Reduction of oxidative stress suppresses poly-GR mediated toxicity in zebrafish embryos

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Summary statement: Toxicity of C9ALS/FTD poly-GR in zebrafish embryos was suppressed by Trolox (reactive oxygen species inhibitor) and can be detected and quantified in protein homogenates in our model and in the frontal cortex of C9FTD/ALS cases.

Abstract
The hexanucleotide repeat expansion (G₄C₂) in the C9ORF72 gene is the most common pathogenic cause of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS). This repeat expansion can be translated into dipeptide repeat proteins (DPRs) and distribution of the poly-GR DPR correlates with neurodegeneration in postmortem C9FTD/ALS brains. Here, we assessed poly-GR toxicity in zebrafish...
embryos, using an annexin A5-based fluorescent transgenic line (secA5) that allows for detection and quantification of apoptosis in vivo. Microinjection of RNA encoding poly-GR into fertilized oocytes evoked apoptosis in the brain and abnormal motor neuron morphology in the trunk of 1-4 days post fertilization (dpf) embryos. Poly-GR can be specifically detected in protein homogenates from injected zebrafish and in frontal cortexes of C9FTD/ALS cases. Poly-GR expression further elevated MitoSOX levels in zebrafish embryos, indicative of oxidative stress. Inhibition of reactive oxygen species using Trolox showed full suppression of poly-GR toxicity. Our study indicates that poly-GR can exert its toxicity via oxidative stress. This zebrafish model can be used to find suppressors of poly-GR toxicity and identify its molecular targets underlying neurodegeneration observed in C9FTD/ALS.

Keywords: C9orf72, ALS, FTD, poly-GR, neurodegeneration, oxidative stress

List of abbreviations:
FTD: frontotemporal dementia
ALS: amyotrophic lateral sclerosis
G₄C₂: hexanucleotide repeat expansion
DPRs: dipeptide repeat proteins
RAN: repeat-associated non-AUG
C9FTD/ALS: C9ORF72-linked FTD and ALS
Sec-A5: annexin A5-based
dpf: days post fertilization
hpf: hours post fertilization
poly-GR: poly-Glycine-Arginine
poly-GA: poly-glycine-alanine
poly-GP: poly-glycine-proline
poly-PR: poly-proline-arginine
poly-PA: poly-proline-alanine
poly-PG: poly-proline-glycine
ROS: reactive oxygen species
LCDs: low-complexity domains
ER: endoplasmic reticulum
NCT: nucleocytoplasmic transport
iPSCs: induced pluripotent stem cells
GRN: progranulin
MAPT: microtubule-associated protein tau
VCP: valosin-containing protein
PTU: Propylthiouracil
SV2: Synaptic Vesicle 2
ELISA: Enzyme Linked Immuno Sorbent Assay
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
α-BTX: α-Bungarotoxin
IF: immunofluorescent
DMSO: dimethyl sulfoxide

Introduction:
Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are two neurological disorders that are characterized by the degeneration of cortical neurons in the frontal and temporal cortices and motor neurons in the motor cortex and spinal cord [1]. The hexanucleotide (G_4C_2) repeat expansion in the C9ORF72 gene is a shared genetic factor that has been linked to both FTD and ALS [2,3]. Patients carrying repeat expansions can develop symptoms of both disorders [1]. The G_4C_2 repeat expansion can cause neurodegeneration via both loss- and gain-of-function mechanisms or a combination of both [4]. Methylation of the repeat and surrounding CpG islands can silence the C9ORF72 gene leading to haploinsufficiency [4]. Sense and antisense RNA containing the expanded repeat accumulate in RNA foci and sequester multiple RNA binding proteins [4]. Furthermore, DPRs are produced by unconventional repeat-associated non-AUG (RAN) translation of the repeat sequences in both sense and antisense direction, creating different DPRs: poly-glycine-alanine (GA), -glycine-proline (GP), -glycine-arginine (GR), -proline-arginine (PR), –proline-alanine (PA) and -proline-glycine (PG) [5-8]. These DPRs are found throughout postmortem brain tissue of patients [9], but only poly-GR pathology correlates with neurodegeneration [10-12].

Notably, the arginine-containing DPRs, poly-GR and –PR, have been reported to be very toxic in both cell and animal models [13-19]. Poly-GR and -PR interact with ribosomal proteins, heterochromatin, nucleolar proteins, RNA-binding proteins and
proteins containing low-complexity domains (LCDs) [18,20,16,17,19,21]. LCD proteins can form membrane-less organelles such as nucleoli, the nuclear pore and stress granules. Poly-GR and -PR have been shown to alter the dynamics and assembly of these organelles, leading to reduced mRNA translation, ribosomal stress, endoplasmic reticulum (ER) stress and aberrant nucleocytoplasmic transport (NCT) [22,23,18,24,19]. In addition, overexpression of either poly-GR and -PR resulted in reduced translation in NSC34 and HeLa cells [17,25], and in a mouse model for poly-GR [19]. Furthermore, poly-GR can interact with mitochondrial ribosomal proteins and consequently impair mitochondrial function in C9FTD/ALS iPSC-derived neurons and in a recent mouse model [26-28]. Finally, poly-GR increases the amount of oxidative stress which can cause DNA damage in motor neurons differentiated from iPSCs [26]. Importantly, since all these processes can influence each other and lead to general cellular malfunctioning, the primary start of the toxicity cascade is still unknown and warrants further investigation [23,29].

