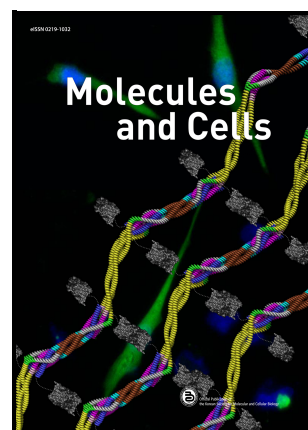


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A comparison study of pathological features and drug efficacy between *Drosophila* models of C9orf72 ALS/FTD

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease with a complex genetic basis, presenting both in familial and sporadic forms. The hexanucleotide (G₄C₂) repeat expansion in the C9orf72 gene, which triggers distinct

pathogenic mechanisms, has been identified as a major contributor to familial and sporadic ALS cases. Animal models have proven pivotal in understanding these mechanisms; however, discrepancies between models due to variable transgene sequence, expression levels, and toxicity profiles complicate the translation of findings. Herein, we provide a systematic comparison of seven publicly available *Drosophila* transgenes modeling the G₄C₂ expansion under uniform conditions, evaluating variations in their toxicity profiles. Further, we tested three previously characterized disease modifying drugs in selected lines to uncover discrepancies among the tested strains. Our study not only deepens our understanding of the *C9orf72* G₄C₂ mutations but also presents a framework for comparing constructs with minute structural differences. This work may be used to inform experimental designs to better model disease mechanisms and help guide the development of targeted interventions for neurodegenerative diseases, thus bridging the gap between model-based research and therapeutic application.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder that primarily affects motor neurons in the brain and spinal cord, resulting in muscle weakness, paralysis and eventual death (DeJesus-Hernandez et al., 2011). Central to ongoing research efforts is the determination to better comprehend the genetic contributors of ALS (Byrne et al., 2013; Kwiatkowski et al., 2009; Rosen et al., 1993; Shatunov et al., 2010). Although only 5-10% of ALS cases are familial (Majounie et al., 2012), a significant percentage of sporadic cases also present genetic cause (Van Daele et al., 2023), prompting extensive inquiries into unraveling the genetic underpinnings and complex pathogenesis of ALS.

One of the major breakthroughs in ALS research is the identification of the hexanucleotide (G₄C₂) repeat expansion in the *C9orf72* gene, which accounts for the largest proportion of familial ALS cases (DeJesus-Hernandez et al., 2011). The expansion results in reduced expression of the *C9orf72* gene (Shi et al., 2018) as well as the formation of RNA foci, which are accumulations of transcribed repeat RNA (Zu et al., 2013). These RNA foci are found to sequester RNA-binding proteins and disrupt RNA processing and splicing (Lee et al., 2013). Furthermore, the transcribed RNA from the G₄C₂ expansion forms stable G-quadruplex structures, which may modulate translation frequency (Conlon et al., 2016). Interestingly, the repeat expansion triggers an unusual repeat-associated non-AUG (RAN) translation, producing dipeptide repeat (DPR) proteins that are prone to aggregation and toxic to various cellular components (Ash et al., 2013). Notably, this same mutation is also observed in frontotemporal dementia (FTD) (Ash et al., 2013; Devenney et al., 2014), suggesting that advancements made in G₄C₂ pathogenesis could have broader implications for understanding and treating both ALS and FTD.

Given the complex characteristics of the G₄C₂ repeat expansion in the *C9orf72* gene, numerous animal models have been generated to accurately represent the disease (Batra & Lee, 2017). In fact, animal models expressing disease-associated transgenes

have been instrumental in elucidating the complexities of G₄C₂-mediated disease pathogenesis. These models enabled us to study how ALS or other G₄C₂-associated neurodegenerative diseases progress *in vivo* and discover potential therapeutic interventions. However, these transgenic animal models are not without limitations.

In many animal models, transgenes are usually overexpressed to a nonphysiological level due to the usage of strong promoters and the absence of regulatory sequences that are normally present in the untranslated regions (UTRs). This can lead to exaggerated phenotypical results, creating disparities between animal models and human disease. Importantly, minute differences in the transgene configuration can have consequential effects on the observed disease phenotype. For example, while most G₄C₂ transgene constructs use uninterrupted pure G₄C₂ repeats, the (G₄C₂)₄₄ construct includes an intronic 5' leader sequence (LDS), an attempt to more closely mimic the genetic configuration observed in ALS patients, leading to notable differences in DPR expression and toxicity (Goodman, Prudencio, Srinivasan, et al., 2019; Kearse et al., 2016). This diversity, while valuable for studying specific aspects of the disease, complicates the comparison and interpretation of results across studies. Further confounding the issue is the variability in toxic profiles, such as the expression of DPR(s), across models, which makes it difficult to delineate the relative contributions of each pathogenic mechanism and identify potential therapeutic targets.

Therefore, it is imperative to undertake an unbiased and systematic comparison of available transgenes of G₄C₂, assessing the toxic profiles and variations in transgene constructs. This endeavor not only enhances our understanding of C9orf72-G₄C₂ mutations but also establishes a framework for assessing constructs with minute structural differences that can have significant consequences. Such a framework is essential for informing experimental designs that accurately model disease mechanisms and for guiding the development of targeted therapeutic interventions. In this article, we contribute to this vital area by providing a comparative analysis of seven publicly available *Drosophila* transgenes that model the G₄C₂ expansion. We also establish a framework wherein we conducted phenotype testing under uniform conditions, ensuring that the transgenes are subjected to comparable environments, thereby enabling a more precise and controlled analysis of their toxicity profiles. Through our analysis, we aim to offer insights into the molecular consequences of variations in transgene constructs, and underscore the nuances that should be considered in future studies involving G₄C₂ expansion modeling. Ultimately, these comparative analyses and the framework developed may help underpin a more comprehensive understanding of the molecular mechanisms at play, which is fundamental for the advancement of targeted strategies in the management and treatment of neurodegenerative diseases.

MATERIALS AND METHODS

Drosophila stocks

All flies were maintained at 27°C and 25% humidity. The following lines were obtained from Bloomington *Drosophila* Stock Center (USA): *Elav-Gal4* (8765), *GMR-Gal4*

(1104), *UAS-luciferase* (35788), *UAS-(G₄C₂)₃₆* (58688), *UAS-LDS-(G₄C₂)₄₄.GR-GFP/TM3* (84723), *UAS-(G₄C₂)₄₉/TM6C* (84726), *UAS-(G₄C₂)₄₉* (84727). Using a transposon-based approach, the *GMR-Gal4* (III) was generated by relocating transgene from its original position on the 2nd chromosome to the 3rd chromosome. *UAS-(G₄C₂)₃₀/cyo* and *UAS-(G₄C₂)₃₀/Tm3* were provided by Peng Jin (Emory University, USA). *UAS-(G₄C₂)₁₆₀* line was provided by Fen-Biao Gao (University of Massachusetts, USA).

Molecular cloning and generation of transgenic flies

UAS-HA-Rhau was synthesized and subcloned into pACU2 vectors using NOTI and XbaI, and the HA epitope was added at the N-terminus. Transgenic flies were generated by BestGene, Inc. Nucleotide sequences are available upon request.

Immunohistochemistry (IHC) for fly brain

As previously described by Park et al. (Cho et al., 2022; Park et al., 2020; Ryu et al., 2022), adult flies were dissected in Schneider's insect medium (Cat No. S0146; Sigma, USA) to obtain brain samples for immunohistochemical analysis. Brains were fixed with 3.7% formaldehyde for 20 min at room temperature. After washing with PBST (0.3% Triton-X100 in phosphate-buffered saline), brains were incubated in a blocking buffer (5% Normal donkey serum in 0.3% PBST) for 1 h at room temperature (RT). Next, the brains were incubated in the primary antibody overnight at 4°C. The following primary antibodies were used in this study: α-GP rabbit (proteintech, 24494-I-AP) at 1:200 concentration, α-rRNA(Y10b) mouse (NOVUS, NB100-662) at 1:200 concentration, α-dTDP-Gly rabbit (gift by James Shen at Taipei Medical University) at 1:200 concentration. Brains were then washed with washing buffer three times for 10 min each. Then, brains were further incubated in the secondary antibody for 2 h at room temperature. The following secondary antibodies were used: α-rb alexa555 (Thermo Fisher Scientific, Z25305) at 1:200 concentration, α-rb alexa647 (Invitrogen, A21244) at 1:200 concentration, α-mouse alexa647 (Invitrogen, A21236) at 1:200 concentration, α-rb alexa488 (Abcam, A11034) at 1:200 concentration. Brains were washed five times with washing buffer prior to mounting on a slide glass for imaging. DAPI was used as a mounting medium. The posterior regions of mushroom body Kenyon cells were used for DPR analysis.

