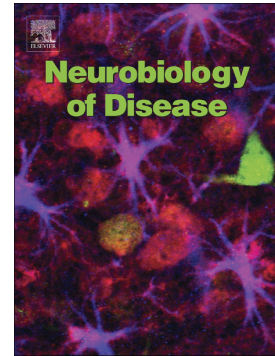


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PII: S0969-9961(18)30750-2
DOI: <https://doi.org/10.1016/j.nbd.2018.11.019>
Reference: YNBDI 4334
To appear in: *Neurobiology of Disease*
Received date: 1 June 2018
Revised date: 21 October 2018
Accepted date: 19 November 2018

Please cite this article as: Vietxuan Phan, Dan Cox, Silvia Cipriani, Sally Spendiff, Stephan Buchkremer, Emily O'Connor, Rita Horvath, Hans Hilmar Goebel, Denisa Hathazi, Hanns Lochmüller, Tatjana Straka, Rüdiger Rudolf, Joachim Weis, Andreas Roos, SIL1 deficiency causes degenerative changes of peripheral nerves and neuromuscular junctions in fish, mice and human. *Ynbdi* (2018), <https://doi.org/10.1016/j.nbd.2018.11.019>

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SIL1 deficiency causes degenerative changes of peripheral nerves and neuromuscular junctions in fish, mice and human

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Abstract

Background

Marinesco-Sjögren Syndrome (MSS) is a rare neuromuscular condition caused by recessive mutations in the *SIL1* gene resulting in the absence of functional SIL1 protein, a co-chaperone for the major ER chaperone, BiP. As BiP is decisive for proper protein processing, loss of SIL1 results in the accumulation of misshaped proteins. This accumulation likely damages and destroys cells in vulnerable tissues, leading to congenital cataracts, cerebellar ataxia, vacuolar myopathy and other MSS phenotypes. Whether the peripheral nervous system (PNS) is affected in MSS has not been conclusively shown.

Methods

To study PNS vulnerability in MSS, intramuscular nerves fibres from MSS patients and from SIL1-deficient mice (*woozy*) as well as sciatic nerves and neuromuscular junctions (NMJ) from these mice have been investigated via transmission electron microscopic and immunofluorescence studies accompanied by transcript studies and unbiased proteomic profiling. In addition, PNS and NMJ integrity were analyzed via immunofluorescence studies in an MSS-zebrafish model which has been generated for that purpose.

Results

Electron microscopy revealed morphological changes indicative of impaired autophagy and mitochondrial maintenance in distal axons and in Schwann cells. Moreover, changes of the morphology of NMJs as well as of transcripts encoding proteins important for NMJ function were detected in *woozy* mice. These findings were in line with a grossly abnormal structure of NMJs in SIL1-deficient zebrafish embryos. Proteome profiling of sciatic nerve specimens from *woozy*

mice revealed altered levels of proteins implicated in neuronal maintenance suggesting the activation of compensatory mechanisms.

Conclusion

Taken together, our combined data expand the spectrum of tissues affected by SIL1-loss and suggest that impaired neuromuscular transmission might be part of MSS pathophysiology.

Keywords

SIL1, Marinesco-Sjögren syndrome, woozy, PNS pathology, neuromuscular junction

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Background

Marinesco-Sjögren syndrome (MSS; MIM:248800) is a rare autosomal recessive disorder that affects multiple organ systems. Common symptoms of MSS are congenital cataracts, cerebellar ataxia, hypotonia, progressive muscle weakness, and delayed psychomotor development [1-4]. The disease affects males and females equally; however, the exact incidence of the disorder in the general population is unknown. MSS is often caused by mutations of the *SIL1* gene which locates on the long arm of chromosome 5 (5q31.2) [5, 6]. SIL1, an adenine nucleotide exchange factor, binds to the 78 kDa glucose-regulated protein (GRP78; BiP) and thereby regulates its ATPase cycle. BiP belongs to the heat shock protein (HSP) 70 chaperone family and plays a key role in protein quality control within the endoplasmic reticulum (ER) [7, 8]. Loss of functional SIL1 results in the build-up of misfolded proteins in the ER and thus to activation of the unfolded protein response (UPR) and likely damages cells in many tissues, leading to congenital or infantile cataracts, Purkinje cell degeneration and ataxia, progressive vacuolar myopathy and other phenotypes such as MSS complicated by Dandy-Walker syndrome [9], by spastic paraplegia [10] or by motor neuronopathy and bradykinetic movement disorder [11]. Overall, this phenotype highlights an important role of SIL1 in neuronal function and maintenance. In wozy mice, loss of functional SIL1 results in ER-stress, UPR activation and Purkinje cell degeneration and vacuolar myopathy with signs of altered autophagy and typical alterations of the myonuclear envelope, indicating that the wozy mouse is a suitable phenocopy of the human disease [12-15].

In the nervous system, ER stress has been identified as a key process in multiple neurodegenerative conditions such as Alzheimer's and Parkinson's disease, Amyotrophic

Lateral Sclerosis (ALS) and prion diseases [16, 17]. In this context, it is important to note that apart from the above described MSS phenotype, a prominent role of SIL1 in maintaining integrity and function of the nervous system is suggested by the following observations: (i) SIL1 has been described as a disease-modifying protein in ALS and Alzheimer's disease, respectively [18, 19], (ii) *in vitro* overexpression of SIL1 resulted in elevation of a variety of proteins with neuroprotective functions [20], (iii) *in vitro* depletion of SIL1 affects several proteins important for neuronal function [21] and (iv) morphological studies of *Sil1*-mutant mice revealed pathological alterations of nerve terminals and neuromuscular junctions along with signs of neurogenic muscular atrophy [18]. Although Horvers and co-workers found no clear evidence of peripheral neuropathy in their cohort of four Dutch MSS patients with proven *SIL1* mutations [22]; still, the evidence discussed above suggests a vulnerability of the PNS against loss of functional SIL1. However, so far there is no dedicated study focusing on the effect of SIL1 deficiency on peripheral nerves and neuromuscular junctions (NMJs). To systematically address this question, we investigated intramuscular nerve fascicles in MSS-patients and *woozy* mice as well as the sciatic nerves of these mice by transmission electron microscopy. Moreover, proteomic profiling of the murine sciatic nerve was performed to obtain insights into the biochemical consequences of loss of the SIL1 protein in the PNS. Finally, NMJs were systematically studied by immunofluorescence in *Sil1*-mutant and wildtype mice as well as in an MSS-zebrafish model.

Materials and Methods

Electron microscopy

Ultrathin sections of archived glutaraldehyde-fixed, resin-embedded *M. quadriceps* biopsies obtained from two MSS-patients with proven *SIL1* mutations [6, 23] were examined. In addition, sciatic nerve specimens derived from *woozy* [15] (3 animals aged 16 weeks and 3 animals aged

26 weeks) and wildtype mice (3 animals aged 16 weeks and 3 animals aged 26 weeks) were fixed in 3.9 % buffered glutaraldehyde. Samples were osmicated in 1 % phosphate-buffered osmium tetroxide, dehydrated and embedded in epoxy resin. Ultrathin sections (100 nm) of transversely and longitudinally embedded nerve fascicles were contrasted with uranyl acetate and lead citrate. EM images were obtained using a CM10 transmission electron microscope (Philips, Amsterdam, The Netherlands).

Proteomic profiling

Tissue lysis and carbamidomethylation

Sciatic nerves derived from three *woozy* and three wild-type animals were used for comparative proteome profiling utilizing a label-free approach. Each sample was ground and lysed in 0.5 mL of 50 mM Tris-HCl (pH 7.8) buffer containing 150 mM NaCl, 1 % SDS and Complete Mini. Afterwards, extracts were centrifuged at 13,500 x g for 30 min at 4 °C and protein lysate was collected. The protein concentration of each sample was determined by BCA assay according to the manufacturer's protocol. Then, cysteines of the proteins were reduced by addition of 10 mM DTT at 56°C for 30 min, followed by alkylation of free thiol groups with 30 mM IAA at room temperature (RT) in the dark for 30 min.

Sample preparation and trypsin digestion

Sample preparation and proteolysis were performed using filter-aided sample preparation (FASP). Briefly, 100 µg of protein was diluted 10-fold with freshly prepared 8 M urea/100 mM Tris-HCl (pH 8.5) buffer and placed on a centrifugal device Nanosep 30 KDa Omega (Life Science). The device was centrifuged at 13,800 g at RT for 20 min for all centrifugation steps. First, to eliminate residual SDS, three washing steps were carried out with 100 µL of 8 M urea/100 mM Tris-HCl (pH 8.5). Then, for buffer exchange, the device was washed thrice with 100 µL of 50 mM NH₄HCO₃ (pH 7.8). Next, 100 µL of proteolysis buffer comprising of trypsin

(Promega) (1:25 w/w, protease to substrate), 0.2 M GuHCl and 2 mM CaCl₂ in 50 mM NH₄HCO₃ (pH 7.8), was added to the device and incubated at 37°C for 14 h. Afterwards, the generated tryptic peptides were recovered by centrifugation with 50 µL of 50 mM NH₄HCO₃ followed by 50 µL of ultra-pure water. Finally, peptides were acidified by addition of 10 % TFA (v/v) and digests were quality-controlled in a reversed-phase HPLC.

