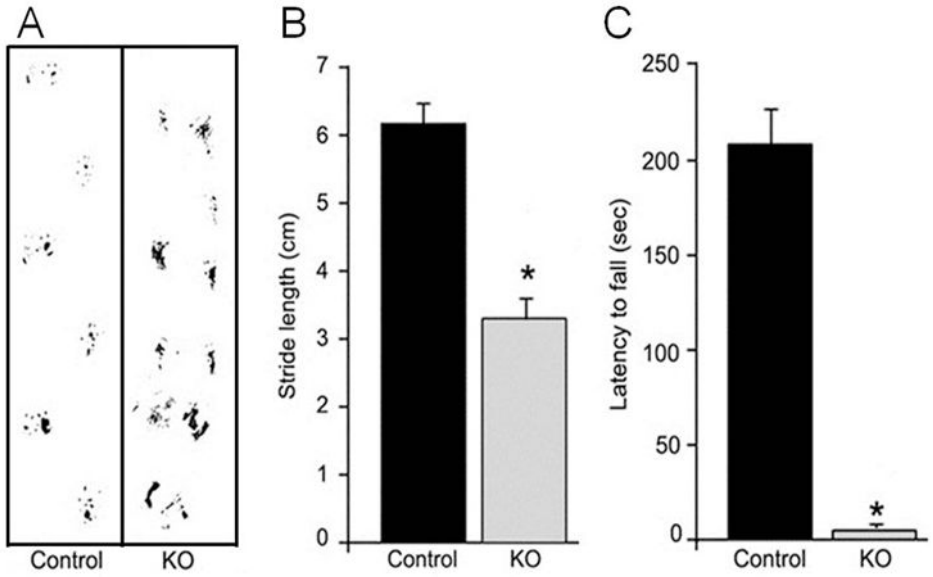

Figure 2. TOG KO mice have severe motor deficits

Seventy five days old wild type (n = 10) and 68 days old TOG KO (n = 7) were evaluated for gait (A) and stride (B) using a paw print pattern test, and for motor coordination and strength using a rotarod test as described in Materials and Methods. Values are expressed as mean ± SEM, Student's *t*-test **p* = 0.05.

