Optineurin defects cause TDP43-pathology with autophagic vacuolar formation

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ABSTRACT

We previously showed that optineurin (OPTN) mutations lead to the development of amyotrophic lateral sclerosis. The association between OPTN mutations and the pathogenesis of amyotrophic lateral sclerosis remains unclear. To investigate the mechanism underlying its pathogenesis, we generated Optn knockout mice. We evaluated histopathological observations of these mice and compared with those of OPTN-amyotrophic lateral sclerosis cases to investigate the mechanism underlying the pathogenesis of amyotrophic lateral sclerosis caused by OPTN mutations. The Optn (−/−) mice presented neuronal autophagic vacuoles immunopositive for charged multivesicular body protein 2b, one of the hallmarks of granulovacuolar degenerations, in the cytoplasm of spinal cord motor neurons at the age of 8 months and the OPTN-amyotrophic lateral sclerosis case with homozygous Q398X mutation. In addition, Optn (−/−) mice showed TAR-DNA binding protein 43/sequestosome1/p62-positive cytoplasmic inclusions and the clearance of nuclear TAR-DNA binding protein 43. The axonal degeneration of the sciatic nerves was observed in Optn (−/−) mice. However, we could not observe significant differences in survival time, body weight, and motor functions, at 24 months. Our findings suggest that homozygous OPTN deletion or mutations might result in autophagic dysfunction and TAR-DNA binding protein 43 mislocalization, thereby leading to neurodegeneration of motor neurons. These findings indicate that the Optn (−/−) mice recapitulate both common and specific pathogenesis of amyotrophic lateral sclerosis associated with autophagic abnormalities. Optn (−/−) mice could serve as a mouse model for the development of therapeutic strategies.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that primarily affects motor neurons in the motor cortex, brain stem, and spinal cord. Most ALS patients die or need artificial respiratory supports within 1–5 years of its onset. Most of them are sporadic, and only approximately 10% have a family history (Robberecht and Philips, 2013). They provided pathological findings regarding frontotemporal lobar degenerations (FTLD) (Neumann and Mackenzie, 2019). More than 20 genes, including superoxide dismutase 1 (SOD1) (Rosen et al., 1993), TAR-DNA binding protein 43 (TARDBP) (Sreedharan et al., 2008; Rutherford et al., 2008), fused in sarcoma/translocated in liposarcoma (FUS/TLS) (Kwiatkowski et al., 2009; Vance et al., 2009), optineurin (OPTN) (Maruyama et al., 2010), and chromosome 9 open reading frame

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72 (C9ORF72) (DeJesus-Hernandez et al., 2011; Renton et al., 2011), have been identified as causative genes for ALS. However, how these gene mutations lead to the pathogenesis of ALS remains unclear.

We previously reported OPTN mutations in ALS cases that are inherited in both recessive and dominant manners (Maruyama et al., 2010; Maruyama and Kawakami, 2013). Recessive inheritance cases involve deletion of the exon 5 or a Q398X nonsense mutation, whereas dominant inheritance cases have an E478G missense mutation. Based on our report, several studies have documented various mutations of OPTN in ALS cases (Del Bo et al., 2011; van Blitterswijk et al., 2012; Iida et al., 2012; Cirulli et al., 2015; Goldstein et al., 2016).

OPTN protein, consisting of 577 amino acids, was originally referred to as FIP-2 because it interacts with adenoviral E3-14.7 K protein (Maruyama and Kawakami, 2013; Li et al., 1998). OPTN protein has two coiled-coil domains separated by microtubule-associated protein 1 light chain 3-interacting region, a ubiquitin binding domain, and a zinc-finger domain at its C-terminus. Moreover, OPTN protein has multiple binding partners, including Rab8 and TANK-binding kinase 1, which bind to the N-terminus, and receptor interacting protein 1 kinase (RIPK1), huntingtin, and myosin VI, which bind to the C-terminus. The amino acid sequence of OPTN is similar to that of nuclear factor kappa B (NF-κB) essential modulator (NEMO), and OPTN protein competes with NEMO for binding to ubiquitinated RIPK1 (Zhu et al., 2007). In addition, OPTN acts as an adaptor protein for autophagic clearance of invading bacteria and damaged mitochondria (Wild et al., 2011; Wong and Holzbaur, 2014). As described above, OPTN protein has multi-functions in a wide variety of biochemical processes, suggesting that the OPTN protein defect leads to neurodegeneration through deregulated cellular processes.

