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Supplementary Materials for

C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits

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Revisions 15 May 2015: Lillian Daugherity's initial is M. The scale on the *y*-axis label for Figure S8B has been changed.

Materials and Methods

Approvals

All animal procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by the Mayo Clinic Institutional Animal Care and Use Committee. For studies utilizing human post-mortem tissues, autopsy consent was given by the next-of-kin and studies were conducted with Institutional Review Board approval.

Generation of AAV9-$(G_4C_2)_2$ and AAV9-$(G_4C_2)_{66}$ vectors

$(G_4C_2)_2$ and $(G_4C_2)_{66}$ repeats were generated as described previously (*27*). We inserted the $(G_4C_2)_2$ and $(G_4C_2)_{66}$ repeats with 119 base pairs of the 5' flanking region and 100 base pairs of 3' flanking region of the *C9ORF72* gene into the HindIII and XhoI restriction sites of the adeno-associated virus (AAV) expression vector pAM/CBA-pl-WPRE-BGH containing inverted repeats of serotype 2. AAV-$(G_4C_2)_2$ and AAV-$(G_4C_2)_{66}$ particles were packaged into serotype 9 type capsid and purified using standard methods (*28*). Briefly, AAV vectors containing $(G_4C_2)_2$ and $(G_4C_2)_{66}$ repeats were generated by plasmid transfection with helper plasmids into HEK293T cells. Forty-eight hours after transfection, the cells were harvested and lysed in the presence of 0.5 % sodium deoxycholate and 50 Units/ml Benzonase (Sigma-Aldrich) by freeze thawing, and the virus was isolated using a discontinuous iodixanol gradient. The genomic titer of each virus was determined by qPCR. The solutions of AAV were diluted with sterile phosphate-buffered saline (PBS).

Neonatal viral injections

Intracerebroventricular (ICV) injections of AAV were performed as previously described with some minor modifications (*22, 29*). Briefly, a 32-gauge needle (Hamilton Company) attached to a 10 µl syringe (Hamilton Company) was inserted at approximately two-fifths the distance between lambda suture and each eye of C57BL/6J pups at post-natal day 0 after they were cryoanesthetized on ice for approximately 3 minutes or until pups exhibited no movement. The needle was inserted at a 30-degree angle from the surface of the head and held at a depth of approximately two millimeters. Two microliters (1E10 genomes/µl) of AAV2/9-$(G_4C_2)_2$ or AAV2/9-$(G_4C_2)_{66}$ solution was manually injected into each cerebral ventricle. After injections, pups were placed on a heat pad until they completely recovered from anesthesia and then were placed back into their home cages.

Behavioral tests

Six-month-old mice expressing AAV9-$(G_4C_2)_2$ (n=12) or AAV9$(G_4C_2)_{66}$ (n=11) underwent a battery of behavioral tests, including the open field assay, social interaction test and rotarod test (*30*). All mice were acclimated to the testing room for 1-2 hours prior to testing. All behavioral equipment was cleaned with 30% ethanol prior to use with each animal. All mice were returned to their home cages and home room after each test.

Open field assay

The apparatus consists of a square Perspex box (40x40x30cm, WxLxH) with side mounted photobeams raised 7.6 cm above the floor to measure rearing. Mice were placed in the center of an open field area and were allowed to explore the area for 15 minutes. Movement was monitored through the use of an overhead camera with AnyMaze software (Stoelting Co.). Mice were tracked for multiple parameters, including total distance traveled, average speed, time mobile and distance traveled in an imaginary "center" zone (20x20 cm), which was illuminated by an overhanging light fixture. Anxiety levels in the mice was measured by comparing the distance traveled in the "center zone" vs. the total distance traveled.

Social interaction test

The social interaction test apparatus consists of a rectangular box subdivided into three chambers: two 17x40 cm chambers and a small 5x40 cm middle chamber are connected by a small 8x5 cm opening that allows free access between each chamber. Two empty, inverted wire-mesh cylinders were placed in opposing corners of each chamber. In the first trial, mice were placed in one chamber and allowed to explore the apparatus freely for four minutes and then placed back in temporary holding cages. Next, a probe mouse of the same sex and strain was placed in the cylinder for three minutes prior to the test mouse being placed back into the chamber. Their interactions were analyzed and monitored for 10 minutes by an overhead camera with Anymaze software (Stoelting Co.). The time the test mouse spent within the area containing the cylinder with the probe mouse (Time mouse cylinder) minus the time spent within the area with the empty cylinder (Time empty cylinder) over the total time (Time mouse cylinder + Time empty cylinder) was used to compute the interaction score.

Rotarod test

Motor coordination and balance was measured using an automated rotarod system (Med Associates, Inc). Each mouse was placed on an accelerating spindle (4-40 rpm) for five minutes for three consecutive trials with at least 20 minutes of rest in between trials. The latency to fall time was recorded when the mouse fell off the spindle, triggering a sensor that automatically stops the timer located underneath the spindle. In instances when the mouse clung to and rolled around the rod three consecutive times, the mouse was manually removed from the spindle and the corresponding time was recorded. This test was repeated for four days.

Statistical analysis for behavioral studies

AnyMaze data from all behavioral tests were analyzed with Prism GraphPad 6.0 (Graphpad Software). AAV9-$(G_4C2)_2$ (n=12) and AAV9-$(G_4C_2)_{66}$ (n=11) mice were compared for each behavioral task. Unpaired, two-tailed Student's t-test was used to detect behavioral differences (P values <0.05). Rotarod data was analyzed with Prism GraphPad 6.0 (GraphPad Software) using two-way ANOVA followed by Sidak's multiple comparisons test to detect motor differences (P values <0.05).

