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1 **Curcumin–cyclodextrin/cellulose Nanocrystals Improve the**
2 **Phenotype of Charcot-Marie-Tooth-1A Transgenic Rats through**
3 **the Reduction of Oxidative Stress**

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1 **Key words:** Curcumin-cyclodextrin/cellulose nanocrystals, Charcot-Marie-Tooth-1A,
2 oxidative stress, myelination, peripheral neuropathy, PMP22, endoplasmic reticulum.

3

4 **Highlights:**

- 5 • Oxidative stress is an important component of CMT1A pathophysiology
- 6 • Curcumin-cyclodextrin/cellulose nanocrystals improve curcumin's bioavailability
- 7 • Nano-Cur improves the functional recovery of CMT1A rats
- 8 • Nano-Cur improves electrophysiological and histological aspects of CMT1A rats
- 9 • Nano-Cur reduces oxidative stress which likely promotes myelination

10

11 **Graphical abstract:**

1 **Abstract**

2 The most prevalent form of Charcot-Marie-Tooth disease (CMT type 1A) is characterized by
3 duplication of the *PMP22* gene, peripheral dysmyelination and decreased nerve conduction
4 velocities leading to muscle weakness. Recently, oxidative stress was reported as a feature in
5 CMT1A patients. Curcumin exhibits antioxidant activities and has shown beneficial
6 properties on peripheral nerves. However, curcumin presents unfavorable pharmacokinetics.
7 We developed curcumin-cyclodextrin/cellulose nanocrystals (Nano-Cur) to bypass this
8 limitation. The present study investigated the therapeutic potential of Nano-Cur *in vitro* in
9 Schwann cells (SCs) and *in vivo* in the transgenic CMT1A rat model. *In vitro*, Nano-Cur
10 treatment (0.01 μ M for 8h) reduced reactive oxygen species and improved mitochondrial
11 membrane potential in CMT1A SCs. Moreover, Nano-Cur treatment (0.01 μ M for 1 week)
12 increased the expression of myelin basic protein in SC/neuron co-cultures. Preliminary *in vivo*
13 experiments carried out in WT rats showed that intraperitoneal (i.p.) injection of Nano-Cur
14 treatment containing 0.2 mg/kg of curcumin strongly enhanced the bioavailability of
15 curcumin. Afterwards, in 1-month-old male CMT1A rats, Nano-Cur treatment (0.2
16 mg/kg/day, i.p. for 8 weeks) significantly improved sensori-motor functions (grip strength,
17 balance performance, and mechanical and thermal sensitivities). Importantly, sensory and
18 motor nerve conduction velocities were improved. Further histological and biochemical
19 analyses indicated that myelin sheath thickness and myelin protein expression (myelin protein
20 zero and PMP22) were increased. In addition, oxidative stress markers were decreased in the
21 sciatic nerve and gastrocnemius muscle. Finally, Nrf2 expression and some major antioxidant
22 enzymes were increased in sciatic nerve. Therefore, Nano-Cur significantly improved
23 cellular, electrophysiological, and functional features of CMT1A rats.

1 **Abbreviations**

- 2 ARE: Antioxidant response element
- 3 BiP: Binding immunoglobulin protein
- 4 CMAP: Compound muscle action potential
- 5 CMT1A: Charcot–Marie–Tooth disease type 1A
- 6 CNCx: Cellulose nanocrystals
- 7 CNX: Calnexin
- 8 COX-SDH: Cytochrome oxidase and succinate dehydrogenase
- 9 CPK: Creatine phosphokinase
- 10 CRL: Calreticulin
- 11 ERAD: Endoplasmic-reticulum-associated protein degradation
- 12 GPx1: Glutathione peroxidase-1
- 13 GRP94: Glucose-regulated protein 94
- 14 GST: Glutathione S-transferase
- 15 HSC70: Heat shock cognate 70 kDa protein
- 16 HSP 90 alpha: Heat shock protein 90 alpha
- 17 HSR: Heat shock response
- 18 Keap1: Kelch-like ECH-associated protein 1
- 19 MBP: Myelin basic protein
- 20 MDA: Malondialdehyde

- 1 MNCV: Motor nerve conduction velocity
- 2 MPZ: Myelin protein zero
- 3 Nano-Cur: Curcumin-cyclodextrin/cellulose nanocrystals
- 4 NFM: Neurofilament medium polypeptide
- 5 Nrf2: Nuclear factor (erythroid-derived 2)-like 2
- 6 PMP22: Peripheral myelin protein 22
- 7 PRDX: Peroxiredoxin
- 8 SC: Schwann cell
- 9 SNCV: Sensitive nerve conduction velocity
- 10 TBARS: Thiobarbituric acid reactive substances
- 11 TRX1: Thioredoxin-1
- 12 UBC: Polyubiquitin-C
- 13 UPR: Unfolded protein response
- 14 VCP: Valosin-containing protein
- 15 XDH: Xanthine dehydrogenase
- 16 β -CD: β -cyclodextrin
- 17 $\Delta\Psi_m$: Mitochondrial membrane potential

1 **Introduction**

2 Charcot–Marie–Tooth disease (CMT) is the most common human inherited peripheral
3 neuropathy with an estimated prevalence of 1/2500 [1]. CMT1A is the autosomal dominant
4 demyelinating form, accounting for 60% of CMT cases [2]. It is caused by a duplication of a
5 region (1.4-Mb) on chromosome 17 of the gene encoding the peripheral myelin protein 22
6 (PMP22) [3]. PMP22 is a glycoprotein expressed in compact myelin formed by Schwann cells
7 (SCs) in the peripheral nervous system (PNS), where it is essential for the homeostasis of
8 myelin sheath [4]. Conformational stability of PMP22 is crucial for mediating its specific
9 location in SC membrane [5]. In the case of gene mutation, PMP22 correct folding, and thus
10 its function, can be compromised. CMT1A leads to leg and hand muscle weakness and a
11 decrease in nerve conduction velocity [1,6]. Loss of mechanical and thermal sensitivities has
12 also been described [1]. Recent studies indicate that oxidative stress is a feature of CMT
13 disease [8] [9], especially in CMT1A patients plasma [7]. Indeed, a modulation of oxidative
14 stress status has been shown in CMT1A patients characterized by an increased activity of
15 antioxidant enzymes, e.g. superoxide dismutase (SOD), glutathione-S transferase (GST),
16 glutathione peroxidase (GPx) and reductase (GRd). In addition, blood levels of other
17 oxidative markers, such as lipid peroxidation (LPO), nitrites (NOx) and GSSG/GSH ratio,
18 were also increased [7]. Moreover, there is increasing evidence in neurological diseases that
19 there is a strong link between ER stress, and thus protein folding, and increased oxidative
20 stress [10].

21 Currently, there is no effective therapy for CMT1A disease. Ascorbic acid, although
22 promising in a mouse model [11], failed to show a significant effect in clinical trials [12,13].
23 Antisense oligonucleotides were found to suppress *PMP22* mRNA levels and improve
24 functional features in mouse and rat CMT1A models [14]. However, *PMP22* mRNA levels

1 vary significantly over time in patients and do not necessarily correlate with the neurological
2 disabilities. It is possible that stabilizing the fluctuations in PMP22 protein or mRNA rather
3 than simply downregulating their absolute values is necessary for disease modulation [15,16].

4 Curcumin, a polyphenol from the curcuminoid family, is extracted from rhizomes of *Curcuma*
5 *longa*. Curcumin possesses multiple pharmacological properties, including neuroprotective,
6 anti-inflammatory and antioxidant effects [17]. This antioxidant effect is frequently associated
7 with increased antioxidant transcription factor Nuclear factor (erythroid-derived 2)-like 2
8 (Nrf2). In addition, curcumin has been shown to increase expression of detoxification
9 enzymes such as glutathione S-transferase P (GST-P), NADPH quinone oxidoreductase-1
10 (NQO1) and antioxidant enzymes such as heme-oxygenase 1 (HO-1) [17]. Moreover, recently
11 we have shown that a local low dose of curcumin, delivered near a sciatic nerve crush site,
12 accelerated rat functional recovery and nerve repair [18]. The effects of curcumin have been
13 attributed to its antioxidant properties, including reduction of total and mitochondrial reactive
14 oxygen species (ROS), decrease of lipoperoxidation and activation of Nrf2 [18]. In addition,
15 curcumin can reduce SC apoptosis and promote myelination [19]. Moreover, experiments in
16 different animal disease models suggest that curcumin improves the translocation of proteins
17 from the ER to the cellular membrane, thus reducing their cytotoxicity [20] [21] [22] .

18 Taken together, because of its pluripotency, oral safety, and low cost, curcumin represents a
19 promising candidate for drug development especially for neurological disorders. However, its
20 systemic administration is limited by its poor aqueous solubility, poor stability, low
21 bioavailability, and rapid metabolism [23]. Nanotechnology-based drug delivery systems have
22 proven to be effective approaches for hydrophobic drug delivery and alternatives to
23 potentially toxic solubilizing agents [24]. To this end, cellulose nanocrystals (CNCx) carrying
24 curcumin were developed [25]. They have a uniform nanorod shape 100-200 nm long, 10-20

1 nm wide and 5-10 nm thick [25]. They are biocompatible and well-tolerated [26]. Curcumin
2 was incorporated into the hydrophobic cavity of β -cyclodextrin (β -CD) that is bound to CNCx
3 surfaces [25]. CDs prevent the decomposition of drugs before entering tissue and are included
4 in various pharmacopeias [27]. The final compound is termed curcumin-CD/CNCx or Nano-
5 Cur. It has been shown that, *in vitro*, Nano-Cur enhanced curcumin delivery into cancerous
6 cells [25].

