**SUPPLEMENTARY METHODS**

**Gene Panel Sequencing**

Targeted next generation sequencing (NGS) of disease genes for CMT was performed using the HaloPlex Target Enrichment Kit (Agilent Technologies) as previously described.1 *NEFL* variants were confirmed in the family samples by Sanger sequencing with primers specific for exon 2.

**Induced Pluripotent Stem Cells**

Human skin fibroblasts from three healthy controls and from P1 were reprogrammed into pluripotent stem cells at Biomedicum Stem Cell Center (University of Helsinki, Finland) by overexpression of *OCT4*, *SOX2*, *KLF4* and *MYC*, and all cell lines were validated for proper growth, iPSC morphology and pluripotency gene expression by immunocytochemistry and quantitative PCR as previously described.2 Patient and Control 3 fibroblasts were transduced with episomal, Control 1 with Sendai viral and Control 2 with Retroviral vectors. Two iPSC clones from P1 and one clone from each control were used in further experiments. iPSC were cultured in Matrigel-coated (Corning) plates with E8-medium (Gibco) supplemented with E8-supplement (Gibco). Cells were passaged when confluent with 0.5 mM EDTA (Invitrogen) in phosphate-buffer saline (PBS).

**Motor neuron differentiation**

Motor neurons were generated according to Du et al3 with minor modifications.iPSC were passaged 1:4-1:6 with 0.5 mM EDTA into Matrigel-coated plates. The next day iPSC-media was replaced with MNDB (motor neuron differentiation base) containing DMEM/F12 with Glutamax (Gibco), Neurobasal medium (Gibco) at 1:1, 0.5x N2 (Gibco), 0.5x B27 (Gibco), 0.1 mM ascorbic acid (Santa Cruz) and 1x penicillin/streptomycin (Lonza). MNDB was supplemented with 3 µM GSK-3 inhibitor XVI/Chir99021 (Merck), 2 µM BMP Inhibitor II/DMH1 (Merck) and 2 µM TGF-B RI kinase inhibitor VI/SB431524 (Merck). iPSC were differentiated into neuronal progenitors in 6 days, changing media every 2 days.

Neuronal progenitors were passaged 1:4-1:6 into Matrigel-coated plates and cultured in MND2 containing MNDB supplemented with 0.1 µM retinoic-acid (Fisher), 0.5 µM purmorphamine (Stemgent), 1 µM GSK-3 inhibitor XVI, 2 µM BMP Inhibitor II/DMH1, 2 µM TGF-B RI kinase inhibitor VI/SB431524 for 6 days changing media every 2 days to generate motor neuron progenitors (MNP).

MNPs were matured as spheroids in suspension by dissociating the progenitors with Accutase (Innovative Cell Technologies) and culturing them in ultra-low-attachment plates (Corning) with MND4-media, MNDB supplemented with 0.5 µM retinoic-acid and 0.1 µM purmorphamine. Suspension culture was maintained for 6 days and media was changed every 2 days by centrifugation (500rpm 5min).

Finally, differentiated motor neurons were obtained by dissociating spheroids with Accutase into small aggregates/single cells, plating them down into poly-D-lysine 50 µg/ml (Merck Millipore) and laminin 10 µg/ml (Sigma-Aldrich) coated plates, and supplementing MNDB with 0.5 µM retinoic-acid, 0.1 µM purmorphamine and 0.1 µM Compound E (Labnet) as well as growth factors 10ng/ml BDNF (recombinant, Sigma-Aldrich), IGF-1 (recombinant, Calbiochem) and CNTF (recombinant, Thermo Scientific). In total neurons were differentiated for 35±1 days before sampling in all experiments.