TAR-DNA binding protein 43 (TDP43)-positive inclusion observed in the central nervous system of ALS and FTLD patients is considered a hallmark of both disorders (Neumann et al., 2006; Arai et al., 2006). Pathological examination of ALS cases harboring OPTN mutations has
revealed TDP43-positive deposits in the neuronal and glial cytoplasm in neural tissue (Ito et al., 2011; Kamada et al., 2014). In addition, the aggregates of the microtubule-associated protein tau and alpha-synuclein were observed in OPTN-ALS cases (Yamaguchi et al., 2019; Ayaki et al., 2018). These observations suggest that OPTN-ALS might have the same mechanism as FTLD-TDP and FTLD-tau neurodegeneration.

In this study, to address the mechanism underlying ALS pathogenesis, we generated Optn knockout (KO) mice. As a result of extensive examination, we found pathological changes, including autophagic vacuoles in spinal cord motor neurons in Optn (−/−) mice and the ALS case with homozygous OPTN mutation. Optn (−/−) mice also showed axonal degeneration of sciatic nerves. These findings indicate that homozygous OPTN mutations have both common and specific pathogenesis causing ALS.

2. Materials and methods

2.1. Ethics statement

All experiments using animals and human materials were conducted according to the guidelines of Hiroshima University and approved by the Hiroshima University Animal Research Committee and Institutional Animal Care and Use Committee of RIKEN Kobe Branch for the care and use of experimental animals.

2.2. Human materials and animals

The Optn KO mouse (Accession No. CDB0949K: (LARGE, RIKEN Center for Life Science Technologies: The RIKEN Center for Life Science Technologies Resister. http://www2.clst.riken.jp/arug/mutant%20mice%20list.html) was established as follows. The Optn-targeting vector was constructed by replacing the exon 8–10 of the Optn gene with a neomycin cassette (Neo) flanked byloxP sites (Fig. 1a). After electroporation of the targeting vector into TT2 embryonic stem cells derived from F1 embryos by crossing male CBA with female C57BL/6 (Yagi et al., 1993), neomycin-resistant colonies were picked up and screened for proper recombinants by Southern blotting. Two independent clones were used to microinject into eight-cell-stage embryos of ICR mice, and two independent clones were used to microinject into eight-cell-stage embryos of ICR mice, and the ALS chimeric offspring were mated with wild-type (WT) C57BL/6 mice. Germline transmission was confirmed by polymerase chain reaction (PCR) using tail DNA. The following primers were used to detect the WT and Optn KO alleles: 5′-AGTCTTGGCGAATACTACTAC-3′ (forward common, P1), 5′-AGGGTGTTAATGCCAAGTACAGC-3′ (reverse WT allele, P2), and 5′-GTGCCCAGTCATAGCCGAATAGC-3′ (reverse Optn KO allele, P3). The expected sizes of the PCR products were 526 and 870 bp, and the genotyping of the Optn (−/−) was performed by PCR using mouse tail DNA as a template (Fig. 1b). The absence of Optn protein in the mutant mice was confirmed by immunoblotting of the lumbar spinal cords (Fig. 1c). Male and female heterozygotes from at least five back-crossings were used to obtain littermates of the male WT and male Optn KO offspring used in this study.

Brain tissues were obtained from two autopsy ALS patients. Patient 1, who died at the age of 52, had the homozygous OPTN variant (p.Q398X) (Kamada et al., 2014). Patient 2, who died at the age of 81, had the heterozygous OPTN variant (p.E478G) (Ito et al., 2011). Their postmortem examinations were performed at the Department of Neurology, Kyoto University Graduate School of Medicine, and they were assessed according to Braak staging of neurofibrillary tangles (NFT) and the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) plaque score (Yamaguchi et al., 2019; Ayaki et al., 2018).

