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Myelinating glia-specific deletion of Fbxo7 in mice triggers axonal degeneration in the central nervous system together with peripheral neuropathy

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Abstract

Myelination of axons facilitates the rapid propagation of electrical signals and the long-term integrity of axons. The ubiquitin-proteasome system (UPS) is essential for proper protein homeostasis, which is particularly crucial for interactions of post-mitotic cells. In our study, we examined how the E3 ubiquitin ligase FBXO7-SCF (SKP1, Cul1, F-box protein) expressed in myelinating cells affects the axon-myelin unit. Deletion of Fbxo7 in oligodendrocytes and Schwann cells in mice using the Cnp1-Cre driver line led to motor impairment due to hind limb paresis. It did not result in apoptosis of myelinating cells, nor did it affect the proper myelination of axons or lead to demyelination. It however triggered axonal degeneration in the central nervous system (CNS) and resulted in the severe degeneration of axons in the peripheral nervous system (PNS), inducing a full-blown neuropathy. Both the CNS and PNS displayed inflammation, while the PNS was also characterized by fibrosis, massive infiltration of macrophages, and edema. Tamoxifen-induced deletion of Fbxo7 after myelination using the Plp1-CreERT2 line, led to a small number of degenerated axons and hence a very mild peripheral neuropathy. Interestingly, loss of Fbxo7 also resulted in reduced proteasome activity in Schwann cells but not in cerebellar granule neurons, indicating a specific sensitivity of the former cell type. Taken together our results demonstrate an essential role for FBXO7 in myelinating cells to support associated axons, which is fundamental to the proper developmental establishment and the long-term integrity of the axon-myelin unit.
The myelination of axons facilitates the fast propagation of electrical signals and the trophic support of the myelin-axon unit. Here, we report that deletion of $Fbxo7$ in myelinating cells in mice triggered motor impairment, but had no effect on myelin biogenesis. Loss of $Fbxo7$ in myelinating glia however led to axonal degeneration in the central nervous system and peripheral neuropathy of the axonal type. In addition, we found that Schwann cells were particularly sensitive to $Fbxo7$ deficiency reflected by reduced proteasome activity. Based on these findings, we conclude that $Fbxo7$ is essential for the support of the axon-myelin unit and long-term axonal health.
Introduction

Oligodendrocytes and Schwann cells are the myelinating cells of the central and peripheral nervous system, respectively. They engage in a unique cell-cell interaction with axons and facilitate the rapid propagation of action potentials. A new line of research also established the trophic support of axons by myelinating cells. This idea was sparked by mouse models that lack the major myelin proteins PLP (myelin proteolipid protein) or CNP (CNPase). Both mutant mouse lines show axonal pathology with little or no demyelination (Griffiths et al., 1998; Lappe-Siefke et al., 2003).

Mechanistic insight came from Fünfschilling and colleagues who demonstrated that absence of the mitochondrial complex IV protein COX10 in myelinating cells, and thus the abolishment of their respiration leads to a peripheral neuropathy but no considerable axonal damage in the brain (Funfschilling et al., 2012). These experiments indicate that oligodendrocytes provide an energy source to support axons, but Schwann cells apparently fail to do so. Lee et al., showed evidence that the monocarboxylate transporter MCT1, which is predominantly expressed by oligodendrocytes and responsible for lactate transport, may provide the metabolic connection as Mct1+/- mice display axonopathy without loss of myelin (Lee et al., 2012). Hence, the integrity of axons is critically dependent on the metabolic support of interacting myelinating cells.

In addition to energy support, it remains elusive if further mechanisms in myelinating cells are crucial for the proper support of the axon-myelin unit. There are indications that the ubiquitin proteasome system may play such a role: The Trembler mouse, expressing a mutant myelin protein PMP22, models Charcot Marie Tooth (CMT) disease type 1, which is characterized by hypomyelination and secondary axonal damage.
In this mouse however, Schwann cells show reduced proteasome activity and fail to degrade mutant, aggregation-prone PMP22 (Fortun et al., 2005; Fortun et al., 2006).

Peripheral neuropathy is also triggered by systemic proteasome inhibition using Bortezomib, a drug typically applied in cancer treatment of Multiple myeloma (Cavaletti and Jakubowiak, 2010). In a rat model exposed to Bortezomib, both damage to Schwann cells and axonal degeneration was observed (Cavaletti et al., 2007). This raises the question to which extent axons depend on the proper functioning of the UPS in myelinating cells?

We have recently identified the F-box protein FBXO7 as a regulator of proteasome activity. FBXO7-SCF (Skp1, Cul1, F-box protein) is a multisubunit E3 ubiquitin ligase that binds to the proteasome holoenzyme by directly interacting with the proteasomal subunit α2 (PSMA2), (Vingill et al., 2016). Here, FBXO7 supports the assembly of core particle and regulatory particles. Consequently, FBXO7 knockout mice show reduced proteasome activity in the brain (Vingill et al., 2016). In addition to FBXO7’s role as a proliferation factor in cancer cells (Laman et al., 2005), which is negligible in post-mitotic cells, FBXO7 is instrumental in the initiation of mitochondrial turnover, an event referred to as mitophagy (Burchell et al., 2013). Collectively, FBXO7 is biochemically connected with both mitochondria and proteasomes and the functioning of these multi-protein complexes are intimately intertwined (Joseph et al., 2018).

Deletion of Fbxo7 in neurons leads to a parkinsonism-related phenotype characterized by mild to severe motor impairment but no cell death or axonal pathology in mice (Vingill et al., 2016). Interestingly, FBXO7 shows also a prominent expression in white matter (Zhang et al., 2014; Zeisel et al., 2015; Vingill et al., 2016) and in Schwann
cells (this study). These findings prompted us to test the hypothesis that deletion of $Fbxo7$ from myelinating cells negatively affects proteasome activity and may affect the integrity of myelinating cells and potentially interfere with the axon-myelin interaction.

In this study, we show that deletion of $Fbxo7$ in myelinating cells causes motor impairment. Interestingly, in the central nervous system (CNS), $Fbxo7$ deletion does not affect myelination but causes mild axonal degeneration and inflammation. In the PNS, deletion of $Fbxo7$ in Schwann cells also does not impair myelination, but, strikingly, it induces a marked, progressive peripheral neuropathy of the axonal type.

**Materials and methods**

**Mouse experiments**

All mice used for this study had a pure C57BL/6N background. The Cnp1-Cre/+ and Plp1-CreERT2/+ mouse lines were strictly kept heterozygous. The mice were housed under standard 12h light-dark cycle with sufficient food and water in the mouse facility of the Max-Planck-Institute for Experimental Medicine in Göttingen or the mouse facility of the RWTH university hospital in Aachen. Experiments were performed according to the guidelines for German animal welfare and were approved by the “Landesamt für Verbraucherschutz und Lebensmittelsicherheit” of Lower Saxony, Germany and the “Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen”, Germany.

**Generation of conditional Fbxo7-knockout mouse lines**

The conventional Fbxo7 knockout mice line was generated as described before (Vingill et al., 2016). To establish new conditional mouse lines, homozygous $Fbxo7^{fl/fl}$ mice, in which exon 4 was flanked by loxP sites, were either mated with heterozygous Cnp1-Cre/+ mice (Lappe-Siefke et al., 2003) or with heterozygous Tamoxifen-inducible Plp1-
CreERT2/+ mice (Leone et al., 2003). In these conditional knockout mice, FBXO7 was deleted from myelinating cells. For further reference, Cnp1-Cre/+;Fbxo7fl/fl and Plp1-CreERT2/+;Fbxo7fl/fl mice are referred to as Cnp1-Cre;fl/fl and Plp1-CreERT2;fl/fl respectively. For the genotyping of male and female mice, following primers were used:

- LacZ cassette (fwd): 5’-attccagctgagcgccggtgc-3’,
- (rev): 5’-gctgtatgaaggaagtgctatt-3’
- Fbxo7 floxed (fwd): 5’-tcagcatgggtttaaacatctacta-3’,
- (rev): 5’-ggtctagatatctcgacataactcgtata-3’
- Fbxo7 wild type (fwd): 5’-gggctgtaggaaggtatgtctatt-3’,
- (rev): 5’-ccctgaggaaggtgtctttgc-3’

- Cre allele (fwd): 5’-caggggaggttaacatccc-3’,
- (rev): 5’-cctgaaatgetttgttccg-3’

Mice were euthanized at 4 months of age as they reached the underweight criteria (weight loss <20%) established in the animal protocol.

Tamoxifen-induced knock down of Fbxo7

In the inducible conditional Plp1-CreERT2;fl/fl mouse line, Cre recombinase expression is only activated upon Tamoxifen injection. Hence, 100μl of 20mg/ml Tamoxifen dissolved in corn oil was intraperitoneally injected in mice for 5 consecutive days. The total amount of injected Tamoxifen was 10mg. The mice were injected with Tamoxifen at two months of age, when myelination was completed. Control mice lacking the Plp1-CreERT2 insertion were also injected with the same amount of Tamoxifen.

Electrophysiological measurement
Electrophysiology was performed on 2 months old, male and female Cnp1-Cre;fl/fl and control mice. Mice were anaesthetized with ketamine hydrochloride/ xylazine hydrochloride (100mg/kg BW/ 8mg/kg BW (Medistar)). A pair of steel needle electrodes (Schuler Medizintechnik, Freiburg, Germany) was placed subcutaneously along the nerve at sciatic notch (proximal stimulation). A second pair of electrodes was placed along the tibial nerve above the ankle (distal stimulation). Supramaximal square wave pulses lasting 100ms were delivered using a Toennies Neuroscreen® (Jaeger, Hoechsberg, Germany). Compound muscle action potential (CMAP) was recorded from the intrinsic foot muscles using steel electrodes. Both amplitudes and latencies of CMAP were determined. The distance between the two sites of stimulation was measured alongside the skin surface with fully extended legs and nerve conduction velocities (NCVs) were calculated automatically from sciatic nerve latency measurements. The distal motor latency (DML) was measured from the delay between initiating a stimulus and the evoked potential. Statistical analyses were performed by applying the student’s t-test. The collaborating researchers were given the mice unaware of genotype to perform analyses in a blinded manner.

