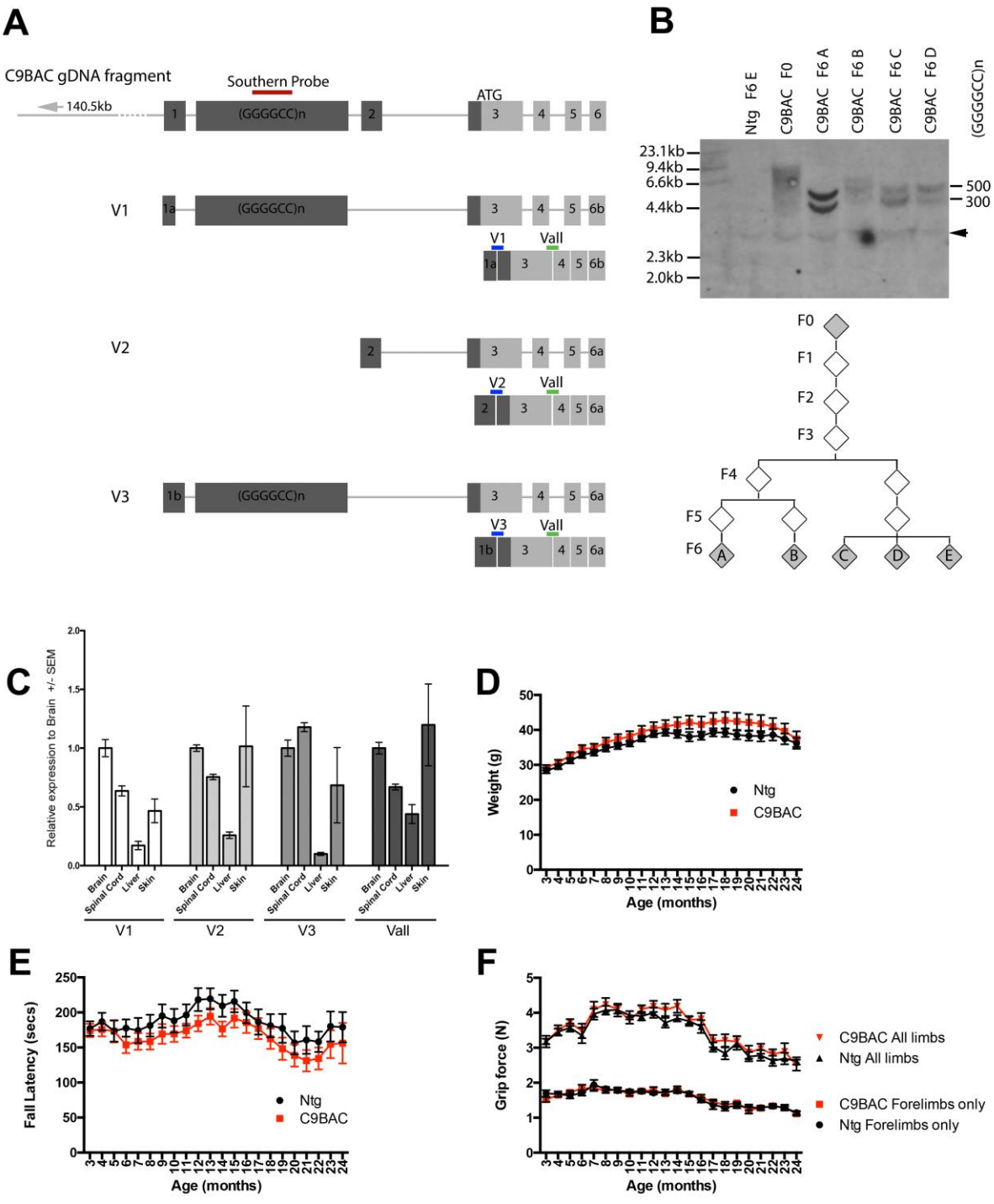


Figure S1. Description and characterization of C9BAC transgenic mice. Schematic showing potential RNA transcripts generated from the *C9ORF72* gene fragment used to

produce the C9BAC mice. The insertion contains exons 1 to 6 of human *C9ORF72*, a $(GGGCC)_{500}$ repeat motif and 140.5kb of upstream non-coding sequence. Three splice variant isoforms are produced from the gene fragment; V1 and V3 contain the hexanucleotide expansion. Nomenclature is based on the current NCBI reference sequences. For each isoform, the upper image shows the unspliced pre-mRNA, while the lower image at right is the corresponding spliced mRNA. The red bar denotes the target site of the southern blot probe. Blue bars show the locations of isoform specific Taqman probes. The green bar shows the location of a Taqman probe designed to recognize all isoforms (Vall). (B) Southern blot of genomic DNA extracted from liver, digested with HindIII, SacI, and TaqI and probed with a 5'-DIG-$(GGGGCC)_5$-DIG-3' DNA probe. Two bands of ~4.4kb and ~6.0kb, are stable in size after 6 generations in the pedigree shown, representing expansions of 300 and 500 repeats. The arrowhead indicates a non-specific band. (C) qPCR analysis of expression of all isoforms (Vall), and isoforms V1, V2 and V3 relative to mouse HPRT and normalized to brain (mean±SEM, n=5). (D) C9BAC mice and Ntg littermates show no differences in weight from 3 to 24-months of age (mean±SEM, 2-way ANOVA; Bonferroni's multiple comparison, Ntg n=17, C9BAC n=15). (E) The performance of the C9BAC mice on a 5-minute accelerating rotarod task did not differ significantly from Ntg controls (mean±SEM, 2-way ANOVA, Bonferroni's multiple comparison). (F) The grip force of forelimb only and all limbs from 3 to 24-months of age showed no difference in mice of either genotype (mean±SEM, 2-way ANOVA, Bonferroni's multiple comparison, Ntg n=17, C9BAC n=15).

