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Chemical screen reveals small molecules suppressing fragile X premutation rCGG repeat-mediated neurodegeneration in *Drosophila*

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Fragile X-associated tremor/ataxia syndrome (FXTAS) is a progressive neurodegenerative disorder recognized in fragile X premutation carriers. Using *Drosophila*, we previously identified elongated non-coding CGG repeats in *FMR1* allele as the pathogenic cause of FXTAS. Here, we use this same FXTAS *Drosophila* model to conduct a chemical screen that reveals small molecules that can ameliorate the toxic effects of fragile X premutation ribo-CGG (rCGG) repeats, among them several known phospholipase A_2 (PLA_2) inhibitors. We show that specific inhibition of PLA_2 activity could mitigate the neuronal deficits caused by fragile X premutation rCGG repeats, including lethality and locomotion deficits. Furthermore, through a genetic screen, we identified a PLA_2 *Drosophila* ortholog that specifically modulates rCGG repeat-mediated neuronal toxicity. Our results demonstrate the utility of *Drosophila* models for unbiased small molecule screens and point to PLA_2 as a possible therapeutic target to treat FXTAS.

INTRODUCTION

The fragile X mental retardation 1 (*FMR1*) gene contains a highly polymorphic CGG repeat in the 5′-untranslated region (1). Whereas normal individuals generally possess between 5 and 54 repeats, individuals with more than 200 CGG repeats, referred to as the full mutation, develop fragile X syndrome (FXS) (2). Premutation alleles (55–200 CGG repeats) of the *FMR1* gene are known to contribute to the FXS phenotype through genetic instability, as these alleles can expand into the full mutation during germline transmission (3). Within the last decade, fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder distinct from the neurodevelopmental disorder, FXS, has been recognized mainly among many male premutation carriers in or beyond their fifth decade of life (4).

The most common clinical feature of FXTAS is a progressive tremor with ataxia. More advanced or severe cases may show a progressive cognitive decline that ranges from executive and memory deficits to dementia (5). Patients may also present with common psychiatric symptoms, such as increased anxiety, mood liability and depression (6,7). Magnetic resonance imaging of adult male patients affected with FXTAS shows mild to moderate global brain atrophy, most common in the fontal and parietal regions, as well as the pons and the cerebellum (8). Nearly, all autopsy studies on the brains of symptomatic premutation carriers show degeneration in the cerebellum, which includes Purkinje neuronal cell loss, Bergman gliosis, spongiosis of the deep cerebellar white matter and swollen axons (9,10). The major neuropathological hallmark and postmortem criterion for definitive FXTAS is eosinophilic, ubiquitin-positive intranuclear inclusions broadly distributed throughout the brain in neurons, astrocytes and in the spinal column (9). Although it is mainly male premutation carriers who are affected, female premutation carriers can also experience the same neurological symptoms, but with less severity, which is likely due to the *FMR1* gene being located on the X chromosome and random X-chromosome inactivation. So far, there is no effective therapeutic intervention for FXTAS.

An RNA gain-of-function mechanism has been suggested as the culprit in FXTAS based on the observation of increased levels of CGG-containing *FMR1* mRNA, along with either no detectable change in FMRP or slightly reduced FMRP

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levels in premutation carriers (8,10–13). The lack of FXS, which results from the loss of function of the *FMR1* gene product, in FXTAS patients, along with the absence of FXTAS symptoms in older individuals with FXS, suggest a role for the expanded ribo-CGG (rCGG) repeat in FXTAS pathology. Using *in situ* hybridization, Tassone *et al.* (14) demonstrated the presence of expanded *FMR1* RNA transcripts in the FXTAS inclusions of a 70-year-old male who died with FXTAS.