RNA Fluorescence In situ Hybridization (FISH)

Mice were euthanized by carbon dioxide overdose and brains and spinal cords were harvested. Half brains and spinal cords were fixed in 4% paraformaldehyde for at least 48 hours, embedded in paraffin, sectioned (5 μm; sagittal sections for brain, coronal sections for spinal cord), mounted on positively charged glass slides and dried overnight. The RNA FISH protocol was performed on mouse or human c9FTD/ALS tissue sections as previously described with some modifications (*12*). Briefly, paraformaldehyde fixed-paraffin embedded (FFPE) brain sections were deparaffinized in xylene and rehydrated through a series of ethanol solutions. Sections were permeabilized with ice cold 2% acetone/1x DEPC-phosphate buffered saline (PBS) for five minutes and washed twice with DEPC-water and dehydrated through a series of ethanol solutions. The sections were incubated with pre-hybridization buffer (50% formamide (Midsci), 10% dextran sulfate (Millipore), 2x saline-sodium citrate buffer (SSC), 50mM sodium phosphate buffer pH 7.0) for 20-30 minutes at 66°C and then hybridized with a fluorescently labeled locked nucleic acid (LNA) probe (*12*) , TYE563-(CCCCGGCCCCGGCCCC; Exiqon 500150, batch: 612968) diluted to final concentration of 40 nM, for 24 hours at 66°C in a dark, humidified chamber. Next, sections were washed once with 2x SSC/0.1% Tween-20 at room temperature for 5 minutes and washed twice with pre-warmed 0.2x SSC at 66°C for 10 minutes in the dark. Slides were mounted with Vectashield mounting media with DAPI (Vector Laboratories). Representative images of RNA foci were taken with Zeiss Axio Imager Z1 fluorescent microscope (Carl Zeiss MicroImaging) under 63x magnification. Semi-quantitative analysis of cells containing RNA foci in AAV9-$(G_4C_2)_{66}$ (n=6) and AAV9-$(G_4C_2)_2$ (n=6) mice were assessed in the following regions: cortex, hippocampus, thalamus, cerebellum, amygdala, and the ventral horn of the spinal cord. All sections were analyzed on a Zeiss Axio Imager Z1 fluorescent microscope (Carl Zeiss MicroImaging) under 63x magnification. RNA foci burden was assessed on a 4 point scale: rare (+/-), low (+), moderate (++) and high (+++) occurrence levels. Quantitative analysis of cells containing RNA foci in AAV9-$(G_4C_2)_{66}$ (n=11) mice were assessed in these regions: cortex (n=500 cells), hippocampus (n=500 cells), motor cortex (n=500), and cerebellar Purkinje cells (n=100 cells).

Co-RNA FISH and immunofluorescence staining

Briefly, paraformaldehyde fixed-paraffin embedded (FFPE) brain sections were deparaffinized in xylene and rehydrated through a series of ethanol solutions. Antigen retrieval was performed in sodium citrate buffer [10mM citrate with 0.05% Tween-20 (pH 6.0)] for 20 minutes. Slides were cooled by running distilled water into the slide holder for 10 minutes. Then sections were permeabilized with 70% ethanol/1x DEPC-water for one hour at room temperature and dehydrated through 85%, 95%, 100% and 100% ethanol solutions for two minutes. Sections were allowed to dry for 15 minutes. All of the following steps were performed in a light protected chamber. The sections were incubated with pre-hybridization buffer for 20-30 minutes at 66°C and then hybridized with a fluorescently labeled locked nucleic acid (LNA) probe (*12*), TYE563-(CCCCGGCCCCGGCCCC; Exiqon 500150, batch: 612968) diluted to final concentration of 80 nM, for 24 hours at 66°C in a dark, humidified chamber. Next, sections were washed once with 2x SSC/0.1% Tween-20 and washed once with

1xDEPC-PBS for 5 minutes at room temperature. Sections were then blocked with 2mg/mL albumin, acetylated from bovine serum (ac-BSA, Sigma) dissolved in 1xDEPC-PBS for one hour at room temperature. Sections were incubated overnight at 4°C with rabbit polyclonal pTDP43 (serines 409/410) or rabbit polyclonal poly(GP) antibody diluted to 1:500 with ac-BSA. Sections were washed three times with 1xPBS at room temperature. Sections were then incubated with Alexa 488-conjugated donkey anti-rabbit (1:1000, Thermo Fisher Scientific, Inc) diluted with ac-BSA for 1.5-2 hours at room temperature Slides were washed three times with 1xDEPC-PBS. Finally slides were mounted with Vectashield mounting media with DAPI (Vector Laboratories).

RNA FISH followed by immunofluorescence staining for pTDP43 or poly(GP) were conducted on brain sections of $(G_4C_2)_{66}$-mouse. A total of 250 pTDP-43-positive cortical cells among five mice were evaluated to determine whether they also contained at least one RNA focus. Likewise, a total of 150 pTDP-43-positive cortical cells among 3 mice were evaluated for the presence of poly(GP) inclusions. Images were taken with Zeiss Axio Imager Z1 fluorescent microscope (Carl Zeiss MicroImaging) under 63x magnification.