**Behavioral analyses**

Only male mice were used for behavioral analyses. Male Cnp1-Cre;fl/fl and control mice were tested at 6 weeks of age, whereas male Plp1-CreERT2;fl/fl and control mice were tested at 5, 7, and 9 months of age. All tests were conducted under standardized conditions, at fixed light intensity of 90 lux and protected from disturbance. Prior to each session, mice were allowed to acclimate for 30min in the test room. Afterwards the weight of the animals was determined and the behavioral tests were performed in a
distinct order: first elevated plus maze for assessing anxiety, then open field test for ambulation and exploratory behavior, followed by tail suspension test, inverted grid, pole test, wire hang, balance beam and Rotarod for motor endurance and coordination. For sensory assessment the hot plate test was performed. The animals were monitored using the software Viewer from Biobserve and obtained data were analyzed using Microsoft Excel and GraphPad Prism. The researcher tested the mice’s behavior in a blinded manner. No genotypes were written on cage cards. Ear tag numbers were matched with genotypes following the behavior tests and statistical analyses.

Elevated plus maze

In order to evaluate the anxiety of mice, an elevated plus maze test was conducted according to the published protocol (Pellow et al., 1985). Mice were placed in the center of the plus maze (5x30cm, 15cm wall height, 40cm elevated from ground) facing an open arm, and both activity and exploratory behavior were recorded for 5min with the Viewer software. The time spent in open and closed arms was analyzed, as intensive time spent in an open arm indicates reduced anxiety.

Open field

General locomotion and exploratory behavior was measured in an open field test, as previously demonstrated (Hall and Ballachey, 1932). The round arena had a diameter of 60cm and was surrounded by a 20cm high wall. The mice were placed in the center and movement was monitored for 7min with the Viewer animal tracking system. The travelled distance and time spent in different zones were analyzed.

Hind limb clasping test
To assess signs of neurological deficits and neurodegenerative disease progression, a modified form of the tail suspension test described by Steru et al. (Steru et al., 1985) was performed. Mice were lifted by the proximal tail for 10 seconds. The position of the hind limbs were scored from 0-3, with 3 representing normal, splayed hind limbs and 0 indicating strong clasping of hind paws. The average of 3 trials represents the final score. In-between repeated measurements a 30sec pause was given.

**Inverted grid**

Muscle strength was assessed by the inverted grid test. Therefore mice were placed onto the grid of their home cage and the grid was inverted. The time mice could hold on to the inverted grid, was measured with a cut-off time at 60sec. 3 repeated measurements were performed with a break of 1min in-between and the average of all measurements were calculated.

**Pole test**

The pole test has been previously described as an assessment for basal ganglia related motor function (Ogawa et al., 1985; Matsuura et al., 1997). Mice were placed onto a vertical pole (50cm long, 1cm diameter) facing upwards and had to turn around to climb down the pole. The time needed to reach the bottom of the pole was measured. Three trails with a 1min break were performed. The average of all trails was calculated.

**Wire hang**

Another test for muscle strength is the wire hang test, as previously described (Aartsma-Rus and van Putten, 2014). Mice were placed with their front paws in the middle of a wire (80cm long, 1mm diameter and 30cm above surface) suspended between two poles.
The time needed to reach one of the poles was measured. 3 repeats were performed with an interval of 1min and the average time was calculated.

**Balance beam**

For testing balance and coordination, a balance beam test was conducted as previously described (Luong et al., 2011). The mice were placed on a 6mm or a 12mm wide and 80cm long beam. The beam is located 50 cm above the ground, with a black box (15x15x15 cm) attached on one end. Once the mice were placed on the opposite end of the beam, time as well as numbers of slips were counted until the black box was reached. For each beam, 3 repetitions were performed, with 1min break in between and 10min resting time in-between beams. The average time required to cross the beam, as well as average number of slips were calculated.

**Rotarod**

While subjecting the mice to a Rotarod test, the motor coordination and endurance was tested. The accelerating protocol of the Rotarod (Ugo Basile) was set to 4 - 40 rpm in 3min. The average time spent on the rotating rod was measured and each mouse was tested 3 times, with a 10min break in between. The cut-off time was set for 5min. The test was performed at 3 time points (0h, 3h and 24h).

**Hot plate**

To assess sensory deficits, a hot plate test was conducted (Eddy and Leimbach, 1953). Mice were placed in a 1000ml round glass beaker on a 55°C metal plate. Once the animals showed any sign of irritation due to the heat, the time was stopped and the mice were immediately removed from the plate. A cut-off time of 30sec was set. 3 repeated
measurements were taken with a 10min break in between. The average time was calculated.

Transcardial perfusion and fixation

Mice were anesthetized by intraperitoneal injection of 10% Ketamine (CP Pharma) and 5% Xylazine (Medistar Arzneimittelvertrieb GmbH). The animals were then transcardially perfused with sterile PBS, followed by 4% PFA/PBS or Karlsson and Schultz buffer (Karlsson and Schultz, 1965; Schultz and Karlsson, 1965), prior to tissue electron microscopy analyses. The tissue of interest was dissected and incubated in 4% PFA/PBS/4% Sucrose or Karlsson and Schultz buffer over night at 4°C and then further processed, depending on the experimental procedure.

Immunohistochemistry of cryo-sections

The PFA-fixated samples of Cnp1-Cre;fl/fl mice and controls were incubated in 30% sucrose/PBS over night at 4°C, embedded it OCT (Tissue Tek, Sakura, Torrance, USA) and cut in 20µm thin cross sections (spinal cord) or longitudinal sections (sciatic nerve) using a cryostat (CM3050 S, Leica). For fluorescent staining, free-floating cryo-sections were washed twice with PBST, permeabilized with 0.5% Triton®X-100/PBS for 30min and blocked for 1h in blocking solution (10% goat serum, 2% BSA, 0.5% TX-100 in PBS). After washing the samples with PBST, they were incubated over night at 4°C in antibody solution (10% goat serum, 2% BSA in PBS) containing the respective dilution of the primary antibody. The samples were washed thrice with PBST and incubated for 1h at RT in a secondary antibody diluted in antibody solution and DAPI. The samples were washed three times with PBST. Finally, the sections were mounted onto a glass slide and mounted with Mowiol mounting medium (6g glycerol, 2.4g Mowiol 4-88, 6ml
H2O, 12ml 0.2M Tris-HCl pH 8.5, 25mg/ml DABCO). Pictures were taken using the Olympus BX51 and analyzed using ImageJ or Fiji (Schindelin et al., 2012; Schneider et al., 2012). The pictures were quantified using a customized macro written by Dr. Miso Mitkovski, which quantified the area stained in pixel, as well as the total area, in order to calculate the percentage of area stained. To minimize bias, several immunostained sections of each brain were blinded and subjected to analyses. For statistical analyses GraphPad Prism was used.

**Immunohistochemistry of paraffin-sections**

PFA-fixated brains of male Cnp1-Cre;fl/fl mice and controls were embedded in paraffin using the standard protocol. Brains were cut into 5µm sagittal sections using a microtome (HM 430, Thermo Fischer Scientific), mounted on glass slides and stained using the LSAB2 detection system. After deparaffinization, the sections were boiled in citrate buffer for antigen retrieval for 10min, cooled down and incubated in Tris buffer + 2% milk for 5 min. The slides were incubated for 5min in 3% H2O2 to inactivate endogenous peroxidase. After rinsing with Tris-buffer + milk, the sections were blocked in blocking buffer (20% goat serum in BSA/PBS) for 10min and incubated in primary antibody diluted in BSA/PBS at 4°C over night. The slides were rinsed with Tris buffer + milk and subjected to DAB staining using the LSAB2 kit (Dako). Following DAB staining, the sections were counter-stained with haematoxylin and Scott’s solution (Thermo Fisher Scientific). The sections were dehydrated and mounted using Eukitt mounting medium (Kindler). Images were taken with the Axiophot microscope (Zeiss) and pictures were analyzed using ImageJ and a custom-made macro provided by Dr. Miso Mitkovski. Here, the DAB-positive areas were converted into a distinct signal to measure the percentage of
DAB-positive area, with regard to the total area of interest. To minimize bias, several
stained sections of each brain were blinded and subjected to analyses. Graphs and
statistical analyses were carried out using GraphPad Prism.

**TUNEL assays**

Sagittal brain sections of 5μm thickness from Cnp1-Cre;fl/fl mice and controls were
stained for apoptotic cells using the DeadEnd™ Colorimetric TUNEL System kit
(Promega). The sections were mounted with Aqua-Poly/Mount (Polysciences) and
quantified with the same ImageJ macro by Dr. Mitkovski as described before.

**Gallyas silver impregnation**

Myelin fibers were stained using the Gallyas protocol (Gallyas, 1979). 5μm sagittal brain
sections of male Cnp-Cre;fl/fl and control mice were de-paraffinized and incubated in a
2:1 mixture of pyridine and acetic anhydride for 30min. The samples were rinsed thrice
with H2O and incubated in incubating solution (12.5mM NH₄NO₃, 5.9mM AgNO₃, 3mM
NaOH) for 10min. Slides were washed 3x with 0.5% acetic acid and incubated in
developing solution (50% of [472mM Na₂CO₃], 35% of [25mM NH₄NO₃, 11.8mM
AgNO₃, 3.5mM Silicotungstic acid], 15% of [25mM NH₄NO₃, 11.8mM AgNO₃, 3.5mM
Silicotungstic acid, 1.89% formaldehyde]) for 3-15min until the desired intensity of
staining was reached. The reaction was stopped by applying 1% acetic acid. The samples
were washed twice with H2O and incubated in 2% sodium thiosulfate solution for 5min.
After rinsing twice with water, the samples were dehydrated and mounted using Eukitt
(Kindler). Quantification was performed with ImageJ and statistical analyses were
conducted with GraphPad Prism.

