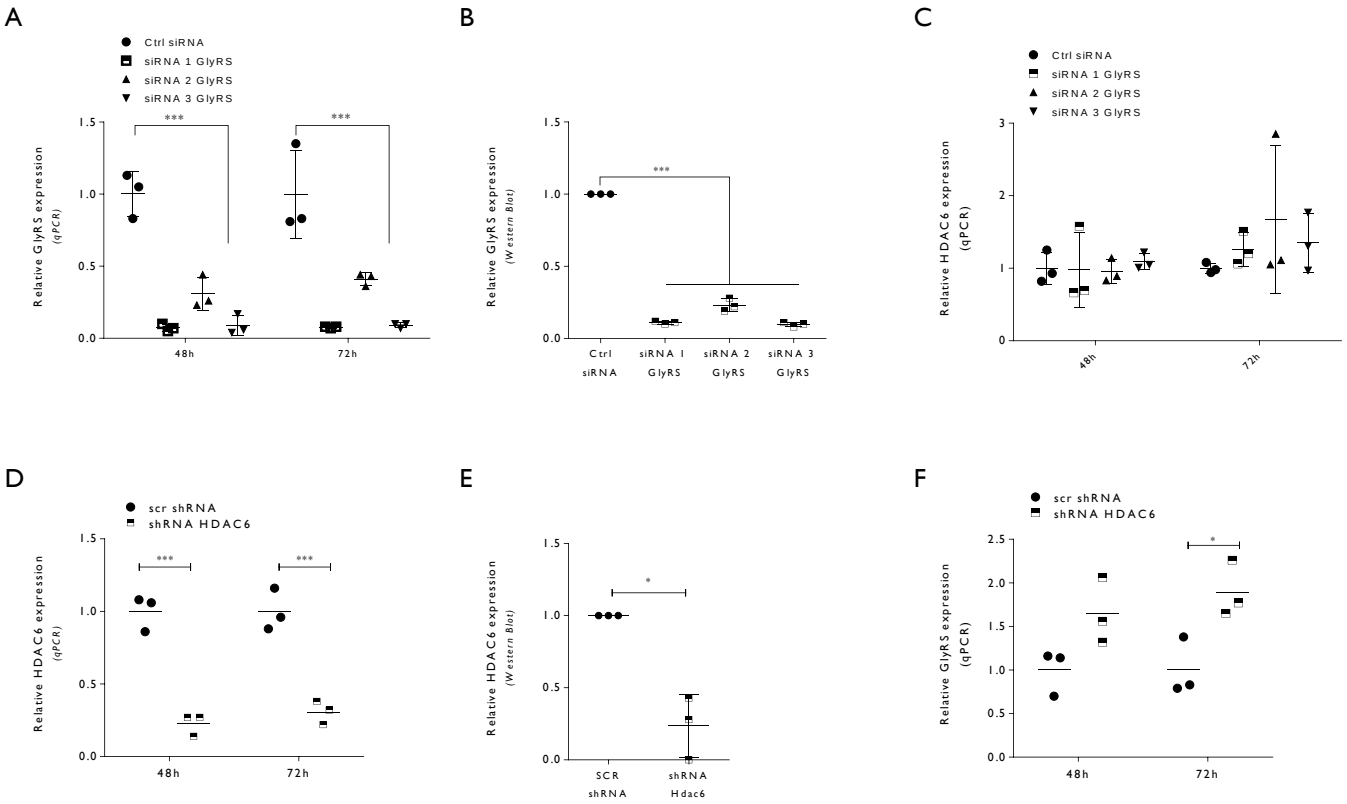
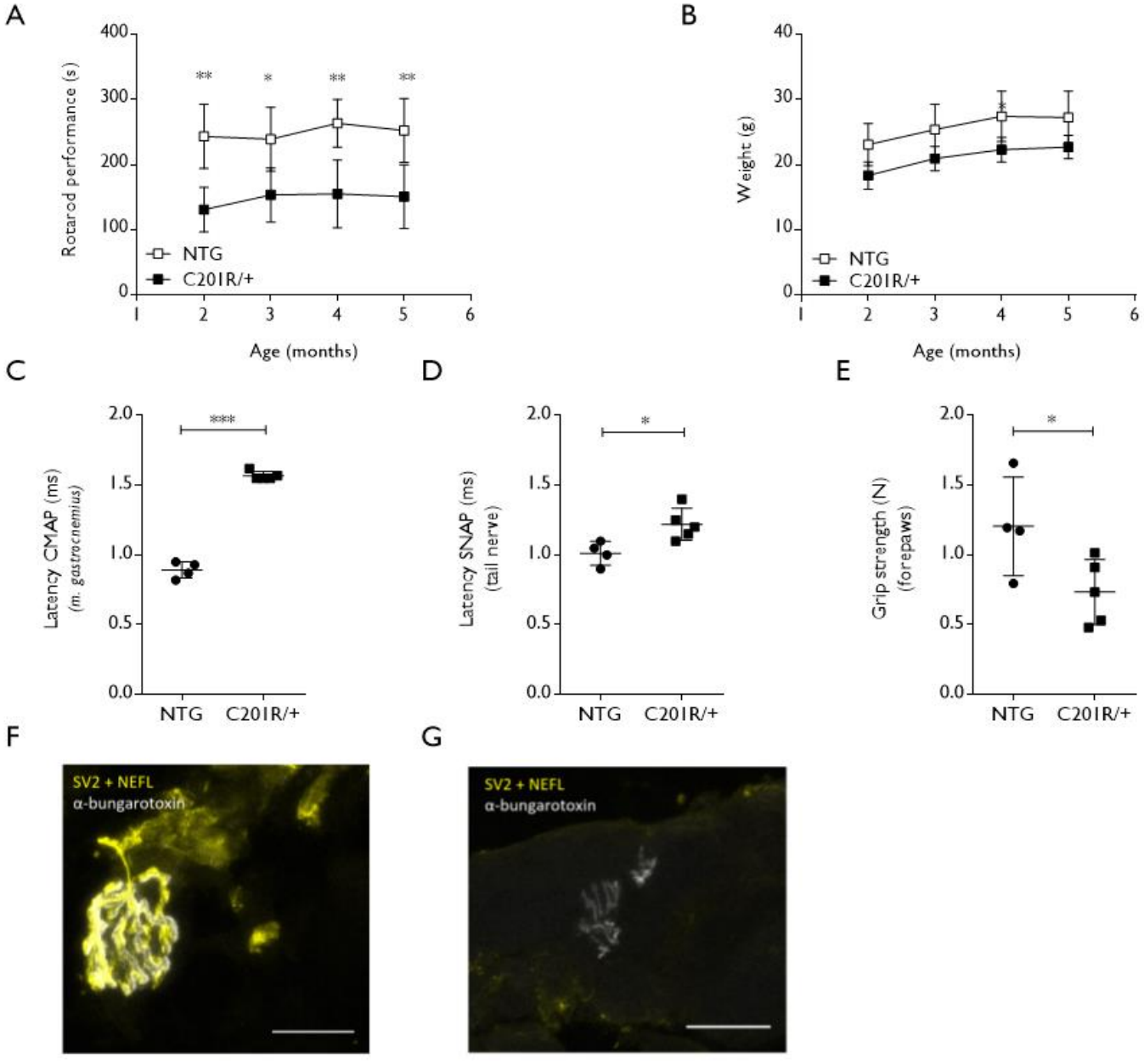
SUPPLEMENTARY FIGURE 1 Knock down using RNA interference reduces HDAC6 and GlyRS expression.