7 The goal of this study was to investigate the effects of Nano-Cur in CMT1A transgenic rats
8 which present three additional murine copies of *Pmp22* gene. Similar to human patients, these
9 rats manifest decreased nerve conduction velocity, muscle weakness, and impaired distal
10 sensation [28]. To provide an *in vitro* proof-of-concept of Nano-Cur effects on oxidative
11 stress and myelin protein production, experiments on SCs and SC/neuron co-cultures were
12 carried out. Thereafter, *in vivo* experiments showed that Nano-Cur improved the functional
13 recovery of CMT1A rats mainly by reducing oxidative stress and promoting myelination.

1 **Materials and Methods**

2 Cyclodextrin-cellulose nanocrystals (Nano) and curcumin-cyclodextrin/cellulose nanocrystals
3 (Nano-Cur) were synthesized according to a previously published procedure [25].
4 Methodologies used for cell culture, mechanical sensitivity and grip strength evaluation,
5 electrophysiology, nerve light microscopy, Western blotting, TBARS, ROS and
6 mitochondrial membrane potential ($\Delta\Psi_m$) evaluation have been previously described in detail
7 [18].

8 *Schwann cell culture*

9 SCs were isolated from sciatic nerves of either WT or CMT1A male rats (8 weeks-old, body
10 weight 200-220 g) [29]. SCs were cultured in SC culture medium as previously described:
11 DMEM D-valine + 2 mM glutamine (Gibco) + 10 % fetal calf serum (Gibco) + 1 % N2
12 Supplement (Gibco) + 20 $\mu\text{g/mL}$ bovine pituitary extract + 5 μM forskolin (Gibco) + 100
13 U/mL penicillin/streptomycin (Gibco) + 250 $\mu\text{g/ml}$ fungizone (Gibco). SCs were positively
14 identified using immunofluorescence staining for S-100 β rabbit pAb, (1:400, Dako, Z0311).

15 *In vitro evaluation of total ROS and mitochondrial superoxide in SCs*

16 WT SCs were pre-treated with curcumin (0.001, 0.01, 0.1, 1 or 10 μM) (Sigma) or with Nano-
17 Cur (0.001, 0.01, 0.1, 1 or 10 μM) diluted in physiological saline solution for 8 h. To induce
18 oxidative stress injury, SCs were then subjected to H_2O_2 (0.1 mM) for 8 h. Total ROS and
19 mitochondrial superoxide were evaluated using CellROX-Green reagent (Life Technologies
20 GmbH) and MitoSOX Red mitochondrial superoxide indicator (Life Technologies GmbH)
21 respectively, according to manufacturer's recommendations. Nuclei were counterstained
22 using DAPI (Sigma Aldrich). ROS production, evaluated by green fluorescence intensity, was
23 measured by fluorescence microscopy (Nikon optical microscope 277 H600L) and pictures

1 were captured using a Nikon digital camera (Nikon, Champigny-sur-Marne, France). All
2 images were processed using the NIS-element software (Nikon, Champigny-sur-Marne,
3 France). To quantify positive cells for each marker, 7 fields were counted using ImageJ
4 software (NIH, USA). The "stained cells/number of total cells" ratio was calculated for each
5 field to evaluate the percentage of ROS-positive cells.

6 *In vitro evaluation of mitochondrial membrane potential ($\Delta\Psi_m$) in SCs*

7 The effects of curcumin and Nano-Cur on oxidative stress induced by H₂O₂ were evaluated by
8 examining mitochondrial membrane potential ($\Delta\Psi_m$) that is a marker of mitochondrial
9 function. Incubation of SCs with rhodamine123 (Rho123, Sigma) was previously described
10 [18]. The "stained cells (specific staining)/number of total cells (DAPI staining)" ratio was
11 calculated for each field (n= 7) to evaluate the percentage of positive cells.

12 *MBP (Myelin Basic Protein) expression in SC/neuron co-cultures*

13 Co-cultures of sensory neurons (from WT rat lumbar dorsal root ganglia) and SCs from WT
14 rats were established according to previously published methods [30]. Briefly, co-cultures
15 were maintained for 30 days in neurobasal medium (Invitrogen, Srl) supplemented with 15%
16 newborn calf serum (NCS) (Invitrogen, Srl) and 5 ng/mL nerve growth factor (NGF)
17 (Invitrogen, Srl). After that, co-cultures were treated during 1 week with 0.01 μ M Nano-Cur
18 solubilized in saline, and the latter was given to control cells. The 0.01 μ M concentration was
19 selected depending on the results of the previous ROS production experiment. MBP
20 (expressed in SCs) and NFM (Neurofilament medium polypeptide, expressed in neurites)
21 were studied by immunofluorescence. Cells were fixed with 4% Formaldehyde in PBS at RT
22 for 15 min and then washed with PBS three times. After permeabilizing with 0.1% Triton in
23 PBS and washing, cells were blocked with 1% BSA in PBS for 15 min and incubated with
24 primary antibodies: rabbit anti-NFM Ab (1:1000, Abcam, ab8135) and mouse anti-MBP Ab

1 (1:1000, Sigma-Aldrich, SMI 99) overnight at 4°C. Anti-rabbit Alexa Fluor-488 and anti-
2 mouse Alexa Fluor-594 fluorescent secondary antibodies were used for primary Ab detection
3 (1:800, Thermo Fisher Scientific) and were incubated for 1h at RT. After three washes with
4 PBS and once with distilled water, coverslips were mounted using mounting solution with
5 DAPI for nuclei staining. For each sample, images were acquired using a Nikon optical
6 microscope H600L (Nikon, Tokyo, Japan). Fluorescence intensity was quantified by ImageJ
7 software (NIH, USA).

8 *Animals*

9 The generation of heterozygous CMT1A rats has been previously described by Prof Michael
10 Sereda [28]. These rats harbor 3 copies of murine *Pmp22* gene. Experiments were performed
11 on 60 one-month-old male rats (30 heterozygous CMT1A rats and 30 Sprague Dawley wild-
12 type rats, body weight 200-220 g). Animals were housed in cages with an enriched
13 environment and maintained on a 12h light/dark cycle, at RT of 22°C and with *ad libitum*
14 access to food and water. All rats were observed daily for general well-being, and their weight
15 was measured weekly (data not shown). All animal experiments were performed according to
16 recommendations of the European Directive of 22 September 2010 (2010/63/EU) on the
17 protection of animals used for scientific purposes. Experimental protocols were specifically
18 approved by the Regional Animal Experimentation Ethics committee (CREEAL n° 16-2013-
19 16). Animal studies are reported in compliance with ARRIVE guidelines [31]. All efforts
20 were made to reduce the number of animals used in this study and to ensure optimal
21 conditions of well-being before, during and after each experiment.

22 *Genotyping*

23 Genotyping of rats was performed by qRT-PCR analysis of DNA samples. For this purpose,
24 genomic DNA was purified from tail biopsies using the DNeasy blood and tissue kit (Qiagen,

1 Hilden, Germany) according to the manufacturer's guidelines. The genotype of either
2 CMT1A rats or WT rats was confirmed by the respective presence or absence of *Pmp22*
3 transgenes detected by qRT-PCR using mouse-specific *Pmp22* primers listed in Table 1.

4 *Curcumin and Nano-Cur bioavailability test*

5 Blood samples were collected at 1h, 2h, 8h and 12h, after an intraperitoneal (i.p.) injection of
6 curcumin alone solubilized in saline (50 mg/kg), or Nano-Cur solubilized in saline (0.2
7 mg/kg). 180 μ L of plasma was spiked with 20 μ L of deuterated curcumin solution
8 (500ng/mL) and extracted using 3CC 60 mg HLB cartridge (Waters Corporation, Milford,
9 USA) with the following steps: conditioning with 1mL 0.05% acetic acid in methanol,
10 equilibration with 0.05% acetic acid in water, loading of sample diluted in 0.05% acetic acid
11 in water, 2 washes with 0.5% acetic acid in water and 3 elutions with 1 mL acetonitrile
12 (ACN)/Methanol 1:1 (v/v). After evaporation under nitrogen, samples were solubilized in 50
13 μ L of water/ACN/acetic acid 50:50:0.1 (v/v/v).

14 Calibration standards were prepared separately by spiking 160 μ L of blank plasma with 20 μ L
15 of the curcumin calibration solutions, to give six calibration standards in the range of 0.05–2
16 μ g/mL. The remaining procedure was the same as that described above.

17 The HPLC system consisted of a Shimadzu LC 10 ADvp pump with an SIL-Ht autoinjector
18 and a CTO-10ASvp column oven. The chromatographic separation was performed on an
19 Xterra C18 (150x2.1mm i.d.; Waters). The flow rate was 0.2 mL/min. The mobile phase was
20 a gradient of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in ACN (solvent B)
21 programmed as follows: initial 50% solvent B, increased to 100% in 6.5 min, maintained at
22 100% for 1.5 min, and finally, decreased to 10% solvent B in the last 2 min. Injection volume
23 was 10 μ L/sample. Detection was carried out with a 4000 QTRAP hybrid triple quadrupole
24 linear ion trap mass spectrometer (Sciex) equipped with a Turbo Ion Spray electrospray

1 ionization (ESI) source and controlled by the Analyst software version 1.6.2 (Sciex).
2 Ionization was achieved with electrospray in the negative ionization mode. The MRM
3 transitions of m/z ($367.1 \rightarrow 133.9$, $367.1 \rightarrow 149.1$ and $367.1 \rightarrow 216.9$ for curcumin and 374.3
4 $\rightarrow 134$, $374.3 \rightarrow 152.1$ and $374.3 \rightarrow 220.1$ for deuterated curcumin) were simultaneously
5 monitored. The concentrations of curcumin were determined by calculating its corresponding
6 peak area ratio to that of the IS (internal standard) using a quadratic regression with $1/x$
7 weighting to the calibration curve.

