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Disease modifying mitochondrial uncouplers, MP101, and a slow release ProDrug, MP201, in models of Multiple Sclerosis

Yoshio Bando[a,b,*], John G. Geisler[c]

[a] Department of Functional Anatomy and Neuroscience, Asahikawa Medical University, Asahikawa, 078-8510, Japan
[b] Department of Anatomy, Akita University Graduate School of Medicine, Akita, 010-08543, Japan
[c] Mitochon Pharmaceuticals, Inc., 970 Cross Lane, Blue Bell, PA, 19422, USA

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ABSTRACT

Mitochondrial dysfunction is thought to be involved in the pathogenesis of MS and here we tested if brain penetrant mitochondrial uncouplers, DNP (MP101) and a novel prodrug of DNP (MP201), have the pharmacology to suppress demyelination and axonal loss in two independent models of MS by modulating the entire organelle's physiology. First, the gold standard EAE mouse model for MS was evaluated by daily oral treatment Day 7–21 with either MP101 or MP201 post-immunization. Both MP101/MP201 significantly suppressed progression of paralysis with limited infiltration of inflammatory cells. Strikingly, although mitochondrial uncouplers do increase energy expenditure even at the low doses provided here, they paradoxically preserved body weight at all doses in comparison to wasting in advanced paralysis of the placebos. Second, the effects of the compounds on suppressing inflammation were also evaluated in the cuprizone model, independent of the immune system. MP101/MP201 had a striking effect preserving both myelination and protecting the axons, in comparison to the placebos where both were destroyed. Both MP101/MP201 induced a significant and sustained increase in neurotrophin, BDNF, in the spinal cords. Both MP101/MP201 suppressed the expression of inflammatory cytokines including IL-1β, TNF-α and iNOS. Results indicate that MP101/MP201 may be a "disease modifying" treatment for MS by specifically modulating mitochondrial physiology. This would be a completely novel treatment for MS, targeting the mitochondria directly using a unique platform, mitochondrial uncouplers, that initially act non-genomically based upon biophysics, but cascades into cellular remodeling, neuroprotection and *pro-survival*. Clinical Phase I testing of MP101 in Normal Healthy Volunteers (NHV) is currently underway allowing for the potential to subsequently evaluate translation in MS patients and other insidious diseases, at expected weight neutral doses.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease in which myelin-reactive autoantibody and lymphocytes migrate out of lymph nodes into the circulation, cross the blood–brain barrier (BBB), and aggressively target putative myelin antigens in the central nervous system (CNS), causing inflammation, demyelination, axonal injury, astrogliosis, and ultimately, neurodegeneration (Bando et al., 2018; Dhib-Jalbut, 2007; Hafler et al., 2005; McFarland and Martin, 2007). However, the molecular mechanism of the pathogenesis of MS remains unknown and current therapies show limited effect on modulating disease progression. Therefore, new therapies for the treatment of MS are required. Experimental autoimmune encephalomyelitis (EAE) is the most intensively investigated experimental animal model of the human inflammatory demyelinating disease such as MS. The mechanism of EAE progression is thought to be involved either or both T cells and B cells (Iglesias et al., 2001).

Our previous study clearly demonstrated that morphological abnormalities of myelin preceded axonal degeneration and morphological changes of axonal organelles such as axoplasmic reticulum and mitochondria during demyelination and axonal degeneration in EAE (Bando et al., 2015). In addition, several studies of autopsied human MS brains and *in vitro* models also reported mitochondrial dysfunction might be a cause of axonal degeneration (Dutta et al., 2006; Kiryu-Seo et al., 2010; Ohno et al., 2011). In fact, axonal loss in MS and EAE is caused by impairment of axonal axoplasmic reticulum (AR) and

Abbreviations: BDNF, Brain Derived Neurotrophic Factor; DNP, 2,4-dinitrophenol; BBB, Blood brain barrier; EAE, Experimental autoimmune encephalomyelitis; AR, axonal axoplasmic reticulum; ROS, reactive oxygen species; MOA, mechanism of action; CNS, central nervous system

* Corresponding author. Department of Anatomy, Akita University Graduate School of Medicine, Akita, 010-08543, Akita, Japan.
E-mail address: ybando@med.akita-u.ac.jp (Y. Bando).

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mitochondrial function followed by increasing axonal Ca^{2+} levels released from AR and mitochondria. Stirling et al. also reported that axoplasmic reticulum Ca^{2+} release caused secondary degeneration of spinal axons (Stirling et al., 2014; Stirling and Stys, 2010).

Mitochondria are important organelles for energy metabolism in the cells and calcium storage. It is known that mitochondrial dysfunction plays an important role in the neurodegeneration of MS, manifested through excessive reactive oxygen species (ROS) and unusual morphology of "Y" shaped mitochondria (Bando et al., 2015; Dutta et al., 2006). MS also commonly affects the optic nerve, causing optic neuritis. Previous studies have shown that optic neuritis and progression to vision loss (retinal ganglion cell survival) can be attenuated with modulation of mitochondrial physiology in the EAE model (Khan et al., 2017). In addition, studies have demonstrated compounds that modulate mitochondrial activity, can attenuate ROS levels and disease progression by improved cellular function (Caldeira da Silva et al., 2008; Wu et al., 2017). Here we focus on testing the merits of mitochondrial uncouplers to protect against paralysis caused by demyelination in the brain and spinal cord.

Mitochondrial uncoupler 2,4-dinitrophenol (DNP) aka MP101, and a novel prodrug of DNP, MP201, with longer residency time, both harness the power of the mitochondria by increasing energy expenditure that results in strengthening cellular survival (Geisler, 2019; Hubbard et al., 2018; Khan et al., 2017; Mitchell, 1961), similar to the positive effects seen with fasting and exercise (Duan et al., 2003; Halagappa et al., 2007; Maalouf et al., 2009; Stranahan and Mattson, 2012). Mitochondrial uncoupling mimics and enhances the naturally occurring phenomena of which ~25% of potential energy is lost as heat, with the leaking of protons across the membrane without going through the ATP synthase channel to make ATP (Rolfe and Brand, 1996). The resultant effect is an increase in metabolism of glucose by glycolysis and of lipids by beta-oxidation (Geisler, 2011). Mitochondrial uncouplers are unique as a therapeutic platform as the mechanism of action (MOA) is "non-genomic" in that the target is not a protein or receptor, but a location, the mitochondrial matrix (Geisler, 2019). The mitochondria are the only organelle within the cell with a pH basic environment. Mitochondrial uncouplers, DNP and its prodrug MP201, are weak acids. Since acids are attracted to bases, acidic DNP is drawn specifically into the basic environment of the mitochondrial matrix (Geisler, 2011). Therefore "off-target" effects are likely to be low, especially at low doses. Recent studies also have shown that low-dose pharmacological agents that induce mild mitochondrial uncoupling have tremendous therapeutic potential in a range of acute and chronic neurodegenerative conditions, whether from known and unknown genetic causes (Geisler et al., 2017; Hubbard et al., 2018; Khan et al., 2017; Wu et al., 2017). DNP is a transporter of a proton (hydrogen) to the mitochondrial matrix, that lowers the mitochondrial membrane potential and thereby lowering ROS production and improving calcium handling (Geisler, 2019). In progressive MS patients, it was shown that moderate exercise for 30-min stimulates brain derived neurotrophic factor (BDNF) production (Briken et al., 2016). Others have suggested that BDNF may be a promising therapeutic for MS (Linker et al., 2010; VonDran et al., 2011). Curiously, it has been shown that DNP can also increase endogenous levels of BDNF in the brain as an oral small molecule. DNP as an inducer of endogenous BDNF, however has not been previously studied in the context of MS (Geisler et al., 2017; Hubbard et al., 2018; Liu et al., 2015). So, the idea of testing DNP to lower ROS and as well as induce BDNF to attenuate MS could be a breakthrough therapy, especially for progressive MS. The severity of progressive MS comes from the destruction of not only the myelin sheaths, but also the axons as well (Ontaneda, 2019), which can in part be seen in the cuprizone model tested here.

Here, we hypothesized that MP101/201 may have neuroprotective properties that suppress demyelination and axonal loss in EAE. Therefore, the potential ability of MP101/201 to suppress EAE was evaluated in EAE mice, and followed up in the cuprizone mouse model that causes demyelination by direct copper chelation toxicity to oligodendrocytes, independent from the immune system.