Immunohistochemistry and semi-quantitative analysis of poly(GA), poly(GP) and poly(GR) inclusions

FFPE sections were deparaffinized in xylene and rehydrated through a series of ethanol solutions. Antigen retrieval was performed in distilled water for 30 minutes. Tissues were immunostained with polyclonal antibodies against poly(GA) (1:50,000), poly(GP) (1:10,000) or poly(GR) (1:2500) using the DAKO Autostainer (Universal Staining System) and the DAKO+ HRP system. Other antibodies used for immunohistochemical analysis were those for the detection of glial fibrillary acidic protein (GFAP, 1:2500, Biogenex) and Neuronal Nuclei (NeuN, 1:5000, Chemicon International). Sections were then counterstained with hematoxylin, dehydrated through a series of ethanol washes and xylene and mounted with Cytoseal mounting media (Thermo Fisher Scientific, Inc). Slides were scanned with a ScanScope® AT2 (Leica Biosystems) at 40x magnification. Representative images of poly(GA), poly(GP), poly(GR) inclusions, NeuN-positive neurons, and GFAP positive astrocytes were taken with ImageScope® software (v12.1; Leica Biosystems) at 40x magnification. Semi-quantitative analysis of the poly(GA), poly(GP), and poly(GR) protein distribution in cortex, hippocampus, thalamus, amygdala, cerebellum and spinal cord was performed on AAV9-$(G_4C_2)_{66}$ (n=6) and AAV9-$(G_4C_2)_2$ (n=6) FFPE brains sections. Individual c9RAN protein expression/inclusions were assessed on a 4 point scale: rare (+/-), low (+) moderate (++) and high (+++) occurrence levels.

Immunohistochemistry on c9FTD brain sections

Immunohistochemistry on formalin-fixed paraffin-embedded frontal cortex tissue from c9FTD/ALS cases positive for the *C9ORF72* repeat expansion, as assessed by repeat-primed PCR and Southern blot (*1, 2*), was performed on a Dako Autostainer with Dako EnVision™+ reagents (Agilent Technologies). Five micron-thick slides were rehydrated in a graded series of xylene and alcohol prior to antigen retrieval for 30 minutes in water. Antibodies used on these two cases included anti-GA (1:50,000), anti-GP (1:10,000), and anti-GR (1:2,500) (*21*). The chromogen used was 3,3'-

diaminobenzidine. Following immunohistochemistry, slides were counterstained with hematoxylin, dehydrated in a graded series of alcohol and xylene, and coverslipped. Slides were visualized using a Zeiss AxioImager Z1 microscope (Carl Zeiss MicroImaging).

Digital pathology

The percentage burden of immunohistochemical GFAP staining and NeuN-positive neuronal density was quantified using Aperio® ePathology technology (Leica Biosystems). We used a ScanScope® AT2 (Leica Biosystems) to obtain high-resolution digitized images of immunostained slides. ImageScope® software (v12.1; Leica Biosystems) was used to annotate the cortex on mid-sagittal serial sections stained for GFAP or NeuN for each mouse. A custom-designed Positive Pixel Count algorithm was designed to quantify the brown chromagen 3,3'-Diaminobenzine, which positively labelled GFAP-positive astrocytes (*31*). The output parameter was a percentage burden of positively stained pixels per given annotation area. A custom-designed Nuclear Algorithm was designed to detect NeuN-positive neurons in the cortex and motor cortex given their rounded appearance and staining intensity. The output parameter was the number of NeuN-positive neurons per given mm^2 area annotated, which was calculated as a density measure.

Hematoxylin and Eosin (H&E) staining

Mid-sagittal H&E stained hemi-brain sections of AAV9-$(G_4C_2)_2$ (n=12) and AAV9-$(G_4C_2)_{66}$ (n=11) mice were scanned using ScanScope® AT2 (Leica Biosystems) to obtain high-resolution digitized images of the slides. Using the ruler function of ImageScope® software (v12.1; Leica Biosystems), a length totaling 10 mm of the Purkinje layer of the cerebellum was delineated for each mouse and the number of Purkinje cells within it counted. Data is presented as the average number of Purkinje cells per 1 mm within each cohort.

Immunofluorescence staining

FFPE brain sections used for immunofluorescence were deparaffinized and rehydrated as stated above. Antigen retrieval was performed in sodium citrate buffer [10mM citrate with 0.05% Tween-20 (pH 6.0)] for 30 minutes. Slides were cooled by running distilled water into the slide holder for 20 minutes. Next, sections were blocked with Protein Block plus Serum Free (DAKO) for 1 hour and incubated with either rabbit polyclonal anti-poly(GA) (1:500), anti-poly(GP) (1:1000) or anti-poly(GR) (1:1000), mouse monoclonal anti-poly(GA) (1:500, (*32*)) anti-ubiquitin-1 (1:1000, Millipore), anti-MAP2 (1:1000, Sigma-Aldrich) or anti-GFAP (1:1000, Biogenex) diluted with Antibody Diluent with Background-Reducing Components (DAKO) overnight at 4°C. Sections were washed three times with 1xPBS at room temperature. Sections were then incubated with Alexa Fluor 594 (1:2000, Thermo Fisher Scientific, Inc.) and Alexa Fluor 488 (1:2000, Thermo Fisher Scientific, Inc.) diluted with Antibody Diluent with Background-Reducing Components (DAKO) for 1.5 hours at room temperature in a light protected chamber. Sections were washed three times with 1xPBS at room temperature, incubated with Sudan Black for 2 minute, washed with distilled water and mounted with Vectashield mounting media with DAPI (Vector Laboratories). The same procedure was