LC-MS/MS analysis

Replicates were measured using an UltiMate 3000 nano RSLC System coupled to a Lumos Dionex Mass Spectrometer (both from Thermo Scientific). Peptides were preconcentrated on a 100 µm x 2 cm, C18, 5 µm, 100 Å trapping column for 10 min using 0.1 % TFA (v/v) at a flow rate of 20 µL/min followed by separation on 75 µm x 50 cm, C18, 2 µm, 100 Å main column (both from Acclaim Pepmap, Thermo Scientific) with a 120 min LC gradient ranging from 3-35 % of 84 % ACN, 0.1 % FA (v/v) at a flow rate of 250 nL/min. MS survey scans were acquired in the Orbitrap from m/z 300 to 1500 at a resolution of 120,000 using the ambient air (protonated (Si(CH₃)₂O))₆ ion at m/z 445.12002 as lock mass [24]. The most intense signals were subjected to high collision induced dissociation (HCD), and the detection was archived in the ion trap, taking into account a dynamic exclusion of 30 s. HCD spectra were acquired with a normalized collision energy of 30 % and an activation time of 10 ms. AGC target values were set to 2 x 10⁶ for Orbitrap MS and 2 x 10⁴ for ion trap MSn scans. Maximum injection times were set to 50 ms and 300 ms for both full MS and MSn scans, respectively.

Label free data analysis

Data analysis of the acquired label free quantitative MS data was performed using the Progenesis Qi software from Nonlinear Dynamics (Newcastle upon Tyne, U.K.) in which alignment of MS raw data was conducted by automatically selecting one of the LC-MS files as reference. After peak picking, only features within retention time and m/z windows from 0-120

min and 300-1500 m/z, with charge states +2, +3, and +4 were considered for peptide statistics and analysis of variance (ANOVA). MS/MS spectra were exported in an mgf file as peak lists.

The mgf peak lists were searched against a concatenated target/decoy version of the mouse Uniprot database, (downloaded in July 2015, containing 16,716 target sequences) using Mascot 2.4.0 (Matrix Science), X! TANDEM Vengeance (2015.12.15.2) and MS-GF+ Beta (v10282) (12/19/2014) with the help of searchGUI 2.8.4. Trypsin with a maximum of two missed cleavages was selected as enzyme. Carbamidomethylation of cysteine was set as fixed and oxidation of methionine was selected as variable modification. MS and MS/MS tolerances were set to 10 ppm and 0.5 Da, respectively.

PeptideShaker software 1.10.2 was used for interpretation of peptide and protein identifications from searchGUI and Mascot. Combined search results were filtered at a false discovery rate (FDR) of 1 % on the protein level and exported using the advanced PeptideShaker features that allow direct re-import of the quality-controlled data into Progenesis Qi. Peptide sequences containing oxidized methionines were excluded for further analysis. Only proteins that were quantified with unique peptides were exported. Then, for each protein, the average of the normalized abundances (obtained from Progenesis Qi) from the replicate analyses was calculated to determine the ratios between the woozy and the wildtype mice. Only proteins which were (i) commonly quantified in all the replicates with (ii) at least a unique peptide, (iii) an ANOVA p-value of <0.05 (Progenesis Qi) and (iv) an average log₂ ratio of which protein that was either higher than the up-regulated cut-off or lower than the down-regulated cut-off was considered as regulated. The cut-off values were determined based on the 2x standard deviation and the normal distribution from all identified protein's log₂ ratio in which the bell curve is symmetric around the mean. Therefore, an average log₂ ratio of a protein which < -1.02 or > 1.05 (corresponding to ~2.09-fold regulation; log₂ ratios of 1.01) for comparative global profile were considered as regulated.

Morphological studies of neuromuscular junctions in woozy mice

Immunofluorescence staining of 100 µm-thick free-floating longitudinal sections of *Extensor Digitorum Longus* muscles were done as previously described [25]. Briefly, muscles were incubated in 1x PBS/0.5 % Tween-20 with 10 µg/mL Heparin (1x PTwH) for 24 h at room temperature. Then, muscles were transferred to 1x PBS/1x PTwH/0.5 % Triton X-100/10 % (vol/vol) DMSO/6 % (vol/vol) BSA (1x BnP) at room temperature for 5 days. Followed by incubation with Bungarotoxin 647 (Invitrogen/B35450) in 1 x BnP solution for 5 d at 37 °C. After the staining, muscles were washed with PTwH solution for 5d at RT before imaging. Muscles were placed in a 35mm glass bottom dish and imaged with an inverted Leica SP2 (Leica Microsystems, Mannheim, Germany) confocal microscope equipped with a HC PL APO 20x/0.75 IMM CORR UV objective. 3D stacks were taken at 8-bit, 1024x1024 pixel resolution, and 400 Hz scan frequency. Images were electronically processed using ImageJ software (NIH, Bethesda, MD). NMJ area was determined using the region of interest (ROI) upon thresholding at 30 – 255 greyscale values.

Transcript studies in *Sil1*-mutant and wildtype mice

To further examine the integrity of the NMJ gene expression investigations were performed for targets previously shown to be involved in NMJ structure [26]. RNA was extracted from the muscle of 26-week-old mice. TRIzol Reagent (Ambion), 0.1 mL per 50-100 mg of muscle weight, was added to each sample. The samples were homogenised on ice using a TissueRuptor (Qiagen) and incubated for 5 min at room temperature before centrifuging for 10 min at 12,000g (4°C). 0.2 mL of Chloroform per 1 mL of Trizol was added to the cleared

homogenate solution and the sample vigorously shaken by hand for 15 sec. Samples were incubated for 3 min at room temperature, centrifuged for 15 min at 12,000g (4°C), and the RNA in the aqueous phase transferred to a new tube. 0.5 mL per 1mL of Trizol of isopropyl alcohol was added to each sample which was again shaken by hand before incubation at room temperature for 10 min. Samples were centrifuged for 15 min to obtain the RNA pellet, which was then washed in 75% ETOH and centrifuged for 5 min. The supernatant was removed, and the pellet dried in a heat block at 55-60°C. The RNA was re-suspended in RNAase-free-water (Ambion) and incubated at 55-60°C for 10 min. Sample purity (A_{260}/A_{280} ratio) and concentration ($\mu\text{g}/\mu\text{l}$) were assessed using a Nanodrop2000 (ThermoFisher).

DNAase treatment and reverse transcription was performed according to manufacturer's instructions using the DNA-free™ DNA Removal Kit (ThermoFisher) and the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) respectively. Samples were stored at -20°C until use.

Real-time PCR (qPCR) was performed on a BioRad CFX96 using Power SYBR Green Master Mix (ThermoFisher). For each gene examined, a temperature gradient and dilution calibration curve were performed to determine optimum conditions for the primer pair (Table 1) and check the reaction efficiency. A master mix composed of 10 μL of SYBR Green, 6 μL of DEPC-H₂O, 1 μL of forward and reverse primer (Table 1) was added to 2 μL of cDNA. Samples were run in triplicate along with a no-template control (NTC), with only wells that were at least 3 Cq cycles away from the NTC being used for analysis. Data was analysed using the $\Delta\Delta\text{Cq}$ method.

Gene	Forward Sequence	Reverse Sequence	Annealing temp (°C)	NCBI Ref
<i>AChRγ</i> [26]	GACCAACCTCATCTCCCTGA	GAGAGCCACCTCGAAGACAC	60	NM_009604.3
<i>NCAM</i> [26]	AAGGGGAAGGCACTGAATT	TCTCCTGCCACTTGACACAG	60	NM_001081445.1
<i>AchRα</i> [26]	TCCCTTCGATGAGCAGAACT	GGGCAGCAGGAGTAGAACAC	60	NM_007389.5
<i>NGF</i> [26]	GCAGTGAGGTGCATAGCGTA	CTGTGTCAAGGGAATGCTGA	60	NM_001112698.2
<i>TATA</i>	TGCCCAGCATCACTATTTCA	CCGTAAGGCATCATTGGACT	60	NM_013684.3

<i>BOX BP</i> [26]				
<i>AchRe</i> [26]	GCAGCTTTTACCGAGAATGG	CGTCAGTTTCTCCAGGACC	60	NC_000077.6
<i>MuSK</i> [26]	TTCAGCGGGACTGAGAAACT	TGTCTTCCACGCTCAGAATG	61.5	NM_001037127.2
<i>BDNF</i> [26]	TAATGCAGCATGATGGGAAA	TCACAGTGAAAGCACCTTGC	60	NM_001048139.1
<i>NGF</i> [26]	GCAGTGAGGTGCATAGCGTA	CTGTGTCAAGGGAATGCTGA	60	NM_001112698.2
<i>NTRK2</i> [27]	CGGCACATAAATTTACACG	GTGAGGTTAGGAGCAGCCAG	63.9	NM_008745.2
<i>NTRK3</i> [27]	AAGTAACCGGCTCACACAC	GATGCAGTAAAGGCTCTGGC	63.9	NM_182809.2
<i>P75</i> [27]	CAACCAGACCGTGTGTGAAC	GAGAACACGAGTCCTGAGCC	63.9	NM_033217.3

Table 1: Oligonucleotides used for the transcription studies

Generation of a MSS-zebrafish model and studies of NMJ integrity and myelination

To examine NMJ integrity and myelination, knockdowns were performed in zygotes of the Golden (*slc24a5b1/+*) *Danio rerio* strain (ZIRC, OR, USA). Zebrafish embryos and larvae were raised and staged according to standard procedures [28].