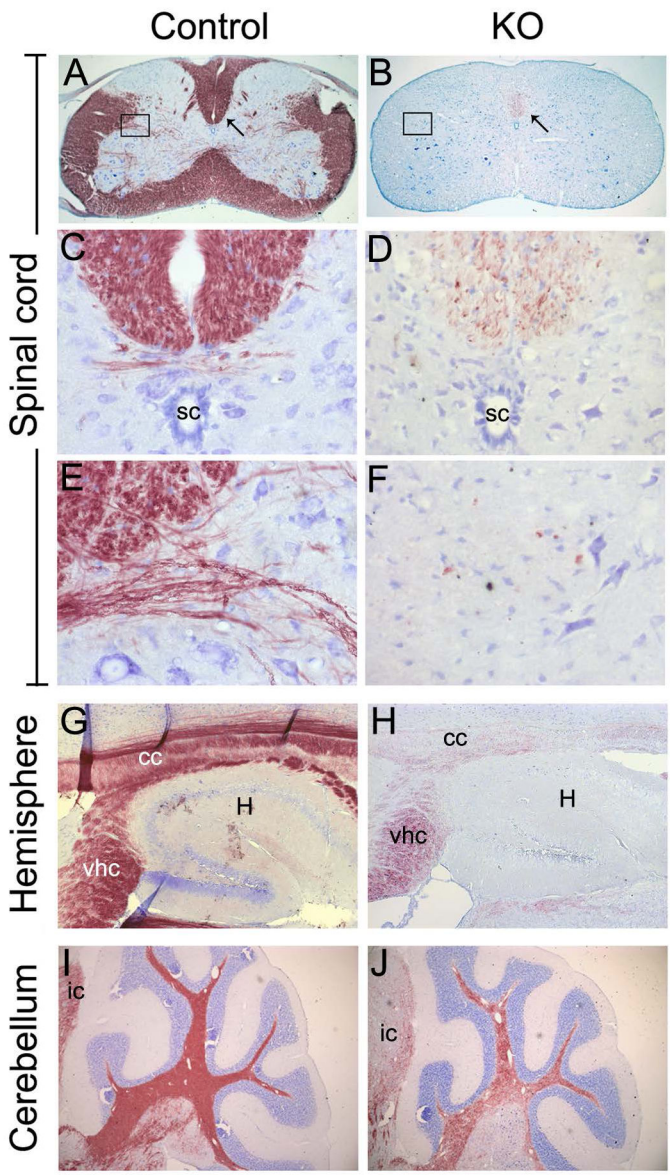

Figure 3. Absence of TOG results in CNS myelin deficit

Cervical spinal cord sections and sagittal brain and cerebellum sections from 25 days old control and TOG KO mice (n = 3 for each genotype) were stained with Black-Gold II myelin stain (red) and counterstained with cresyl violet (blue). Spinal cord, brain and cerebellum show reduced staining intensity with Black-Gold II indicating myelin deficit in the TOG KO ranging from almost complete (spinal cord) to strong but incomplete (cerebellum). Spinal cord of control (A, C, E) and TOG KO (B, D, F); C and D are magnified images of the region of the dorsal corticospinal tract indicated by an arrow in A and B, respectively; E and F are magnified images of the inset (boxed area) in A and B, respectively. G and H are sagittal brain sections of control and TOG KO, respectively. I and J are sagittal sections of the cerebellum of control and TOG KO, respectively. sc: spinal

canal; cc: corpus callosum; vhc: ventral hippocampal commissure; H: hippocampus; ic: inferior colliculus.

