PCSK9 deficiency promotes the development of peripheral neuropathy

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Conflicts of interest -

Financial support - AKJ is the recipient of a PhD scholarship from La Fondation pour la Recherche Médicale (Paris, France) and from the European Union and Région Réunion (Saint-Denis, Réunion); GL is the recipient of a project grant KRINGLE2 ANR-20-CE14-0009 funded by the Agence Nationale de la Recherche (Paris, France); SB is the recipient of a program grant ATIP-AVENIR funded by Inserm (Paris, France).

Abbreviations

ApoER2: Apolipoprotein E receptor 2

ASCVD: Atherosclerotic cardiovascular diseases

CD36: Cluster of differentiation 36

DRG: Dorsal root ganglia

IENFD: Intraepidermal nerve fiber density

LDLR: Low-density lipoprotein receptor

LRP1: LDLR-related protein 1

NARC-1: Neural Apoptosis-Regulated Convertase-1

NCV: Nerve conduction velocity

PCSK9: Proprotein convertase subtilisin/kexin type 9

PNS: Peripheral nervous system

SCs: Schwann cells

VLDLR: Very-low-density lipoprotein receptor

Abstract

PCSK9 is a serine protease primarily produced and secreted by the liver. Its best-known and studied function is to induce the hepatic degradation of the low-density lipoprotein (LDL) receptor (LDLR), thereby increasing the concentration of LDL-cholesterol (LDL-C) in the blood. Beyond its effects on LDL-C metabolism, recent studies have reported pleiotropic biological roles for PCSK9 notably in septic shock, vascular inflammation, viral infection, and cancer. While the function and the structural integrity of peripheral nerves are critically influenced by circulating lipids and cholesterol levels, the impact of PCSK9 in the peripheral nervous system is still unexplored. In this study, we investigated the consequences of PCSK9 deficiency on the physiology of peripheral nerves. We found that PCSK9 deletion in mice leads to peripheral neuropathy characterized by a reduction of mechanical pain sensations. PCSK9 deficient mice also presented skin structural changes with a reduction of number of terminal nociceptive Schwann cells, Remak fiber axonal swelling, as well as hypomyelination of small nerve fibers. Interestingly, peripheral nerves of PCSK9 deficient mice presented an upregulation of the fatty acid transporter CD36 expression which correlated with an increase in nerve lipid contents and structural mitochondrial abnormalities. Our findings demonstrate that PCSK9 plays a critical role in the peripheral nerves by regulating lipid homeostasis, and through its impact on CD36, PCSK9 deficiency could lead to the development of symptoms related to peripheral neuropathy.

Key words: PCSK9, CD36, peripheral neuropathy

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a natural circulating inhibitor of the low-density lipoprotein receptor (LDLR) (1). It binds to the LDLR epidermal growth factor-A (EGFA) domain, and targets it for lysosomal degradation, which prevents LDLR recycling at the cell surface. As a result, PCSK9 increases the circulating levels of LDL-cholesterol (LDL-C). PCSK9 loss-of-function mutation carriers have reduced LDL-C levels and are protected against atherosclerotic cardiovascular diseases (ASCVD) (2), whereas PCSK9 gain-of-function mutation carriers present with familial hypercholesterolemia and are at high ASCVD risk (3). These observations led to the successful development of PCSK9 inhibitors, a new class of LDL lowering drugs, prescribed on top of statins, that have demonstrated their efficacy in large cardiovascular outcome trials (4, 5). In addition, PCSK9 also reduces the abundance of LDLR related receptors, including the very-low density lipoprotein receptor (VLDLR), the LDLR related proteins 1 (LRP1) and 8 (LRP8, also known as apoER2), as well as the fatty acid transporter and scavenger receptor CD36 in different tissues (1).

PCSK9 is primarily expressed by the liver and circulating PCSK9 is almost exclusively of hepatic origin (6), but the protein is expressed in several other tissues, including the intestine, kidneys, pancreas, and the nervous system (7, 8). PCSK9 was initially cloned in cerebellar neurons undergoing apoptosis and originally called Neural Apoptosis-Regulated Convertase-1 (NARC-1) (7). There is evidence linking PCSK9 to lipid homeostasis as well as neuronal differentiation and inflammation in the central nervous system (CNS) (9). In the peripheral nervous system (PNS), PCSK9 expression has been observed in a rat Schwann cell (SC) line (7) and in human tibial nerves at levels deemed physiologically relevant (10), but the exact functions of PCSK9 in the PNS are still unknown.

Lipids play a key role in maintaining the structural and functional integrity of peripheral nerves (11). Despite their capacity to endogenously synthesize lipids, (12, 13), neurons (14) and SC (15) can take up lipids from the circulation, which is underlined by the abundant expression of lipoprotein receptors in the PNS. For instance, SC express the LDLR, LRP1, and LRP8 (16–18). These receptors are also expressed in the dorsal root ganglia (DRG) along with the VLDLR (19). Given the importance of lipid homeostasis in peripheral nerves, the central role of PCSK9 in lipid metabolism, and the abundant expression of its target receptors, we hypothesized that PCSK9 could play an important role in regulating lipid metabolism in the PNS. In the present study, we show that PCSK9 is expressed in peripheral sensory neurons and in SCs. We found that PCSK9 deficiency in mice markedly reduces their behavioral responses to light and acute

mechanical pain stimuli. We also observed a significant increase in CD36 protein levels and lipids content in peripheral nerves of mice lacking PCSK9. Lipid metabolism and energy production alteration were associated with mitochondrial dysfunction and axonal swelling in these mice. Together, our data demonstrate that PCSK9 plays a critical role in the regulation of lipid homeostasis in the peripheral nervous system by regulating the expression and function of CD36.

Materials and methods

Animals

All *in vivo* experiments were conducted in accordance with the European Community Guidelines for the Use of Animals in Research (86/609/EEC and 2010/63/EU) and approved by the local Ethics Committee for animal experimentation (APAFIS#3209-2015111215451823v2). *B6;129S6-Pcsk9tm1Jdh/J* (*Pcsk9* KO) (Jackson Laboratory, Bar Harbor, ME) and *C57BL/6J* wild type (WT) mice were kept under a controlled light/dark cycle (12 hours of light/12 hours of dark), under temperature-controlled conditions (21°C) and standard humidity. Mice were fed chow diet ad libitum with free access to water. Mice were anesthetized with isofluorane and euthanized by intracardiac puncture. Blood samples were collected for plasma isolation by centrifugation and tissues (liver, DRG, sciatic nerves, foot pads) were snap frozen in liquid nitrogen. Plasma and tissue samples were stored at -80°C until use. All mice experiments were performed at 24 weeks of age unless mentioned otherwise.

Immunohistochemistry

Right after euthanasia, mice were perfused with 20 mL 1X PBS followed by 15 mL of 4% paraformaldehyde (PFA) dissolved in 1X PBS. Sciatic nerves, foot pads, and DRGs were dissected and fixed overnight in 4% PFA at 4°C. Tissues samples were washed 3 times (10 min) in cold PBS, cryoprotected overnight in a PBS solution containing 30% sucrose, and embedded in OCT compound, and stored at -80 °C, until use. Frozen tissues were cut into 14-µm sections (20-µm for foot pads), placed onto glass slides, dried on a heating plate for 10 mins. The slides were stored at -20°C until use. Sections were rehydrated in PBS, permeabilized with PBT (PBS + 0.1% Triton X-100), blocked in donkey serum (Sigma-Aldrich, Saint-Quentin-Fallavier,France), prior to an overnight incubation with primary antibodies (Supplemental Table). For PCSK9 immunostaining, we added an antigen retrieval step by incubating the sections for 10 min in 10 mM citrate buffer, pH 6.0 at 95°C. Slides were washed 3 times in PBT for 10 mins, incubated with fluorescent secondary antibodies (Supplemental Table) for 1 hour, and washed again as above. Nuclei were counterstained with DAPI (0.5 µg/ml), slides were washed again and then mounted and kept at 4 °C.

Rnascope

RNA in situ hybridization was performed using the RNAscope Multiplex Fluorescent Reagent Kit Assay (Advanced Cell Diagnostics, Inc., Newark, CA) according to the manufacturer's instructions. RNAscope probes used were as follows: Pcsk9 (Cat No. 498111), Sox10 (Cat No.

435931), Rbfox3 (NeuN) Cat No. 313311-C2 and Ldlr (Cat No. 443701). Nuclei were ultimately counterstained with DAPI.

Image acquisition and analysis

Images were acquired on a laser scanning confocal microscope Eclipse Ti2 (Nikon) with identical parameters (laser power, iris diameter, and gain) to acquire images from all sections.