2. Material and methods

2.1. Animals

Adult C57BL/6J female mice (6–8 week old) were purchased from Sankyo laboratory (Sapporo, Japan) and used in all experiments. The experimental procedure was approved by the Institutional Committee for Experimental Animals (Asahikawa Medical University).

2.2. Experimental autoimmune encephalomyelitis (EAE) model

EAE induction was performed as previously described (Bando et al., 2015, 2018). In brief, myelin protein peptides including MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Scrum (Tokyo, Japan). Female C57BL/6J mice, 6-8-weeks old (N = 12/treatment), were immunized s.c. in the flank with the emulsion made of 75 μl of Ag peptide (150 μg of MOG_{35-55}) and 75 μl of complete Freund's adjuvant containing 0.4 mg of heat-inactivated *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories). Each animal also received 200 ng of pertussis toxin (Sigma Aldrich) through i.p. injection on days 0 and 2 post-immunization. EAE clinical score was determined and recorded every day as described previously (Bando et al., 2015). Treatment with MP101 and MP201 started 7-days post-immunization and continued for 14-days and which time treatment stopped and the mice were monitored for an additional 3-weeks. Behavior was also monitored for gait at weekly intervals. At day 42, the mice were sacrificed to evaluate the lumber spinal cord for degree of demyelination, and the spinal cord was removed for RT-PCR and immunoblotting. Body weights were daily recorded and mean clinical score was assigned to each group as previously described (Bando et al., 2015, 2018).

2.3. Immunoblotting

Tissues were lysed in RIPA buffer (1% Nonidet P-40, PMSF, EDTA in PBS). After determination of the protein concentration (DC protein assay kit; BioRad), 10–20 μg of protein extract was separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF paper (Millipore, Bedford, MA) and immunostained with anti-MBP antibody (clone SMI-94; BioLegend, 1:1000), anti-MBP antibody (QD9; Millipore, 1:1000), anti-CNPase antibody (sigma, 1:1000), anti-SMI32 antibody (BioLegend, 1:1000), anti-ATF3 antibody (Santa Cruz, 1:1000), anti-COX4 antibody (Abcam, 1:1000), anti-synaptophysin (Sigma, 1:1000), anti-BDNF antibody (Santa Cruz, 1:500) or anti-GAPDH antibody (Sigma, 1:1000). QD9 can specifically recognize degenerating MBP indicating degenerating myelin (Matsuo et al., 1997, 1998; Weil et al., 2016). For detection, HRP-conjugated secondary antibodies (1:1000, GE Healthcare) were used followed by ECL chemiluminescence development (GE Healthcare) with a Lumino Image Analyzer (LAS-3000; Fuji, Tokyo, Japan).

2.4. Cuprizone-induced demyelination model

Cuprizone (CPZ) purchased from Sigma, the special chow containing 0.3% CPZ was synthesized (Oriental Yeast Co. LTD, Chiba, Japan). Female C57BL/6J mice (N = 10/treatment) at 8-weeks of age were provided the CPZ chow and simultaneously treatment started with MP101/MP201 with daily oral once-per-day delivery at 0.5, 1 and 5 mg/kg and 8, 16, and 80 mg/kg respectively from 4-weeks in the CPZ-induced demyelination period. Weekly monitoring of body weights was also continued. At the end of 4weeks, necropsy was performed to evaluate demyelination of the corpus callosum by histology.

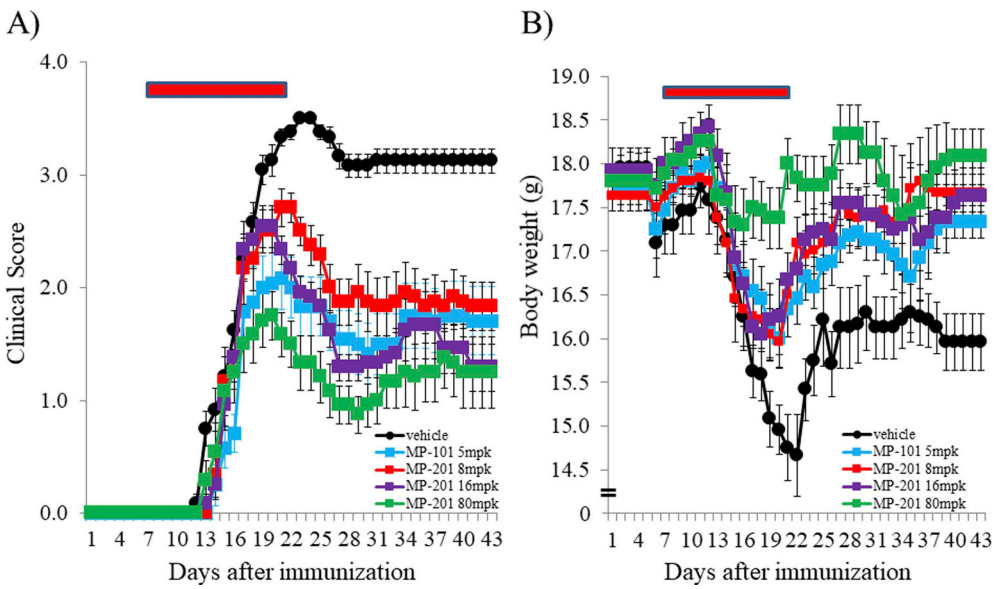

Fig. 1. MP101 and MP201 can Ameliorates MOG_{35-55}-Induced EAE.
(A) Progression of EAE was daily monitored and scored as disease severity on a clinical scale (vehicle, closed circle; MP101 5mpk, closed square shown in blue; MP201 8mpk, closed square shown in red; MP201 16mpk, closed square shown in purple; MP201 80mpk, closed square shown in green). Mice were treated with either MP101 or MP201 for 2 weeks (day 7–21 after immunization, red bar). *P values: MP101 5mpk = 0.019, MP201 8mpk = < 0.001, 16mpk = < 0.0001 and 80mpk = < 0.0001. **(B)** Body weights were also measured daily. *P values: MP101 5mpk = 0.018, MP201 8mpk = < 0.0001, 16mpk = 0.044, and 80mpk = < 0.0001. *P values by Logistic regression model using ordinal package from cran project (www.cran.r-project.org).

2.5. Treatment of MP101/201

MP101/MP201 (gift from Mitochon Pharmaceuticals, Inc., Blue Bell, PA, USA) was dissolved in DMSO (1% final) and Q.S. with 0.5% methylcellulose. MP201 is a prodrug of DNP and has been described previously (Geisler, 2019; Hubbard et al., 2018; Khan et al., 2017). For the EAE model, mice were treated by oral gavage (10 mL/kg) daily with either MP101 (5 mg/kg) or MP201 (8, 16 or 80 mg/kg) as indicated or vehicle, starting from Day-7 post immunization until peak disease (Day-21). In the cuprizone model, mice were provided 0.5, 1 and 5 mg/kg of MP101 and 8, 16, and 80 mg/kg of MP201.

2.6. Immunohistochemistry and SEM analysis

Animals under deep anesthesia were sacrificed and perfused with 0.1 M phosphate buffer (PB, pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M PB (Bando et al., 2015). Spinal cords were removed and immersed in 30% sucrose in 0.1 M PB for 1–2 days. Spinal cords were then frozen in OCT medium. Frozen 14 μm sections were prepared on a cryostat, and then stored at −30 °C until use. In some experiments, the sections were stained with Fluoromyelin (Molecular Probes) to assess demyelination as described previously. For immunohistochemistry, the sections were immunostained with anti-GFAP antibody (1:1000, Sigma), anti-Iba 1 antibody (1:1000, WAKO), anti-2H3 antibody (Developmental Studies Hybridoma Bank) (Namikawa et al., 2006). Immunostaining was performed following a standard fluorescein protocol as described previously (Bando et al., 2015). The sections were analyzed with a confocal laser microscope (FV-1000D, OLYMPUS) with Fluoview (OLYMPUS). Scanning electron microscope (SEM) analysis with osmium-maceration method was applied as described previously (Bando et al., 2015).

Inflammatory foci was defined as presence of > 20 inflammatory cells in the perivascular space of a given blood vessel on the LFB/CV staining (Bando Y. et al., 2018; Balabanov et al., 2007). The degree of demyelination (% demyelination) was also measured (Bando Y. et al., 2018; Farez et al., 2009). Each pathology group consisted of tissue sections from three to four animals.