**Electron microscopic analysis**
The optic and sciatic nerve of male and female Cnp1-Cre;fl/fl and control mice, respectively, as well as the sciatic nerve of male Plp1-CreERT2;fl/fl and control mice were dissected and post-fixed in Karlsson-Schulz fixative. The tissue was embedded in epon according to a modified method described by Luft et al. (Luft, 1961). Staining of semi-thin sections: The embedded samples were sectioned using a microtome (Ultracut S, Leica), while cutting with a diamond knife (Diatome Ultra 45°) semi-thin section of 500nm thickness. The semi-thin sections were stained using methylene blue and Azure II staining solution (1:1) for 1min at 60°C. Semi-thin sections were observed under a light microscope. Contrasting of ultra-thin sections: 50nm ultra-thin sections were cut. The samples were placed onto formvar-polyvinyl coated cupper grids (2mm-1mm, AGRA scientific) and contrasted with a 1% uranyl acetate solution and Reynolds lead citrate (Reynolds, 1963). Images were taken with the CM10 electron microscope by Philips combined with a wide-angle dual speed 2K-CCD camera Veleta by Olympus. ImageJ, Microsoft Excel and GraphPad Prism were used for further analyses. For g-ratio measurement, 100 axons of 10 images (3000x magnification) per animal were analyzed in a blinded manner using ImageJ. The g-ratio is defined by the inner to the outer diameter of the myelin sheath. The g-ratio was determined by measuring the outer circumference of the myelin sheath, as well as the inner circumference (c) from which the respective diameter (d) was derived (d=c/π). A baseline g-ratio value is approximately 0.77 in the central nervous system and 0.6 in the peripheral nervous system. Values higher indicate a hypomyelination, whereas smaller values indicate hypermyelination. For analyses, electron micrographs were blinded and pathological abnormalities were assessed.
Plasmids and antibodies

For generating short hairpin RNA, the pSUPER RNAi System was used. Target sequences against homologous sequences of human, mouse and rat were used:

- Fbxo7 shRNA #1 (functional): 5’-gaagagaccttgcttcata-3’
- Fbxo7 shRNA ctrl (non-functional): 5’-gaaactacgcatcttccgac-3’

Antibodies used for immunoblot analysis:
- ms α-pan 14-3-3 (Santa Cruz, sc-1657, 1:1000 dilution), ms α-FBXO7 (Santa Cruz, sc-271763, 1:50 dilution), ms α-GSTπ1 (BD Biosciences, 610719, 1:250 dilution), ms α-NF-M (Santa Cruz, sc-51683, 1:200 dilution),
- ms α-PLP (kindly provided by Prof. Dr. Mikael Simons, 1:50 dilution), ms α-Sox10 (Santa Cruz, sc-365692, 1:200 dilution), HRP-coupled secondary goat α-mouse IgG (Dianova, 113-035-146, 1:10000 dilution).

Antibodies used for immunohistochemistry:
- ms α-APP 6E10 (Biolegend, 803004, 1:200 dilution), ms α-GFAP (Nova Castra, NCL-GFAP-GA5, 1:200), ms α-GSTπ1 (BD Biosciences, 610719, 1:200 dilution), rb α-Iba1 (Wako, 019-19741, 1:1000 dilution), rb α-MBP (Cell Signaling, 78896T, 1:200 dilution), ms α-PLP (kindly provided by Prof. Dr. Mikael Simons, 1:000 dilution), Cy3/Alexa 555 coupled secondary antibodies goat α-mouse IgG and goat α-rabbit IgG (Dianova, 1:1000 dilution), Cy2/Alexa 488 coupled secondary antibodies goat α-mouse IgG and goat α-rabbit IgG (Dianova, 1:1000 dilution).

Quantitative real-time (RT) PCR

To measure the relative expression of different mRNA levels, first RNA was isolated from male Cnp-Cre; fl/fl and control cerebella using the TRIZOL reagent (Invitrogen). The respective cDNA was synthesized using the SuperScript III First-strand Synthesis
System (Invitrogen). A quantitative real-time PCR was performed using the Power SYBR
Green system and PCR thermo cycler (Thermo Fischer Scientific). Microsoft Excel was
used for analysis, applying the Livak-delta-delta CT method for quantification. β-Actin
was used as a reference gene, in addition to the average of all control samples. Following
primers were used for quantitative RT-PCR:

- IL-10 (fwd): 5’-atgcaggactttaagggttacttg-3’, (rev): 5’-tagacacctttgtcttgagacct-3’
- IL-1RA (fwd): 5’-cattccacctgggaaggt-3’, (rev): 5’-gacgggtgaggttaaagcg-3’
- IL-1β (fwd): 5’-cctgcagctggagatgtgga-3’, (rev): 5’-cccatacaggcaaggagga-3’
- IL-6 (fwd): 5’-cgagagagacttccagacagagga-3’, (rev): 5’-ggagagcatgggaatgtgggg-3’
- Tnf-α (fwd): 5’-gcgtgtcctatgtctcagcc-3’, (rev): 5’-tgaggacacgtactgggg-3’
- Ip-10 (fwd): 5’-ctgctccttctgtctgggtctg-3’, (rev): 5’-atagctcgcagggatgtccagccg-3’
- Mep1 (fwd): 5’-gtcctgtctgtctgggtcc-3’, (rev): 5’-gcacgggtgctccaaagaacctgtcg-3’
- β-Actin (fwd): 5’-ctgctgctgacatcaaaagagaacgtg-3’, (rev): 5’-ggatgccacagattccatccaaag-
  3’
- Fbxo7 (fwd): 5’-tggagaagtacatgttacat-3’, (rev): 5’-tactccacagcaacgtacag-3’

**Transduction of MSC80 cell line**

To generate a stable cell line expression of the Fbxo7 shRNAs, an adeno-associated viral
transduction was performed. HEK293T cells were transfected with the pLentiLox 3.7
system (pll3.7/fbxo7 puro, pVSVG, pREV-REV and pRRE), (Rubinson et al., 2003).
Three days after transfection, the supernatant, containing lentiviral particles, was
collected and added to cultured MSC80 cells for transduction. Transduced MSC80 cells
were treated with 0.75μg/ml puromycin for selection. Cells resistant to puromycin
incorporated the construct of interest and showed a stable expression of the Fbxo7 shRNAs. These cells were cultured and passaged like normal immortalized cell lines.

**Primary cell culture**

Primary Schwann cell and cerebellar granule neurons (CGN) were cultured from male and female postnatal day 5 (P5) mouse sciatic nerves and brachial plexus or cerebella as previously described (Dong et al., 1999; Holubowska et al., 2014).

**Proteasome activity assay**

The proteasome activity assay was carried out as described by Kisselev and Goldberg et al. (Kisselev and Goldberg, 2005). The assay was performed with lysates of transduced MSC80 cells, primary Schwann cells as well as primary cerebellar granule neurons (CGN). Cells were lysed in proteasome lysis buffer (50mM Tris-HCl pH 7.5, 250mM sucrose, 5mM MgCl\(_2\), 0.5mM EDTA, 2mM ATP, 1mM DTT, 0.025% Digitonin). 100\(\mu\)g BSA and 100\(\mu\)M of fluorogenic peptide substrate Suc-LLVY-AMC was added to the proteasome assay buffer (50mM Tris-HCl pH 7.5, 40mM KCl, 5mM MgCl\(_2\), 0.5mM ATP, 1mM DTT) and was incubated for 10 min at 37°C. 12\(\mu\)g of cell lysate was added per well. The fluorescent signal was measured at 355/460nm and 0, 30, 60, 90 and 120 min using the Tecan microplate reader. To verify the reduction of FBXO7 induced by shRNA or FBXO7 knockout, part of the cell lysate was set aside and subjected to western-blot analysis.

**Myelin purification**

Myelin was purified from female brain samples by density centrifugation using a sucrose gradient. According to Norton and Poduslo’s previously described method (Norton and Poduslo, 1973; Larocca and Norton, 2007) the samples were homogenized in 0.32M sucrose.
sucrose containing protease inhibitors and using the ultraturrax (IKA). The homogenate was carefully pipetted onto 0.85M sucrose, supplemented with protease inhibitors and transferred in an Ultra-Clear Tube (Beckman no. 344060). The sucrose gradient was centrifuged for 30min at 75000xg in the Beckman XL-70 ultracentrifuge, using a swing out rotor (SW40Ti Beckman rotor, 24400rpm). The interphase was transferred into a new tube and washed with water. After centrifugation at 75000xg for 15min, an osmotic shock was induced by resuspending the pellet in H_{2}O, incubated for 15min and centrifuged at 12000xg for 15min (SW40Ti Beckman rotor, 9700rpm). This osmotic shock procedure was repeated once. Subsequently, the pellet was resuspended in 0.32M sucrose and transferred onto 0.85M sucrose layer, which represents the second sucrose gradient. After centrifugation for 30min at 75000xg, the interphase was collected in a new tube, H_{2}O was added and centrifuged at 75000xg for 15min. This final pellet consisted of enriched myelin and was resuspended in 200μl of TBS containing protease inhibitors and was stored at -80°C.

For myelin purification of sciatic nerve isolated from male and female animals were homogenized in 0.29M sucrose containing protease inhibitors using an ultraturrax (IKA). The lysate was added onto 0.85M sucrose in an Ultra-Clear Tube (Beckman no. 344062). The samples were centrifuged for 30min at 75000xg (26900rpm) in a TH660 Beckman rotor. The interphase was washed with water as described before and only one osmotic shock procedure was carried out. The pellet was resuspended in 100μl of TBS containing protease inhibitors and stored at -80°C.