Mouse and human primary Schwann cell culture

Primary Schwann cell cultures were isolated as follows: Sciatic nerves of 6 mouse pups between postnatal days 2 and 4 (P2 and P4) were collected in L15 medium (Thermofisher, Massachusetts, USA) and incubated for 30 min in 2 mg/mL collagenase II and then 10 min in 0.25 % trypsin containing EDTA at 37 °C. Nerves were dissociated into single-cell suspensions with a 1mL pipet and then through an 18-G/21-G needle. Schwann cells were resuspended in primary Schwann cell medium (ScienCell #1701, California, USA) supplemented with 1 µM insulin, 2 µM forskolin, and 10ng/mL Nrg1ß (20).

Human primary Schwann cells isolated from human spinal nerve were commercially obtained from Sciencell (California, USA) Catalog No. 1700. Cells were maintained in primary Schwann cell medium.

Mouse primary sensory neurons culture

Primary sensory neuron cultures were isolated as follows: dorsal root ganglia of 6 mouse pups between postnatal days 2 and 4 (P2 and P4) were collected in PBS-Glucose (0.3 µM) and incubated for 30 min in 0.125% collagenase D (Roche, Switzerland) and then 10 min in 0.25 % trypsin at 37 °C. Ganglia were dissociated into single-cell suspensions with a glass Pasteur pipette. Sensory neurons cells were resuspended in Neurobasal (NB) Gibco™21103049 (Thermofisher, Massachusetts, USA) supplemented with B27 Gibco™ 17504044 (Thermofisher, Massachusetts, USA).

Biochemistry and western blot analyses

Cholesterol and triglyceride contents in plasma and tissue samples were determined using colorimetric assays (Diasys, Holzheim, Germany). Concentration of PCSK9 in the plasma and tissue samples were assessed by ELISA (Quantikine MPC900, R&D Systems, Minneapolis, MN). Protein extracts were obtained after lysis of tissues in 350-µL Tris HCl buffer pH=9.5 (10 mM) containing EDTA (1mM), NaCl (150 mM) supplemented with the Halt™ Protease and

Phosphatase Inhibitor Cocktail (Thermofisher, Massachusetts, USA). Proteins (≈20µg) were separated by sodium dodecyl sulfate 7.5% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were blocked in tris-buffer saline containing 5% (w/v) non-fat dry milk, 0.1% (v/v) Tween 20 (TBS) for 1 h at room temperature before incubation with the primary antibodies overnight at 4 °C. Membranes were washed in TBS-T and incubated for 90 min at 25°C with HRP-conjugated secondary antibodies. Immunoreactive bands were detected by chemiluminescence (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare Life Sciences, Vélizy-Villacoublay, France) and images were acquired with an Amersham Imager 600 (GE Healthcare, Vélizy-Villacoublay, France) and analyzed with the ImageJ software (Maryland, USA). Throughout, results were normalized to β-actin expression.

Behavioral assessment of peripheral neuropathy

von Frey test

Mice were placed in a plastic chamber (10 x 10 x 14 cm) on an elevated wire grid. They were habituated to the testing apparatus for 1h/day during a week before behavioral testing. The plantar surface (glabrous) of the hind paw was stimulated with a set of calibrated von Frey filaments (0.008 - 6 g) (Bioseb - *In Vivo* Research Instruments, Vitrolles, France). The paw withdrawal threshold (PWT) was determined as described previously (21). Von Frey responses were determined as the percentage of withdrawal using specific filament (0.07, 0.16, 0.4, 0.6, 1,1.4 g). Each filament was tested five times in increasing order.

Pinprick test

Mice were placed in a plastic chamber on an elevated wire grid. The plantar surface of the hind paw was stimulated with a pin gently applied without moving the paw or penetrating the skin. Pin stimulation was repeated 5 times on different areas with a 1–2 min interval between each stimulation. The percentage of positive attempts (paw withdrawal) was calculated.

Hargreaves test

In the Hargreaves test, we measured the response to radiant heat. Mice were placed in a plastic chamber on a glass floor and the infrared heat stimulus from the plantar test (Ugo Basile, France) was applied to the hind paw. The latency for the animal to withdraw the hind paw was

measured. The beam intensity was adjusted at 20% so that control mice displayed a latency of 8–12 s. A cutoff time of 30 seconds was used to avoid tissue damage.

Brush test

To assess sensitivity to dynamic light touch, we used a brush test. The plantar surface of the hind paw was stimulated with a soft paintbrush by gently stroking from heel to toe. The brush stimulation was repeated 5 times with an interval of 1 min and the percentage of paw withdrawals from 5 stimuli was calculated.

Rotarod test

Gross motor ability and coordination were assessed using a rotarod apparatus (Bioseb-Vitrolles, France). Mice were first placed on the apparatus for 30 s with no rotation and thereafter for 2 min at a constant low-speed rotation (4 rpm). Those that fell from the rod at 4 rpm were placed again on it until they were able to stay for 1 min. Mice were tested in accelerating conditions from 4 to 40 rpm over 2 min period. The time during which the animal walked on the rod before falling was collected (maximum value: 120 s). The occurrence of two consecutive passive rotations, i.e., without walking but accompanying the rod, was considered as a fall. Each mouse was tested three times per day with inter-trial intervals of 5 min.

Nerve conduction studies (NCV)

Mice (24-week-old) were anesthetized with isoflurane at doses of 4–5% for induction and 1–2% for maintenance. The core temperature was maintained at 34 °C with a heating pad. Stainless steel needle electrodes (Natus Biomedical, Middleton, Wisconsin) were cleaned with 70% alcohol between animals. Using the Natus UltraPro S100 EMG/NCS system and the Keypoint 4 software, sensory NCV was determined by dorsal paw recordings and stimulating electrodes on the ankle. For sensory NCV, the latency of onset (milliseconds) of the sensory nerve action potential after supramaximal antidromic stimulation of the sural nerve at the ankle was divided by the distance between the recording and stimulation electrodes (measured in millimeters using a Vernier caliper). Motor NCV was calculated by subtracting the distal from the proximal latency (measured in milliseconds) from the stimulus artifact of the take-off of the evoked potential, and the difference was divided by the distance between the two stimulating electrodes (measured in millimeters using a Vernier caliper).

Intra epidermal nerve fiber density (IENFD) and Nociceptive SC quantification

IENFD quantification was performed on WT and PCSK9 KO mice. Both right and left plantar surface of the hind paw were collected, fixed overnight with 4% PFA. Tissues were rinsed with 1X PBS and then incubated in 30% sucrose PBS solution overnight. Tissues were cryoembedded in mounting media (OCT), and sectioned at 20 µm thickness before being processed for immunohistochemistry with an antibody against PGP9.5 and L1CAM (supplementary table). Fluorescent images were collected by confocal microscopy. Approximately 10 images per stack were flattened using the ImageJ software. Six different skin sections were measured for each footpad. The number of intraepidermal nerve fibers and nociceptive Schwann cells (PGP9.5+/DAPI+) or (L1CAM+/DAPI+) were presented as the mean number of fibers and cells per linear millimeter of epidermis. All analyses were performed by double-blinded quantification.

Quantifications of immunolabeled LDLR and CD36

Sciatic nerve sections (14 µm) were stained with an antibody against LDLR and CD36 (supplementary table). An average of 10 images were measured for every mouse, and intensity expression was calculated using ImageJ software and normalized to the DAPI intensity of each image. All analyses were performed by double-blinded quantification.

Electron Microscopy

Mice were perfused with 20 mL 1XPBS followed by 15mL of 4% PFA (Electron Microscopy Sciences # 15172) diluted in PHEM buffer (0.1 M; pH 7.4) (Electron Microscopy Sciences # 11163). Sciatic and sural nerves were dissected and fixed in 4% PFA + 2.5% glutaraldehyde (Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA) diluted in PHEM buffer overnight at 4°C. Tissues were stored in 0.5% glutaraldehyde / PHEM buffer at 4°C until use. After being washed in 0.2 M PBS buffer, the nerves were incubated with 0.5% osmic acid + 0.8% potassium hexacyanoferrate trihydrate in 0.1 M phosphate buffer for 120 minutes at room temperature. After two rinses in PHEM buffer, the nerves were dehydrated in a graded series of ethanol solutions (30-100%). The nerves were embedded in EmBed 812 using an Automated Microwave Tissue Processor for Electronic Microscopy, Leica EM AMW. Thin sections (70 nm; Leica-Reichert Ultracut E) were collected at different levels of each block. These sections were counterstained with 1.5% uranyl acetate in 70% Ethanol and lead citrate before observation using a Tecnai F20 transmission electron microscope at 120KV in the Institut des

Neurosciences de Montpellier: Electronic Microscopy facilities, INSERM U 1298, Université de Montpellier, Montpellier France.
The axonal diameter was manually and blindly measured using the MyelTracer software (22). Mitochondria were manually and double-blindly quantified. TEM images of sciatic nerve sections were acquired at (x8,500, x10,000) magnifications of 20 randomly selected fields.