A. N2a cells were transfected with siRNA molecules directed against GlyRS mRNA or a control siRNA. At 48h and 72h post transfection, cells were collected to check mRNA expression levels by qPCR analysis. GAPDH and HPRT mRNA expression levels were used as a reference: ctrl siRNA 1.00±0.01, n=3 vs siRNA 1 GlyRS 0.08±0.01, n=3 vs siRNA 2 GlyRS 0.36±0.07, n=3 vs siRNA 3 GlyRS 0.09±0.00, n=3; two-way ANOVA, siRNA $F(3,16)=64.90$, $p<0.0001$. **B.** HDAC6 mRNA expression was checked by qPCR at 48h and 72h post transfection with siRNA molecules. GAPDH and HPRT mRNA expression levels were used as a reference: ctrl siRNA 1.00±0.00, n=3 vs siRNA 1 GlyRS 1.12±0.19, n=3 vs siRNA 2 GlyRS 1.31±0.51, n=3 vs siRNA 3 GlyRS 1.22±0.18; two-way ANOVA, siRNA $F(3,16)=0.5335$, $p=0.6659$. **C.** pCMV-dsRed plasmids containing either scrambled shRNA or shRNA molecules directed against HDAC6 mRNA were transfected in N2a cells. At 48h and 72h post transfection, cells were collected to check HDAC6 mRNA expression levels by qPCR analysis. GAPDH and HPRT mRNA expression levels were used as a reference: scr shRNA 1.00±0.00, n=3 vs shRNA HDAC6 0.27±0.06, n=3; two-way ANOVA, shRNA $F(1,8)=0.4020$, $p<0.001$. **D.** GlyRS mRNA expression was assessed by qPCR at 48h and 72h post transfection with shRNA-mediated knockdown of HDAC6. GAPDH and HPRT mRNA expression levels were used as a reference: scr shRNA 1.00±0.00, n=3 vs shRNA HDAC6 1.77±0.17, n=3; two-way ANOVA, shRNA $F(1,8)=16.81$, $p=0.0034$.