2.7. Mouse ES-Derived OLs

Cultured oligodendroglial progenitor cells (OPCs) were originally generated by mouse embryonic stem cells (Yamashita et al., 2017). Then, ES-derived OPCs were differentiated into oligodendrocytes (OLs) stimulated with 30 ng/ml triiodothyronine (T3, Sigma-Aldrich) for their maturation. At 7 days, the mature OLs were exposed to Lysolecitin (LPC, Sigma-Aldrich) to induce oligodendroglial cell death for 24hr. For TUNEL staining, Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoKine, Germany) was applied.

2.8. Mouse primary cultured microglia

Primary cultured mouse microglia were prepared from C57BL/6J mice and cultured in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum. The cells were stimulated with 1 μg/ml lipopolysaccharide (LPS, sigma, USA). The cells were also treated with/without MP101 (0–40 μM) for 18hr.

2.9. RT-PCR

Total RNA was extracted from primary cultured microglia using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Two microgram of total RNA in each sample was subjected to first-strand cDNA synthesis with AMV reverse transcriptase (Promega, Madison, WI). Aliquots from the RT reaction were used for PCR amplification using specific primer pairs as described previously (Bando et al., 2018).

2.10. Statistical analysis

The data in this text represents the mean ± SEM, and the error bars in figures also represent SEM. Unpaired *t* tests were used to compare the significance of differences between two groups, and one-way ANOVA followed by Bonferroni tests were used to analyze data with more than two groups. $P < 0.05$ was considered statistically significant. Clinical score analysis from logistic regression model using ordinal package from cran project (www.cran.r-project.org).

3. Results

3.1. MP201 Treatment suppresses EAE induction

Eight-week old female C57BL/6J mice were immunized with MOG_{35-55} peptide to induce EAE. Mice were treated with either MP101 (5 mg/kg) or MP201 (8, 16 or 80 mg/kg respectively), daily by oral gavage beginning at Day 7–21 post-immunization. Their clinical score and body weight were measured daily until sacrifice at day 42. The vehicle treated-control mice showed typical clinical symptoms by EAE,

Table 1
The effect of MP101 and MP201 on the progression of EAE.

	Incidence	Mortality	Day of Onset	Mean max Grade	Grade at the peak		Ordinal Regression†	
					Max	Min	Clinical Scores	Body Weight
Vehicle	12/12	-	12.3 ± 0.3	3.5 ± 0.0	3.5	3.5	-	-
MP101 5mpk	12/12	-	15.9 ± 1.6**	2.4 ± 0.2**	3.5	0	0.01	0.01
MP201 8mpk	12/12	-	14.1 ± 0.3	2.8 ± 0.1	3.5	1.5	0.001	< 0.0001
MP201 16mpk	12/12	-	13.8 ± 0.2	2.7 ± 0.2*	3.5	1.5	0.0001	0.04
MP201 80mpk	12/12	-	13.7 ± 0.4	2.0 ± 0.2**	**3.0**	1.0	0.0001	< 0.0001

*P < 0.05 vs vehicle, **P < 0.01 vs vehicle.
†Logistic regression using ordinal package from cran project (www.cran.r-project.org) to show P-value from Fig. 1A (clinical scores) and Fig. 1B (body weight preservation).

likely to reach peak disease at days 16–20 (Fig. 1). Treatment with MP101 significantly delayed onset of EAE symptoms. Compared to the vehicle-treated mice, EAE mice treated with either MP101 or MP201 (80 mg/kg) showed a significant milder EAE and rapid recovery in a dose dependent manner (Fig. 1A, Table 1). Although 5 mg/kg of MP101 was significant (P value 0.01), the effect of MP201 on the progression of EAE was statistically more powerful (P value 0.001–0.0001) suggesting that the longer residency time with the prodrug may have advantages in pharmacology (Geisler, 2019). On the other hand, rapid recovery from EAE was observed in all groups of both MP101 or MP201-treated EAE mice, but not in the vehicle treated EAE mice. These results were also supported by the result of their body weight preservation at all doses compared to placebo, which showed signs of wasting (Fig. 1B). To clearly illustrate the effect of MP101 and MP201 on preventing visible paralysis relative to placebo, mice were videoed at Day 15 (peak) (see Movie. S1).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.neuint.2019.104561.

3.2. MP101/MP201 Treatment reduces hindlimbs paralysis

To address the clinical assessment of the effect of MP101 and MP201 on hindlimbs paralysis, gait analysis was performed. The hindlimbs only of the EAE mice, at day-20 after MOG immunization, were brushed by India ink. The mice then walked on white paper. If their hindlimbs were not paralyzed, the footsteps of their hindlimbs would be seen on the white paper. Normal footsteps were observed in naïve mice (Fig. 2). By contrast, the footsteps were absent in the vehicle-treated EAE mice, due to hindlimb paralysis. Both MP101 or MP201-treated EAE mice clearly showed their footsteps. Notably, the effect of MP201 was more pronounced than that of MP101, in an increasing dose-dependent manner.

3.3. MP101/MP201 Treatment reduces demyelination and axonal degeneration in EAE

To examine whether either MP101 or MP201-treatment could reduce EAE-induced demyelination and axonal degeneration, immunohistochemistry was performed. The lumber spinal cords in EAE mice were examined (Fig. 3). Fluoromyelin dye was used for myelin staining (Fig. 3A–C). 2H3 dye was also used for neurofilament staining (Fig. 3D–G). Fluoromyelin staining clearly demonstrated that the intensity of the dye in either MP101 or MP201 treated mice was greater than the vehicle-treated EAE mice. This suggests that both MP101 and MP201 reduce demyelination in the EAE spinal cord (Fig. 3H). In addition, 2H3 staining showed similar result with reduced axonal degeneration in comparison to vehicle (Fig. 3B–E).

3.4. MP101/MP201 Treatment reduces inflammatory demyelination and gliosis in EAE

The number of inflammatory foci in the EAE mice by DAPI staining was also examined. Both MP101 or MP201-treated mice showed a significant decrease of inflammatory foci compared to the vehicle-treated EAE mice (Fig. 3J). These observations suggest that inflammatory demyelination is significantly reduced in both MP101 or MP201-treated mice (Fig. 3J–K). In addition, immunohistochemistry demonstrated that MP101 reduced astrocytes/microglia-mediated gliosis during EAE. Although activation of astrocytes/microglia was not markedly diminished by MP201 treatment, MP201 could reduce infiltration of inflammatory cells from the periphery.

3.5. MP101/MP201 Treatment reduces abnormal myelin morphology and axonal pathology in EAE

We previously established the methods for scanning electron microscopy (SEM) analysis to observe morphological changes of myelin and axonal organelles during demyelination (Bando et al., 2015). This method enables us to observe abnormal myelin morphology from the initial step of demyelination (detachment of myelin with compact lamination, from the axon) to the subsequent axonal degeneration, which produces the “ovoid”, typically found at the distal end of an axonal transection. The ovoid is a pathological axonal structure generated in response to axotomy, by the development of axonal smooth ER (axoplasmic reticulum, AR) and localized accumulation of mitochondria within a short section of the distal severed axon (Bando et al., 2015). In the vehicle treated-EAE mice, detachment of myelin from the axons was observed in the white matter of the lumber spinal cord (Fig. 4A, white arrowheads). On the other hand, both MP101 and MP201 suppressed formation of abnormal myelin at the initial step of demyelination (Fig. 4B–C). Further studies demonstrated that development of AR and accumulation of mitochondria was also observed (Fig. 4). Besides of these observations, sagittal section of SEM image demonstrated that both MP101 or MP201-treated mice had reduced axon pathology, such as ovoid formation (Fig. 5C and D), which was observed in the vehicle-treated EAE mice (Fig. 5A and B). Moreover, there were no Y-shaped mitochondria in the axons of either MP101 or MP201-treated mice (Fig. 5C and D), compared to vehicle-treated EAE mice (Fig. 5B and inset). Quantitative analysis also showed a significant difference of mitochondrial length in the axon, and also the number of Y-shaped mitochondria (abnormal morphology of mitochondria) between vehicle-treated EAE mice and either MP101 or MP201-treated mice (Fig. 5E and F).