Toluidine Blue Staining and myelinated fiber analysis.
Slides were stained with 1% toluidine blue solution containing 2% borax, washed with water, and then mounted in Entellan mounting medium (EMS, Hatfield, USA). Reconstituted tiles of the whole nerves were imaged at ×63 magnification. To determine g-ratio, images were cropped into random fields. Axon and fiber (axon+myelin) diameters for at least 1162 axons per genotype (on average ≈300 axons per mouse) were calculated using MyelTracer software (22). All quantifications were performed by an individual blinded to the animal genotype.

Lipid Droplet Staining
For lipid droplets staining, sections were fixed in 4% paraformaldehyde, washed with 1X PBS and incubated for 45 min at 20°C with 1 µg/mL of the fluorescent neutral lipid stain Bodipy 493/503 (Life Technologies, California, USA). After washing, sections were counterstained with DAPI and mounted in vectashield (Vector Laboratories).

Proteomics
Nerves from PCSK9 KO mice (n=4 WT mice and n=3 PCSK9 KO) were pooled until reaching 10 mg and lysed with RIPA buffer (50 mM Tris-HCl pH 7.2, 5 mM EDTA, 0.1%SDS) and Halt™ Protease Inhibitor Cocktail (Thermofisher, Massachusetts, USA) for 60 minutes at 4°C with constant shaking. Next, samples were centrifuged at 13,000 g for 5 minutes, the supernatant containing the protein extract was collected and the total protein concentration was determined (BCA Protein Assay Kit, Thermofisher). Samples were reduced with DTT (15 mM) and protein alkylation was then performed at RT, by incubating with iodoacetamide (final concentration 15 mM), for 30 minutes in the dark. Trypsin digestion (enzyme-to-protein ratio of 1:20), was performed overnight at 37°C, and the tryptic peptide digests were resuspended in 7 µL of 4% acetonitrile (ACN) in 0.1% TFA and analyzed by nano-LC using a Thermo Fisher Ultimate 3000 series NCS-3500 RS coupled with NSI-Q-Orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, Bremen, Germany). 5 µL of sample (each sample was injected in triplicate) were separated on a LC-EASY-spray C18 column (2.6 µm, 100 Å,

75 µm × 25 cm, Thermo Fisher Scientific). The mass spectrometry analysis was performed with the following conditions: spray voltage: 2 kV, heated capillary temperature: 275 °C, and S-lens RF level: 30%. Mass spectra were acquired with XCalibur 4.2.47 software (Thermo Fisher Scientific) and registered in data-dependent acquisition with the mass spectrometer operating in positive mode. Survey full scan mass spectra were acquired in the 350 to 2,000 m/*z* range at a resolving power of 70,000 (at m/z 400) with automatic gain control (AGC) target of $1e^6$ and maximum injection time (max IT) of 120 ms. Injection of blank (4% acetonitrile in 0.1% TFA) was performed before and after samples to prevent carry-over. The Orbitrap performance was evaluated weekly and external calibration of the mass spectrometer was performed prior to analysis with an LTQ ESI positive ion calibration solution (Pierce).

Protein identification and quantification

Raw mass spectrometry data were automatically processed using Proteome discoverer software (version 2.2.2.2.0, Thermo Fisher Scientific) for protein identification and quantification. MS and MS/MS spectra were searched against the UniProt mouse reference proteome database with canonical and isoform sequences. The database search was performed with the following parameters: oxidized methionine and protein N-terminal acetylation were set as variable modifications, and Cysteine carbamidomethylation was set as a fixed modification. Trypsin was set as enzyme-specific and two missed cleavages were allowed. A mass tolerance of 10 ppm was used for precursor ions and 0.02 Da for-product ions. The false-discovery rate (FDR) was fixed at 1% at the level of proteins and peptides using a target-reversed decoy database search strategy. A minimum of one unique peptide sequence with a Sequest score (Xcorr) ≥ 2 was used. For peptides with an Xcorr < 2, identification was confirmed by manual interpretation of the corresponding MS/MS spectrum.

Statistical analysis

Graph Pad-Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used for graphic presentation and statistical analysis. Results are expressed as the mean per group ± SD. We considered *$p<0.05$, **$p<0.01$, and ***$p<0.001$ as significant. For a two-group comparison, a T-test was used. For multiple comparisons, a one-way ANOVA followed by Tukey's multiple comparisons test was used. For the integration of the proteome for functional analysis, protein abundance was calculated with the generation of spectral feature by the node Feature Finder Multiplex followed by PIA-assisted FDR-multiple scores estimation and filtering (combined FDR score<0.01), their ID mapping and combination with peptide IDs, their subsequent

alignment, grouping, and normalization. DAVID (The Database for Annotation, Visualization, and Integrated Discovery) platform was used for gene ontology (GO) enrichment analysis. GO was performed in our protein LFQ dataset and results for significant terms associated with the gene set were selected based on the fold discovery rate FDR<0.05.

Supplementary table 1 *List of all antibodies used* (IF: immunohistology, WB: western blot)

Target antigen	**Vendor or source**	**Catalog #**	**Dilution factor**
Rabbit anti human pro-PCSK9 N-terminal domain	**Abcam**	**ab135647**	**IF 1:250**
Goat anti human PCSK9 C-terminal domain	**Abcam**	**ab28770**	**IF 1:250**
Rabbit anti-mouse PCSK9	**Cayman**	**10240**	**IF 1:200**
Mouse anti-S100β	**Sigma-Aldrich**	**S2532**	**IF 1:1000**
Rat anti- L1CAM	**Merck milipore**	**MAB5272**	**IF 1:500**
Goat anti-LDLR	**R&D**	**AF2258**	**IF,WB 1:500**
Mouse anti-ApoER2	**Abnova**	**H00007804-M01**	**IF,WB 1:500**
Rabbit anti-LRP1	**Abcam**	**ab92544**	**IF,WB 1:500**
Goat anti-VLDLR	**R&D**	**AF2258**	**IF,WB 1:500**
Chicken anti-NF	**Merck milipore**	**AB5539**	**IF 1:1000**
Rabbit anti-β-actin	**Abcam**	**ab8227**	**WB 1:5000**
Rabbit anti-PGP9.5	**Merck milipore**	**AB1761**	**IF 1:500**
Rabbit anti-CD36	**Abcam**	**ab124515**	**IF,WB 1:250**

Results

PCSK9 is expressed in the peripheral nervous system **-** We assessed the expression of PCSK9 in DRG and sciatic nerves from adult wild-type mice. Using multiplex *in situ* hybridization we detected PCSK9 mRNA transcripts in both sections of DRG neurons and Schwann cells (SC) of the sciatic nerve where they respectively co-localized with the neuronal marker NeuN and the SC marker Sox10 (Figure 1A-B). Consistently, PCSK9 protein was also detected in mouse DRG neurons expressing Neurofilament (NF) (Figure 1C) and in the sciatic nerves, where it colocalized both with the pan-SC marker S100B (Figure 1D) and the non-myelinating SC marker L1CAM (Supplementary figure 1A). These results were confirmed by analyzing PCSK9 expression in cultured primary SCs and sensory neurons isolated from P5 wild-type mice (Figure 1E-F). Next, we quantified PCSK9 protein levels in wild-type spinal cord, DRG, sciatic nerves, and liver extracts by ELISA, using PCSK9 KO mice as negative controls. We determined that while PCSK9 protein was not detectable inspinal cord extracts, significant levels were detected in DRG (0.46±0.11pg/µg) and sciatic nerve (0.29±0.03pg/µg) extracts, at lower concentrations than those found in the liver (0.92±0.11pg/µg) and in plasma (158±54 ng/ml) (Figure 1G). Finally, we assessed whether PCSK9 was also present in human nerves, using transverse sections of human sciatic nerves biopsies. We observed a co-localization between PCSK9 (both precursor and mature forms) and S100B in SCs (Supplementary figure 1B-C). Pro-PCSK9 was exclusively localized within SCs (Supplementary figure 1B), whereas mature PCSK9 exhibited a more spread pattern, and was localized in SCs as well as in axons (Supplementary figure 1C). Expression of PCSK9 in Human SCs was also confirmed using Human primary SC culture (Supplementary figure 1D). *All together, these observations demonstrate that PCSK9 is endogenously expressed in sensory neurons and SC of the PNS.*