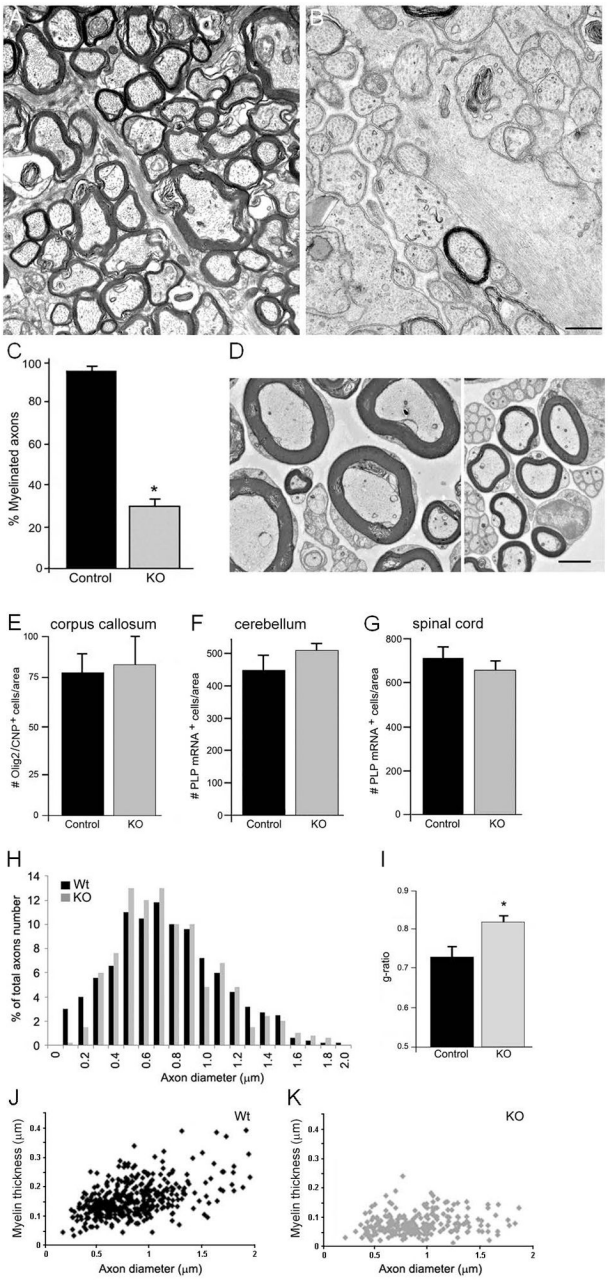

Figure 4. TOG KO optic nerves show dysmyelination and hypomyelination

TEM visualization of optic nerves cross-sections of control (A) and TOG KO (B); scale bar = 500 nm. (C) Percentage of myelinated axons in micrographs of optic nerve cross sections; n = 3 mice of each genotype and > 1,000 axons analyzed for each genotype. Values are expressed as mean ± SEM, Student's *t*-test **p* = 0.05. (D) TEM visualization of sciatic nerves cross-section of TOG KO large axons (left panel) and small axons (right panel); scale bar = 2 μm.

(E) Olig2 and CNP immuno-positive cell counts in the corpus callosum of control and TOG KO mice. (F) PLP mRNA-positive cell counts in half-sections of cerebellum and (G) spinal cord from control and TOG KO mice. Three sections each from three mice of each genotype

were analyzed. Values are expressed as mean ± SEM, Student's *t*-test **p* = 0.05. (H) Histogram of the distribution of optic nerve axons according to their caliber. The population of axons diameter is distributed in bins of 0.1 μm in control (black) and TOG KO (grey); n > 450 axons for each genotype. (I) g ratio determined from measurements of myelinated axons in optic nerves of control and TOG KO mice. Values are expressed as mean ± SEM, Student's *t*-test **p* = 0.05. (J) Representation of myelin thickness as a function of axonal diameter in the optic nerve in control and (K) TOG KO.

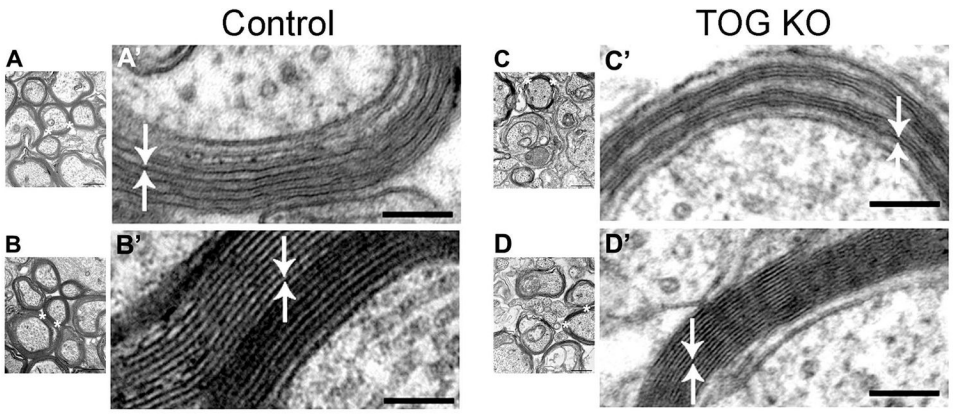

Figure 5. Myelin compaction in the TOG KO optic nerves

TEM cross sections of optic nerves from control (A-A′, B-B′) and TOG KO (C-C′, D-D′). White asterisks in A, B, C and D delineate myelin segments magnified in A′, B′, C′ and D′, respectively. Major dense lines (MDL) in A′, B′, C′ and D′ identified with white arrows pointing in opposite directions. Scale bars in A, B, C and D: 500 nm; scale bars in A′, B′, C′ and D′: 100 nm.