performed for pTDP-43 immunofluorescence staining using pTDP-43 antibody (Serine 409/410, 1:500). Quantitative analysis of pTDP-43 positive cells in brain sections of $(G_4C_2)_{66}$-mice (n=6) were assessed in regions: cortex (n=500), motor cortex (n=500) and hippocampus (n=500). Quantitative analysis of co-immunofluorescence staining for pTDP43 and poly(GA)(*32*) was conducted on brain sections from $(G_4C_2)_{66}$-mice. A total of 250 pTDP-43-positive cortical cells among five mice were examined for the presence of poly(GA) inclusions. Representative images were taken with a Zeiss Axio Imager Z1 fluorescent microscope (Carl Zeiss MicroImaging) under 63x magnification.

Preparation of urea fractions of frozen hemi-brains of $(G_4C_2)_2$ and $(G_4C_2)_{66}$-mice and c9FTD cortical tissue

The urea fractions of both mouse and human brain tissues were prepared as previously described with minor modifications (***33, 34***). Briefly, ~100 mg frozen postmortem cortical tissue from a FTD patient verified to be a carrier of the *C9ORF72* repeat expansion, as assessed by repeat-primed PCR and Southern blot (*35*), and whole hemi-brains of $(G_4C_2)_2$-mice (n=3) and $(G_4C_2)_{66}$-mice (n=3) were subjected to a sequential extraction protocol using Tris-EDTA buffer (TE, 50mM Tris pH 7.4, 50mM NaCl, 1mM EDTA), high salt-Triton X-100 buffer, myelin floatation buffer, and sarkosyl buffer. Sarkosyl-insoluble material was further extracted in urea buffer and supernatant was saved as the urea fraction for Western blot analysis.

Western blot analysis

Western blot was performed as previously described (*34*). In brief, urea fraction of whole hemi-brains of $(G_4C_2)_2$ (n=3), $(G_4C_2)_{66}$-mice (n=3) and cortical tissue from a c9FTD case were mixed with Laemmli's buffer and analyzed by SDS-PAGE using a 4-20% Tris-glycine gel (Invitrogen). The gel was transferred to a PVDF membrane followed by blocking with 5% nonfat dry milk in TBST for 1 hour. The blot was then incubated with a rabbit polyclonal pTDP-43 antibody (phosphoSer409/410, 1:1000) or rabbit polyclonal full-length TDP-43 antibody (1:1000, Proteintech) overnight at 4°C. Membranes were washed in TBST and incubated with donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:3000, Jackson ImmunoResearch) for 1 hour at room temperature. Target protein levels were detected by treatment with enhanced chemiluminescence substrate and exposure in Luminescent Image Analyzer LAS-4000 Imaging System (Fuji Photo Film Co.).

Poly(GP) immunoassay

Frozen half brains of AAV9-$(G_4C_2)_2$ (n=12) and AAV9-$(G_4C_2)_{66}$ (n=11) mice were sonicated in ice cold Tris-EDTA buffer (TE, 50mM Tris pH 7.4, 50mM NaCl, 1mM EDTA) with protease and phosphatase inhibitors for five to ten pulses at two second intervals until all large pieces of tissue were dissolved. Two-hundred microliters of each brain homogenate was diluted in 200 µl of 2x lysis buffer (50mM Tris pH 7.4, 250mM NaCl, 2% Triton X-100, 4% SDS). Brain lysates were spun at 13,000 RPM for 20 minutes at 4°C. Supernatant was collected and stored at -20°C. Protein concentrations of the brain lysates were determined with bicinchoninic acid protein assay (Pierce Biotechnology). Poly(GP) protein levels in lysates were then measured using a previously described sandwich immunoassay that utilizes Meso Scale Discovery (MSD)

electrochemiluminescence detection technology (*27*). Lysates were diluted in Tris-buffered saline (TBS) and tested using 5 µg of protein per well in duplicate, and 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 prior to interpolation of poly(GP) levels using the standard curve. Statistical analysis using a non-paired, two-tailed t-test was performed on Prism GraphPad 6.0 (GraphPad Software) to detect significant differences in poly(GP) levels between groups ($P<0.0001$).

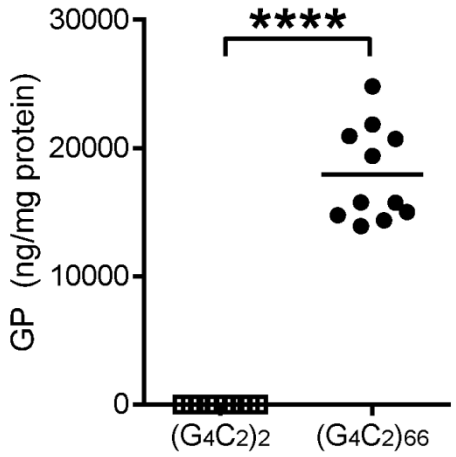

Fig. S1. Poly(GP) c9RAN proteins were detected by immunoassay in $(G_4C_2)_{66}$-mice. Poly(GP) levels in brain homogenates of 6 month old control $(G_4C_2)_2$-mice (n=12) and $(G_4C_2)_{66}$-mice (n=11) were evaluated by immunoassay. Poly(GP) proteins were specifically detected in brains of $(G_4C_2)_{66}$-mice but not $(G_4C_2)_2$-mice. ****$P<0.0001$, unpaired, two-tailed Student's *t*-test.