PCSK9 deficiency impairs mechanical pain sensitivity **-** To unravel the physiological role of PCSK9 in the PNS, we conducted a series of behavioral analyses to comparatively assess the sensory acuity of wild-type and PCSK9 knockout (PCSK9 KO) mice. At 10 weeks of age, PCSK9 KO mice exhibited reduced sensitivity to mechanical stimuli compared to wild-type animals, evidenced by an increase in paw withdrawal threshold and a decrease frequency of paw withdrawal in response to an innocuous stimulus with von Frey filaments (Figure 2A-B). In contrast, at this stage, no clear defects were detected in their responses to noxious stimuli using either large-diameter von Frey filaments or the pinprick test (Figure 2B-C). At 24 weeks of age, in addition to the reduction of light mechanical pain sensation evidenced by an increased paw withdrawal threshold and the reduction of response to innocuous von Frey filaments

(Figure 2D-E), PCSK9 KO mice also showed a significant reduction in responses to acute mechanical pain stimuli (Figure 2E-F). It is noteworthy that PCSK9 KO mice at both ages did not show any significant impairment in response to thermal pain or dynamic touch sensations, nor did they present with impaired motor coordination (Supplementary figure 2A-F). *Taken together, these observations indicate that the absence of PCSK9 in mice impairs mechanical pain sensations.*

PCSK9 deficiency alters the morphology and the conduction velocity of peripheral nerve fibers - Given that pain sensation is transmitted by small nerve fibers innervating the epidermis, we comparatively assessed intraepidermal nerve fiber density (IENFD) in the foot pads of wild-type and PCSK9 KO mice using PGP9.5 as a marker of terminal nerve fibers. Although we found similar IENFD between control and PCSK9 KO mice at 13±5 and 11±6 fibers/mm respectively, $p= 0.3903$ (Figure 3A-B), we noticed a significant decrease in the number of specialized SC called nociceptive SC (23) (nSC) in PCSK9 knockout mice compared with wild-type, at 3±1 and 7±1 cells/mm, respectively, $p< 0.0001$ (Figure 3C-D) by using an immunostaining for L1CAM protein that we found highly expressed in these cell types. This observation was confirmed by the quantification of nSCs expressing PGP9.5/DAPI (Supplementary figure 3A-C). We then performed electron microcopy analyses of sciatic nerve sections from these animals and we observed axonal swelling of small C-fibers at the level of Remak bundles in PCSK9 KO mice (Figure 3E). Quantitative analyses of Remak bundles revealed a shift in the proportion of these C-fibers towards an increase in axon diameter (Figure 3F). This was characterized by a significant increase in axons diameter in PCSK9 KO mice with 2.5±2% of their axons having a diameter above 1.5µm, compared with only 0.2±0.2% for wild-type mice, $p= 0.0221$ (Figure 3G). Similar morphological differences between these mice were also observed in their sural nerve that mostly contain non-myelinated axons (data not shown). Remak bundles normally consist in a single non-myelinating SC that ensheaths several small C-type axon fibers separately (24). In PCSK9 knockouts, most axons within Remak bundles appeared to fasciculate together (Figure 3E), with an abnormal presence of vacuoles within these bundles (Supplementary figure 3D). We next comparatively assessed myelinated axons number and morphology in sciatic nerve sections from WT and PCSK9 KO mice. We did not observe any significant difference in the number of myelinated small-diameter axons (1 to 5µm) and myelinated large diameter axons ($\geq 5\ \mu m$) between WT and PCSK9 KO mice (Figure 4A).

Interestingly, our analysis of the myelination status of these fibers revealed that small myelinated A-fibers *(≤ 5 μm)* in PCSK9 KO mice had a significant increase in their G-ratio reflecting a decrease in myelin thickness, compared with wild-type mice (Figure 4B-C). Importantly, these morphological differences observed in PCSK9 KO mice translate at the functional level into a significantly reduced sensory nerve conduction velocity (9.36 ± 0.39 vs. 11.45 ± 1.04 m/s, $p=0.0031$) but similar motor nerve conduction velocity (21.38 ± 3.04 vs. 21.56 ± 0.61 m/s, $p=0.9$), compared with wild-type mice, respectively (Figure 4D). *Altogether, these data show that although PCSK9 knockout mice did not exhibit any change in the density of terminal nerve fibers in the skin, the number of nociceptive SCs was significantly reduced. Moreover, while the peripheral nerves of mutant mice contain normal numbers of axons, they exhibit axonal swelling of C-fibers present in the Remak bundles, and a decrease in myelin thickness of small A-fibers paralleling the reduction of sensory nerve conduction velocity. Thus, PCSK9 KO mice present with alterations in peripheral nerve morphology and conduction properties.*

PCSK9 modulates CD36 but not LDLR expression in peripheral nerves – To gain insights into the molecular mechanism(s) underlying the sensory and morphological phenotypes observed in PCSK9 knockout mice, we assessed the expression of the main PCSK9 targets in the sciatic nerves of these animals. Surprisingly, we did not detect any increase of the level of LDLR in the peripheral nerves of *PCSK9* knockout mice compared to WT animals (Figure 5A-D), in contrast to what was observed in the liver (Supplementary figure 5A, B). Similar results were obtained by analyzing the expression of VLDLR, LRP1 and apoER2 receptors (Supplementary figure 4A-C). In contrast, we strikingly observed a significant increase in the expression of CD36 in sciatic nerves of PCSK9 KO compared with wild-types (Figure 5E-H), as in the liver (Supplementary figure 5A, C). We established that up-regulation of CD36 in the peripheral sciatic nerve of PCSK9 knockout mice was prominent in nerve fibers as well as myelinating and non-myelinating SC (Supplementary figure 6A-B). Of note, in the DRG, CD36 was expressed at a very low levels, and its expression as well as that of the LDLR were not significantly altered in PCSK9 KO mice (Supplementary figure 7A-D). *Our findings thus demonstrate that the absence of PCSK9 specifically induces the upregulation of CD36 but not that of the LDLR or other target receptors in peripheral nerves.*

PCSK9 deficiency is associated with lipid accumulation in peripheral nerves **-** We next evaluated whether the upregulation of the fatty acid transporter CD36 in peripheral nerves

altered their lipid contents. We measured cholesterol and triglyceride concentrations in sciatic nerve extracts and observed an increase of 30% and 50% respectively, in PCSK9 knockout comparatively to wild-type mice (Figure 6A-B). Liver lipid contents were also measured and were consistent with the results of previous studies (25) (Supplementary figure 5D-E). In contrast, we did not observe any change in cholesterol and triglyceride concentration in DRG extracts from control and PCSK9 KO mice (Supplementary figure 7E-F). In peripheral nerves, cholesterol and triglyceride contents remarkably correlated with CD36 protein expression (Supplementary figure 8A-B). Furthermore, neutral lipid staining of sciatic nerve sections showed an accumulation of lipid droplets in PCSK9 knockout compared to control animals (Figure 6C-D). Structurally, by electron microscopy analysis we observed that PCSK9 KO peripheral nerves displayed lipid droplets within Remak bundles (Figure 6E). *These results indicate that PCSK9 deficiency leads to an abnormal lipid accumulation within peripheral nerves.*

***Mitochondrial defects in peripheral nerves* of *PCSK9 KO mice* -** To fully unravel the pathophysiological onset of the sensory phenotype of PCSK9 knockout mice, we next performed an untargeted proteomic investigation of their sciatic nerves. We observed that PCSK9 knockout mice showed a significant upregulation of several proteins involved in lipid mitochondrial metabolism and a downregulation of cytoskeletal proteins compared to wild-type mice (Figure 7A). Major upregulated proteins are implicated in β-oxidation such as Carnitine O acetyl transferase (Crat) and Trifunctional enzyme subunit alpha (Hadha), enzymes in the TCA cycle like Isocitrate dehydrogenase [NAD] subunit alpha (Idh3a), and proteins of the Electron Transport Chain including Complex I (Ndufs5), Complex III (Uqcrc2, Uqcrb) and Complex IV (Cox5b). Interestingly, Caveolin-1, a protein necessary for the localization and functioning of CD36 at the plasma membrane, was also upregulated in PCSK9 KO mice, in line with CD36 expression results. The proteomic analysis also revealed a downregulation of proteins involved in proteasomal catabolic processes such as Psma1, Psmb2, and Psma5, as well as microtubule organization proteins including Map4, Mapt, Msn, and Sorbs3 as well as the myelin P2 protein (PMP2) constitutive of peripheral nerve myelin (Figure 7A). Biological processes enrichment analysis of PCSK9 knockout nerves proteome indicated increased overall processes of lipid metabolism and electron transport chain processes and downregulation of processes involved in microtubule cytoskeleton organization and the capacity to respond to oxidative stress (Figure 7B) and most of the identified upregulated proteins are clustered as mitochondrial components (Supplementary figure 8C). To corroborate these results, we

analyzed mitochondria number and morphology in section of sciatic nerve by electron microscopy. Interestingly, we observed an increase number of mitochondria in Remak bundle nerve fibers of PCSK9 KO mice compared to control (Figure 7C-D) and an increased proportion of abnormal mitochondria, which is representative of mitophagic processes (Figure 7D-E). *Taken together, these observations indicate that PCSK9 knockout mice present with structural mitochondrial defects in the peripheral nerves.*

