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Inhibition of Drp1 hyper-activation is protective in animal models of experimental multiple sclerosis

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Abstract

Multiple Sclerosis (MS), a leading neurological disorder of young adults, is characterized by the loss of oligodendrocytes (OLs), demyelination, inflammation and neuronal degeneration. Here we show that dynamin-related protein 1 (Drp1), a mitochondrial fission protein, is activated in primary OL cells exposed to TNF- α induced inflammation or oxidative stress, as well as in EAE-immunized and cuprizone toxicity-induced demyelinating mouse models. Inhibition of Drp1 hyper-activation by the selective inhibitor P110 abolishes Drp1 translocation to the mitochondria, reduces mitochondrial fragmentation and stems necrosis in primary OLs exposed to TNF- α and H₂O₂. Notably, in both types of mouse models, treatment with P110 significantly reduces the loss of mature OLs and demyelination, attenuates the number of active microglial cells and astrocytes, yet has no effect on the differentiation of oligodendrocyte precursor cells. Drp1 activation appears to be mediated through the RIPK1/RIPK3/MLKL/PGAM5 pathway during TNF- α -induced oligodendroglia necroptosis. Our results demonstrate a critical role of Drp1 hyper-activation in OL cell death and suggest that an inhibitor of Drp1 hyper-activation such as P110 is worth exploring for its ability to halt or slow the progression of MS.

Keywords

Drp1; Oligodendrocytes; Necroptosis; Multiple sclerosis

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Conflict of interest

The authors have declared that no conflict of interest exists.

1. Introduction

Multiple Sclerosis (MS) is a chronic autoimmune-mediated demyelinating disease of the central nervous system (CNS), which is the leading cause of neurological disability in young adults. The pathological hallmark of MS is dramatic loss of clusters of oligodendrocytes (OLs), leading in turn to demyelination, neuronal axon degeneration and ultimately irreversible neurological disability (Patrikios et al., 2006). During the early phase of the disease, although remyelination occurs it is not sustained resulting in chronic demyelinated axons. The etiology and pathogenesis of MS are enigmatic. Although currently available immunomodulatory therapy is effective in reducing disease relapses and a few clinical trials (Simvastatin and Ocrelizumab) suggest its potential in reducing disability progression in primary progressive MS, there is evidence that the pathogenic process can still advance (Chataway et al., 2014; Montalban et al., 2016).

Mitochondrial dysfunction is a known correlate of the axonal damage in MS lesions. Respiratory deficiency (Mahad et al., 2009), mitochondrial DNA depletion (Campbell et al., 2011), oxidative damage (Campbell and Mahad, 2012; Mahad et al., 2015) and mitochondria-associated apoptosis (Ghafourifar et al., 2008), are manifested in both human MS pathological specimens and MS experimental animal models. However, the factors that cause mitochondrial damage in MS and the extent that mitochondrial impairment contributes to oligodendrocyte cell death and demyelination remain poorly understood. Mitochondria are highly dynamic organelles whose size and structure are regulated by a balance between fusion and fission. This dynamic process maintains normal mitochondrial function and enables mitochondrial recruitment to critical subcellular compartments and mitochondrial quality control (Detmer and Chan, 2007). Defects in either fusion or fission lead to mitochondrial fragmentation and result in cell death (Jahani-Asl et al., 2010; Dorn, 2015). Such mitochondrial malfunctions have been increasingly implicated in human diseases such as neurodegenerative and inflammatory disorders.

The regulation of mitochondrial dynamics is controlled by a set of conserved GTPases. Mitofusin1/2 and optic atrophy 1 (OPA1) regulate fusion whereas dynamin-related protein 1 (Drp1) mediates fission (Chan, 2006). Drp1 is primarily located in the cellular cytosol. Upon activation, it translocates to the mitochondria where it binds to mitochondrial adaptor proteins, such as Fis1, Mff and Mid49/51, to drive fission (Chang and Blackstone, 2010a, 2010b). The activity of Drp1 is regulated by posttranslational modifications. Phosphorylation of Drp1 at serine 637 inhibits both Drp1 enzyme activity and translocation to mitochondria, whereas phosphorylation at serine 616 promotes Drp1-mediated mitochondrial fission (Jahani-Asl and Slack, 2007; Chang and Blackstone, 2010b). Physiological levels of Drp1 are essential for cell survival and brain development (Wakabayashi et al., 2009). Hyper-activation of Drp1 causes a wide range of damage, including mitochondrial depolarization, oxidative stress and bioenergetic defects, ultimately leading to cellular apoptosis (Estaquier and Arnoult, 2007; Cassidy-Stone et al., 2008), necrosis (Wang et al., 2012; Guo et al., 2014) or autophagic cell death (Su and Qi, 2013; Zuo et al., 2014). Inhibition of Drp1 activation by either genetic manipulation or pharmacological reagents has been reported to be protective in neurodegenerative diseases, such as Parkinson's disease (Su and Qi, 2013; Rappold et al., 2014), Huntington's disease (Song et

al., 2011; Guo et al., 2013), cardiac injuries (Ong et al., 2010; Disatnik et al., 2013) and cancer (Xie et al., 2015). However, whether Drp1 activation plays a role in the pathogenesis of MS and whether its inhibition might reduce MS pathology are unknown.

Tumor necrosis factor α (TNF- α)-induced necroptosis is a type of oligodendrocyte cell death that is associated with the severity of MS lesions and disease progression (Ofengeim et al., 2015). Necroptosis depends on the kinase activity of receptor-interacting kinase-1 and -3 (RIPK1/RIPK3) (Vanlangenakker et al., 2012). In the absence of apoptosis, RIPK1 and RIPK 3 form a complex, known as the necrosome, which subsequently phosphorylates and activates mixed-lineage kinase domain-like (MLKL) protein, thus initiating necroptosis (Pasparakis and Vandenabeele, 2015). RIPK1/3 and MLKL, the hallmarks of necroptosis, are activated in the cortical tissues of MS patients and MS experimental mouse models (Ofengeim et al., 2015). Recently, Wang et al. (2012) reported that RIPK1/3 activates mitochondrial phosphoglycerate mutase/protein phosphatase – PGAM5 – which subsequently dephosphorylates Drp1 at ser637 and promotes Drp1 fission activity, triggering TNF- α -induced necrosis. This line of evidence places Drp1 downstream of RIPK1/3 in the pathway leading to TNF- α -induced necroptosis. Further, in the experimental autoimmune encephalomyelitis (EAE) demyelinating model, scanning electron microscopy revealed an increased number of shorter mitochondria accumulating in degenerating axons, and this abnormal morphology of mitochondria was associated with increased immunostaining of the Drp1 fission protein (Bando et al., 2015). Yet whether Drp1 is activated in MS and what exact role it plays in the progression of MS lesions remain unknown.

We recently developed a peptide inhibitor P110 that selectively blocks Drp1 translocation to the mitochondria by interfering with Drp1/Fis1 interaction under stress conditions (Qi et al., 2013). P110 is a seven amino acid peptide conjugated to the cell permeating TAT peptide, TAT_{47–57}, which enables in vivo delivery (Guo et al., 2013; Qi et al., 2013). It targets Drp1, preventing the interaction of Drp1 and Fis1, thus inhibiting the Drp1 hyperactivation (Qi et al., 2013). Treatment with P110 reduces mitochondrial damage and organ injury without affecting Drp1 physiological function in the animal models cited above. P110 can pass through blood-brain-barrier and is non-toxic (Guo et al., 2013). The treatment efficacy of P110 requires the presence of Drp1 (Guo et al., 2013; Qi et al., 2013), but does not affect other fusion-related proteins or mitochondrial fission related proteins including Mff and Mid49/51 (Disatnik et al., 2013; Guo et al., 2013; Qi et al., 2013). These characteristics of P110 make it a unique and specific inhibitor to modulate Drp1 activation under pathological conditions.