Multiple cell and animal models, including zebrafish [30], have been generated for C9FTD/ALS, many of which support DPR toxicity and a gain-of-function hypothesis (reviewed in [4,31,32,30]). New therapeutical strategies are under development, which depend on reliable in vivo drug screens [33]. However, not all C9orf72 animal models show a robust phenotype, including neurodegeneration, which is needed for quantifiable pharmaceutical outcome measures. Here, we generated a zebrafish model to quantify and screen suppressors of poly-GR toxicity in vivo. We choose zebrafish (Danio rerio) as vertebrate animal model. Zebrafish have been extensively used in (neuro)toxicity studies for multiple reasons [34,35]. Zebrafish embryos are transparent and develop externally, allowing for easy detection of body and organ abnormalities. Basic (neuro)biological processes are conserved between vertebrates, making identification of aberrant cellular and molecular pathways in the pathogenesis of human disorders feasible [34,36]. Finally, they are well-suited for drug screens because they readily take up small molecules from the water [35].

To address poly-GR toxicity in zebrafish embryos, we injected RNA encoding ATG-mediated codon-optimized 100xGR. High concentrations led to dead and malformed fish, while low concentrations of only 10pg poly-GR were sufficient to evoke apoptosis in the brain and caused aberrant motor neuron morphology at 1-4 days post
fertilization (dpf). In order to visualize and quantify apoptosis, we made use of transgenic zebrafish expressing fluorescently labeled Sec-A5 that labels apoptotic clusters with YFP [37]. This transgenic model allows screening for drugs that modify poly-GR toxicity, and as a proof of concept we identify Trolox as a suppressor of GR-toxicity. Trolox is an antioxidant that reduces reactive oxygen species (ROS), which are free radicals that can evoke oxidative stress and cause mitochondrial and DNA damage, both of which are linked to many neurodegenerative diseases [38]. In this study, we highlight the involvement of this cellular stress pathway in the pathogenesis of C9ORF72-linked ALS and FTD.

**Methods:**

*Sample collection*
FTD/ALS and non-demented control human brain sections were provided by the Dutch Brain Bank. Patients or relatives gave informed consent for autopsy and use of brain tissue for research purposes. Frozen frontal cortex samples consisted of 7 C9FTD/ALS patients, 2 GRN FTD, 1 MAPT FTD, 2 VCP FTD and 5 non-C9ORF72 non-demented controls. Patient information regarding age-of-onset and disease duration can be found in supplementary table 1.

*Constructs*
Plasmids containing the pcDNA3.1+Peredox-mCherry-NLS (addgene cat. Nr 32384) or pcDNA3.1+100xGlycine-Arginine (kind gift of Isaacs lab [39]) were transformed into Top10 competent cells followed by DNA isolation with NucleoBond®Xtra maxi kit (BioKé). Constructs were linearized by digestion with Apal (NEB) and/or NheI (NEB), purified by phenol-chloroform extraction and quantified on Nanodrop (Thermofisher). To obtain control constructs for injection of RNA we performed site directed mutagenesis to destroy the ATG initiation codon using the QuickChange Lightning Site-Directed mutagenesis Kit (ThermoFisher). The following primers were used to obtain the mutated TAG-stop codon: forward: 5’-CTCGTCCACG TCCCTAGGTGGGATCCG – 3’ reverse: 5’-GCTCGGATCCACCTAGGGACGTGGAC– 3’. To denature the DNA template and anneal the mutagenic primers containing the desired mutation we used a thermal cycling protocol (1x(2 min at 95°C), 18x (20sec at 95°C, 10 sec at 60°C, 3 min at 68°C), 1x (5 min at 68°C)). After mutant strand synthesis the template DNA was
digested by adding 1µl of the Dpn1 enzyme, mixtures were incubated for 5 minutes at 37°C. Mutated DNA constructs were transformed and isolated as described above.

**RNA synthesis**

For the production of RNA the mMessage mMachine T7 kit (Invitrogen) was used. 400 µg of linear template DNA was mixed with 2 µL reaction buffer, 10 µl NTP/CAP, 0.1 µl RNAse out and nuclease free water. The mixture was incubated at 37°C for 2 hours. After the incubation 1 µl TURBO DNase was added to remove template DNA at 37°C for 15 minutes. 30 µl lithium chloride and 30 µl nuclease free water was added for precipitation and mixtures were placed at -20°C for at least 30 minutes. RNA was subsequently centrifuged at 4°C, washed with 70% ethanol, re-centrifuged, dried to air and dissolved in DEPC water and quantified using Nanodrop (ThermoFisher). Samples were stored at -80°C in aliquots.

**Injections and fish maintenance**

For all experiments one-cell-stage zebrafish embryos (Danio rerio) of the wildtype (AB) or Sec-A5 YFP zebrafish reporter line [37] were injected in the yolk sac within 30 minutes after egg fertilization. No fish older than 4 days post fertilization were used and at this time zebrafish embryos are not sexually different. Injection mixtures contained a standard amount of 400 pg/nl mCherry-mRNA and 10% phenol red. The poly-GR mRNA was added to this mixture with a final concentration of 1 to 100 pg/nl. All zebrafish embryos were injected with 2 nl of injection mix. After injection, embryos were kept in a 28°C incubator for 1 till 4 days in E3-medium and on the first day with additional methylene blue as antifungal aid. Propylthiouracil (PTU) (1:40) was added to E3-media to prevent pigmentation and keep the fish optically transparent. At 24 hours post fertilization (hpf) the embryos underwent visual inspection for dysmorphic features. Morphologically abnormal embryos and unfertilized eggs were excluded from all further experiments. Embryos were selected at 1 dpf time point based on their mCherry signal intensity to select only correctly injected embryos. The non – mCherry fluorescent embryos and mosaic embryos were disposed. All experiments were approved by the local animal welfare committee.
**Apoptotic cluster count**

The apoptotic cluster quantification study was performed in the Sec-A5 YFP zebrafish reporter line [37] at 1 – 4 dpf with 3 separate injection rounds for each time point and minimal 10 fish per group per time point were imaged (total N is 30 per group per timepoint). Prior to imaging, embryos were dechorionated, anesthetized with 10% tricaine and embedded in 1.8% low melting point agarose. A Leica SP5 AOBS confocal microscope and an HCX L 20x NA 1.00 water dipping objective were used for the apoptotic cluster count. A z-stack step size of 4.2 micron was used ranging from the first till the last YFP+ cell on the Z-axis. Automatic quantification of apoptotic cells was done using Fiji software (free available at https://imagej.net/software/fiji/) with the 3D object counter application with automated threshold and a minimum of 3 voxols. Statistical analysis was done using Prism (https://www.graphpad.com). 1-way ANOVA with Bartlett’s test for equal variances showed a significant difference in the variance per group, so we performed a Kruskal-Wallis test that does not assume equal variances and post Dunn’s multiple comparison test to compare groups.

