# CMT2D Neuropathy is Influenced by Vitamin D-mediated Environmental Pathway

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**Supplementary Materials and Methods**

***Mice***

Both male and female wild-type C57BL/6J (JAX# 000664 and Salccas), VDR KO (JAX# 006133), and P234KY-*Gars*$^{CMT2D}$ mutant (JAX# 033165) mice were used in this study. Mice were housed in a 12 h light/dark cycle with food and water ad libitum. Animal experiments were under the guidelines of the Institutional Animal Care and Use Committee animal protocols.

***Vitamin D-deficient diet treatment***

WT or CMT2D neonates were housed with their mothers till weaning (postnatally 3 weeks old). Animals were fed with VD adequate diet or VD deficient diet (Envigo) right after weaning and lasted for 4 weeks. The VD deficient diet contains no VD but higher Calcium and Phosphorus (Teklad, TD. 120322) to prevent developmental abnormalities. Behavioral performance was tested at 7 weeks of age, then mouse tissues were collected for pathological analysis. VD level in mouse serum was determined by liquid chromatography-mass spectrometry (LC-MS) (Heartland Assays, Iowa, USA).

***Calcipotrial administration***

CMT2D mice and their WT littermates were injected intraperitoneally (i.p) with Cal (Tocris, #2700, 60 μg/kg) or vehicle (0.1% DMSO in saline) 5 days a week starting at postnatal day 6 (P6). Behavioral performance was tested at 8 weeks of age, then mouse tissues were collected for pathological analysis.

***RT-qPCR***

The total RNA was extracted from the isolated mouse tissues by using Trizol reagent (Thermofisher, 15596018) and RNeasy mini column (Qiagen, 217004). cDNA was synthesized by using HiScript II Q Select RT SuperMix (Vazyme Biotech, R232-01). The VEGF expression was determined by real-time

quantitative PCR (RT-qPCR) with GAPDH as the internal control. The primer sequence of mouse *GAPDH*: Forward 5'-TCAACAGCAACTCCCACTCTTCCA-3'; Reverse 5'-TTGTCATTGAGAGCAATGCCAG CC-3'. The primer sequence of mouse *VEGF-$A_{164}$*: Forward 5'- GAGAGCAGAAGTCCCATGAAG -3'; Reverse 5'- TCTCCTATGTGCTGGCTTTG-3'. The primer sequence of mouse *GARS*: Forward 5'-TCCAAAACGTCCTATGGCTGG-3'; Reverse 5'- TGTAGCACTCATCACAGGCG-3'. The primer sequence of mouse *Nrp1*: Forward 5'- AAGCGCAAGGCTAAGTCGTT-3'; Reverse 5'- GGAAGTCAT CACCTGTGCCA-3'.

***Hindlimb extension test***

Hindlimb extension was tested as previously described (He et al., 2015). Mice were hung by the tail tip for about 10 sec, and the extent of hindlimb extension was scored between 0 (clasped hindlimb) and 2 (normal extension). Each mouse was tested for 3 consecutive trials with intervals of 5 sec.

***Rotarod test***

The rotarod test was performed as previously described (He et al., 2015). Briefly, the mouse was firstly trained on a rotating rod with the speed of 1 r.p.m. after a short period of acclimation. The training session lasted for 3 min or until the mouse fell. In the testing session, the rotarod rotated with speed accelerating from 0 r.p.m. at the rate of 0.1 r.p.m./min. Each mouse was tested for 3 trials at 20 min intervals. Motor coordination was measured as the average time spent on the rotarod of the 3 trials.

***NMJ (neuromuscular junction) immunostaining and imaging***

Gastrocnemius muscles were dissected, fixed with 2% paraformaldehyde, and compressed for immunostaining. Primary antibodies that were used included: rabbit anti-NF145 (1:500, AB1987, Millipore) and rabbit anti-synaptophysin (1:200, sc-9116, Santa Cruz). Secondary antibodies used were Alexa488-conjugated anti-rabbit antibodies (1:1000, A32731, Molecular Probes/Invitrogen). Tetramethylrhodamine-conjugated α-bungarotoxin (1:1000, T-1175, Molecular Probes/Invitrogen) was used to stain acetylcholine

receptors (AchRs). Images were acquired using Olympus Fluoview 3000 confocal microscope. The overlap of motor nerve terminal (cyan) and muscle endplate (magenta) was examined to indicate occupancy of NMJs.

***Nerve histology and imaging***

Sciatic nerves were dissected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer in 4 ℃ overnight. After washing, tissues were stained in 1% osmic acid for 1 h, followed 0.5% uranile acetate (pH 4) for 1 h at room temperature, then dehydrated through graded alcohols, and embedded in Araldite resin. To visualize axons, 1 μm transverse sections were cut on a Leica EM UC6 microtome, stained with methylene blue Azure II, and examined on a transmission electron microscope (TECNAI-10, Philip, Netherland). The axon numbers were determined from at least 5 non-overlapping fields (50×50 μm) for each sample. The axon diameters were measured by Image J. The percentage of large-diameter axons was determined by analyzing the numbers of large-diameter axons (> 2 μm) relative to total axon numbers for each mouse. The presented data were from the average of at least 3 animals.

***Cell culture and Calcipotrial treatment***

C2C12 mouse adherent myoblasts were maintained in DMEM (Gibco, C11965500BT) with 10% heat-inactivated fetal bovine serum (Biological industries, 04-001-1ACS) and penicillin-streptomycin (Gibco, 15140-122) under sterile conditions in an incubator at 37 °C with 5% CO2. On the day of the experiment, cells were treated with 10 nM Calcipotriol or vehicle (0.1% DMSO) for 1 h and collected for RNA purification.