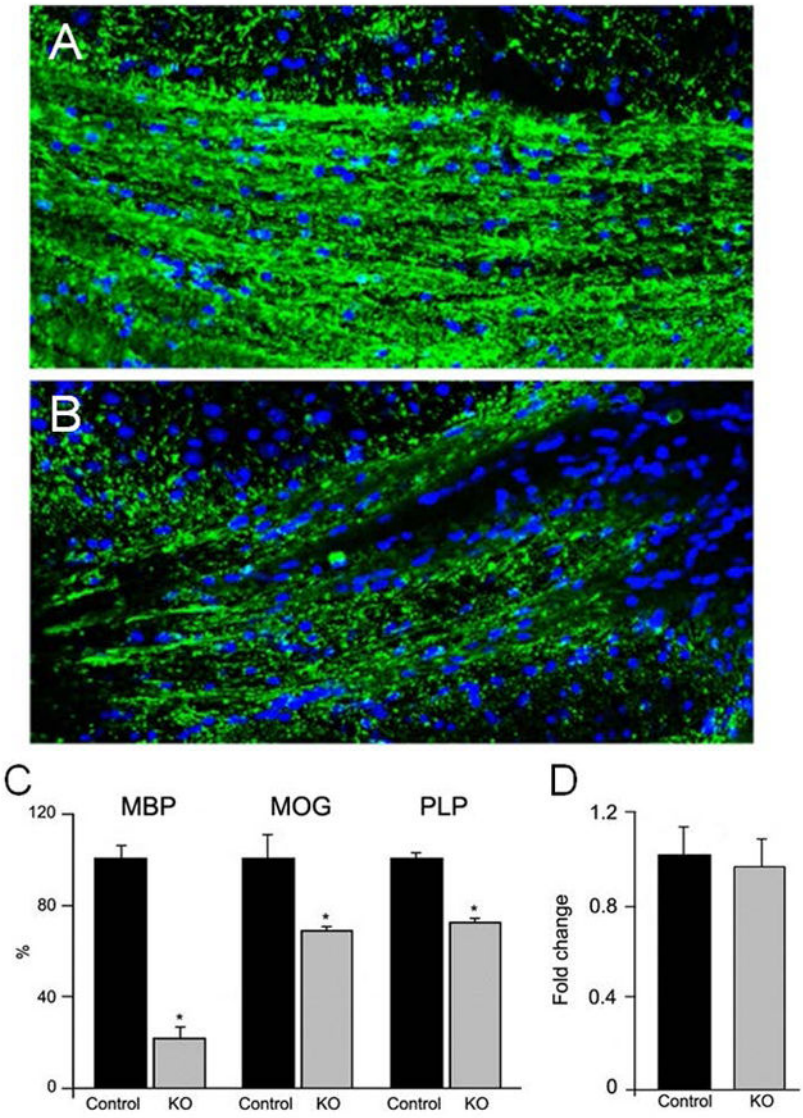

Figure 6. MBP translation requires TOG

(A) Coronal sections of 45 days old control and (B) TOG KO mouse brain immunostained with anti-MBP (green). Cell nuclei were visualized with TO-PRO-3 (blue). (C) Western blot analysis of brain homogenates from 45 days old control (black) and TOG KO (grey) mice for MBP, MOG and PLP proteins (n = 3 for each). (D) Quantitative RT PCR of MBP transcripts in brains of 45 days old control (black, n = 5) and TOG KO (grey, n = 5) mice. C–D: all data are the mean ± SEM; Student's *t*-test **p* = 0.05.