Antisense morpholino oligonucleotides (MOs) were purchased from Gene Tools (Pilomath, OR). We obtained a previously published [33] *Sil1* splice-blocking MO directed against the splice acceptor site of exon 2 (5'-GGTGACTGTGTAACAGAACAATC-3'). The Gene Tools standard control-MO targeting a human β -hemoglobin gene (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a negative control for the effects of MO injection. Zygotes were injected with 6ng of either *Sil1* MO or control-MO following standard protocols.

Bright field microscopy images of larvae were captured using a Leica dissection stereomicroscope equipped with a Leica digital camera (model DFC 420C). For immunofluorescent staining of whole mount zebrafish, 5-day post fertilization zebrafish embryos were dechorionated using Pronase E (Sigma Aldrich) and euthanized by anesthetic overdose. Whole mount staining was performed as described previously [29], utilizing a mouse anti-SV2

antibody to visualize the motor neurons (1:200, Developmental Studies Hybridoma Bank) and Alexa Fluor 594- α -bungarotoxin conjugate to visualize acetylcholine receptors (1:1000, Thermo Fisher). A Claudin K antibody (1:200) was used for myelin-staining. Z-stack images encompassing the entire zebrafish tail were obtained using a 20x air objective on a Nikon A1R confocal microscope.

Results

Electron microscopy

EM of intramuscular nerves found in quadriceps muscles of MSS patients revealed accumulations of membranous autophagic/mitophagic material as well as spheroid-like accumulations of cytoskeletal elements and of organelles associated with a thinning of the myelin sheaths in axons; a similar buildup of autophagic material was also present in Schwann cells of myelinated and unmyelinated nerve fibers (Fig. 1).

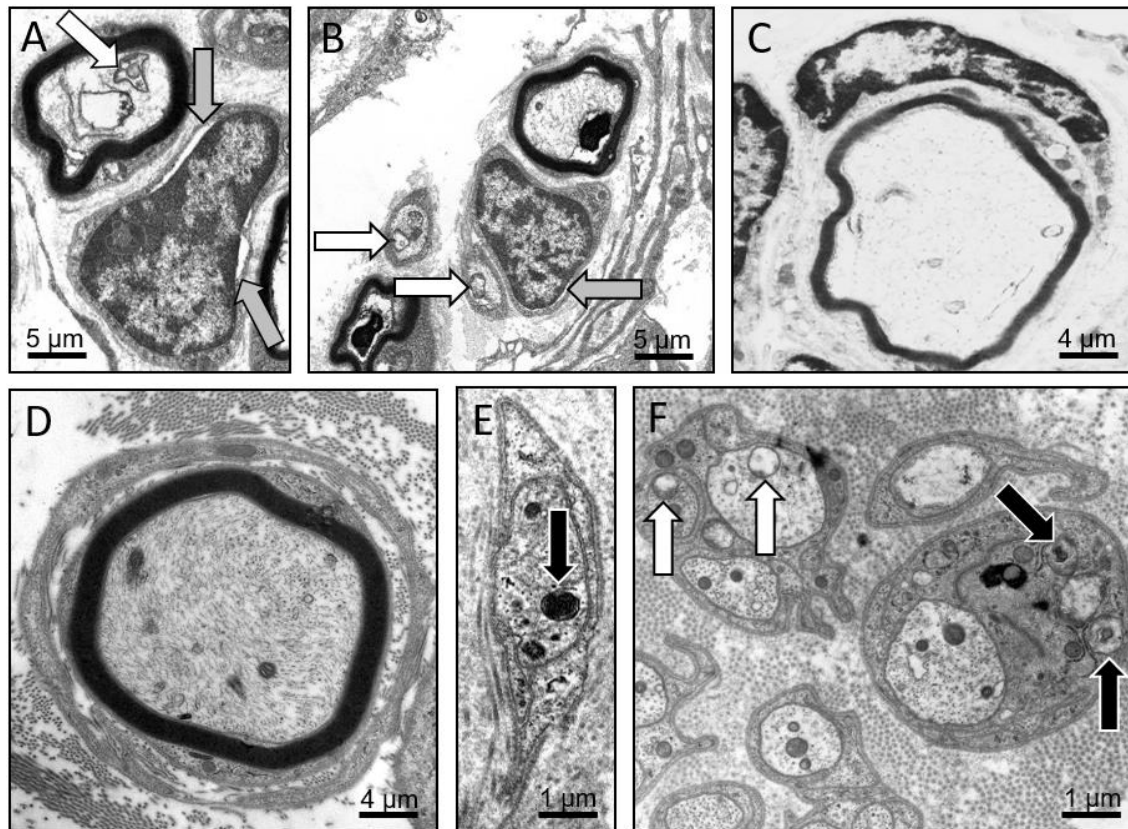


Figure 1: EM of intramuscular nerve fibres in MSS patient skeletal muscle. (A, B) Membranous material and swollen/degenerated mitochondria (white arrows) and nuclear envelope widening combined with chromatin condensation in Schwann cells (grey arrows). **(C, D)** Spheroid-like accumulation of cytoskeletal elements and of organelles associated with a thinning of the myelin sheaths. The nerve fibre depicted in **(D)** is surrounded by a surplus Schwann cell process. **(E)** Autophagic material (black arrow) in an unmyelinated axon. **(F)** Autophagic material (black arrows) in the cytoplasm of a Schwann cell; swollen mitochondria in unmyelinated axons (white arrows).

Similar alterations were found in quadriceps intramuscular nerves of 26-week-old (Fig. 2) and in sciatic nerves of 16 and 26-week-old *woozy* mice (Figs. 3, 4) but not in nerves derived from respective wildtype littermates (data not shown). In addition, prominent widenings of the space between the inner and the outer leaflets of the nuclear envelope in Schwann cells was observed in MSS-patients and these mice; these vacuolar structures often contained granular or membranous electron-dense material (Figs. 1, 2). Aberrant, often widened endoplasmic reticulum structures were observed in both human patient and *woozy* mouse Schwann cells (Figs. 1-4). Axons with disproportionately thin myelin sheaths could be occasionally found in

both, MSS-patients and woozy mice (Figs. 1-3). Notably, even though the deposits of electron dense autophagic material became more prominent in myelinated and non-myelinated axons (11% in 16-week-old and 21% in 26-week-old mutant animals on average) as well as in Schwann cells in 26-week-old animals (10% in 16-week-old and 16% in 26-week-old mutant animals on average) (Figs. 3, 4), the mild perturbations of myelination did not increase with age. Perturbations of the nuclear envelope are detectable in 7% of Schwann cell nuclei on average. The mitochondrial changes occurred with 12% on average in the PNS of 16-week-old mutant mice and with 26% on average in the PNS of 26-week-old woozy animals.

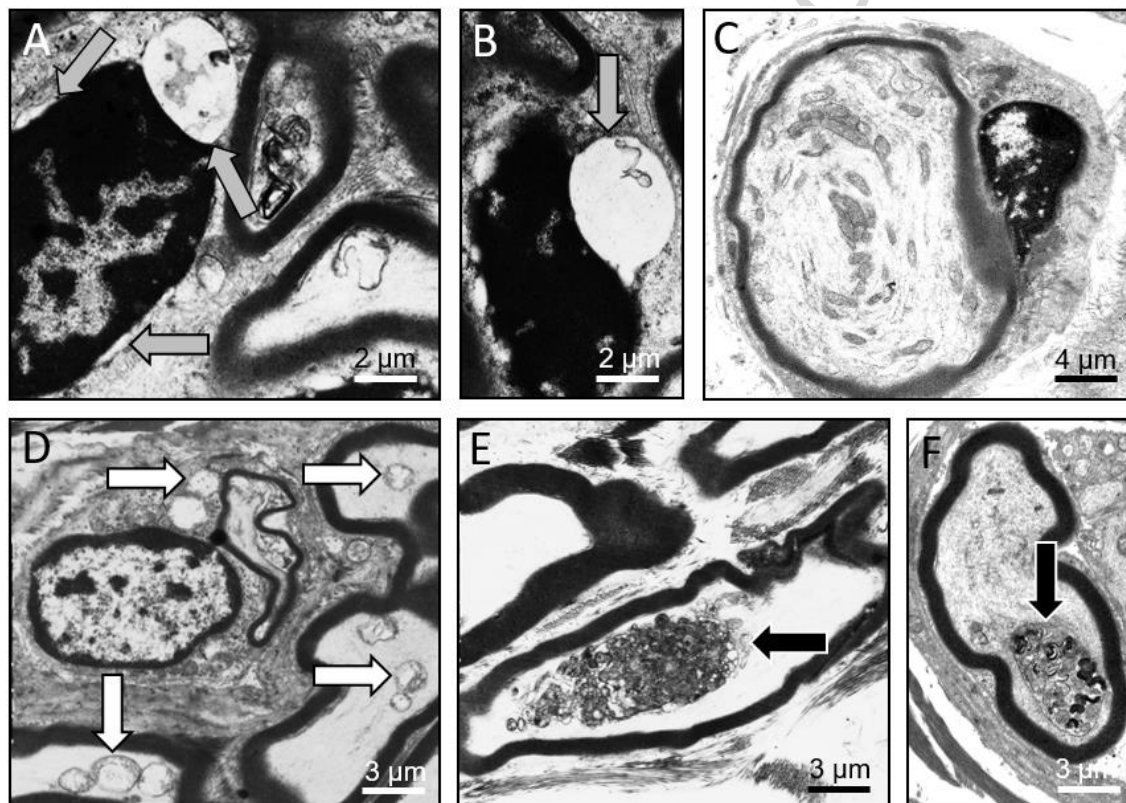


Figure 2: Ultrastructural alterations in 26-week-old woozy mouse intramuscular (quadriceps muscle) nerve fascicles. (A, B) Focal widening (grey arrows) of the nuclear envelope of Schwann cells. (C) Spheroid-like swelling of a myelinated nerve fiber due to intraaxonal accumulation of cytoskeletal elements and organelles. (D) Swollen intraaxonal mitochondria (white arrows). (E, F) Large accumulations of granular and membranous autophagic material in the axons and/or in the adaxonal Schwann cell cytoplasm (black arrows).