3.6. MP101/MP201 suppressed functions of myelin and axon during EAE

We examined the expression levels of various proteins in the white

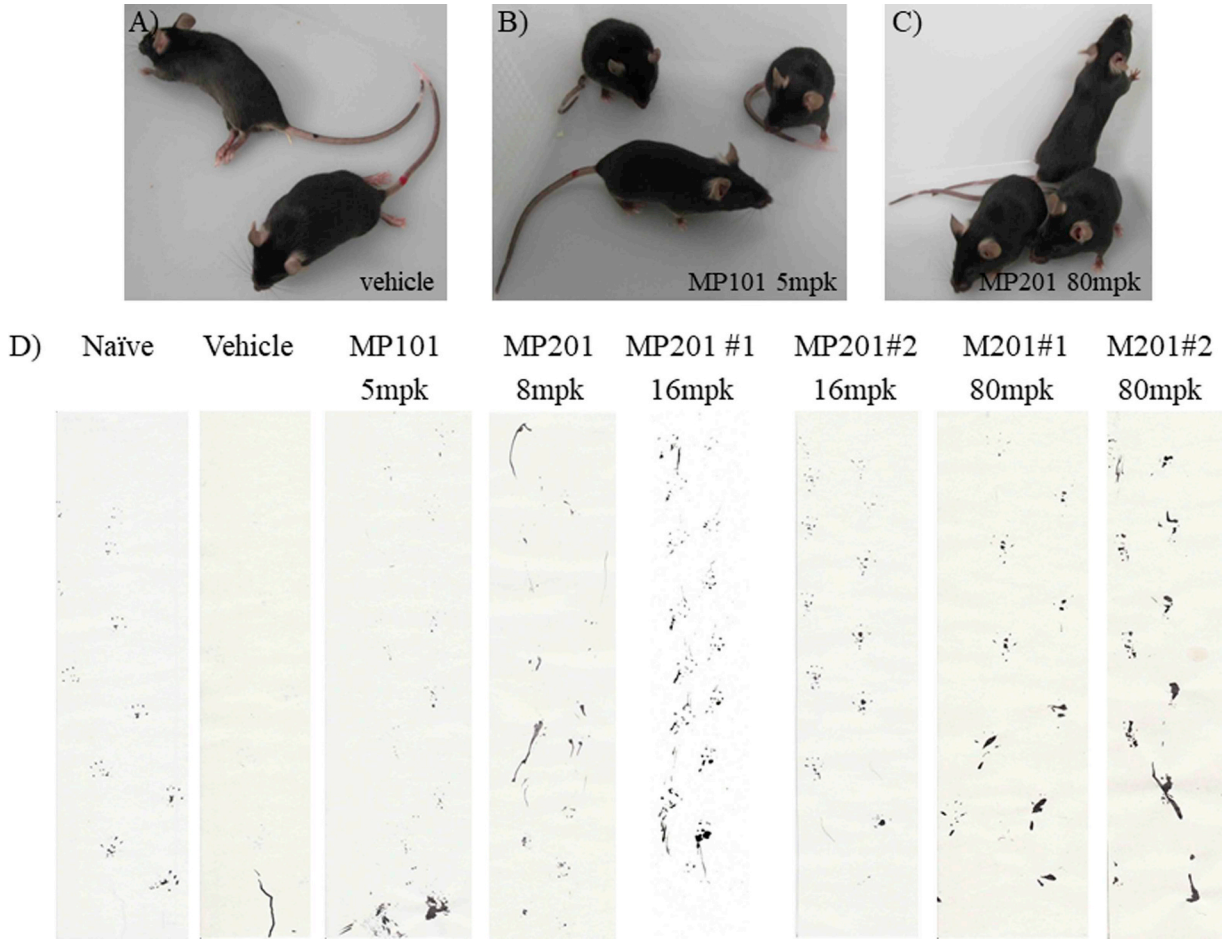

Fig. 2. Gait analysis at peak disease of EAE in either MP101 and MP201-treated mice.
Representative pictures in the vehicle-treated EAE mice **(A),** the MP101 5mpk-treated EAE mice **(B)** and the MP201 80mpk-treated EAE mice **(C)** are shown, respectively. **(D)** representative foot stamps by gait analysis in the EAE mice (MP101 5mpk), MP201 8mpk, MP201 16mpk, MP201 80mpk) are shown, respectively. Especially, in MP201-treated EAE mice, gait analysis obtained from the two different mice are shown (MP201-16mpk, MP201-80mpk, respectively).

matter of the EAE spinal cords by immunoblotting (Fig. 6). Although the vehicle-treated mice showed a significant reduction of MBP expression, both MP101 or MP201 treatments maintained higher levels of MBP expression during EAE induction (Fig. 6A). On the other hand, QD9, which is a marker for degenerating MBP was expressed in only the vehicle-treated EAE mice, but not in either MP101 or MP201-treated mice. Consistent with these results, expression levels of CNPase (a marker for oligodendrocytes) in both MP101 or MP201-treated mice was much higher than that in the vehicle-treated mice. These results suggested that both MP101 and MP201 reduced demyelination by maintaining oligodendroglial function. The expression levels of SMI32 (a marker for axonal injury), ATF3 (a marker for neuronal injury), COX4 (a marker for mitochondria in axons, neurons and glial cells, respectively), synaptophysin (a marker for the synapse) and BDNF were examined by immunoblotting. Both MP101 or MP201-treated mice showed no expression of SMI32 and ATF3, while vehicle-treated EAE mice did. By contrast, higher expression levels of COX4, synaptophysin and BDNF were observed in both MP101 and MP201-treated mice, and not in the vehicle-treated EAE mice. These results indicated that both MP101 and MP201 treatment suppressed neuronal and axonal degeneration by maintaining mitochondrial function in neurons and the axons. Furthermore, RT-PCR analysis also demonstrated lower expression of inflammatory cytokines such as IL-1beta, TNF-alpha and iNOS in both MP101 and MP201-treated mice, compared to the vehicle-treated mice (Fig. 6B). Consistent of the results by immunoblotting, RT-PCR analysis showed similar results for MBP and ATF3 expression.

3.7. MP101/MP201 also suppressed cuprizone-induced demyelination in mice

We next examined the effect of MP101 and MP201 on a different demyelination model, which is independent of the immune system using Cuprizone (CPZ). CPZ is a chelator of copper that is added to the chow. Cuprizone slowly induces demyelination by oligodendroglial cell death, mainly at the corpus callosum of the mouse brain. In the placebo group, CPZ-administration decreased body weights, as an indirect indicator that demyelination was successfully induced in the corpus callosum (Fig. S1). Treatment with MP101 and MP201 started with oral daily doses (q.d.). However, neither MP101 or MP201-treated mice showed loss of body weight with CPZ-treatment. Treatment MP101/MP201 and CPZ in the chow continued for 4-weeks and then the mice were sacrificed to evaluate the brain. The corpus callosum was examined by histology to determine the effect of MP101 and MP201 on CPZ-induce demyelination. Fluoromyelin staining demonstrated that severe demyelination was observed in the corpus callosum of the vehicle-treated mice during CPZ-induced demyelination (Fig. 7). In contrast, both MP101 and MP201-treated mice showed suppression of demyelination in the corpus callosum. Furthermore, 2H3 staining also demonstrated that suppression of axonal loss in the corpus callosum was observed in both MP101 or MP201-treated mice, but not in vehicle-treated mice during CPZ-induced demyelination. While DAPI staining of the corpus callosum demonstrated a significant increase in the number of glial cells, which are probably astrocytes or microglia, in the