**Quantitative PCR**

RNA from differentiated motor neuronal cultures was harvested with NucleoSpin RNA kit (Macherey-Nagel). RNA was reverse transcribed with the Maxima first strand cDNA synthesis kit (Thermo scientific). cDNA levels of *CHAT, GAPDH, ISL1, MAP2, MNX1, NEFH, NEFL,* *NEFM* and *TUBB3* were analyzed by qPCR amplification in CFX Real-time system C1000Touch (Bio-Rad) with SYBR-green Flash (Thermo Fisher) by transcript specific primers; CHAT (Forward: GGAGGCGTGGAGCTCAGCGACACC, reverse: CGGGGAGCTCGCTGACGGAGTCTG), GAPDH (Forward: CGCTCTCTGCTCCTCCTGTT, reverse: CCATGGTGTCTGAGCGATGT), ISL1 (Forward: GTTACCAGCCACCTTGGAAA, reverse: GGACTGGCTACCATGCTGTT), MAP2 (Forward: CCAATGGATTCCCATACAGG, reverse: CTGCTACAGCCTCAGCAGTG), MNX1 (Forward: GTCCACCGCGGGCATGATCC, reverse: TCTTCACCTGGGTCTCGGTGAGC), NEFH (Forward: AGTCCGAGGAGTGGTTCCGA, reverse: GTCCAGCTGCTGAATGGCTTC), NEFL (Forward: CAAGACCCTGGAAATCGAAG, reverse: TGAAACTGAGTCGGGTCTCC), NEFM (Forward: TGCAGTCCAAGAGCATCGAGC, reverse: AGTCTCTTCACCCTCCAGGAGTT) and TUBB3 (Forward: GCCAAGTTCTGGGAAGTCAT, reverse: CCACTCTGACCAAAGATGAA). Nonsense mediated mRNA decay was inhibited with 200ug/ml Cycloheximide (CHX) for 18h in cultured neurons before mRNA extraction as above.

**Western blotting**

Proteins from differentiated motor neuronal cultures were harvested with RIPA buffer (Thermo Fisher). 10 µg of protein/sample was run on TGX-stainfree 10-12% acrylamide gels (Bio-Rad) and transferred to 0.2 µm Nitrocellulose/PVDF-membrane (Bio-Rad) with the TransBlot Turbo transfer system (Bio-Rad). Membranes were blocked with 5%-non-fat dry milk (Valio) TBS-T and incubated in primary antibodies with 3%-BSA TBS-T. The following primary antibodies were used (1:1000): mouse mAb NEFL N-terminal (sc-390732, Santa Cruz), mouse pAb NEFL (N5139, Sigma), rabbit pAb NEFM (20664-1-AP, Proteintech), rabbit GAPDH (14C10, Cell Signaling), mouse mAb TUJ1 (801201, Biosite) and mouse mAb ChAT (AMAb91129, Sigma), and secondary antibodies: Goat-anti-mouse (Merck) 1:5000 or Goat-anti-rabbit (Merck) 1:10,000. Secondary antibody signal was detected with ECL reagent (Thermo Scientific). Membranes were imaged with Chemidoc XRS+ (Bio-Rad).

**Immunocytochemistry**

Motor neuronal cultures in Chamber slides (Labnet) were fixed with 4% paraformaldehyde (PFA) for 15 min in RT, and then permeabilized with PBS 0.2% Triton 100x (Fisher) for 10 min. The cells were blocked with protease-free 5% BSA (Jackson ImmunoReseach) in phosphate buffer saline 0.1% Tween20 (PBS-T) for 2 h in RT. The following primary antibodies were incubated overnight at 4°C (1:50-1000): mouse mAb NEFL N-terminal (sc-390732, Santa Cruz), mouse pAb NEFL (N5139, Sigma), rabbit pAb NEFM (20664-1-AP, Proteintech), mouse mAb TUJ1 (801201, Biosite), chicken pAb MAP2 (AB5543, Millipore) and mouse mAb ISL-1 (40.2D6, DSHB). As secondary antibodies the following were used: DyLight Fluorophore (Thermo Scientific) goat-anti-mouse 550 and goat-anti-rabbit 488, Alexa Fluorophore (Thermo Scientific) goat-anti-mouse 594, goat-anti-chicken 488 and goat-anti-rabbit 488. Cover glasses were applied on chamber slides with Vectashield DAPI (Vectorlabs), and imaged with Axio Observer Z1 (Zeiss). The gamma for manuscript figures was set to 0.45 with Zeiss Zen- software for visualization. Neurite signal was quantified from TUJ1, MAP2 and NEFM immunostaining images from a single z-plane with the ImageJ software and normalized to nuclei count in the specific image.