In this study, we extend these results by showing that Drp1 is hyper-activated in both primary OLs cultures exposed to extracellular stressors and two mouse models of demyelination that recapitulate the pathological features of human MS. Using the Drp1 peptide inhibitor P110, we demonstrate that inhibition of Drp1 hyper-activation by P110 reduces mitochondrial fragmentation and necrosis in primary OLs, and attenuates demyelination lesions in both cuprizone and EAE animal models. We further report that Drp1 activation is mediated through the RIPK1/RIPK3/MLKL/PGAM5 pathway in TNF- α -exposed OLs. Our findings thus demonstrate a critical role of Drp1 hyper-activation that involves in necroptosis of OLs in the progression of MS lesions.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies used in the study were listed as follow: antibodies of TOM20 (sc-11415), CD3 (sc-20047) and β -actin (sc-47778) were purchased from Santa Cruz Biotechnology. Myelin basic protein (MBP, SMI-99P) antibody was from Covance. Cell markers for early OPC (Olig2, AB9610), mature OL (CC1, OP80), neuronal NeuN (MAB377) and GFAP (MAB360) for astrocyte were purchased from Millipore. Neurofilament 200 (N4142, sigma) was used for immunostaining of neuronal axons. Antibodies against phospho-Drp1 (Ser616, #3455), phospho-Drp1 (Ser637, #6319), Caspase-3 (#9662), MLKL (#28640), phospho-MLKL (Thr357/Ser358, #14516) and VDAC (#4866) were purchased from Cell Signaling. Anti-DLP1/Drp1 (#611113) antibody was from BD biosciences. Iba1 (#019-19741) antibody was from Wako Chemicals to identify active microglia. PGAM5 (ab126534) antibody was from Abcam. HMGB1 (#10829-1-AP) antibody was from Proteintech. 7N-1 (RIP1 inhibitor II) was purchased from Millipore. TNF- α was from Peprotech.

2.2. Animals

C57BL/6 mice were purchased from Jackson Laboratory (Stock No: 000664) and housed at Animal Research Center of Case Western Reserve University. Mice were maintained with a 12-hour light/12-hour dark cycle (on at 6:00 am, off at 6:00 pm). All animal care and animal experimental procedures were done in compliance with approved animal policies of the Institutional Animal Care and Use Committee of Case Western Reserve University School of Medicine.

2.3. Drp1 peptide inhibitor P110

The Drp1 peptide inhibitor P110 and control peptide TAT were synthesized by American Peptide Company (now called Bachem Americas Inc., Torrance, CA, USA) (Product # 368000, Lot # 1311151T). As previously described (Guo et al., 2013; Qi et al., 2013), the peptides are synthesized as one polypeptide with TAT₄₇₋₅₇ carrier in the following order: N-terminus–TAT–spacer (Gly-Gly)–cargo (Drp1₄₉₋₅₅)–C-terminus. The C-termini of the peptides are modified to C (O)-NH₂ using Rink Amide AM resin to increase stability. Peptides are analyzed by analytical reverse-phase high-pressure liquid chromatography (RP-HPLC) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) and purified by preparative RP-HPLC. The purity of peptides is >90% measured by RP-HPLC Chromatogram. Lyophilized peptides are stored at –80 °C freezer and are dissolved in sterile water before use. P110 has shown efficacy and no toxicity both in vitro and in animal models in vivo (Filichia et al., 2016; Guo et al., 2013).

In our previous study we have found that P110 treatment in the dosage of 0.5–3 mg/kg/day range were effective in a Huntington's disease mouse model (Guo et al., 2013), Parkinson's disease mouse model (Filichia et al., 2016) and ischemic brain injury in rat (Guo et al., 2014). We tested the dosages of P110 at 0.5 and 1.0 mg/kg/day in our MS mouse model and found that P110 at 1.0 mg/kg/day provided better protection. Thus, in the current study, mice were i.p. injected with P110 at 1 mg/kg once daily.

2.4. Experimental autoimmune encephalomyelitis (EAE) model

For induction of EAE, C57BL6/J female mice at 10-week-old of age were immunized with MOG_{35–55} together with complete Freund's adjuvant emulsion (Hooke Laboratories, MOG_{35–55} EAE Induction kit, EK-2110) according to the manufacturers' instruction. Using the EAE Induction Kit result in 98% successful disease induction. All EAE-induced animals were monitored daily and randomly divided into four groups: 1) A group of animals with P110 treatment starting at the first induction day of EAE (day 0); 2) A group of animals with P110 treatment started at the before the EAE symptom appearing (day 7) after EAE induction; 3) A group of animals in which peptide P110 was given at the beginning of sickness (score 1, day 11–12) after EAE induction; 4) A group of animals treated with vehicle as control animals. For each group, at least 8–12 animals/group were used for clinical scoring to determine functional impairment. Subsequently, 3–4 independent cohorts of each group were used for immunohistochemistry, biochemical analysis and EM analyses at the end of the P110 treatment.

For the treatment groups, all EAE-induced animals were administrated once daily systemic i.p delivery of P110 at a dosage of 1 mg/kg/day at various days of 0, 6, or 11–12 after immunization. According to previous studies (Guo et al., 2013; Guo et al., 2014), at this dosage, P110 does not show any overt evidence of toxicity to any of the animals. A daily treatment period of 29 days was tested in this study. All EAE induced mice were blinded, monitored closely and video recorded and clinically assessed daily according to the well accepted clinical scoring 0 to 5, by which 0 represents no symptoms; 1 = limp tail; 2 = limp tail and hind limb weakness; 3 = hind limb paralysis; 4 = hind limb paralysis and forelimb weakness; and 5 = moribund.

2.5. Chronic cuprizone-induced demyelination model

To induce demyelination in corpus callosum, 8-week of age male C57BL6/J mice were fed with a standard 0.2% cuprizone diet in chow (Harlan Laboratories) for a period of six weeks. The successful cuprizone-induced demyelination rate is 98% of animals in our laboratory. All the cuprizone-induced animals were randomly grouped prior to P110-treatment, no-treatment following 6-week of a cuprizone-chow diet. P110 treated mice were received i.p injection of P110 (1 mg/kg/day) either at the beginning of cuprizone-chow feeding for 6-week treatment or P110 injection following 3-week cuprizone-fed chow and for a period of 3-week P110 treatment. A parallel group of animals with vehicle treatment served as controls. All mice were assessed for motor deficits using Rotarod test in a double-blinded manner in every other three days. The times and traveled distances on rotarod until mice fell off were measured.