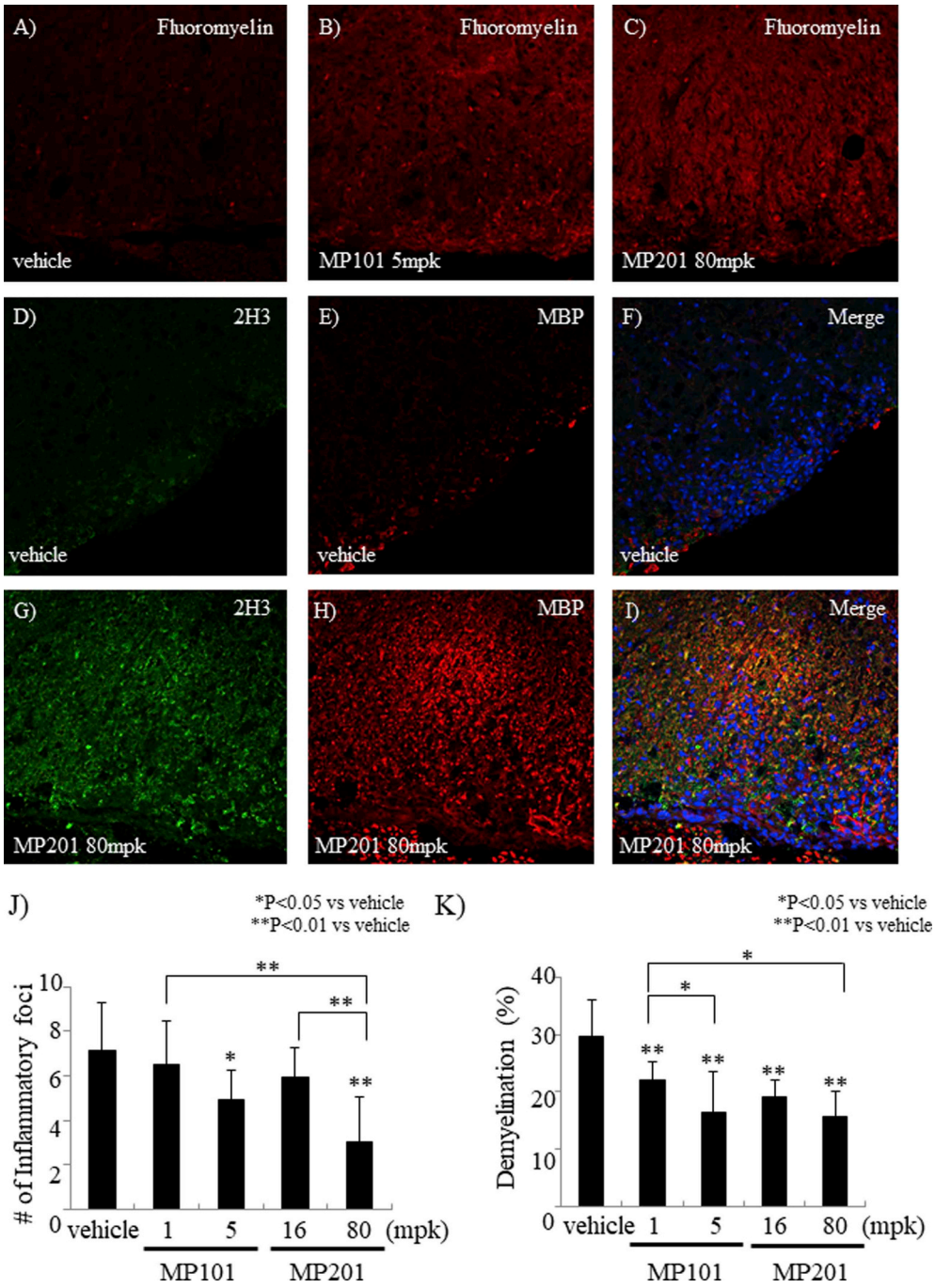

Fig. 3. Suppression of Demyelination and Axonal Injury in the MP101 and MP201-Treated EAE Mice.
Frozen cross section of lumber spinal cords from either vehicle (**A, D-F**), MP101 (**B**) or MP201 (**C, G-I**)-treated mice taken on day 20 post immunization (peak disease) were stained with fluoromyelin (**A-C**), 2H3 (**D, G**), or MBP (**E, H**). Merge image are also shown (**F** and **I**). (**A-C**) Fluoromyelin staining in either vehicle (**A**) MP101 5mpk, (**B**) MP201 80mpk, (**C**)-treated EAE mice are shown. Dual staining with 2H3 (green, **D** and **G**) and MBP (red, **E** and **H**) in either vehicle (**D-F**) or MP201 80mpk-treated mice are shown. (**F** and **I**) DAPI staining (blue) was also applied. Quantitative analysis of the number of inflammatory foci (**J**) and % demyelination (**K**). *P < 0.05, **P < 0.01.

vehicle-treated mice, there were only a limited number of glial cells found in the corpus callosum in both MP101 and MP201-treated mice, suggesting protection from CPZ-induced demyelination.

3.8. MP101/MP201 suppressed gliosis in CPZ-Induced demyelination

To examine what types of cells accumulated in the corpus callosum in CPZ-treated mice, immunohistochemistry was performed. Previous studies demonstrated that GFAP-positive astrocytes and Iba-1-positive microglia accumulated in the corpus callosum in CPZ-treated mice (Nomura et al., 2017). In this model, the blood brain barrier is not injured by CPZ. Therefore, inflammatory cells from the periphery are basically not observed. That is, the Iba-1-positive cells are microglia from the brain, and not macrophages from the periphery. Consistent with previous studies in the vehicle treated mice, GFAP-positive astrocytes and Iba-1-positive microglia accumulated in the corpus callosum in CPZ-induced demyelination (Fig. S2). On the other hand, both MP101 and MP201-treated mice showed that only limited numbers of astrocytes and microglia were observed to be present in the corpus callosum. These results indicate that MP101/MP201 blocked demyelination by reducing glial activation in mice with CPZ-induced demyelination.

3.9. MP101 suppressed activation of microglia in vitro

To assess the suppressive effect of MP101 on microglia, expression levels of inflammatory cytokines in primary cultured microglia were measured by RT-PCR. LPS increased expression levels of IL-1β and iNOS in microglia (Fig. 8). In distinction, MP101-treated cultured microglia showed suppression of these cytokine expressions. This result suggests that MP101 suppresses activation of microglia, *in vitro*.

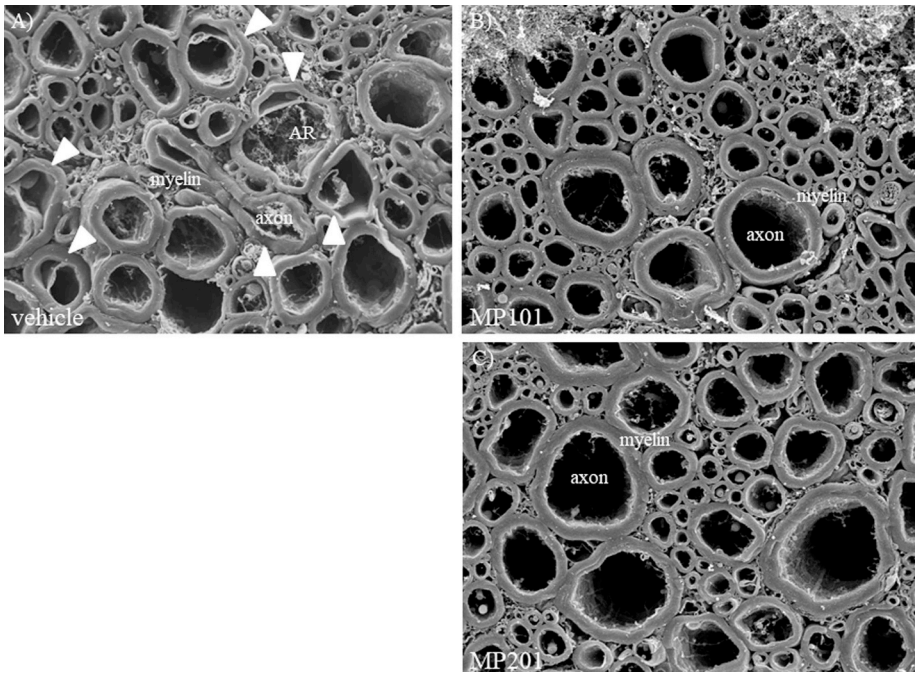

Fig. 4. Progression of Abnormal Myelin Morphology in Spinal Cord of EAE.
Representative images of abnormal myelin morphology in either vehicle (**A**), MP101 5mpk (**B**) or MP201 80mpk (**C**)-treated mice at peak disease (day 20 after immunization) are shown. Arrows indicate detachment of myelin from the axon (initial step of demyelination as described in ref. 6). Both MP101 and MP201 can suppress progression of abnormal myelin morphology in EAE.