8 *Cellulose nanocrystal body distribution*

9 Dylight 800 derivatization procedure was performed as has been described in the literature
10 [32] Briefly, 50 μL amantadine (1 mg/mL) in dimethylformamide (DMF) were added to 100
11 μL of commercial Dylight 800 NHS (1 mg/mL, Thermofisher) in DMF. The mixture was
12 maintained over-night at RT in the dark. The reaction was monitored by TLC using 30%
13 methanol in chloroform as solvent. The resulting compound was immediately mixed with 10
14 mL of CD/CNCx (2 mg/mL) to obtain Dylight 800-CD/CNCx complexes. The solution was
15 then left unstirred over-night at RT in the dark. Two centrifugations were carried out to
16 eliminate excess Dylight 800. Dylight 800-CD/CNCx complexes were injected i.p. into
17 juvenile rats (1.5 month-old) and fluorescence intensity was measured at the following times:
18 T2, T8, T12, T24 and T48 by IVIS Lumina LT (Perkin Elmer, Wellesley, USA).

19 *Rat Treatments*

20 Rats were treated daily for 8 weeks by i.p. injection of Nano-Cur containing 0.2 mg/Kg/day of
21 curcumin. The rats were randomly divided into 6 groups: WT rats treated with saline
22 (WT/Saline, $n= 10$); WT rats treated with Nano solubilized in saline (WT/Nano, $n= 10$); WT
23 rats treated with Nano-Cur solubilized in saline (WT/Nano-Cur, $n= 10$); CMT1A rats treated
24 with saline (CMT1A/Saline, $n= 10$); CMT1A rats treated with Nano solubilized in saline

1 (CMT1A/Nano, n= 10); CMT1A rats treated with Nano-Cur solubilized in saline
2 (CMT1A/Nano-Cur, n= 10). The number of animals has been established at 10 per group in
3 order to comply with the "3Rs rule" and in particular the reduction and refinement
4 instructions. Treatments were administered by one experimenter, who did not then conduct
5 the experiments.

6 *Grip strength test*

7 Grip strength tests were performed every week during the 8 weeks of treatment. Briefly, the
8 rats were allowed to grasp the T bar of a grip strength meter (BIO-GS3, Bioseb, France) by
9 their hind paws and were then pulled backwards in the horizontal plane. The force applied to
10 the bar at the point when the animal lost grip was recorded as the peak tension. Three tests
11 separated by 10 min were performed for each rat and the mean value of these tests was
12 calculated to represent its grip strength.

13 *Balance test*

14 The bar test evaluated the muscular strength of the four paws and the equilibrium
15 performance of the animals when placed on a fixed rod. This test was performed every week
16 during the 8 weeks of treatment. The rat was placed on its four paws on the middle of a
17 wooden rod (diameter: 2.5 cm; length: 50 cm; height: 30 cm). The time spent on the bar (fall
18 latency) in each trial was recorded. Three tests separated by 10 min were performed for each
19 rat and the mean value of these tests was calculated to represent its equilibrium performance.

20 *Mechanical sensitivity test*

21 The hind paw withdrawal threshold in response to a mechanical stimulus was measured every
22 week, during the 8 weeks of treatment, using a series of von Frey's filaments (Bio-VF-M,
23 Bioseb, France). Briefly, after rat acclimatization in a plastic cage with a mesh floor, von Frey

1 filaments (1g to 60g) were applied to the mid-plantar surface of the left hind-limb for 10s.
2 Filaments were applied in ascending order, and the smallest filament to produce a paw
3 withdrawal response was considered as the threshold stimulus. Three tests separated by 5 min
4 were performed for each rat and the mean value of these tests was calculated to represent its
5 mechanical withdrawal threshold.

6 *Thermal nociception test*

7 Thermal withdrawal latencies were measured using a 52°C hotplate (Bioseb, Vitrolles,
8 France) every week during the 8 weeks of treatment. After 10 min of acclimatization, the
9 animal was placed on the hotplate, and the time was recorded until any expression of
10 nociceptive behaviors such as hind paw shaking or jumping occurred. The cut-off time was
11 fixed at 30s to avoid tissue damage. Three tests separated by 10 min were performed on each
12 rat and the mean value was used to represent its thermal nociception threshold.

13 *Electrophysiology*

14 Electrophysiological parameters of the sciatic nerve were recorded at W8 just before rat
15 euthanasia. Animals were anesthetized by i.p. injection of a solution containing 100 mg/Kg of
16 ketamine (Imalgene 1000, Merial, Lyon, France) and 4 mg/Kg of xylazine (Rompun 2%,
17 Bayer, Lyon, France). Body temperature was maintained using a heated mat (CMA450,
18 Harvard Apparatus, Les Ullis, France). Briefly, both the M wave (or CMAP amplitude) and H
19 reflex were measured with the PowerLab/26T (ADInstruments, Paris, France) after
20 stimulation of the distal and proximal regions of the sciatic nerve with supramaximal
21 impulses. Motor nerve conduction velocity (MNCV) was calculated from the latency
22 difference between the M wave after successive distal and proximal stimulations at two sites.
23 Sensory nerve conduction velocity (SNCV) was calculated from the latency difference
24 between the H reflex after successive distal and proximal stimulation at two sites [33].

1 *Gastrocnemius muscle histoenzymology*

2 Gastrocnemius muscle fragments were embedded in OCT medium, frozen in liquid nitrogen
3 and stored at -80°C. Transverse sections of muscle (5 µm) were taken using a Leica CM 1850
4 UV cryotome at -25°C and collected onto glass slides. Three separate stainings were
5 performed. ATPase staining was used to evaluate the distribution of skeletal muscular fiber
6 types and subtypes. Sections were stained using the m-ATPase reaction with alkaline and acid
7 pre-incubation, by the method of Padykula and Herman (1955) [34]. Nicotinamide adenine
8 dinucleotide (NADH) staining was used to evaluate the pattern of muscular fiber types,
9 distribution of mitochondria and disruption of myofibrils. The NADH tetrazolium reductase
10 reaction was carried out according to the method of Pearse (1968) [35], modified by
11 Dubowitz and Brooke (1973) [36]. Finally, combined cytochrome oxidase and succinic
12 dehydrogenase (COX-SDH) staining was used to show the pattern of muscular fiber types,
13 fibers with abnormal mitochondria and fibers devoid of cytochrome oxidase. This reaction
14 was described by Nachlas et al. (1957) [37] and modified by Wegman and Tordet-Coidroit
15 (1960) [38] . For each sample, images were acquired using a Nikon optical microscope
16 H600L (Nikon, Tokyo, Japan) and were taken from three randomly-chosen fields.

17 *Light microscopy and morphometric analysis*

18 Sciatic nerve samples were collected, fixed in 2.5 % glutaraldehyde and embedded in epoxy
19 resin (Euromedex, Souffelweyersheim, France). Semi-thin transverse sections (1.5µm) were
20 stained with toluidine blue and used for morphometric evaluations, which were conducted by
21 one blinded examiner from random images. Damage to tissue was estimated by the total
22 number of myelinated axons per nerve and the mean diameter of nerve fibers (µm). Myelin
23 sheath thickness was calculated by the formula: (fiber diameter - axon diameter)/2. The
24 degree of myelination was estimated by the axon to fiber diameter ratio (g-ratio).

1 *Western blotting*

2 In brief, equal amounts of sciatic nerve homogenates (20 µg of total protein) were loaded onto
3 a 4-20 % polyacrylamide mini-gel, which was then separated by SDS-PAGE and electro-
4 blotted onto a nitrocellulose membrane. Blots were then blocked with 0.1 % Tween 20 and 5
5 % skimmed milk in TBST, and incubated overnight with mouse monoclonal antibodies
6 recognizing either β cytoplasmic actin (1:1000, Sigma Aldrich, A5441), MPZ (1:5000, Gift of
7 Joan J. ARCHELOS Univ. of Graz, Austria), or MBP (1:200, BioLegend, 808401), or with
8 rabbit polyclonal antibodies recognizing PMP22 (1:200, Sigma-Aldrich, SAB4502217) or
9 Nrf2 (1:500, Proteintech, 16396-1-AP). Membranes were washed thrice with TBST and
10 probed for 1h at RT with anti-rabbit or anti-mouse secondary antibodies conjugated to
11 horseradish peroxidase (HRP) (1:1000, Dako, Rabbit: P0448, Mouse: P0447). Protein bands
12 were revealed using the ECL system (Bio-Rad) and captured by G-box chemi XT4 (Syngene,
13 Cambridge, UK). Protein band intensities were then quantified using ImageJ software (NIH,
14 USA).

15 *Pmp22 mRNA quantification by qRT-PCR*

16 Sciatic nerves were collected from rats for genotyping and were dissected into small
17 fragments. Fragments were immediately frozen in liquid nitrogen. RNA isolation was
18 performed using the RNeasy plus universal tissue kit (Qiagen, Courtaboeuf, France) and
19 QIAzol lysis reagent (Qiagen, Courtaboeuf, France). The RNA concentration was measured
20 using a QuBit 3.0 fluorimeter and purity was evaluated by migration on agarose gel and
21 separation by electrophoresis (18S/28S ratio). In order to obtain cDNA, reverse transcription
22 was performed on 500 ng of RNA suspended in 3 µL gDNA-free water, using the Quantitect
23 reverse transcription kit (Qiagen, Courtaboeuf, France). qRT-PCR was used to quantify
24 *Pmp22* gene expression in sciatic nerve samples from heterozygous or WT rats. Expression of

1 *Pmp22* gene was normalized using two reference genes: *Hprt* (hypoxanthine guanine
2 phosphoribosyl transferase) and *Rictor* (RPTOR independent companion of MTOR complex
3 2) which have been described previously as good candidates for normalizing qRT-PCR assays
4 in peripheral nerves [39]. qRT-PCR was performed using a Rotor-gene 6000 real-time PCR
5 cycler (RG; Corbett life Science, Sydney, Australia) and SYBR green RT-PCR (Qiagen,
6 Courtaboeuf, France). All primers used are listed in Table 1. Quantification cycle (C_q) for
7 each vial was determined using a threshold of 10^{-1} . This value was chosen as it was clearly
8 above the background fluorescence level. A melting curve analysis was performed which
9 showed single product-specific melting temperatures. No primer-dimers were generated
10 during the 40 RT-PCR amplification cycles employed. All samples were quantified in
11 triplicate for analysis. All values were standardized according to the reference genes.