2.3. Behavior analysis

To test motor functions, mice were tested with a rotarod device (MK-610A, Muromachi Kikai, Tokyo, Japan) and a grip strength meter (1027SM, Bio Research Center Co. Ltd., Nagoya, Japan). The rotarod was initially run at 4 rpm and was accelerated to 40 rpm after 300 s. Four trials of this test were performed per day, and the interval time between trials was more than 20 min. Five trials of the test for grip strength were conducted per day.

2.4. Histology and immunohistochemistry

Mice were deeply anesthetized with pentobarbital (75 mg/kg, intraperitoneally) and perfused with 4% paraformaldehyde in phosphate buffer. The brain, spinal cord, and sciatic nerve from the mid-thigh region were postfixed in the same solution overnight and for 4 h, respectively. Dissected tissue was cryoprotected in sucrose solution for histological and immunohistochemical analysis, and 10-μm-thick frozen sections were made with cryostat. Moreover, 8-μm-thick paraffin-embedded sections were made from OPTN-ALS autopsy cases.

For Klüver-Barrera staining visualizing myelin, the frozen sections and deparaffinized sections were washed in double distilled water (DDW). Washed sections were subjected in 0.1% luxol fast blue solution overnight at 50 °C. Next, after washing sections in DDW, these sections were de-colored in 0.05% lithium carbonate solution and 70% ethanol. After washing in DDW, sections were subjected in 0.1% cresyl violet solution for 10 min at 37 °C. These sections were de-colored in 95% ethanol until only nuclei and Nissl bodies are blue purple before dehydration and coverslipping.

The frozen sections and deparaffinized sections were immersed in 5% normal swine serum in phosphate-buffered saline (PBS) for 1 h and then immersed with primary antibodies in PBS overnight at 4 °C. Antibody binding was visualized by horseradish peroxidase (HRP)-labeled goat anti-rabbit antibodies (1:100, both DAKO, Glostrup, Denmark) for 30 min at room temperature. The sections were incubated at room temperature with 3,3′-diaminobenzidine (DAB; DAKO) after washing three times in PBS and then counterstained with hematoxylin to visualize the localization of epitopes.

To count the number of motor neurons, every 10th section of the lumbar spinal cord was immunostained for ChAT, and a total of 12 sections were used for counting. After primary antibody reaction, the sections were incubated with the appropriate biotinylated secondary antibody (1:500, Vector Laboratories, Inc.). For avidin-biotin complex formation, a VECTASTAIN Elite ABC Kit (Vector Laboratories, Inc.) was used. For the peroxidase-chromogen reaction, the sections were stained with DAB and then counterstained with hematoxylin.

The primary antibodies used in this study were as follows: rabbit anti-TDP43 (10782-2-AP; 1:1000, Proteintech Group, Inc., Rosemont, IL, USA), goat anti-choline acetyltransferase (ChAT; BA144P, 1:200, EMD Millipore Corporation, Billerica, MA, USA), rabbit anti-seques somatic 1/p62 (p62; ab91526, 1:500, Abcam, Cambridge, UK), rabbit anti-ubiquitin (Ub; EPR8830, 1:200, Abcam, Cambridge, UK), and rabbit anti-charged multivesicular body protein 2B (CHMP2B; ab33174, 1:600, Abcam, Cambridge, UK).

The immunohistochemically stained sections and hematoxylin-eosin staining sections were examined and digitized with a Nikon digital charge-coupled device camera (DS-Ri1) mounted on a Nikon E1000M microscope.