Protein extraction and western blotting

Total protein extraction and Western blot analysis was performed as previously described with minor modification (Park et al., 2020). 30 adult fly heads were suspended in lysis buffer (50 mM Tris-buffered saline (Tris-HCL) pH 7.5, 150 mM NaCl, 1% Triton ×100) containing protease inhibitor cocktail (Thermo Scientific, #87786; 1:100) and homogenized. 30 µg of lysate was mixed with Laemmli buffer (Bio-Rad, #161-0747) to β-mercaptoethanol (BIOSESANG, #60-24-2) and boiled at 95°C for 5 min. The lysates were loaded on 10% SDS-PAGE gel for 2 hours at 70 V and then transferred to the nitrocellulose membrane (Bio-Rad, BR170-4270) for 1 hour at 100 V. The membrane was incubated in blocking buffer [1X TBS (BIO-RAD, #1706435), 0.1 % Tween-20, and 5% skim milk] for 1 hour at room temperature (RT). Primary antibodies were used as follows: mouse anti-MAP Kinase (Sigma, M8159, 1:1000), rabbit anti-P-SAPK/JNK (Cell signaling, #4668S, 1:1000), rabbit anti-GRP78 (BiP) antibody (StressMarq Biosciences, SPC-180); and mouse anti-alpha tubulin (DSHB, E7-s, 1:2000). The secondary antibodies were used: goat anti-mouse-IgGk BP-HRP

(santa cruz, sc-516102, 1:2000); and goat anti-rabbit IgG, F(ab')₂-HRP (santa cruz, sc-3837, 1:2000). Lumigen ECL Ultra (Lumigen, TMA-6) was treated prior to detection at ChemiDoc™ XRS+.

RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

RNA extraction and cDNA synthesis of fly samples were performed as previously described (Chung et al., 2017; Lee et al., 2023; Lee et al., 2022). Total RNAs were extracted from adult fly heads or adult whole bodies using Easy-Blue system (iNtRON Biotechnology). cDNAs were synthesized from 3 µg of total RNAs using GoScript Reverse Transcription (Promega, A2791) according to the manufacturer's standard protocol. Quantitative PCR (qPCR) experiments were performed using the synthesized cDNAs with QuantiSpeed SYBR Green kit (PhilKorea, QS105-10) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Gene specific primers were listed in Supplementary Figure S5a (Tran et al., 2015; Xu et al., 2013). rp49 was used as a housekeeping gene when analyzing fly samples. mRNA fold change of each gene was calculated using the comparative Ct method. Gene-specific primers were designed with Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast) and *Drosophila* RNAi Screening Center (DRSC) FlyPrimerBank (flyrnai.org/flyprimerbank). Some primer sequences were obtained from published studies.

Conditions for acquiring and imaging eye phenotypes

Depending on the data, eye images of 1d, 7d or 10d adult flies expressing G4C₂ repeats under the control of *GMR-Gal4* at 25°C or 27°C and 25% humidity were acquired with a Nikon Eclips 90i.

Negative geotaxis assay

Right after eclosion from pupa, 15 flies of each genotype were collected and reared for, depending on the experiment, 3d or 10d at 27°C with 25% humidity. After the assigned days, these flies were transferred to an acrylic cylinder closed at one end (3-cm diameter, 18-cm height) without CO₂ anesthesia, while the top of the cylinder was sealed with sponges to block escape. After 20min acclimation at room temperature, the flies were placed on the bottom of the cylinder by tapping the cylinder against a table, and climbing was recorded for 1 min. Climbing ability was measured with a climbing index (proportion of 15 flies climbing >10 cm from the bottom of cylinder within 10 s) in each experimental group of flies.

Lifespan assay

Lifespan assay were performed with guidance from a previous study (Pradhan et al., 2023). In each experiment, at least 100 male flies of each genotype were collected within 24 h after pupal eclosion (APE). Flies were maintained at low density (15 males per wide vial) at 27°C on a 12-hr light/12-hr dark cycle and transferred to new vials every 2 days. At each transfer, the number of dead flies was counted. Lifespan results are shown as Kaplan-Meier survival curves. Statistical differences were assessed with the log-rank test.

Image acquisition and processing

All IHC samples were taken at 400x magnification using 40x water immersion objective, acquired by Zeiss LSM780 confocal microscopy (Zeiss, Germany) at 20°C. Kenyon cells in the fly brains were taken to detect DPRs' expression. After image acquisition,

maximum-intensity projections of each image were obtained using Zeiss Zen software, and additional image processing (pseudo-color filters or deconvolution) was applied in Adobe Photoshop CC. The posterior regions of mushroom body Kenyon cells were imaged with uniform conditions and the pixel intensity was taken using histogram analysis.

Statistical analyses

Statistical analysis was done using GraphPad Prism 7 (GraphPad Software, USA). Depending on the data, we applied Student's t-test, Log-rank(Mantel-Cox) test or one-way ANOVA followed by Tukey's post-hoc analysis. In all figures, n.s., *, **, ***, and **** represent $P > 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively. Error bars are SEM.

RESULTS

Characteristics of G₄C₂ constructs used in the study

Table 1. Characteristics of G₄C₂ constructs used in the study. For each line, the transgene information, distinguishing features, the presence of AUG codons, the chromosome in which the construct is inserted, the presence of RNA foci (literature based), the presence of dipeptide repeat (DPR) proteins (literature based), the observation of toxicity (literature based), and relevant references are provided.

Identification			Transgene information		Reference-based toxicity				
BL number	Transgene	Repeats	Distinguishing features	AUG codon	Inserted chromosome	RNA foci	DPR	Phenotype(s)	Reference(s)
na	(G ₄ C ₂) ₁₆₀	160 repeats	Intronic 160 G ₄ C ₂ repeat minigenes flanked by human intronic and exonic sequences	None	Chr 2.	Found (Tran et al., 2015)	Found GP (Tran et al., 2015)	Modest life-span toxicity at 29°C (Tran et al., 2015)	(Tran et al., 2015), (Yuva-Aydemir et al., 2019)
na	(G ₄ C ₂) _{30 (III)}	30 repeats	Uninterrupted pure repeats	None	Chr 3.	n.a	n.a	Locomotion defects	(Xu et al., 2013), (Dubey et al., 2022)
na	(G ₄ C ₂) _{30 (II)}	30 repeats		Eye degeneration (d14) (Xu et al., 2013)					
58688	(G ₄ C ₂) ₃₆	36 repeats	Uninterrupted pure repeats	None	Chr 2, 25C6, 2L:5108448.	Found (Mizielinska et al., 2014)	Found GR (Mizielinska et al., 2014)	Eye degeneration (d15) (Dubey et al., 2022)	(Mizielinska et al., 2014)
								Eye degeneration, Life-span, Egg-to-Adult viability (Mizielinska et al., 2014) [**strongly suggest the role of DPR toxicity]	

847 23	LD S- (G ₄ C ₂) 44	44 repeats	5' leader sequence (LDS) inserted immediately upstream of the G ₄ C ₂ repeats, 114 bp of sequence upstream of the repeat in intron 1 of <i>C9orf72</i> in patients, and a 3' GFP tag in the GR reading frame.	No ne	Chr 3.	n.a	GFP- tagged GR	Eye degeneration (d15) (Cunningham et al., 2020) [*potential RNA toxicity involvement]	(Goodman, Prudencio, Srinivasan, et al., 2019), (Cunningham et al., 2020)
847 26	(G ₄ C ₂) 49 (III)	49 repeats	Uninterrupted pure repeats	No ne	Chr 3.	Found (Kramer et al., 2016)	Found GP (Kramer et al., 2016)	Eye degeneration, Life-span (Kramer et al., 2016)	(Goodman, Prudencio, Kramer, et al., 2019), (Kramer et al., 2016)
847 27	(G ₄ C ₂) 49 (II)			No ne	Chr 2.				