Label-Free Quantitative Mass spectrometry
Protein quantification: Proteolytic digestion. Protein fractions corresponding to 10 μg myelin protein were processed according to a filter-aided sample preparation (FASP) protocol modified essentially as described by Distler et al. (Distler et al., 2014; Distler et al., 2016). Myelin protein samples were lysed and reduced in lysis buffer (7 M urea, 2 M thiourea, 10 mM DTT, 0.1 M Tris pH 8.5) containing 1 % ASB-14 by shaking for 30 min at 37 °C. Subsequently, the sample was diluted with 10 volumes lysis buffer containing 2 % CHAPS to reduce the ASB-14 concentration and loaded on centrifugal filter units (30 kDa MWCO, Millipore). Unless stated otherwise, all steps were automated on a liquid-handling workstation equipped with a vacuum manifold (Freedom EVO 150, Tecan) by using an adaptor device constructed in-house. After removal of the detergents by washing twice with wash buffer (8 M urea, 10 mM DTT, 0.1 M Tris pH 8.5), proteins were alkylated with 50 mM iodoacetamide in 8 M urea/0.1 M Tris pH 8.5 (20 min at RT), followed by two washes with wash buffer to remove excess reagent. Buffer was exchanged by washing three times with 50 mM ammonium bicarbonate (ABC) containing 10 % acetonitrile. After three additional washes with 50 mM ABC/10 % acetonitrile, which were performed by centrifugation to ensure quantitative removal of liquids, proteins were digested overnight at 37 °C with 500 ng trypsin in 40 μl of the same buffer. Tryptic peptides were recovered by centrifugation followed by two additional extraction steps with 40 μl of 50 mM ABC and 40 μl of 1 % trifluoroacetic acid (TFA), respectively. Aliquots of the combined flow-throughs were spiked with 10 fmol/μl of yeast enolase 1 tryptic digest standard (Waters Corporation) for quantification purposes (Silva et al., 2006) and directly subjected to analysis by liquid chromatography coupled to electrospray mass spectrometry (LC-MS).
LC-MS analysis: Nanoscale reversed-phase UPLC separation of tryptic peptides was performed with a nanoAcquity UPLC system equipped with a Symmetry C18 5 μm, 180 μm × 20 mm trap column and a HSS T3 C18 1.8 μm, 75 μm × 250 mm analytical column maintained at 45 °C (Waters Corporation). Injected peptides were trapped for 4 min at a flow rate of 8 μl/min 0.1 % TFA and then separated over 120 min at a flow rate of 300 nl/min with a gradient comprising two linear steps of 3-35 % mobile phase B in 105 min and 35-60 % mobile phase B in 15 min, respectively. Mobile phase A was water containing 0.1 % formic acid while mobile phase B was acetonitrile containing 0.1 % formic acid. Mass spectrometric analysis of tryptic peptides was performed using a Synapt G2-S quadruple time-of-flight mass spectrometer equipped with ion mobility option (Waters Corporation). Positive ions in the mass range m/z 50 to 2000 were acquired with a typical resolution of at least 20,000 FWHM (full width at half maximum) and data were lock mass corrected post-acquisition. Analyses were performed in the ion mobility-enhanced data-independent acquisition mode drift time-specific collision energies as described in detail by Distler et al. (Distler et al., 2014; Distler et al., 2016). Specifically, a novel data acquisition strategy with dynamic range enhancement (DRE) was used, in which a deflection lens cycles between full and reduced ion transmission during one scan. This method provides an optimal trade-off between identification rate (i.e. proteome depth) and dynamic range for correct quantification of high-abundant myelin proteins. Continuum LC-MS data were processed for signal detection, peak picking, and isotope and charge state deconvolution using Waters ProteinLynx Global Server (PLGS) version 3.0.2 (Li et al., 2009). For protein identification, a custom database was compiled by adding the sequence information for yeast enolase 1 and
porcine trypsin to the UniProtKB/Swiss-Prot mouse proteome and by appending the reversed sequence of each entry to enable the determination of false discovery rate (FDR). Precursor and fragment ion mass tolerances were automatically determined by PLGS 3.0.2 and were typically below 5 ppm for precursor ions and below 10 ppm (root mean square) for fragment ions. Carbamidomethylation of cysteine was specified as fixed and oxidation of methionine as variable modification. One missed trypsin cleavage was allowed. Minimal ion matching requirements were two fragments per peptide, five fragments per protein, and one peptide per protein. The FDR for protein identification was set to 1% threshold.

Experimental design and data analysis: Myelin was purified from the CNS and PNS of Cnp1-Cre;fl/fl and control mice. Myelin protein fractions from the CNS of five animals per condition (Ctrl, KO) and from the PNS of 9 animals per condition, each pooled into 3 samples, were processed with replicate digestion, resulting in two technical replicates per biological replicate and thus in a total of 20 (CNS) /12 (PNS) LC-MS runs to be compared, respectively. The freely available software ISOQuant (http://www.isoquant.net) was used for post-identification analysis including retention time alignment, exact mass and retention time (EMRT) and ion mobility clustering, data normalization, isoform/homology filtering, and calculation of absolute in-sample amounts for each detected protein according to the TOP3 quantification approach (Distler et al., 2014; Kuharev et al., 2015; Distler et al., 2016). Only peptides with a minimum length of seven amino acids, which were identified with PLGS scores above or equal to 5.5 in at least two runs, were considered. FDR for both peptides and proteins was set to 1% threshold and only proteins reported by at least two peptides were quantified using the
TOP3 method. The parts per million (ppm) abundance values (i.e. the relative amount \( \text{w/w} \) of each protein in respect to the sum over all detected proteins) were log2-transformed and normalized by subtraction of the median derived from all data points for the given protein. Significant changes in protein abundance were detected by moderated t-statistics with an empirical Bayes approach and false discovery (FDR)-based correction for multiple comparisons (Kammers et al., 2015). For this purpose, the Bioconductor packages "limma" (Ritchie et al., 2015) and "q-value" (Storey and Tibshirani, 2003) were used in RStudio, an integrated development environment for the open source programming language R. Detected proteins were displayed in a volcano plot, high lighting those candidates that showed a significance of \( q<0.01 \) and a 25% up and down regulation as compared to control.

**Experimental design and statistical analysis**

ImageJ and Microsoft Excel were used to quantify data. For statistical analyses, GraphPad Prism was used. Depending on the experimental designs and requirements, Student’s unpaired t-test; one- or two-way ANOVA, followed by Bonferroni’s multiple comparison test; Kruskal-Wallis, followed by Dunn’s multiple comparison test, or Chi-squared test was applied. Statistical differences were considered significant if \( p<0.05 \) \((*p<0.05, **p<0.01, ***p<0.001)\). Whenever possible the exact p-value was indicated.

All data were displayed as standard error of the mean (=SEM). Each figure legend contains elaborate information on experiments (gender, number of animals or independent experiments included in the analyses, statistics, p-values).

**Results**
In our previous study by Vingill et al., we generated a reporter mouse line, in which the lacZ cassette was controlled by the endogenous FBXO7 promoter. β-Galactosidase expression revealed a prominent expression of FBXO7 in the corpus callosum and in the cerebellar white matter (Vingill et al., 2016). To examine the functional relevance of FBXO7 in myelinating cells *in vivo*, we mated *Fbxo7fl/fl* mice with the Cnp1-Cre driver line (Lappe-Siefke et al., 2003) to delete *Fbxo7* in predominantly myelinating cells of the central and peripheral nervous system (CNS and PNS).

Genotyping of these animals revealed living conditional knockout mice at the time of weaning (*Fig 1a*). A reduction in mRNA was clearly detectable in cerebellar tissue with large areas of white matter (*Fig 1b*) At 6 weeks of age, the conditional knockout mice (Cnp1-Cre;*Fbxo7fl/fl*, short: Cnp1-Cre;*fl/fl*) were visibly different from control mice (Cnp1-Cre). The mice were characterized by kyphosis (*Fig 1c*), reduced weight (*Fig 1d*) and a slightly reduced hind limb clasping score (*Fig 1e*). At 4 months of age, the knockout mice deteriorated quickly as the hind limb weakness progressed to a more severe hind limb paresis. Autopsies revealed atrophy of the muscles in the hind limbs (*Fig 1f*) and atrophic lungs (*Fig 1g*). The mice also became very emaciated and had to be euthanized.

To characterize the motor phenotype, we subjected 6 week-old Cnp1-Cre;*fl/fl* and control mice to motor tests and found that knockout mice were significantly impaired in holding on to an inverted grid as compared to control groups (*Fbxo7fl/fl* or Cnp1-Cre) (*Fig 1h*). In addition, the track length of the knockout mice in the open field arena was significantly reduced as compared to control groups (*Fig 1i*). We also analyzed the motor learning and coordination using the rotarod and the balance beam, respectively. We found
that Cnp1-Cre;fl/fl mice were unable to improve their performance on the rotarod (Fig 1j) and lacked the proper motor coordination to keep up with control mice on the balance beam both with respect to the time and the coordination while traversing the beam (Fig 1k). These results demonstrate that loss of Fbxo7 in myelinating cells negatively affects the overall morphology and posture of the mice in addition to their motor performance.

To investigate if deletion of Fbxo7 has any effects on myelination in the CNS and PNS in three month-old mice, we first examined if there are any gross morphological changes in the myelinated regions in the brain upon Gallyas staining, but we did not detect any obvious difference in the pattern of the tracts (Fig 2a). In addition, we did not observe an increase in TUNEL+ or APP+ cells in the white matter of the cerebellum or the corpus callosum, indicating no cell death or axonal impairment in white matter areas at the time of analysis (Fig 2b, c, d,e).

Next, we turned to a more in-depth histological analysis of the CNS and performed electron microscopy of the optic nerve of control (Cnp1-Cre) and Cnp1-Cre;fl/fl mice. We first examined semi-thin sections of the nerve, which did not reveal any gross morphological changes (Fig 3a). Using higher magnification electron micrographs (Fig 3b), we determined the g-ratio, which is defined by the inner to the outer diameter of the myelin sheath, of control and knockout mice and found a similar distribution of the values on the scatter plot (Fig 3c). Consequently, there was no significant difference in the average g-ratio in both groups (Fig 3d). We did however observe a redistribution of axon size. Interestingly, we found a significantly lower number of small diameter axons (< 1μm) and an increase in large diameter axons (>1μm), (Fig 3e). On counting oligodendrocyte nuclei, we did not find any significant
change in the knockout optic nerve (Fig 3f). We also did not observe any significant difference in the number of axons in the optic nerve (Fig 3g). We did however find a significant increase in degenerated axons in the knockout optic nerve (Fig 3h). Owing the humane endpoint, we could not examine whether or not the degeneration of axons would progress in older animals.

With further histological analyses of the white matter in the cerebellum and the corpus callosum, we found a significantly larger number of Iba+ microglial and GFAP+ astroglial cells in the former but not in the latter (Fig 4a, b, c, d). The lumbar section of the spinal cord however displayed a strong increase in Iba+ and GFAP+ cells (Fig 4e, f). Of note, neither microglia nor astrocytes accumulated in a focal manner in the spinal cord. In addition, we carried out quantitative PCR on cerebellar mRNA transcribed into cDNA to investigate the presence of anti- and pro-inflammatory markers. Among the factors examined, we found an increase of both anti- and pro-inflammatory indicators but only the pro-inflammatory cytokine Ip-10/Cxcl10 (interferon gamma–induced protein 10) was significantly upregulated (Fig 4g, h).