The sciatic nerves of WT and Optn (−/−) mice were fixed using 2.5% glutaraldehyde, washed in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide, and embedded in TAAB epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (60 nm) were cut with a Reichert Ultracut-S microtome and visualized with Toluidine-blue staining. To evaluate the axonal diameter of sciatic nerves, 10 serial sections were digitalized using a Nikon digital charge-coupled device camera (DS-Ri1) mounted on a Nikon E1000M microscope.

The digital image processing and analysis were performed using ZEN (Carl Zeiss AG), Image J (Rasband, 1997–2018; Schneider et al., 2012), and Adobe Photoshop CS 5.1 (Adobe Systems Incorporated, San Jose, CA, USA).
Fig. 2. The number of motor neurons in the Optn (−/−) spinal cord decreased from the age of 6 months. (a) Immunohistochemical staining of the WT (left) and Optn (−/−) (right) mice with an antibody against ChAT. (b) Quantitative analysis of the anterior horn motor neurons of the lumbar spinal cord showed that the number of ChAT-positive anterior horn neurons in the WT mice (n = 5) was significantly lower than that in Optn (−/−) mice (n = 5) at the age of 6 months, though there was no significant difference between the WT and Optn (−/−) mice at the age of 3 months. In addition, the number of ChAT-positive neurons decreased significantly at the age of 24 months. (c) All anterior horn motor neurons of the lumbar spinal cord also had the same tendency of that of ChAT-positive neurons in the WT mice (n = 5) and Optn (−/−) mice (n = 5). The number of all anterior horn neurons decreased as significantly at the age of 24 months as that of ChAT-positive neurons. (d) Lumbar spinal cord sections were stained with Klüver-Barrera stain. The white matter of the spinal cords of the WT and Optn (−/−) mice at the age of 24 months. Scale bars = a, 100 μm; c, 500 μm. Error bars = standard error of the mean (SEM). (*p < 0.02; **p < 0.01; n.s., not significant).
2.5. Statistical analysis

The data analysis, including Student’s t-tests of unpaired data, was performed with Prism 6 software (GraphPad Software, La Jolla, CA). p-Values less than 0.05 were considered statistically significant.

3. Results

3.1. Anterior horn neurons of the lumbar spinal cord in Optn (−/−) mice decreased at the age of 6 months

We first examined whether anterior horn neurons were lost in the Optn (−/−) mice by counting ChAT-positive neurons at the lumbar spinal cord (Fig. 2a). At the age of 3 months, there was no significant difference between WT and Optn (−/−) mice. At the age of 6 months, the number of ChAT-positive neurons in Optn (−/−) mice was significantly lower than that of WT mice (p < 0.02). This difference between WT and Optn (−/−) mice persisted afterward. The number of ChAT-positive neurons in Optn (−/−) mice at the age of 24 months decreased to about a half of that of WT and Optn (−/−) mice at the age of 3 months (Fig. 2b). The number of all anterior horn neurons showed the same tendency of that of ChAT-positive neurons (Fig. 2c). In contrast, Klüver-Barrera staining did not show any obvious changes in the ventral parts of the white matter of the spinal cord in WT and Optn (−/−) mice until they were 24 months old (Fig. 2d).

We also examined the hippocampus, cortex, and optic nerve in Optn (−/−) mice.
 Welch number of neurons in the CA3 region of the hippocampus (Fig. 3a). Statistical analysis showed no significant differences in the

T. Kurashige et al. WT: ± 22.5/mm² and the layer V of the cortex (p > 0.999, Welch’s t-test, WT: n = 5, 648.6 ± 72.0/mm², Optn (−/−): n = 5, 651.5 ± 92.7/mm²) between WT and Optn (−/−) mice. (Fig. 3b). Similarly, although histopathological changes appeared at 6 months of age, Optn (−/−) mice did not display any behavioral phenotypes until the age of 24 months. Kaplan-Meier survival curves of the WT and Optn (−/−) mice (p = 0.73, log-rank test, WT: n = 11, 658.0 ± 45.9 days, Optn (−/−): n = 10, 688.0 ± 5.6 days) (Fig. 4a). At the age of 24 month. The WT and Optn (−/−) mice did not show any difference in their body weights (t-test, WT: n = 6, Optn (−/−): n = 7) (Fig. 4b). There were no differences in their performances measured by the rotarod test (p = 0.588, Welch’s t-test, WT: n = 6, Optn (−/−): n = 6) (Fig. 4c). The mean grip strengths of (d) the forelimbs and (e) four limbs in the 22-24-month-old WT and Optn (−/−) mice (p: p = 0.2135, f: p = 0.764, t-test, WT: n = 8, Optn (−/−): n = 7). Error bars = standard error of the mean (SEM).