**TUNEL assay**

Wildtype AB strain fish injected with 10 pg poly-GR or only 400pg mCherry mRNA were used for TUNEL assay analysis. Fish were fixed at 2 dpf for 4h in 4% paraformaldehyde followed by washing in PBS. Subsequently, fish were washed and kept in 100% MeOH at -20°C until further processing. Rehydration was obtained by incubating fish in decreasing concentrations of MeOH: 75% - 50% - 25% MeOH followed by PBS. After washing in PBS-T (0.2% Triton X-100; Sigma in 0.1M PBS) fish were treated with 10µg/ml Proteinase K in PBS-T for 15 minutes and refixed with 4% paraformaldehyde for 20 min. After washing fish three times in PBS-T for 10 min, fish were incubated for 30 minutes with 100 µl TdT buffer (Click-iT Plus TUNEL Assay 647 dye; Invitrogen). Following initial incubation, fish were incubated overnight with 100 µl TdT reaction mix (94 µl TdT buffer; 2 µl EdUTP; 4 µl TdT enzyme). After incubation fish were washed with 3% bovine serum albumin (BSA; Sigma) in PBS-T three times followed by incubation with 100 µl Click-iT reaction mix for 3 h and washed three times with 3% BSA in PBS-T. Fish were mounted in 1.8% low melting point agarose (Invitrogen). Imaging was performed using a SP5
Intravital microscope equipped with HCX-APO L 20x objective (Leica). N is 9 fish per group.

**Synaptic Vesicle 2 whole mount staining**
At 48h post injection with 10pg ATG poly-GR, 10pg TAG poly-GR, or only 400 pg mCherry mRNA, wildtype AB zebrafish were fixed overnight in 4% paraformaldehyde. Fish were permeabilized with acetone for 1h at -20°C and blocked with 1% BSA / 1% DMSO / PBS-T (0.2% Triton X-100; Sigma in 0.1M PBS) for 1 h at room temperature. Subsequently, fish were stained for 30 min with 1µg/ml α-bungarotoxin-TRITC (Invitrogen), washed with PBS and incubated overnight at 4°C with the anti-mouse SV2 antibody (1:200, AB231587, developmental studies hybridoma bank, university of Iowa). After incubation fish were washed 10 times in 1.5% PBS-Triton X-100 (Sigma) and incubated overnight with the secondary antibody Anti-mouse-Cy5 (1:200, Sigma Aldrich). The following day the fish were washed 6 times in 1.5% PBS-Triton X-100 and mounted with 1.8% low melting point agarose (ThermoFisher). Imaging was done with Leica SP5 AOBS confocal microscope and an HCX L 20.0x1.00 water dipping objective. N is 10 fish per group.

The intensity of the SV2 staining was measured for n=5 independent fish per group, across 3 neurites per fish, and corrected for background fluorescence. We performed a one-tailed unpaired t-test with Welch correction.

**Fluorescent immunohistochemistry**
For immunohistochemical analysis, fish from the AB wildtype strain were injected with 10pg poly-GR or only 400pg mCherry mRNA. Fish were fixed at 1-4 dpf (n = 30 per group) overnight in 4% paraformaldehyde and subsequently embedded in paraffin. Tissues were cut into 6 µm thick sections using a rotary microtome. Sections were deparaffinized using xylene and rehydrated in an alcohol series (100%-96%-90%-80%-70%-50%). Antigen retrieval was done in 0.01 M sodium citrate, pH 6.0 using pressure cooker treatment. Endogenous peroxidase activity was blocked with 3% H₂O₂ and 1,25% sodiumazide in 0.1 M PBS. Immunostaining was performed overnight at 4°C in PSB block buffer (0.1 M PBS / 0.5% protifar / 0.15% glycine) and with primary antibodies (anti-GR 1:5000 LifeTein, anti-yH2AX 1:750 GeneTEX) at 4°C. After incubation with the primary antibody, sections were washed with PBS block buffer and incubated with secondary anti-mouse/rabbit Cy2/3 linked antibodies.
(1:500, Jackson). To remove background staining, a 10 min incubation with Sudan Black (Sigma, 0.1 gr in 100ml 70% ethanol, filtered) was done. To visualize nuclei, slides were incubated for 10 min with Hoechst 33342 (Invitrogen). Slides were mounted with ProLongGold (Invitrogen) and kept at 4°C until imaging at a Zeiss LSM700 Confocal microscope with the 40x and 63x lens.

**Protein isolation from frozen frontal cortex**

Prior to lysing, frontal cortex samples were thawed on ice and supplied with RIPA buffer containing 0.05% protease inhibitors (Roche) and 0.3% 1M DTT (Invitrogen). Samples were mechanically lysed, followed by 30 min incubation on ice. After 30 min incubation, mechanical lysing was repeated and samples were centrifuged for 20 min at 4°C, followed by 3x 1 min sonication. After sonication, samples were centrifuged for 20 min at 4°C and the supernatant was used for ELISA. Whole protein content was determined using BCA assay (Thermo Fisher Scientific). The pellet was incubated at 95°C for 2.5 hours in 150 µl 20% SDS with every 30 min 5 sec vortexing at max intensity. After incubation, samples were added to 500 µl with 2% SDS and run on ELISA.