To get an insight into the global changes of the myelin proteome in the CNS upon loss of Fbxo7, we isolated myelin from brain and subjected the material to quantitative mass spectrometry and determined the up- and downregulated proteins. Mitochondrial structural proteins and metabolism-associated proteins, in particular those, which are responsible for mitochondrial functions, were the largest groups of the significantly down-regulated proteins (Fig 5a, b, Fig 5-1). Among the top score upregulated proteins, we identified glutathione S-transferase pi 1 (GSTπ1) (Fig 5c, Fig 5-1). GSTπ1 is a detoxification enzyme for reactive oxygen species that is exclusively expressed by
oligodendrocytes (Tansey and Cammer, 1991). With immunostainings of the spinal cord, we confirmed the upregulation of GSTπ1 in the CNS of knockout mice as compared to control animals (Fig 5d). In contrast, we did not find any change in the level of major myelin proteins such as PLP (proteolipid protein) (Fig 5e). In summary, we found that while deletion of Fbxo7 in oligodendrocytes did not affect myelination or triggered the demise of these cells, it led to subliminal cell stress indicated by micro- and astrogliosis, the upregulation of pro-inflammatory markers and detoxifying enzymes in oligodendrocytes.

Since the knockout mouse was characterized by a progressive paresis of the hind limbs, we focused our analyses on the PNS. We first turned to electron microscopy to examine the integrity of the sciatic nerve of control and knockout mice. Semi-thin sections already revealed a dramatic pathology with large areas void of healthy sciatic nerve components (Fig 6a), while other regions were seemingly unaffected. With higher magnification electron micrographs, we discovered obvious pathologies, which are described in the following analyses (Fig 6b). We first determined whether the pathology included defects in myelination, but we did not find any change in the distribution of the g-ratio on the scatter plot (Fig 6c) or any significant difference in the average g-ratio of control (Cnp1-Cre) and Cnp1-Cre;fl/fl mice (Fig 6d), indicating that myelination per se was not affected. The scatter plot however revealed a smaller population of large diameter axons in the knockout mice as compared to control animals. Analysis of the distribution showed a significant reduction of axons larger than 4μm (Fig 6e).

While we discovered only a downward trend in the average number of Schwann cell nuclei in the knockout sciatic nerve as compared to control (Fig 6f), we found a
significant decrease in the number of myelinated axons (Fig 6g), reflected in the loss of sciatic nerve components in the severely affected areas. The electron micrographs also displayed a significant increase in degenerated axons of the sciatic nerve of Cnp1-Cre;fl/fl mice (Fig 6h). In contrast to the healthy axons, we find accumulations of organelles such as mitochondria in the degenerated axons. The affected areas also displayed a significantly increased number of degenerated Remak bundles at various stages of demise (Fig 6i). Consistently, the number of intact Remak bundles was slightly reduced (Fig 6j). We did not observe any onion bulb formations that would indicate degeneration of Schwann cells.

In line with the increase in degenerated axons, we found a clear and significant increase in APP+ accumulations in immunohistochemical analyses of the sciatic nerve (Fig 7a), underscoring the compromised axonal health. In contrast, our analyses revealed little or no difference in the major myelin protein MBP in the sciatic nerve (Fig 7b). The axonal degeneration was also accompanied by an increase in Iba1 reactivity, indicating the presence of peripheral immune cells (Fig 7c). We then tested the idea that Schwann cells respond to the axonal degeneration by the upregulation of the cytoskeletal protein GFAP, typically expressed by non-myelinating Schwann cell and by cells, which have lost axonal contact (Jessen et al., 1990; Jessen and Mirsky, 2016). While there was little to no GFAP reactivity in control mice, Cnp1-Cre;fl/fl sciatic nerves showed a significant upregulation of GFAP (Fig 7d).

Consistent with the Iba1 reactivity, electron micrographs of the sciatic nerve of Cnp1-Cre;fl/fl mice confirmed the significant invasion of macrophages (Fig 7e), which entered through blood vessels (Fig 7f). We found macrophages that endocytosed entire
axon-myelin units (Fig 7g) as well as foamy macrophages that were packed with endocytosed material (Fig 7h). Furthermore, we detected fibrosis (Fig 7i), a close contact between endocytosing macrophages and fibroblasts (Fig 7j), and edema (Fig 7k). Interestingly, we also found macrophages and fibroblasts mingling with healthy myelinated axons and Remak bundles in the seemingly unaffected areas of the sciatic nerve. Taken together, while loss of FBXO7 in Schwann cells had no effect on myelination, it resulted in a peripheral neuropathy characterized by the focal degeneration of axons and Remak bundles and fibrosis together with the invasion of macrophages.

With electrophysiological examinations of the sciatic nerve in the living knockout mice, we measured a dramatic reduction in compound muscle action potential (CMAP), (Fig 8a) and a moderate yet significant reduction of the nerve conductance velocity (NCV), (Fig 8b), but no difference in distal motor latency (DML), (Fig 8c), suggesting an axonal defect without major damage to the myelin. These results are consistent with an axonal but not demyelinating phenotype and in line with the mild changes in NCV of CMT types 2 versus type 1 patients or in respective animal models (Sereda and Nave, 2006).

To determine the global protein changes in the knockout sciatic nerve, we carried out a quantitative mass spectrometry analysis of purified myelin isolated from control (Cnp1-Cre;fl/+), and Cnp1-Cre;fl/fl sciatic nerves. Among the significantly down-regulated proteins, we found mostly a similar change in mitochondrial and metabolism-associated proteins, in addition to cytoskeletal proteins and protein modification enzymes (Fig 8d, e), consistent with the loss of myelinated axons. The results of the up-regulated proteins such as immunoglobulins showed a massive increase in immune response (Fig 8f, Fig 8-
In our previous study, we learned that loss of FBXO7 affects proteasome activity in the brain and in heterologous HEK293T cells (Vingill et al., 2016), but we did not further dissect the activity of the proteasome in different cell types of the nervous system. To determine if loss of FBXO7 in Schwann cells affects their proteasome activity, we first generated an FBXO7 RNAi cell line based on the murine Schwann cell line MSC80 (Boutry et al., 1992), and validated the efficient knock down of FBXO7 (Fig 9a). Using the control and FBXO7 knockdown cell line, we performed proteasome activity assays. Here, cell lysates are mixed with an artificial substrate conjugated to a fluorophore. The measured fluorescence reflects the chymotrypsin-like activity of the 20S core subunit PSMB5. We found a 40% reduction in the knockdown cell line as compared to control cell lines (Fig 9b). Having the Fbxo7-/- mouse line available, we cultured primary Schwann cells from Fbxo7-/-, Fbxo7+/- and Fbxo7+/+ mice. With immunoblotting, we subsequently confirmed the loss or reduction of FBXO7 in homozygous and heterozygous cells, respectively (Fig 9c). Additionally, we detected the expression of the Schwann cell marker SOX10 but no expression of non-Schwann cell cytoskeletal proteins (Fig 9c). In Fbxo7-/- Schwann cells, we found a significantly reduced proteasome activity when compared to Fbxo7+/- and Fbxo7+/+ cells (Fig 9d). Since the time window of the Schwann cell culture protocol allowed us to isolate cerebella from the same animals, we simultaneously cultured cerebellar granule neurons (CGN) and subjected them to the proteasome activity assay. Interestingly, we found that loss of
Fbxo7 had little or no effect on their proteasome activity (Fig 9e), suggesting that in contrast to CGNs, Schwann cells are sensitive to FBXO7 loss.

Having identified FBXO7 as a critical factor for axon-glia interaction in the PNS, we examined the loss of Fbxo7 on the axon-glia interaction after development in the adult animal. Here, we used an inducible system to breed Fbxo7fl/fl mice with the Plp1-CreERT2 driver line. We injected these mice and the control group with tamoxifen at 2 months of age when myelination was completed and assessed the motor skills at 5, 7 and 9 months of age (Fig 10a), followed by histological analyses. Genotyping was carried out to identify the knockout and control group (Fig 10b). In addition, we carried out immunoblotting analysis to establish a reduction in FBXO7 levels. Here, cortical tissue with little or no white matter contamination or cerebellar tissue that harbors large white matter areas were lysed and examined using the FBXO7 antibody. FBXO7 reactivity was reduced in cerebellar lysates but not in cortical lysates of PLP-CreERT2;fl/fl mice (Fig 10c), indicating the successful tamoxifen-triggered recombination. We also determined the weight of control and Plp1-CreERT2;fl/fl mice and found that starting at 5 months of age, the knockout mice were significantly lighter as compared to 3 month-old mice (Fig 10d). We first tested the control and knockout group for hind limb clasping, which showed a slight decrease of the score at the latest time point of analysis (Fig 10e). The knockout mice showed no difference in their performance on the balance beam, in the open field arena or in the wire hang test (Fig 10f, g, h, i, j), suggesting that the mice are fully ambulant and with proper forelimb/hind limb coordination. In addition, the knockout mice showed no deficit in the pole test or on the inverted grid (Fig 11a, b), nor did they show any signs of anxiety in the elevated plus maze or sensory deficits on the
hot plate (Fig 11c, d). Plp1-CreERT2;fl/fl mice did however perform significantly worse on the rotarod as compared to control mice at every time point tested (Fig 11e), suggesting a persisting impairment that affects the motor endurance.

To determine if the sciatic nerve revealed a similar pathology as compared to the Cnp1-Cre;fl/fl mice, we examined electron micrographs. The overall integrity of the sciatic nerve was not compromised (Fig 12a). The analysis of the g-ratio revealed no difference between knockout and control mice (Fig 12b, c) and no redistribution of axonal diameters as observed in the Cnp1-Cre;fl/fl mice (Fig 12d). We also did not observe an increase in fibrosis (Fig 12e) or degenerated Remak bundles (Fig 12f). We did however observe a slight increase in infiltrating macrophages into the sciatic nerve in the Plp1-CreERT2;fl/fl mice (Fig 12g) and a significant increase in degenerated axons (Fig 12h), although to a less severe degree as compared to Cnp1-Cre;fl/fl mice. These data indicate that the disruption of Fbxo7 expression in Schwann cells of the mature PNS leads to a mild, late-onset neuropathy.
Discussion

Myelinating cells are fundamental for the fast saltatory conduction and for the trophic support of axons (Saab et al., 2013; Saab and Nave, 2017). In this study, we described a critical role for the F-box protein FBXO7 in the axon-myelin unit. FBXO7 is widely expressed in the nervous system including myelinating cells. While deletion of Fbxo7 in the oligodendrocytes or Schwann cells does not affect myelin biogenesis, we observe signs of degeneration and inflammation in the CNS and an even more dramatic phenotype in the PNS. Here, loss of Fbxo7 triggers peripheral neuropathy characterized by axonal degeneration and macrophage invasion. This phenotype is particularly severe if Fbxo7 is ablated early on and thus in contrast to a mild version of neuropathy caused by the post-myelination deficiency of Fbxo7.