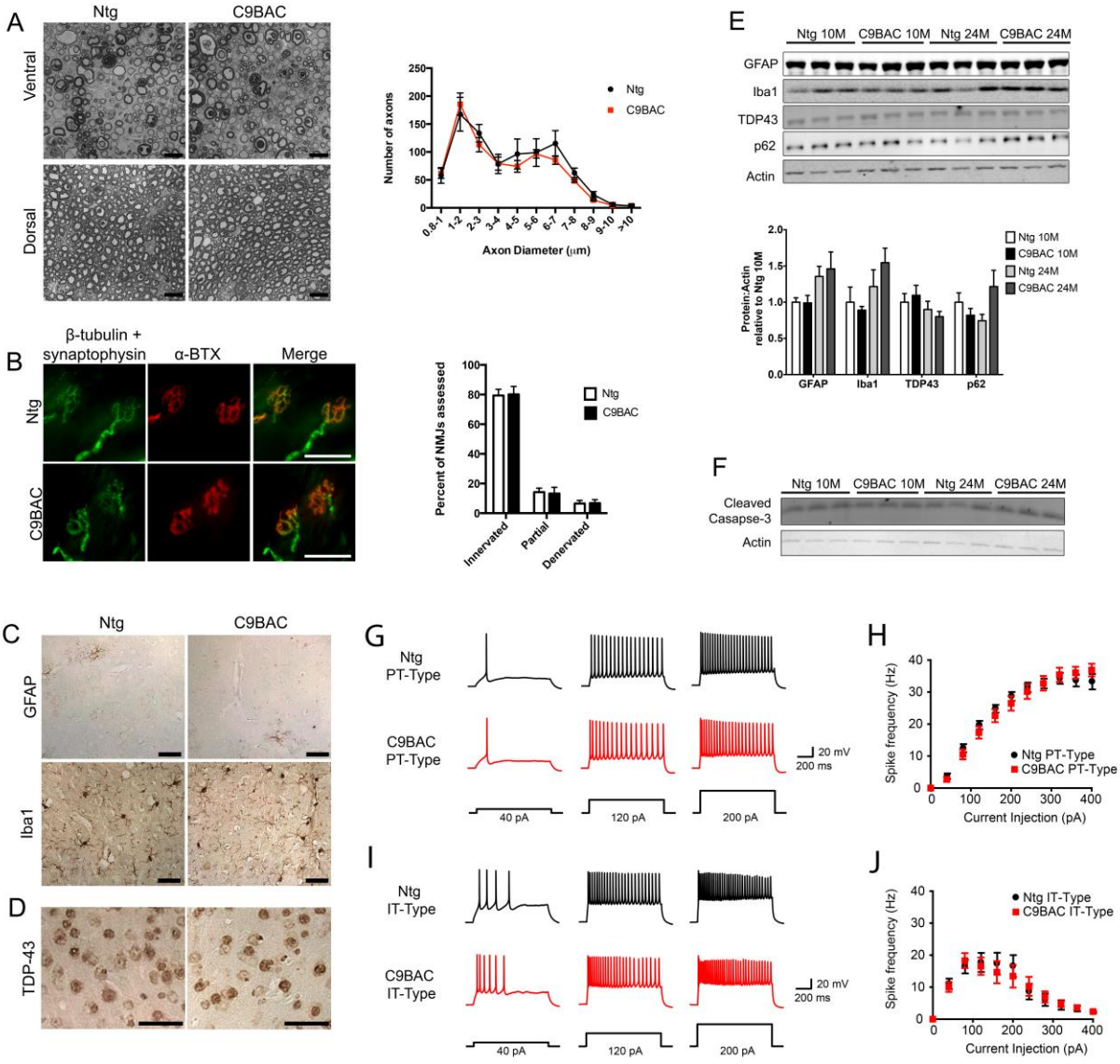
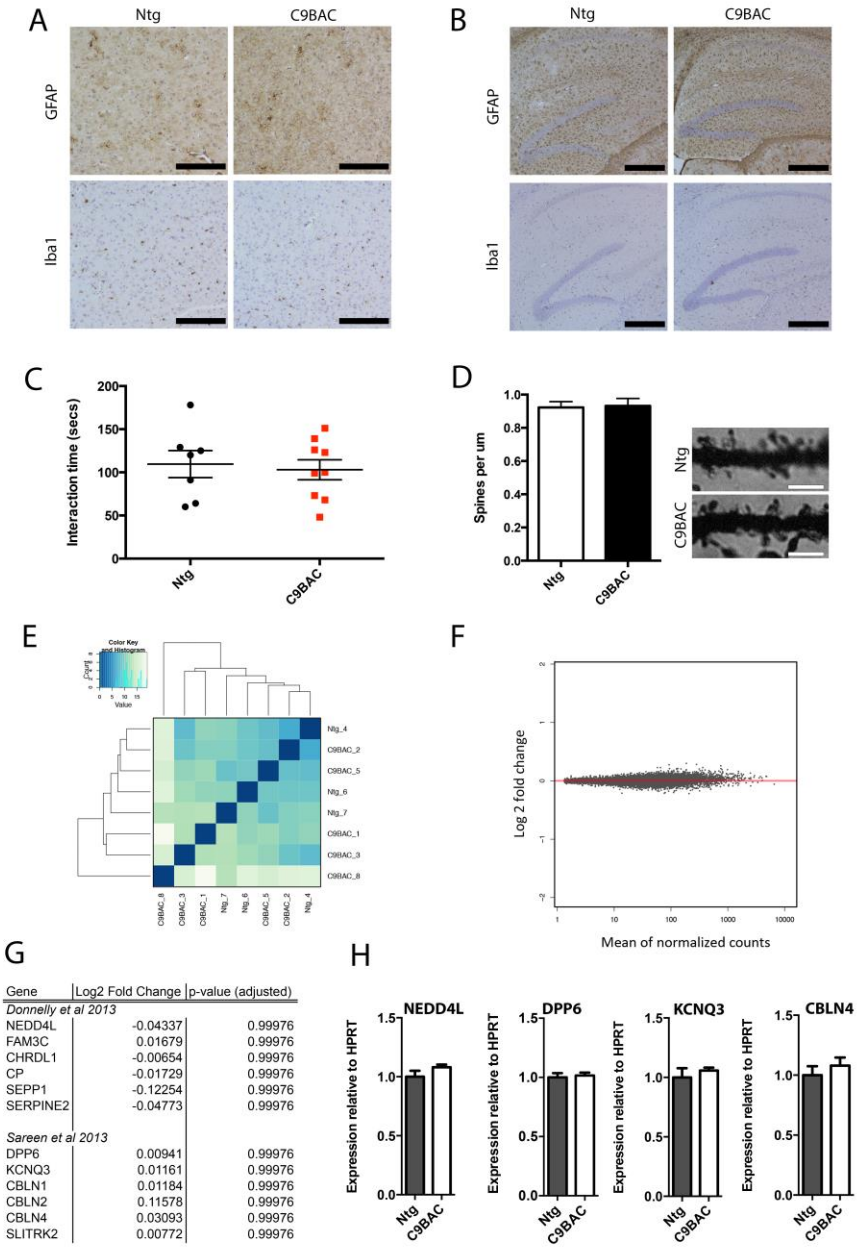