**Enzyme Linked Immuno Sorbent Assay (ELISA)**

Poly-GR sandwich ELISA was performed at 1 – 4 dpf with 3 separate experiments for each time point and 30 fish per group per time point. Prior to lysing, fish were dechorionated and euthanized. Mechanical lysis of fish was performed in RIPA buffer containing 0.05% protease inhibitors (Roche) and 0.3% 1 M DTT (Invitrogen). After 30 min incubation, mechanical lysis was repeated and samples were centrifuged at 13.000xg for 15 min at 4°C, followed by 3x 1 min sonication. After sonication, samples were centrifuged at 13.000xg for 20 min at 4°C. Whole protein content was determined using BCA assay (Thermo Fisher Scientific). MaxiSorp 96 well F-bottom plates (Thermo Fisher) were coated for 2h with 5.0 µg/ml monoclonal GR antibody (LifeTein Services) followed by overnight blocking with 1% BSA in PBS-Tween (0.05% Tween-20 in 0.1M PBS) at 4°C. After washing, samples were added at 300µg total protein in one well and 2-fold diluted in PBS in a second well. Standard curve made with 15x GR synthetic peptide was added in duplo. All samples were incubated for 1h on the plate. After washing, all wells were incubated for 1h with biotinylated monoclonal anti-GR antibody (LifeTein) at a final concentration of 0.25 µg/ml in
PBS-Tween/1% BSA. After washing, samples were incubated for 20 min with Streptavidin-HRP conjugate (R&D Sciences) diluted 1:200 in PBS-Tween/1% BSA. Following extensive washing, samples were incubated with substrate reaction mix (R&D Sciences) for 15 min and stopped using 2 N H$_2$SO$_4$. Read-out was performed at 450nm and 570nm (Varioskan). We performed a two-way ANOVA with post Bonferroni test for differences between groups over time.

**Rescue experiments**

Trolox (Sigma) was dissolved in DMSO and diluted in E3 egg medium to a final concentration of 50μM. Dissolved Trolox or similar volumes of DMSO as a control were added to the egg water directly after micro-injections and refreshed every day. The number of apoptotic clusters was determined at 1-4 dpf as described above in Sec-A5 YFP zebrafish (n=30 per experiment from 3 independent experiments). 1-way ANOVA with Barlett’s test for equal variances showed a significant difference in the variance per group, so we performed a Kruskal-Wallis test that does not assume equal variances and post Dunn’s multiple comparison test to compare groups.

**MitoSOX Assay**

Wildtype AB strain fish injected with 10pg poly-GR or only 400pg mCherry mRNA were used for MitoSOX (Invitrogen) assay analysis. As a positive control for mitochondrial stress, wildtype AB strain fish at 2 dpf treated with 100μM H$_2$O$_2$ were used for MitoSOX (Invitrogen) assay analyses. Fish were collected and sorted into groups of 10-15 fish. Subsequently, fish were dechorionated and washed once with HBSS (HBSS Calcium Magnesium; Gibco). Fish were incubated with 100μM H$_2$O$_2$ in HBSS for 20 min at 28°C to induce stress. Following H$_2$O$_2$ incubation, all fish were washed 3 times with warmed HBSS media (28°C). For MitoSOX staining all fish were dechorionated and washed once with pre-warmed HBSS (28°C) and incubated with 400μl of 5μM MitoSOX stock reagent diluted in HBSS at 28°C for 20 min. Following MitoSOX incubation, all fish were washed 3 times with warmed HBSS media (28°C) and anesthetized with 1x Tricaine for 5 min. Fish were mounted in 1.8% low melting point agarose (Invitrogen). Imaging was performed using a SP5 Intravital microscope equipped with HCX-APO L 20x/100W objective (Leica). F-test for equal variances was p=0.914 for mCherry vs poly-GR, so variances are not significantly different and we performed a students’ t-test to test for differences. For
poly-GR with DMSO vs poly-GR with Trolox the F-test was p=0.045 so variances are significantly different, so we proceeded with a Student’s t-test with Welch correction.

Q-PCR

Transcript levels of poly-GR mRNA in injected zebrafish embryos were determined using SYBR Green fluorescence (iTaq Universal SYBR Supermix; BioRad). Wildtype AB strain fish un-injected controls or injected with 10pg poly-GR were used, with n = 30 fish per group. All samples were run in triplo. Primers for the poly-GR mRNA: FW primer: 5’- CCAAGCTGGCTAGCGTTTA -3’, and RV primer: 5’- TACCTCGTCCACGTCCCAT-3’ and β-actin gene: FW primer: 5’- GCTGTTTTC CCCTCCATTGTT – 3’; RV primer: 5’- TCCCATGCCAACCATCACT – 3’. Analysis was performed using the 2-ΔΔCt method with data normalized to β-actin.

Results:

Injection of poly-GR encoding RNA evokes apoptosis and aberrant motor neuron axon morphology in 1-4 dpf zebrafish embryos.