12 *Immunohistochemistry on frozen sections of sciatic nerve*

13 Sciatic nerve samples were embedded in OCT medium and frozen in liquid nitrogen. Frozen
14 sections (5 μ m) were dried for 1h on Superfrost/+ slides, followed by fixation with 4%
15 paraformaldehyde in PBS for 10 min at RT. Antigen retrieval was performed by incubation in
16 0.1% Tween in PBS for 15 min. Non-specific binding was blocked using 10% BSA in PBS
17 for 1h. Rabbit anti-PMP22 (1:200, Sigma-Aldrich, SAB4502217) was added in 4% BSA in
18 PBS, overnight at 4°C. The samples were then rinsed for 15 min in PBS at RT and incubated
19 with the secondary antibody, donkey anti-rabbit Alexa-fluor 594 (1:200, Dako) in 4% BSA in
20 PBS for 2h at RT. Coverslips were mounted using Dako fluorescence mounting medium
21 (Dako France S.A.S, Trappes, France). Red fluorescence was viewed and its intensity was
22 measured by fluorescence microscopy (Nikon optical microscope H600L). Images were
23 captured using a Nikon digital camera (Nikon, Champigny-sur-Marne, France).

24 *Detection of reactive oxygen species in sciatic nerves*

1 Briefly, frozen transverse sections of sciatic nerves (5 μm) were washed with PBS and
2 incubated with 5 μM CellROX-Green reagent (Life Technologies GmbH, 64293 Darmstadt,
3 Germany) for 30 min at 37 $^{\circ}\text{C}$. Nuclei were counterstained using DAPI (Sigma Aldrich).
4 ROS, shown by green fluorescence, were evaluated by fluorescence intensity using
5 fluorescence microscopy.

6 *Determination of GSSG/GSH ratio in gastrocnemius muscles*

7 GSH and GSSG content in gastrocnemius muscle were evaluated using the glutathione assay
8 kit (Cayman Chemical Co., Ann Arbor, MI). Briefly, muscles were washed with ice-cold PBS
9 and homogenized by grinding in PBS and sonicated at 0 $^{\circ}\text{C}$. The homogenates were
10 centrifuged (12000 g, 10 min), and supernatants were then incubated for 5 min at RT with
11 10% metaphosphoric acid (v/v). The mixture was centrifuged (15000 g, 5 min), and the
12 supernatant was analyzed for GSH and GSSG content using the GSH disulphide reductase
13 5,5'-dithiobis (2-nitrobenzoic acid) recycling method employing the procedure described in
14 the glutathione assay kit (Cayman Chemicals). Results were expressed as a ratio of
15 GSSG/GSH.

16 *Evaluation of thiobarbituric acid reactive substance*

17 In brief, rat urine samples were collected after the 8 weeks of treatment (for 12h before
18 animal euthanasia) and were stored at -80 $^{\circ}\text{C}$ until analyses. Lipid peroxidation was evaluated
19 by quantification of TBARS, specifically MDA. The standards were obtained by a dilution
20 range of MDA in distilled water (5, 2.5, 1.25, 0.62, 0.31, 0.16, 0.08 and 0 μM). To precipitate
21 the proteins, trichloroacetic acid 10% was added to urine samples and standards.
22 Subsequently, thiobarbituric acid (TBA) reagent (0.67% TBA, 30% Tris 0.1M, 6.6% NaOH
23 2N) was added to urine samples and standards. Urine samples and standards were heated at 95
24 $^{\circ}\text{C}$ in a water bath for 30 min, cooled in ice water, and then centrifuged for 10 min at 2000

1 rpm. Absorbance of the supernatant was read at 532 nm, and the concentration of TBA-MDA
2 equivalents was calculated from a standard curve derived from MDA. Urinary creatinine
3 concentrations were measured using Cobas C701 automatic analyzer (Roche Diagnostics,
4 Meylan, France). Results were expressed as a ratio of MDA/Creatinine ($\mu\text{M}/\text{mM}$).

5 *Proteomic analysis of sciatic nerve*

6 Proteomic analysis (SWATH-MS/MS) and statistics were performed using standard
7 procedures described in the Supplementary Material. Uniprot entry ID and fold changes of the
8 selected proteins are displayed in Supplementary Table S2.

9 *Data and Statistical Analysis*

10 All data are expressed as mean \pm SEM (Standard Error of the Mean). If normality (Shapiro-
11 Wilk test) and equal variance (Brown-Forsythe test) were observed, data were then compared
12 using either one or two-way analysis of variance (ANOVAs) with repeated measures,
13 followed by post-hoc Tukey's or Dunnett's comparison test. If normality and equal variance
14 were not observed, data were compared using the Kruskal-Wallis test followed by post-hoc
15 Dunn's test. All statistical analyses were performed with GraphPad statistical software
16 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered significant when
17 p was <0.05 , <0.01 , or <0.001 .

1 Results

2 *Nano-Cur improves oxidative stress markers in vitro in rat SCs culture*

3 ROS may attack intracellular biological macromolecules, including nuclear acids, lipids and
4 proteins, finally inducing mitochondrial dysfunction and cell apoptosis [40]. ROS are released
5 from mitochondria, and their excess can disrupt the mitochondrial membrane potential ($\Delta\Psi_m$)
6 [41]. The effects of Nano-Cur or curcumin alone on ROS production and $\Delta\Psi_m$ were
7 investigated *in vitro* using primary cultured WT rat SCs. H₂O₂ addition (0.1 mM) induced a
8 significant increase in total and mitochondrial ROS levels and reduced the index of
9 mitochondrial membrane integrity ($\Delta\Psi_m$) compared to control SCs not subjected to H₂O₂
10 (Figure 1A-C). To investigate the most effective dose, cells were pre-treated over a
11 concentration range of 0.001, 0.01, 0.1, 1 and 10 μ M of free curcumin or Nano-Cur, and H₂O₂
12 was added afterwards. 1 μ M curcumin reduced total ROS levels induced by H₂O₂, while
13 doses of 0.001, 0.01 and 10 μ M had no effect. Nano-Cur doses of 0.01 μ M and 0.1 μ M
14 showed the greatest antioxidant effect (Figure 1A). Concerning mitochondrial ROS
15 production, no significant difference was found between the control group and the group
16 treated with 1 μ M curcumin (Figure 1B). However, Nano-Cur doses of 0.01 μ M and 0.1 μ M
17 significantly reduced H₂O₂-induced mitochondrial ROS (Figure 1B). Likewise, no significant
18 difference was found in $\Delta\Psi_m$ between the control and curcumin groups at doses of 0.1, 1 or
19 10 μ M, whereas Nano-Cur doses of 0.01 μ M, 0.1 μ M and 1 μ M significantly increased $\Delta\Psi_m$
20 (Figure 1C). These results suggest that low doses of Nano-Cur (0.01 and 0.1 μ M) have the
21 most potent antioxidant effect.

22 ROS production and $\Delta\Psi_m$ were also evaluated *in vitro* using primary SC cultures from WT
23 and CMT1A rats. Results indicated a significant increase in total and mitochondrial ROS
24 production and a significant decrease in $\Delta\Psi_m$ in CMT1A SCs in comparison to WT SCs

1 (Figure 1A-F). Compared to untreated CMT1A SCs, a Nano-Cur concentration of 0.01 μM
2 induced a significant reduction in total ($p < 0.001$) and mitochondrial ($p < 0.001$) ROS, and a
3 significant increase in $\Delta\Psi\text{m}$ ($p < 0.05$). A ten times higher concentration of Nano-Cur (0.1 μM)
4 showed a weaker effect. These results suggest the presence of oxidative stress in CMT1A SCs
5 and indicate that a low concentration Nano-Cur (0.01 μM) has the most potent antioxidant
6 effect.

7

8 *Nano-Cur improves MBP expression in vitro in SC/neuron co-culture*

9 Nano-Cur concentration of 0.01 μM was selected based on the results of the previous
10 experiment. Co-cultures of SCs and neurons from WT rats were treated with saline (Figure
11 2A,C) or 0.01 μM Nano-Cur (Figure 2B,D) and stained for MBP showing myelin and NFM
12 detecting neurites. In comparison to control conditions, Nano-Cur treatment induced a
13 significant increase in MBP expression (Figure 2E), while no significant effect was observed
14 on NFM expression (Figure 2F). The increase in MBP/NFM ratio in Nano-Cur co-culture
15 reflects a potential to initiate myelination in comparison to the control co-culture (Figure 2G)
16 given that MBP constitutes around 8% of the total proteins in PNS myelin and is one of the
17 first proteins expressed in the myelination process [42].

18

19 *Exploratory experiments of CD/CNCx biodistribution and curcumin bioavailability*

20 The i.p. injection of fluorophore-labelled nanocrystals (Dylight 800-CD/CNCx) in a rat
21 showed a wide distribution in the body, including the limbs (Figure 3). The peak of
22 distribution was at $t = 2\text{h}$, and the nanocrystals were detectable until 72h post-injection. In
23 addition, the plasma concentration-time relationship was studied (Supplementary Table S1).
24 Nano-Cur, at a dose 250 times lower than that of free curcumin (0.2 versus 50 mg kg^{-1} i.p.),

1 still achieved a higher average plasma concentration of curcumin at t= 1h (26.3 ± 16.1 versus
2 24.9 ± 0.2 ng mL⁻¹ respectively) and t= 2h (5.3 ± 0.7 versus 4.3 ± 0.7 ng mL⁻¹ respectively).
3 In addition, Nano-Cur maintained a prolonged release of curcumin in the plasma for at least
4 12h afterwards, while curcumin was not detectable after 8h.