Discussion

In this study, we described the expression of PCSK9 in the peripheral nervous system at the level of DRG neurons and Schwann cells. We found that PCSK9 gene deletion induces a neuropathic phenotype characterized by a reduction in mechanical pain sensations. These sensory deficits were associated with a reduction in sensory nerve conduction properties without significant alteration of the number of sensory axons in the nerve nor in the density of their terminal endings in the skin. In contrast there was a reduction in the number of terminal nociceptive SCs. We established that PCSK9 gene deletion induces an upregulation of the fatty acid transporter CD36 associated with an abnormal accumulation of lipids in peripheral nerves. We also showed by proteomic and histological analysis that PCSK9 deficiency leads to structural mitochondrial defects. In light of these observations, we propose a model in which PCSK9 through the regulation of CD36 limits the internalization of fatty acid and accumulation of triglyceride storage in the peripheral nerves (Graphical abstract).

Here we show for the first time that PCSK9 is physiologically expressed in the PNS at the level of the DRG neurons and in both myelinating and non-myelinating SCs, which had initially been suggested by the detection of PCSK9 mRNA in a rat SC line (7) and in human tibial nerves (10). We also show that mice lacking PCSK9 initially manifest a decrease in their ability to perceive light mechanical pain at 10 weeks of age, and subsequently become less sensitive to acute mechanical pain stimuli at 24 weeks.

In line with their neuropathic phenotype, we observed structural alterations at the level of terminal endings of the peripheral nerves in the skin of PCSK9 knockout mice. Intriguingly, while we did not observe any significant decrease in IENFD in the skin, we noticed a significant reduction in the number of nociceptive Schwann cells, a non-neuronal cutaneous cell subtype in close relationship with sensory endings known to play a critical role in tactile sensations (23). At the level of sciatic nerves, we observed axonal swelling of unmyelinated C-fibers in Remak bundles, as well as a reduction in the thickness of the myelin sheath around small diameter A-myelinated axons in PCSK9 KO mice. These two types of fibers, referred to as unmyelinated C-fibers and myelinated Aδ-fibers, respectively, are specifically involved in the transmission of mechanical pain (26, 27). The combination of these structural abnormalities, including the loss of nociceptive Schwann cells, the swelling of small C fiber axons, and the hypomyelination of A-δ fibers, appears to be at the origin of the sensory deficits observed in PCSK9 KO mice.

We also showed that PCSK9 knockout mice present an increased expression of CD36 in peripheral nerve SCs and axons, whereas the expression of the LDLR, VLDLR, ApoER2 and LRP1 were unaltered in these tissues comparatively to wild-type mice. The absence of modulation of LDLR abundance in PCSK9 knockout mice peripheral nerves is surprising, given the overt role that PCSK9 plays in LDLR intracellular degradation in hepatocytes and many other cell types (28, 29). Nevertheless, the lack of LDLR modulation in the brain and cerebellum of PCSK9 KO mice has previously been reported by others (30–32). Although the mechanism is still unclear, several proteins have been proposed as intermediates to either enhance or impede PCSK9-mediated degradation of LDLR. Indeed, Annexin 2 (33, 34) and glypican-3 (35) were shown to inhibit PCSK9 binding to LDLR, whereas cyclase-associated protein 1 (36) enhances its binding capacity. The differential expression of these proteins between the hepatic and nervous system could potentially explain the distinct regulatory functions of PCSK9 on LDLR between these tissues. In contrast, the increase in CD36 expression has been reported in many organs of PCSK9 knockout mice, including the liver, adipose tissue, heart, and kidneys (37, 25, 38, 39). The increase in CD36 expression resulting from PCSK9 gene deletion in these tissues was systematically associated with an abnormal accumulation of lipid droplets(37, 25, 38, 39). Accordingly, we also found an accumulation of lipids in the peripheral nerves of PCSK9 knockout mice proportional to the levels of CD36 expression. Of note, the deteriorating sensory phenotype from 10 to 24 weeks discussed earlier could potentially be attributed to an increase in toxicity caused by the gradual accumulation of lipids over time. Collectively, these observations support a role for PCSK9 in controlling CD36 levels in peripheral nerves, thereby protecting nerve cells against excessive lipid uptake and triglyceride accumulation that can lead to neuronal lipotoxicity.

In line with this hypothesis, we found that PCSK9 knockout mice nerves present with an increased expression of mitochondrial enzymes involved in β-oxidation, enzymes of the TCA cycle, as well as proteins of the electron transport chain. CD36 induced accumulation of lipids in peripheral nerves may indeed overwhelm the capacity of SCs and axons to metabolize these compounds through mitochondrial β-oxidation, the TCA cycle and oxidative phosphorylation to generate ATP. We also found that proteins involved in proteasomal catabolic processes and microtubule biology were downregulated, both parameters being indicative of neurodegenerative processes (40, 41). Furthermore, we found structurally abnormal and dysfunctional mitochondria within unmyelinated Remak bundle fibers of PCSK9 deficient mice. In line with these observations, an increased proportion of dense depolarized

mitochondria in peripheral axons has also been reported in mouse models of diabetic neuropathy (42, 43) and cardiac mitochondrial metabolism was found altered in cardiomyocytes of PCSK9 knockout mice, in addition to enhanced CD36 expression and the presence of lipid droplets in the heart of these animals (38). These observations show that in the absence of PCSK9, peripheral nerves exhibit increased CD36 expression and an abnormal accumulation of lipids. We propose that this excess of lipids leads to mitochondrial dysfunction in small nerve fibers, and can contribute to the sensory neuropathic phenotype observed in these animals.

Interestingly, these results contrast with the fact that over the last decade only one documented case of peripheral neuropathy has been to be linked to PCSK9 inhibitors usage (44). Long term trials conducted with evolocumab and alirocumab, two PCSK9 inhibitor antibodies have not demonstrated any link to negative neurocognitive events (4, 45). However, there was no specific evaluation of sensory motor endpoints. Of note, these drugs specifically antagonize the domain of interaction between PCSK9 and the LDLR but not with the fatty acid transporter CD36 (1, 39), and theoretically would thus not alter the regulatory effect of PCSK9 on CD36. In addition, these drugs as well as the novel anti-PCSK9 siRNA inclisiran only target circulating PCSK9 (6, 46). Given that PCSK9 KO mice totally lack PCSK9 in the circulation as well as in all tissues, we cannot rule out that the neuropathic phenotype of PCSK9 knockout mice might stem from the absence of local PCSK9 within SCs and/or sensory neurons rather than from the absence of circulating PCSK9.

Nevertheless, the discovery of a role for PCSK9 in peripheral nerve lipid homeostasis, and its modulation through the CD36 regulation open a new avenue of research in certain cases of acquired demyelinating disorders of peripheral nerves (i.e. Guillain-Barre Syndrome, Anti-Myelin Associated Glycoprotein Neuropathy) as well as in nerve injury and remyelination, where lipids are considered limiting resources (47, 48). Furture investigations are necessary to better understand the underlying mechanisms of modulation of PCSK9 and of its target receptors in such pathologies in order to design news targeted therapeutical approaches for these diseases.