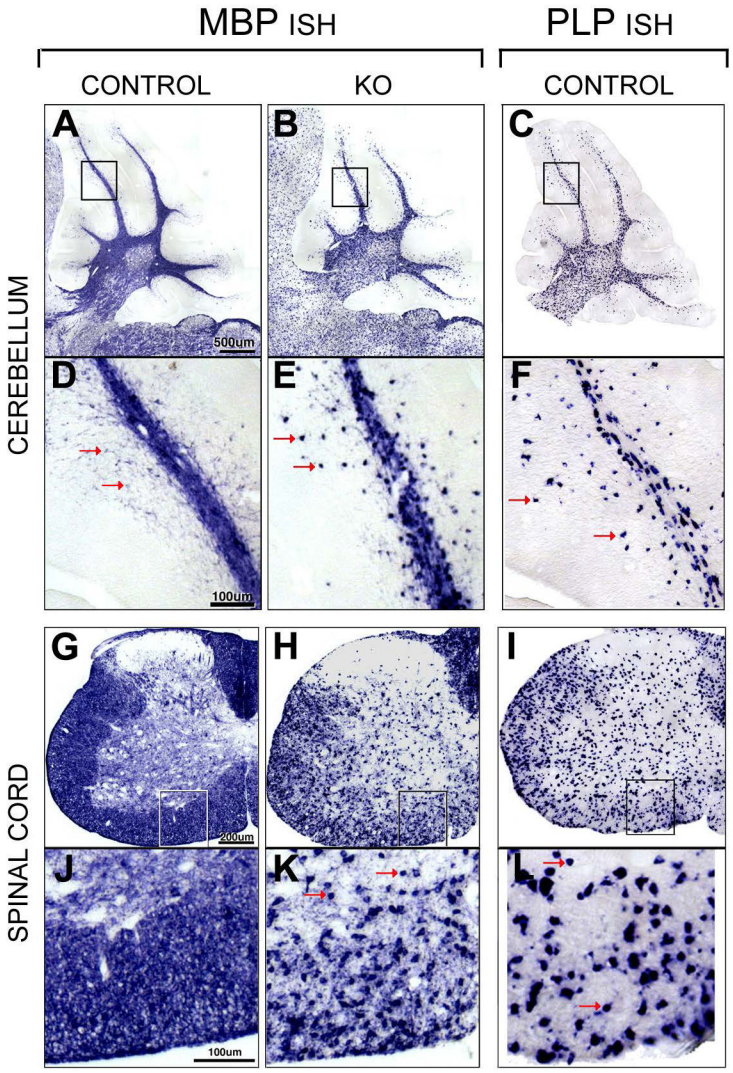

Figure 7. MBP transcripts are mislocalized in the TOG KO mice

ISH for MBP mRNA was performed in the cerebellum (A, B) and spinal cord (G, H) of control (A, G) and TOG KO (B, H) 25 days old mice. (D) High magnification of box area in (A). (E) High magnification of box area in (B). (J) High magnification of box area in (G) and (K). High magnification of box area in (H). D, E, K: red arrows identify cell bodies. ISH for PLP transcripts was performed in the cerebellum (C) and spinal cord (I) of control 25 days old mice. (F) High magnification of the box area in (C). (L) High magnification of the box area in (I). F, L: red arrows identify cell bodies. Scale bar of (A, B, C) as indicated in (A); scale bar of (D, E, F) as indicated in (D); scale bar of (G, H, I) as indicated in (G); scale bar of (J, K, L) as indicated in (J). Images are from matched sections of cerebellum and spinal cord from control and TOG KO mice.

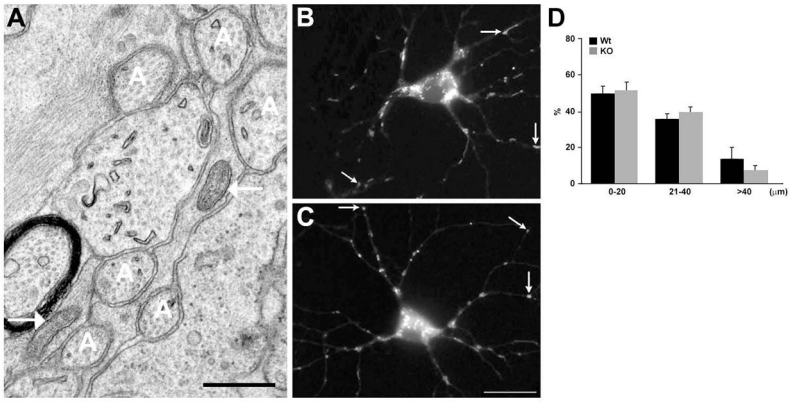

Figure 8. Mitochondria are normally distributed in the TOG KO oligodendrocytes

(A) Electron micrograph of TOG KO optic nerve showing mitochondria (indicated by arrows) in processes abutting axons labeled “A”. Oligodendrocytes from control (B) and TOG KO (C) mice cultured in the presence of MitoTracker Red CM-H_2Ros to label mitochondria. Arrows point at mitochondria located distally in the cell processes. (D) Distribution of mitochondria in the processes of oligodendrocytes in culture. Mitochondria were binned according to their distance from the cell body.