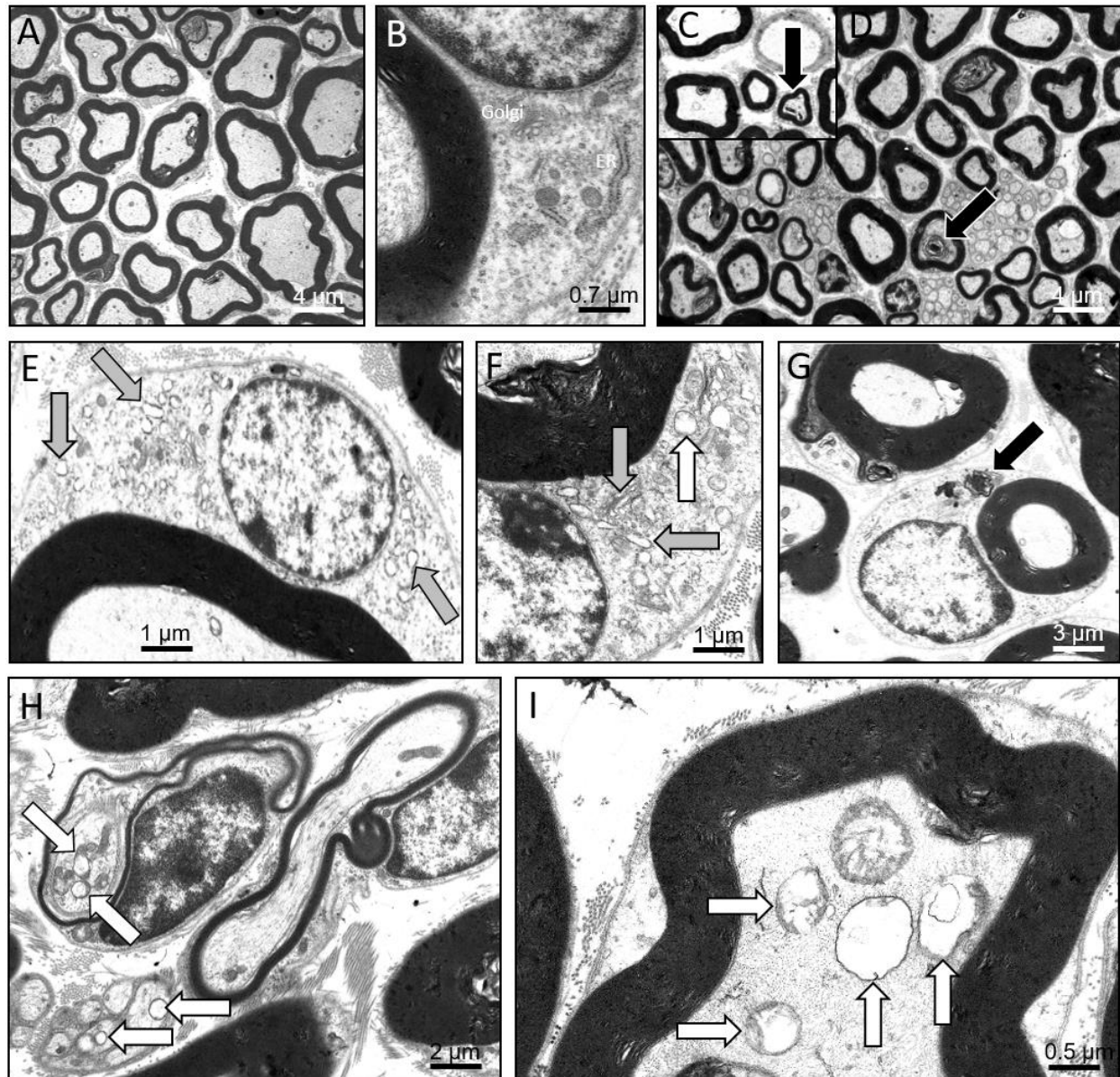


Figure 3: EM of 16-week-old control and woolly mouse sciatic nerves. (A, B) Normal control nerve fibres. **(C, D)** Membranous cytoplasmic bodies (black arrows) in woolly mouse nerve fibres. **(E, F)** Prominent ER/Golgi structures in the cytoplasm of Schwann cells of myelinated fibres (grey arrows) and degenerating mitochondrion (white arrow). **G** Autophagic vacuole in a Schwann cell (black arrow). **(H)** Disproportional thin myelinated axons and **(H, I)** prominent, swollen intraaxonal mitochondria (white arrows).

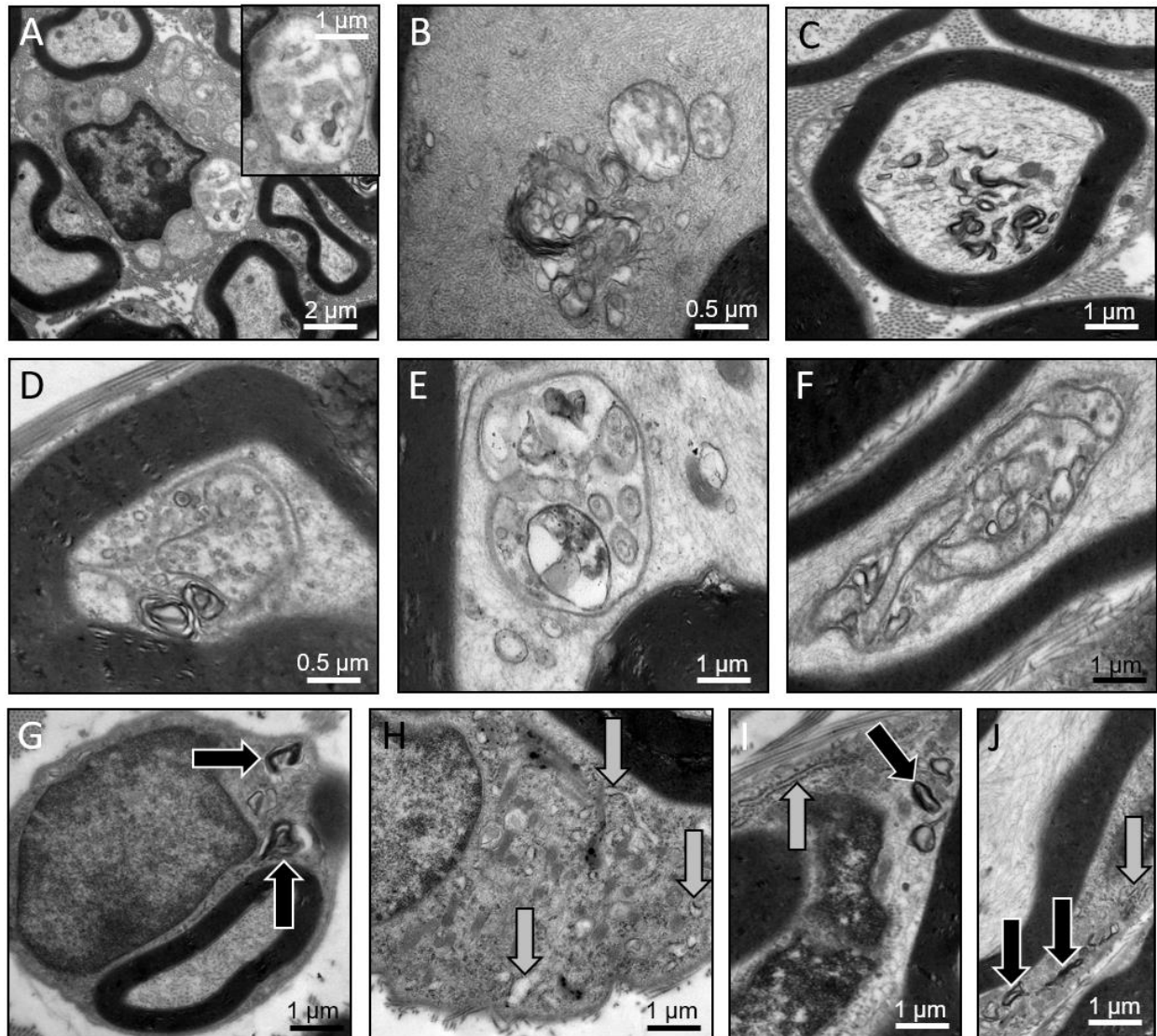


Figure 4: Ultrastructural findings in sciatic nerves of 26-week-old woozy mice. (A) and inset in (A) degenerating unmyelinated axon in Remak bundle. (B, C) Accumulations of autophagic material in axons of myelinated nerve fibres. (D, E, F) Invaginations of the axolemma associated with accumulated autophagic material within the axons and in the adaxonal Schwann cell cytoplasm. (G) Autophagic vacuoles containing membranous material in the cytoplasm of a Schwann cell of a myelinated nerve fibre (black arrows). (H-J) Prominent ER structures (grey arrows) merging with membranous autophagic material (black arrows) in the cytoplasm of Schwann cells.