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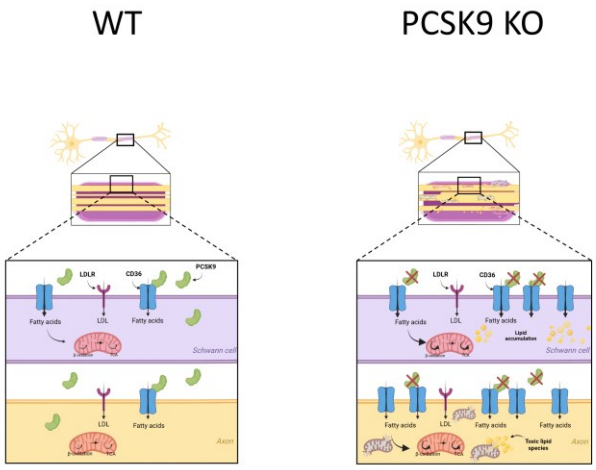
Figures:



Graphical abstract. PCSK9 modulates nerve energy metabolism and health.

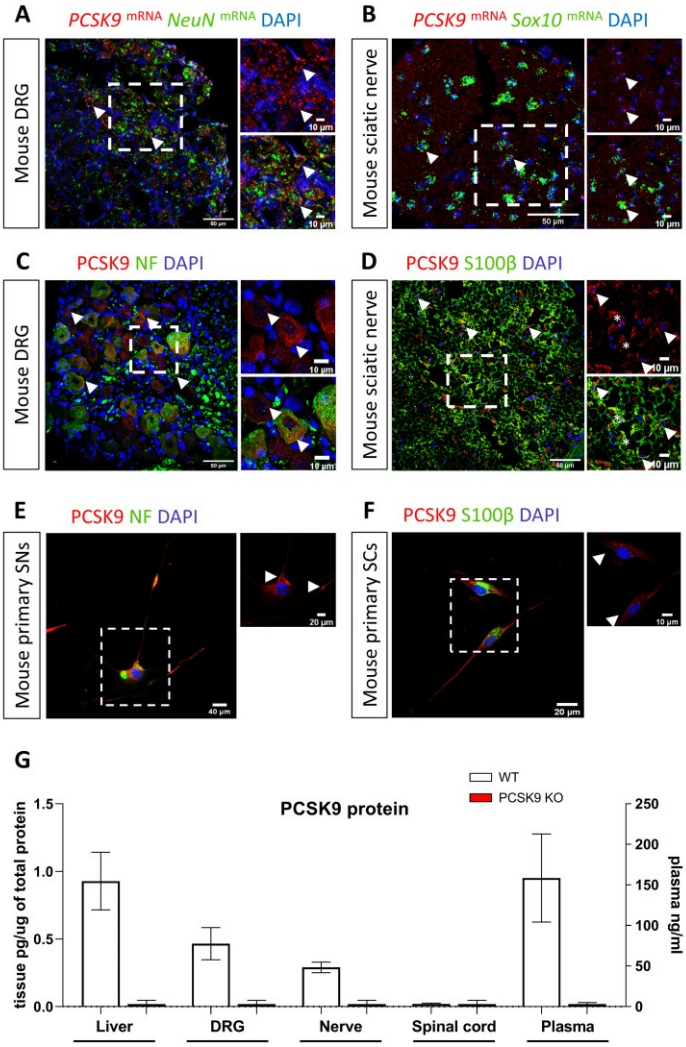


Figure 1. **PCSK9 expression in the peripheral nervous system of mice.** **(A)** PCSK9 mRNA expression in mouse dorsal root ganglia and **(B)** sciatic nerve sections. **(C)** PCSK9 protein expression in mouse dorsal root ganglia and **(D)** sciatic nerve (arrowhead: PCSK9 expression; asterisk: axonal PCSK9 expression. **(E)** PCSK9 expression in mouse primary sensory neurons and **(F)** Schwann cells (arrowhead: PCSK9 expression). **(G)** PCSK9 protein concentration in spinal cord, nerve, DRG, liver and plasma in WT mice with the corresponding negative controls from PCSK9 KO mice; $n = 3$ mice per group. Data are shown as mean ± standard deviation.

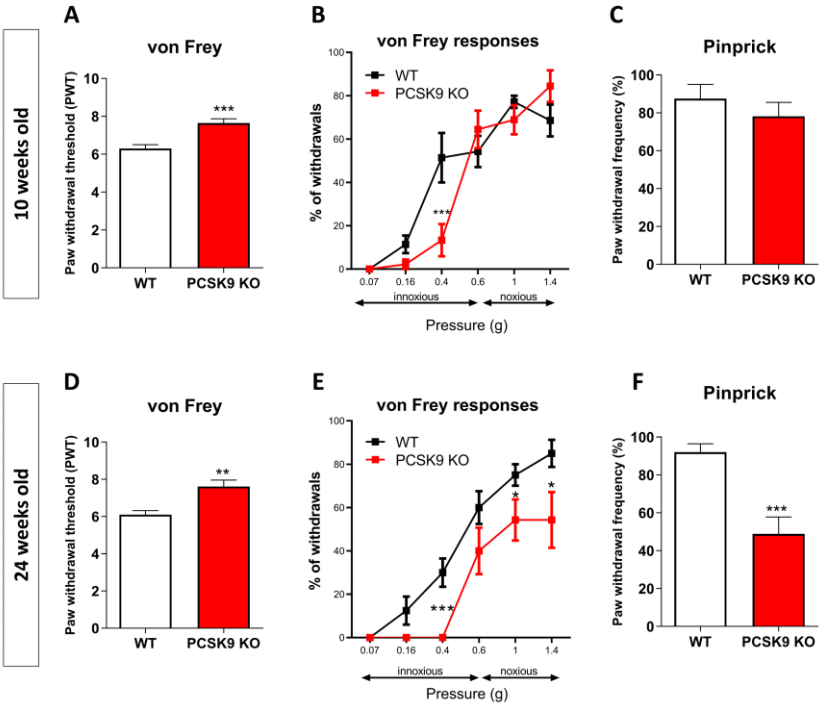


Figure 2. **PCSK9 deficiency is associated with sensory behavioral abnormalities. (A)** 10-week-old WT (n=10) and PCSK9 KO (n=14) mice were tested for mechanical pain sensation with the von Frey test presented as paw withdrawal threshold and **(B)** withdrawal percentage. **(C)** 10-week-old WT (n=8) and PCSK9 KO (n=11) mice were tested for acute pain sensation with the pinprick test. **(D)** 24-week_old WT (n=10) and PCSK9 KO (n=9) mice were tested for mechanical pain sensation with the von Frey test presented as paw withdrawal threshold and **(E)** withdrawal percentage. **(F)** 24-week-old WT (n=10) and PCSK9 KO (n=9) mice were tested for acute pain sensation with the pinprick test. Data are shown as mean ± standard deviation, ***P*≤0.005 and ***≤0.001.