FBXO7 is a subunit of the SCF E3 ubiquitin ligase, encoded by PARK15 and has been implicated in an atypical form of Parkinson Disease (PD). This movement disorder was termed Parkinsonian-Pyramidal Syndrome and is characterized by juvenile onset and a spectrum of symptoms and signs, which include among others bradykinesia, rigidity, Babinski sign and cognitive deficits (Shojae et al., 2008; Di Fonzo et al., 2009; Gunduz et al., 2014; Yalcin-Cakmakli et al., 2014; Lohmann et al., 2015). A peripheral neuropathy accompanying this particular syndrome has not been reported yet, but other reports have suggested that axonal neuropathy correlates with idiopathic Parkinson disease to a much higher extent as compared to control individuals (Manca et al., 2009; Toth et al., 2010; Rajabally and Martey, 2011). Further genetic studies investigated the prevalence of asymptomatic peripheral neuropathy in patients suffering from juvenile parkinsonism. Here, peripheral neuropathy characterized by denervation, abnormalities in
motor conduction of peroneal nerve, in sural sensory conduction and sympathetic skin
response, was diagnosed in more than 50% of the PD patients with juvenile onset (Taly
and Muthane, 1992). Individuals with Parkin mutations also showed an asymptomatic
sensory axonal neuropathy (Ohsawa et al., 2005), supporting the view of peripheral
neuropathy as a co-existing disorder of PD. Hence, it might be interesting to examine
PARK15 patients for clinical features of peripheral neuropathy.

The milder pathology in the CNS stands in contrast to the strong pathology in the
PNS. Increased axonal degeneration in the optic nerve together with the inflammation
and astrogliosis in the brain and spinal cord upon FBXO7 deletion may however reflect
the initial phase of a progressive degeneration of CNS axons. Owing to the humane end
point dictated by the peripheral neuropathy, we can only speculate about the pathological
course in older mice. Whether or not the axon-myelin unit in the CNS will ever be as
severely affected as in the PNS requires a more specific genetic approach. What is
obvious though is that the loss of Fbxo7 during development contributes strongly to
peripheral neuropathy, given that the conditional deletion of Fbxo7 in adult mice triggers
a milder phenotype. Deletion of Fbxo7 in Schwann cells causes a full-blown neuropathy
with degenerated axons but no apparent effect on myelin biogenesis. However, what can
be observed is that myelin together with degenerating axons is co-phagocytosed by
macrophages, which contributes to the secondary loss of myelin. Furthermore, our
findings lead to the conclusion that the loss of contact with axons likely results in
Schwann cells adopting a non-myelinating state, indicated by the upregulation of GFAP
in the sciatic nerve. GFAP is typically expressed by non-myelinating Schwann cells and
upregulated in Schwann cells that are deprived of axonal contact (Jessen et al., 1990). In
conclusion, this sign of Schwann cell dedifferentiation is in line with observations in CMT mouse models characterized by damaged nerve fibers (Fledrich et al., 2014; Klein et al., 2014; Groh et al., 2015).

The neuropathology of the PNS displays a quite striking phenotype. We find a focal destruction of the sciatic nerve that is characterized by degenerated axons and Remak bundles, which are in turn characterized by empty collagen pockets or reduced to Remak cells only (Weis et al., 2012). We further observe an increase in fibroblasts, which are in close proximity with infiltrating macrophages, a scenario which has been previously found in CMT models (Groh et al., 2012). The macrophages are clearly responsible for the removal of the degenerating axons together with the myelin, which subsequently leaves edema in the inflammation foci. Other parts of the sciatic nerve appear completely normal with healthy myelinated axons and Remak bundles, but the impending infiltration of macrophages can already be observed. As we do not observe lymphocytes at the time of analysis, the macrophage invasion should foremost be viewed as a clean-up action following Wallerian degeneration of the axons. Focal inflammatory mechanisms that include colony stimulating factor (CSF) 1-induced increase in macrophage number may also amplify the devastating clearance of myelinated fibers (Groh et al., 2012; Groh et al., 2015).

The focal breakdown of the myelin-axon units in the sciatic nerve is a remarkable feature that may induce the focal activation of the innate immune response. Focal inflammation is however rather atypical for genetic neuropathies. For example, while Charcot Marie Tooth disease type 2 (CMT2), harboring certain MPZ mutations, is characterized by a prominent axonal degeneration, mild or no demyelination and only
moderate reduction of nerve conduction velocity (Chapon et al., 1999; Shy et al., 2004; Finsterer et al., 2006), peripheral nerve biopsies of these CMT2 patients show a rather uniform pathology (Chapon et al., 1999; Senderek et al., 2000). Consistent with CMT2, we also find the depletion of large myelinated fibers.

FBXO7 plays also an important role in neurons for regulating motor functions (Vingill et al., 2016). In contrast to myelinating cells, loss of Fbxo7 in neurons does not lead to any obvious signs of axonal degeneration (Vingill et al., 2016). These findings suggest that while absence of Fbxo7 in neurons has no effect on axonal health, its loss in myelinating cells is detrimental to axonal integrity. This raises the question of which mechanism in myelinating cells is dispensable for myelination but indispensable for the support of the axon-myelin unit? As of now, this is quite challenging to answer as E3 ubiquitin ligases such as FBXO7-SCF are versatile enzymes implicated in multiple pathways. Our current understanding of FBXO7 is its regulatory role in proteasome assembly and activity (Vingill et al., 2016). As a consequence, systemic deletion of Fbxo7 in the brain results in a decrease in proteasome activity. Interestingly, we found that loss of Fbxo7 affects different cell types to a variable extent. Schwann cells appear to be very sensitive to proteasome inhibition. In line with this finding, the use of Bortezomib, a reversible inhibitor of the catalytic proteasomal subunit PSMB5, has been applied to experimentally induce neuropathy in rats that display Schwann cells pathology and axonal damage (Cavaletti et al., 2007). Multiple Myeloma patients treated with bortezomib develop a peripheral neuropathy, which has emerged as dose-limiting toxicity (Cavaletti and Jakubowiak, 2010; Cavaletti, 2011). Based on these reports, it is conceivable that reduced proteasome activity in Schwann cells caused by loss of Fbxo7
contributes to or even triggers peripheral neuropathy. Also, these findings suggest that it is not just the mere presence of Schwann cells that stimulates the survival of peripheral neurons as shown e.g. in ErbB3-deficient mice (Riethmacher et al., 1997), but likely the intact UPS in Schwann cells that is required to fully support axonal health. Our proteome analyses of the PNS also revealed the downregulation of E2 ubiquitin-conjugating enzymes, indicating that the UPS in the sciatic nerve is compromised beyond the loss of Fbxo7.

FBXO7 also is required for mitophagy, a clearance mechanism that is essential for mitochondrial homeostasis (Burchell et al., 2013). Whether or not mitophagy is impaired in oligodendrocytes or in Schwann cells of CNP1-Cre;fl/fl mice remains to be determined. The proteome analyses however show the significant downregulation of enzymes required for glycolysis and mitochondrial proteins involved in oxidative phosphorylation in the CNS of these mice. Along with the mitochondrial changes, we observed the increase in GSTπ1. This is consistent with the increased levels of GSTπ1 in MPTP-induced Parkinson model exhibiting oxidative stress (Castro-Caldas et al., 2009). Oligodendrocytes appear to be rather resistant to mitochondrial dysfunction. Ablation of mitochondrial respiration has little or no effect of oligodendrocyte and their associated axons in CNP1-Cre;Cox10fl/fl mice, Fünfschilling and colleagues demonstrated however that loss of respiration in Schwann cells leads to peripheral neuropathy (Funfschilling et al., 2012), indicating a sensitivity of Schwann cells to mitochondrial dysfunction. The authors described a progressive loss of myelinated axons in the PNS but no effect on myelination itself, which is comparable to the phenotype we have discovered in our conditional knockout mice.
Taken together, we show that myelination is entirely independent of FBXO7, the support of axons by myelinating cells however is not. This dependence is particularly striking when Fbxo7 is already ablated during development. The loss of Fbxo7 triggers a peripheral neuropathy and signs of degeneration in the central nervous system. The global assessment of protein changes in myelinating cells also supports the notion that FBXO7’s role in UPS regulation and mitochondrial function contributes to the inability of these cells to support axonal health, identifying FBXO7 as an essential factor of the axon-myelin unit.

References


Hall CS, Ballachey EL (1932) A study of the rat's behavior in a field; a contribution to method in comparative psychology: Berkeley, University of California Press.


Neuromolecular Med 8:205-216.


Figure legends

Figure 1: Loss of Fbxo7 in myelinating cells triggers motor impairment together with organic pathologies

a Genotyping PCR of control (Fbxo7fl/fl, Cnp1-Cre) and conditional knockout mice (Cnp1-Cre;fl/fl).

b Quantitative RT-PCR of fbxo7 and β-actin in cerebella isolated from 2 month-old, male Fbxo7fl/fl and Cnp1-Cre;fl/fl mice, 4 animals per genotype were included in the analysis. Student’s unpaired t-test, ** p=0.0029, mean +SEM.

c Representative images of 2 month-old, male control and Cnp1-Cre;fl/fl mice. Arrow indicates kyphosis.

d Average weight of 8 male Fbxo7fl/fl, 9 Cnp1-Cre and 10 Cnp1-Cre;fl/fl mice. One-way ANOVA, Bonferroni’s, *** p<0.001, mean +SEM.

e 8 Fbxo7fl/fl, 9 Cnp1-Cre and 10 Cnp1-Cre;fl/fl mice (all male) were subjected to hind limb clasping test. Kruskal–Wallis Test, Dunn’s, *p<0.05, mean +SEM.

f Exposed skeletal muscles of 3 month-old, male Cnp1-Cre;fl/fl and control mouse (Cnp1-Cre).

g Isolated lungs of 3 month-old Cnp1-Cre;fl/fl and control mouse (Cnp1-Cre).
h-k 8 male Fbxo7fl/fl, 9 Cnp1-Cre and 10 Cnp1-Cre:fl/fl mice were subjected inverted grid test, Kruskal –Wallis Test, Dunn’s, ***p<0.001, mean+SEM (h), open field test, One-way ANOVA, Bonferroni’s, *p<0.05, mean+SEM (i), rotarod test, Two-way ANOVA, Bonferroni’s, *p<0.05, ***p<0.001, mean+SEM (j) and balance beam test, Two-way ANOVA, Bonferroni’s, , **p<0.01, ***p<0.001, mean+SEM (k).