3.10. MP101 suppressed lysolecithin-induced oligodendroglial cell death in vitro

To assess the protective effect of MP101 on oligodendrocytes, cell viability was measured in cultured oligodendrocytes exposed to lysolecithin (LPC) for 24hr. LPC induced oligodendroglial cell death *in vitro* (Fig. S3). In contrast, MP101-treated cultured oligodendrocytes were protected from LPC-induced cell death. This result suggests that MP101 suppresses LPC-induced oligodendroglial cell death, *in vitro*.

4. Discussion

The results from the current studies indicate that oral administration of MP101/MP201 provides significant neuroprotective benefits in two experimental models of MS. MP101/MP201 treatment suppressed not only demyelination followed by axonal damage in EAE, but also CPZ-induced demyelination, which is a completely different animal model independent of the immune system. In addition, *in vitro* studies also demonstrated that these compounds suppressed both LPC-induced oligodendroglial cell death, and pathological microglial activation, which suggests possible explanation for their neuroprotective effects on demyelinating diseases such as MS.

The mitochondrial population is an important intracellular composite of many individual mitochondria organelles working symbiotically within the cell, responding to cellular energy demands. The heterogeneous mitochondria can be mosaic in performance. Mitochondrial damage and dysfunction by hypoxia, oxidative stress, toxins, calcium overload, etc., can result in cell death in many neuromuscular and neurodegenerative diseases (Bowling and Beal, 1995; Correia et al., 2012; Floyd, 1999; Grosso et al., 2008, 2011; Kim et al., 2004; Lim et al., 2015; Mattson, 2012; Millay et al., 2008; Nicholls, 2008; Pandya et al., 2013; Tankersley et al., 2007). In fact, mitochondrial oxidative stress plays a crucial role and may be upstream in the pathogenesis of MS, similar perhaps to Alzheimer's Disease (Pratico et al., 2001). Therefore, a meaningful strategy for new treatments for MS, could be drug therapies that modulate mitochondrial physiology in favor of significantly reducing oxidative stress and as well as increasing critical growth factors for both neuronal protection and repair (Binder and Scharfman, 2004; Zuccato and Cattaneo, 2009).

In the current study, the mitochondrial uncoupler, DNP (MP101) and the prodrug of DNP with extended elimination time, MP201, were evaluated (Geisler, 2019). Both MP101/MP201 suppressed demyelination followed by axonal injury in EAE and CPZ-induced demyelination in a dose dependent manner. In addition, gait analysis also demonstrated that MP101/MP201 could functionally suppress development of hindlimb paralysis in the progression of EAE (Fig. 2). Although these compounds could suppress at least partly the increasing number of inflammatory foci during EAE (Fig. 3J), there are many inflammatory cells in the pia matter from the periphery (Fig. 3 and Fig. S1). This finding indicates that the protective effect of MP101/MP201 on the progression of EAE was much stronger in the CNS rather than the peripheral organs of immune system including lymphoid tissues and spleen. In addition, MP201 may have another beneficial effect on GFAP + ve astrocytes and Iba-1 + ve microglia. In general, it has been hypothesized that astrocytes and microglia implicate progression of EAE by producing some inflammatory cytokines (Ponath et al., 2018). Interestingly, however, MP201 seemed to increase the levels of GFAP and Iba-1 (Fig. S2). In addition, the intensity of 2H3 and MBP was still higher in these treated mice, indicating milder demyelination and axonal injury. These observations suggest that MP201 drives a neuroprotective pathway in astrocytes and microglia. Further studies will be required to clarify the mechanism of effect of MP101 and MP201 on astrocytes and microglia.

Surprisingly, treatment of EAE mice with either MP101 or MP201 demonstrated that these compounds could suppress induction of abnormal myelin morphology and pathological changes of axonal organelles such as AR and mitochondria during EAE (Figs. 4–5). In addition, both MP101/MP201 markedly inhibited demyelination at the initial step of demyelination (Fig. 4). We previously reported that detachment of myelin from the axon is the initial step of demyelination in MS and EAE, and that well-developed AR and accumulated mitochondria can be observed in the axonal ovoids in the EAE spinal cord, characteristic of degenerating axons in MS and EAE. In addition, Y-shaped abnormal mitochondria are also found in the ovoid (Bando Y. et al., 2015). However, treatment of EAE mice with either MP101 or MP201 suppressed Y-shaped mitochondrion formation in the axon (Fig. 5). Taken together, these observations suggest that MP101/MP201 could play a crucial role in protecting neuronal cells by protecting the myelin and axons.

In addition to the *in vivo* findings, the *in vitro* studies reveal that the

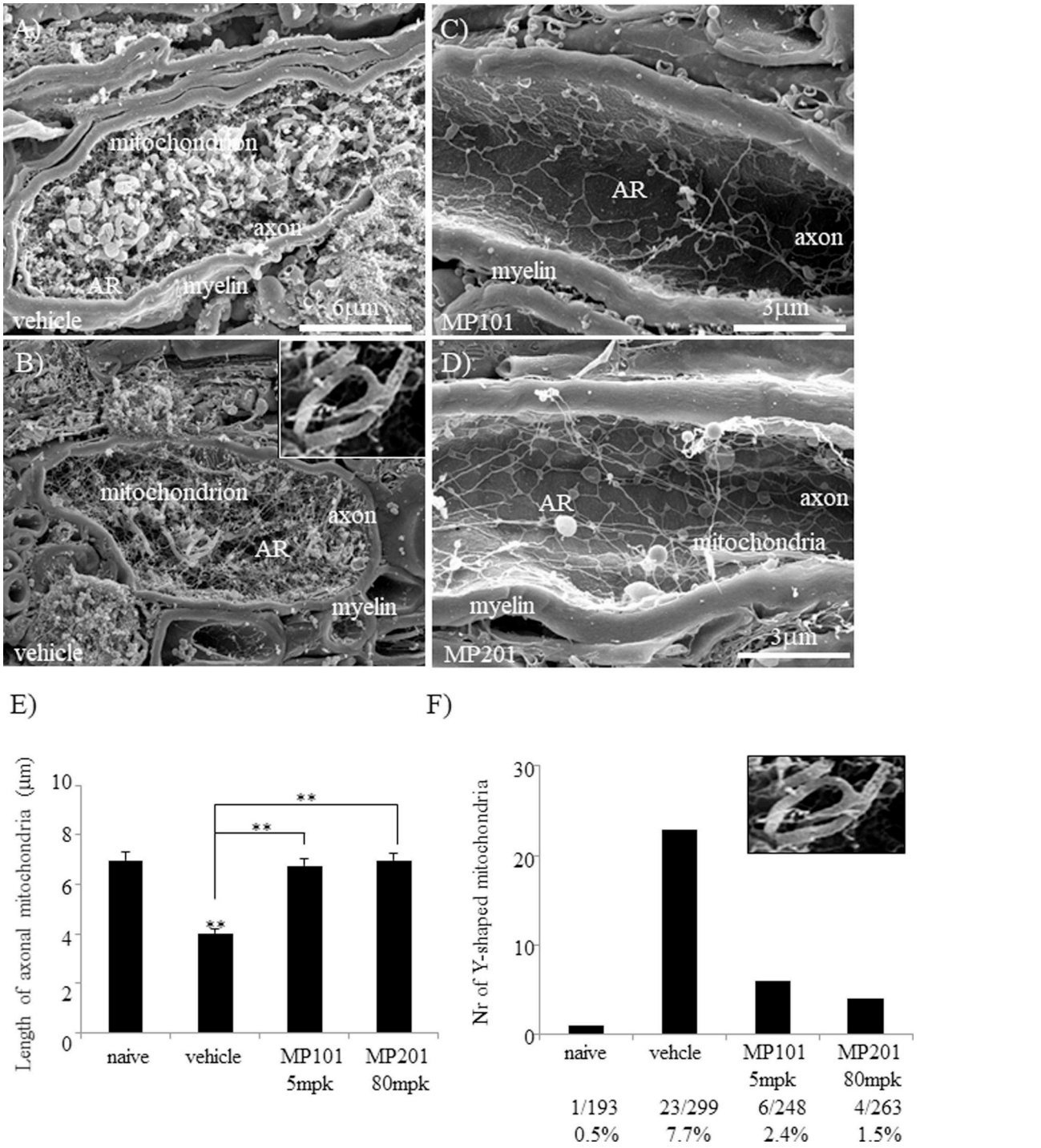

Fig. 5. Spatial Distribution of Axoplasmic Reticulum and Axonal Mitochondria in Spinal Cord of EAE.
Longitudinal sections were observed from either vehicle (**A-B**), MP101 5mpk (**C**) or MP201 80mpk (**D**)-treated EAE mice at peak disease. (**A-B**) well-developed axoplasmic reticulum (AR) and accumulating mitochondria in degenerating axon "ovoid" are shown. (**A**) accumulation of many round-shaped mitochondria rather than well-developed AR are observed in the ovoid. (**B**) well-developed AR rather than accumulation of mitochondria are observed in the ovoid. In some axons, Y-shaped abnormal mitochondria are also observed in the ovoid (inset of panel **B**). On the other hand, both MP101 (**C**) and MP201 (**D**)-treated mice showed no well-developed AR nor accumulation of mitochondria. Quantitative analysis of length of axonal mitochondria (**E**) and the number of Y-shaped mitochondria in the axon of the EAE spinal cord. **P < 0.01.

compounds also suppressed LPC-induced oligodendroglial cell death (Fig. S3) and microglial activation (Fig. 8). These results indicate that MP101/MP201 have neuroprotective effects on demyelination and axonal degeneration in demyelinating diseases.