In addition to the increased levels of CGG-containing *FMR1* mRNA seen in fragile X premutation carriers, several lines of evidence further support an RNA-mediated gain-of-function toxicity model for FXTAS. First, in a 'knock-in' mouse model, in which the endogenous CGG repeats (five CGG repeats in the wild-type mouse *Fmr1* gene) were replaced with a ~100 CGG repeat fragment, intranuclear inclusions were found to be present throughout the brain, with the exception of cerebellar Purkinje cells (13). An increase in both the number and size of the inclusions was seen during the life course, which correlates with the progressive character of the phenotype observed in humans (9). Secondly, neuropathological studies in humans have revealed a highly significant association between length of the CGG tract and frequency of intranuclear inclusions in both neurons and astrocytes, indicating that the CGG repeat length is a powerful predictor of neurological involvement clinically (age of death), as well as neuropathologically (number of inclusions) (9). Thirdly, intranuclear inclusions can be formed in both primary neural progenitor cells and established neural cell lines, as was revealed using a reporter construct with an *FMR1* 5′-UTR harboring expanded (premutation) CGG repeats (15). Finally, we have described a model of FXTAS using *Drosophila* expressing the *FMR1* untranslated-CGG repeats 5′ to the enhanced green fluorescent protein (EGFP) coding sequence and demonstrated that premutation-length rCGG repeats are both toxic and sufficient to cause neurodegeneration (11). These observations led us to propose that transcription of the CGG_{90} repeats leads to an RNA-mediated neurodegenerative disease.

Within the last decade, *Drosophila* has emerged as a premiere model system for the study of human neurodegenerative diseases, due to the realization that flies and humans share many structurally and functionally related gene families (16–18). Development of such disease models in the fly allows genetic approaches to be applied to address specific hypotheses concerning disease progression and to test candidate modifier genes or therapeutic drug compounds (16–18). Here, we used our FXTAS *Drosophila* model to conduct a chemical screen and identified the small molecules that can ameliorate the toxic effects of fragile X premutation rCGG repeats, among them several known phospholipase A_2 (PLA_2) inhibitors. We show that specific inhibition of PLA_2 activity could indeed suppress the neuronal toxicity caused by fragile X premutation rCGG repeats. An additional genetic screen led us to identify a PLA_2 *Drosophila* ortholog that could specifically modulate rCGG repeat-mediated neuronal toxicity. Our results reveal a previously unrecognized pathway and druggable target for FXTAS and highlight the general potential of using *Drosophila* for unbiased chemical screens in drug development.

RESULTS

Identification of small molecules that suppress the toxicity caused by fragile X premutation rCGG repeats through a chemical screen

We have previously reported a *Drosophila* disease model of FXTAS and provided experimental evidence that fragile X premutation rCGG repeats alone are sufficient to cause neurodegeneration in a repeat-dosage manner (11). We discovered that flies with modest expression of fragile X premutation $rCGG_{90}$ repeats exclusively in the neurons do not reach adulthood. Lethality occurs primarily during embryonic development before larval formation. Taking advantage of this lethality, we designed a high-throughput strategy to screen chemical libraries for small molecules relevant to FXTAS. We first balanced the UAS-CGG_{90}-EGFP transgene with a chromosome carrying a curly wing (Cyo) marker recombined with a heat shock-inducible apoptotic *hid* (*hs-hid)* transgene (w^{1118}, UAS-CGG_{90}-EGFP/TM2 Cyo:hs-hid). Next, we tested the viability of adult progeny using the *Drosophila* GAL4/UAS system. We performed crosses of UAS-CGG_{90}-EGFP/TM2 Cyo:hs-hid with homozygous pan-neuronal *elav*-Gal4 driver. As expected, there was an absence of heterozygous non-Cyo adult progeny (*elav*-GAL4/+; UAS-CGG_{90}-EGFP/+), which confirmed that $rCGG_{90}$ repeat expression in neurons leads to lethality. The only living progenies would carry *elav*-GAL4/+; +/TM2 Cyo:hs-hid. Furthermore, upon heat shock, the progenies carrying *elav*-GAL4/+; +/TM2 Cyo:hs-hid also died, because the expression of *hid* leads to cell death and lethality at embryonic stages that are otherwise viable.