2.6. Black Gold myelin staining

Black Gold myelin staining was performed according to the manufacturer's instruction (#AG105, Millipore). Briefly, 20 µm sections were incubated in prewarmed 0.3% Black-Gold II solution at 60 °C for 12 min. After rinsing, sections were incubated with 1% sodium thiosulfate solution for 3 min at 60 °C and rinsed, dehydrated in a series of gradated alcohols, air-dried, and mounted in mounting media. Images were captured from matched areas of stained sections using Leica DFC500 fluorescence microscope. To quantify myelin,

the extent of demyelination was scored as previous study (Steelman et al., 2012): demyelination range from 0% to 10% of the total anatomical structure was scored as 0; 11%–30% represented a score of 1; 31–60% represented a score of 2; 61–90% represented a score of 3; and 91–100% of demyelination represented a score of 4.

2.7. Purified OPCs culture and OPC differentiation

Cell culture plates were pre-coated with secondary antibodies IgM (10 µg/ml) (Millipore, Cat #55460) in 50 mM Tris-HCL and followed primary mouse antibody A2B5. Dissociated cells were incubated in the pre-coated culture dishes for 30 min at 37 °C and then non-adherent cells were gently removed. OPCs were released by 0.05% Trypsin in DMEM at a purity of approximately 96%. Cells were expanded in DMEM/F12 medium supplemented with 5 ng/ml NT-3, 10 ng/ml CNTF, 20 ng/ml bFGF and 20 ng/ml PDGF-AA.

To induce OPC differentiation cells were switched to media lacking bFGF and PDGF-AA, with the addition of thyroid hormone (T3, sigma, 40 ng/ml). OPCs were allowed to differentiate for 2 and 5 days and labeled with O4⁺ and MBP antibodies. The percentage of positive O4⁺ and MBP⁺ cells were calculated and compared.

2.8. Isolation of mitochondrial-enriched fraction and lysate preparation

Mitochondria were isolated as described in previous studies. Cells were washed with ice-cold PBS and incubated on ice in lysis buffer (250 mM sucrose, 20 mM HEPES-NaOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, protease inhibitor cocktail and phosphatase inhibitor cocktail) for 30 min. Cells were scraped and disrupted by repeated aspiration through a 25-gauge and 30-gauge needles, respectively. Mouse brain tissue or spinal cord was minced and homogenized. The homogenates were spun at 800 g for 10 min at 4 °C, and the resulting supernatants were spun at 10,000 g for 20 min at 4 °C. The pellets were washed with lysis buffer and spun at 10,000 g for 20 min at 4 °C. The final pellets were suspended in lysis buffer containing 1% (v/v) Triton X-100 noted as mitochondrial-enriched fractions. Mitochondrial membrane protein voltage-dependent anion channel (VDAC) was used as a loading control.

2.9. Immunohistochemical staining of frozen sections

Mice were anesthetized with Avertin and perfused with PBS and 4% paraformaldehyde. Dissected brains were post-fixed in 4% paraformaldehyde overnight at 4 °C and equilibrated in 20% sucrose. 20 µm coronal cryosections were sectioned and pre-treated with Reveal Decloaker Solution (Biocare Medical) for antigen retrieval according to the manufacturer instruction. After blocking, sections were incubated with the primary antibodies and followed by appropriate secondary antibodies conjugated with Alexa fluorescence 488 or 594. Sections were counter-stained with DAPI (1:1000, Sigma) and mounted with mounting medium (Vector Laboratories). All Images were taken and analyzed using a Leica DFC500 fluorescence microscope.

2.10. Cell viability measurement

Purified OPCs/OLs were pre-treated with Drp1 peptide inhibitor P110 for 30 min and then followed by incubation with or without either H₂O₂ (200 µM) or mouse TNF-α (20 ng/ml)

for 24 h. Cell viability was measured using MTT-based kit (Cat. #11465007001, Roche) according to manufacturer's instruction. Cell death was determined by measuring released LDH in the culture medium according to manufacturer's instruction (#4744926001, Roche).

2.11. HMGB1 release assay

HMGB1 (high mobility Group B1) protein released from necrotic cells has been considered as classical biochemical hallmarks for necrotic cell death. Cultured medium was collected from P110 pretreated OPC/OL cells following exposure to H₂O₂ or TNF- α . Proteins in the medium were purified and concentrated using Amicon Ultra 0.5 ml centrifugal filters (Millipore). HMGB1 released into cell culture supernatants was evaluated by Western blotting with anti-HMGB1 antibody.

2.12. Luxol Fast Blue (LFB) staining

LFB staining was performed according to the manufacturer's instruction (#26681, Electron Microscopy Sciences). 20 μ m coronal sections were immersed in LFB solution at 56 °C overnight and then washed sequentially with 95% ethanol, distilled water, 0.1% lithium carbonate solution, 70% ethanol and water. The sections were then dehydrated with a series of gradated ethanol, cleaned with HistoClear and mounted. A set of serial sections were stained and analyzed; images (5 to 6 sections/animal) were captured under light microscopy. The demyelinated areas (lack of LFB staining) were measured using Image J software. The percentage of demyelinated areas of spinal cord was calculated.

2.13. Immunoprecipitation

Whole cell lysates were extracted from purified OPC cells using cell lysate buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, and protease inhibitor). Equal amounts of soluble protein was immunoprecipitated overnight with indicated antibody overnight at 4 °C and followed incubation with protein A/G beads for 1 h. Negative controls were used with isotype-matched IgG. Immunoprecipitates were washed and separated by SDS-PAGE and immunoblotted with indicated antibodies.

2.14. Western blot analysis

Purified OPC cells or tissue samples of spinal cord or corpus callosum were homogenized and protein concentration was determined by Pierce BCA protein assay kit (Thermo Fisher). Then, equal amounts of protein were applied to 12% or 15% SDS-PAGE gels, and electrophoretically transferred onto PVDF membranes (Millipore). The membranes were blocked in blocking buffer and probed with indicated primary antibodies overnight at 4 °C and followed by secondary antibodies conjugated to HRP. Enhanced chemiluminescence was performed with a West Pico Kit (Thermo Fisher). The density of bands was quantified using ImageJ software (NIH).

2.15. Immunocytochemistry

OPCs and OLs were immunostained with O4 or MBP (SMI-99P, 1:500, Covance) antibodies and followed by secondary antibody goat anti-mouse IgM Alexa 488 or 594 (1:500; Invitrogen). For double labeling, cells were incubated with the second primary antibodies

against TOM20 overnight at 4 °C, followed by secondary antibodies conjugated Alexa Fluor 488 or 594. After fixation, cells were mounted with Vecta Shield mounting medium with DAPI (Vector Laboratories).

2.16. Tissue preparation for electron microscopy (EM) analysis

For ultrastructural analyses of myelination, anesthetized animals were perfused with 2% glutaraldehyde/4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences). Tissues were post-fixed in 1% OsO₄ for 2 h and spinal cord or brain coronal sections (500 µm) containing corpus callosum were prepared (Leica, Vibratome), dehydrated, stained with saturated uranyl acetate and embedded in a Poly/Bed812 resin (Polysciences Inc.). The 1 µm thick sections were cut and stained with toluidine blue and matched area were selected for EM. For ultrastructure analysis, ultrathin sections (0.1 µm) were cut and visualized using an electron microscope (JEOL100CX). G ratios were calculated from at least 50–100 randomly selected myelinated axons.

2.17. Statistical analysis

All data analyses were performed using GraphPad Prism 6.00. Data are shown as mean ± SEM. $p < 0.05$ is deemed statistically significant. Statistical analysis was performed by two-tailed unpaired Student's *t*-tests, one-way or two-way ANOVA with post-hoc analysis by Tukey's multiple comparison test or Sidak's multiple comparisons test. Quantifications were performed in a blinded fashion. No statistical tests were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Data distribution was assumed to be normal, but this was not formally tested. All experiments were performed at least three times independently.