A previous study demonstrated that MP201, a mitochondrial uncoupler prodrug of 2,4-dinitrophenol (DNP), has potential neuroprotective effects in experimental optic neuritis, by preventing retinal ganglion cell loss with intervention starting even later than here to mirror the human scenario of when a patient would likely see their doctor (Khan et al., 2017).

MP101 or MP201 have also been studied as an effective mitochondrial uncoupling agent in experimental models of neurodegeneration (Geisler

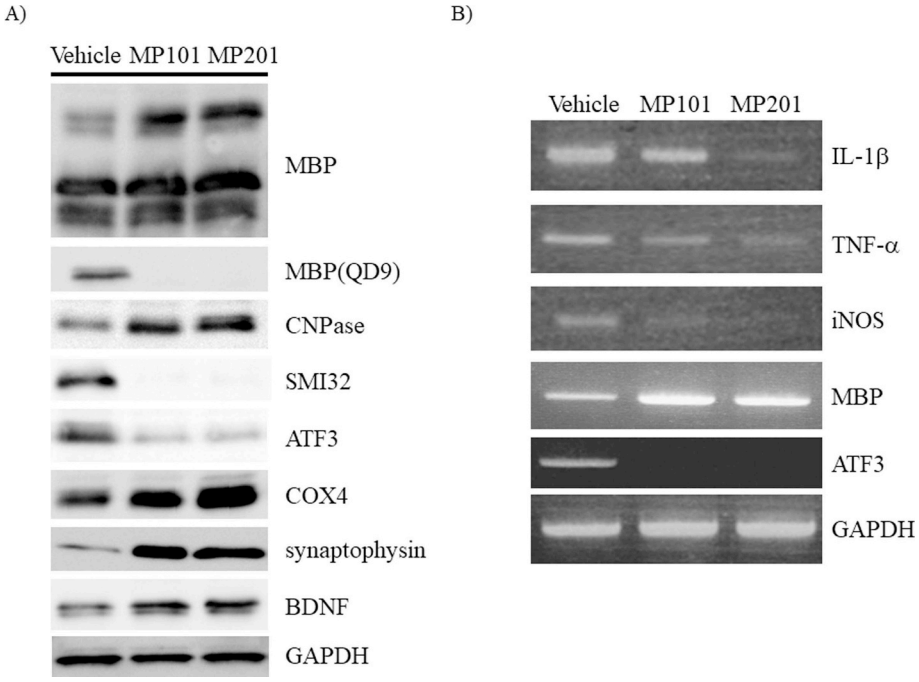

Fig. 6. MP101 and MP201 Can Suppress Oligodendroglial and Neuronal Dysfunction.
(**A**) Immunoblot analysis of the EAE spinal cord from either vehicle, MP101 or MP201-treated EAE mice at peak disease. MBP (a myelin component protein), MBP (QD9, degenerating MBP), CNPase (a marker for oligodendrocyte), SMI32 (a marker for degenerating axon), ATF3 (a marker for injured neuron), COX4 (a marker for mitochondrial function), synaptophysin (a marker for synapse function), BDNF (a neuroprotective factor) were examined, respectively. (**B**) RT-PCR analysis was performed for IL-1β, TNF-α, iNOS, MBP and ATF3. GAPDH was used for internal control.

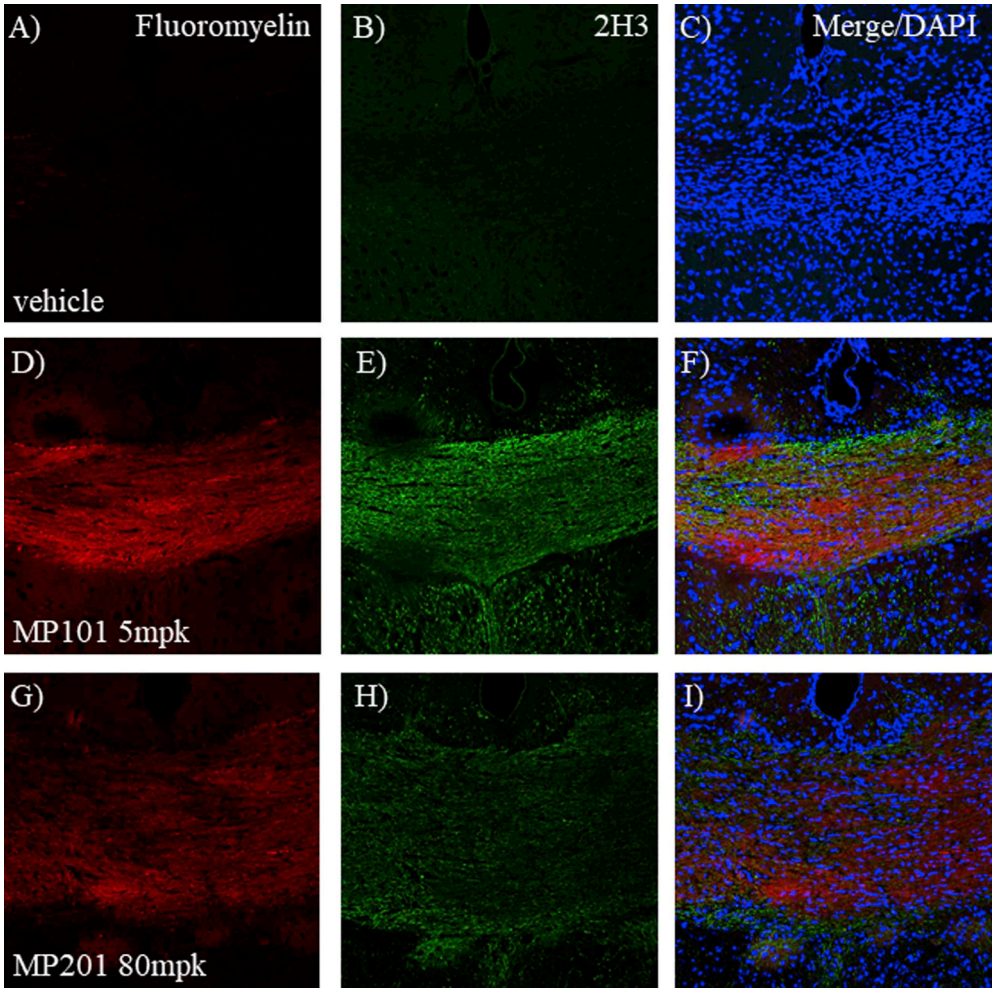

Fig. 7. Suppression of Demyelination and Axonal Injury in the MP101 and MP201-Treated CPZ-Induced Demyelination.
Frozen cross section of corpus callosum from either vehicle (**A-C**), MP101 (**B–F**) or MP201 (**G-I**)-treated mice taken on 4 weeks of 0.3% CPZ-administration were stained with either fluoromyelin (**A, D** and **G**) or 2H3 (**B, E** and **H**). DAPI staining (blue) was also applied. Merge image are also shown (**C, F** and **I**).