Comparative analysis of the neurotoxicity and pathological effects of various G₄C₂ constructs in *Drosophila* models

To evaluate the respective toxicities and pathologies of publicly available G₄C₂ constructs (Table 1. and Supplementary Figure S1a), we performed an in-depth comparative analysis in *Drosophila* using two different GAL4 drivers: *Elav-Gal4* and *GMR-Gal4*. Initially, we employed the *GMR-Gal4* driver to express the G₄C₂ constructs in the *Drosophila* eye, a well-established model for studying neurodegeneration. Using the degeneration severity criteria detailed in Supplementary Figure S1b, degeneration scores revealed that two lines had significantly higher scores compared to the other constructs (Figures 1a and 1b). While most G₄C₂ constructs, apart from (G₄C₂)₁₆₀, generated significant degenerative eye phenotypes, flies expressing (G₄C₂)₃₀ (II) and (G₄C₂)₃₆ demonstrated the most pronounced degeneration. Note that two (G₄C₂)₄₉ lines were excluded from the experiment due to their lethality when expressed in neurons or the eye at 27°C, thereby precluding further analysis.

Subsequently, we examined the climbing ability of the flies expressing different G₄C₂ constructs neuronally with the *Elav-Gal4* driver on days 3 and 10 to assess the progression of motor behavior impairments over time. As pan-neuronal expression of (G₄C₂)₃₀ transgenes using *Elav-Gal4* (I) caused developmental lethality (Xu et al., 2013), we used the *Elav-Gal4* (II) driver, a previously described non-lethal *Elav-Gal4* alternative (Wen et al., 2020), for our investigations. On day 3, both (G₄C₂)₃₀ lines demonstrated substantial toxicity, significantly impairing the climbing ability of the flies (Figure 1c). By day 10, the LDS-(G₄C₂)₄₄ line was the most affected among the flies that survived until adulthood, showing the steepest decline in motor function over time among flies observed (Figure 1d).

Finally, we assessed the lifespan of the flies expressing these constructs for a period of up to 30 days to measure the overall organismal health, complementing the relative short-term phenotypic effects observed in the eye and motor behavior assays. This revealed significant variations in the lifespan, with different constructs displaying differing levels of toxicity (Figure 1e). Importantly, the LDS-(G₄C₂)₄₄ line was found to be the most toxic, evidenced by the shortest lifespan among all the constructs studied. Additionally, the (G₄C₂)₃₆ construct showed a moderate effect on lifespan when expressed in neurons using *Elav-Gal4*.

Our results illustrate that the expression of G₄C₂ repeat expansion constructs in *Drosophila* leads to notable neurotoxic effects, with varying levels of toxicity among different constructs. Particularly, the *LDS*-(G₄C₂)₄₄ line was highly toxic, exhibiting detrimental effects on both climbing and lifespan phenotypes, while demonstrating a milder phenotype in eye degeneration. Moreover, the (G₄C₂)₃₀ (II) line was highly toxic in the climbing assay and eye degeneration, indicating impaired neuronal function, while the (G₄C₂)₃₆ showed consistent moderate toxicity in performed assays. These findings elucidate the neurotoxic impact of G₄C₂ repeat expansion constructs in *Drosophila*, highlighting the varying degrees of toxicity among different constructs.

Detection of GP DPRs and TBPH in the brains of G₄C₂-expressing *Drosophila*

We found that the expression of seven publicly available G₄C₂ repeat transgenes resulted in differing degrees of toxicity. Existing literature indicates a strong correlation between DPRs and neuronal toxicity (Lee et al., 2016; Mizielska et al., 2014), which led us to hypothesize that elevated concentrations of DPR proteins could be observed in flies exhibiting the highest behavioral or cellular degeneration.

To test this hypothesis, we utilized a well-established DPR antibody specific to polyglycine-proline (GP) repeats (Dafinca et al., 2016) as a representative DPR marker and observed its expression in the *Drosophila* CNS. In line with our hypothesis, we observed considerable GP DPRs in both (G₄C₂)₃₀ (II) and *LDS*-(G₄C₂)₄₄ expressing fly lines, whereas other lines exhibited relatively low GP DPR expression levels (Figure 2a and 2b). Surprisingly, despite showing moderate phenotypic toxicity, the fly brains expressing (G₄C₂)₃₆ showed little-to-no evidence of GP DPR expression, possibly suggesting involvement of other toxic DPRs, such as GR DPR (Mizielska et al., 2014). Interestingly, the GP DPRs largely lacked the co-localization with DAPI staining, suggesting that GP DPRs, similar with previous findings (Goodman, Prudencio, Srinivasan, et al., 2019), were expressed in the cytoplasm of Kenyon cells. In addition, to validate that signals from GP staining can be used as a representative marker for DPRs, we utilized the *LDS*-(G₄C₂)₄₄ transgene and tracked the localization of GR DPR by detecting green fluorescent protein (GFP) signals in the brain. While significant GR expression was, as expected, observed, the GFP signals were predominantly in the Kenyon cell nucleus, contrary to the GP DPRs (Supplementary Figure S2a). This suggests that DPR proteins predominantly localize in distinct subcellular localizations, as previously indicated by Wen et al (Wen et al., 2014).

In our investigation into the pathological markers associated with G₄C₂ repeats expansion, we turned our attention to the mislocalization of TDP-43 (TBPH) in *Drosophila*, a phenomenon well-documented in various neurodegenerative disorders as well as C9orf72 repeat-associated expansion (Haeusler et al., 2016; Park et al., 2020; Solomon et al., 2018). Utilizing a previously used TBPH-specific antibody (Lin et al., 2011; Park et al., 2020), we analyzed the localization patterns of this protein across our fly models within the Kenyon cells. Our observations revealed a shift in TBPH localization under the influence of G₄C₂ expression (Supplementary Figure S2b). Contrary to the predominant nuclear presence of TBPH in control cells, models

expressing G₄C₂ exhibited a discernible decrease in nucleus TBPH while an increase in cytoplasmic TBPH inclusion, indicating a potential involvement of alterations in the nuclear-cytoplasmic transport mechanisms, a finding that aligns with current scientific discourse on neurodegenerative pathologies (Gasset-Rosa et al., 2019; Park et al., 2020; Solomon et al., 2018).

Due to previous reports indicating that repeat-associated RNA toxicity may be involved in cellular or behavioral defects (Cunningham et al., 2020; Goodman, Prudencio, Srinivasan, et al., 2019; Kim et al., 2021; McEachin et al., 2020), we quantified the transgene-specific RNA levels in various transgene expressions through quantitative reverse transcription PCR (qRT-PCR) analysis (Supplementary Figure S5a-d). Our findings demonstrated a marked increase in RNA transgenes across all measured fly lines, with specific RNA measures correlating with our earlier toxicity profiles (Figure 1a-e). This correlation, however, should be approached with a degree of caution due to the methodological challenges in comparing RNA levels across different transgenes (see discussion).

Collectively, these results not only support the notion that DPRs, such as GP and GR DPRs, exhibit distinct subcellular localizations and potentially possess varying mechanisms of action but also highlight the consistency of our data with findings from other literature underscoring the reliability of our platform to model G₄C₂-related diseases. Future research is essential to delve deeper into these mechanisms and to investigate possible therapeutic strategies to counter G₄C₂-related neurotoxicity.

Evaluating drug efficacy against G₄C₂ toxicity: Impacts on lifespan, mobility, and eye degeneration in *Drosophila* models

In the present investigation, our attention was concentrated primarily on the *LDS*-(G₄C₂)₄₄ and (G₄C₂)₃₀ (II) transgene constructs. This choice was guided by several factors. Primarily, these models displayed high toxicity levels in the three preceding tests (eye degeneration analysis, climbing ability analysis, and lifespan analysis) without reaching lethality, a critical attribute for deriving measurable results. Constructs such as (G₄C₂)₄₉ were eliminated from consideration due to their extreme toxicity levels, which led to lethality and thereby precluded further analysis. Conversely, constructs that exhibited weak to mere moderate toxicity lacked the sensitivity and robustness required for a valuable model to evaluate the potential therapeutic interventions. Similarly, we selected constructs that demonstrated DPR expression, intrigued to investigate potential alterations in DPR expression or localization induced by our therapeutic agents.

We next sought to investigate the potential of specific therapeutic candidates in mitigating these effects (Supplementary Figure S3a). As such, we chose three compounds, TMPyP4, PJ34, and KPT-276, for evaluation due to their distinct mechanisms of action in relation to different stages of G₄C₂ toxicity. TMPyP4 is known to bind to and destabilize G-quadruplexes, potentially affecting the stability of G₄C₂ repeats and thereby reducing both RNA and protein toxicity (Zamiri et al., 2014). PJ34

is an inhibitor of poly (ADP-ribose) polymerase and is specifically considered for attenuating protein toxicity (Gao et al., 2022). KPT-276 inhibits the nuclear export of proteins and RNA, and thus might affect G₄C₂-related pathologies through modulating nucleocytoplasmic trafficking (Chou et al., 2018; Schmidt et al., 2013). This selection of drugs allowed us to target and dissect various aspects of G₄C₂ toxicity.