SIL1 deficiency perturbs integrity of neuromuscular junctions in woozy mice and MSS zebrafish

Prompted by our previous observation that in *woozy* mice, integrity of the NMJs is disturbed and that this is associated with signs of neurogenic atrophy of muscle fibers [18], we investigated NMJs in *extensor digitorum longus* muscles from 26-week-old *woozy* and wild-type mice and in a MSS-zebrafish model. Morphological investigation of a total of 100 NMJs stained with bungarotoxin revealed a statistically significant reduction in the size of NMJs in the *woozy* animals (Fig. 5A, B). Moreover, in mutant mice, the fluorescence intensity of α -bungarotoxin appeared weaker thus suggesting reduced presence of AChR compared to wildtype NMJs (Fig. 5A). Immunofluorescence of NMJs in zebrafish *Sil1* morphants illustrated a disruption of synapse formation along the vertical myosepta with a striking disorganization of presynaptic branching across the muscle fibres of the myotomes compared to those observed in control fish (Fig. 6A). Further studies of myelinating Schwann cells using a Claudin K antibody did not show significant differences between control-morpholino and *Sil1*-morpholino injected fish (Fig. 6B).

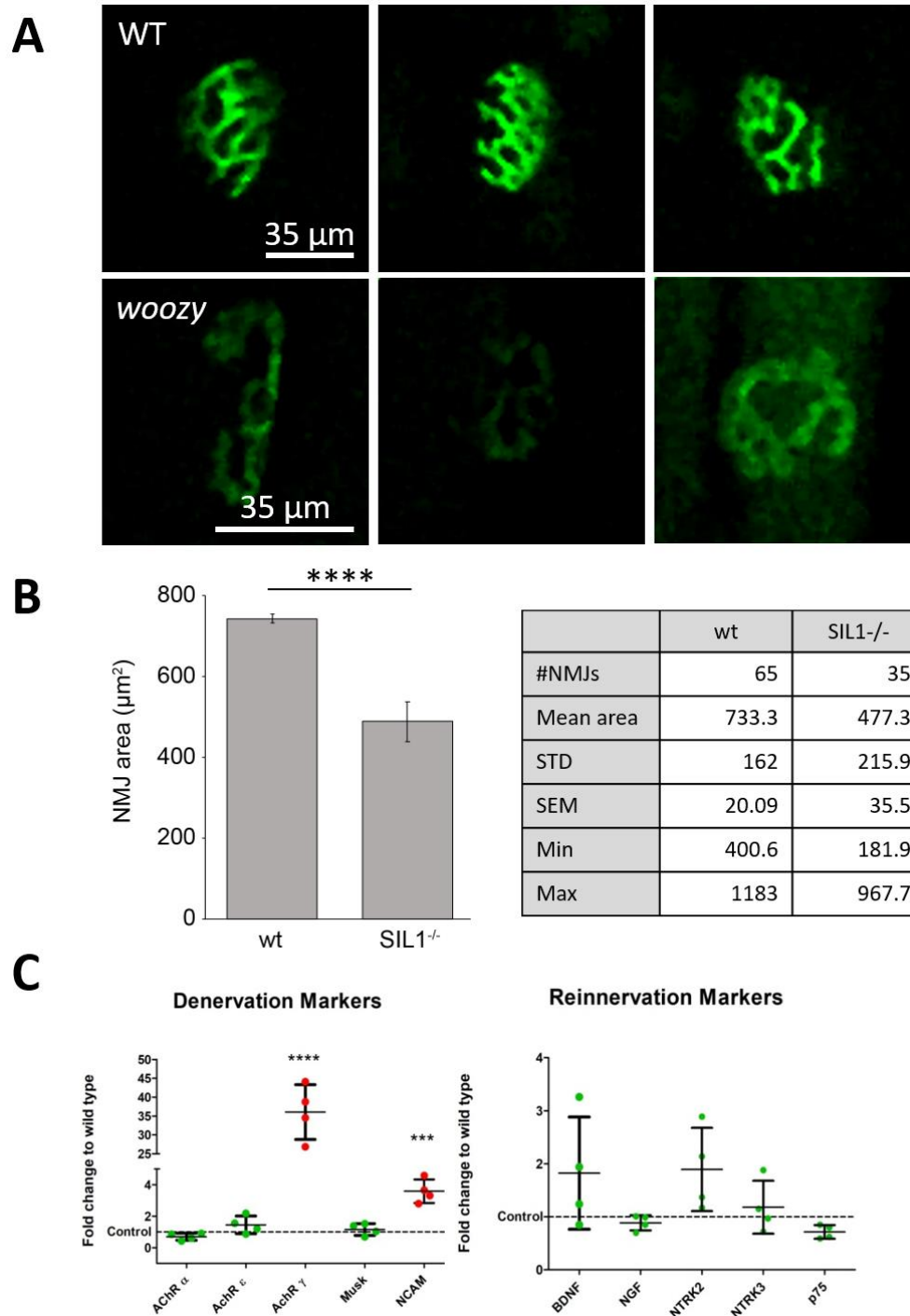


Figure 5: NMJ-analysis of *Sil1*-mutant and wild-type mice (26 weeks of age). (A) Visualization of NMJs via fluorescence-dye conjugated α -bungarotoxin binding to acetylcholine receptors revealed an overall reduced fluorescence intensity in the mutant animals compared to control suggesting reduced level of the receptor upon *SIL1*-deficiency. (B) Analysis of the size of wildtype and *SIL1*-mutant NMJs. (C) Studies of abundances of transcripts encoding for de-innervation (*AChR α* , *AChR ϵ* , *AChR γ* , *Musk*, *NCAM*) and re-innervation markers (*BDNF*, *NGF*, *NTRK2*, *NTRK3*, *p75*) revealed significantly increased abundances of *AChR γ* and *NCAM* transcripts in *Sil1*-mutant animals compared to wildtype littermate controls. *AChR* = acetylcholine receptors; *Musk* = muscle, skeletal receptor tyrosine-protein kinase; *NCAM* = neural cell adhesion molecule; brain-derived neurotrophic factor; *NGF* = β -nerve growth factor; *NTRK* = high affinity nerve growth factor receptor; *p75* = low affinity neurotrophin receptor *p75NTR*.

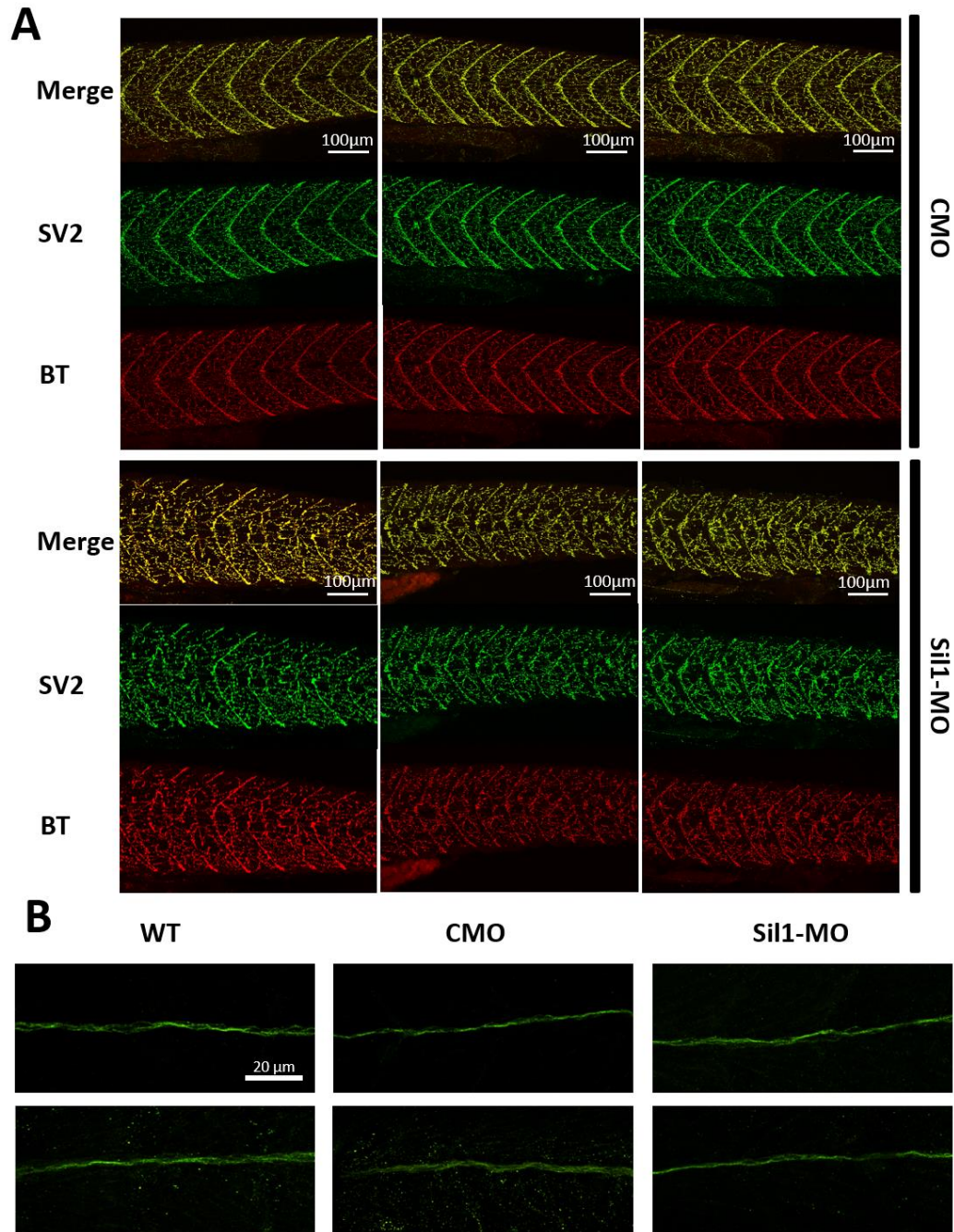


Figure 6: Immunofluorescence studies in 48 hpf MSS-zebrafish. (A) Immunofluorescent studies of NMJs in control-MO (6A) & Si1-MO (6B) injected embryos (6ng of MO-injection respectively). SV2 stains presynaptic motor neurons and α -bungarotoxin detects the postsynaptic AChRs. Si1-MO injected fish display disorganised branching of nerves and of synapses along the vertical myosepta. MO=morpholino, hpf = hours post fertilisation, AChRs = acetelycholine receptors, SV2 = synaptic vesicle protein 2. (B) Staining of myelinating Schwann cells utilizing an anti-Claudin K antibody did not show significant differences between wildtype, control-morpholino (CMO) and Si1-morpholino-injected fish (6C). Scale bar = 20 μ m.