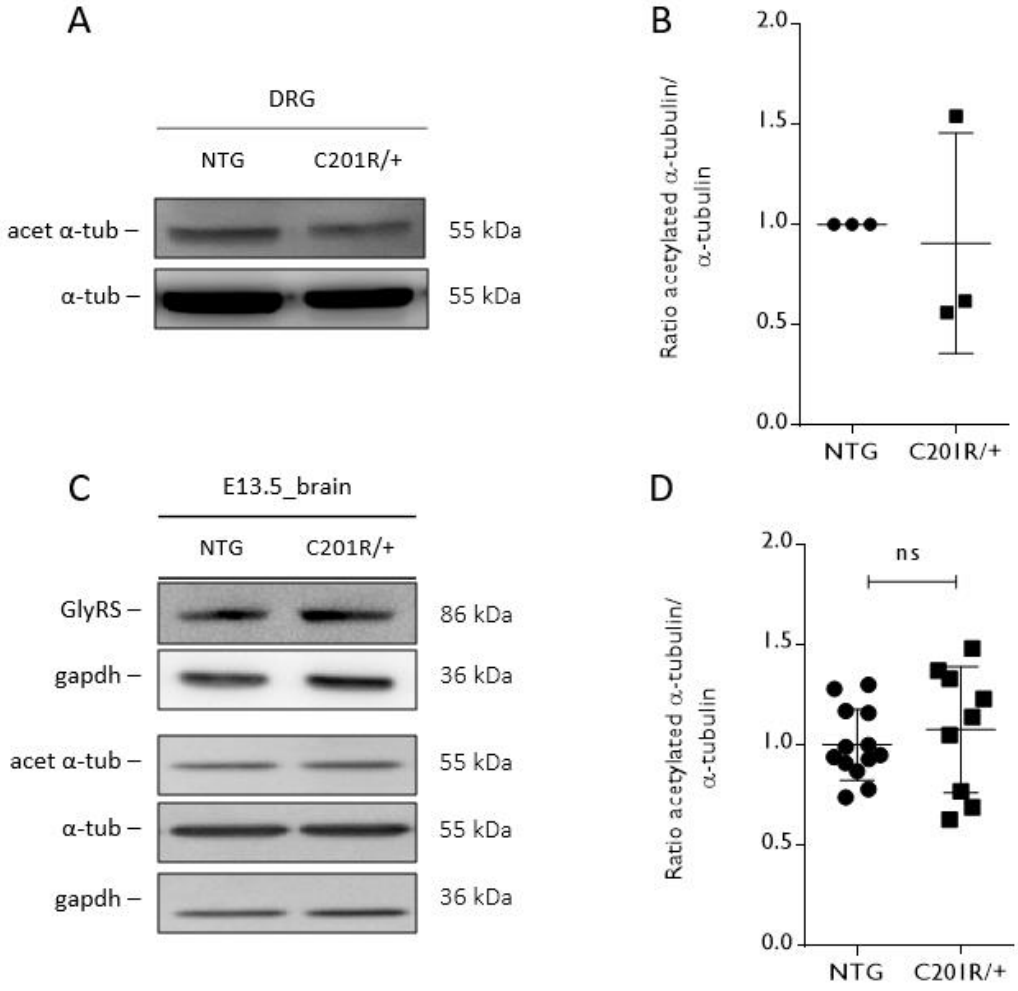
SUPPLEMENTARY FIGURE 2 Gars$^{C201R/+}$ mice show motor and sensory deficits reminiscent of CMT2D.