We first assessed the impact of TMPyP4, PJ34, and KPT-276 on the lifespan of flies expressing the *LDS-(G₄C₂)₄₄* line using *Elav-Gal4* (Figures 3a, 3b, Supplementary Figure S3b). The dosages administered were based on established concentrations from prior studies using the *Drosophila* ALS models, ensuring methodological consistency and comparability of our results with existing literature (Chou et al., 2018; Gao et al., 2022; Schmidt et al., 2013; Zamiri et al., 2014). Both TMPyP4 and PJ34 treatments significantly extended the lifespan of these flies compared to controls. However, KPT-276 did not exhibit a notable effect on lifespan (Supplementary Figure S3b).

Next, we investigated the effects of three above-mentioned drugs on the climbing ability of flies expressing the *(G₄C₂)₃₀* (II) and *LDS-(G₄C₂)₄₄* lines (Figures 3c, 3d, Supplementary Figure S3c). We observed that TMPyP4 and PJ34 had beneficial effects on the climbing ability, but only in the *(G₄C₂)₃₀* (II) expressing flies. No significant improvement in the climbing ability of the *LDS-(G₄C₂)₄₄* line was seen. KPT-276 did not affect the climbing ability of flies expressing the two G₄C₂ transgenes (Supplementary Figure S3c).

Lastly, we used *GMR-Gal4* to express *(G₄C₂)₃₀* (II) and *LDS-(G₄C₂)₄₄* in the eyes of flies and evaluated the effects of TMPyP4, PJ34, and KPT-276 on eye degeneration (Figures 3e-h, Supplementary Figure S3d, S3e) using the degeneration severity criteria detailed in Supplementary Figure S1b and S1c. Remarkably, all three drugs showed a beneficial effect in reducing the degeneration scores for both G₄C₂ constructs, indicating their potential in alleviating eye pathologies associated with G₄C₂ toxicity (with the exception of KPT-276, which alleviated the eye degeneration in *(G₄C₂)₃₀* (II) but not *LDS-(G₄C₂)₄₄*).

Our results indicate that TMPyP4 ameliorates disease phenotype, reinforcing the notion that destabilizing RNA G₄C₂ G-quadruplex structures can inhibit associated toxicity (Li et al., 2021; Mishra et al., 2019). Recent studies have unveiled that an RNA helicase, *DHX36*, can modulate the toxicity of G₄C₂ by acting on repeat RNA G-quadruplexes and facilitating RAN translation (Liu et al., 2021; Tseng et al., 2021). Given these findings, we were interested to explore whether genetic modulation of G-quadruplex regulators can affect disease phenotype in our experimental platform. To address this, we generated transgene RNA helicase associated with AU-rich element (*Rhau*), the *Drosophila* homologue of *DHX36* and a potent G-quadruplex unwinder, and co-expressed it with *(G₄C₂)₃₀* (II) and observed the *Drosophila* eye degeneration intensity. Our results confirm that the co-expression of *Rhau* with *(G₄C₂)₃₀* (II) did in fact amplify the toxicity, suggesting a role for *Rhau* in exacerbating the disease phenotype (Supplementary Figure S3f, S3g), a finding consistent with previous literatures (Liu et al., 2021; Tseng et al., 2021). These results imply that our platform

may be applied not only for the testing of pharmacological drugs but also for exploring genetic modulations.

Our data indicate that TMPyP4 and PJ34 can ameliorate some of the toxic effects associated with G_4C_2 constructs, particularly in extending lifespan and improving climbing ability in specific constructs. Moreover, TMPyP4, PJ34, and KPT-276 were all effective in reducing eye degeneration associated with G_4C_2 toxicity. These findings provide valuable insights into potential therapeutic strategies for conditions associated with G_4C_2 repeat expansions and underscore the importance of targeting different aspects of G_4C_2 toxicity.

Impact of representative G_4C_2 -associated drugs on GP DPR in *Drosophila* models

Given that the three previously characterized disease modifying drugs - TMPyP4, PJ34, and KPT-276 – were able to elicit amelioration of G_4C_2 -associated toxicity in $(G_4C_2)_{30}$ (II) and $LDS-(G_4C_2)_{44}$ expressing flies, we decided to investigate the expression and localization of DPR proteins. We hypothesized that the beneficial effects observed in Figure 3 could potentially result from a change in the localization of -, or the reduction of DPR proteins.

In accordance with this hypothesis, we found that treatment with TMPyP4 at 100 μ M led to a reduction in DPR expression in both $(G_4C_2)_{30}$ (II) and $LDS-(G_4C_2)_{44}$ lines (Figure 4a and 4c). TMPyP4 is a known G-quadruplex destabilizer, and its ability to reduce the expression of DPR proteins suggests that it may be acting through destabilization of the G-quadruplex structures in the G_4C_2 region, thereby inhibiting the aberrant translation process that generates the toxic DPRs (Wang et al., 2019). Similarly, treatment with PJ34 also led to a decrease in GP signals but only in $LDS-(G_4C_2)_{44}$ lines (Figure 4b and 4d). However, PJ34's effects were not as potent as TMPyP4's and both were no observable changes in the localization of GP proteins. Interestingly, KPT-276, a selective nuclear export inhibitor, fed flies showed no difference in GP signals in comparison to the control group (Supplementary Figure S4a and S4b). It is important to note that all three drugs were unable to significantly reduce (or alter the localization of-; data not shown) GR DPRs, as measured by GFP signals in $LDS-(G_4C_2)_{44}$ lines (Supplementary Figure S4c-f).

Taken together, these data provide evidence that TMPyP4, PJ34, can reduce or ameliorate the toxic effects of G_4C_2 transgenes. Because our study primarily concentrates on the reduction of GP, a commonly recognized non-toxic DPR, it is crucial to extend research to include the distribution of other DPR proteins, such as GA, PR, or even chimeric DPRs. In addition, it remains inconclusive whether these drugs can reduce GR DPRs in other flies expressing transgenes other than $LDS-(G_4C_2)_{44}$. This exploration would provide a firmer understanding of the effects of the three-representative disease-modifying drugs.

DISCUSSION

In this study, we utilized available *Drosophila* transgenic models of C9orf72-G₄C₂ mutations for a comprehensive and unbiased examination of the neurotoxic and pathological consequences. Our systematic comparative analysis indicates that these constructs are associated with distinct toxicity profiles, which encompass degenerative effects on the eye, impairment of climbing ability, and a reduction in lifespan, thereby suggesting a well-substantiated model for the exploration of neurodegeneration. Moreover, we present an experimental framework that aids in comprehending how factors, such as subtle sequential differences, may engender significant disparities in both toxicity profiles and drug treatment outcome (Figure 5). Although it remains elusive which specific constructs are responsible for higher G₄C₂ expression levels, their differential expression patterns warrant investigation to elucidate the reasons behind variable toxicities among constructs. This knowledge may be pivotal in shaping future research directions.

In addition to DPR toxicity, RNA toxicity may play a role in neurodegeneration. To test this hypothesis, we performed qRT-PCR using construct-specific primers to quantify G₄C₂ RNA levels (Supplementary Figure S5a-d) within the same category of G₄C₂ transgenes. We found that all tested transgenes showed significant increase in probed RNA. Interestingly, we found that the (G₄C₂)₃₀ (II) line exhibited higher RNA levels compared to (G₄C₂)₃₀ (III) line, consistent with our toxicity profile data (Supplementary Figure S5c). However, interpreting these RNA results necessitates caution, primarily since a direct quantitative comparison of RNA across different transgenes is methodologically constrained due to inherent variations in used primers for qRT-PCR. These findings suggest the need for further comprehensive studies to delineate the potential toxicity caused by RNA in the context of G₄C₂ expansions and neurodegeneration. Nonetheless, the successful conduction of qRT-PCR using different primers offers promising prospects for advancing our understanding of this complex area of research.