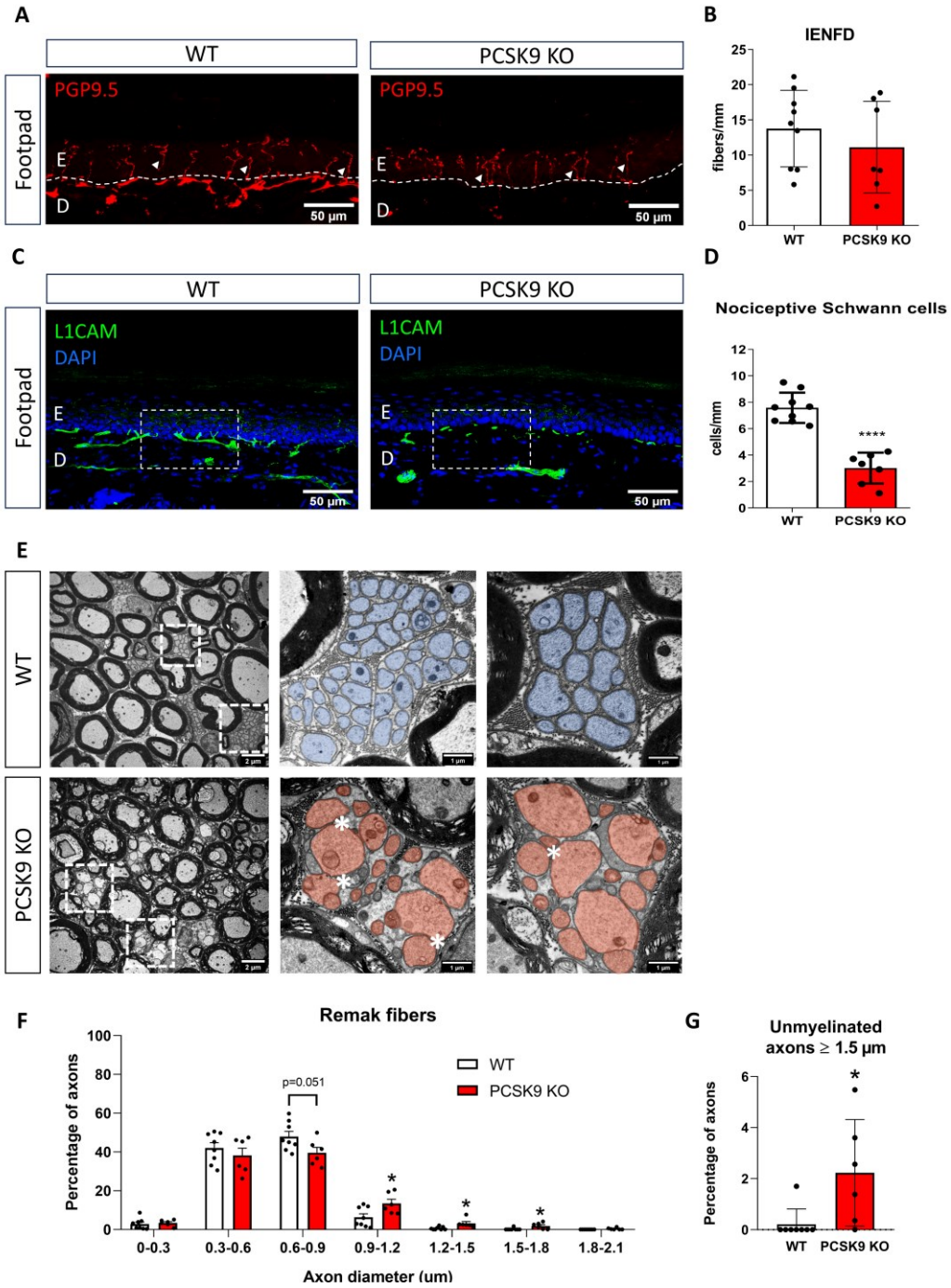
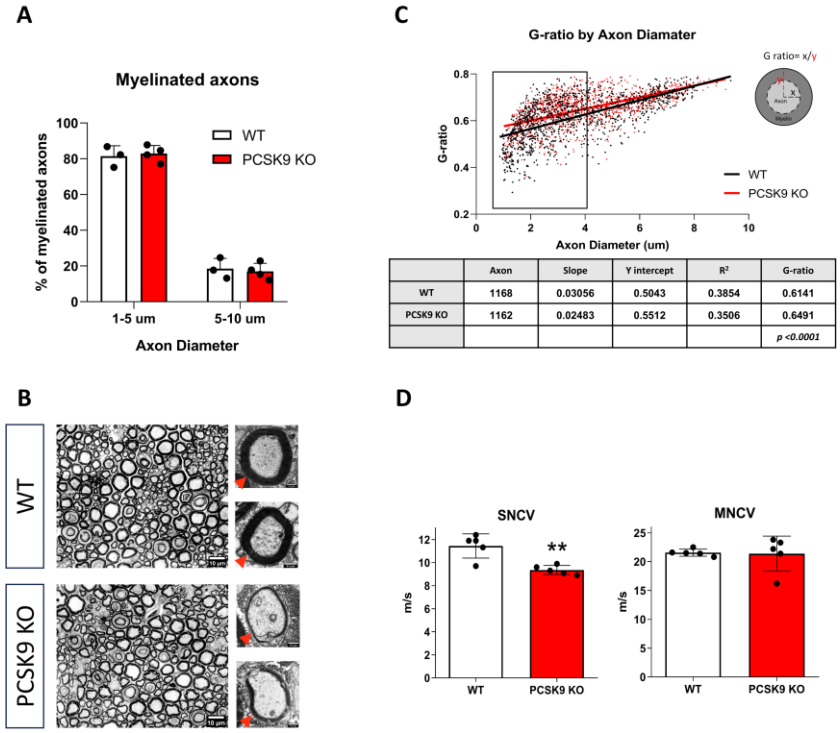


Figure 3. **PCSK9 deficiency is associated with loss of nociceptive Schwann cells and C-fiber axonal swelling**. **(A)** Representative image of intraepidermal nerve fiber density (IENFD) and **(B)** Quantification in WT (n=9) and PCSK9 KO (n=7). **(C)** Representative image of nociceptive terminal Schwann cells in the skin and **(D)** Quantification of these cell numbers between WT (n=9) and PCSK9 KO (n=7) mice. **(E)** Representative photomicrographs of Remak bundle structure by transmission electron microscopy in WT (small fibers are highlighted in blue) and PCSK9 KO mice (small fibers are highlighted in pink); (*) indicates

axon-axon touching. **(F)** Distribution of axon diameter in Remak bundles between WT (n=8) and PCSK9 KO (n=6) mice. **(G)** Percentage of small unmyelinated C-fibers with a diameter greater than 1.5 µm in WT vs. PCSK9 KO, (WT n=8, ≥2035 axons were analyzed) vs. (PCSK9 KO n=6, ≥1727 axons were analyzed). Data are shown as mean ± standard deviation, *$P < 0.05$.



	Axon	Slope	Y intercept	R^2	G-ratio
WT	1168	0.03056	0.5043	0.3854	0.6141
PCSK9 KO	1162	0.02483	0.5512	0.3506	0.6491
					p <0.0001

Figure 4. **PCSK9 deficiency is associated with structural and physiological changes in myelinated A fibers. (A)** Proportions of myelinated axons distributed according to axon diameter in both WT (n=3) and PCSK9 KO (n=4) mice; *n* = 3-4 mice per group. **(B)** Representative images of toluidine blue staining of myelin in WT and PCSK9 KO mice (arrow indicates myelin). **(C)** G-ratio analysis with a summary table in WT (n=4), (≥1168 axons were analyzed) vs. in PCSK9 KO (n=3), (≥1162 axons were analyzed). **(D)** Sensory nerve conduction velocity (SNCV) and motor nerve conduction velocity (MNCV) compared between WT (n=5) and PCSK9 KO (n=5) mice. Data are shown as mean ± standard deviation, ***P*≤0.005 and ****≤0.0001.

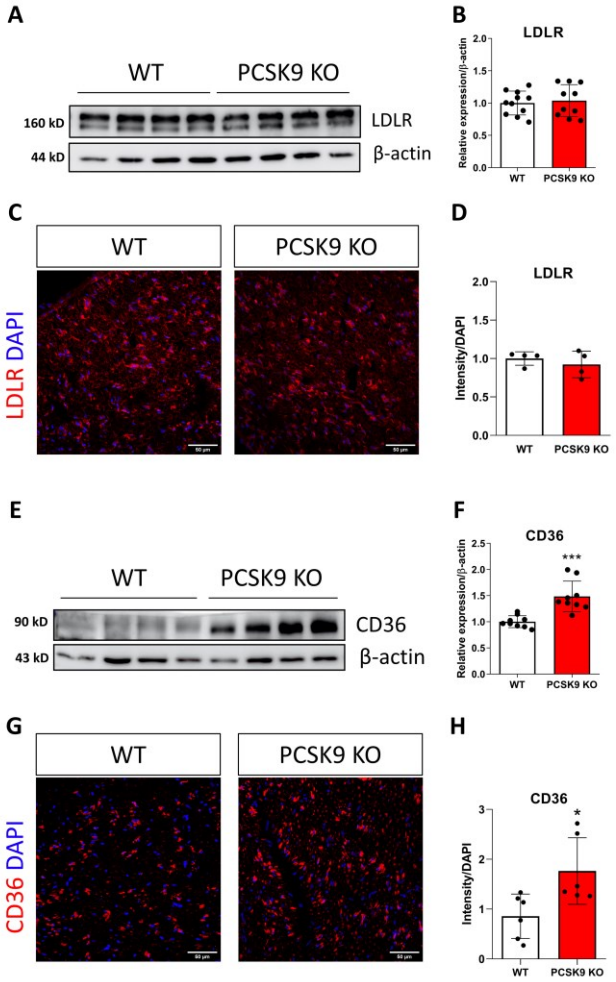


Figure 5**. PCSK9 deficiency results in increased CD36 expression in peripheral nerves**. **(A)** LDLR expression in the sciatic nerve by immunoblotting. **(B)** Quantification of LDLR expression in 24-week-old WT (n=11) and PCSK9 KO (n=10). **(C)** Immunohistochemistry for LDLR in sciatic nerve sections of 24-week-old WT and PCSK9 KO mice. **(D)** Immunohistochemistry quantification of LDLR expression in 24-week-old WT (n=4) and PCSK9 KO (n=4) nerve sections. **(E)** CD36 expression in the sciatic nerve by immunoblotting. **(F)** Quantification of CD36 expression in 24-week-old WT (n=9) and PCSK9 KO (n=9). **(G)** Immunohistochemistry for CD36 in sciatic nerve sections of 24-week-old WT and PCSK9 KO mice. **(H)** Immunohistochemistry quantification of CD36 expression in 24-week-old WT (n=6) and PCSK9 KO (n=6) nerve sections. Data are shown as mean ± standard deviation, *$P < 0.05$ and ****≤0.0001.