A. Body weight of Gars$^{C201R/+}$ mice was monitored with age, starting from 2 months of age on: NTG 25.81±2.03 g, n=4 mice vs Gars$^{C201R/+}$ 21.08±1.97 g, n=4 mice; two-way ANOVA factor age: F(3,32)=4.998, p=0.0059; factor genotype: F(1,32)=28.09, p<0.0001. **B.** At different ages, the motor performance of Gars$^{C201R/+}$ mice and littermate controls (Non-Transgenic, NTG) was assessed by a rotarod test accelerating from 4 to 40 rpm during 5 min: NTG 249±11 sec, n=4 mice vs Gars$^{C201R/+}$ 147±11.26 sec, n=4 mice; two-way ANOVA factor age: F(3,32)=0.4097, p=0.7471; factor genotype: F(1,32)=48.72, p<0.0001. **C.** Grip strength was measured in the forepaws using a triangle with a dynamometer. Data shown for one year old mice. NTG 1.21±0.35 N, n=4 mice vs Gars$^{C201R/+}$ 0.73±0.23 N, n=5 mice; unpaired t-test: t=2.423, p=0.0459. **D-E.** Latencies of the Compound Muscle Action Potential (CMAPs) were assessed by stimulation at the level of the sciatic notch and measurements at the *m. gastrocnemius*: NTG 0.89±0.06 ms, n=4 mice vs Gars$^{C201R/+}$ 1.57±0.03 ms, n=5 mice; unpaired t-test: t=22.39, p<0.0001. Latencies of the Sensory Nerve Action Potentials (SNAPs) were recorded from the tail nerve. NTG 1.01±0.09 ms, n=4 mice vs Gars$^{C201R/+}$ 1.22±0.12 ms, n=5 mice; unpaired t-test: t=2.991, p=0.0202. **F-G.** Innervated (G) and denervated (H) neuromuscular junctions (NMJ) in the *m. gastrocnemius* were visualized by immunofluorescence (yellow: neurofilament light, NEFL + synaptic vesicle glycoprotein 2A, SV2; gray: α-bungarotoxin). Confocal images are represented. Scale bar: 20 µm.