Since the rescue of the lethality was an easily scored phenotype, we were able to use this assay to conduct a chemical screen to identify the small molecules that can improve/restore the viability of the flies expressing fragile X premutation rCGG repeats. As depicted in schematic representation (Fig. 1A), we raised crosses between *elav*-GAL4 and UAS-CGG_{90}-EGFP/TM2 Cyo:hs-hid as well as control flies into food supplemented with 40 μM of individual unique compounds from a library of 2000 US Food and Drug Administration (FDA)-approved drugs and natural products (The Spectrum Collection, MicroSource Discovery Systems, Inc.). At day 2, crosses were subsequently heat shocked to eliminate (*elav*-GAL4/+; +/TM2 Cyo:hs-hid) progenies, and the vials were then kept at 25°C for 10–15 days to score for any of the viable (*elav*-GAL4/+; UAS-CGG_{90}-EGFP/+) flies that are otherwise lethal. Among the 2000 compounds initially screened, 58 were found to result in either puparium formation or the emergence of adult non-Cyo progeny (*elav*-GAL4/+; UAS-CGG_{90}-EGFP/+). Since the puparium formation could also represent dead (*elav*-GAL4/+; +/TM2 Cyo:hs-hid) progeny simply due to heat shock, we selected the top 35% (20 of 58 compounds) based on the percentage of recovered flies or pupae from the crosses for further validation. Among these 20 compounds, 11 were validated using large-scale viability assays (Fig. 1B). The confirmed compounds belong to several biochemically distinct pathways, with several of them having the potential to target inflammatory pathways (Fig. 1B).