Figure S2: Aged C9BAC mice show no gross motor phenotypic abnormalities. (A) Toluidine blue stained semi-thin section of L5 ventral (upper panels) and dorsal (lower panels) nerve roots of 24-month old C9BAC mice and age-matched Ntg mice. Age-related alterations in axon and Schwann cell morphology were observed in motor fibers in both Ntg and C9BAC mice, while sensory fibers appeared normal. A plot of the numbers of myelinated motor axons in L5 ventral nerve roots as a function of axon diameter (μm) revealed no significant change in size distribution in 24-month-old C9BAC mice compared to Ntg controls (mean±SEM). (B) Immunofluorescent staining (Green=synaptophysin, beta-III-tubulin, Red=alpha-bungarotoxin

Alexa Fluor-555) and quantification of neuromuscular junctions in the gastrocnemius muscle of 24-month old C9BAC mice revealed no significant change in innervation (mean±SEM). (C) Immunostaining of motor cortex from 24-month old C9BAC mice revealed no pronounced increase in number of GFAP-labeled activated astrocytes or Iba1-labeled microglia, (D) nor was TDP-43 found to mislocalize in these neurons (E) Quantitative western blotting revealed no difference in levels of GFAP, Iba1, TDP-43 or p62 in the spinal cord of age matched Ntg and C9BAC mice (mean±SEM, n=3, Kruskal-Wallis test). (F) Western blotting for cleaved caspase-3 levels did not detect activation of apoptosis in aged C9BAC mice. (G) Representative voltage traces recorded from pyramidal tract (PT)-type neurons in layer 5 of motor cortex from control and C9BAC mice evoked by 40, 120, and 200 pA current steps as indicated. (H) A summary graph for the current-spike frequency relationship measured from PT-type neurons (Ntg: 23 cells, 7 mice; C9BAC: 15 cells, 5 mice; $P > 0.4$, 2-way ANOVA, Bonferroni corrected for multiple comparisons). Representative voltage traces recorded from intratelencephalic (IT)-type neurons in layer 5 of motor cortex (I) and a summary graph of the current-spike frequency relationship for mutant and control neurons (J) (Ntg: 19 cells, 7 mice; C9BAC: 13 cells, 5 mice; $P > 0.6$, 2-way ANOVA, Bonferroni corrected for multiple comparisons). Scale bars A = 20 µm, C = 50 µm, E =50 µm, H = 50 µm.



Gene	Log2 Fold Change	p-value (adjusted)
Donnelly et al 2013		
NEDD4L	-0.04337	0.99976
FAM3C	0.01679	0.99976
CHRDL1	-0.00654	0.99976
CP	-0.01729	0.99976
SEPP1	-0.12254	0.99976
SERPINE2	-0.04773	0.99976
Sareen et al 2013		
DPP6	0.00941	0.99976
KCNQ3	0.01161	0.99976
CBLN1	0.01184	0.99976
CBLN2	0.11578	0.99976
CBLN4	0.03093	0.99976
SLITRK2	0.00772	0.99976