***Statistical analysis***

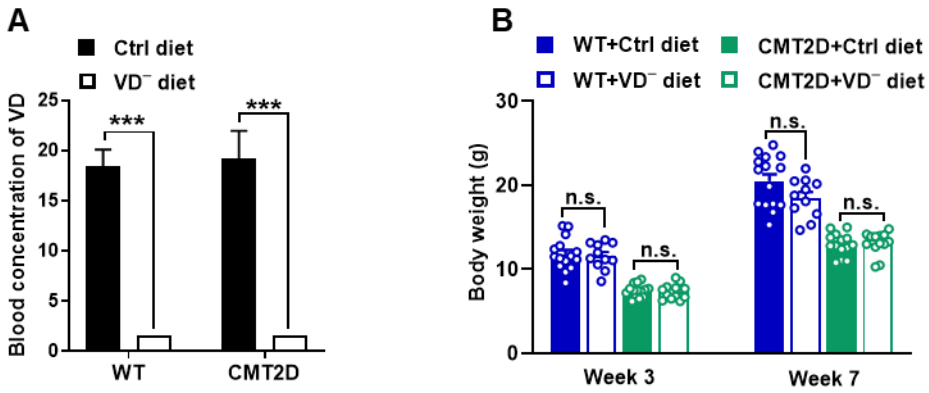
All statistical analyses were performed using GraphPad Prism software. Statistical significance was assessed using Student's *t*-test for the comparation of two independent experimental groups, one-way

ANOVA for the comparation of groups with a single independent variable, or two-way ANOVA for the comparation of groups with two independent variables (genotype and treatment). All the statistical testing was performed on at least three biological replicates. $P < 0.05$ was considered significant. Graphs were presented as mean ± SEM unless otherwise stated.

**Supplementary Reference**

He, W., Bai, G., Zhou, H., et al. (2015). CMT2D neuropathy is linked to the neomorphic binding activity of glycyl-tRNA synthetase. Nature *526*, 710-714.

## Supplementary Figures

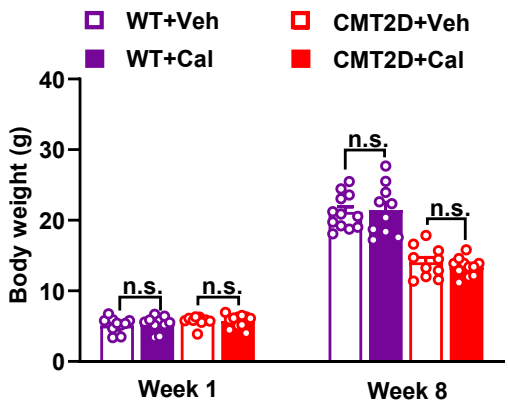



**Supplementary Figure S1** VD deficiency had no impact on the mouse body weights.

(**A**) The blood VD level was decreased after 4 weeks of VD deficient diet (VD$^{-}$ diet) treatment compared to their littermates fed with VD adequate diet (Ctrl diet) ($n \geq 3$).

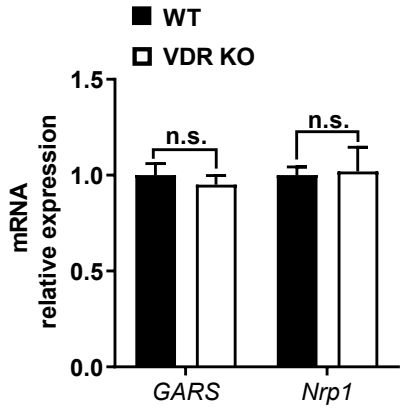
(**B**) VD deficiency had little effect on the mouse's body weight ($n \geq 11$).

Data are presented as mean ± SEM. Statistical analyses were performed with two-way ANOVA. n.s., no significance, ***$P < 0.001$.



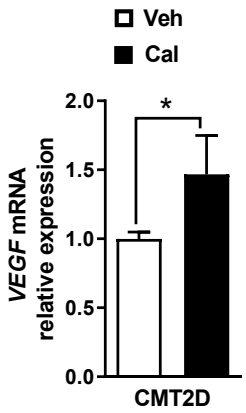

**Supplementary Figure S2** Cal treatment did not affect the mouse body weights. Cal treatment had no significant effect on the body weight of both WT and CMT2D mice ($n \geq 10$).

Data are presented as mean ± SEM. Statistical analyses were performed with two-way ANOVA. n.s., no significance.




**Supplementary Figure S3** Knockout of VDR did not change the expression of GARS and Nrp1 in mice. RT-qPCR analysis showing that VDR KO had no significant impact on the expression of GARS and Nrp1 in the mouse spinal cords ($n = 4$).

Data are presented as mean ± SEM. Statistical analyses were performed with Student's *t*-test. n.s., no significance.




**Supplementary Figure S4** Cal treatment increased the VEGF expression in CMT2D mice. RT-qPCR analysis showing that *VEGF* (*VEGF-$A_{164}$* isoform) expression was upregulated in CMT2D mice 24 hours after a single injection of Calcipotriol (Cal, 60 μg/kg i.p) ($n \geq 3$).

Data are presented as mean ± SEM. Statistical analyses were performed with Student's *t*-test. *$P < 0.05$.