We started with assessing the toxicity of poly-GR at the systemic level. RNA was transcribed in vitro from an ATG-100xGR construct, using alternative codons without the G₄C₂ repeat sequence. This allows for testing poly-GR DPR toxicity independent of possible RNA toxicity caused by pure G₄C₂ repeats. A concentration series ranging from 1-200pg RNA was injected into the yolk-sac of fertilized zebrafish oocytes at the one-cell stage and showed a dose-dependent toxicity (supplementary figure 1), with high concentrations leading to dead and undeveloped embryos and lower concentrations leading to malformations including heart edema and bent tails. For further experiments we chose a dose of 10pg poly-GR encoding RNA that resulted in a robust and reproducible phenotype without causing severe malformations. 10pg of poly-GR injections still resulted in 30% of injected embryos with visible deformity (including heart edema and bent tails), but only those with lack of visible deformity were utilized. Lower concentrations did not show a clear and reproducible phenotype on apoptotic cluster count (data not shown). To determine the effect of poly-GR at the cellular level, we used the Sec-A5 reporter zebrafish line that fluorescently labels apoptotic clusters in vivo by YFP [37]. Cells labeled with secA5-YFP are apoptotic and exhibit several hallmarks of apoptotic cells, including DNA fragmentation, nuclear condensation, altered morphology, and loss of membrane asymmetry [37]. As
Injections by themselves already cause a slight increase of cell death, we used RNA from an mCherry-only construct as control. This marker was also used to select for correctly injected fish. Co-injection of mCherry RNA together with low concentrations of poly-GR encoding RNA evoked a 1.5-2.5-fold increase in apoptosis in zebrafish embryos at all stages (1-4 dpf) (figure 1A upper panel). The increase in apoptosis was confirmed by a TUNEL staining (figure 1A lower panel). To quantify the number of apoptotic clusters, we imaged zebrafish heads in vivo and automatically quantified the YFP-positive clusters in z-stack images. 10pg of poly-GR RNA was sufficient to cause a significant increase in the number of apoptotic clusters in the brain of zebrafish embryos at 1-4 dpf compared to mCherry control injected fish (figure 1B, Kruskal-Wallis test with post Dunn’s multiple comparison test p = < 0.0001) without generating other gross morphological abnormalities nor affecting viability. TUNEL quantification also showed a significant difference between poly-GR injected fish versus mCherry only (supplementary figure 2). To further characterize the effect of poly-GR overexpression, we stained embryos for synaptic vesicle 2 (SV2) in combination with α-Bungarotoxin (α-BTX), to visualize axons and the neuromuscular junction. This revealed that poly-GR injected zebrafish embryos display aberrant neuromuscular morphology with fewer axonal protrusions of the motor neuron axonal structure in the trunk (figure 1C). SV2 intensity was significantly reduced in poly-GR injected embryos (figure 1D, one-tailed t-test with Welch correction, p = < 0.0001).

Injection of poly-GR RNA is translated in poly-GR peptide which remains detectable for at least 4 days in vivo.

To confirm the translation of injected RNA encoding poly-GR, peptides were visualized using immunofluorescent (IF) staining of whole-body zebrafish at 1-4dpf. Poly-GR peptides were detected as nuclear and perinuclear puncta in post-mortem frontal cortex of C9ORF72 FTD cases and in embryos injected with 10pg poly-GR but were absent in mCherry only injected embryos and in human frontal cortex from non-demented controls (figure 2A and supplementary figure 3). To quantify poly-GR peptide expression, we developed an Enzyme-Linked Immuno Sorbent Assay (ELISA) using a synthetic 15xGR peptide (LifeTein). This ELISA showed high specificity for poly-GR and did not show any signal for a 15xPR synthetic peptide (LifeTein) (supplementary figure 3). Next, we diluted the 15xGR peptide to make a
dose-response curve in the high and low range (supplementary figure 3), which revealed a sensitivity of 200pg/ml (supplementary figure 3). To further validate the ELISA, we isolated protein from the frontal cortex of seven C9FTD/ALS cases, five FTD cases due to other genetic causes (GRN, VCP or MAPT) and five non-demented controls (figure 2B). Poly-GR could only be detected in frontal cortex samples of C9FTD/ALS cases, illustrating the specificity of our assay (one-way ANOVA test p = 0.0024 with post Tukey’s test indicating a difference of the C9FTD/ALS group with all other groups). The calculated amount of poly-GR was on average 22.7 ng/ml in frontal cortices of C9FTD/ALS patients. Additional protein isolation using 20% SDS and 95°C incubation to dissolve more poly-GR from the insoluble fraction only yielded 1.28 ng/ml extra poly-GR (data not shown). Zebrafish embryos injected with 10 pg of RNA encoding poly-GR showed a signal of 10-20 pg/ml in the ELISA that was significantly different from Cherry-only injected fish (2-way-ANOVA p = 0.0003) and remained high for 1-4 days after injection (figure 2C), while poly-GR mRNA was mostly gone after 2dpf (supplementary figure 3F). Together, the ELISA data confirmed that our poly-GR zebrafish model mimics physiological poly-GR levels observed in C9FTD/ALS patient material.

To further study the specificity of the observed protein-toxicity, we generated a translation-defective 100xGR construct by mutating the ATG-start codon into a TAG-stop codon. This construct prevents generation of poly-GR production and can be considered as a control for RNA toxicity per se. We confirmed that this TAG-100xGR did not produce any poly-GR peptides using our ELISA for poly-GR (supplementary figure 3). Injection of high amounts (50-200pg) of RNA of the TAG-100xGR construct caused an increase in dead and malformed embryos (supplementary figure 1). Apparently, high concentration of non-coding RNA can have a small toxic effect on its own, even though this RNA does not contain a G\textsubscript{4}C\textsubscript{2} repeat sequence. Low amounts (2-10pg) of TAG-mediated poly-GR RNA was only slightly toxic compared to mCherry only (supplementary figure 1), while low amounts of ATG-mediated poly-GR RNA evoked abundant apoptosis (figure 1). In summary, injection of poly-GR RNA is translated into poly-GR peptides that present as (peri)nuclear puncta throughout the zebrafish body and remain detectable for at least 4 days. Expression of poly-GR peptides causes abundant apoptosis in the developing zebrafish.
Inhibition of oxidative stress rescues poly-GR mediated toxicity in vivo.