Figure S3: Aged C9BAC mice do not recapitulate FTD-like pathology nor show changes in cortical neuron electrophysiology. Immunostaining for markers of astrogliosis (GFAP) and microgliosis (Iba1) in (A) prefrontal cortex and (B) hippocampus of 24-month-old C9BAC and Ntg control mice. (C) During a 5-minute test period, the interaction times of 18-22 month old Ntg or C9BAC mice with a juvenile male mouse were not different (mean±SEM, unpaired t-test, Ntg n=7, C9BAC n=9). (D) Golgi staining and quantification of dendritic spine density of layer 2-3

pyramidal neuron apical dendrites found no difference between 18-month old C9BAC and Ntg mice (mean±SEM, Mann-Whitney U-test, n=3 animals per genotype, 3-12 dendrites per brain, total length of dendrite sampled: Ntg = 1943.5μm, C9BAC = 1266.1μm). (D) During a 5-minute test period, the interaction times of 18-22 month old Ntg or C9BAC mice with a juvenile male mouse were not different (mean±SEM, unpaired t-test, Ntg n=7, C9BAC n=9). (E) Heatmap showing the Euclidean distance between sample conditions and replicates (frontal cortex, 6-month old, n=5 C9BAC and n=3 Ntg) as calculated from the regularized log transformation of the gene expression counts. Based on these values, no distinct hierarchical clustering was observed, indicating that the overall gene expression patterns of C9BAC and Ntg mice were similar. (F) Differentially expressed genes (DEG). MA-plot of log2 fold changes from the C9BAC over the mean of normalized counts. No DEG were identified using DESeq2 negative-binomial Wald test. (G) Table of genes of interest selected from the DESeq2 dataset showing adjusted p-value. (H) ddPCR quantification of NEDD4L, DPP6, KCNQ3 and CBLN4 from the RNA library used for RNAseq analysis (mean±SEM, Mann-Whitney U-test).

Table 1
Electrophysiological properties of pyramidal tract (PT)- and intratelencephalic (IT)-type projection neurons in layer 5 of motor cortex recorded from control or C9BAC mice.

PT-type neurons	**Ntg** (23 cells, 7 mice)	**C9BAC** (15 cells, 5 mice)	***P*-value**
Resting membrane potential (mV)	-66.69 ± 0.61	-67.42 ± 0.68	P > 0.3
Input resistance (mΩ)	218.34 ± 6.89	223.04 ± 14.5	P > 0.8
Sag amplitude (mV)	5.92 ± 0.29	5.35 ± 0.43	P > 0.09
Spike threshold (mV)	-37.84 ± 0.61	-37.96 ± 0.81	P > 0.7
Spike amplitude (mV)	69.43 ± 0.89	69.76 ± 1.4	P > 0.7
Spike half-width (ms)	1.85 ± 0.04	1.9 ± 0.05	P > 0.3
Afterhyperpolarization (mV)	21.62 ± 0.49	21.87 ± 0.76	P > 0.8
Rheobase (pA)	38.73 ± 3.07	40.6 ± 4.21	P > 0.7
IT-type neurons	**Ntg** (19 cells, 7 mice)	**C9BAC** (13 cells, 5 mice)	***P*-value**
Resting membrane potential (mV)	-69.01 ± 0.82	-68.5 ± 1.06	P > 0.7
Input resistance (mΩ)	840.71 ± 35.79	775.61 ± 53.62	P > 0.4
Sag amplitude (mV)	1.52 ± 0.27	1.51 ± 0.33	P > 0.8
Spike threshold (mV)	-33.02 ± 1.08	-32.37 ± 0.95	P > 0.5
Spike amplitude (mV)	50.11 ± 2.26	47.92 ± 2.18	P > 0.2
Spike half-width (ms)	2.21 ± 0.08	2.38 ± 0.13	P > 0.2
Afterhyperpolarization	18.62 ± 0.76	17.75 ± 0.79	P > 0.5

(mV)			
Rheobase (pA)	17.94 ± 2.32	19.84 ± 1.65	P > 0.3

Supplemental Experimental Procedures

Motor behavior testing

Motor function was assessed in F1 male C9BAC mice and non-transgenic (Ntg) littermates monthly from 3- to 24-months of age using rotarod (Med-Associates Inc., USA). Mice were tested three times on a 5-minute, 4-40rpm accelerating rotarod task with latency to fall for each individual test recorded. The mean score for each animal was used for statistical analysis. Limb grip strength was recorded using a digital force gauge (Mark-10, USA) fitted with a metal grid. Mice were placed on the grid, allowing either all paws to grip or forepaws only, followed by being gently pulled horizontal to the apparatus until grip was released. Each animal was tested 5 times for fore- or all limb grip, with peak strength for each recorded for statistical analysis. Data from longitudinal behavior testing was statistically analyzed by two-way ANOVA and Bonferroni‘s multiple comparison test.

Intruder testing

To assess social interaction behavior, an unfamiliar 6 week old, juvenile C57Bl6/J wild type mouse was introduced into the home cage of an individually housed 18-22 month-old male C9BAC or Ntg mouse. Direct physical interaction by the home cage male with the juvenile was recorded over a 5-minute period.

Electrophysiological recordings: Motor Unit Number Estimate

Electrophysiological recordings were as described [1]. Motor conduction studies and motor unit number estimate (MUNE) were performed using a portable electrodiagnostic system (Cardinal Synergy). For the motor nerve conduction studies, the lowpass filter was set at 30 HZ, and the high-pass filter was set at 10 kHZ. The nerve was stimulated with single square-wave pulses of 0.1-ms duration. Supramaximal responses were gradually generated, and maximal responses were obtained with stimulus currents <20 mA (most often <10 mA). The distal latency, distal and proximal compound motor action potential (CMAP) amplitudes, distal and proximal CMAP durations (measured from onset of initial negative deflection to initial return to baseline), and conduction velocity were determined for each nerve studied. For the MUNE recordings, the incremental technique was used [2-4]. For electromyography potentials were recorded from several sites of the hindlimb muscles with a concentric needle electrode (30G) [5].