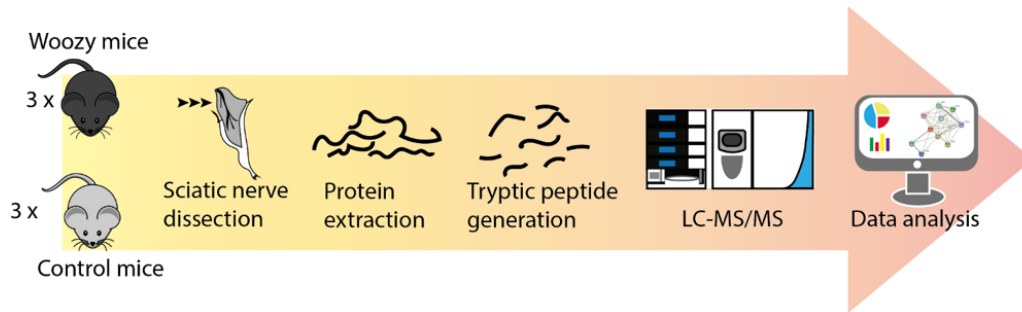
Gene expression analysis of de-innervation and re-innervation

The gene expression analysis of de-innervation and re-innervation markers, performed on *Sil1* mutant and wildtype mice, revealed significant changes supporting the concept of perturbed NMJ integrity and function: among the investigated de-innervation markers, transcripts of *AChR γ* and *N-CAM* were respectively 36.05-fold and 3.58-fold higher in mutant than in wildtype animals whereas the other transcript marker (*AChR α* , *AChR ϵ* , *MuSK*) values were not statistically significant changed in abundances. Re-innervation transcript markers (*BDNF*, *NGF*, *NTRK2*, *NTRK*, *p75*) did not show significant changes in transcript abundances (Fig. 5D).

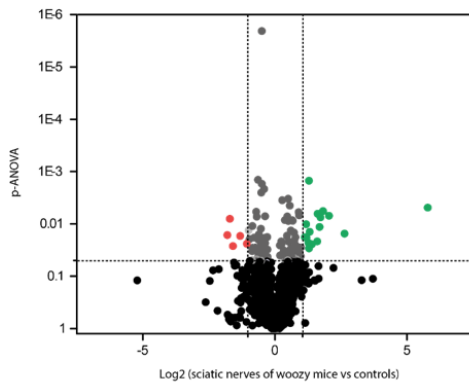
SIL1-mutant sciatic nerves display changes in protein abundances

Proteomics is a powerful tool for the unbiased investigation of pathophysiological processes [30, 31]. Here, we compared *Sil1*-mutant and wildtype sciatic nerves using quantitative mass spectrometry. We found that 1.22% of the quantified proteins (20 out of 1632) were differentially expressed upon loss of functional SIL1 in this tissue: 15 (0.92%) of these proteins were upregulated and 5 (0.3%) downregulated (Fig. 7A-C). The affected proteins are located to the cytoplasm, cellular membranes and the extracellular space (Fig. 7D). For an overview on the regulated proteins and proposed functions, see supplemental table 1. To provide insight into SIL1 neuronal cytopathology, the spectrum of affected proteins was analyzed for enriched gene ontology (GO) terms using STRING [32] (Suppl. Fig. 1).

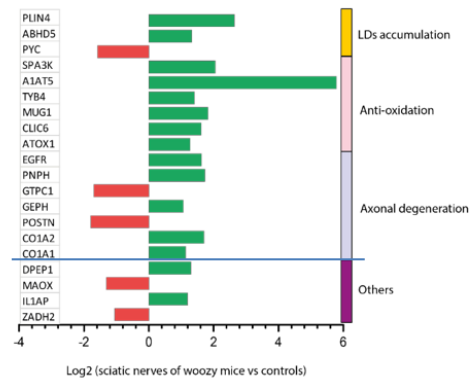
A



B



C



D

down-regulated protein (with at least 2 unique peptides)
down-regulated protein (with 1 unique peptide)
up-regulated protein (with at least 2 unique peptides)
up-degulated protein (with 1 unique peptide)

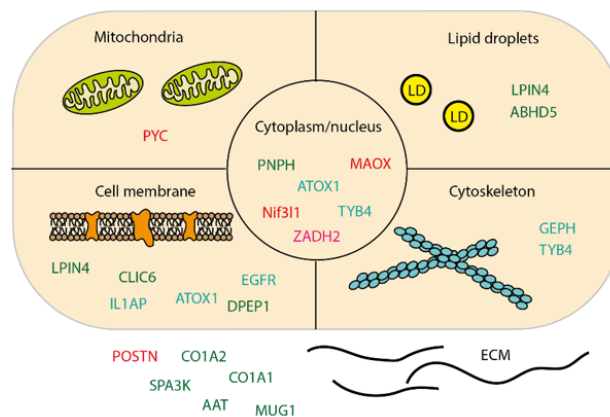


Figure 7: Proteomic profiling of *Sil1*-mutant nerves. (A) Applied workflow. (B) Volcano plot of obtained proteomic results. (C) Diagram presenting the fold of protein-dysregulation and the function of the affected proteins. (D) Schematic presentation of the subcellular localization of the proteins altered in abundances upon the loss of functional *SIL1* in murine sciatic nerves.

Discussion

PNS is vulnerable against the loss of SIL1

Recessive SIL1 mutations cause MSS in human patients and the *woozy* phenotype in mice [13, 15, 23], both with degeneration of Purkinje cells and ataxia. Some MSS patients present with additional Dandy-Walker syndrome [9], spastic paraplegia [10] or motor neuropathy associated with bradykinetic movement disorder [11]. Moreover, *Sil1* knock down in zebrafish by two different antisense oligo morpholinos resulted in loss of Purkinje cells [33]. Hence, a profound role of SIL1 in neuronal function and maintenance can be postulated. This assumption is also supported by the crucial role of (functional) BiP levels for motor neuron survival [34] and observations that SIL1 elevation (*i*) attenuates motor neuron vulnerability in a mouse model of ALS [18] and (*ii*) triggers the expression of proteins with neuroprotective properties [20]. To address the vulnerability of the PNS against SIL1 loss, we performed combined electron microscopic, immunofluorescence, proteomic and selected transcript studies. Results of our ultra-morphological studies on intra-muscular nerve fascicles from two MSS-patients and three *woozy* and wildtype mice revealed affection of myelinating and non-myelinating Schwann cells as well as of axons engulfed by both types of Schwann cells. Hereby, changes in the architecture of the nuclear envelope were observed accompanied by mitochondrial degeneration and aggregates of autophagic material. These aggregates were remarkably prominent in myelinated axons of *Sil1*-mutant mice and were found to be more pronounced in 26- compared to 16-week-old animals. Interestingly, abnormal aggregates of autophagic material and perturbations of nuclear envelope structures as well as mitochondrial degeneration have already been extensively described in MSS-patient and *woozy* mouse muscle as well as in *in vitro* models of the disease [13, 21] suggesting that these ultra-morphological changes are a consistent feature in tissues vulnerable for SIL1 loss. Moreover, this finding is in line with the

results of a study linking levels of functional BiP to the activation of autophagy as a mechanism acting towards the breakdown of protein aggregates and to axonal degeneration [34].

The major differential diagnosis of MSS is the congenital cataracts, facial dysmorphism and peripheral neuropathy (CCFDN) syndrome due to a recurrent recessive mutation in *CTDP1*. CCFDN is associated with a symmetric, distal peripheral neuropathy with a predominant motor phenotype. Secondary scoliosis and foot deformities are common. Sensory neuropathy develops after age ten years [35]. Interestingly, scoliosis has also been described in 61% of patients with *SIL1* mutations [36]. Our present findings suggest that MSS is phenotypically even more reminiscent to CCFDN, as neuropathy appears to be a consistent feature of *SIL1* mutation in patients and mice. However, the buildup of autophagic material in MSS and *woozy* mice was present predominantly in axons, whereas the neuropathy in CCFDN was described to be hypo/demyelinating [35]. Moreover, myelination also seemed to be normal in the MSS-zebrafish model.