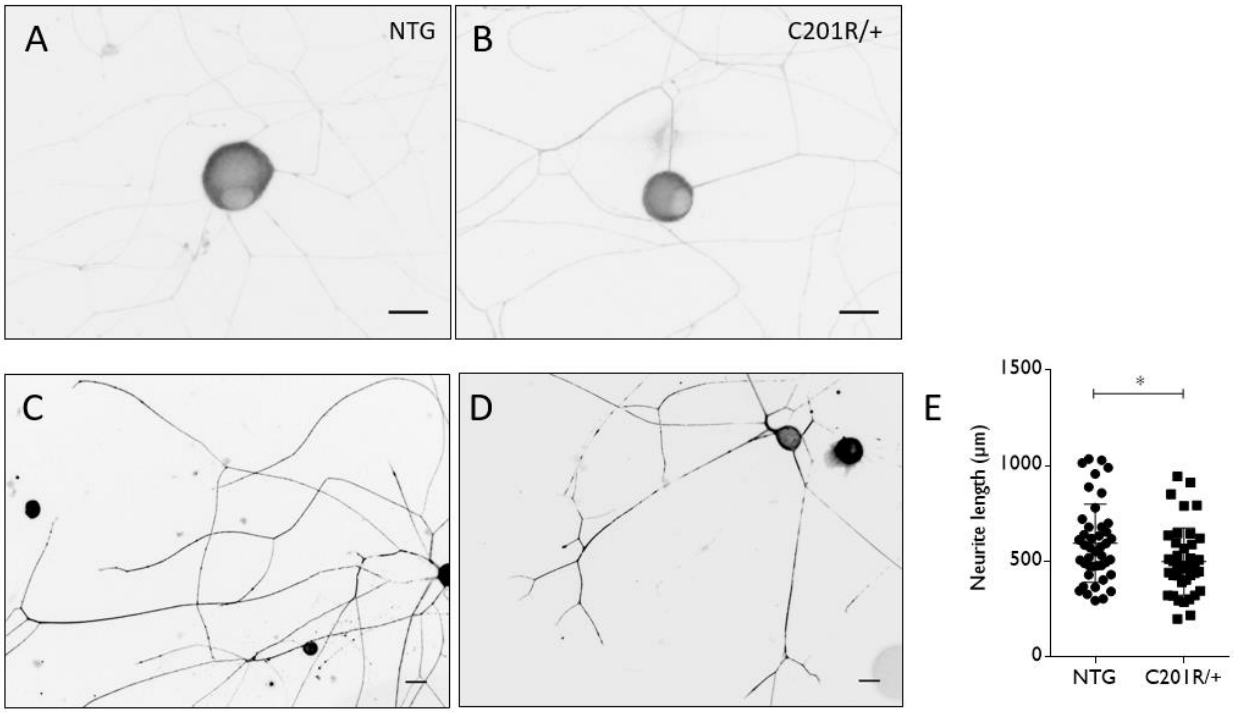


SUPPLEMENTARY FIGURE 3. Sensory neuron functionality in DRG neurons cultured from one-year old Gars$^{C201R/+}$ mice.

A-B. Immunofluorescence was applied to visualize endogenous GlyRS in cultured DRG neurons from Gars$^{C201R/+}$ and littermate control (NTG) mice. Scale bar 40 µm. **C-D.** The length of the neurites was assessed by visualizing the neurites using an antibody detecting β3-tubulin in Gars$^{C201R/+}$ NTG DRG neuron cultures. Scale bar: 40 µm. **E.** The length of the neurites was measured by quantifying the length of β3-tubulin staining in one neurite from each cell in Gars$^{C201R/+}$ NTG DRG neuron cultures: NTG 594.7±204.3 µm, n=41 cells from 4 different DRG primary cultures vs Gars$^{C201R/+}$ 499.4±175.3 µm, n=41 cells from 4 different DRG primary cultures; unpaired t-test: t=2.289, p=0.0246.