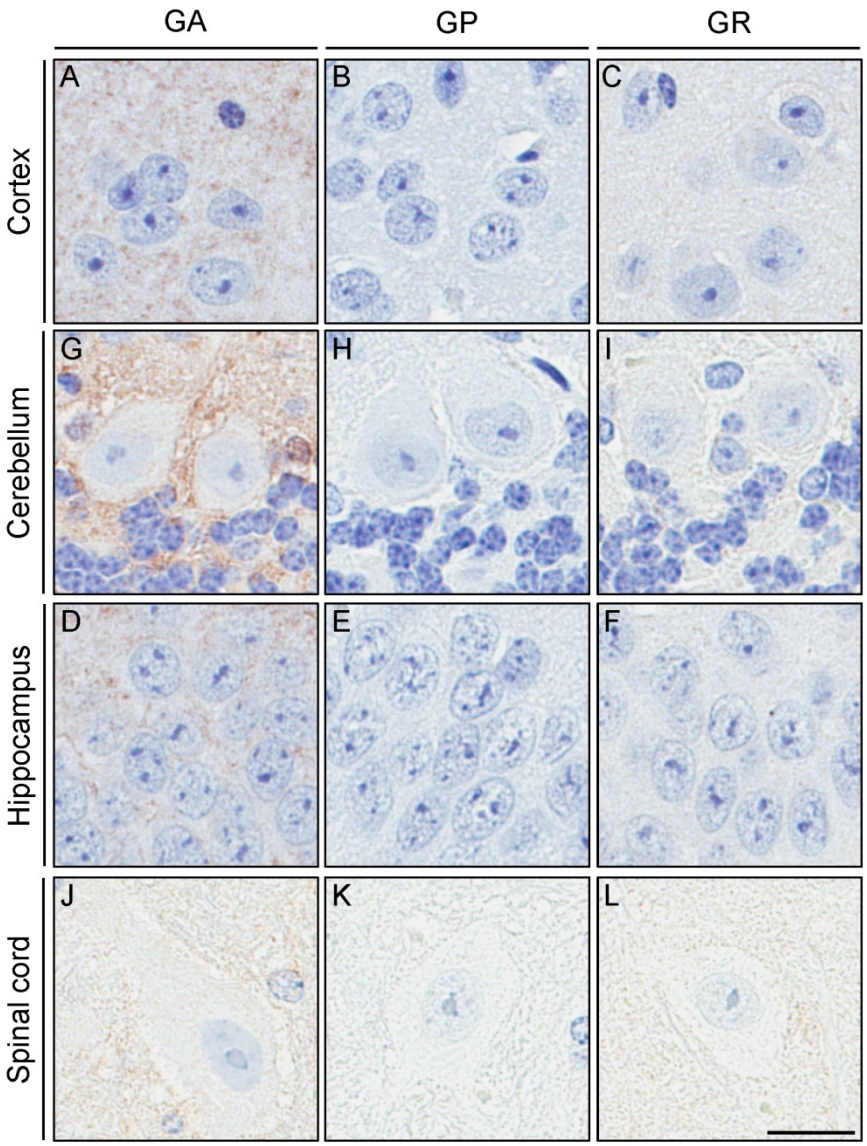

Fig. S2. c9RAN protein inclusions were not detected in the central nervous system of control $(G_4C_2)_2$-mice. Brain and spinal cord sections of 6 month old control mice expressing $(G_4C_2)_2$ (n=6) were immunostained with antibodies for the detection of poly(GA), poly(GP) or poly(GR) c9RAN proteins. No c9RAN proteins were detected. Shown are representative images of cortex (**A-C**), cerebellum (**D-F**), hippocampus (**G-I**) and spinal cord (**J-L**). Scale bar: 20 µm.

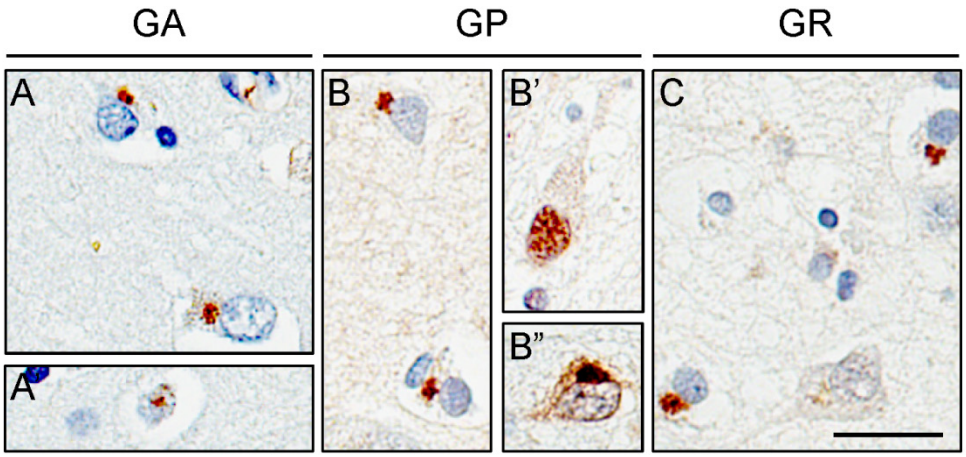

Fig. S3. c9RAN protein immunostaining in c9FTD/ALS patient cortex. Sections of c9FTD/ALS patient frontal cortex were subjected to immunohistochemical analyses for poly(GA) (**A, A'**), poly(GP) (**B-B''**) or poly(GR) (**C**). Scale bars: 20 µm.