Since all fly brains expressing G₄C₂ repeat-associated transgenes showed increased expression level of G₄C₂ RNA compared to control, we were curious to see whether aberrant G₄C₂ expression might precipitate global RNA irregularities. To examine this, we employed ribosomal RNA (rRNA) antibodies, which revealed a significant augmentation in cytoplasmic rRNA foci in both (G₄C₂)₃₀ (II) and LDS-(G₄C₂)₄₄ lines (Supplementary Figure S5e and S5f). This phenotype is particularly interesting in light of prior studies that have underscored that G₄C₂ expression contributes not just to TDP-43 aggregation but also leads to the formation of RNA foci in the cytoplasm. Nevertheless, we must tread carefully in interpreting the rRNA foci data, as the precise genesis of these structures remains undetermined, and their functional implications within cellular pathology are still to be unraveled. Future studies employing molecular investigations to elucidate the origins and consequential ramifications of rRNA foci formation in relation to G₄C₂ expression will be beneficial in understanding G₄C₂ repeat expansion-associated RNA toxicity.

Beyond the insights gained regarding G₄C₂ toxicity, our *Drosophila* platform

demonstrates a promising utility in therapeutic development. As well as providing an effective model for screening potential pharmacological interventions such as TMPyP4, PJ34, and KPT-276, this platform may also serve as a powerful tool for genetic interactor screenings, as exemplified by our findings from *Rhau* expression studies. Genetic screenings can offer critical insights into the molecular mechanisms underlying G₄C₂ toxicity and the pathology of associated diseases. They can identify genetic modifiers, potential risk factors, and potential therapeutic targets, by help pinpointing genes or genetic interactions that modulate the toxicity of G₄C₂ repeats. Hence, our platform could serve as a resource in the wider scientific community, not only for testing therapeutic efficacy but also for uncovering novel molecular pathways involved in G₄C₂-related pathologies.

The results from our study also shed light on the controversial efficacy of KPT-276. Recent studies have questioned the effectiveness of KPT-276 in improving quality-of-life in various diseases (Boeynaems et al., 2016; Lee et al., 2016). Our study corroborates these findings, as KPT-276 was found to be ineffective in extending the lifespan or improving the climbing ability of flies expressing *LDS*-(G₄C₂)₄₄. However, we observed a beneficial effect of KPT-276 in reducing eye degeneration in flies expressing the (G₄C₂)₃₀ construct as depicted in a previous study (Zhang et al., 2015). This suggests that while KPT-276 may have a role in certain facets of G₄C₂ toxicity, its therapeutic potential may be limited or context-dependent (Vanneste & Van Den Bosch, 2021). These results lend additional credibility to our platform, demonstrating its capacity to reflect real-world clinical data, and thus reinforcing its reliability as a tool for drug efficacy evaluation.

Our study accentuates the necessity for strategic selection of G₄C₂ constructs in ALS research. Researchers would benefit from consideration of the particular aspect of toxicity or pathological feature they seek to investigate. For instance, a construct producing abundant DPRs would be more suitable for studies focusing on protein aggregation, whereas those forming G-quadruplexes might be apt for RNA toxicity studies. By strategically selecting G₄C₂ constructs, researchers can design experiments that more accurately model the specific disease mechanisms and evaluate the efficacy of targeted therapeutic interventions.

In addressing the variations observed in GP amounts between different G₄C₂ models, it is important to note crucial technical limitations of our study. Our primary evidence for comparing GP expression levels are the GP signal differences measured through IHC-based imaging techniques, which can be misleading in quantifying protein levels (Figure 2a and 2b). Therefore, we performed a western blot analysis using both GP- and GR-specific antibodies in fly brains expressing G₄C₂ repeat constructs to 1) validate the GP- and GR-specific antibodies used for IHC and 2) compare DPR protein quantities between different G₄C₂ models. While we were able to detect GP or GR in *LDS*-(G₄C₂)₄₄ expressing flies that harbors a GFP tag in the GR repeat frame (or a single off-framed GFP in the GP repeat frame), thereby suggesting that the GP- and GR-specific antibodies are likely specific and effective, we were unable to detect measurable amount of GP or GR DPRs in other lines (data not shown). We speculate that the detection of DPRs in *LDS*-(G₄C₂)₄₄ expressing flies may stem from the nature

of fused GFP, which resulted in increased size and stabilization of *LDS-(G₄C₂)₄₄*-derived GP or GR protein which made possible to overcome detection threshold of western blot analysis. Additionally, we speculate that the inability to detect GP or GR DPRs in other transgenes expressing flies may be due to the constitution of the *G₄C₂* repeat constructs employed in our models. Aside from *(G₄C₂)₁₆₀*, the constructs used for western blotting analysis were of lengths no greater than 44 repeats, which are likely insufficient for the production of detectable levels of GP or GR proteins. Boivin et al., encountered similar challenges, reporting an inability to detect DPRs derived from very short *G₄C₂* repeats, often less than 10 repeats, even when these sequences were tagged. They hypothesized that such minute DPR proteins likely possess inherent instability, resulting in rapid turnover, which significantly hinders their detectability in standard assay conditions (Boivin et al., 2020). It is also possible that conventionally used gels or membranes for Western blotting is insufficient in separating and retaining small proteins. This variability necessitates cautious interpretation of our results. Future studies aiming to quantify DPR levels will benefit from developing enhanced methodologies that can reliably detect and measure smaller or less stable DPR proteins, thereby providing a more comprehensive understanding of their role in ALS/FTD pathology.

It also remains unclear whether these differences in GP signal changes are due to variations in RAN translation efficiency or discrepancies in the transcription levels of the transgenes. It is important to acknowledge that the process of RNA translation is subject to a multitude of influencing factors, including, but not limited to, availability of ribosomes, the engagement of various initiation factors, specific configurations of mRNA secondary structures, interactions with diverse RNA-binding proteins, and a range of post-transcriptional modifications. Each of these elements can significantly impact the translation process, adding layers of complexity to our understanding of the observed phenomena. Such intricacies in the mechanisms governing RNA translation necessitate a more detailed investigative approach, incorporating advanced kinetics and comprehensive biochemical assays, to unravel the underlying causes of the observed differences in protein levels. Future studies should be conducted to delve deeper into these aspects, potentially utilizing advanced methodologies capable of dissecting the nuanced interplay of factors impacting RNA translation in the context of *G₄C₂* repeat expansion.

Our investigation provides a framework for future studies into *G₄C₂*-associated ALS. Future directions should include evaluating the interplay between G-quadruplexes and DPRs in neuronal toxicity, and how these features can be modulated for therapeutic gains. There is a need for more comprehensive toxicity profiling of the candidate compounds in mammalian models of ALS, including pharmacokinetic and pharmacodynamic assessments. Additionally, the development of high-throughput screening assays utilizing the standardized *Drosophila* constructs could facilitate the identification of novel therapeutic candidates. In conclusion, while the *Drosophila* model offers promising insights and a platform for *G₄C₂*-associated ALS research, it is essential to acknowledge the complexities and heterogeneity of human pathology. The translation of these findings to human therapeutics, although potentially transformative,

necessitates careful and robust validation through in-depth molecular studies and comprehensive clinical trials.

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AUTHOR CONTRIBUTIONS

D.L., H.C.J., E.S.K, and S.B.L. wrote the manuscript. H.C.J. and E.S.K. performed experiments. D.L., H.C.J., and E.S.K. analyzed the data. S.Y.K., J.Y.C., S.H.C., K.A.K., J.H.C., B.S.K, I.J.C., C.G.C., E.S.K., and S.B.L. provided expertise and feedback. E.S.K. and S.B.L. supervised the research.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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FIGURE LEGENDS

Figure 1. Comparative analysis of the neurotoxicity and pathological effects of various G₄C₂ constructs in *Drosophila* models