3.2. Cytoplasmic vacuoles appear in the Optn (−/−) mice and the OPTN-ALS case with the homozygous OPTN mutation

Lumbar spinal cord sections were stained by hematoxylin-eosin staining (Fig. 5a). Cytoplasmic vacuoles were observed in neurons of Optn (−/−) mice from 8 to 24 months of age, whereas they were not observed in WT mice until the age of 24 months. Lumbar spinal cord sections were also immunostained with an antibody against CHMP2B, which was already known as a granulovacuolar degeneration marker (Yamazaki et al., 2010; Yamazaki et al., 2011), in the WT mice and Optn (−/−) mice (Fig. 5b). The rims of cytoplasmic vacuoles observed by hematoxylin-eosin staining were immunopositive for CHMP2B, which meant that these vacuoles were formed by the abnormal autophagosomes.

In addition, we performed histopathological examinations of OPTN-ALS cases. Their tau-pathologies were evaluated as Braak NFT stage III-IV, and the distributions of amyloid beta were almost none (CERAD plaque score 0) (Ito et al., 2011; Kamada et al., 2014; Yamaguchi et al., 2019; Ayaki et al., 2018). Interestingly, in patient 1, who exhibited homozygous p.Q398X mutation of OPTN, neurons in anterior horn neurons of the cervical spinal cord and the hippocampus showed CHMP2B-positive cytoplasmic vacuoles (Fig. 6a). In contrast, there were no vacuoles except for in the CA1 region of the hippocampus in patient 2, who exhibited heterozygous p.E478G mutation of OPTN (Fig. 6b).

3.3. Nuclear TDP43 loss and the TDP43/p62-positive cytoplasmic inclusions in the lower motor neurons of the Optn (−/−) mice

The nucleus of lumbar spinal cord neurons was immunopositive for TDP43 in WT mice until the age of 24 months and Optn (−/−) mice at the age of 4 months (Fig. 7a, b). At the age of 8 months, Optn (−/−) mice presented cytoplasmic vacuoles with TDP43-positive inclusions in spinal cord neurons (Fig. 7c). At the age of 14 months, TDP43 immunoreactivities disappeared in the nucleus of all anterior lumbar spinal cord neurons of Optn (−/−) mice (Fig. 7d). At the age of 18 months and later, Optn (−/−) mice showed an increase of TDP43-positive cytoplasmic inclusions in the anterior horn neurons (Fig. 7e, f). The number of neurons with TDP43-positive aggregates (Fig. 8a) and the proportion of these neurons to all neurons in ventral horns (Fig. 8b) increased at the age of 8 months and later (p < 0.01).