Electrophysiological recordings: Whole-cell cortical neuron recordings

Brain slice preparation for electrophysiological recordings. After anesthetizing mice with isoflurane, brains were rapidly removed and chilled in an ice-cold sucrose solution containing 76mM NaCl, 25mM NaHCO3, 25mM glucose, 75mM sucrose, 2.5mM KCl, 1.25mM NaH2PO4, 0.5mM CaCl2, and 7mM MgSO4; pH 7.3. Acute brain slices (300 µm) were prepared (VT-1200s, Leica) tilted anteriorally 15° in the coronal plane. Slices were then incubated in warm (32-35°C) sucrose solution for 30 minutes and then transferred to warm (32-34°C) artificial cerebrospinal fluid (aCSF) composed of 125mM NaCl, 26mM NaHCO3, 2.5mM KCl, 1.25mM NaH2PO4, 1mM MgSO4-7H2O, 20mM D-(+)-glucose, 2mM CaCl2-2H2O, 0.4mM ascorbic acid, 2mM pyruvic acid, and 4mM L-(+)-lactic acid; pH 7.3, 315 mOsm. All solutions were continuously bubbled with 95% O_2/5% CO_2.

Slices were transferred to a submersion chamber on an upright microscope (Zeiss AxioExaminer, 5x and 40x objectives lenses, 0.16 and 1.015 NA respectively) and continuously superfused (2-4 ml/min) with warm oxygenated aCSF (32-34°C). Neurons were visualized with a digital camera (Sensicam QE, Cooke) using transmitted light with infrared differential interference contrast optics. PT-type neurons were distinguished by their large, pyramidal shaped cell bodies and thick apical dendrite. IT-type neurons were identified by their small oval shaped cell bodies and a thinner apical dendrite. The identity of each cell was further confirmed by analyzing its intrinsic membrane properties including sag amplitude, input resistance, and response to current injections as these are significantly different between the two types (Table 1). Glass recording electrodes (2-4 MΩ) were filled with internal solution containing 2.7mM KCl, 120mM $KMeSO_4$, 9mM HEPES, 0.18mM EGTA, 4mM MgATP, 0.3mM NaGTP, 20mM phosphocreatine(Na), pH 7.3, 295 mOsm with 0.25% biocytin. Whole-cell patch clamp recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices) and digitized using an ITC-18 (Instrutech) controlled by custom software written in Igor Pro (Wavemetrics). All signals were low-pass filtered at 10 kHz and sampled at 20-100 kHz. The access resistance averaged 9.6 ± 0.7 MΩ. Cells with an R_a of 30 MΩ or more were eliminated from the analysis. The R_a was not compensated during recordings. To measure intrinsic excitability, cells were recorded in the presence of the synaptic blockers: NBQX (AMPA receptor antagonist, 5 µM), CPP (NMDA receptor antagonist, 5 µM), and SR95531 ($GABA_A$ receptor antagonist, 10 µM, all from Tocris). The resting membrane potential (RMP) was measured after whole-cell configuration was achieved. Neurons exhibiting an RMP greater than -60 mV were excluded from the analysis. The input resistance was determined by measuring the voltage change in response to hyperpolarizing current steps and the amplitude of the sag response was calculated as the difference in minimum potential at the early phase of a hyperpolarizing current step and the steady-state potential at the end of the 1 s step. The current-spike frequency relationship was measured with depolarizing current steps (1 s) applied in 40 pA increments. The rheobase

was measured similarly using 2 pA increments. Action potential properties were measured from single spikes evoked by rheobase current injections. The amplitude of the afterhyperpolarizations (AHP) was measured from the spike threshold to the minimum membrane potential during the AHP.

All data analyses were performed in Igor Pro (Wavemetrics), Excel (Microsoft), and Origin (OriginLab). The Mann-Whitney test was used to compare the rheobase, input resistance, RMP, sag amplitude, and spike properties recorded from control and C9BAC mice. A 2-way ANOVA with Bonferroni correction was used to test statistical differences in the current-frequency relationships between groups.

Primary cortical culture and drug susceptibility analysis

Cortical neurons were harvested and cultured from individual E15 embryos generated from C9BAC x WT crosses. For Experiment 2 of the knock down experiment, all transgenic embryos from a litter were maintained in Hibernate media (Thermo-Fischer) at 4C for approximately 5 hours whilst genotypes were established, and were subsequently pooled and cultured. Cells from individual embryos were seeded onto poly-L-ornithine coated plates or cc2 coated chamber slides at 50,000/cm^2. Neurons were maintained in neurobasal media supplemented with Glutamax, B27, and pen/strep (all from Invitrogen) with one half of the media replaced every 3-4 days. To inhibit glial cell proliferation, the anti-mitotic AraC (1 µM, Sigma) was added at DIV3 to the cultures used for assessment of neuronal susceptibility to cytotoxic insult. Susceptibility of DIV15 neurons to 24 hr treatment with chloroquine (CQ) and 3-methyladenine (3-MA) (all from Sigma) at the indicated doses was assessed by lactate dehydrogenase release assay (Promega).

For treatment with silencing vectors, cells were plated in 6-well dishes at 50,000/cm^2 and feed every fourth day me. At day in vitro four, 18µl of rAAV-GFP (titre = 1E13 genome copies/ml) or rAAV-GFP-miRC9 (titre = 2.4 E13 gc/ml) were added to 1.5 ml of media and the cultures were maintained for a further 6-days. For protein analysis, cells were scrapped from the well, washed with PBS and pelleted. For quantification of *C9ORF72* transcripts, total RNA was collected using Trizol as per the manufacturers instructions.