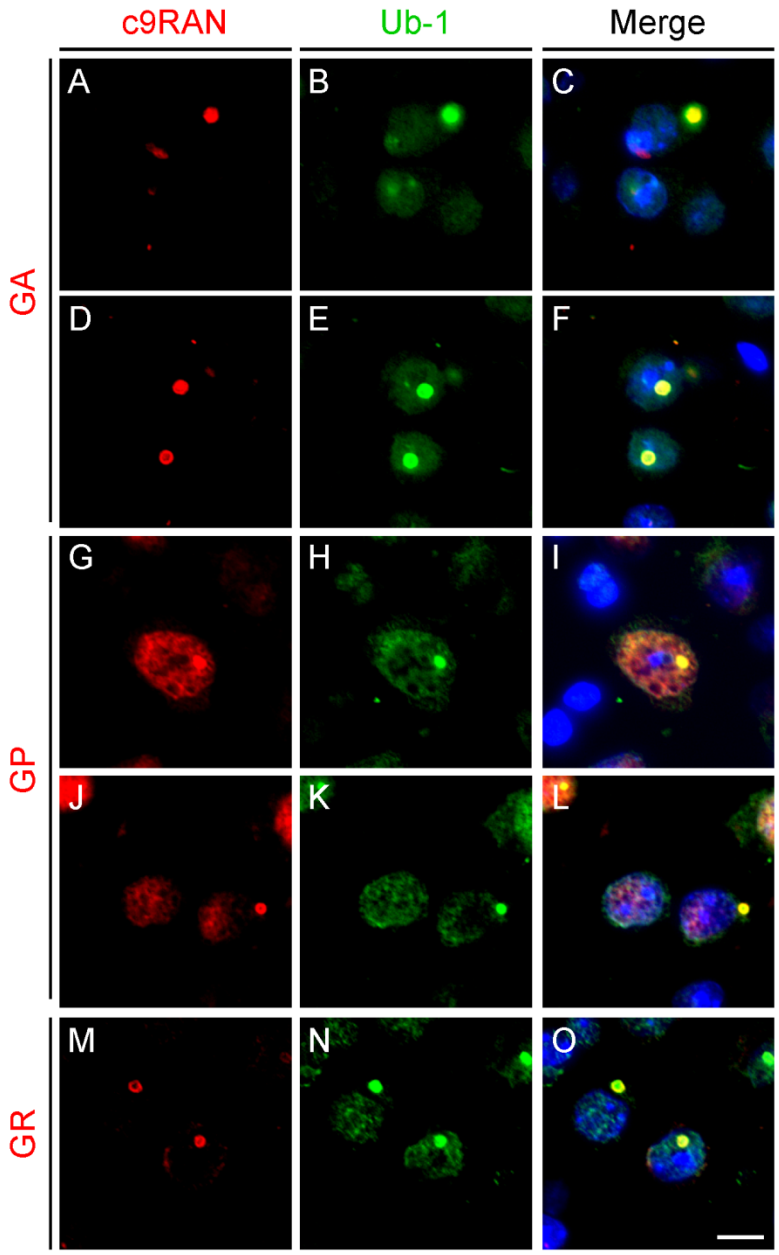

Fig. S4. c9RAN protein inclusions in the cortex of $(G_4C_2)_{66}$-mice were ubiquitin-positive. Co-immunofluorescence staining for poly(GA), poly(GP) or poly(GR) proteins and ubiquitin-1 were carried-out to determine whether c9RAN protein inclusions were ubiquitin-positive. The majority of intranuclear and cytoplasmic inclusions composed of poly(GA) (**A-F**), poly(GP) (**G-L**) or poly(GR) (**J-O**) observed throughout the central nervous system of 6 month old $(G_4C_2)_{66}$-mice were immunopositive for ubiquitin. Scale bar: 10 µm.