Poly-GR peptides have been shown to disturb many cellular processes and pathways [4,33,32], but their primary target is still unknown. To discriminate between primary and secondary effects, we used a pharmacological approach. In C9ORF72 patient iPSC-derived motor neurons that express both poly-GR and -PR, decreased cell survival is correlated with dysfunction in Ca^{2+} homeostasis, increased ER stress, and reduced mitochondrial membrane potential [27]. Trolox, an antioxidant that reduces ROS, partially rescued toxicity in an iPSC-induced motor neuron model of C9FTD/ALS [26]. In our in vivo study, Trolox significantly reduced poly-GR-mediated apoptosis in Sec-A5 transgenic embryos (figure 3A and B) (Kruskal-Wallis test p = <0.0001. Post Dunn’s multiple comparison test *** = p < 0.0001 difference between DMSO vs Trolox treated fish at 2-4dpf). Quantification of TUNEL staining also showed a reduction of apoptotic cells in zebrafish embryos treated with Trolox (figure 3A lower panel and supplementary figure 2 one-way ANOVA p=<0.0001. Post Tukey’s test shows a significant difference between poly-GR injected fish treated with Trolox compared to DMSO). As Trolox is known to inhibit the formation of ROS, we used a whole-mount protocol to quantify the ROS in alive zebrafish by MitoSOX staining. As pigmented cells are highly reactive to this assay, hydrogen peroxide was used as positive control to distinguish specific signal (supplementary figure 4). Indeed, 10pg poly-GR injected fish showed an increase in MitoSOX staining compared to mCherry-only injected fish (figure 3C and D and supplementary figure 4). Trolox is dissolved in DMSO, so we treated poly-GR injected fish with Trolox or equal amount of DMSO and found that DMSO increased the amount of ROS even further, which was subsequently reduced by Trolox (figure 3C and E). Surprisingly, SecA5 and TUNEL apoptotic cell counts in Trolox treated embryos were reduced to baseline level of mCherry-only injections, consistent with complete suppression of poly-GR toxicity by Trolox.

Discussion:

In this study, we use zebrafish embryos to visualize and quantify apoptosis in brain tissue evoked by injection of RNA encoding poly-GR. Poly-GR peptides were detectable as small (peri)nuclear puncta throughout the zebrafish body and their expression level slowly increased form 10-20ng/ml over 4 days as quantified by ELISA, which mimics physiological levels seen in C9FTD/ALS patients. Poly-GR
evoked formation of ROS, which we were able to suppress using Trolox, an inhibitor of oxidative stress. Our study indicates the importance of this specific cellular stress pathway in the toxicity of poly-GR in vivo, and potentially also in the pathogenesis of C9FTD/ALS.

Our model only focusses on toxicity induced by a single DPR and does not take into account the effects of G₄C₂ repeat RNA-toxicity, haploinsufficiency of the normal C9ORF72 protein or simultaneous expression of different DPRs. The construct is ATG-mediated and uses alternative codons to encode DPRs to circumvent G₄C₂-RNA toxicity, and as a control we used an ATG-mutated translation deficient construct to control for toxicity mediated by RNA alone. Injection of high RNA concentrations of this TAG-construct was toxic in embryos, showing that high expression of RNA molecules per se has a toxic effect. The TAG-construct used in our study does not give any information on RNA toxicity of the pure G₄C₂ repeat structure. Thus, our model only provides information about poly-GR toxicity, which was clearly higher than the sole effect of injections of the TAG-construct.

In this study we show that poly-GR can be specifically detected in brain sections and protein isolates from frontal cortex of C9FTD/ALS cases using a monoclonal antibody against poly-GR. This antibody is able to recognize both the cytoplasmic form of poly-GR and (peri)nuclear poly-GR inclusions in post-mortem brain tissue (supplementary figure 3). After injections, poly-GR mRNA is present for 2 days and thereafter decreases (as expected for transient injections), but the amount of poly-GR protein slowly increases until 4dpf. We think that poly-GR peptides that have formed in the previous days are more stable over time (the half-life of the poly-GR protein is longer than that of the mRNA). Possible aggregation of poly-GR might also affect the ELISA read-out. In our injected zebrafish, poly-GR was mainly detected as nuclear puncta in the brain, while the observed poly-GR pathology in post-mortem brain tissue of C9FTD/ALS patients mainly consists of cytoplasmic and perinuclear aggregation. Interestingly, solubility of DPRs can differ per brain region, and soluble DPRs are less abundant in clinically affected areas [12]. Arginine methylation of poly-GR may also influence its cellular targets and might affect disease course [40]. Furthermore, poly-GR aggregation can be influenced by co-expression of poly-GA [41], that is not expressed in our model. For poly-GA, toxicity was directly linked to
its cytoplasmic aggregation in a mouse model and neuronal cell culture [42]. For poly-GR, cytoplasmic aggregation does not seem to be necessary to exert its toxic effect in our model and in published mouse models [19,28], making this antibody and zebrafish model applicable to study toxicity of both aggregated and soluble poly-GR forms.

In the adult brain, apoptosis is linked to neurodegeneration [43] and signs of apoptosis have been shown in spinal cord motor neurons of ALS patients [44,45] and in neurons and astrocytes in brain tissue from FTD patients [46]. We observed increased apoptosis in zebrafish brain tissue upon expression of poly-GR. The Sec-A5 quantification in our model was higher than the TUNEL quantification, possibly because Sec-A5 labels the phospholipid phosphatidylserine (PS) layer that is exposed early during apoptosis. In the final stages of apoptosis where DNA fragmentation is greatest, apoptotic cells lack a defined cell membrane and nuclear architecture and debris of multiple secA5-YFP+ cells can be observed near high-intensity TUNEL staining, making it possible to count more earlier apoptotic YPF+ cells than late apoptotic TUNEL+ cells [37]. Both quantifications showed a clear increase in poly-GR injected zebrafish, confirming the toxicity of this peptide. Next to apoptosis in brain tissue, overexpression of DPRs in zebrafish models has been reported to cause motor axon outgrowth defects such as shorter axons and aberrant branching [15,47,48]. Our study confirms these findings and shows similarities to motor axonal phenotypes in zebrafish models for other genetic causes for FTD and ALS [49,50]. Interestingly, overexpression of poly-GA did evoke toxicity but no defects in motor neurons or motility in zebrafish [51,47], indicating that this phenotype cannot be generalized to all DPRs.