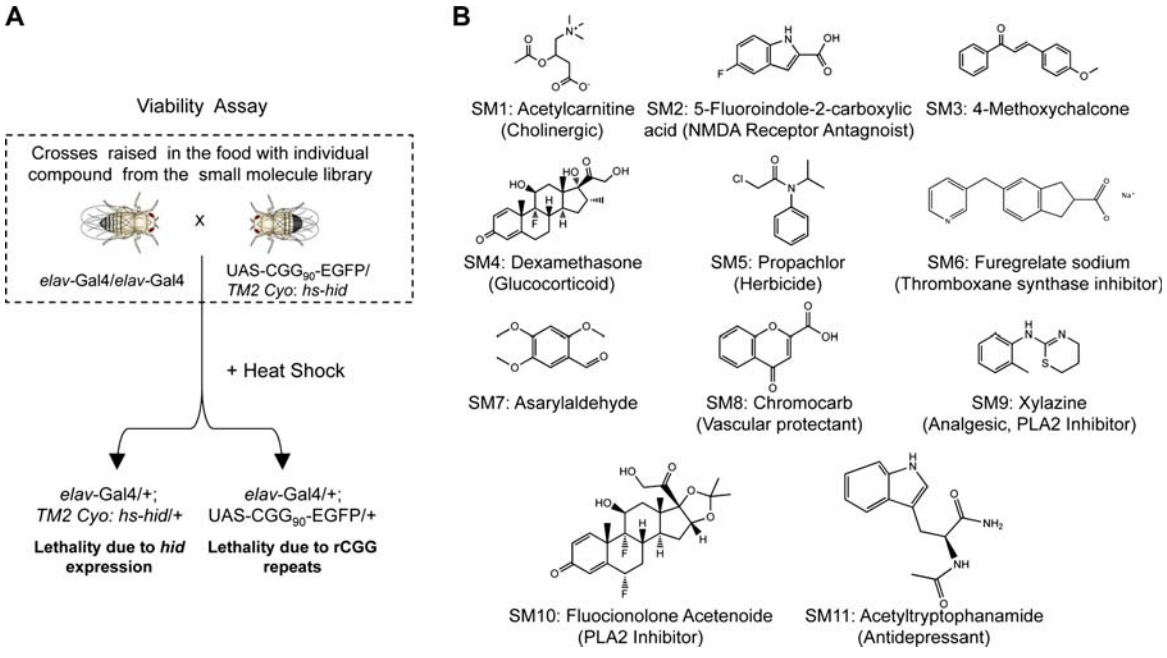

Figure 1. Identification of small molecules suppressing the toxicity caused by fragile X premutation rCGG repeats through a chemical screen. (**A**) Schematic representation illustrating a chemical genetic screen for small molecules that can suppress fragile X premutation rCGG repeat-mediated lethality. *Elav-Gal4* driver and UAS-CGG_{90}-EGFP/Cyo:hs-hid were crossed to produce progeny embryos on food supplemented with or without individual unique compounds from a library of 2000 small molecules. The progeny embryos continued to develop on food supplemented with individual drug until progeny eclosed. Relative viability was obtained by comparing numbers of adult progeny or pupae based on their genotype after heat shock was administered. (**B**) Chemical structures of 11 small molecules that could suppress the toxicity caused by fragile X premutation rCGG repeats are shown.

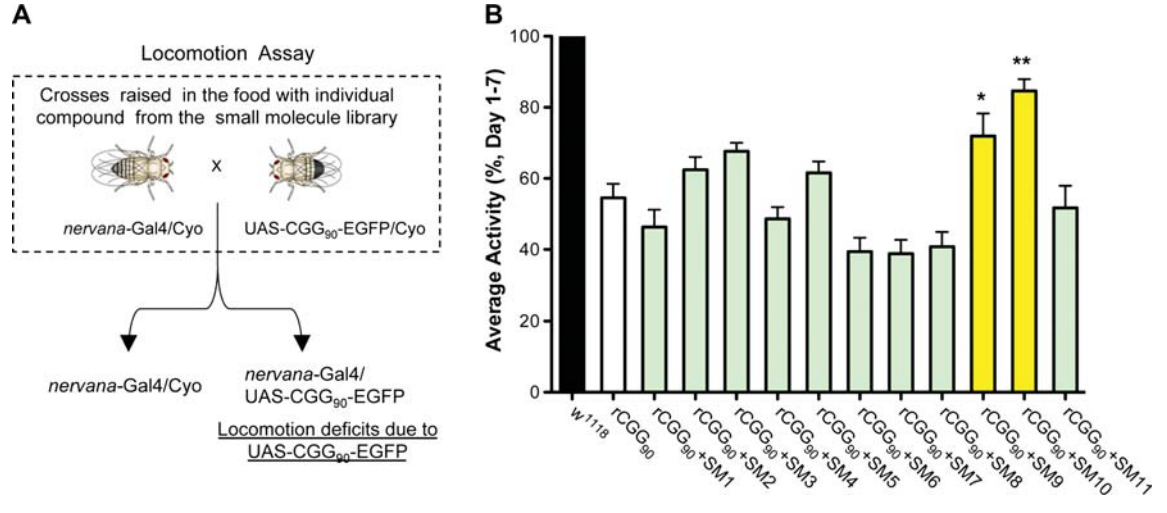

Figure 2. Identification of small molecules that can ameliorate the locomotion deficits caused by fragile X premutation rCGG repeats. (**A**) *nervana*-GAL4 driver and UAS-CGG_{90}-EGFP/Cyo were crossed to produce progeny embryos on food supplemented with or without selected compounds as indicated. Adult progeny of genotype UAS-CGG_{90}-EGFP/*nervana* were selected and 32 female virgins 48 h old were subsequently monitored for locomotor activity continuously for several weeks. We simultaneously monitored the locomotion of 32 flies that were fed with fly food containing the compound each time. (**B**) The percentage of mean locomotor activity over a period of 7 days was plotted for selected drugs. Error bars indicate SEM. $^{**}P < 0.001$, $^{*}P < 0.01$.