3. Results

3.1. Increased Drp1 activity in EAE and cuprizone-induced demyelination mouse models

Drp1 phosphorylation at specific serine sites (Ser616) increases Drp1 activity (Jahani-Asl and Slack, 2007). To examine whether Drp1 is activated in MS demyelinating lesions, we first examined the MOG_{35–55}-induced EAE demyelination model of MS. We harvested spinal cords of one group of mice at 16 days following MOG_{35–55} immunization (the peak of sickness) and a second group at 29 days; we determined Drp1 phosphorylation using antibodies that specifically recognize phospho-Drp1 at Ser616 or Ser637. Western blot analysis of total protein lysates showed an increase in Drp1 phosphorylation at Ser616 (p-Drp1-Ser616) combined with a decrease in phosphorylation at Ser637 (p-Drp1-Ser637) after MOG_{35–55} immunization (Fig. 1A). An increase in Drp1 total protein level was observed under the same conditions (Fig. 1A). Immunohistochemistry consistently revealed an increase of both Drp1 (Fig. 1B) and p-Drp1-Ser616 in the mature oligodendrocytes (CC1⁺, Fig. 1C), neurons (NeuN⁺, Fig. 1C), oligodendrocyte precursor cells (OPCs, olig2⁺, Supplementary Fig. 1A) and microglial cells (Iba1⁺, Supplementary Fig. 1A) in the EAE-lesioned spinal cords. These data suggest that Drp1 is activated in most cell types of lesioned spinal cords.

Next we examined the extent of Drp1 translocation to mitochondria, an essential step in the initiation of Drp1-dependent mitochondrial fission (Chang and Blackstone, 2010a, 2010b). We isolated mitochondrial fractions from the spinal cords of EAE-immunized mice and performed Western blot analysis (Fig. 1D). We observed increased levels of mitochondrial associated Drp1 at 16 and 29 days following MOG_{35–55} immunization, further demonstrating Drp1 activation in EAE. Under the same experimental conditions as above, we also examined the protein levels of other mitochondrial fusion-related proteins. We found that the level of fusion protein OPA1 but not mitofusin 1 (Mfn1) was decreased under these conditions (Supplementary Fig. 1B). These results indicate that a disruption in the balance between fusion and fission occurs in the EAE mouse model that favors increased fission.

To ensure that the effects we were seeing were not related to an artifact associated with the EAE model of MS, we repeated our observations in mice with a cuprizone-induced demyelination. In this model, the death of mature OLs is caused by cuprizone-induced inhibition of copper-dependent mitochondrial enzymes (Matsushima and Morell, 2001; Kipp et al., 2009). As in the EAE model, at 1, 2 and 3 weeks after cuprizone treatment, we found increased p-Drp1-Ser616 but decreased p-Drp1-Ser637 in the demyelinating corpus callosum (Fig. 1E). The increased expression of p-Drp1-Ser616 in demyelinating corpus callosum was validated by immunostaining (Supplementary Fig. 1C). The only significant differences in the two model systems were that, first, after cuprizone treatment, there were no changes in the total protein levels of Drp1 (Fig. 1E). The level of Drp1 associated with mitochondria was significantly increased during cuprizone treatment when compared to control animals (Fig. 1F). Second, unlike the situation in EAE, the level of OPA1 was not altered in the corpus callosum following cuprizone treatment (Supplementary Fig. 1D). Collectively, these results demonstrate that Drp1 is hyper-activated during demyelinating lesions caused by either EAE or cuprizone.

3.2. Increased Drp1 activation and mitochondrial fragmentation in response to extracellular stress

OLs cell death causes demyelination and subsequent axonal degeneration in MS (Watzlawik et al., 2010). Using immunopurified primary OLs culture, we examined Drp1 translocation to the mitochondria, a marker of Drp1 activation, in response to either oxidative stress (H₂O₂) or TNF- α , two factors that can cause OLs death (Selmaj and Raine, 1988; Jurewicz et al., 2005; Mammucari and Rizzuto, 2010). Western blot analysis revealed an increase of 1.5- to 2.5-fold in mitochondria-associated Drp1 protein levels in OPC/OL cultures exposed to H₂O₂ or TNF- α (Fig. 2A–D), indicating Drp1 translocation under these conditions of stress. Mitochondrial morphology of OL cells was determined by immunostaining of the outer membrane protein, Tom20. Following exposure to H₂O₂ or TNF- α , mitochondria in cells immunopositive for MBP displayed a punctate staining pattern (Fig. 2E) suggestive of mitochondrial fragmentation. The percentage of MBP⁺ cells exhibiting damaged mitochondria was increased approximately 70% and 60% in response to H₂O₂ and TNF- α , respectively, compared to that in control cells (~10%) (Fig. 2F). Thus, Drp1 translocates to the mitochondria in OLs in response to MS-related insults, leading to increased mitochondrial fragmentation.

3.3. Inhibition of Drp1 hyperactivation prevents functional impairment and OL degeneration

Because Drp1 is hyper-activated both in OLs cultures exposed to extracellular stresses and in MS mouse models (Figs. 1 and 2), we next set out to determine whether inhibition of Drp1 activation by a selective Drp1 peptide inhibitor P110 (Qi et al., 2013) could be protective in our MS models. MOG_{33–35} immunized EAE mice were treated once daily with P110 by intraperitoneal injection for 29 days, starting at different phases of diseases: 1) immediately after immunization (day 0), 2) before of onset of sickness (day 7), and 3) at the onset of sickness (day 12). Treatment with P110 in either of the first two situations prevented the emergence of functional deficits and reduced the severity of EAE-induced symptom compared to that of vehicle-treated EAE mice (Fig. 3A). Mice treated after the onset of symptoms (situation 3) also improved, exhibiting a trend towards partial prevention. This smaller effect, however, was not statistically significant (Fig. 3A). In line with the protection in the clinical score, treatment with P110 also reduced the EAE-associated weight loss (Supplementary Fig. 2A). P110 also could decrease the rate of death after immunization (EAE: 18.2%; EAE-P110 Day0: 9.1%; EAE-P110 Day 7: 9.1%; EAE-P110 Day 12: 16.7%). Western blot analysis showed that treatment with P110 at beginning of EAE-induction significantly reduced Drp1 translocation to OLs mitochondria after EAE induction (Fig. 3B), validating the *in vivo* inhibition of Drp1 by P110. Together, these data suggest that inhibition of Drp1 activation by P110 ameliorates the severity of EAE-induced sickness, especially when treatment starts at earlier phases of disease.

We found that P110 treatment at beginning of EAE-induction increased MBP myelin expression and reduced the areas of lesion in demyelinating spinal cords of EAE mice, as determined by immunostaining of anti-MBP antibody and Luxol Fast Blue (LFB) myelin staining (Fig. 3C and D). Western blot analysis further demonstrated that the loss of MBP protein in EAE-lesioned spinal cords was significantly reduced by P110 treatment (Fig. 3E). Importantly, P110 treatment had no effects on the MBP-labeled myelin expression in control mice (Fig. 3C and E). Toluidine blue myelin staining and ultrastructural electron microscopy (EM) analysis showed increased percentages of myelinated/remyelinated axons throughout the extent of lesions in P110-treated mice relative to that in vehicle-treated EAE mice ($70.5 \pm 4.4\%$ vs $33.1 \pm 1.7\%$, Fig. 3F and G, Supplementary Fig. 2B). Quantification of the G-ratio revealed thicker myelin in P110-treated EAE mice than that in vehicle-treated EAE mice ($p < 0.001$; two-tailed unpaired Student's *t*-test, Fig. 3H).