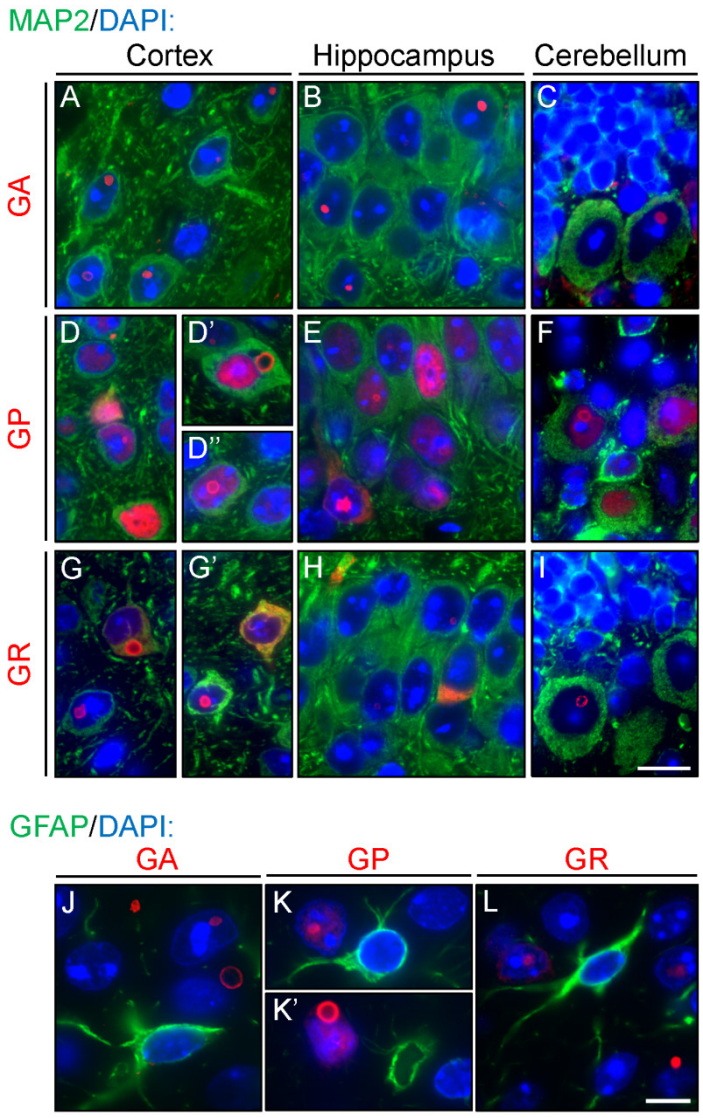

Fig. S5. c9RAN protein inclusions predominantly localized to neurons in the central nervous system of $(G_4C_2)_{66}$-mice. (**A-I**) Intranuclear and cytoplasmic inclusions composed of c9RAN proteins were observed in cells positive for the neuronal marker, MAP2, in 6 month old $(G_4C_2)_{66}$-mice. Poly(GA) (**A-C**), poly(GP) (**D-F**), and poly(GR) (**G-I**) inclusions present in MAP2-positive cells were observed in the cortex, hippocampus and cerebellum. (**J-L**) In contrast, poly(GA) (**J**), poly(GP) (**K** and **K'**), and poly(GR) (**L**) inclusions were rarely detected in GFAP-positive astrocytes, as shown in these representative images of the cortex. Scale bar: 10 μm.

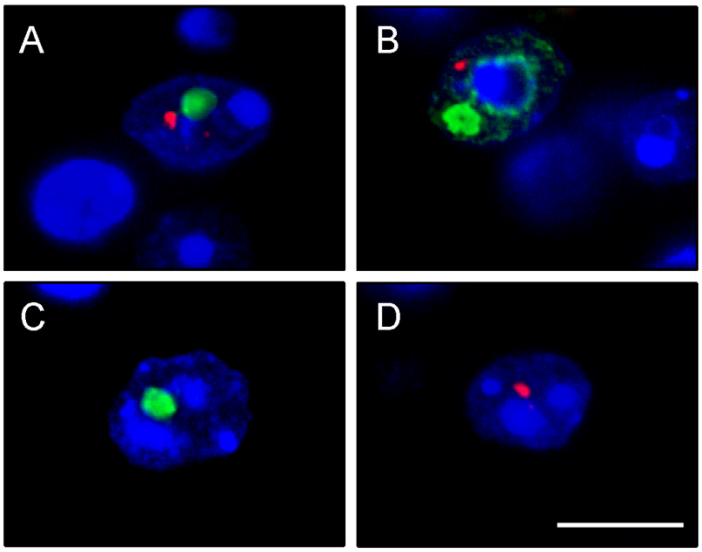

Fig. S6. Poly(GP) and foci were frequently observed within the same cell in the cortex of 6 month old $(G_4C_2)_{66}$-mice. (A-D) RNA FISH followed by immunofluorescence staining for poly(GP) was carried-out on $(G_4C_2)_{66}$-mouse brain sections (n=3). Of 150 cortical cells immunopositive for poly(GP) inclusions, ~70% also contained at least one RNA focus. Scale bar: 10 µm.