Figure 2: Histological analyses of the CNS of Cnp1-Cre:fl/fl mice

a Sagittal brain sections of male control and conditional knockout mice were subjected to Gallyas’ silver impregnation of myelin. Cb = Cerebellum, CC = Corpus callosum, scale bar = 0.5mm.

b, c Sagittal cerebellar sections of 3 month-old, male control mice (Fbxo7fl/fl or Cnp1-Cre) and Cnp1-Cre:fl/fl mice were subjected to TUNEL staining (arrow heads), 3 animals per genotype were included in the analysis. Two-way ANOVA, Bonferroni’s, mean +SEM (b) or to immunostaining with the APP antibody (c). Scale bar = 100µm.

d, e Sagittal forebrain sections of 3 month-old, male control mice and Cnp1-Cre:fl/fl mice were subjected to TUNEL staining (arrow heads), 3 animals per genotype were included in the analysis. Two-way ANOVA, Bonferroni’s, mean +SEM (d) or to immunostaining with the APP antibody (e). Scale bar = 100µm.

Figure 3: Effects of Fbxo7 deletion on myelination in the CNS

a Semi-thin section of control and knockout optic nerve. Scale bar = 20µm

b Representative electron micrographs of cross sections of optic nerve of control (Cnp1-Cre) and Cnp1-Cre:fl/fl mice. Scale bar = 2µm
c, d Optic nerves isolated from control and Cnp1-Cre;fl/fl mice were subjected to electron microscopy analysis. Three male animals per genotype and 200 axons per mouse were included in the g-ratio measurements displayed in scatter plot (c) and as average values (d). Student’s unpaired t-test, mean +SEM.

e Distribution of axon caliber in optic nerve of control and Cnp1-Cre;fl/fl mice. The percentage of axons per group was analyzed (N-1 Chi-squared test, ***p<0.001).

f-h Average number of oligodendrocyte nuclei/mm². Student’s unpaired t-test, mean +SEM (f), axons/mm² Student’s unpaired t-test, mean +SEM (g) and degenerated axons/mm² Student’s unpaired t-test, * p=0.0437, mean +SEM (h) were determined.

Representative electron micrographs of healthy and degenerated axons. Scale bar = 0.5μm in (g), 2μm in (h).

Figure 4: Pathological changes in the CNS white matter upon Fbxo7 deletion

a, b Sagittal cerebellar sections of 3 month-old, male Fbxo7fl/fl, Cnp1-Cre and Cnp1-Cre;fl/fl mice were subjected to immunostaining with the Iba1 or GFAP antibodies (arrow indicates Iba1+ cell, asterisks indicates GFAP+ cells), 3 animals per genotype were included in the analysis. One-way ANOVA, Bonferroni’s, *p<0.05, **p<0.01, mean +SEM. Scale bar = 50μm.

c, d Sagittal forebrain sections of 3 month-old, male control and Cnp1-Cre;fl/fl mice were subjected to immunostaining with the Iba1 (arrows = Iba1+ cells) or GFAP antibodies (asterisks = GFAP+ cells), 3 animals per genotype were included in the analysis. One-way ANOVA, Bonferroni’s, mean +SEM. Scale bar = 50μm.
Cross sections of the spinal cord (approx. lumbar region L3-L5) isolated from male and female control and Cnp1-Cre;fl/fl mice were immunostained with the Iba1 or GFAP antibodies, 3 animals per genotype were included in the analysis. One-way ANOVA, Bonferroni’s, ***p<0.001, mean ±SEM. Scale bar = 50 μm.

RT-PCR analysis of anti- and proinflammatory cytokines in the cerebellar white matter of male control and Cnp1-Cre;fl/fl mice, 4 animals per genotype were included in the analysis. Student’s unpaired t-test, **p=0.0055, mean ±SEM.

Figure 5: Histological and CNS mass spectrometry analyses of the Cnp1-Cre;fl/fl mice

Purified myelin from brain of female control and Cnp1-Cre;fl/fl mice was analyzed using quantitative mass spectrometry. Down-regulated functional protein groups displayed in pie chart were determined based on the number of proteins yielded in each term. Proteins displayed in pie charts adhere to the following criteria: q<0.01 and 25% decrease in fold change.

Purified myelin from 3-month-old, female control and Cnp1-Cre;fl/fl brains (CNS) was analyzed by quantitative mass spectrometry. The mean of five biological replicates per genotype and two technical replicates per animal was analyzed using the log2 cKO/control ratio (= fold change). Cnp1-Cre;fl/fl values were normalized to control. Volcano plot displays down- and up-regulated proteins from Cnp1-Cre;fl/fl as compared to control. Blue vertical and horizontal lines reflect the filtering criteria (q< 0.01, fold change (FC) -1.25 and 1.25). FC ±1.25 indicates peptide levels decreased or increased by 25%, respectively. Red and green dots represent proteins that adhere to these criteria.
Purified myelin from brain of female control and Cnp1-Cre;fl/fl mice was analyzed using quantitative mass spectrometry. Up-regulated functional protein groups displayed in pie chart were determined based on the number of proteins yielded in each term. Proteins displayed in pie charts adhere to the following criteria: q<0.01 and 25% increase in fold change.

Immunohistochemical staining of GSTπ1 in spinal cord of male and female Cnp1-Cre;fl/fl mice and control groups, 3 animals per genotype were included in the analysis. One-way ANOVA, Bonferroni’s, **p<0.01, ***p<0.001, mean +SEM. Scale bar = 50μm.

Immunohistochemical staining of PLP in spinal cord of male and female Cnp1-Cre;fl/fl mice and control groups, 3 animals per genotype were included in the analysis. Student’s unpaired t-test, mean +SEM. Scale bar = 50μm.

Figure 6: Loss of Fbxo7 triggers peripheral neuropathy without effects on myelination

Semi-thin sections of the sciatic nerve of female control and Cnp1-Cre;fl/fl mice. Scale bar = 20μm.

Electron micrographs of sciatic nerve isolated from female control and Cnp1-Cre;fl/fl mice. * = macrophages, # = fibroblasts. Scale bar = 5μm.

Sciatic nerve cross-sections of 3-month-old, female Cnp1-Cre and Cnp1-Cre;fl/fl mice were analyzed. Four mice per genotype and 100 axons per mouse were included in the g-ratio analyses displayed in a scatter plot (c) or as average values, Student’s unpaired t-test, mean +SEM (d).
e Distribution of axon caliber in sciatic nerve of control and Cnp1-\textit{Cre;}\textit{fl/fl} mice. The percentage of axons per group was analyzed (N-I Chi-squared test, *p<0.05, ***p<0.001).

f Electron microscopy analysis of average number of Schwann cell nuclei per/mm$^2$. Student’s unpaired t-test, mean +SEM.

g Electron microscopy analysis of average number of myelinated axons (Student’s unpaired t-test, *** p=0.0002, mean +SEM), together with representative electron micrograph. Scale bar = 2μm.

h Electron microscopy analysis of average number of degenerated axons and representative electron micrograph thereof. Student’s unpaired t-test, ** p=0.001, mean +SEM. Scale bar = 2μm.

i, j, Electron microscopy analysis of average number of degenerated Remak bundles/mm$^2$, Student’s unpaired t-test, *** p=0.0002, mean +SEM (i) and healthy Remak bundles/mm$^2$, Student’s unpaired t-test, mean +SEM (j). Scale bar = 1μm (i), 2μm (j).

Figure 7: Pathological changes in the sciatic nerve

a, b Longitudinal sections of sciatic nerves isolated from male control and Cnp1-\textit{Cre;}\textit{fl/fl} mice were subjected to immunohistochemistry with the APP, One-way ANOVA, Bonferroni’s * p<0.05, mean +SEM (a) or MBP antibody, Student’s unpaired t-test, mean +SEM (b). Scale bar = 50μm.

c, d Longitudinal sections of sciatic nerves isolated from male control and Cnp1-\textit{Cre;}\textit{fl/fl} mice were subjected to immunohistochemistry with the Iba1, One-way ANOVA, Bonferroni’s, **p<0.01, mean +SEM (c) or GFAP antibody, One-way ANOVA,
e-h Macrophages were counted in female wild type and knockout mice (Student’s unpaired t-test, *** p<0.0001, mean +SEM). Among those were macrophages invading via the blood vessels (f, scale bar = 2µm), macrophages that have completed the engulfment of myelin-axon segments (g, scale bar = 1µm) and foamy macrophages (h, scale bar = 5µm). Mp = macrophage, Fb = fibroblast, BV = blood vessel, f Mp = foamy macrophage.

i, j Fibroblasts were counted in female wild type and knockout mice (Student’s unpaired t-test, *** p< 0.0001, mean +SEM). Representative electron micrograph of fibroblast in close contact with macrophages in the sciatic nerve of Cnp1-Cre;fl/fl mice (j). Mp = macrophage, Fb = fibroblast. Scale bar = 2µm.

k Semi-thin section of the sciatic nerve of female Cnp1-Cre;fl/fl mice displaying edema (arrow heads). Scale bar = 20µm.