In EAE-lesioned-spinal cords the number of Olig2⁺ oligodendrocyte lineage cells and CC1⁺ OLs markedly decreased at 29 days, and treatment with P110 significantly blunted this loss of cells (Fig. 4A,B and C). Significantly, P110 had no effects on OPC proliferation and/or differentiation of purified WT oligodendrocyte progenitor cell (OPC) cell culture, as measured by changes in the percentages of O4⁺ or MBP⁺ cells (Supplementary Fig. 3). Thus, the increased number of mature OL cells (CC1⁺) in the MS models is likely due to an improvement in mature OL survival, rather than the stimulation of OPC differentiation.

As in our previous studies (Moore et al., 2014; Ofengeim et al., 2015), we observed an increase in the number of Iba1⁺ microglia and GFAP⁺ astrocytes in the EAE-lesioned cords, consistent with an early induction of inflammation by EAE. Treatment with P110 reduced

the number of these inflammatory cells significantly (Fig. 4D, E and F). No changes were seen in the number of infiltrating T lymphocytes marked by immunolabeling with anti-CD3 antibody in the EAE-lesioned cords (Supplementary Fig. 4). There was also no difference in spleen size between P110-treated and vehicle-treated animals (Fig. 4G and H). Thus, P110 treatment reduced the local inflammation in demyelinating lesions without affecting T cell infiltration.

Collectively, our findings show that inhibition of Drp1 hyper-activation by P110 treatment reduced mature OL cell death and prevent demyelination of EAE-immunized mice.

3.4. P110 attenuates OL cell death and inflammation in cuprizone-treated animals

Since cuprizone treatment results in a remarkable demyelination of corpus callosum and other regions without the involvement of the peripheral immune system (Matsushima and Morell, 2001; Denic et al., 2011), we asked whether the effectiveness of P110 treatment could also be seen in this second MS model. Administration of P110 simultaneously with cuprizone, significantly protected against the degradation of motor performance on the Rota-rod (Fig. 5A). Unlike the situation in the EAE model, however, delaying administration of P110 for 3 weeks after cuprizone treatment was also effective and led to significant improvements in motor function.

By both black gold myelin staining and immunostaining with anti-MBP antibody, we observed marked demyelination in the corpus callosum of cuprizone-treated mice at 6 weeks after treatment (Fig. 5B). P110 treatment significantly increased the expression level of MBP in the corpus callosum of cuprizone-fed animals (Fig. 5B and C). These findings were further validated by Western blot analysis; cuprizone led to decreased MBP protein level in corpus callosum and P110 treatment blocked this reduction (Fig. 5D). In agreement with our hypothesis, P110 treatment also abolished Drp1 translocation to the mitochondria of oligodendroglia (Fig. 5E). P110 was also effective in providing myelin protection in cortex and hippocampus, as evaluated by black gold myelin staining (Supplementary Fig. 5A and B) and by Western blot analysis (Supplementary Fig. 5C). EM analysis revealed both an increased percentage of myelinated axons as well as an increased myelin thickness, as quantified by G-ratio (G-ratio: $p < 0.01$ (Cupr versus Cupr/P110); one-way ANOVA, Fig. 5F, G and H).

Cuprizone treatment led to a decreased number of CC1⁺ OLs in corpus callosum consistent with the damage we found (Fig. 6A, B and C). Treatment with P110 significantly but not completely blocked the decrease in CC1⁺ OLs (Fig. 6A and C) suggesting that the mature, myelin forming OLs were protected by the drug treatment. Although the response to cuprizone does not engage the peripheral immune system, it does involve an activation of brain astrocytes as well as Iba1-positive microglia. P110 also reduced both responses in the corpus callosum of cuprizone-lesioned mice (Fig. 6D–G). These findings are consistent with our observations in the EAE mouse model and point to a model in which Drp1 hyperactivation causes damage to mature OLs, leading to subsequent demyelination and neurological deficits in MS.

3.5. P110 protects against H₂O₂- or TNF- α -induced mitochondria fragmentation and necrosis

To better understand the cell death mechanism induced by Drp1 hyper-activation, we treated cultured OL cells with P110 (1 μ M) following the exposure of H₂O₂ (an oxidative stress) and TNF- α (a proinflammatory cytokine implicated in MS (Sharief and Hentges, 1991)). Treatment with P110 significantly attenuated the resulting increase in Drp1 association with mitochondria in MBP⁺-cells (Fig. 7A) and led to significantly less mitochondrial fragmentation after H₂O₂ (35% of cells with fragmented mitochondria; down from 70%) and TNF- α (29%, down from 61% – Fig. 7B and C). Again, P110 treatment alone had no affect mitochondrial network integrity (Fig. 7B and C), a finding consistent with previous observations (Guo et al., 2013; Qi et al., 2013; Su and Qi, 2013). The rescue of mitochondrial integrity after P110 addition was also associated with significantly increased OLs viability after an H₂O₂ or TNF- α challenge (Fig. 7D). P110 treatment also reduced the release of HMGB1 (high mobility group box 1 protein) and LDH (lactate dehydrogenase) (Fig. 7E and F) – classical biochemical hallmarks specific for necrotic cell death (Chen et al., 2001; Bianchi and Manfredi, 2004; Rovere-Querini et al., 2004) – yet had no significant effect on the levels of pro-caspase 3, a marker of apoptosis. Thus, we conclude that inhibition of Drp1 by P110 treatment reduces TNF- α or H₂O₂-induced mitochondrial damage in OLs, which leads to increased OL survival.

3.6. Drp1 activation is mediated by the RIPK1/RIPK3/MLKL/PGAM5 pathway

Finally, we searched for the mechanism by which Drp1 is activated in these demyelinating conditions. Several kinases including PI3K/Akt/mTOR, GSK-3 β , ERK1/2, Cdk5 and RIPK1/RIPK3 are activated in human MS lesions as well as in various animal models of MS (Flores et al., 2008; Narayanan et al., 2009; Galimberti et al., 2011; Yang et al., 2013; Luo et al., 2014; Ofengeim et al., 2015). To determine whether these kinases regulate Drp1 activation after an TNF- α -related insult, we treated OLs with a number of different kinase inhibitors in the presence of TNF- α , and determined the level of Drp1 associated with mitochondria. We found that treatment with 7N-1, a RIPK1 kinase inhibitor, greatly suppressed the translocation of Drp1 to mitochondria in TNF- α -treated OLs (Fig. 8A). In contrast PD98059 (an ERK1/2 inhibitor), LiCl (a GSK-3 β inhibitor) and roscovitine (a Cdk inhibitor) were without effect (Supplementary Fig. 6). Treatment with 7N-1 reduced the extent of mitochondria fragmentation (Fig. 8B and C) and increased cell viability (Fig. 8D) of OLs exposed to TNF- α . The addition of 7N-1 did not affect the integrity of the mitochondrial network or cell survival in unstimulated OLs (Fig. 8B, C and D). Further, we found that treatment with 7N-1 blocked the release of HMGB1 and LDH after TNF- α exposure (Fig. 8E and F), consistent with previous findings that 7N-1 mainly inhibits TNF- α -induced necroptosis (Wang et al., 2012; Ofengeim et al., 2015). As shown above, 7N-1 treatment alone did not directly affect any of these pathways in control cells.