Our results suggest that inhibition of oxidative stress can suppress poly-GR toxicity. We were surprised to find a full rescue of poly-GR toxicity, indicating a primary or central role of oxidative stress in the pathogenesis evoked by poly-GR. Poly-GR can probably evoke oxidative stress via multiple ways. A recent poly-GR mouse model confirmed direct poly-GR binding to ATP5A1, a subunit of mitochondrial respiratory chain complex V [28], and poly-GR can act as a mitochondria-targeting signal [52]. In drosophila, poly-GR impairs mitochondrial inner membrane structure, ion homeostasis, mitochondrial metabolism, and muscle integrity [53]. Mitochondrial
and/or oxidative stress can worsen ER stress by reducing the efficiency of protein folding pathways and thereby increasing the amount of misfolded proteins [54]. The other way around, ER stress can cause ROS production [55] and disrupt the membrane of mitochondria, since the two organelles are interconnected [56]. Recent reports on mouse models for poly-GR and –PR show a downregulation of genes involved in ribosome biogenesis [21] and poly-GR and –PR inhibit translation in cell culture [19,17,57,58]. Enhanced phosphorylation of eIF2α, increased levels of ER foldase PDIA1 and upregulation of CHOP have been found in postmortem brain sections of ALS patients [59-62], all indicating ER stress. Finally, our observed effect of Trolox mimics that of Edaravone, an FDA approved drug for ALS that is believed to act as a ROS scavenger and decrease the generation of ROS [63]. The effectivity of these drugs and the widespread ER stress indicators found in multiple ALS models and patient material all point towards the involvement of these pathways in the pathogenesis of C9FTD/ALS.

The ability of poly-GR to induce abundant apoptosis in the absence of the other DPRs, G4C2 repeat RNA toxicity and haploinsufficiency, illustrates the high toxicity of this specific DPR. To date, the effect of Trolox on the toxicity of DPRs has only been investigated in cell culture [26,23,64], and our results suggest Trolox can also rescue DPR toxicity in vivo. Previous studies indicate that poly-GR might share cellular targets with poly-PR, including mitochondrial and ribosomal proteins [26,27,57,17]. It would therefore be interesting to test whether Trolox can suppress both DPRs. Further screening experiments in poly-GR expressing zebrafish embryos could yield additional small molecule suppressors of poly-GR toxicity. Our study shows that reducing oxidative stress can suppress poly-GR toxicity in zebrafish embryos and indicates a possible role of oxidative stress in the neurodegeneration observed in C9FTD/ALS patients.

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Conflicts of interest/Competing interests:
The authors declare that they have no competing interests.

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Availability of data and material:
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Authors' contributions:
F.W.R. designed experiments, generated data, interpreted data, supervised the project, and wrote the manuscript. R.F.M.V., E.C.v.d.T., D.J.S., W.H.Q. and H.C.v.d.L. designed experiments, generated data, and interpreted data. T.J.v.H. designed experiments and substantively revised the manuscript. R.W. designed experiments, interpreted data, supervised the project, and substantively revised the manuscript. All authors approved the submitted version and have agreed to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.
FTD/ALS and non-demented control human brain sections were provided by the Dutch Brain Bank. Patients or relatives gave informed consent for autopsy and use of brain tissue for research purposes. As required by Dutch legislation, all animal experiments were approved in advance by the institutional Animal Welfare Committee (Erasmus MC, Rotterdam, The Netherlands).

References:


Figure 1: Injection of ATG poly-GR evokes apoptosis and aberrant motor neuron axon morphology in 1-4 dpf zebrafish embryos.