Figure 8: Electrophysiological and mass spectrometry analyses of the Cnp1-Cre;fl/fl mice

a, b, c Electrophysiological recordings of sciatic nerves of 2 month-old, male and female control and Cnp1-Cre;fl/fl mice assessing compound muscle action potential (CMAP), Student’s unpaired t-test, *** p<0.0001, mean +SEM (a), nerve conduction velocity (NCV), Student’s unpaired t-test, ** p=0.0017, mean +SEM (b) and distal motor latency (DML), Student’s unpaired t-test, mean +SEM c). Six mice per condition were included in the analyses.
d Purified myelin from sciatic nerve of male and female control and Cnp1-Cre;fl/fl mice was analyzed using quantitative mass spectrometry. Down-regulated functional protein groups displayed in pie chart were determined based on the number of proteins yielded in each term. Proteins displayed in pie charts adhere to the following criteria: q<0.01 and 25% decrease in fold change.

e Purified myelin from 3-month-old, male and female control and Cnp1-Cre;fl/fl sciatic nerves (PNS) was analyzed by quantitative mass spectrometry. Nine pairs of sciatic nerves were pooled into 3 groups per genotype, each measured in duplicates and analyzed using the log2 cKO/control ratio. Values of Cnp1-Cre;fl/fl myelin were normalized to control. Volcano plot displays down and up-regulated proteins of Cnp1-Cre;fl/fl myelin as compared to control. Blue vertical and horizontal lines reflect the filtering criteria (q<0.01, fold change (FC) -1.25 and 1.25). FC ±1.25 indicates peptide levels decreased or increased by 25%, respectively. Red and green dots represent proteins that adhere to these criteria.

f Purified myelin from sciatic nerve of male and female control and Cnp1-Cre;fl/fl mice was analyzed using quantitative mass spectrometry. Up-regulated functional protein groups displayed in pie chart were determined based on the number of proteins yielded in each term. Proteins displayed in pie charts adhere to the following criteria: q<0.01 and 25% increase in fold change.

Figure 9: Fbxo7 knockdown or deletion leads to reduced proteasome activity in Schwann cells
a Lysates of MSC80 Schwann cells transduced with control vector (pSuper), FBXO7 RNAi control (non-functional) or FBXO7 RNAi were subjected to immunoblotting with the FBXO7 and 14-3-3 antibodies. The latter served as loading control.

b Lysates of MSC80 Schwann cells (control vector, control FBXO7 RNAi control (non-functional) or FBXO7 RNAi) were subjected to proteasome activity assay. Four independent experiments were included in the analyses. Two-way ANOVA, Bonferroni’s, ** p<0.01, *** p< 0.001, **** p< 0.0001, mean +SEM.

c Lysates of cultured primary Schwann cells from male and female wild type, Fbxo7+/-, Fbxo7-/- mice and Fbxo7+/+ cerebellar lysates were subjected to immunoblotting with the FBXO7, SOX10, NFM, GFAP and 14-3-3 antibodies. The latter served as loading control.

d Lysates of cultured primary Schwann cells from postnatal day 5 male and female Fbxo7+/+, Fbxo7+/- and Fbxo7-/ mice were subjected to proteasome activity assay. Four independent experiments were included in the analyses. Two-way ANOVA, Bonferroni’s, * p<0.05, *** p< 0.001, mean +SEM.

e Lysates of cultured primary cerebellar granule neurons from male and female Fbxo7+/+, Fbxo7+/- and Fbxo7-/ mice were subjected to proteasome activity assay. Five independent experiments were included in the analyses. Two-way ANOVA, Bonferroni’s, mean +SEM.

Figure 10: Behavioral analyses of Plp1-CreERT2;fl/fl and control cohorts I
a Schematic of experimental design. Plp1-\textit{CreERT2};\textit{fl/fl} and control cohorts were injected with tamoxifen at 2 months of age. Analyses were carried out at 5, 7 and 9 months of age.

b Genotyping PCR of control \textit{Fbxo7fl/fl} and Plp1-\textit{CreERT2};\textit{fl/fl} mice.

c Cortical and cerebellar lysates from male and female control and Plp1-\textit{CreERT2};\textit{fl/fl} mice were immunoblotted with the FBXO7 and 14-3-3 antibodies. The latter served as loading control.

d, e The weight (d) and hind limb clasping score (e) of 10 male \textit{Fbxo7fl/fl} and 11 male Plp1-\textit{CreERT2};\textit{fl/fl} mice were determined. Two-way ANOVA, Bonferroni’s, *p<0.05, mean +SEM.

f-j 10 male \textit{Fbxo7fl/fl} and 11 male Plp1-\textit{CreERT2};\textit{fl/fl} mice were subjected to balance beam test (f, g), open field test (h, i), or wire hang test (j). Two-way ANOVA, Bonferroni’s, mean +SEM.

Figure 11: Behavioral analyses of Plp1-\textit{CreERT2};\textit{fl/fl} mice control cohorts II

a-e 10 male \textit{Fbxo7fl/fl} and 11 male Plp1-\textit{CreERT2};\textit{fl/fl} mice were subjected to pole test (a), inverted grid test (b), elevated plus maze test (c), hotplate test (d) and rotarod test (e). Two-way ANOVA, Bonferroni’s, *p<0.05, mean +SEM.

Figure 12: Electron microscopy analysis of sciatic nerves of Plp1-\textit{CreERT2};\textit{fl/fl} and control mice

a Representative electron micrographs of sciatic nerves isolated from 10-month-old \textit{Fbxo7fl/fl} and Plp1-\textit{CreERT2};\textit{fl/fl} mice. Scale bar = 5μm.
Sciatic nerve cross-sections of 10-month-old, male Fbxo7fl/fl and Plp1-CrERT2;fl/fl mice were analyzed. Four mice per genotype and 100 axons per mouse were included in the g-ratio analyses displayed in a scatter plot (b) or as average values (c), Student’s unpaired t-test, mean +SEM.

Distribution of axon caliber in sciatic nerve of control and Plp1-CrERT2;fl/fl mice. The percentage of axons per group was analyzed (N-1 Chi-squared test).

Electron microscopy analysis of average number of fibroblasts/mm². Student’s unpaired t-test, mean +SEM. Fb = fibroblast. Scale bar = 2µm.

Electron microscopy analysis of average number of degenerated Remak bundles/mm². Student’s unpaired t-test, mean +SEM. Scale bar = 2µm.

Electron microscopy analysis of average number of macrophages/mm². Student’s unpaired t-test, mean +SEM. Representative electron micrographs of invading macrophages (Mp), infiltrating sciatic nerve from blood vessels (BV). Scale bar = 2µm.

Electron microscopy analysis of average number of degenerated axons/mm². Student’s unpaired t-test, * p=0.0449, mean +SEM. Representative electron micrograph of degenerated shrunken axon (asterisk) and accumulation of pleomorphic, probably autophagic material in the adaxonal Schwann cell cytoplasm in Plp1-CrERT2;fl/fl mice. Scale bar = 2µm.
Figure 1

(a) Western blot analysis of Cre, floxed Fbxo7, and wild type Fbxo7. bp = base pairs.

(b) Cerebellum mRNA expression:
- Fbxo7 mRNA
- β-actin mRNA

Fbxo7 flop/flop (gray) vs. Cnp1-Cre; flop/flop (green).

(c) Images of Cnp1-Cre; flop/flop and Cnp1-Cre.

(d) Weight:
- Fbxo7 flop/flop
- Cnp1-Cre
- Cnp1-Cre; flop/flop

(e) Hind limb clasiaing:
- Fbxo7 flop/flop
- Cnp1-Cre
- Cnp1-Cre; flop/flop

(f) Skeletal muscle images of Cnp1-Cre; flop/flop and Cnp1-Cre.

(g) Lung images of Cnp1-Cre; flop/flop and Cnp1-Cre.

(h) Inverted grid:
- Time, seconds

(i) Open field:
- Track length, cm

(j) Rotarod:
- Time, seconds

(k) Balance beam:
- Time, seconds
- Coordination score

Width: 12mm, 6mm
Figure 4

a

Iba1

Cerebellum

b

GFAP

Cerebellum

c

Iba1

Corpus callosum

d

GFAP

Corpus callosum

e

Iba1

Spinal cord

f

GFAP

Spinal cord

g

anti-inflammatory markers

Cerebellum

h

pro-inflammatory markers

Cerebellum

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Figure 5

a) Down-regulated proteins in CNS myelin

b) KO vs. Ctrl myelin proteins

- Log2 (p-value)
- Log2 (fold change)

KO: significantly up regulated
Ctrl: significantly down regulated
non-significant

detected CNS myelin proteins: 522 total
up-regulated proteins: 232 total
down-regulated proteins: 290 total

---

c) Up-regulated proteins in CNS myelin

---

d) Fbxo7 fl/fl, Cnp1-Cre, Cnp1-Cre; fl/fl

GSTπ1

- Spinal cord

---

e) Cnp1-Cre; fl/+, Cnp1-Cre; fl/fl

PLP

- Spinal cord

---
Figure 8

(a) CMAP
(b) NCV
(c) DML

(d) Down-regulated proteins in PNS myelin
(e) KO vs. Ctrl myelin proteins

(f) Up-regulated proteins in PNS myelin

detected PNS myelin proteins: 368 total
up-regulated proteins: 131 total
down-regulated proteins: 237 total
Figure 10

a) Plp1-CreERT2; Fbxo7 fl/fl - mouse line

b) Western blot analysis showing Cre, floxed Fbxo7, wild type Fbxo7, and Fbxo7

b) Weight

Cortex

Cerebellum

d) Body weight

Hind limb clasp

f) 6mm Balance beam

Clasping score

Time, seconds

Coordination score

6mm Balance beam

Time, seconds

Coordination score

h) Open field

Track length, cm

Open field

Center

Intermediate

Periphery

Open field

i) Wire hang

Time, seconds

months post Tamoxifen injection

j) Wire hang
**Figure 11**

**a**

Pole test

Time, seconds

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**b**

Inverted grid

Time, seconds

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**c**

Elevated plus maze

3 month

Time, seconds

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5 month

Time, seconds

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7 month

Time, seconds

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**d**

Hotplate

Time, seconds

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**e**

Rotarod

Time, seconds

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Figure 12

a. Comparison of Fbxo7 fl/fl and Plp1-CreERT2; fl/fl sciatic nerves.

b. Scatter plot showing G-ratio vs. axon diameter in sciatic nerve for Fbxo7 fl/fl and Plp1-CreERT2; fl/fl.

c. Bar graphs comparing average G-ratio between Fbxo7 fl/fl and Plp1-CreERT2; fl/fl.

d. Bar graphs showing distribution of axon diameter in sciatic nerve.

e. Fibrosis comparison between Fbxo7 fl/fl and Plp1-CreERT2; fl/fl.

f. Bar graphs showing number of degenerated Renal bundles/mm².

h. Bar graphs showing number of degenerated axons/mm².