To determine whether Drp1 is activated by the RIPK pathway via phosphorylation, we exposed OLs to TNF- α then assayed the levels of phospho-Ser616- (P-S616-) and P-S637- Drp1 in the presence or absence of 7N-1. Consistent with the inhibition of RIPK1 activation, TNF- α up-regulated phosphorylation of MLKL, a substrate of RIPK; and this was attenuated by 7N-1 (Fig. 8G and H). A TNF- α challenge to mature OLs caused a decrease in

P-S637-Drp1 and an increase in P-S616-Drp1 leading to Drp1 activation. We found that the addition of 7N-1 blocked the decrease in P-S637-Drp1, but had no effect on the P-S616-Drp1 increase (Fig. 8G and H). This selective interference with Drp1 posttranslational modifications has not been reported before and may speak to the relative importance of the two phosphorylation sites in the mitochondrial actions of Drp1. PGAM5, a downstream component of RIPK1/RIPK3/MLKL pathway, has been shown to recruit and dephosphorylate Drp1 at Ser637 on the mitochondria upon TNF- α -mediated necrosis (Wang et al., 2012). Drp1 immunoprecipitates, immunoblotted with PGAM5 revealed a strong physical interaction between Drp1 and PGAM5 (Fig. 8I); this interaction was abolished by 7N-1 (Fig. 8I). Taken together, our data suggest that Drp1 is activated through RIPK1/RIPK3/MLKL/PGAM5 signaling pathway during TNF- α -mediated necroptosis in OLs.

4. Discussion

OLs are the myelin producing cells of the CNS and are essential for proper brain function (Lopez Juarez et al., 2016; Simons and Nave, 2016). Mitochondria have been found to be located in different compartments of the OLs including soma, primary processes and the cytoplasmic channels of the myelin sheath (Rinholm et al., 2016). The proper functioning of mitochondria in OLs is critical for their cellular integrity. They provide ATP of course, but they also buffer Ca^{2+} and insure proper fatty acid metabolism and lipid synthesis all of which are critical for the maintenance of the myelin sheath (Haak et al., 2000; Carvalho, 2013). Though Drp1 activation and mitochondrial dysfunction have been extensively studied in neurons (Uo et al., 2009; Jahani-Asl et al., 2015) and astrocytes (Motori et al., 2013), our study is the first to report the role of Drp1-mediated mitochondrial fragmentation in oligodendroglial cell death. Our results suggest that Drp1 activation is a key part of the mechanism leading to necroptosis of OLs in demyelinating conditions in MS animal models similar to those found in MS. Not only do we find a strong mechanistic linkage between Drp1 localization and OLs cell death, we also demonstrate that either TNF- α -induced inflammatory or H_2O_2 -induced oxidative stress can be a direct trigger of the hyperactivation of Drp1. We further demonstrate that this activation occurs through the RIPK1/RIPK3/PGAM5 pathway. Drawing on our past experience, we show that inhibition of Drp1 activation by the P110 inhibitor peptide reduced oligodendrocyte cell death in vitro and in vivo and produced a clinically significant rescue of motor function in two different animal models of MS. Our findings reveal a previously unidentified role for Drp1 in the pathogenesis of OL damage that occurs during demyelination, and suggest that targeting Drp1 might be attractive as a novel treatment strategy for MS and other demyelination diseases.

In this study, we used EAE (a widely used autoimmune-based model) and cuprizone-induced demyelination as models of human MS. In both models, we observed the activation of Drp1 including Drp1 recruitment to the mitochondria following phosphorylation at Ser616 and dephosphorylation at Ser637. These changes have clear clinical implications since inhibition of the activation and subsequent translocation of Drp1 led to significant OL protection in both models. The number of mature OLs remained similar to sham treated controls and evidence of brain inflammation was reduced in the demyelinated regions. In

addition to this cellular protection, P110 treatment significantly attenuated the motor deficits found in both mouse models.

Because Drp1 can be activated in various cell types, in the current study, we cannot exclude the possibility that P110 treatment in vivo may inhibit Drp1 activation in other cell types, such as neurons and astrocytes, and that these contribute to the protection of the myelin sheath. We also noticed different protein expression of Drp1 in the two mouse models; the total protein level of Drp1 increased following EAE but was not changed in cuprizone-induced toxicity model. Despite this, an increase in active phosphorylated Drp1 was found in both. This points to the importance of Drp1 activation as a final common pathway in a number of different demyelinating conditions. It also suggests that there may be additional mechanisms that drive the induction of Drp1 in the EAE mouse model that would be profitable to investigate.

The disruption of mitochondrial fusion and fission has been observed in a number of human diseases. However, whether and how impairment of mitochondrial dynamics contributes to the progression of MS remains unknown. In the EAE mouse model, in addition to Drp1 activation, we observed a decrease in fusion protein OPA1, suggesting that the process of fusion might be disturbed in EAE. Because there was no change in OPA1 observed in demyelinating corpus callosum of cuprizone-induced MS mouse model, it would appear that the changes of fusion-related proteins might be an indirect effect of OLs impairment, at least in the EAE mouse model.

Mitochondrial adaptors including Fis1, Mff and MiD49/51, mediate Drp1-dependent mitochondrial fission (Otera et al., 2010b; Palmer et al., 2011; Zhao et al., 2011b). We previously found that Drp1 binding to Fis1 mainly occurs under stress while only weak interactions between Drp1 and Fis1 are observed in normal cells. One study reported that Mff recruits Drp1 to induce mitochondrial fission independent of Fis1 (Otera et al., 2010a), whereas MiD49/51 promotes mitochondrial fusion by inhibition of Drp1 (Zhao et al., 2011a). These lines of evidence suggest that these fission adaptors may play different roles in basal mitochondrial fission and the fission that occurs under pathological conditions. The findings reported here are the first to explore these cellular events in oligodendrocytes. The findings are completely consistent with our previous studies showing that blocking Drp1/Fis1 interaction by P110 treatment abolishes Drp1 translocation to mitochondria, reduces mitochondrial fragmentation, suppresses mitochondrial oxidative stress, and reduces cell death in a number of disease models in vitro and in vivo (Disatnik et al., 2013; Guo et al., 2013; Qi et al., 2013; Su and Qi, 2013; Guo et al., 2014). It is highly significant, in particular with regard to potential clinical applications, that P110 had minimal effects on Drp1 mitochondrial levels, mitochondrial morphology, mitochondrial function and cell survival in normal cells (Disatnik et al., 2013; Guo et al., 2013; Qi et al., 2013; Su and Qi, 2013; Guo et al., 2014), despite the presumed reduction in Drp1/Fis1 interactions.