Identification of small molecules that can ameliorate the locomotion deficits caused by fragile X premutation rCGG repeats

To further validate these compounds, we developed a secondary behavioral assay. In this assay, UAS-CGG_{90}-EGFP transgenic flies were crossed with *nervana-GAL4*, which could drive the expression of fragile X rCGG repeats in CNS, albeit weakly, allowing us to analyze adult flies for locomotion deficits. The expression of rCGG repeats leads to locomotion defects (Fig. 2). As shown in Figure 2A, we set up the cross between UAS-CGG_{90}-EGFP/TM2 Cyo and *nervana-GAL4* in food that was supplemented with each compound identified above. The progeny flies (UAS-CGG_{90}-EGFP/*nervana-GAL4*) were collected to monitor locomotion. To continuously

monitor the locomotion for a given fly, we have established a *Drosophila* Activity Monitor (DAM) system for this purpose (19). For each individual compound, we simultaneously monitored the locomotion of 32 flies that were fed with fly food containing the compound each time. The data were collected and analyzed for locomotor activity (Fig. 2B). Each drug treatment was repeated at least three times. Through this secondary testing, we found that fluocinolone acetonide and xylazine could significantly ameliorate the locomotion defects induced by fragile X premutation rCGG repeats (Fig. 2B). The other compounds had either mild effects or no effect on locomotion deficits induced by rCGG repeats.

Phospholipase A_2 inhibitors can specifically suppress locomotion deficits caused by fragile X premutation rCGG repeats

Intriguingly, fluocinolone acetonide is a well-known inhibitor of phospholipase A2 (PLA_2) (20). The PLA_2 family includes secretory phospholipase A2, cytosolic phospholipase A2, plasmalogen-selective phospholipase A2 and calcium-independent phospholipase A2 (21,22). It is generally thought that the release of arachidonic acid by cytosolic PLA_2 is the rate-limiting step in the generation of eicosanoids and platelet-activating factor. These lipid mediators play critical roles in the initiation and modulation of inflammation and oxidative stress (21,22). The tight regulation of PLA_2 activity is necessary for maintaining basal levels of arachidonic acid and eicosanoids for performing normal function (21,22). Our finding that fluocinolone acetonide could suppress both lethality and locomotion deficits caused by fragile X premutation rCGG repeats raised the possibility that PLA_2 inhibitors in general could suppress rCGG-mediated neurodegeneration. To test this hypothesis, we used other known PLA_2 inhibitors for locomotion assays. Cytidine 5'diphospho-choline (citicoline) inhibits $cPLA_2$ activity and lowers the concentration of free fatty acids in a dose- and time-dependent manner (23). Likewise, release of arachidonic acid can be blocked by quinacrine and arachidonyl trifluoromethylketone (Fig. 3) (24,25). We observed significant improvements in locomotion in flies expressing rCGG repeats for all the PLA_2 inhibitors, whereas these compounds had no significant impact on the locomotor activity of wild-type flies (Fig. 3). These observations strongly suggest that PLA_2 activity is altered by fragile X premutation rCGG repeats, and PLA_2 inhibitors could suppress rCGG-mediated neurodegeneration.

Candidate genetic screen identifies the *Drosophila* ortholog of the phospholipase A2 gene, CG1583, as the modulator of rCGG repeat-mediated neuronal toxicity

Given our findings from the chemical screen, we sought to harness the power of *Drosophila* genetics to explore the role of PLA_2 isoforms in mediating fragile X premutation rCGG repeat-mediated neuronal toxicity. In *Drosophila,* the genes for some PLA_2 isoforms have been mapped, but none has been cloned and fully characterized; therefore, to examine the role of the PLA_2 pathway in FXTAS, we used the Kyoto encyclopedia of genes and genomes (KEGG) human database to identify and collect known pathways that are related to lipid