RT-qPCR

Frozen tissue samples were homogenized in a gentleMACS Dissociator (Miltenyi Biotec, USA) before total RNA extraction with Trizol (Life Technologies). Reverse transcription to cDNA was performed using random hexamers and MultiScribe reverse transcriptase (High capacity RNA-to-cDNA Kit, Life Technologies). Real time quantitative PCR was performed on an Applied biosystems StepOnePlus Real-Time PCR system using the Fast TaqMan Advanced master mix following the manufacturer instructions. A commercial mouse HPRT assay (Life Technologies, Mm01545399_m1) was used as an endogenous control. Data was analyzed using the Delta Delta CT method. Probes used to detect each variant are as follows: Vall commercial assay (Life Technologies Hs00376619_m1), probe AGAATATGGATGCATAAGGAAAGAC; V1 commercial assay (Life Technologies Hs00331877_m1), probe AGATGACGCTTGATATCTCCGGAGC (Life Technologies assay, # Hs00331877_m1); V2 forward primer AGGCGGTGGCGAGTGGATA, reverse primer TTGGAGCCCAAATGTGCCTTA, probe CGACTCTTTGCCCACCG; V3 forward primer GCGGGGTCTAGCAAGAGCAG, reverse primer TTGGAGCCCAAATGTGCCTTA, probe CCACCGCCATCTC. Position of probes is shown in Figure S1A.

Droplet Digital PCR (ddPCR)

To assess the absolute concentration of transcripts, droplet digital PCR (ddPCR Bio-rad) was performed on both mouse samples and human frontal cortex samples from c9ALS/FTD (age, 61.3±4.5 years; sex, 3 male, 1 female), SOD1 fALS (age, 46.8±3.5 years; sex, 3 male, 1 female), and non-neurological disease control patients (age, 62.8±7.2 years; sex, 2 male, 2 female). Mouse brain tissue was obtained from 2 month old C9BAC transgenic mice (n=4) and wild type littermate controls (n=4). Total RNA was extracted by homogenization in Trizol (Life Technologies, USA) in a gentleMACS Dissociator (Miltenyi Biotec). cDNA was synthesized from 2μg/μl RNA using High-Capacity RNA-to-cDNA Kit (Life Technologies, USA). For reactions, cDNA was diluted 1:5 in nuclease free water. 20μl reactions solution were prepared for each sample, containing ddPCR Supermix for Probes (Bio-Rad, USA), 2μl of diluted cDNA, 1μl of each primer and 1μl C9ORF72 variant specific FAM-labeled probes. 20μl samples and 70μl of ddPCR-oil was loaded into a DG8 cartridge (Bio-Rad, USA) and droplet generated using the QX100 droplet generator (Bio-Rad, USA). PCR was then run using 40μl of the sample droplet solution on a standard thermocycler (Eppendorf, USA), and absolute quantification calculated using the QX100 ddPCR reader (Bio-Rad, USA). Primers and probes used to detect each variant are as follows: C9ORF72 all variants (Vall) forward primer ATCCTTCGAAATGCAGAGAG, Vall reverse primer TGAAGTGGGAGGTAGAAACT, Vall probe CTGGAATGGGGATCGCAGCACA; C9ORF72 variant 1 (NM_145005.6) commercial assay, probe AGATGACGCTTGATATCTCCGGAGC (Life Technologies assay, # Hs00331877_m1); C9ORF72 variant 2 (NM_018325.4) forward primer AGGCGGTGGCGAGTGGATA, reverse primer TTGGAGCCCAAATGTGCCTTA, probe CGACTCTTTGCCCACCG; C9ORF72 variant 3 (NM_001256054.2) forward primer GCGGGGTCTAGCAAGAGCAG, reverse primer TTGGAGCCCAAATGTGCCTTA, probe CCACCGCCATCTC. Position of probes is shown in Figure S1A. Endogenous mouse C9ORF72 (Rik_3110043O21, NM_001081343.1) commercial

assay, probe GCAGCGGCGAGTGGCTATTGCAAGC (Life Technologies Mm01216837_m1). Probes for detection of RNAseq candidate genes were Biorad PrimePCR ddPCR Expression Probe Assays: Cbln4, Mouse dMmuCPE5122216 DDPCR GEX FAM ASSAY 500R; Dpp6, Mouse dMmuCPE5105460 DDPCR GEX FAM ASSAY 500R; Kcnq3, Mouse dMmuCPE5117670 DDPCR GEX FAM ASSAY 500R; Nedd4l, Mouse dMmuCPE5124352 DDPCR GEX FAM ASSAY 500R; Hprt, Mouse dMmuCPE5095493.

For detection of the mature microRNA product of rAAV-GFP-miRC9, RNA was diluted to 10ng/µl and the Taqman microRNA reverse transcription kit (Life Technologies assay #4366597) was used to generate cDNA. A custom Taqman small RNA assay was designed to detect the mature microRNA product of rAAV-GFP-miRC9 (probe ATAGCACCACTCTCTGCATT, Life Technologies). Control RNA snoRNA135 was detected using a commercially available probe (Life Techonolgies # 4427975). Droplet digital PCR was performed as indicated above.

Antibodies

Primary antibodies: Goat anti-beta Actin (Abcam, USA), Rabbit anti-Actin (Sigma, USA), Rabbit anti-GFAP (IHC 1:500 Abcam, USA), Rabbit anti-Beta-III-tubulin (IHC 1:1000, Covance, USA), rabbit anti-ChAT (IHC 1:100, Abcam, USA), rabbit anti-cleaved caspase-3 (AB3623, Millipore, USA), Rabbit anti-IBA1 (IHC 1:1000, WAKO, Japan), mouse anti-NF200 (IHC 1:100, clone SMI-32 Abcam, USA), Guinea Pig anti-P62 (Progen, Germany), Rabbit anti-Synaptophysin (IHC 1:5, Life Technologies, USA), Rabbit anti-TDP43 (IHC 1:500 Proteintech, USA), Rabbit anti-TDP43 N-terminus (IHC 1:500, kind gift from Zuoshang Xu) [6]. Secondary antibodies: Donkey anti-Rabbit Alexa Fluor-488 (IHC 1:1000, Life Technologies, USA), Donkey anti-Mouse Alexa Fluor-488 (IHC 1:1000, Life Technologies, USA), Biotinylated Goat anti-Rabbit (IHC 1:1000, Vector Labs, USA), Biotinylated Horse anti-Mouse (IHC 1:1000, Vector Labs, USA)**.** Stated dilutions

are for immunohistochemistry or immunofluorescent staining. For western blotting all antibodies were used at the manufacturers recommended dilution.