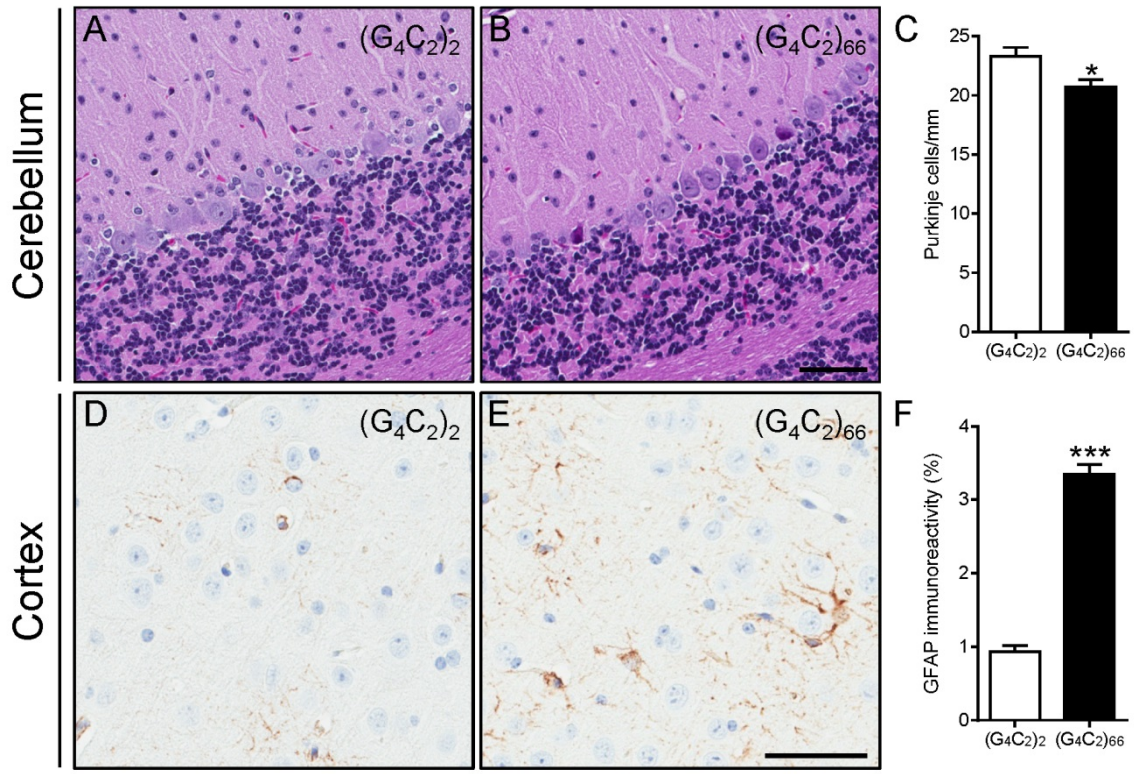

Fig. S7. $(G_4C_2)_{66}$-mice developed Purkinje cell loss and astrogliosis. (**A, B**) Representative images of hematoxylin and eosin staining of the cerebellum of control $(G_4C_2)_2$-mice and $(G_4C_2)_{66}$-mice at 6 months of age. (**C**) The number of Purkinje cells per 10 mm of the Purkinje layer in the cerebellum of each mouse was counted [$(G_4C_2)_2$-mice, n=12; $(G_4C_2)_{66}$-mice, n=11], and the average number of Purkinje cells/mm was calculated for each group. This revealed a statistically significant decrease of 11% in $(G_4C_2)_{66}$-mice. (**D, E**) Representative images of GFAP immunostaining in the cortex of control $(G_4C_2)_2$-mice and $(G_4C_2)_{66}$-mice at 6 months of age. (**F**) Quantification of the percent burden of GFAP staining in the whole cortex revealed a significant increase in GFAP burden in $(G_4C_2)_{66}$-mice (n=6) compared to $(G_4C_2)_2$-mice (n=6). Data are presented as mean ± S.E.M. *$P<0.05$, ***$P<0.001$, unpaired, two-tailed Student's *t*-test. Scale bars: 60 μm.

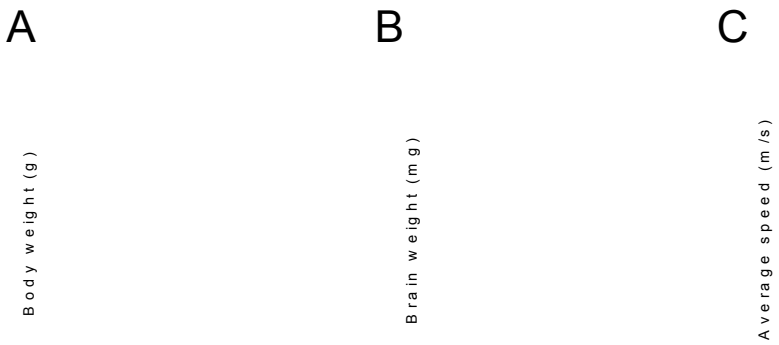

Fig. S8. $(G_4C_2)_{66}$-mice exhibited a decrease in brain weight and increased speed in the open field assay. (**A, B**) Brain and body weight of 6 month old control $(G_4C_2)_2$-mice (n=12: 6 males, 6 females) and $(G_4C_2)_{66}$-mice (n=11: 6 males, 5 females). (**A**) No significant changes were observed in the body weight between males expressing either 2 or 66 G_4C_2 repeats, however a decrease in body weight was observed in females expressing $(G_4C_2)_{66}$ compared to females expressing $(G_4C_2)_2$. (**B**) A significant decrease in the brain weight of $(G_4C_2)_{66}$ mice was observed suggestive of brain atrophy. (**C**) At 6 months of age, $(G_4C_2)_{66}$-mice had an increased average speed in the open field assay compared to $(G_4C_2)_2$-mice, indicative of hyperactivity (see also Fig. 4). Data are presented as mean ± S.E.M. ***$P<0.001$, *$P<0.05$, unpaired, two-tailed Student's *t*-test.

Table S1. Semi-quantitative analysis of RNA foci and c9RAN proteins in various regions of the central nervous system of 6 month old $(G_4C_2)_{66}$-mice (n=6)

		c9RAN proteins			
	Foci	GA	GP	GR	pTDP-43
Cortex	+++	+++	+++	+	+
Hippocampus					
CA1-CA3	++	+++	++	+	+
Dentate gyrus	+	+	+	+	-
Cerebellum					
Purkinje cell layer	++	+	+	+/-	-
Molecular cell layer	+/-	+/-	+/-	+/-	-
Granule cell layer	+/-	+/-	+/-	+/-	-
Thalamus	++	+++	++	++	n/a
Amygdala	+/-	+/-	+/-	-	n/a
Spinal cord	+	+	+	+/-	n/a

+++ High, ++ Moderate, + Low, +/- Rare, n/a: not assessed