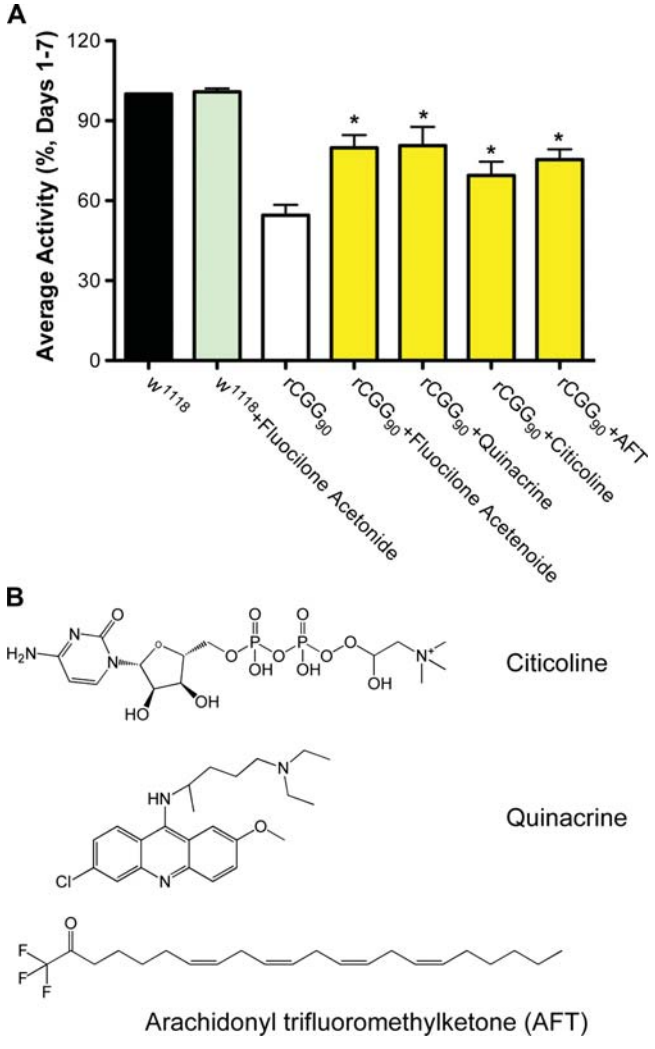

Figure 3. Phospholipase A_2 inhibitors can specifically suppress locomotion deficits caused by fragile X premutation rCGG repeats. (**A**) The percentage of mean locomotor activity over a period of 7 days was plotted for multiple PLA_2 inhibitors. Error bars indicate SEM; $^*P < 0.001$. (**B**) Chemical structures of additional PLA_2 inhibitors used for locomotion assays are shown.

metabolism and PLA_2 activity (26). We then created reference maps for the well-known PLA_2 pathways and looked for the genes and pathways that are related in flies (Table 1). We identified mutations in some of the genes of the pathway and conducted a genetic screen based on the fragile X premutation rCGG repeat-mediated neurodegenerative eye phenotype that we saw previously. The screen involved directing the expression of fragile X premutation-length rCGG repeats to the eye with the *gmr-GAL4* driver. This was followed by crossing *gmr-GAL4, UAS-(CGG)$_{90}$-EGFP* transgenic flies with flies mutant in genes coding for lipid metabolism and PLA_2 activity identified from the reference KEGG map. The progenies were examined for potential suppression or enhancement of the disorganized eye phenotype by comparison with control rCGG flies. Through this screen, we identified an EP line, *CG1583*^{EP1516} (a *Drosophila* homolog of PLA_2 gene), that could modulate the rCGG-mediated neurodegeneration (Fig. 4A). Since *CG1583*^{EP1516} is an EP insertion that is supposed to activate the transcription of the adjacent gene, we