Ubiquitin and p62 immunostaining did not show any cytoplasmic aggregates in lumbar spinal cord neurons in WT mice until the age of 24 months, whereas this was the case in Optn (−/−) mice only at the age of 4 months. (Fig. 7g, h). At the age of 8 months, Optn (−/−) mice presented p62-positive aggregates in spinal cord neurons (Fig. 7i) but did not have any vacuoles with ubiquitin-positive aggregates (Fig. 7j). In Optn (−/−) mice, cytoplasmic vacuoles appeared with ubiquitin-positive aggregates in the cytoplasm at the age of 14 months (Fig. 7p).
Fig. 5. Cytoplasmic vacuolar formations in neurons of the Optn (−/−) mice. (a) Lumbar spinal cord sections were stained by hematoxylin-eosin staining. Cytoplasmic vacuoles (arrow) were observed in neurons of Optn (−/−) mice from the age of 8 months. (b) Lumbar spinal cord sections were immunostained with an antibody against CHMP2B in the WT mice and Optn (−/−) mice. The rims of cytoplasmic vacuoles were immunopositive for CHMP2B (arrow). (c) The number of neurons with cytoplasmic vacuoles significantly increased to three per section in the lumbar spinal cord neuron of Optn (−/−) mice at 8 months of age. After the age of 8 months, cytoplasmic vacuoles were observed in about four neurons per section in Optn (−/−) mice. (d) The ratio of neurons containing cytoplasmic vacuoles in Optn (−/−) mice increased at the age of 8 months. Scale bars = a. 100 μm (left column), 20 μm (right column), b. 20 μm. Error bars = standard error of the mean (SEM). (*p < 0.01).
Fig. 6. Cytoplasmic vacuolar formations appeared in the OPTN-ALS case with the homozygous OPTN mutation.

Cytoplasmic vacuolar formations described by hematoxylin-eosin staining and CHMP2B immunostaining in the OPTN-ALS cases. (a) In the OPTN-ALS case with homozygous Q398X mutation, neuronal cytoplasmic vacuoles that were similar to granulovacuolar degenerations were observed in the spinal cord and the hippocampus. (b) In the OPTN-ALS case with heterozygous E478G mutation, the distribution of neuronal cytoplasmic vacuoles was limited in the hippocampus. Scale bars = 20 μm.
(caption on next page)
At the age of 14 months and later, Optn (−/−) mice showed an increase of p62- and ubiquitin-positive cytoplasmic inclusions in the anterior horn neurons (Fig. 7j–l, p–r). The number of neurons with p62-positive aggregates (Fig. 8c) and the proportion of these neurons to all neurons in ventral horns (Fig. 8d) also increased at the age of 8 months and later (p < 0.01). The number of neurons with Ub-positive aggregates (Fig. 8e) and the percentage of these neurons in all ventral horn neurons (Fig. 8f) increased at the age of 14 months and later (p < 0.01).

3.4. The degeneration and inclusion formation of the sciatic nerve of Optn (−/−) mice preceded inclusion appearance in the spinal cord

At the age of 24 months, most of the myelinated nerve fibers were maintained and clusters of small myelinated fibers were preserved in both the WT mice and Optn (−/−) mice (Fig. 9a). However, statistical analysis revealed that the Optn (−/−) mice showed a decrease in the density of large myelinated nerve fibers and an increase in the density of small myelinated nerve fibers (Fig. 9b).

4. Discussion

We previously reported on mutations of the OPTN gene in human ALS (8), but the mechanism underlying its pathogenesis is largely unknown. In this study, we established and examined Optn (−/−) mice to understand the molecular mechanism of neurodegeneration. Optn (−/−) mice presented the formation of cytoplasmic TDP43-positive inclusions in the lumbar spinal cord neurons at the age of 8 months and the clearance of nuclear TDP43 at the age of 14 months. The cytoplasmic TDP43-positive inclusions and the clearance of nuclear TDP43 are hallmarks of human ALS cases presenting FTLD-TDP pathology. The OPTN-ALS model mice harboring VCP R155H mutation also had cytoplasmic enlarged vacuoles in the anterior horn neurons, which suggested that primary defects in UPS/autophagy pathways (as may occur with VCP, UBQLN2, SQSTM1, or optineurin mutations) may similarly promote aggregate formation (Yin et al., 2012). Our results suggest that the autophagic dysfunction might be a mechanism of OPTN-ALS preceding the FTLD-TDP pathology.

Because it has been reported that autophagosomes are generated in the distal axons (Maday and Holzbaur, 2014; Maday et al., 2012), we evaluated the sciatic nerves of WT and Optn (−/−) mice. Optn (−/−) mice showed a decrease of large myelinated nerve fibers of the sciatic nerve but did not present pathological findings associated with the demyelination. This finding was similar to the previously reported axonal degeneration of the lateral column of the lumbar spinal cord (Ito et al., 2016). In addition, we could not find changes in the white matter of the lumbar spinal cord using Klüver-Barrera staining, which indicates that demyelinating changes were absent in the lateral column. This finding is consistent with those regarding the sciatic nerve in this study and those regarding the lateral column reported previously (Ito et al., 2016).