Western Blotting

Tissue samples were lysed in RIPA buffer containing Complete Protease Inhibitor Tablets (Roche, USA) using a Dounce homogenizer followed by sonication on ice. Samples were centrifuged for 10 min at 14,000×g at 4˚C and the supernatant collected. Total protein concentration was quantified using the BCA Protein Assay kit (Thermo, USA). Five micrograms of protein lysate were run on Novex 12% Tris-Glycine gels (Life Technologies, USA) using Tris-Glycine SDS running buffer (Invitrogen, USA). Proteins were transferred to nitrocellulose membranes using an i-Blot transfer device (Invitrogen, USA). Membranes were blocked for 1 hour at room temperature with Odyssey Blocking Buffer (LiCor, USA) before being probed overnight with primary antibodies. IR labeled secondary antibodies (LiCor, USA) were applied, and blots visualized using the Odyssey Infrared imaging system (LiCor, USA).

Poly(GP) immunoassay

An immunoassay for poly(GP) dipeptides was performed as published [36]. Tissues were homogenized in 10% (w/v) buffer containing 50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1% Triton X-100, 5 mM EDTA, as well as protease (EMD Millipore, USA) and phosphatase inhibitors (Sigma-Aldrich, USA). Sodium dodecyl sulfate was then added to a portion of homogenate at a final concentration of 2% and samples were vortexed, re-homogenized and sonicated. After sonication, samples were centrifuged at 16,000 x g for 20 min and supernatants collected. The protein concentration of lysates was determined by BCA assay (Thermo Scientific, USA). Poly(GP) levels in lysates were measured using a previously described sandwich immunoassay that utilizes Meso Scale Discovery (MSD) electrochemiluminescence detection technology [7]. Lysates were diluted in Tris-buffered saline (TBS) and tested using 35µg of protein per well in

duplicate wells. Serial dilutions of recombinant $(GP)_8$ in TBS were used to prepare the standard curve. Response values corresponding to the intensity of emitted light upon electrochemical stimulation of the assay plate using the MSD QUICKPLEX SQ120 were acquired and background corrected using the average response from lysates obtained from non-transgenic mice prior to interpolation of poly(GP) levels using the standard curve.

RNA fluorescence in situ hybridization (FISH)

Cryosections (20µm) were fixed in 4% PFA for 20 min, followed by five 20 min washes in DEPC-treated PBS containing 0.1% Tween-20. Sections were permeabilzed with 0.5% Triton X-100 before pre-hybridization for 1 hour at 55C in 2x saline sodium citrate (SSC) containing 0.1% Tween-20, 50% of dextran sulfate, 50µg/ml heparin, 1mg/ml heat-denatured salmon sperm and 40% formamide. For hybridization, samples were incubated overnight at 55°C in pre-hybridization buffer containing a 1:500 dilution of 1µM DNA probe (Sense: $(GGCCCC)_4$, Anti-sense: $(GGGGCC)_4$, DM1 $(GAC)_6$, DM2 $(GGAC)_6$) 5' end Cy3 labeled. After hybridization the samples were washed in a pre-warmed mix 1:1 of hybridization buffer and 2XSSC for 30 min at 55°C. Followed by 2 washes in pre-warmed 2XSSC for 30 min at 55°C, 2 washes with 0.2XSSC for 30 min at 55°C, and 2 washes in 1XPBS+ 0.1% Tween-20 for 30 min, at room temperature. Sections were stained with DAPI (1:10,000) for 5 min, and autofluorescence quenched by incubation in 0.5% Sudan Black in 70% ethanol for 7 min. Glass coverslips were mounted using Lab Vision PermaFluor (Thermo Scientific Shandon, USA)

For FISH coupled with fluorescent immunohistochemistry, after probe hybridization and washes, sections were blocked by incubation with 10% serum donkey-serum in PBS with 0.4% TritonX-100 for 1hr, followed by incubation with primary antibodies diluted in blocking solution overnight

at 4°C. After PBS washes, sections were incubated with an Alexa Fluor-488 conjugated secondary antibodies diluted 1:1000 in PBS for 1hr at room temperature. Autofluorescence was blocked by submersion in 0.5% Sudan black B in 70% ethanol for 7 mins, followed by staining of nuclei by incubation in DAPI (1:10,000). Glass coverslips were applied using ImmuMount (Thermo Scientific Shandon, USA).

For quantification of motor cortex external pyramidal layer nuclei 8-10 10µm z-stack images were collected from three sections per animal. Lumbar spinal motor neurons were identified by large nuclei, with 20-40 nuclei assessed from 8 sections per animal. The number of foci per nucleus was counted using ImageJ, with each nucleus categorized as containing 0 foci, 1-5 foci, 6-10 foci or >10.