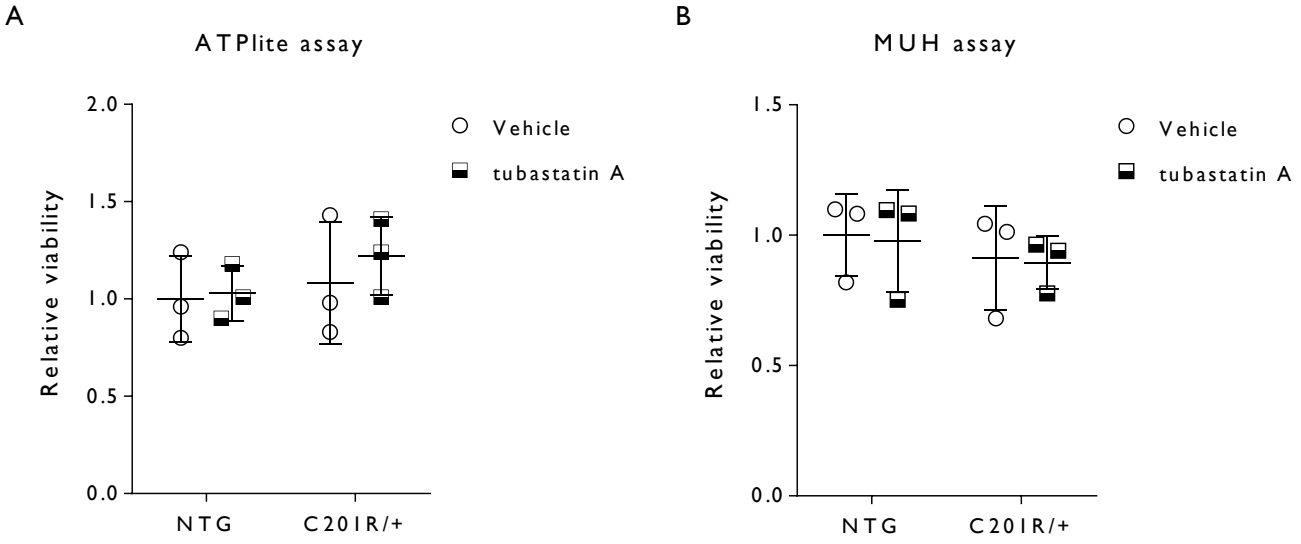


SUPPLEMENTARY FIGURE 4 Cell viability in DRG neuron cultures from one year old Gars$^{C201R/+}$ and littermate control mice is normal.
A. The ATP concentration was monitored using a luminescence-based assay in DRG neuron cultures from Gars$^{C201R/+}$ and littermate control (NTG) mice, treated with vehicle or 1 µM tubastatin A. Graphs (mean +/- SD) demonstrate viability relative to littermate control , vehicle-treated DRG cultures. N = three independent DRG neuron cultures. **B.** Fluorimetric assay measuring the conversion of MUH by intracellular esterases of viable cells in in DRG neuron cultures from Gars$^{C201R/+}$ and littermate control (NTG) mice, treated with vehicle or 1 µM tubastatin A. Graphs (mean +/- SD) demonstrate viability relative to littermate control, vehicle-treated DRG cultures. N = three independent DRG neuron cultures.



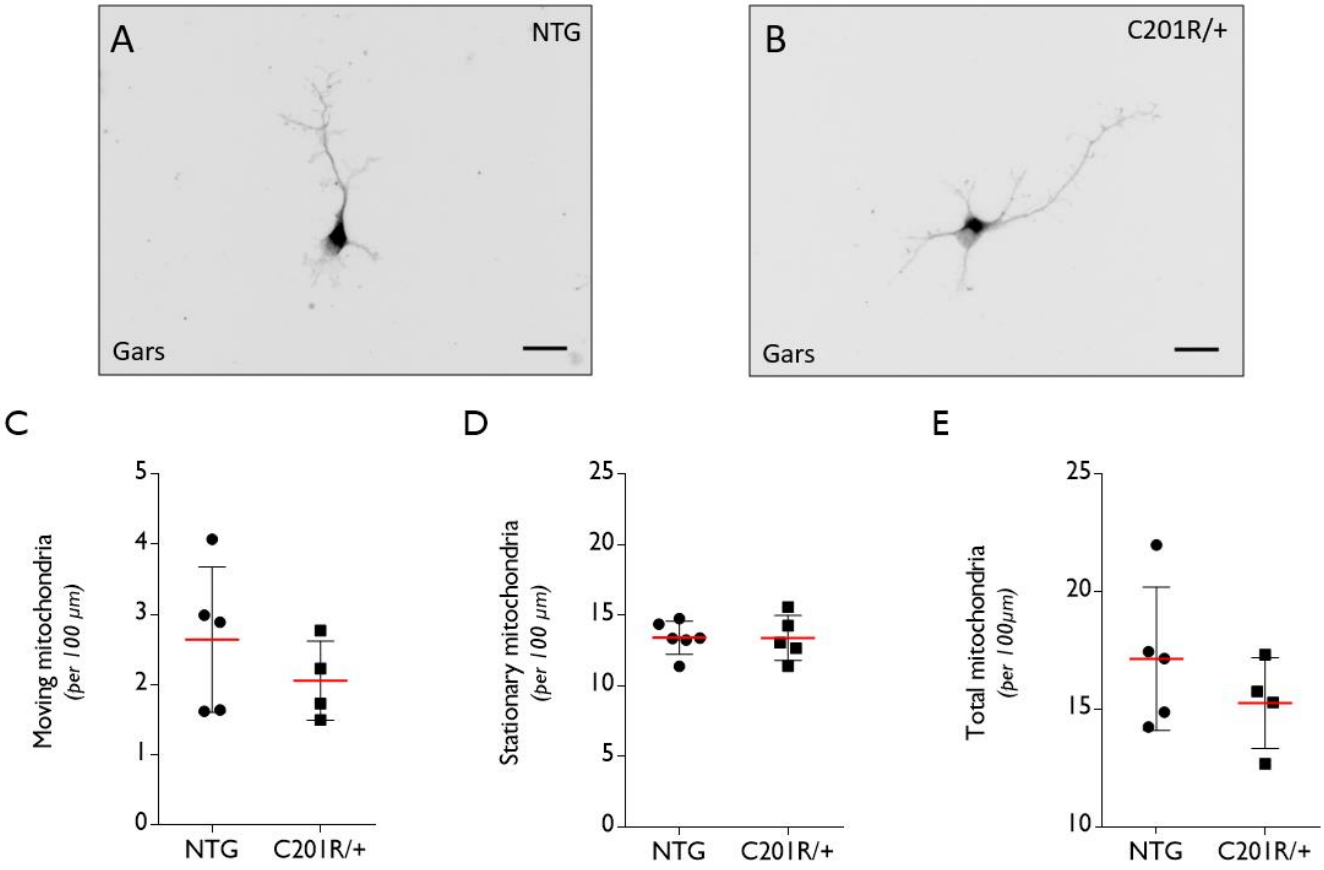
SUPPLEMENTARY FIGURE 5 Mitochondrial axonal transport in embryonic primary motor neuron cultures from Gars$^{C201R/+}$ mice is normal.