5

6 *Nano-Cur treatment improves sensori-motor performances and nerve conduction in CMT1A* 7 *rats*

8 Three WT groups and three CMT1A groups treated daily with i.p. saline, CD/CNCx (Nano)
9 or curcumin-CD/CNCx (Nano-Cur 0.2 mg/Kg/day) for 8 weeks were studied. For each
10 behavioral test, statistical analysis showed no significant difference between the three WT
11 groups during the 8 weeks. Similarly, no difference was observed between CMT1A/Saline
12 and CMT1A/Nano groups.

13 The hind paw grip performance of the animals was evaluated using the grip strength test
14 (Figure 4A). No significant difference was reported between all groups at week 0 (W0). As
15 expected, the grip strength of CMT1A/Saline rats was significantly lower than that of
16 WT/Saline rats during the 8 weeks of the study. Likewise, grip performance in CMT1A/Nano
17 rats was lower than that of WT/Saline animals at W4-8. However, no significant difference
18 was found between CMT1A/Nano-Cur and WT/Saline rats over the first 4 weeks of
19 treatment, although it was significantly lower from W5-8. Interestingly, analyses indicated a
20 significant improvement in grip strength in CMT1A/Nano-Cur rats compared to
21 CMT1A/Saline animals at W6-8. Similarly, a significant increase of grip strength was
22 revealed in CMT1A/Nano-Cur animals versus CMT1A/Nano rats at W4 and W6-8. This
23 suggests that Nano-Cur treatment limits the loss of grip strength in CMT1A rats.

1 Animal balance performance was evaluated using the bar test (Figure 4B). The balance time
2 values of CMT1A/Saline rats were significantly less than that of WT/Saline rats during the 8
3 weeks of study. Similarly, a significant decrease in balance performance was observed in
4 CMT1A/Nano rats compared to WT/Saline animals. CMT1A/Nano-Cur rats exhibited a
5 significantly lower balance time during the first two weeks of therapy when compared to
6 WT/Saline rats. Interestingly, in the latter 6 weeks, no significant difference was observed. In
7 addition, a significant improvement in balance performance was observed in CMT1A/Nano-
8 Cur rats at W6, 7 and 8 in comparison to CMT1A/Saline or CMT1A/Nano rats. Taken
9 together, Nano-Cur treatment has a beneficial effect on the balance performance of CMT1A
10 rats.

11 The response of rats to mechanical stimuli was evaluated using von Frey filament test (Figure
12 4C). At W8, a significant increase in the pressure that induced paw withdrawal, indicating a
13 decrease in tactile sensitivity, was found in CMT1A/Saline animals compared to WT/Saline
14 animals, while a tendency to increase was observed at W6 and W7. Similarly, at W6, W7 and
15 W8, a significant increase in the mechanical withdrawal pressure was observed in
16 CMT1A/Nano rats in comparison with WT/Saline. Interestingly, a significant improvement of
17 tactile sensitivity was shown in CMT1A/Nano-Cur animals at W6 and W7 compared to
18 CMT1A/Nano rats. Finally, no significant difference during the 8 weeks was observed
19 between CMT1A/Nano-Cur and WT/Saline groups. These results suggest that Nano-Cur
20 treatment limits the loss of tactile sensitivity in CMT1A rats.

21 The response to a thermal stimulus was evaluated using the hotplate test (Figure 4D). At W7
22 and W8, a significant increase in the time for paw withdrawal was noticed in CMT1A/Saline
23 and CMT1A/Nano rats compared to WT/Saline rats, thus indicating a deterioration in thermal
24 sensitivity in CMT1A rats. In contrast, CMT1A/ Nano-Cur rats at W7 and W8, showed no

1 significant difference in thermal withdrawal threshold compared to WT/Saline. Accordingly,
2 Nano-Cur was able to limit the loss of the thermal sensitivity in CMT1A rats.

3 Evoked electromyography was performed at W8 (Table 2) to measure both motor and sensory
4 nerve conduction velocities (MNCV and SNCV respectively) in sciatic nerves. The MNCVs
5 in CMT1A/Saline and CMT1A/Nano rats were 14.1 m.s^{-1} and 14.9 m.s^{-1} respectively,
6 markedly less than that of the WT rats which was approximately 40 m.s^{-1} . Interestingly, the
7 mean of MNCVs for CMT1A/Nano-Cur rats increased to 25 m.s^{-1} , representing an
8 improvement of more than 65% (compared to CMT1A/Saline and CMT1A/Nano groups).
9 Similarly, the mean of M wave amplitudes (compound muscle action potential CMAP) was
10 increased by more than 180% in CMT1A/Nano-Cur rats compared to CMT1A/Saline and
11 CMT1A/Nano rats, although this increase did not achieve full restoration. Expectedly, in
12 CMT1A/Saline and CMT1A/Nano groups, it was not possible to record the amplitude of H
13 reflex, and thus calculate SNCVs. On the other hand, in CMT1A/Nano-Cur rats, H reflex was
14 detectable, allowing SNCVs to be calculated as 12.5 m.s^{-1} , while that of WT/Saline rats was
15 35.9 m.s^{-1} . Taken together, Nano-Cur was found to ameliorate nerve conduction in CMT1A
16 rats.

17

18 *Nano-Cur treatment limits demyelination in vivo*

19 Morphometric analysis of sciatic nerves was carried out at W8. Quantification of histological
20 features are presented in Table 3, and corresponding representative images are shown in
21 Figure 5. No significant difference was found in the determined parameters among the three
22 WT groups. The total number of myelinated axons was similar in all groups (WT and
23 CMT1A). The mean diameter of myelinated axons and mean diameter of myelinated fibers
24 (axon + myelin) were significantly decreased in each of the CMT1A groups in comparison to

1 WT/Saline. However, in CMT1A/Nano-Cur group, a significant increase in the diameter of
2 myelinated fibers was observed when compared to CMT1A/Nano group. G-ratio (axon
3 diameter/ fiber diameter) was found to be significantly higher in each of the three CMT1A
4 groups compared to the WT/Saline group suggesting that the degree of myelination is lower
5 in CMT1A rats. Likewise, myelin thickness was significantly lower in each of the CMT1A
6 groups compared to the WT/Saline group. Interestingly, an approximate 35% increase in
7 myelin thickness was observed in CMT1A/Nano-Cur versus CMT1A/Saline and
8 CMT1A/Nano animals.

9 The expression levels of myelin proteins MPZ, MBP, and PMP22 were evaluated in sciatic
10 nerve homogenates at W8 (Figure 6A-C). For each protein, no significant difference was
11 observed between each of the WT groups. With respect to MPZ expression (Figure 6A), a
12 significant decrease was found in each of the three CMT1A groups when compared to
13 WT/Saline. Interestingly, its expression was increased in CMT1A/Nano-Cur rats compared to
14 CMT1A/Saline animals. Likewise, a decreased expression of MBP (Figure 6B) was also
15 found in all CMT1A groups when compared to WT/Saline rats, while no beneficial effect of
16 Nano-Cur was observed. Regarding PMP22 (Figure 6C), decreased expression was observed
17 in CMT1A/Saline and CMT1A/Nano homogenates when compared with those of the
18 WT/Saline animals. However, no difference in PMP22 expression levels was found between
19 CMT1A/Nano-Cur and WT/Saline animals, while its expression increased in CMT1A/Nano-
20 Cur group compared with CMT1A/Nano group. These results were confirmed by
21 immunofluorescent staining of PMP22 in sciatic nerve slices at W8. Representative images
22 (Figure 6E) show an increase in PMP22 staining in CMT1A/Nano-Cur slices compared to the
23 other CMT1A groups, which in turn have a lower expression than WT groups. While no
24 significant difference was seen in *Pmp22* mRNA expression levels when comparing each of
25 the WT groups, a significant increase was found in the CMT1A/Saline rats compared to

1 WT/Saline animals (Figure 6D). A trend to decrease in *Pmp22* mRNA expression was
2 observed in CMT1A/Nano-Cur group in comparison to CMT1A/Saline rats. Taken together,
3 these results suggest that Nano-Cur treatment promotes myelination in CMT1A rats.

4

5 *Nano-Cur treatment limits muscle damage in CMT1A rats*

6 Since a hallmark of the disease is muscular atrophy of variable severity, the effect of Nano-
7 Cur on muscle damage was tested in gastrocnemius muscle biopsies of rats at the end of W8.
8 Microscopic analysis of three histochemical stains (ATPase, COX-SDH, NADH; see
9 Methods) did not show any differences in gastrocnemius muscle fiber size or distribution of
10 mitochondria between all groups at W8 (Figure 7A). The clustering of type I and/or type II
11 muscle fibers is an early sign of muscle damage [43]. A trend of such clustering was observed
12 in biopsies of CMT1A/Saline and CMT1A/Nano when compared to WT/Saline biopsies
13 (Figure 7A, white arrows). However, fiber clustering was less evident in CMT1A/Nano-Cur
14 rat biopsies. In addition, the muscle lysis marker creatine phosphokinase (CPK) was
15 significantly increased in CMT1A/Saline rats and CMT1A/Nano rats compared to the
16 WT/Saline group (Figure 7B). Concerning creatinine, another muscle lysis marker, a
17 significant decrease was observed in CMT1A/Saline rats compared to the WT/Saline group.
18 Interestingly, CPK and creatinine plasma levels were not different between CMT1A/Nano-
19 Cur and WT/Saline groups (Figure 7B,C), suggesting a beneficial effect of Nano-Cur on
20 CMT1A gastrocnemius muscle integrity.