Table 1. Genetic interaction between Gmr:CGG90-EGFP/+ and candidate mutant alleles corresponding to lipid and arachidonic acid metabolism

Annotation/gene	Orthology	Pathway	Alleles analyzed	Phenotypic effect on gmr:CGG90-EGFP/+
CG10706/SK	Phospholipase A2	Glycerophospholipid metabolism, ether lipid metabolism, arachidonic acid metabolism, linoleic acid metabolism, alpha-linolenic acid metabolism	$SK^{BG01378}$	—
			SK^{d11567}	—
CG11124/sPLA2			$sPLA2^{v13911}$	—
CG14507			$CG14507^{v35712}$	—
			$CG14507^{v35713}$	
CG17035/ GXIVsPLA2			$GXIVsPLA2^{G16441}$	—
			$GXIVsPLA2^{f00744}$	
			$GXIVsPLA2^{v44441}$/Cyo	
			$GXIVsPLA2^{v44443}$/Cyo	
CG42628/radish			Rad^{v39931}	—
			Rad^{v39932}	
CG1583			$GIIIsPLA2^{EP325}$	—
			$GIIIsPLA2^{EP1516}$	Suppression
			$GIIIsPLA2^{v50353}$	Suppression
			$GIIIsPLA2^{v8927}$/CyO	—
CG3009			$CG3009^{v12216}$	—
CG10133			$CG10133^{v18014}$	—
			$CG10133^{KG04150}$	
CG6718/ iPLA2-VIA			$iPLA2\text{-}VIA^{EY05103}$	—
			$iPLA2\text{-}VIA^{v260B}$	
CG10672	Carbonyl reductase (NADPH)	Arachidonic acid metabolism	$CG10672^{KG07864}$	—
			$CG10672^{v18661}$	
			$CG10672^{v18662}$	
CG10964/Sniffer			$Sni^{KG00373}$	—
			$Sni^{EY20992}$	
CG10602	Leukotriene-A4 hydrolase		$mRpL13^{f04195}$/CyO	—
			$CG10602^{v31280}$/CyO	
CG12013	Glutathione peroxidase	Glutathione metabolism, arachidonic acid metabolism	$PHGPx^{G7172}$	—
GstD8 (Glutathione S transferase D8)			$GstD8^{v46938}$/CyO	—
			$GstD8^{v46939}$/Cyo	—
CG1492	Gamma-glutamyltranspeptidase	Arachidonic acid metabolism, taurine and hypotaurine metabolism, selenoamino acid metabolism, cyanoamino acid metabolism, glutathione metabolism	$CG1492^{v8319}$/TM3	—
CG6461			$Ggt\text{-}1^{d02194}$; $Ggt\text{-}1^{d06929}$	—