NMJs are vulnerable to the loss of SIL1

Axonal neuropathies often go along with defects of neuromuscular junctions (NMJs) [37]. Therefore, we sought to determine whether perturbed NMJ integrity is a pathophysiological aspect of *SIL1* deficiency. Immunofluorescence of NMJs in *woozy* mice revealed smaller NMJs and weaker fluorescence intensity for α -bungarotoxin, suggesting reduced presence of acetylcholine receptor (AChR) subunits. Immunofluorescence of our MSS zebrafish model confirmed impaired NMJ integrity, supporting our findings obtained in *woozy* mice and demonstrating that this pathophysiological feature manifests across different species. Alterations of AChR density might, at least partially, result from a disturbed *SIL1*-BiP machinery involved in the folding of muscle membrane proteins such as AChRs. In this context, it is important to note that agrin which is secreted by axon endings and crucial for clustering of AChRs is also itself a substrate of a neuronal *SIL1*-BiP machinery. Our findings confirm our

previous report of perturbed NMJ integrity in *woozy* mice [18], and indicate that impairment of neuromuscular transmission is part of MSS pathophysiology. The concept of a pre- and post-synaptic impact is supported by results of our proteomic studies (discussed below in more detail) showing that gephyrin, a synapse/ NMJ-associated protein [38] is affected by Sil1-mutation in murine sciatic nerves and by results of our targeted skeletal muscle transcript studies showing that NCAM as well as AChR γ display altered transcript abundances. Neural cell adhesion molecule (NCAM) plays a crucial role in the development and maturation of NMJs and is required for stability of re-innervated NMJs also by acting as a signal for regenerating axons [39]. Thus, the *NCAM* increase most likely is a compensatory mechanism preventing NMJs from complete breakdown in MSS. The strong increase of *AChR γ* transcript levels might also compensate for decreased AChR presence at the NMJs.

Proteomic changes in sciatic nerves of wozzy mice allow molecular insights into PNS vulnerability

To obtain biochemical insights into PNS vulnerability upon SIL1-loss, comparative proteome profiling via a label-free approach was performed. To this end, the proteomic signature of SIL1-deficient sciatic nerve was examined utilizing three biological replicates derived from *woozy* and wildtype animals aged 26 weeks. Some of the proteins found to be affected prominently such as perilipin are major regulators of lipid homeostasis which is important for mitochondrial integrity and function [40-43]. Pyruvate carboxylase (PYC) is a mitochondrial protein catalyzing ATP-dependent carboxylation. As fatty acid oxidation can be attenuated by PYC-inhibition, a decrease in SIL1-mutant nerves most likely serves as a protective mechanism. However, vulnerability of PYC activity in both non-synaptic and synaptic mitochondria against cellular stress burden has been demonstrated [44] suggesting that decreased abundance of PYC in SIL1-mutant nerve might correspond with mitochondrial perturbations on the morphological level as well as with impaired function of the NMJs discussed above.

Mitochondrial defects promote ROS production [45], a fact which is in line with the detected increase in antioxidant factors upon SIL1 deficiency, exemplified by the up-regulation of Murinoglobulin-1 (MUG1), Serine protease inhibitor A3K (SA3K), Chloride intracellular channel protein 6 (CLIC6), Alpha-1-antitrypsin 1-5 (A1AT5), Thymosin beta-4 (TYB4) and Copper transport protein ATOX1 [46-48]. Interestingly, TYB4 improves neurological functional outcome and axonal remodeling after embolic stroke in rats also suggesting a neuroprotective function of this protein [49]. EGFR promotes intrinsic axonal regeneration [50]. Similarly, as purine nucleoside phosphorylase (PNPH) deficiency results in a disorder characterized by recurrent infections, neurologic symptoms (rigid muscles, ataxia, developmental delay, and intellectual disability) [51, 52], its increased abundance in *Sil1*-mutant PNS may also be a protective mechanism. Collagen nerve guides support axonal regeneration of the peripheral nerve [53] and COL1A1 and COL1A2 have been identified with increased abundance in woody nerves. Gephyrin is well known to promote nerve survival by establishing synaptic specificity at the NMJs [54]. Periostin (POSTN) has been identified to play a key role in axonal regeneration [55]. Additionally, secreted POSTN has been shown to promote nerve regeneration in patients with peripheral neuropathies [56]. Therefore, the down-regulation of POSTN in the global profile either hints for an increasing activity of secreted POSTN in the extracellular space in axonal regeneration or contributes to axonal vulnerability. However, increased abundance of proteins with protective potential does not only indicate a vulnerability of the PNS against loss of functional SIL1, but also might explain why PNS pathology is subtle compared to cerebellar and skeletal muscle phenotype. In this context, it is worth noting that recently one of our molecular studies on organ vulnerability in MSS suggested that the presence of antagonizing factors modifies the vulnerability of cells/ tissues against loss of functional SIL1 [57].

Conclusion

Results of our combined morphological and biochemical studies suggest that the PNS along with the NMJs are vulnerable to SIL1-deficiency in human, mouse, and zebrafish. Only few nerve fibers showed disproportionately thin myelin sheaths. In contrast, axonal mitochondria seem to be affected by the loss of SIL1, and the build-up of autophagic material in axons is progressive. Up-regulation of proteins supporting axonal survival not only support the concept of PNS vulnerability to SIL1-loss but might also explain why other cellular populations are more vulnerable. Moreover, our findings highlight that the presence of PNS pathology might be added to the spectrum of MSS and should be considered when delineating MSS from CCFDN in the clinical setting.

Abbreviations

A1AT5A: alpha-1-antitrypsin 1-5; AChR: acetylcholine receptor; ALS: Amyotrophic Lateral Sclerosis; ANOVA: analysis of variance; ATOX1: copper transport protein; CCFDN: congenital cataracts, facial dysmorphism and peripheral neuropathy; CLIC6: chloride intracellular channel protein 6; COL1A1 and COL1A2: collagen alpha 1 type 1 and 2; ER: endoplasmic reticulum; FASP: filter-aided sample preparation; FDR: false discovery rate; GRP78, BiP: 78 kDa glucose-regulated protein; HCD: high collision induced dissociation; HSP: heat shock protein; GO: gene ontology; MSS: Marinesco-Sjögren Syndrome; MOs: morpholino oligonucleotides; MUG1: murinoglobulin-1; NCAM: neural cell adhesion molecule; NMJs: neuromuscular junctions; NTC: no-template control; PNPH: purine nucleoside phosphorylase; PNS: peripheral nervous system; POSTN: periostin; PYC: pyruvate carboxylase; ROI: region of interest; RT: room temperature; SA3K: serine protease inhibitor A3K, TYB4: thymosin beta-4; UPR: unfolded protein response

Declarations

Ethics approval and consent to participate

Work with human MSS-samples (originally collected for diagnostic purposes) for further research studies has been approved by the ethical review committee of the University Hospital of RWTH-Aachen University (EK104/10). Work with wozy and wildtype mice was reviewed and approved by the Animal Care Committee of the University of Aachen (RWTH-Aachen). All mice were handled according to the guidelines from the Federation for European Laboratory Animal Science Association (FELASA).

Consent for publication

Not applicable

Availability of data and material

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009022.

Competing interests

The authors declare that they do not have any competing interests

Funding

This work was supported by a grant from the START program of RWTH Aachen University (to A. R.; Grant No. 41/12), the French Muscular Dystrophy Association (AFM-Téléthon; #21644; grant to A.R.) and EU Joint Program Neurodegenerative Diseases (JPND), the German Federal Ministry of Education and Research (BMBF; CMT-Net 01GM1511D) and the DGM (MND-Net) to J.W. Financial support by the Ministerium für Innovation, Wissenschaft und Forschung des Landes Nordrhein-Westfalen, the Senatsverwaltung für Wirtschaft, Technologie und Forschung des Landes Berlin and the Bundesministerium für Bildung und Forschung is gratefully acknowledged.

Authors' contributions

A.R., R.H., H.L. and J.W. designed the study. Proteomic profiling was carried out by V.P. and A.R. and transcript studies have been performed by S.C. and S.S.; Animals were prepared by

S.B. and T.S. and studies on murine NMJs were carried out by T.S. E.O. and R.R.; zebrafish models were generated and analysed by D.H. and D.C.; H.H.G. provided images of intramuscular nerve fibres of the MSS-patients and electron microscopic studies of murine muscle and nerve samples have been carried out by A.R. and J.W.

Acknowledgements

We thank Hannelore Mader and Claudia Krude for expert technical assistance.