21

22 *Nano-Cur treatment decreases oxidative stress and induces the expression of antioxidant*
23 *enzymes in CMT1A rats*

1 Oxidative stress was evaluated by quantification of total ROS in sciatic nerve sections at W8
2 (Figure 8A). No significant difference in basal ROS content was observed in the WT groups.
3 However, a significant increase in ROS levels was found in CMT1A/Saline and
4 CMT1A/Nano versus WT/Saline group. Interestingly, ROS content was significantly
5 decreased in CMT1A/Nano-Cur in comparison to CMT1A/Saline and CMT1A/Nano groups,
6 and it was similar to that of WT/Saline group. On the other hand, GSSG/GSH ratio was
7 evaluated in gastrocnemius muscle homogenates at W8 (Figure 8B). A non-significant
8 tendency to increase was observed in the average GSSG/GSH ratio of CMT1A/Saline and
9 CMT1A/Nano groups versus WT/Saline group. In contrast to the variability observed in
10 CMT1A rats, Nano-Cur-treated rats showed a similar profile to WT rats. Total lipid
11 peroxidation was evaluated by quantification of thiobarbituric acid reactive substances
12 (TBARS), specifically malondialdehyde (MDA), in rat urine samples collected at W8 (Figure
13 8C). No significant difference was observed among the WT groups. In each of the three
14 CMT1A groups, urinary MDA levels were significantly higher in comparison to WT/Saline
15 rats, and no difference was shown in CMT1A/Nano-Cur rats compared to CMT1A/Saline
16 group.

17 The expression of Nrf2 protein, a transcription factor involved in the production of
18 antioxidant enzymes, was evaluated in sciatic nerve homogenates at W8 (Figure 8D). No
19 significant difference was found between each of the WT groups as well as in CMT1A/Saline
20 or CMT1A/Nano groups in comparison to the WT/Saline group. Interestingly, in
21 CMT1A/Nano-Cur rats, an increased expression of Nrf2 was shown when compared to each
22 of the other groups (WT/Saline, CMT1A/Saline and CMT1A/Nano).

23 As proteomics technology is widely used in neurodegenerative disease analysis [44], and in
24 order to gain further insight about the role of oxidative stress in CMT1A disease, a MS

1 proteomic analysis was performed on distal sciatic nerve homogenates from WT/Saline,
2 CMT1A/Saline and CMT1A/Nano-Cur rats at W8 (Figure 8E-J). Among the identified
3 proteins, significantly increased expression levels of peroxiredoxin-1 (PRDX1) and
4 peroxiredoxin-5 (PRDX5), two peroxidases that reduce peroxides, were observed in
5 CMT1A/Saline rats versus WT/Saline animals. Interestingly, Nano-Cur treatment
6 significantly increased the expression levels of PRDX1 and PRDX5 in CMT1A/Nano-Cur
7 rats as compared to CMT1A/Saline or to WT/Saline groups. While the expression level of
8 thioredoxin-1 (TRX1), a major component of the disulfide reductase system, remained
9 unchanged in CMT1A/Saline group in comparison to WT/Saline animals, it was significantly
10 increased in CMT1A/Nano-Cur in comparison to CMT1A/Saline or WT/Saline. Concerning
11 superoxide dismutase-1 (SOD1), which catalyzes the dismutation of superoxide anion free
12 radicals into molecular oxygen and hydrogen peroxide, no significant difference was found in
13 CMT1A/Nano-Cur animals in comparison to CMT1A/Saline, while a significantly increased
14 expression was found in CMT1A/Saline and CMT1A/Nano-Cur groups in comparison to
15 WT/Saline rats. The expression level of glutathione peroxidase-1 (GPx1) which acts on
16 peroxides (e.g., lipid peroxide, hydrogen peroxide), was significantly decreased in
17 CMT1A/Saline and CMT1A/Nano-Cur groups in comparison to WT/Saline group, but no
18 significant effect of Nano-Cur treatment was observed. Finally, expression levels of xanthine
19 dehydrogenase (XDH), which catalyzes the oxidation of purines, pterins and aldehydes and
20 can be transformed to xanthine oxidase (XOD), remained unchanged in the three groups.
21 Taken together, these results add that imbalance in the redox status in sciatic nerve is another
22 major feature of CMT1A rats, and that Nano-Cur treatment shows an antioxidant effect *in*
23 *vivo*.

24

1 *Effect of Nano-Cur treatment on protein folding and degradation in CMT1A rats*

2 Our proteomic data provided the opportunity to investigate variations in the expression levels
3 of proteins involved in glycoprotein (*e.g.* PMP22) folding and degradation. The expression
4 levels of heat shock protein 90 alpha (HSP 90 alpha) and heat shock cognate 70 kDa protein
5 (HSC70) involved in correct protein folding and heat shock response (HSR) [45,46], were
6 significantly increased in CMT1A/Saline rats in comparison to WT/Saline rats (Figure 9A-B).
7 Similarly, a significant increase in the expression of the ER chaperones calnexin (CNX) and
8 calreticulin (CRL), which contribute to the proper folding of glycoproteins (such as PMP22)
9 [47], was observed in CMT1A/Saline group when compared to WT/Saline group (Figure
10 9C,D). Terminally misfolded glycoproteins are targeted for degradation by the proteasome in
11 the endoplasmic reticulum-associated degradation (ERAD) pathway involving glucose-
12 regulated protein 94 (GRP94), transitional ER ATPase valosin-containing protein (VCP) and
13 the ubiquitin system [47,48]. The expression levels of GRP94, VCP, polyubiquitin-C (UBC),
14 and proteasome subunit alpha-type4 were significantly elevated in CMT1A/Saline group
15 compared to WT/Saline group (Figure 9E,H). When misfolded proteins build up in the ER,
16 binding immunoglobulin protein (BiP) initiates the unfolded protein response (UPR) cascade
17 to potentiate ER quality control mechanisms [47]. Here, no significant difference in the
18 expression level of BiP was observed between each of the tested groups (Figure 9I).
19 Following Nano-Cur treatment, a significant increase in the expression levels of HSP 90
20 alpha, HSC70, CNX, CRL, GRP94, VCP, UBC and proteasome subunit alpha-type4 was
21 observed in CMT1A/Nano-Cur groups when compared to WT/Saline groups. Interestingly,
22 compared to CMT1A/Saline animals, a significant increase in the expression levels of HSC70
23 and UBC was detected in CMT1A/Nano-Cur rats. However, although a tendency to increase
24 in CNX, CRL, HSP 90 alpha, GRP94, VCP and proteasome subunit alpha expression levels
25 was observed in CMT1A/Nano-Cur group versus CMT1A/Saline group, it was not

1 statistically significant. Taken together, these results suggest that there is activation of HSR
2 and ERAD pathways in CMT1A rats. Moreover, Nano-Cur treatment may mitigate
3 proteotoxicity through ERAD and HSR pathways activation.

1 **Discussion**

2 CMT1A is the most common dysmyelinating peripheral neuropathy for which no specific
3 treatment is unfortunately available to date. Recently, oxidative stress has been described in
4 CMT1A patients carrying *PMP22* duplication [7]. Curcumin is a polyphenol known for its
5 powerful antioxidant properties. Indeed, in a previous study, we showed that local low dose of
6 curcumin (0.2 mg/day for 5 weeks) improved remyelination and functional recovery in a rat
7 sciatic nerve crush model through the reduction of oxidative stress [18]. However, since
8 CMT1A disease is a polyneuropathy, a systemic administration of curcumin is required. Due
9 to the unfavorable pharmacokinetics of curcumin, Nano-Cur was developed as an innovative
10 approach to improve the kinetic properties of curcumin. Nano-Cur was shown previously to
11 enhance cellular delivery of curcumin *in vitro* [25]. Thus, in the scope of our goal to test
12 Nano-Cur for the treatment of CMT1A disease, *in vitro* experiments were carried out first as a
13 proof-of-concept of Nano-Cur therapeutic potential. After that, Nano-Cur efficacy was tested
14 in a CMT1A rat model by evaluating its effect on sensori-motor performances, as well as on
15 myelination, oxidative stress, and ER stress.

16 Our *in vitro* results showed that Nano-Cur improved MBP expression in WT SC/neuron co-
17 culture. On the other hand, ROS accumulation in mitochondria leads to mitochondrial
18 permeability and thus a collapse of mitochondrial membrane potential ($\Delta\Psi_m$) [40]. Here, we
19 showed that Nano-Cur decreased oxidative stress in WT SCs subjected to oxidative stress
20 (induce by H_2O_2), while $\Delta\Psi_m$ was restored to normal. The greatest antioxidant effect of
21 Nano-Cur was observed at low concentration (0.01 μ M) rather than at higher concentrations.
22 Previous studies demonstrated that, although low concentrations of curcumin molecule induce
23 antioxidant effects, higher concentrations (1-50 μ M) increase cellular levels of ROS [49,50].
24 Since Nano-Cur significantly improves the entry of curcumin molecules into the cells [25],

1 the 0.01 μM doses of Nano-Cur seem to be sufficient to deliver the correct amount of
2 curcumin that induces the desired anti-oxidant effect. These results provide an *in vitro* proof-
3 of-concept for Nano-Cur efficacy at low dose. Then, we showed to our knowledge for the first
4 time, the presence of oxidative stress in cultured SCs of CMT1A rats. It has been described
5 that oxidative stress plays a key role in many neurodegenerative diseases such as Multiple
6 Sclerosis, Alzheimer, Parkinson and Huntington diseases [51] as well as in several peripheral
7 neuropathies (PN) especially diabetic PN [52] and chemotherapy-induced PN [53]. The
8 increase in mitochondrial ROS and decrease in mitochondrial membrane potential ($\Delta\Psi\text{m}$)
9 suggest that mitochondrial dysfunction is an important source of ROS in CMT1A SCs. Being
10 vulnerable to oxidative damage, changes in mitochondrial function are linked to several
11 neurological diseases including other CMT mutations [54,55]. Importantly, using the same
12 dose previously determined on WT SCs (0.01 μM), we observed that Nano-Cur significantly
13 reduce total and mitochondrial ROS in CMT1A SCs, also restoring the $\Delta\Psi\text{m}$ to normal. These
14 results thus confirm the antioxidant potential of the Nano-Cur *in vitro*.