- (a) Representative images of eye phenotype in *Drosophila* models expressing denoted G₄C₂ transgenes at day 7 APE (Genotype: Control, *GMR-Gal4/+;UAS-luciferase/+* | (*G₄C₂*)₁₆₀, *GMR-Gal4/UAS-(G₄C₂)₁₆₀;+/+* | (*G₄C₂*)₃₀ (III), *GMR-Gal4/+;UAS-(G₄C₂)₃₀/+* | (*G₄C₂*)₃₀ (II), *GMR-Gal4/UAS-(G₄C₂)₃₀;+/+* | (*G₄C₂*)₃₆, *GMR-Gal4/UAS-(G₄C₂)₃₆;+/+* | *LDS-(G₄C₂)₄₄*, *GMR-Gal4/+;UAS-LDS-(G₄C₂)₄₄.GR-GFP/+* | (*G₄C₂*)₄₉ (III), *GMR-Gal4/+;UAS-(G₄C₂)₄₉/+* | (*G₄C₂*)₄₉ (II), *GMR-Gal4/UAS-(G₄C₂)₄₉;+/+*) (black scale bar, 100 μ m).
- (b) Quantification of eye phenotype in *Drosophila* expressing denoted G₄C₂ transgenes described in Figure 1a. The degeneration score was assigned based on the criteria provided in Supplementary Figure S1b.
- (c) Quantification of climbing ability in *Drosophila* expressing denoted G₄C₂ transgenes at day 3 APE (Genotype: Control, *Elav-Gal4/+;UAS-luciferase/+* | (*G₄C₂*)₁₆₀, *Elav-Gal4/UAS-(G₄C₂)₁₆₀;+/+* | (*G₄C₂*)₃₀ (III), *Elav-Gal4/+;UAS-(G₄C₂)₃₀/+* | (*G₄C₂*)₃₀ (II), *Elav-Gal4/UAS-(G₄C₂)₃₀;+/+* | (*G₄C₂*)₃₆, *Elav-Gal4/UAS-(G₄C₂)₃₆;+/+* | *LDS-(G₄C₂)₄₄*, *Elav-Gal4/+;UAS-LDS-(G₄C₂)₄₄.GR-GFP/+* | (*G₄C₂*)₄₉ (III), *Elav-Gal4/+;UAS-(G₄C₂)₄₉/+* | (*G₄C₂*)₄₉ (II), *Elav-Gal4/UAS-(G₄C₂)₄₉;+/+*).
- (d) Quantification of climbing ability in *Drosophila* expressing denoted G₄C₂ transgenes described in Figure 1c at day 10 APE.
- (e) Lifespan analysis of *Drosophila* expressing denoted G₄C₂ transgenes described in Figure 1c. Survival data were plotted using the Kaplan-Meier method. Both (*G₄C₂*)₄₉ lines were excluded from lifespan analysis due to lethality in our experimental conditions.

Figure 2. Detection of GP DPRs in the brains of G₄C₂-expressing *Drosophila*

- (a) Representative images of GP staining in the Kenyon cells of *Drosophila* brains

expressing G_4C_2 transgenes at day 10 APE. (Genotype: Control, *Elav-Gal4/+;UAS-luciferase/+* | (G_4C_2)₁₆₀, *Elav-Gal4/UAS-(G₄C₂)₁₆₀;+/+* | (G_4C_2)₃₀ (III), *Elav-Gal4/+;UAS-(G₄C₂)₃₀/+* | (G_4C_2)₃₀ (II), *Elav-Gal4/UAS-(G₄C₂)₃₀;+/+* | (G_4C_2)₃₆, *Elav-Gal4/UAS-(G₄C₂)₃₆;+/+* | *LDS-(G₄C₂)₄₄, Elav-Gal4/+;UAS-LDS-(G₄C₂)₄₄.GR-GFP/+*) (white scale bar, 5 μ m).

- (b) Quantification of GP DPR staining in *Drosophila* expressing denoted G_4C_2 transgenes described in Figure 2a.

Figure 3. Evaluating drug efficacy against G_4C_2 toxicity: Impacts on lifespan, mobility, and eye degeneration in *Drosophila* models

- (a) Lifespan assay on *Drosophila* expressing the G_4C_2 transgene *LDS-(G₄C₂)₄₄* (Genotype: *Elav-Gal4/+;UAS-LDS-(G₄C₂)₄₄.GR-GFP/+*) treated either with Control (black) or TMPyP4 at 100 μ M (green). Survival data were plotted using the Kaplan-Meier method.
- (b) Lifespan assay on *Drosophila* expressing the G_4C_2 transgene described in Figure 3b treated either with Control (black line) or PJ34 at 5 μ M (red). Survival data were plotted using the Kaplan-Meier method.
- (c) Quantification of climbing ability in *Drosophila* models at day 10 APE treated either with Control or TMPyP4 at 100 μ M. (Genotype: Control, *Elav-Gal4/+;UAS-luciferase/+* | (G_4C_2)₃₀ (II), *Elav-Gal4/UAS-(G₄C₂)₃₀;+/+* | *LDS-(G₄C₂)₄₄, Elav-Gal4/+;UAS-LDS-(G₄C₂)₄₄.GR-GFP/+*)
- (d) Quantification of climbing ability in *Drosophila* expressing denoted G_4C_2 transgenes described in Figure 3c at day 10 APE treated either with Control or PJ34 at 5 μ M.
- (e) Representative images of *Drosophila* eye expressing denoted G_4C_2 transgenes at day 10 APE (Genotype: Control, *GMR-Gal4/+;UAS-luciferase/+* | (G_4C_2)₃₀ (II), *GMR-Gal4/UAS-(G₄C₂)₃₀;+/+* | *LDS-(G₄C₂)₄₄, GMR-Gal4/+;UAS-LDS-(G₄C₂)₄₄.GR-GFP/+*). The flies were fed with Control (ADCW) or treated with TMPyP4 at 100 μ M (black scale bar, 100 μ m).
- (f) Quantification of eye degeneration scores in *Drosophila* expressing denoted G_4C_2 transgenes, as described in Figure 3e. fed with Control (ADCW) or treated with TMPyP4 at 100 μ M. Eye degeneration was scored based on the criteria provided in Supplementary Figure S1b.
- (g) Representative images of *Drosophila* eye expressing denoted G_4C_2 transgenes described in Figure 3e at day 10 APE. The flies were fed with Control (DMSO 0.01%) or treated with PJ34 at 5 μ M.
- (h) Quantification of the eye phenotype in *Drosophila* expressing denoted G_4C_2 transgenes described in Figure 3e., fed with Control (DMSO 0.01%) or treated with PJ34 at 5 μ M. Eye degeneration was scored based on the criteria provided in Supplementary Figure S1c.

Figure 4. Impact of representative G₄C₂-associated drugs on GP DPR in *Drosophila* models

- (a) Representative images of GP staining in the Kenyon cells of *Drosophila* brains expressing G₄C₂ transgenes at day 10 APE. (Genotype: Control, *Elav-Gal4/+;UAS-luciferase/+* | (*G₄C₂*)₃₀ (II), *Elav-Gal4/UAS-(G₄C₂)₃₀;/+* | *LDS-(G₄C₂)₄₄, Elav-Gal4/+;UAS-LDS-(G₄C₂)₄₄.GR-GFP/+*). The experimental lines were treated with either ACDW or TMPyP4 at 100μM (white scale bar, 5 μm).
- (b) Representative images of GP staining in the Kenyon cells of *Drosophila* brains expressing G₄C₂ transgenes denoted in Figure 4a at day 10 APE. The experimental lines were treated with either DMSO (0.01%) or PJ34 at 5μM (white scale bar, 5 μm).
- (c) Quantification of GP DPR staining in *Drosophila* expressing denoted G₄C₂ transgenes described in Figure 4a treated with either ACDW or TMPyP4 at 100μM.
- (d) Quantification of GP DPR staining in *Drosophila* expressing denoted G₄C₂ transgenes described in Figure 4a treated with either DMSO (0.01%) or PJ34 at 5μM.

Figure 5. Summary table of the disparate phenotypic outcomes in *Drosophila* expressing (*G₄C₂*)₃₀ (II) and *LDS-(G₄C₂)₄₄* treated with disease-modifying drugs

- (a) The table presents the differing responses of *Drosophila* expressing (*G₄C₂*)₃₀ (II) and *LDS-(G₄C₂)₄₄* when treated with three previously characterized disease modifying drugs. The symbols 'O' and 'X' are utilized to denote an ameliorating effect and no observable change respectively.

SUPPLEMENTARY MATERIALS

Supplementary Figure S1. Detailed characterization of G₄C₂ transgenes and scoring criteria for *Drosophila* eye degeneration

- (a) An illustration detailing the seven publicly available G₄C₂ transgenes utilized in this study. The specific characteristics and attributes of each transgene are depicted, providing detailed explanation of their structure and potential implications in the context of our experimental design.
- (b) Representative images of *Drosophila* eye and criteria for scoring *Drosophila* eye degeneration. Scores range from 1 to 5, with 1 representing control eyes showing normal phenotype and 5 indicating severe degeneration. Intermediate scores reflect increasing severity of eye degeneration characterized by ommatidia cell death, pigmentation degeneration, and eye shape deformity. A higher score represents a more severe outcome (black scale bar, 100 μm).

- (c) Representative images of DMSO-fed (0.01%) *Drosophila* eye and criteria for scoring *Drosophila* eye degeneration. Scores range from 1 to 6, with 1 representing control eyes showing normal phenotype and 6 indicating severe degeneration. Intermediate scores reflect increasing severity of eye degeneration characterized by ommatidia cell death, pigmentation degeneration, and eye shape deformity. A higher score represents a more severe outcome.