ACCEPTED MANUSCRIPT

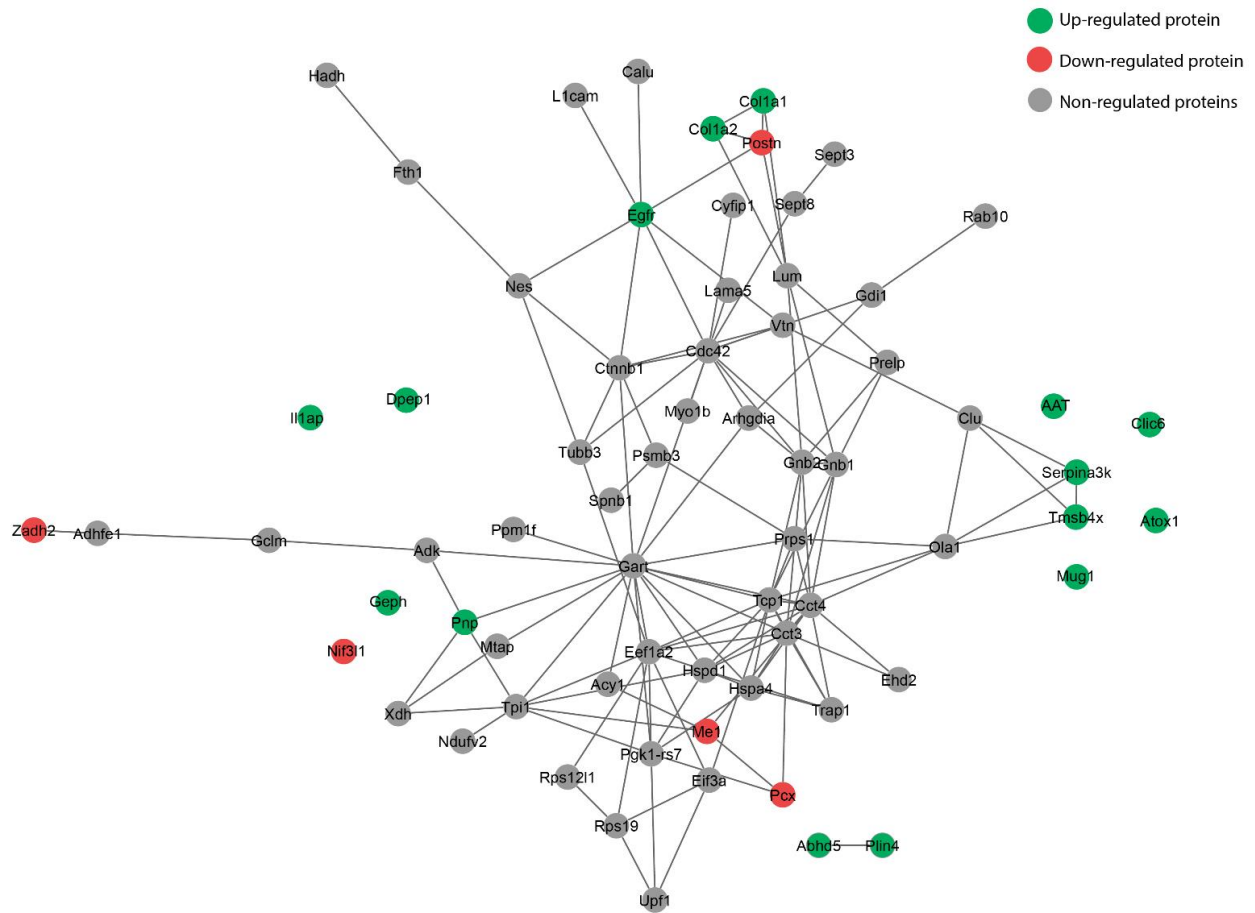
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Supplemental Figure 1: STRING-network analysis of proteomic findings.

Note: Green: up-regulated protein

Red: down-regulated protein

Accession number	Name	Subcellular localization	Function	Ref.
O88492	Perilipin 4	Cell membrane, lipid droplet	Formation of lipid droplets (LDs) by coating their surface and selectively recruiting lipases and other proteins. Inactivation of PLIN4 reduces LD accumulation in mice.	[1, 2]
Q9DBL9	1-acylglycerol-3-phosphate O-acyltransferase (ABHD5)	Lipid droplet	Accumulation of ectopic fat by activating ATGL. Interaction of ABHD5 with ATGL and the mitochondrial PLINs at LD surface enhances mitochondrial LD accumulation.	[3, 4]
Q05920	Pyruvate carboxylase, mitochondrial (PYC)	Mitochondria	PYC decrease causes ATP deficit leading to decrease of cytochrome c oxidase activity and oxidative phosphorylation in turn resulting in a severe mitochondrial damage. However, in response to reduction of ATP production, the energy sensor AMP-activated protein kinase (AMPK) pathway is activated and inhibits energy-consuming processes including protein, carbohydrate and lipid biosynthesis by inactivating lipid metabolic enzymes such as acetyl-CoA carboxylase (ACACA and ACACB).	[5, 6]
P07759	Serine protease inhibitor A3K (SA3K)	Secreted	Protecting cells against oxidative stress. SA3K also elevates SOD, a key antioxidant factor of ROS. Furthermore, SA3K inhibits NADPH oxidase 4 (NOX4) which functions in catalyzing the reduction of molecular oxygen to ROS.	[7, 8]
Q00898	Alpha-1 antitrypsin (AAT)	Secreted	Mutant AAT or AAT deficient mice show inflammation, high ROS-level and a more oxidized, cellular redox state. Moreover, AAT injection/overexpression can not only inhibit oxidative stress but also promote proliferation and migration and suppress apoptosis.	[9-11]
	Thymosin beta-4 (Tβ4)	cytoskeleton	Regulates multiple cell signalling pathways related to cell proliferation, differentiation and modulation of inflammatory mediators by targeting the antioxidant SOD enzymes and increasing their expression	[12, 13]

			therefore reducing the intracellular ROS level. Moreover, T β 4 can increase expression of catalase enzymes and thus protects cells against oxidative damage. In addition, T β 4 is an actin sequestering protein and thus plays a role in organisation of cytoskeleton.	
P28665	Murinoglobulin-1 (MUG1)	Secreted	MUG1 is an inhibitor for Neuropsin , a protein responsible for visual and non-visual photoreception. Notoriously, inactivation of Neuropsin has a positive effect against stress and promoting synaptic plasticity.	[14, 15]
Q8BHB9	Chloride intracellular channel protein 6 (CLIC6)	Cell membrane	CLIC6 has an antioxidant effect by interacting with dopamine D receptors. Moreover, a transcriptome study on chronic mild stress (CMS) linked CMS to deregulation of genes such as <i>CLIC6</i> involved in dopamine receptor mediated signaling pathway. Hence, the up-regulation of CLIC6 in woody nerves might accord with stress protection.	[16]
O08997	Copper transport protein ATOX1 (ATOX1)	Cytoplasm	ATOX1, an antioxidant copper chaperone, binds and transports cytosolic copper to copper transporting ATPase proteins (such as ATP7A) in the trans-Golgi network and various endocytic vesicles. This maintains copper levels in cytosol and mitochondria which is required for maturation of cytochrome c oxidase. ATOX1 deficient mice have lower SOD activity due to low efficiency of copper loading. Many studies demonstrated protective functions of ATOX1 against hydrogen peroxide-induced oxidative damage and the inactivation of ATOX1 greatly increases cell sensitivity to stress.	[17]
Q01279	Epidermal growth factor receptor EGFR	Cell membrane	EGFR promotes axonal regeneration via modulation of the neuronal intrinsic regenerative ability.	[18]
P23492	Purine nucleoside phosphorylase (PNPH)	Cytoplasm	PNPH cleaves inosine into hypoxanthine and ribose-1-phosphate which is necessary for many essential biochemical processes in the nervous system. It has been shown that the uric acid hypoxanthine induces axonal growth and has antioxidant effects in multiple sclerosis, stroke, and other neurodegenerative diseases.	[19, 20]
Q9EQ80	Putative GTP	Cytoplasm,	NIF3L1 negatively regulates expression of genes	[21]

	cyclohydrolase 1 type 2 NIF3L1	nucleus	involved in neuronal differentiation. Hence, its decrease suggests promotion of expression of differentiation-related genes in woody nerves.	
Q8BUV3	Gephyrin (GEPH)	Cell junction, synapse	Microtubule-associated protein involved in membrane protein-cytoskeleton interactions. Anchors the inhibitory glycine receptor (GLYR) to subsynaptic microtubules. Its increase might accord with a cellular strategy compensating the vulnerability of the NMJs.	[22, 23]
Q62009	Periostin (POSTN)	Secreted, extracellular matrix	Expressed in Schwann cell precursors and Schwann cells, and is particularly high in migratory Schwann cells. Stimulation of Schwann cells with NRG1 or TGF β -1 results in elevated periostin expression. Astroglial-derived periostin promotes axonal regeneration after spinal cord injury thus suggesting a protective effect of this secretory protein.	[24, 25]
Q01149	Collagen alpha- 2(I) chain (CO1A2)	Secreted, extracellular matrix	Impaired extracellular matrix (ECM) degradation perturbed nerve regeneration and the transformation into a mature ECM is necessary for efficient nerve regeneration. Upregulation of collagens is supported by increase of the serine protease inhibitor AAT which degrades many extracellular matrix components such as elastin, collagens and fibronectin.	[26-29]
P11087	Collagen alpha- 1(I) chain (CO1A1)	Secreted, extracellular matrix		
P31428	Dipeptidase	Cell membrane	Hydrolyzes a wide range of dipeptides and modulates cell proliferation	http://www.uniprot.org

Supplemental Table 1: Description of subcellular localization and function of proteins affected by SIL1-deficiency in murine sciatic nerve.

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Highlights:

- Electron microscopic studies identified vulnerability of the peripheral nervous system against the loss of functional SIL1 by the build-up of protein aggregates, perturbations of the nuclei of Schwann cells and mitochondrial degeneration
- Proteome profiling of sciatic nerve specimens from woozy mice revealed altered levels of proteins implicated in neuronal maintenance suggesting the activation of compensatory mechanisms
- Perturbed integrity of the neuromuscular junctions (NMJs) could be identified upon the loss of functional SIL1 in mice and zebrafish
- Results of transcript studies support the concept of NMJ-vulnerability against SIL1-loss