Focusing on the situation in MS, it has been shown that TNF- α is one of the most relevant cytokines involved in the pathogenesis of the disease. MS patients have elevated TNF- α levels in the immediate vicinity of active lesions (Bitsch et al., 2000) and TNF- α levels are increased in the CSF and serum of MS patients (Matusevicius et al., 1996). TNF- α impairs

OLs through a mitochondria-dependent pathway (Bonora et al., 2014) and predominantly results into necroptotic cell death via the RIPK/MLKL pathway (Ofengeim et al., 2015). These earlier observations acquire enhanced significance from our finding that inhibition of RIPK by 7N-1 abolishes Drp1 activation. In response to TNF- α , we also observed an increased interaction between Drp1 and PGAM5, consistent with previous work (Wang et al., 2012), and this interaction is blocked by the addition of 7N-1. Though the role of PGAM5 in PIRK/MLKL-related necroptosis is controversial, our results support its role by showing that Drp1/PGAM5 forms a complex downstream of RIPK in response to TNF- α -mediated necroptosis. Thus, we would propose that inhibition of Drp1 by P110 treatment blocks the signal transduction of Drp1-dependent necroptosis, which in turn reduces the demyelinating lesions in the two mouse models studied and, by extension, potentially in MS as well.

From the point of view of MS it is significant that a link between Drp1 activation and inflammation has recently been proposed. In response to RNA viral infection, translocation of Drp1 to the mitochondria triggers mitochondrial damage and activation of the NLPR3 inflammasome (Wang et al., 2014), demonstrating a direct link between Drp1 activation and inflammation. Inhibition of Drp1 by either genetic manipulation or pharmacological reagents has been shown to suppress the production of pro-inflammatory mediators in microglial cells exposed to LPS (Park et al., 2013), reduce mitochondrial fragmentation in primary astrocytes during an inflammatory response (Motori et al., 2013), and attenuate acute liver inflammation (Kang et al., 2015). In MS, inflammation is generally viewed as the most proximal cause of the demyelination, axonal impairment and neuronal damage (Rieckmann and Maurer, 2002). In our current study, inhibition of Drp1 hyperactivation by P110 treatment reduced the number of active microglial cells and astrocytes in both EAE and cuprizone-induced MS mouse models. Thus, the efficacy of P110 may result in part from its effects on the innate immune response, an important argument for its exploration as a treatment for MS.

While these anti-inflammatory effects are significant, we feel that it is noteworthy that the protection provided by P110 administration was in large part independent of the suppression of T-cell infiltration. This is seen most clearly in the EAE mouse model. What our findings show is that to a great extent the primary effect of P110 is directly on the OLs themselves. Part of the evidence for this comes from our observation of a P110-induced protection in the cuprizone model. Cuprizone attacks the mature oligodendrocytes directly; and while it stimulates a local response from the innate immune cells of the brain (microglia and astrocytes), it does not engage the peripheral immune system. The strong suggestion is that P110 has potential not only as an effective MS therapy in its own right, but, because its mechanism of action is different from current immunomodulatory approaches, it could potentially form a part of a combination therapy that attacked MS from two independent angles. Taken together, our data suggest that inhibition of Drp1 protects against oligodendrocyte cell death, and attenuates demyelination and subsequent brain inflammation – all pathological hallmarks of MS. We further show that treatment with a Drp1 peptide inhibitor prevented functional impairment in two different MS mouse models. Because of its potential to be used in combination with current established immunomodulatory approaches,

a better understanding the role of Drp1 in mediating MS lesions may help to halt or slow the progression of MS and other demyelinating diseases.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Drp1 hyperactivation in EAE- and cuprizone-induced demyelination mouse models. (A) Mice were immunized with MOG₃₅₋₅₅ and total protein lysates of mouse spinal cords were harvested at 16 and 29 days following EAE induction. Drp1 phosphorylation was analyzed by Western blot (WB) analysis with anti-p-Drp1 S616 or anti-p-Drp1 S637 antibodies. Quantitative analysis of protein expression levels was performed by intensity measurement of Drp1, p-Drp1 Ser616 and p-Drp1 Ser637 in contrast to β -actin. (B) Representative images of Drp1 and DAPI immunostaining in the spinal cords of control mice and EAE mice (16 days post-immunization). Scale bar: 100 μ m. (C) Double immunostaining of anti-p-Drp1 Ser616 with anti-CC1 (a marker of mature OLs, lower panel) or anti-NeuN (a marker of neuron, upper panel) in the spinal cord sections of EAE mice (16 days post-immunization) or control mice. Scale bar: 50 μ m. (D) Mitochondrial fractions were isolated from spinal cords of EAE mice after 16 or 29 days post-immunization or control mice. Drp1 mitochondrial level was analyzed by WB with anti-Drp1 antibodies. Quantitative analysis of protein amount was performed by intensity measurement of Drp1 in contrast to mitochondrial loading control VDAC. (E) After mice were fed with cuprizone for 1, 2 or 3 weeks, the corpus callosum of mice were harvested. The total protein levels of Drp1, p-Drp1 Ser637 and p-Drp1 Ser616 were examined by WB with the indicated antibodies. Actin was used as a loading control. (F) Mitochondria were isolated and Drp1 mitochondrial level was analyzed by WB with anti-Drp1 antibody. VDAC was used as a mitochondrial loading

control. All of data are expressed as mean $\pm$ SEM, n = 3 animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group. n.s (not significant) vs. control group.

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Fig. 2.

Increased Drp1 activation in cultured oligodendrocytes exposed to H₂O₂ or TNF-α. (A–D) Immunopurified primary OL cells were treated with either H₂O₂ (200 μM, A and C) or TNF-α (20 ng/ml, B and D). Mitochondrial fractions were isolated and Drp1 mitochondrial level was analyzed by WB at the indicated time points. Quantitative analysis of protein amount was performed by intensity measurement of Drp1 in contrast to mitochondrial loading control VDAC. Results are expressed as mean ± SEM of 3 independent experiments. (E) Cultured OLs were treated with either H₂O₂ (200 μM, 2 h) or TNF-α (20 ng/ml, 6 h). Cells were co-stained with anti-Tom20 and MBP antibodies. Mitochondrial morphology in cells immune-positive for anti-MBP antibody was analyzed by microscopy. The boxed areas were enlarged in the right panel. Scale bar = 50 μm. (F) The percentage of MBP⁺-cells with fragmented mitochondria versus total number of MBP⁺-cells was quantitated and expressed as mean ± SEM. At least 3 independent experiments were performed. At least 60 cells/group were counted by the observers blinded to experimental conditions. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. control group.

Fig. 3.

Inhibition of Drp1 hyperactivation by P110 enhances functional recovery and OL survival. (A) Mouse EAE clinical scoring (0 to 5) was used to score the severity of functional deficit of EAE animals. Enhanced functional recovery was assessed in the EAE animals received P110 treatment (1 mg/kg/day, i.p. injection) starting at day 0 (purple dot-line), 7 (orange dot-line) and 12 (black dot-line) after immunization of MOG₃₅₋₅₅, and vehicle-treated animals (green dot-line). $n = 9-12$ animals per group. EAE or control mice were treated with P110 or vehicle starting immediately after MOG₃₅₋₅₅ immunization. Spinal cords were harvested at 16 days after EAE immunization. (B) Drp1 mitochondrial level was analyzed by WB. Quantitative analysis of protein amount was performed by intensity measurement of Drp1 in contrast to mitochondrial loading control VDAC. $n = 3$ mice/group. (C) Representative images of Luxol Fast Blue (LFB) staining (upper), immunostaining of MBP (middle) and neurofilament (NeuF, lower) in the white matter of spinal cords in P110-treated or vehicle-treated EAE mice. Demyelination area was outlined by dash-line. Quantitation of demyelinated area showed in (D). $n = 3-5$ mice/group. (E) MBP expression was determined