We could not see any previously reported phenotypes of Optn (−/−) (Ito et al., 2016). The number of anterior horn neurons in Optn (−/−) mice was comparable to that in WT mice at the age of 3 months. However, we observed a reduced number of motor neurons in Optn (−/−) mice compared to WT mice from 6 to 24 months of age. However, the phenotype of Optn (−/−) mice was spared, and Optn (−/−) mice did not show any obvious motor dysfunction, presumably because the number of motor neurons present in 24-month-old mutant mice is still more than half the number present in 3-month-old WT mice. We speculate that the half number of motor neurons are sufficient to support motor function, because it is thought that in human ALS cases, more than half of the motor neurons are lost by the time the symptoms become noticeable.

In this study, we reported a pathological mouse model of human homozygous OPTN-ALS. We found pathological changes, including autophagic vacuoles and TDP43 pathology, in spinal cord motor neurons in Optn (−/−) mice and the ALS case with homzygous OPTN mutation, although we could not describe any phenotypes of Optn (−/−) mice. Optn (−/−) mice also showed axonal degeneration of sciatic nerves. These findings indicate that homzygous OPTN mutations have the specific pathogenesis.

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Fig. 8. Statistical analysis of the anterior horn neurons stained by TDP43 (upper), p62 (middle), and ubiquitin (lower).

(a) The number of neurons containing TDP43-positive aggregates and vacuoles significantly increased to above two per section in the lumbar spinal cord neuron of Optn (-/-) mice at 8 months of age. After the age of 8 months, cytoplasmic vacuoles were observed in about four neurons per section in Optn (-/-) mice. (b) The proportion of neurons containing TDP43-positive aggregates and cytoplasmic vacuoles to all anterior horn neurons in Optn (-/-) mice increased at the age of 8 months. (c) The number of neurons containing p62-positive aggregates and vacuoles significantly increased to above two per section in the lumbar spinal cord neuron of Optn (-/-) mice at 14 months of age. After the age of 14 months, cytoplasmic vacuoles were observed in about four neurons per section in Optn (-/-) mice. (d) The ratio of neurons containing p62-positive cytoplasmic aggregates and vacuoles to all anterior horn neurons in Optn (-/-) mice statistically increased at the age of 14 months. (e) The number of neurons containing ubiquitin-positive aggregates and vacuoles significantly increased to about 1.5 per section in the lumbar spinal cord neuron of Optn (-/-) mice at 14 months of age. After the age of 14 months, cytoplasmic vacuoles were observed in about 2.5 neurons per section in Optn (-/-) mice. (f) The percentage of neurons containing ubiquitin-positive cytoplasmic aggregates and vacuoles to all neurons in the anterior horn of Optn (-/-) mice statistically increased at the age of 14 months. Error bars = standard error of the mean (SEM). (*p < 0.01).
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Declaration of competing interest

The authors have no conflict of interest directly relevant to the content of this article.

Fig. 9. Large myelinated nerve fibers decreased and small myelinated nerve fibers increased in sciatic nerves. (a) On epon-embedded and toluidine-blue-stained sciatic nerve sections, most of the myelinated nerve fibers were maintained and a cluster of small myelinated fibers were seen in WT and Optn (−/−) mice. Naked fibers and onion bulb formations were not observed in either WT or Optn (−/−) mice. (b) Statistical analysis revealed that the density of large myelinated fibers in Optn (−/−) mice was lower than in WT mice and that the density of small myelinated fibers in Optn (−/−) was higher than in WT mice. Scale bars = 50 μm (upper), 20 μm (lower). Error bars = standard error of the mean (SEM). (p < 0.01).

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