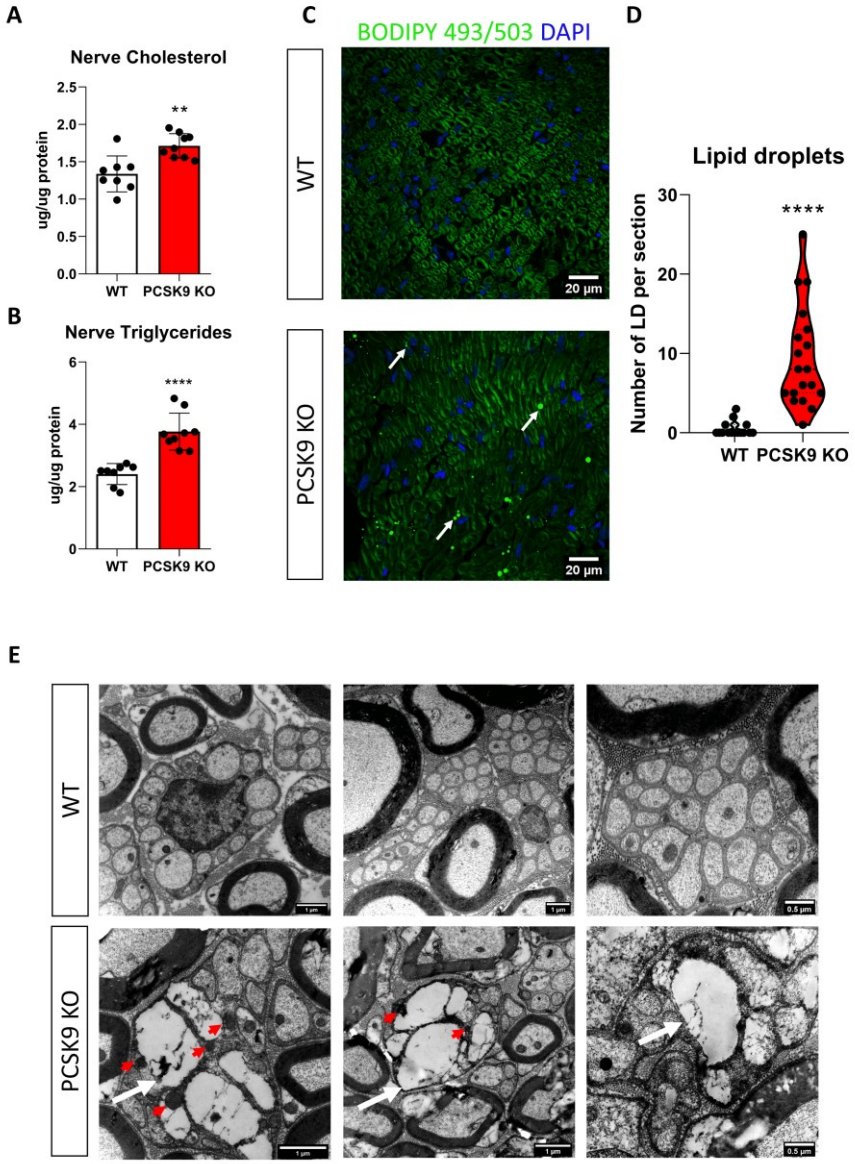


Figure 6. **PCSK9 deficiency results in increased lipids in peripheral nerves. (A)** Cholesterol levels in the sciatic nerve of 24-week-old WT (n=8) and PCSK9 KO (n=9) mice. **(*B*)** Triglyceride levels in the sciatic nerve of 24-week-old WT (n=8) and PCSK9 KO (n=9) mice. **(C)** Visualization of lipid droplets in sciatic nerve sections using BODIPY 493/503 staining (arrows: lipid droplets). **(D)** Quantification of the number of lipid droplets per section from 24-week-old WT (n=3) and PCSK9 KO (n=4) mice. **(E)** Representative photomicrographs of sciatic nerve sections by transmission electron microscopy in WT and PCSK9 KO mice are shown. White arrows indicate lipid droplets; red arrows show the associated mitochondria. Data are shown as mean ± standard deviation, $^{**}P < 0.01$ and ****<0.0001.

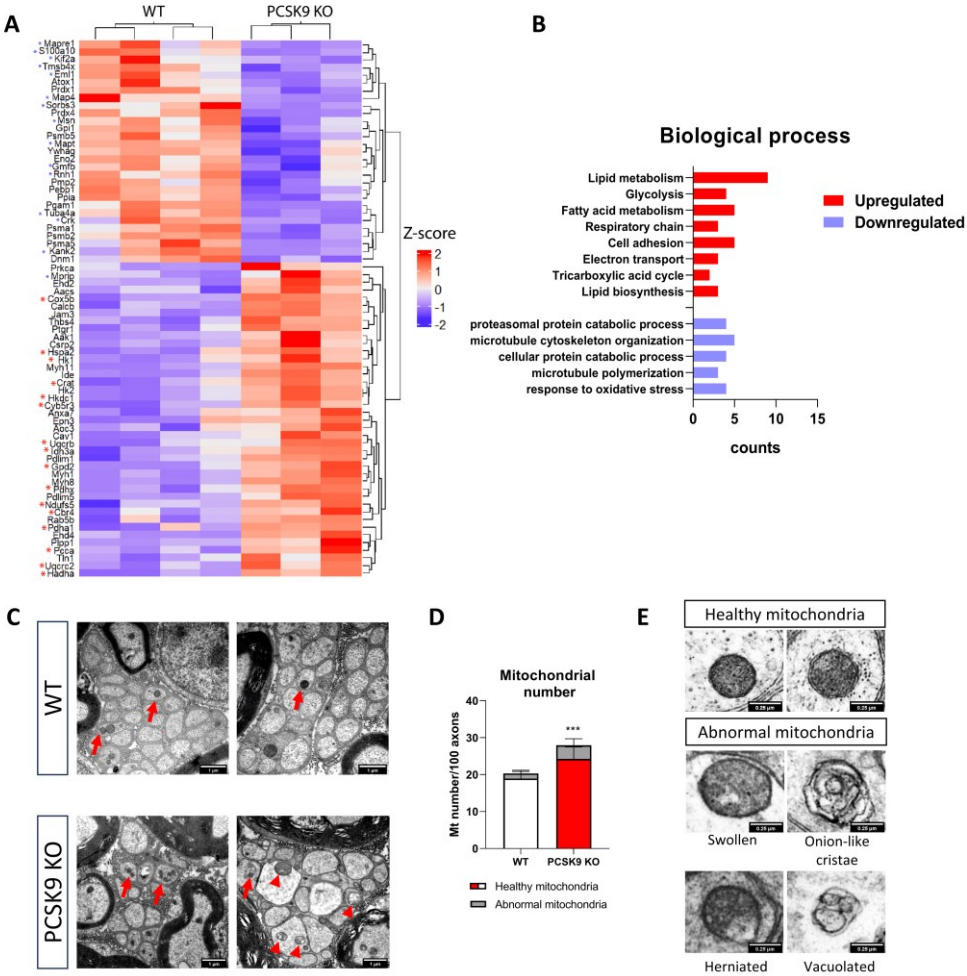


Figure 7. **Proteomic profile and mitochondrial abnormalities of PCSK9 KO nerves. (A)** Heatmap showing relative protein expression values (*z*-score) of *n*=62 proteins that are differentially expressed in the nerves of WT (n=4) compared to PCSK9 KO (n=3) mice (red asterisk represents upregulated mitochondrial proteins, blue asterisk represents downregulated microtubule proteins). **(B)** Gene ontology annotation of the top upregulated (red) and downregulated terms in biological processes. **(C)** Representative TEM images of Remak bundles (red arrows: healthy mitochondria, red arrowheads: abnormal mitochondria). **(D)** Quantification of total mitochondrial numbers with the ratio of abnormal mitochondria per 100 axons in WT (n=8, ≥2035 axons were analyzed) and PCSK9 KO (n=6, ≥1727 axons were analyzed). **(E)** Representative images of healthy mitochondria and abnormal mitochondria. Data are shown as mean ± standard deviation, ***$P \leq 0.001$.