A) Max projection of z-stack images of the Sec-A5 fluorescent reporter line (green) embryos 48 hours after injection with 10pg ATG poly-GR, 10pg TAG poly-GR or 400pg mCherry only. Scale bar is 100 µm. B) Quantification of z-stack images of the Sec-A5 fluorescent reporter line embryos after injection with 10pg ATG poly-GR, 10pg TAG poly-GR or 400pg mCherry only at 1-4 days post fertilization (dpf). N=minimal 10 fish per group (mean ± s.e.m). Kruskal-Wallis test (p = <0.0001). Post Dunn’s multiple comparison test * = p <0.05, ** = p < 0.001 and *** = p < 0.0001 differences of ATG poly-GR injected fish compared to 10pg TAG poly-GR and mCherry only at 1-4 dpf. C) Synaptic vesicle (SV2) in combination with α-Bungarotoxin (BTX) staining visualizes aberrant axonal protrusions in the tail of 2dpf old wildtype AB embryos after injection with 10pg ATG poly-GR compared to 10pg TAG poly-GR and 400pg mCherry only. n = 10 per group (mean ± s.e.m). Scale bar is 100 µm. D) Fluorescence of SV2 staining was measured for 5 fish/group and 3 neurites per fish and was significantly reduced in 10pg ATG poly-GR injected embryos. One-tailed t-test with Welch correction p= < 0.0001.
Figure 2: Poly-GR peptides are detected as (peri)nuclear puncta in zebrafish embryos at 1-4 dpf. A) Immunofluorescence staining for poly-GR (red) in 2 dpf old wt AB embryos after injection with 10 pg poly-GR or 400pg mCherry only. N = 30 per group. C9FTD patient frontal cortex sections were used as positive control. Poly-GR peptides are detected as nuclear or perinuclear dots in all poly-GR injected fish (arrows). Nuclei were stained with Hoechst. Scale bars are 20 µm. B) poly-GR is detected in post-mortem frozen brain samples of n=7 C9ORF72 FTD/ALS cases but not in post-mortem frozen brain samples of FTD patients with mutations in Progranulin (GRN) n=2, Valosin-containing protein (VCP) n=2 or Microtubule-associated protein tau (MAPT) n=1 nor in brain samples of non-demented controls n=5 (mean ± s.e.m). One-way ANOVA test p= 0.0024 with post Tukey’s test indicating a difference of the C9ORF72 FTD/ALS group with all other groups. C) ELISA for the detection of poly-GR shows a signal of 10-20pg peptide in 1-4 dpf wt AB embryos injected with 10pg RNA encoding poly-GR. Two-way ANOVA p = 0.0003 with post Bonferroni test indicating that all time points were significantly different from mCherry-only injected fish. n=90 fish per group per time point divided over 3 independent experiments (30 fish per group x 3 experiments per time point).
Figure 3: Trolox reduces the number of apoptotic clusters and oxidative stress in zebrafish embryos injected with 10pg poly-GR. A) Upper panel: max projection of z-stack images of the Sec-A5 fluorescent reporter line embryos 48 hours after injection with 10pg poly-GR and treated with 50µM Trolox or DMSO only. A) Lower panel: max projection of TUNEL staining of the same treatment groups in wt AB fish
at 2dpf. Scale bars are 100 µm. B) Quantification of Sec-A5 z-stack images (mean ± s.e.m). N = 30 fish per group per day. Kruskal-Wallis test (p = <0.0001). Post Dunn’s multiple comparison test *** = p < 0.0003. C) MitoSOX red staining in 2 dpf old wildtype AB embryos after injection with 10pg poly-GR or 400pg mCherry only and treated with DMSO only or Trolox (dissolved in DMSO). MitoSOX signal in magenta is seen as clusters in the brain and in spinal cord in the tail. MitoSOX staining shows ROS reactivity in magenta in pigmented cells (arrowheads) but also along the tail and head (arrows). Scale bars are 100 µm. D) MitoSOX signal is significantly higher in 10pg poly-GR compared to mCherry only injected zebrafish embryo tails at 2dpf. N = 8 fish per group (mean ± s.e.m). Students’ t-test p=0.0002. F-test for equal variances p=0.914 so variances are not significantly different. E) MitoSOX signal is significantly reduced in 10pg poly-GR injected zebrafish embryo tails treated with Trolox versus DMSO only at 2dpf. N = 9 fish per group (mean ± s.e.m). F-test p=0.045 so variances are significantly different. Students’ t-test with Welch correction p=0.01. Note that H₂O₂ treated embryos as MitoSOX positive control experiments with an additional n = 8 fish per group are shown in supplementary figure 4.
Fig. S1. Concentration series of poly-GR ATG-mediated and TAG-mediated constructs. Constructs were injected in the yolk sac of fertilized oocytes in concentrations ranging from 1-200pg/nl. The amount of dead and malformed fish was assessed 24 hours after injection. Malformed embryos were defined as: severely undeveloped, having no head, a smaller head or only 1 eye. Fish with heart edema and/or tail twists were also scored as malformed. The average amount in mCherry injected fish is about 5% dead and only a few % malformed fish. 10pg poly-GR injections were used for all further analysis (arrows). All malformed fish were taken out of any further analysis. N = 50 fish per construct per concentration.
Fig. S2. Quantification of TUNEL assay at 2dpf. A) Max projection of z-stack images of TUNEL assay in wildtype AB embryos 48 hours after injection with 10pg poly-GR or 400pg mCherry only and treated with DMSO or Trolox (50µM, dissolved in DMSO). TUNEL positive signal is magenta, cell nuclei are stained with DAPI in blue. Scale bar is 50 µm. n = 10 per group. B) Quantification of TUNEL assay on wildtype AB embryos after injection with 10pg poly-GR or 400pg mCherry only, treated with 50µM Trolox or DMSO at 2 days post fertilization (dpf). N = 9 fish/group. 1-way ANOVA p=<0.0001. Post Tukey’s test shows a significant difference between poly-GR injected fish compared to mCherry and between poly-GR injected fish treated with Trolox compared to DMSO.
**Fig. S3. Validation of ELISA for poly-GR.** A) Poly-GR staining on C9FTD and non-demented control frontal cortex section shows poly-GR staining in the whole cell body or as perinuclear aggregate. Scale bars are 20µm. B) ELISA shows a positive selective signal for a synthetic 15xGR peptide over a synthetic 15xPR peptide. The limit of detection in the low range is 200pg. C) Dose-response curves for the synthetic 15xGR peptide in the low (0-3130pg) and high (1-12,500pg) range. D) ELISA signal development of a dilution series of protein samples isolated from wildtype AB embryos injected with 10pg poly-GR, 400pg of mCherry only or 10pg of the poly-GR stop construct. E) Quantification of poly-GR levels in mCherry only, poly-GR injected and poly-GR injected fish treated with Trolox. 1-way ANOVA with post Tukey test shows no significant difference between poly-GR peptide levels in Trolox treated embryos. F) Q-PCR for poly-GR mRNA levels in uninjected controls versus 10pg poly-GR injected fish.
Fig. S4. MitoSOX assay in poly-GR injected embryos resembles levels of 100µM H₂O₂ treated embryos. MitoSox staining shows ROS reactivity in magenta in pigmented cells (arrowheads) but also along the tail (arrows) of H₂O₂ and 10pg poly-GR injected fish. Scale bar is 100µm. B) Quantification of MitoSOX fluorescence signal. N=9 fish per group. 1-way ANOVA p = 0.0001 with post Tukey test indicating both poly-GR injected and H₂O₂ treated embryos are significantly higher than mCherry injected embryos. poly-GR injected vs H₂O₂ treated embryos don’t differ from each other.

Table S1. Patient characteristics

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* Disease duration has been estimated from the onset of first symptoms (retrospectively determined, not date of diagnosis) until death.