Supplementary Figure S2. Detection of GR DPRs and TBPH in the brains of *LDS-(G₄C₂)₄₄*-expressing *Drosophila*

- (a) Representative images of GR-GFP in the Kenyon cells of *Drosophila* brains expressing *LDS-(G₄C₂)₄₄* transgenes denoted in figure 2a at day 10 APE (white scale bar, 5 μ m).
- (b) Representative images of TBPH staining in the Kenyon cells of *Drosophila* brains expressing *G₄C₂* described in Figure 2a (white scale bar, 1 μ m). Outer and inner dashed lines indicate the borders of cell bodies and nuclei, respectively. The intensity profile of fluorescent signals representing TBPH proteins across cell bodies along red lines are presented at the bottom.

Supplementary Figure S3. Detailed experimental design and the evaluation of KPT-276 and *Rhau* expression in *Drosophila* models expressing *G₄C₂* transgenes

- (a) A schematic illustration of the candidate selection process, divided into three main stages: Fly Crossing, Candidate Selection, and Drug Treatment. The diagram visually represents the experimental design and subsequent selection process used to identify and test 3 potential therapeutic compounds: TMPyP4, PJ34, and KPT-276.
- (b) Lifespan quantification of *Drosophila* expressing the *G₄C₂* transgene described in Figure 3a. The flies were fed to either Control (black) or KPT-276 at 1 μ M (purple). Survival data were plotted using the Kaplan-Meier method.
- (c) Climbing ability of *Drosophila* expressing *G₄C₂* transgenes described in Supplementary Figure S3c at day 10 APE. The flies were subjected to either Control (black) or KPT-276 at 1 μ M (purple).
- (d) Representative images of *Drosophila* eye expressing *G₄C₂* transgenes as described in Figure 3e at day 10 APE. The flies were fed with either Control or KPT-276 at 1 μ M (black scale bar, 100 μ m).
- (e) Quantification of eye phenotype degeneration score in *Drosophila* expressing denoted *G₄C₂* transgenes as described in Figure 3e. The severity of the degenerative phenotype is represented as a score based on the criteria provided in Supplementary Figure S1c.

- (f) Representative images of *Drosophila* eye expressing denoted $(G_4C_2)_{30}$ (II) transgenes at day 1 APE (Genotype: *EYFP, UAS-EYFP/UAS-(G₄C₂)₃₀;GMR-Gal4/+* | *Rhau, UAS-HA-Rhau/ UAS-(G₄C₂)₃₀;GMR-Gal4/+*).
- (g) Quantification of eye phenotype degeneration score in *Drosophila* expressing denoted $(G_4C_2)_{30}$ (II) transgenes as described in Figure 3f. The severity of the degenerative phenotype is represented as a score based on the criteria provided in Supplementary Figure S1b.

Supplementary Figure S4. Assessment of representative G_4C_2 -associated drugs on DPR expression in *Drosophila* brain cells

- (a) Representative images of GP staining in the Kenyon cells of *Drosophila* brains expressing G_4C_2 transgenes denoted in Figure 4a at day 10 APE. The experimental lines were treated with either DMSO (0.01%) or KPT-276 at 1 μ M (white scale bar, 5 μ m).
- (b) Quantification of GP DPR staining in *Drosophila* expressing denoted G_4C_2 transgenes described in Figure 4a treated with either DMSO (0.01%) or KPT-276 at 1 μ M.
- (c) Representative images of GR-GFP in the Kenyon cells of *Drosophila* brains expressing G_4C_2 transgenes denoted in figure 4a at day 10 APE. The experimental lines were treated with either ACDW or TMPyP4 at 100 μ M (white scale bar, 5 μ m).
- (d) Quantification of GR-GFP in *Drosophila* expressing denoted G_4C_2 transgenes described in Figure 4a treated with either ACDW or TMPyP4 at 100 μ M.
- (e) Representative images of GR-GFP in the Kenyon cells of *Drosophila* brains expressing G_4C_2 transgenes denoted in figure 4a at day 10 APE. The experimental lines were treated with DMSO (0.01%), PJ34 at 5 μ M, or KPT-276 at 1 μ M (white scale bar, 5 μ m).
- (f) Quantification of GR-GFP in *Drosophila* expressing denoted G_4C_2 transgenes described in Figure 4a treated with DMSO (0.01%), PJ34 at 5 μ M, or KPT-276 at 1 μ M.

Supplementary Figure S5. G_4C_2 RNA quantification and assessment of toxicity through rRNA antibody staining in G_4C_2 -expressing *Drosophila* lines.

- (a) A table outlining the primer sets used, target locations, construct details, and resulting ΔC_t values for each genotype at day 10 APE. (Genotype: Control, *Elav-Gal4/+;UAS-luciferase/+* | $(G_4C_2)_{160}$, *Elav-Gal4/UAS-(G₄C₂)₁₆₀;+/+* | $(G_4C_2)_{30}$ (III), *Elav-Gal4/+;UAS-(G₄C₂)₃₀/+* | $(G_4C_2)_{30}$ (II), *Elav-Gal4/UAS-(G₄C₂)₃₀;+/+* | *LDS-(G₄C₂)₄₄, Elav-Gal4/+;UAS-LDS-(G₄C₂)₄₄.GR-GFP/+*)
- (b) Quantification of $2^{-\Delta\Delta C_t}$ fold changes in *Drosophila* expressing $(G_4C_2)_{160}$ detailed in Supplementary Figure S5a.

- (c) Quantification of $2^{-\Delta\Delta Ct}$ fold changes in *Drosophila* expressing $(G_4C_2)_{30}$ (III) and $(G_4C_2)_{30}$ (II) detailed in Supplementary Figure S5a.
- (d) Quantification of $2^{-\Delta\Delta Ct}$ fold changes in *Drosophila* expressing $LDS-(G_4C_2)_{44}$ detailed in Supplementary Figure S5a.
- (e) Representative images of Kenyon cells from the specified *Drosophila* genotypes detailed in Supplementary Figure S5a stained with an rRNA antibody. Yellow arrowheads indicate rRNA foci (white scale bar, 2 μ m).
- (f) Quantification of the number of cytoplasmic rRNA foci in *Drosophila* expressing genotype detailed in Supplementary Figure S5a.