by WB with anti-MBP antibodies. Quantification of the level of MBP normalized to β -actin is represented in the histogram; $n = 3-4$ animals per group. (F-H) EM and G-ratio analysis confirmed an increase in the number of myelinated axons and thicker myelination after P110 treatment. $n = 3$ animals per group. 10 images per animals and ~ 100 axons were quantified. $**p < 0.01$, $***p < 0.001$ vs. control group. $##p < 0.01$, $###p < 0.001$ vs. EAE group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Inhibition of Drp1 hyperactivation by P110 enhances OL survival and attenuates the activation of microglia and astrocyte. (A–F) EAE-lesioned spinal cord sections were immunostained with antibodies of Olig2 and CC1 (A), Iba1 and GFAP (D). The numbers of Olig2⁺ (B), CC1⁺ (C), Iba1⁺ (E) and GFAP⁺ (F) cells in the lesioned spinal cord were quantitated and shown. $n = 3$ mice per group. (G–H) Spleens of vehicle- and P110-treated mice were weighed and normalized to animal body weight. $n = 6–10$ animals per group. All of data are expressed as mean $\pm$ SEM; *** $p < 0.001$ vs. control group. ** $p < 0.01$ vs. EAE group. n.s (not significant) vs. EAE group. A, D: Scale bar = 100 μ m.

Fig. 5. P110 attenuates OL death and demyelination in cuprizone-treated animals. (A) Mice were fed with control- or 0.2% cuprizone-chow following treatment of P110 (1 mg/kg/day, i.p. injection) or vehicle. Motor performance was assessed by rotarod test. Significantly increased length of the time on rotarod was observed in the group of animals with P110 treatment starting at the time that cuprizone treatment began, only slightly increased the length of time in the group of animals with delayed P110 administration for 3 weeks after cuprizone treatment. $n = 6-8$ per group. Mice were treated with P110 or vehicle right after cuprizone feeding. 6 weeks after treatment, brain of mice were harvested. (B) Representative

images of Black Gold staining and immunostaining of MBP in lesioned corpus callosum after P110 treatment. (C) Quantification of demyelination score showed increased myelination in cuprizone demyelination corpus callosum after P110 treatment. $n = 3-6$ mice/group. (D) MBP protein level was analyzed by WB. Quantification of the level of MBP normalized to β -actin is shown; $n = 3$ animals per group. (E) Mitochondria were isolated from corpus callosum of mice and Drp1 mitochondrial level was analyzed by WB. Quantification of the level of Drp1 normalized to VDAC is shown. $n = 3$ mice per group. (F–H) Toluidine blue myelin staining and EM ultrastructure analysis showed increased myelin thickness in corpus callosum (F) and increased percentage of myelinated axons (G) after P110 treatment compared to vehicle-control. The thickness of myelinated axons was quantified by G-ratio. $n = 3$ animals/group. At least 100 axons were measured for G-ratio. $*p < 0.05$, $***p < 0.001$ vs. control group. $^{##}p < 0.01$, $^{###}p < 0.001$ vs. Cuprizone group.

Fig. 6.

P110 attenuates OL death and the activation of microglia and astrocyte. Immunostaining of Olig2⁺ and CC1⁺ antibodies. (A), Iba1⁺ microglia (D) and GFAP⁺ astrocyte (F). The numbers of Olig2⁺ (B), CC1⁺ (C), Iba1⁺ (E) and GFAP⁺ (G) cells/mm² in the corpus callosum were counted from at least 6–7 fields/section, n = 3 animals/group. All of data are mean ± EM; ****p* < 0.001 vs. control group. ###*p* < 0.001 vs. Cuprizone group. Scale bars in A, D, E = 100 μm.

Fig. 7.

P110 reduces mitochondria fragmentation and necroptosis in OLs exposed to H₂O₂ or TNF-α. Primary OL cells were treated with P110 (1 μM) or vehicle for 30 min followed by exposure of H₂O₂ (200 μM) or TNF-α (20 ng/ml). (A) Mitochondria were isolated after 2- or 6-h incubation of H₂O₂ or TNFα. Drp1 protein level was analyzed by WB. VDAC was used as a mitochondrial loading control. Three independent experiments were conducted. (B) Following 2 h H₂O₂ or 6 h TNF-α exposure, the OL cells were immunostained with anti-Tom20 and anti-MBP antibodies. The boxed area in each micrograph is enlarged in the right panel. Scale bar: 50 μm. (C) The percentage of MBP⁺ OL cells with fragmented mitochondria versus total number of MBP⁺ OLs is quantitated. At least 60 cells/group were

counted by the observer blinded to the experimental conditions. Three independent experiments were performed. (D) Cell viability was measured using MTT assay after H₂O₂ (24 h) or TNF- α (24 h) exposure. Data are presented as mean $\pm$ SEM of percentage relative to control; n = 8 wells per group and repeated three times. (E) Cell culture media were harvested after H₂O₂ or TNF- α incubation. The release of HMGB1 into the medium was analyzed by WB with anti-HMGB1 antibodies. (F) Lactate dehydrogenase (LDH) release was determined by the LDH assay kit. Three independent experiments were conducted. All of data are expressed as mean $\pm$ SEM. *** p < 0.001 vs. control cells; ## p < 0.01, ### p < 0.001 vs. cells exposed to H₂O₂. \$\$\$ p < 0.001 vs. cells exposed to TNF- α .

Fig. 8.

Drp1 activation is mediated by RIPK1/RIPK3/MLKL/PGAM5 signaling pathway. Cultured OLs were pre-treated for 30 min with 7N-1 (10 μ M) or vehicle followed by exposure to TNF- α (20 ng/ml) for 6 h. (A) Drp1 mitochondrial level was analyzed by WB. Quantitative analysis of protein amount was measured by intensity of Drp1 against to mitochondrial loading control VDAC. Data are mean $\pm$ SEM of three independent experiments. (B) OL cells were stained with anti-Tom20 (red) and anti-MBP (green) antibodies. The boxed area in each micrograph is enlarged in the right panel. Scale bar: 50 μ m. (C) Quantification of the percentage of fragmented mitochondria in MBP⁺ OL cells. Results are expressed as mean $\pm$ SEM of three independent experiments. At least 60 cells/group were counted by the observer blinded to experimental conditions. (D) Cell viability was determined by MTT assay. Data are presented as mean $\pm$ SEM of percentage relative to control; n = 8 wells per group and repeated three times. The release of HMGB1 into the medium (E) was analyzed by WB with anti-HMGB1 antibodies. Lactate dehydrogenase (LDH) release (F) was determined by the

LDH assay kit. (G) The total protein levels of p-Drp1 (Ser616), p-Drp1 (Ser637), Drp1, p-MLKL, MLKL were analyzed by WB with the indicated antibodies. (H) Quantification of the protein levels normalized to actin is represented in the histogram as mean $\pm$ SEM. Three independent experiments were performed. (I) Total protein lysates were subjected to immunoprecipitation with anti-Drp1 antibodies followed by immunoblotting with anti-PGAM5 antibodies. The shown blots were from three independent experiments. The 7N-1 lanes shown on each gel are for control purposes to show no effect on cells after treatment with 7N-1 alone. ** $p < 0.01$, *** $p < 0.001$ vs. control cells; ## $p < 0.01$, ### $p < 0.001$ vs. TNF α -treated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)