**Single motor neuron transcriptome profiling**

Motor neurons from P1 clone 1 and control 1 were analyzed using the 10x Genomics platform. Motor neurons from patient 1 (P1) clone 1 and control 1 were dissociated with Accutase (Innovative Cell Technologies) into a single cell suspension. The cells were filtered using Flowmi Cell Strainer (40µm, Bel-Art), counted with Countess automated cell counter (Thermo Fisher Scientific) and loaded into Chromium Single Cell Chip v2 (10x Genomics). The loading of the Chromium Single Cell Chip and the generation of gel beads in emulsion (GEMs) were performed using the Chromium Controller (10x Genomics) according to the manufacturer’s instructions using Chromium Single Cell 3’ v2 Reagent Kit, aiming for a 1000 cell capture per sample. GEM-reverse transcription incubation was performed in 100 µl reaction volume using SuperCycler Trinity (Kyratec) as follows: 53°C 45 min, 85°C 5 min, hold 4°C. Post GEM-reverse transcription cleanup and cDNA amplification were performed according to the manufacturer’s instructions using DynaBeads MyOne Silane Beads (Life Technologies) and following incubation times: initial denaturation 98°C 3 min, 14 cycles of 98°C 15 sec, 67°C 20 sec and 72°C 1 min, final extension 72°C 1 min using DNA Engine Tetrad 2 Thermal Cycler (Biorad). Samples were indexed with Chromium i7 Sample Indexes as follows: initial denaturation 98°C 45 sec, 12 cycles of 98°C 20 sec, 54°C 30 sec and 72°C 20 sec and final extension 72°C 1 min using DNA Engine Tetrad 2 Thermal Cycler (Biorad). Sample libraries were sequenced in Illumina HiSeq 2500 using HiSeq Rapid SBS Kit v2 (Illumina) and HiSeq PE Rapid Cluster Kit v2 (Illumina) and following read lengths: 26bp (Read 1), 8bp (i7 Index), 0 bp (i5 Index) and 98bp (Read 2). The Cell Ranger v1.3 mkfastq and count analysis pipelines (10x Genomics) were used to demultiplex and convert Chromium single cell 3’ RNA-sequencing barcode and read data to FASTQ files and to generate align reads and gene-cell matrices. The Cell Ranger aggr pipeline (10x Genomics) was used to combine sequencing outputs of both samples, equalizing the read depth between libraries before merging.

Altogether, 1430 P1 clone 1 cells and 443 control 1 cells were captured and analyzed with Seurat R package v.2.1.04 using default parameters unless otherwise stated. Genes with at least 5 UMI counts were included to analysis. To exclude low-quality cells from analysis, cells that contained less than 1000 genes, more than 6500 genes or more than 15% UMI counts mapped to mitochondrial DNA were discarded. Gene expression counts in each cell were normalized by the total expression, multiplied with a scale factor of 10000 and transformed to log-space. Highly variable genes were identified by limiting the minimum average expression to 0.0125, maximum average expression to 3 and minimum average dispersion to 0. The effect of exceptional UMI counts and mitochondrial UMI counts were further normalized using linear regression. Principal component analysis (PCA) was used for dimensionality reduction using identified highly variable genes (n=5599). Data was clustered with a shared nearest neighbor modularity optimization based clustering algorithm using resolution 0.4 and first five significant principal components, which were not solely enriched by mitochondrial genes. t-distributed stochastic neighbor embedding (t-SNE) was performed using the same principal components as for clustering setting perplexity to 100 and maximum number of iterations to 5000. The expression of MN-associated markers *CHAT,* *SLC18A3*, *ISL1*, *MNX1*, *LHX1*, *LHX3*, *DCC*, *ONECUT1* and *ONECUT2* were summed to identify iPSC-derived MN cluster from the data. Differentially expressed genes between 349 patient and 96 control iPSC-derived MNs were identified using a likelihood-ratio test for single-cell gene expression.5 Genes with absolute fold change ≥ 1.5 and adjusted p-value < 0.001 were considered significant. Transcripts expressed from sex chromosomes were excluded, because patient cells were from a female and control cells were from a male. Data visualization was performed with ggplot2 R package.

**Transmission electron microscopy (TEM)**

Motor neuronal cultures on glass slides were fixed with 2% PFA + 2% Glutaraldehyde (Sigma) for 30 min at 4°C, washed with PBS and fixing continued with 2% Glutaraldehyde O/N at 4°C. Fixed neurons were prepared according to standard protocols for transmission electron microscopy. Images were captured with the Jeol 1400 TEM microscope at 80,000V. Neurite areas were analyzed with the ImageJ-software from cross-sections of neurites from 10 000x images, and the presence of microtubules and intermediate filament bundles was evaluated.

**Statistical analyses**

Statistical analysis was performed using Prism 7. An unpaired two-tailed *t*-test was performed when comparing neurite sizes between patient and control samples, and when comparing cycloheximide-treated and non-treated individual cell lines. p < 0.05 was considered statistically significant.

**References**

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