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Fig 1

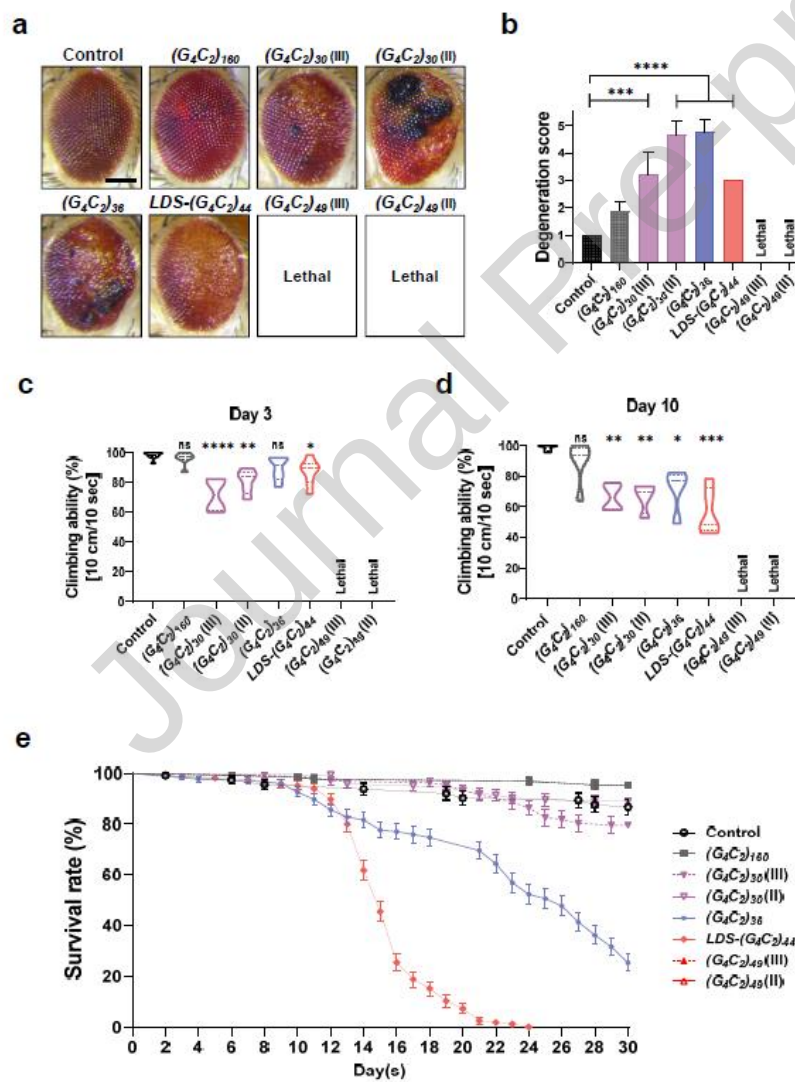


Fig 2

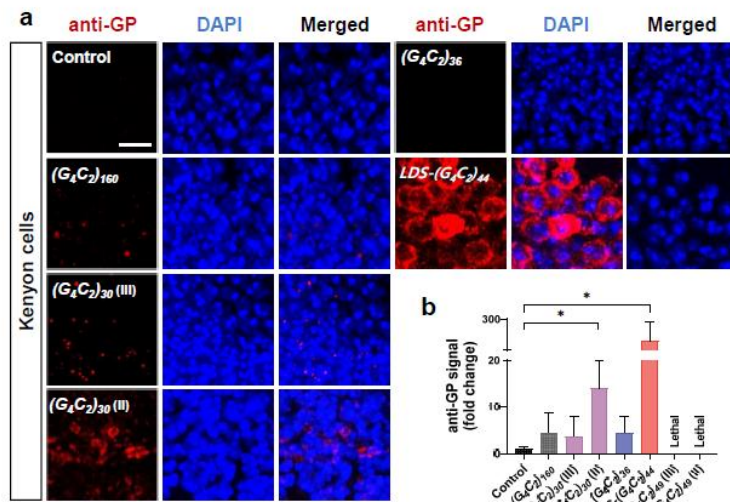


Fig 3

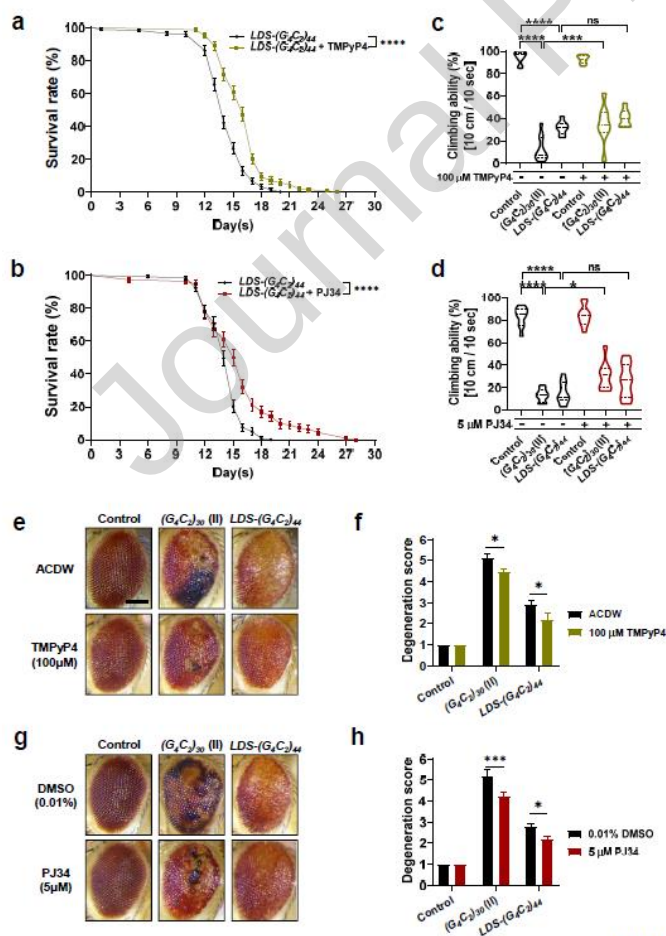


Figure 3.

Fig 4

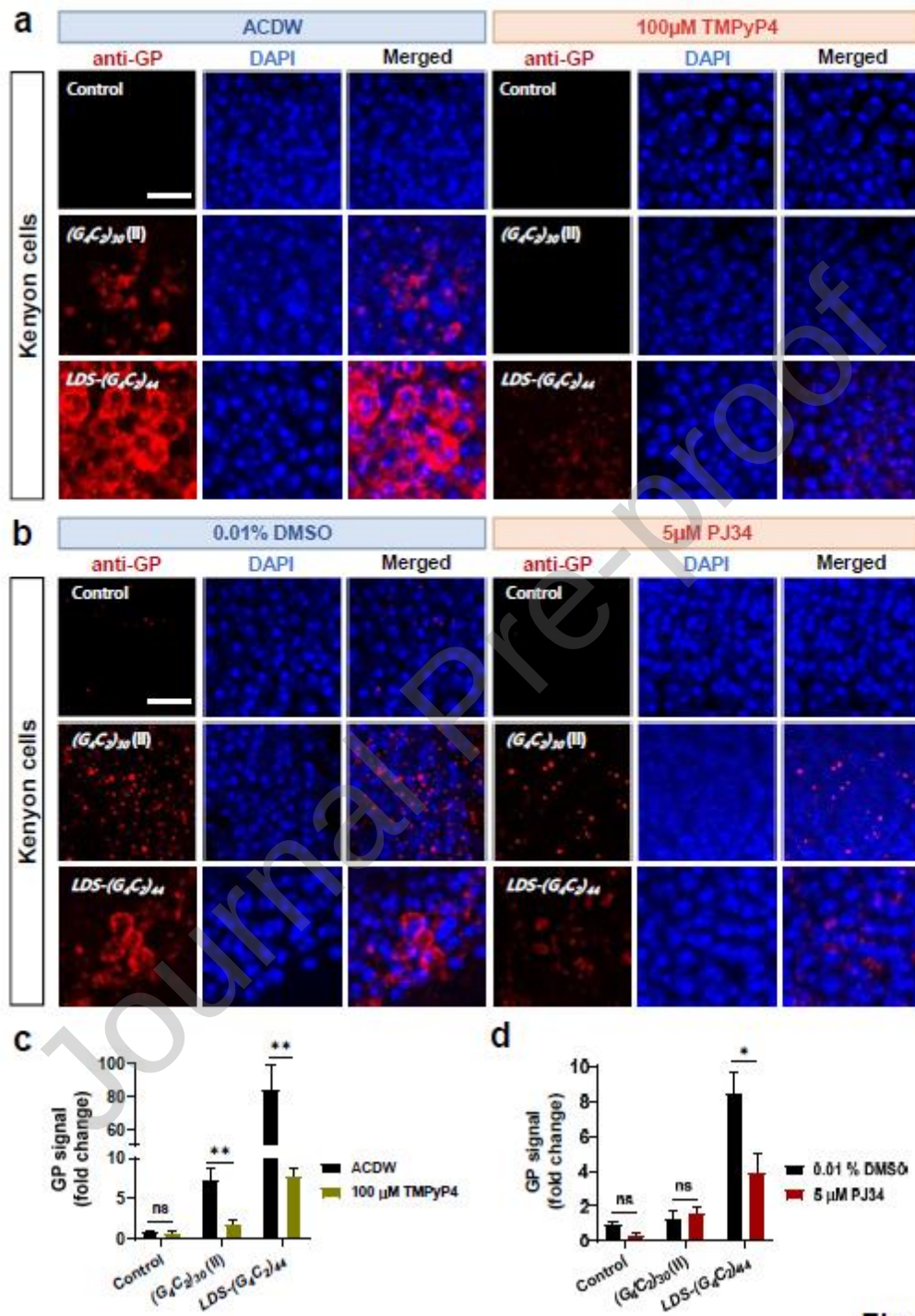


Figure 4.

Fig 5

Reference drugs		TMPyP4		PJ34		KPT-276	
G ₄ C ₂ repeats models		(G ₄ C ₂) ₃₀ (II)	LDS-(G ₄ C ₂) ₄₄	(G ₄ C ₂) ₃₀ (II)	LDS-(G ₄ C ₂) ₄₄	(G ₄ C ₂) ₃₀ (II)	LDS-(G ₄ C ₂) ₄₄
Pathological Outcomes	Eye Degeneration	0	0	0	0	0	X
	Lifespan	N/A	0	N/A	0	N/A	X
	Climbing assay (d3)	0	0	0	X	X	X
	Climbing assay (d10)	0	0	0	X	X	X
Quantity of GP signal		Decrease	Decrease	X	Decrease	X	X
Quantity of GR-GFP signal		N/A	X	N/A	X	N/A	X