15 Based on the encouraging data obtained in *in vitro* studies, an *in vivo* approach was then
16 conducted. Exploratory study of i.p. injection in rat, showed that the nanoparticle vectors are
17 widely distributed throughout the body. Interestingly, Nano-Cur increased the average
18 bioavailability of curcumin by approximately 250 times compared to curcumin alone and
19 maintained a sustained release of curcumin following a single i.p. injection. These results
20 confirm the unfavorable pharmacokinetics of curcumin when administered alone and show its
21 improvement by the new formulation (Nano-Cur).

22 Then, our functional experiments performed in CMT1A rats showed that Nano-Cur treatment
23 (0.2 mg/kg/day) improved sensori-motor coordination and muscular strength, limited the loss
24 of mechanical and thermal sensitivity, and limited muscular damage. In parallel, Nano-Cur

1 markedly increased MNCVs and CMAP amplitudes compared to CMT1A/Saline. Although
2 not fully restored to normal, these values represent around 60% and 45% of the WT values
3 respectively. In addition, Nano-Cur promoted the detection of H reflexes and thus the
4 calculation of SNCVs in CMT1A rats. The treatment also notably improved myelin thickness
5 and the expression of compact myelin proteins MPZ and PMP22 in CMT1A sciatic nerves.
6 This is in agreement with studies showing that curcumin (alone or delivered by nano-vectors)
7 increased myelination in different models of peripheral and central diseases [19,56]. Indeed,
8 the *in vivo* improvement in myelination contributes to the increase in NCV and subsequent
9 functional improvements. Reviewing literature, CMT rats treated with neuregulin-1 showed
10 an improved CMAP (around 45% of the WT value) with an unaltered NCV [57]. An 8-
11 months treatment of CMT1A rats by PXT3003, being currently in phase III clinical trial and
12 composed of baclofen, sorbitol and naltrexone, improved MNCV (64% of WT value), without
13 a significant effect on CMAP amplitudes [58].

14 In order to understand the mechanisms of action by which the Nano-Cur improved
15 myelination and thus the phenotype of CMT1A rats, oxidative stress processes were explored.
16 Thus, concordant with *in vitro* results, oxidative stress was observed in CMT1A rats by an
17 elevated ROS production in sciatic nerves and lipoperoxidation markers in urine. An
18 increasing trend in the GSSG/GSH ratio in the gastrocnemius muscles has also been observed.
19 Oxidative stress is an imbalance between pro-oxidant and antioxidant processes, which results
20 to an excessive production of ROS. This excessive ROS production is deleterious for cells,
21 and since myelin is rich in lipids, SCs are particularly exposed to lipoperoxidation. In
22 addition, chronic oxidative stress can exceed muscle regeneration capacity [59] and accelerate
23 the loss of muscle fibers in CMT patients [7]. The elevated expression of a set of antioxidant
24 proteins (PRDX1, PRDX5, and SOD1) in CMT1A rat sciatic nerves observed here, is likely
25 an adaptive response to counteract this oxidative stress. Interestingly, it has been observed

1 that Nano-Cur decrease ROS production in sciatic nerves and tended to decrease GSSG/GSH
2 ratio in gastrocnemius muscles. However, the fact that the total lipoperoxidation measured in
3 urine was not reduced by Nano-Cur, suggests another source and/or tissue impacted by ROS
4 in CMT1A rats, other than sciatic nerve and gastrocnemius muscle. Nano-Cur treatment also
5 increased the levels of Nrf2 in the sciatic nerve of CMT1A rats. Furthermore, the levels of
6 antioxidant enzymes PRDX1, PRDX5, and TRX1 were significantly increased in sciatic
7 nerve of CMT1A rats treated with Nano-Cur in comparison to CMT1A control rats. Thus, the
8 increased expression of these enzymes induced by Nano-Cur treatment likely contributes to
9 the reduction of oxidative stress in CMT1A sciatic nerve. Previously, we have shown that
10 local treatment with curcumin increased Nrf2 levels in crushed rat sciatic nerves [18]. In
11 normal conditions, Kelch-like ECH-associated protein 1 (Keap1) binds to Nrf2 and tags it for
12 degradation. Under oxidative stress condition, Nrf2 is freed from Keap1 and then is
13 translocated to the nucleus, where it binds to the promoter region of the antioxidant response
14 element (ARE) to regulate the gene expression of antioxidant enzymes [60]. A recent study,
15 in the human neuroblastoma SH-SY5Y cell line, reported that curcumin increases Nrf2
16 protein expression without any effect on the protein levels of Keap1 and the transcription
17 levels of Nrf2 and Keap1 mRNA. Thus, curcumin would interact directly with Keap1,
18 preventing its binding to Nrf2 and, consequently, increasing the amount of Nrf2 translocated
19 to the nucleus, and antioxidant enzymes production [61]. We have also reported that curcumin
20 has an important affinity for lipid membranes, thus allowing the reduction of lipoperoxidation
21 in SCs [18]. Indeed, curcumin, like many polyphenols, has the ability to directly scavenge
22 some ROS [17]. Thus, curcumin, through the activation of Nrf2, the increase of antioxidant
23 enzymes, and the elimination of ROS, represents a powerful antioxidant compound
24 potentially usable in treatment of many pathologies.

1 There is increasing evidence in neurological diseases that there is a strong link between
2 increased oxidative stress and ER stress [10]. Indeed, the ER redox environment dictates the
3 fate of entering proteins in ER. Thus, an increase in intracellular oxidative stress is
4 unfavorable to the proper folding of proteins [10]. Protein misfolding and ER stress have been
5 commonly reported in neurological diseases, including CMT1A [47]. In our study, the
6 expression levels of ER quality control system proteins (CNX and CRL) [47] and HSR
7 proteins (HSP 90 alpha and HSC70) [45] were increased in the sciatic nerves of CMT1A rats.
8 Moreover, the expression levels of ERAD players, namely GRP94, VCP, UBC, and the
9 proteasome [47] were also increased in CMT1A sciatic nerve. Activation of ER quality
10 control system, ERAD, and HSR suggest an ER stress and the induction of adaptive
11 mechanisms to facilitate PMP22 folding/elimination. If the situation cannot be restored, the
12 UPR drives the cell to apoptosis [47]. The unchanged levels of BiP suggest that UPR may not
13 be involved at this stage of the disease (3-month-old rats). Thus, this study adds a further step
14 in understanding the complex mechanisms of ER stress in CMT1A disease which seem to
15 vary in case of *PMP22* point mutation [47]. Finally, Nano-Cur trended to increase the
16 expression levels of CNX, CRL, HSP 90 alpha, GRP94, VCP, and proteasome subunit alpha,
17 with a significant increase regarding HSC70 and UBC. In addition, in a CMT1B model
18 (R98C mice), treatment with curcumin improves the peripheral neuropathy by alleviating ER
19 stress, reducing the activation of UPR, and promoting SC differentiation [62]. Therefore, in
20 our study, oxidative stress reduction and stimulation of ERAD and HSR players could
21 potentially, aid in PMP22 refolding and elimination of misfolded PMP22, explaining the
22 beneficial effects of Nano-Cur on myelination. However, further studies are therefore needed
23 to elucidate the ER adaptive mechanisms resulting from *Pmp22* duplication.

24 **Conclusions**

1 In conclusion, the present study shows that Nano-Cur not only improves curcumin
2 pharmacokinetics, but also CMT1A rat phenotype. Curcumin delivered by CNCx exerts its
3 action mainly by mitigating oxidative stress and potentially alleviating ER stress and thus
4 improving myelination. Significant improvements were observed at the cellular, histological,
5 electrophysiological, and functional levels. Further mechanistic insights into the link between
6 these three pathways will help in understanding the pathology of CMT1A and the mechanism
7 of action of Nano-Cur. Finally, our results support the concept that Nano-Cur holds promise
8 as a potential treatment for peripheral neuropathies.

1 **Author contributions**

2 M.C., F.B., and A.D. designed the experiments. M.C. and Z.M. acquired and analyzed the
3 data. M.C., L.V., L.R., F.F., and P.A.F. conducted the experiments. G.M.A.N., R.G., and V.S.,
4 provided the reagents. M.C., Z.M., and F.B. wrote the original manuscript draft. F.B., A.D.,
5 F.F. reviewed and edited the manuscript. All authors reviewed and approved the manuscript.

6

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17

18 **Conflict of Interest Statement:** The authors declare no conflict of interest.

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2 **Figure 1: Nano-Cur treatment mitigates oxidative stress in SCs *in vitro*.** (A, B, C) Effects
3 of curcumin and Nano-Cur were examined *in vitro* on WT SCs submitted to oxidative stress
4 induced by H₂O₂ (0.1 mM) for 8 h. (D, E, F) Effects of Nano-Cur were examined *in vitro* on
5 WT and CMT1A SCs. (A and D) Fluorescence intensity as a measure of total ROS production
6 using CellROX method. (B and E) Fluorescence intensity as a measure of mitochondria
7 superoxide production using MitoSOX method. (C and F) Fluorescence intensity as a
8 measure of mitochondrial membrane potential using Rho123. Values are expressed as mean ±
9 SEM. n= 7/group. (A, B, C) Results were compared using one-way ANOVA and post-hoc
10 Tukey's test (*: p<0.05, **: p<0.01 and ***: p<0.001 vs. control (no H₂O₂); §: p<0.05, §§:
11 p<0.01 and §§§: p<0.001 vs. H₂O₂). (D, E, F) (*: p<0.05, **: p<0.01 and ***: p<0.001 vs.
12 WT SCs; #: p<0.05, ##: p<0.01 and ###: p<0.001 vs. CMT1A SCs).