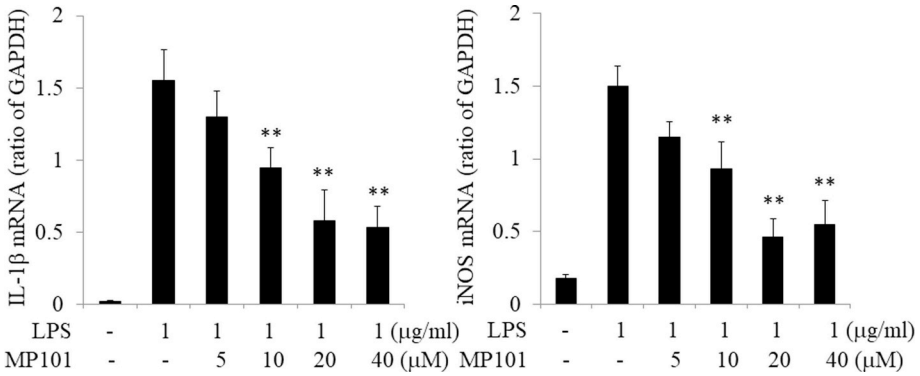

Fig. 8. MP101 can suppress IL-1β and iNOS expression in primary cultured microglia.
Primary microglia were stimulated with LPS (1 μg/ml) for 18hr. RT-PCR analysis was performed for IL-1β and iNOS in the corpus callosum of mouse brain. MP101 can suppress induction of these inflammatory cytokines in a dose dependent manner. *P < 0.01.

et al., 2017; Hubbard et al., 2018; Wu et al., 2017) as well as neuromuscular disease and hearing loss (Geisler, 2019). Accumulating evidence has demonstrated that DNP suppresses calcium accumulation and overload in the mitochondria to produce its neuroprotective effects (Hubbard et al., 2018; Pandya et al., 2007), and also stimulates neurite outgrowth in cultured neuronal cells likely by inducing BDNF (Wasilewska-Sampaio et al., 2005). In addition, DNP ameliorates learning and memory deficits in a mouse model of Alzheimer's disease (Geisler et al., 2017), and protect neurons against dysfunction and degeneration in experimental models of ischemic stroke (Korde et al., 2005), traumatic brain injury (Hubbard et al., 2018; Pandya et al., 2007), Huntington disease (Wu et al., 2017), peripheral nerve injury (da Costa et al., 2010), reduces hepatic insulin resistance (Perry and Shulman, 2013; Samuel et al., 2007) and extends lifespan (Caldeira da Silva et al., 2008).

While antioxidants attempt to neutralize ROSs after they have been made (Smith and Murphy, 2010; Sood and Whitten, 2018), uncouplers actually prevent overt ROS production (Kowaltowski et al., 2009; Miwa and Brand, 2003), a potentially more effective point of intervention. This idea was comprehensively reviewed by Kowaltowski et al. (Kowaltowski et al., 2009), who discussed the collective benefits of enhancing respiratory rates by mild uncoupling, as follows: 1) increasing O_2 consumption prevents formation of superoxide radical anions (O_2^-) by decreasing O_2 tension in the microenvironment, 2) it favors more oxidized levels of respiratory chain intermediates, such as in Complex I and III, known as a substantial source of ROSs, 3) uncoupling keeps NADH levels lower, which prevents ROS formation by mitochondrial matrix flavoproteins, and, 4) it lowers mitochondrial membrane potential ($\Delta\Psi$), a condition that thermodynamically disfavors the reverse flow from Complex II to I, thereby decreasing ROS formation.

DNP pharmacology extends beyond ROS production and calcium handling, in that they induce cAMP production, which activates CREB, a transcription factor for BDNF, an important growth factor that can repair damaged neurons and cognition (Geisler, 2019; Hubbard et al., 2018; Kandel et al., 2014). BDNF has been explored as a modulator and treatment in MS, and the striking results found here in two MS models supports findings that BDNF, produced by astrocytes and microglia may be a significant factor in axonal protection. Interestingly, the BDNF levels in the spinal cords were still significantly elevated *after* 3-weeks of stopping treatment, suggesting a sustain effect (Fig. 6).

In addition to induction of BDNF, both MP101/MP201 suppressed oligodendroglial dysfunction based on the expression levels of MBP, CNPase and COX4, while lowering the expression levels of inflammatory cytokines, IL-1β, TNF-α and iNOS. Furthermore, both MP101/MP201 appeared to sustain neuronal function, because the synaptophysin expression was maintained during EAE, and the expression of neuronal injury markers, SMI-32 and ATF3, was lower than that in the vehicle-treated group. The expression data suggests a pleiotropic protective effect in pathways involving lowering inflammatory demyelination and axonal injury and driving BDNF-mediated neuroprotective pathways. Although not measured here, there may be an additional benefit from improved metabolic endpoints as it was seen that DNP can lower circulating insulin, glucose and triglycerides when provided chronically in wild type mice, in addition to the lowering of hydrogen peroxide (H_2O_2) production, DNA damage and oxidized proteins in the liver, brain and heart (Caldeira da Silva et al., 2008). The collective benefits may push off the requirements for aging to see onset if provided prophylactically and resolve issues related to metabesity (Fuente-Martin et al., 2019; Geisler, 2019).

Although there are many therapies to reduce inflammation, there are current no drugs available that are “disease modifying”, especially for progressive MS. The studies presented here demonstrate a potential novel therapy that specifically targets the mitochondria to provide a striking effect of neuroprotection with MP101/MP201 (see Movie S1. MP101 & MP201 Prevents Paralysis in EAE Model). Paradoxically, both compounds preserved body weight in comparisons to the placebo showing signs of weight loss and wasting. DNP was used originally in the 1930's for weight reductions at high doses on well over 100,000 humans (Cutting et al., 1933; Cutting, 1933; Tainter et al., 1933). However here, the doses are at least 10x lower than the human equivalent dose commonly used in the 1930's for weight loss (~100 mg t.i.d./day) and now repositions DNP as a reinvented drug for neuroprotection without weight loss. Bioenergetics of the mitochondria are well conserved across all mammalian species (Porter and Brand, 1995), suggesting effective translation of the mechanism of action from mouse models to patients. An important factor to consider is that the mechanism of action is initially “non-genomic”, since the target is a physical subcellular location in the mitochondria and primarily based upon acid-base biophysics (Geisler, 2011). This unique “bioenergetics” conserved factor increases the likelihood of successful translation, since proteins/receptors can lose conservation from the animal models to human, altering the pharmacology found in animal models. Furthermore, two different models of MS were evaluated to determine the merits of this approach, the EAE and CPZ models, although no model completely represents the human scenario. In both models, MP101/MP201 attenuated demyelination, protected the axons and improved behavioral changes in paralysis and gait. Similar to metformin and topiramate found by phenotypic screens with currently no known target (Rena et al., 2013; Shank et al., 2000), DNP's pharmacology stemming from biophysics and changing mitochondrial membrane potential will be further studied to delineate the MOA for neuroprotection, while still providing disease modifying medicine to patients in need. MP101/MP201 represent a promising therapy for use in relapse-remitting MS and progressive MS where there are no meaningful treatments available today. FDA recently approved Mitochon Pharmaceuticals an open IND for MP101 (DNP), therefore now clinically staged. Phase I in humans is underway in Normal Healthy Volunteers (NHV) allowing for the *possibility* to subsequently move into patients with MS to demonstrate translation as a breakthrough drug at likely weight neutral/weight preserving doses in the near future. In addition to assessing safety and tolerability as critical primary endpoints, Phase I studies will provide the first ever pharmacokinetics of DNP in humans.

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Authors contributions

All of the experiments were conducted by Y.B. in Japan who co-wrote the manuscript with J.G. J.G. contributed knowledge about the composition of the MP101/201 therapy and its effects in other disease models, but he played no role in the design, conduct, or analysis of the studies described in this manuscript.

Declaration of competing interest

Authors have no conflicts of interest with the material presented in this manuscript, and specifically Y.B. has no financial interests in MP101/201 or Mitochon. Co-author JG is a founder and share-holder of Mitochon Pharmaceuticals, Blue Bell, PA, 19422, USA. MP201 (DNP prodrug) has pending U.S. patent applications (15/451,938 and 62/693,142), as well as DNP/MP101 (15/002,531 and 15/357,412). The FDA has awarded Mitochon Pharmaceuticals Orphan Designation for DNP/MP101 for Huntington's Disease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104561.

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