Immunohistochemistry

For *DAB* immunohistochemistry, 8µm paraffin sections of 4% paraformaldehyde or formalin-fixed sagittal brain or coronal spinal cord were dewaxed in xylene and brought to water through a graded alcohol series. Endogenous peroxidase activity was quenched by incubation in a solution of 3% H_2O_2 in 10% methanol for 20mins at 4^{o}C. Non-specific antibody binding was blocked by incubation with 10% serum goat-serum in PBS with 0.4% TritonX-100 for 1hr. Primary antibodies were diluted in blocking solution and sections incubated overnight at 4^{o}C. Following washing, biotinylated secondary antibodies diluted in PBS were applied for 1hr at room temperature. After washing sections were incubated with an avatin-HRP solution (Vector labs, USA) for 30 mins. Peroxidase labeling was visualized with chromogen solution 3, 3'-diaminobenzidine (Sigma-Aldrich, USA). Sections were counterstained with hemotoxylin, dehydrated and a glass coverslip applied using DPX. For fluorescent immunohistochemistry, Alexa Fluor-488 or -546 conjugated secondary antibodies diluted in PBS for 1hr at room temperature. Autofluorescence was blocked by submersion in 0.5% Sudan black B in 70%

ethanol for 7 mins, followed by staining of nuclei by incubation in DAPI (1:10,000). Glass coverslips were applied using ImmuMount (Thermo Scientific Shandon, USA).

Immunostaining for neuromuscular junctions

Mice were perfused first with a PBS prewash for 2mins followed by 4% PFA in PBS for 5mins. Gastrocnemius muscles were dissected and post-fixed overnight in 1.5% PFA at 4^{o}C. Muscles were washed in PBS, incubated in 25% sucrose in PBS overnight at 4^{o}C and embedded longitudinally in OCT (Thermo Scientific Shandon, USA). 35µm cryosections were collected, mounted on glass slides and stored at -80°C until use. Frozen sections were air dried for 30mins and washed with PBS, followed by blocking in 10% goat-serum in 10% tritonX_{100} for 3hrs. Sections were incubated for 24hrs at 4°C in a primary antibody solution containing a cocktail of rabbit anti-synaptophysin (1:5, Invitrogen) and rabbit anti-Neuronal Class III Beta-Tubulin (1:1000, Covance, USA) diluted in blocking solution. Following washing sections were incubated in PBS containing secondary antibodies Alexa Fluor-488 conjugated donkey anti-Rabbit (1:500) and Alexa Fluor-555 conjugated alpha-bungarotoxin (1:500) overnight at 4°C. Sections were washed, counterstained with DAPI and coverslips mounted using Immumount (Thermo Scientific Shandon, USA).

For quantification, 15µm z-stacks were collected at 40x magnification from two non-adjacent section of gastrocnemius muscle. Approximately 150-200 NMJs were assessed per animal. NMJs were categorized as; innervated, with complete overlap of green and red signal; partial, with incomplete overlap of green and red signals; denervated, with no overlap of green and red signals.

Semi-thin sectioning, toluidine blue staining and axon quantification

Mice were fixed by perfusion with 4% PFA in PBS and L5 ventral nerve roots dissected. Following overnight fixation in 2.5% gluteraldehyde in 0.1M cacodylate buffer nerves were washed and further post-fixed in 1% osmium tetroxide for 1hr. Samples were dehydrated through a graded ethanol series into propylene oxide, followed by overnight infiltration in 1:1 solution of propylene oxide and SPI-Pon 812 resin mixture. Following 3hrs of incubation in SPI-Pon 812 resin samples were polymerized at 68°C for 4 days. Nerves were trimmed, reoriented and 0.6µm semi-thin sections collected. Semi-thin sections were mounted on glass slides, followed incubation in toluidine blue on a hot plate for 30 seconds. Following washing, sections were dried and a coverslip mounted using DPX. Sections were imaged and axon number and diameter quantified using ImageJ.

Dendritic spine analysis

Brains from 18 month old C9BAC and Ntg littermate controls (n=3 per genotype) were prepared for Golgi staining using the FD Rapid GolgiStain Kit (FD NeuroTechnologies Inc, USA) as per the manufacturers instructions. Brains were embedded in low gelling temperature agarose (Boston BioProducts, USA) and 100µm coronal sections were collected using a Leica VT1000 S vibratome (Leica Microsystems Inc, USA). Apical dendrites of layer 2-3 pyramidal neurons were imaged at 60x magnification with 1.5x optical zoom in the prefrontal cortex (3-12 per brain). Number of spines was counted along a recorded length of dendrite (total length of dendrite sampled: Ntg = 1943.5µm, C9BAC = 1266.1µm). Density of spines per dendrite was calculated by dividing the number of spines by the length of dendrite sampled.

RNAseq

Total RNA was extracted from dissected frontal cortex of six months old C9BAC and their Ntg littermate controls by Trizol (Life Technologies). 4µg of high quality total RNA was treated with

RiboZero magnetic beads (Epicentre) to remove most of the ribosomal RNA (rRNA). rRNA depleted total RNA was then treated with Turbo DNAse 1 (Ambion) for 30 min at 37°C to remove possible contamination from genomic DNA. Resulting RNA was purified with RNA clean and Concentrator 5 (Zymo Research), removing RNA fragments shorter than 200 nt and tRNA. Strand-specific libraries were then prepared as described [8]. Validation of cDNA libraries fragment size was performed using an Agilent Technologies 2100 Bioanalyzer. Four barcoded samples were pooled in a single lane and sequenced as 100nt paired-end reads on an Illumina HiSeq 2000 instrument. RNA seq generated greater than 34 million clean reads for each sample. Using TopHAT 2, at least 75% of this total clean reads mapped at a single location of the mouse reference genome (mm10) for each replicate sample. RNA seq reads were then aligned to mouse reference transcripts and their abundance was estimated using RSEM software workflow (RSEM parameters : --bowtie-e 70 --bowtie-chunkmbs 200 --forward-prob 0) [9]. Normalization and differential expression gene analysis was performed with DEseq2 software package, based on the negative binomial model on R/bioconductor statistical framework [10].

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