1

2 **Figure 2: Nano-Cur treatment improves MBP expression in SCs *in vitro*.** Representative
3 images of MBP (red), NFM (green), and DAPI (blue) immunohistochemistry on co-cultured
4 SCs and neurons (WT) maintained for 30 days and treated thereafter for 1 week with (A and
5 C) Saline or (B and D) Nano-Cur (0.01 μ M). (E) MBP fluorescence intensity calculated as
6 ratio MBP/DAPI. (F) NFM fluorescence intensity calculated as ratio NFM/DAPI. (G)
7 MBP/NFM ratio. Results were compared using t test (*: $p < 0.05$, and **: $p < 0.01$ vs. control).
8 $n = 4$ frames/group. Scale bars: 20 μ m.

1

- 2 **Figure 3: Biodistribution of CD/CNCx.** *In vivo* imaging of Dylight 800-CD/CNCx complex
3 dispersal in a rat at different hours after an i.p. injection.

1

2 **Figure 4: Nano-Cur treatment improves the phenotype of CMT1A rats.** (A) Grip strength
3 of hind limbs (g). (B) Balance performance evaluated as fall latency time (s). (C) Mechanical
4 withdrawal threshold using von Frey's filaments (g). (D) Thermal withdrawal threshold
5 evaluated as withdrawal latency time (s) on a hotplate. Data are expressed as mean \pm SEM. n=
6 10 rats/group. Results were compared using two-way ANOVA (repeated measures) and post-
7 hoc Dunnett's test (*: p<0.05, **: p<0.01 and ***: p<0.001 vs. WT/Saline group) and with
8 two-way ANOVA followed by post-hoc Tukey's test (#: p<0.05, ##: p<0.01 and ###:
9 p<0.001 vs. CMT1A/Saline group; \$: p<0.05, \$\$: p<0.01 and \$\$\$: p<0.001 vs. CMT1A/Nano
10 group).

1

2 **Figure 5: Nano-Cur treatment improves myelination in CMT1A rats.** Representative
3 images of transverse semi-thin sections of WT and CMT1A rat sciatic nerves stained with
4 toluidine blue. Scale bars: 100 μm (for inserts: magnification x3). Corresponding
5 quantification of axon number, axon diameter, fiber diameter, G-ratio, and myelin thickness
6 are displayed in Table 3.

1

2 **Figure 6: Nano-Cur treatment limits demyelination *in vivo*.** (A, B and C) Relative protein
3 quantification of MPZ, MBP, and PMP22 by Western blot. n= 7/group. (D) Representative
4 histogram of Pmp22 mRNA quantification by qRT-PCR. n= 10/group. (E) Representative
5 images of PMP22 immunohistochemistry on sciatic nerve slices. Scale bars: 100 μ m (for
6 inserts: magnification x3). Results were compared using one-way ANOVA and post-hoc
7 Dunnett's test (*: p<0.05, **: p<0.01 and ***: p<0.001 vs. WT/Saline group) and with one-
8 way ANOVA followed by post-hoc Tukey's test (#: p<0.05, ##: p<0.01 and ###: p<0.001 vs.
9 CMT1A/Saline group; \$: p<0.05, \$\$: p<0.01 and \$\$\$: p<0.001 vs. CMT1A/Nano group).

1

2 **Figure 7: Nano-Cur treatment limits muscle damage in CMT1A rats.** (A) Representative
3 microscopy images of gastrocnemius muscle biopsies with ATPase; cytochrome COX-SDH;
4 and NADH staining. White arrows indicate clustering of muscle fibers. No fiber clustering is
5 observed in CMT1A/Nano-Cur images. Scale bars: 100 μ m. Plasma assay of muscle lysis
6 markers (UI/L) (B) creatine phosphokinase (CPK) and (C) creatinine. Data are expressed as
7 mean \pm SEM. n= 10/group. Results were compared using Kruskal-Wallis test (*: $p < 0.05$ vs.
8 WT/Saline group).

1

2 **Figure 8: Nano-Cur treatment decreases oxidative stress and improves the expression of**
3 **some antioxidant enzymes in CMT1A rats.** (A) Fluorescence intensity as a measure of total
4 ROS using CellROX method in sciatic nerves. n= 10/group. (B) Determination of
5 GSSG/GSH ratio in gastrocnemius muscle. n= 5/group. (C) Determination of urinary MDA
6 concentration by the assay of TBARS. n= 10/group (D) Relative protein quantification of
7 Nrf2 by western blot of sciatic nerve samples. n= 8/group. Results were compared using one-
8 way ANOVA and post-hoc Dunnett's test (*: p<0.05, **: p<0.01 and ***: p<0.001 vs.
9 WT/Saline group) and with one-way ANOVA followed by post-hoc Tukey's test (#: p<0.05,
10 ##: p<0.01 and ###: p<0.001 vs. CMT1A/Saline group; \$: p<0.05, \$\$: p<0.01 and \$\$\$:
11 p<0.001 vs. CMT1A/Nano group). (E-J) Histograms of anti-oxidant enzymes (PRDX1,
12 PRDX5, TRX1, SOD1, GPx1), and pro-oxidant enzyme (XDH) expression levels after MS.
13 Results were compared using Kruskal-Wallis test and consecutive post-hoc test for all-pairs
14 comparisons with Conover test (*: p<0.05, **: p<0.01 and ***: p<0.001). WT/Saline n= 5,
15 CMT1A/Saline n= 4, CMT1A/Nano-Cur n= 4. Data are expressed as mean ± SEM.

1

2 **Figure 9: The effect of Nano-Cur on proteins involved in ER stress.** (A-I) Histograms of
3 HSP 90 alpha, HSC70, CNX, CRL, GRP94, VCP, UBC, proteasome subunit alpha, and BiP
4 expression levels after MS. Results were compared using Kruskal-Wallis test and consecutive
5 post-hoc test for all-pairs comparisons with Conover test (*: $p < 0.05$, **: $p < 0.01$ and ***:
6 $p < 0.001$). Data are expressed as mean \pm SEM. WT/Saline $n = 5$, CMT1A/Saline $n = 4$,
7 CMT1A/Nano-Cur $n = 4$.

1 **Table 1: Primers used in qRT-PCR analysis.**

Target	Forward primer	Reverse primer
<i>Pmp22</i>	GTTCCCTGTTCTTCTGCCAGC	CCTCATTTCGCGTTTCCGCA
<i>Rictor</i>	CCTTCCGAATACGAGGGCG	ATTCAGATGGCCCAGCTTCT
<i>Hprt</i>	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC

2

1 **Table 2: Electrophysiological recordings of distal and proximal regions of the rat sciatic**
2 **nerves at W8**

	WT/Saline	WT/Nano	WT/Nano-Cur	CMT1A/Saline	CMT1A/Nano	CMT1A/Nano-Cur
MNCV (m/s)	39.9 (±4.7)	41.5 (±5.0)	39.5 (±3.8)	14.1 (±1.2) ^{***}	14.9 (±1.3) ^{***}	25.0 (±2.4) ^{** ### \$\$\$}
CMAP (mV)	15.7 (±0.9)	14.9 (±0.9)	14.8 (±1.2)	2.5 (±0.5) ^{***}	2.3 (±0.5) ^{***}	7.2 (±0.7) ^{*** ### \$\$\$}
SNCV (m/s)	35.9 (±3.9)	37.4 (±3.8)	36.2 (±2.9)	N.D.	N.D.	12.5 (±1.7) ^{***}
H reflex amplitude (mV)	1.6 (±0.3)	1.7 (±0.3)	1.6 (±0.2)	N.D.	N.D.	0.4 (±0.1) ^{***}

3

4 Electrophysiology values are presented as mean ± SEM. Results were compared using one-
5 way ANOVA and post-hoc Dunnett's test (*: p<0.05, **: p<0.01 and ***: p<0.001 vs.
6 WT/Saline group) and with one-way ANOVA followed by post-hoc Tukey's test (#: p<0.05,
7 ##: p<0.01 and ###: p<0.001 vs. CMT1A/Saline group; \$: p<0.05, \$\$: p<0.01 and \$\$\$:
8 p<0.001 vs. CMT1A/Nano group). n= 10/group. N.D.: Not detected.

1 **Table 3: Morphometric features of myelinated axons in sciatic nerves**

	WT/Saline	WT/Nano	WT/Nano-Cur	CMT1A/Saline	CMT1A/Nano	CMT1A/Nano-Cur
Axon number	1796 (\pm 62.1)	1766 (\pm 71.5)	1741 (\pm 58.1)	1636 (\pm 44.2)	1616 (\pm 58.6)	1679 (\pm 64.1)
Axon diameter (μ m)	5.7 (\pm 0.2)	5.6 (\pm 0.2)	5.6 (\pm 0.2)	4.3 (\pm 0.1)***	4.1 (\pm 0.2)***	4.4 (\pm 0.1)***
Fiber diameter (μ m)	9.8 (\pm 0.3)	9.6 (\pm 0.2)	9.9 (\pm 0.3)	5.9 (\pm 0.2)***	5.8 (\pm 0.2)***	6.7 (\pm 0.3)*** \$
G-ratio	0.57 (\pm 0.01)	0.57 (\pm 0.01)	0.56 (\pm 0.01)	0.71 (\pm 0.01)***	0.69 (\pm 0.02)***	0.64 (\pm 0.01)** #
Myelin thickness (μ m)	2.05 (\pm 0.08)	2.00 (\pm 0.04)	2.12 (\pm 0.07)	0.88 (\pm 0.02)***	0.86 (\pm 0.03)***	1.18 (\pm 0.1)*** # \$

2

3 Corresponding representative images of sciatic nerve sections used for quantification are
4 shown in Figure 5. Data are presented as mean \pm SEM. Results were compared using one-way
5 ANOVA and post-hoc Dunnett's test (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ vs. WT/Saline
6 group) and with one-way ANOVA followed by post-hoc Tukey's test (#: $p < 0.05$ vs.
7 CMT1A/Saline group; \$: $p < 0.05$ vs. CMT1A/Nano group). n= 10/group.