A,B. Primary motor neurons were cultured from Gars$^{C201R/+}$ mice and NTG embryo's (E13.5) during 1 week and stained for the presence of GlyRS. Scale bar: 20 µm. **C.** Mitochondrial movement was visualized by loading the DRG neurons with a mitochondrial dye, MitoTracker Red FM. A time-laps movie was recorded during 200 s and the mitochondrial movement was assessed per 100 µm for one neurite per cell. NTG 2.64±1.03 mitochondria, n=5 different cultures vs Gars$^{C201R/+}$ 2.06±0.56 mitochondria, n=4 different cultures; unpaired t-test t=1.008, p=0.3468. **D.** The number of stationary mitochondria was quantified per 100 µm for one neurite per cell. **E.** The total number of mitochondria in the neurites from NTG or Gars$^{C201R/+}$ DRG neurons in the absence or presence of TubA was assessed as the number of moving and stationary mitochondria. NTG 17.14±3.05 mitochondria, n=5 different cultures vs Gars$^{C201R/+}$ 15.26±1.93 mitochondria, n=4 different cultures; unpaired t-test t=1.067, p=0.3213.



SUPPLEMENTARY FIGURE 6. Unaltered levels of acetylated α-tubulin in embryonic brain tissue from Gars$^{C201R/+}$ mice.

A. Brain tissue from E13.5 embryos (Gars$^{C201R/+}$ and NTG) was dissected for Western Blot analysis to detect the acetylation status of α-tubulin. An antibody directed against the endogenous GlyRS protein was used to assess expression levels. Gapdh was used as a loading control. **B.** The ratio between acetylated α-tubulin and α-tubulin was quantified by densitometry. Values were normalized to NTG samples. N = 9-13 embryos.



SUPPLEMENTARY FIGURE 7 HDAC6 inhibition does not alter the nerve conduction in NTG mice.

A-B. CMAP latencies and amplitudes were recorded in the m. gastrocnemius from vehicle and tubA-treated mice C201R littermate control mice. **C-D.** SNAP latencies and amplitudes were measured in the tail nerve from vehicle and tubA-treated C201R littermate control mice.