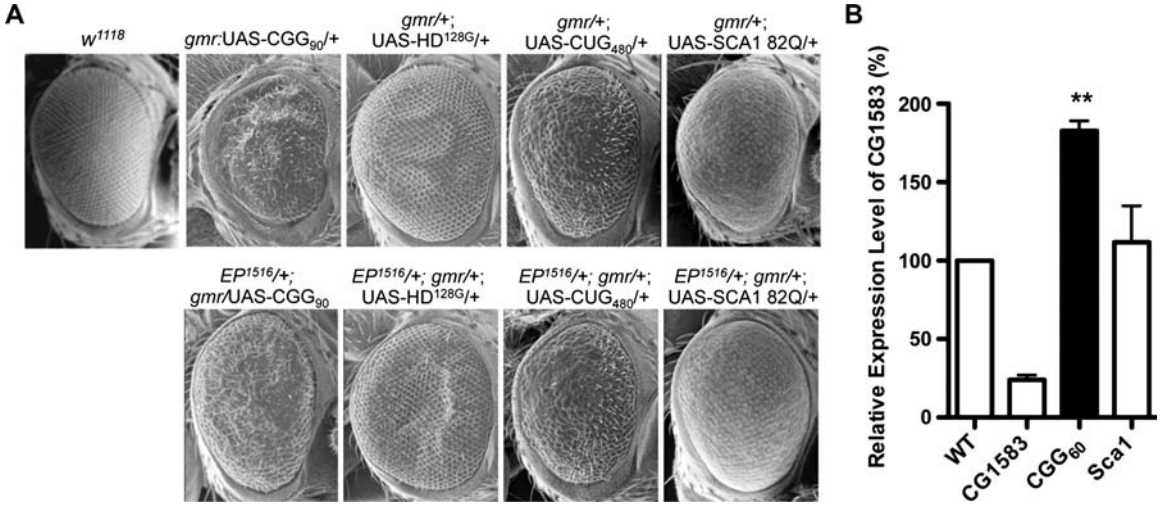

Figure 4. *CG1583*, a predicted *Drosophila* ortholog of PLA_2, specifically modulates fragile X premutation rCGG-mediated neurodegeneration. (**A**) *CG1583* modulates rCGG-mediated neurodegeneration in fly. Top panel shows control (w^{1118}) fly (column 1); and *gmr-GAL4* directed expression of the following transgenes: UAS-$(CGG)_{90}$-EGFP (column 2), UAS-HD^{128G}/+ (column 3); UAS-CUG_{480}/+ (column 4); UAS-SCA1 82Q/+ (column 5). Bottom panel shows the modulation of the eye phenotype in fly expressing CGG_{90}-EGFP with the heterozygous background of $CG1583^{EP1516}$ mutation (column 1); column 2: fly expressing UAS-HD^{128G}/+ in the heterozygous background of $CG1583^{EP1516}$ mutation (column 2); fly expressing UAS-CUG_{480}/+ in the heterozygous background of $CG1583^{EP1516}$ mutation (column 3); fly expressing UAS-SCA1 82Q/+ in the heterozygous background of $CG1583^{EP1516}$ mutation (column 4). The suppression between the genotypes was scored blindly as normal, moderate or severe. Scanning electron microscopic eye images are shown. (**B**) Quantitative analysis of the *CG1583* mRNA levels by real-time PCR from the adult brains of flies with the following genotypes: wild-type (w^{1118}); $CG1583^{EP1516}$; elav; $rCGG_{60}$-EGFP; and UAS-SCA1 82Q/+. Housekeeping ribosomal protein 32 (*Rpl32*) mRNA was used as an internal control (mean ± SEM; $n = 3$).

performed quantitative reverse transcription polymerase chain reaction (RT–PCR) using the RNAs isolated from age-matched brains to determine the expression of CG1583 in both $CG1583^{EP1516}$ and control (w^{1118}) flies. We found that $CG1583^{EP1516}$ specifically disrupted the *CG1583* gene and led to the partial loss of gene expression (Fig. 4B). We also found that an adjacent gene, *Traf 6*, did not interact genetically with rCGG-mediated neurodegeneration. This modulation was further confirmed using an RNAi UAS line ($GIIIsPLA2^{v50353}$) that could express dsRNAs against *CG1583* in the presence of a GAL4 driver (Table 1).

CG1583 is a specific modifier of rCGG-mediated neurodegeneration

Since PLA_2 has been linked to several neurological disorders (21,22,27–30), we sought to determine whether CG1583 is a specific modifier of rCGG-mediated toxicity. We crossed $CG1583^{EP151}$ with fly models of other neurodegenerative disorders. Like fragile X premutation rCGG repeats, transgenic flies expressing mutant Huntington's disease (HD), cytosine uracil guanine (CUG) repeats or SCA1 show rough eye phenotype when crossed with *gmr*-Gal4 driver (18,31,32). As shown in Figure 4A, the partial loss of CG1583 had no effect on the neuronal eye degeneration caused by mutant HD, CUG repeats or SCA1. We further determined the expression of CG1583 using RNAs isolated from the age- and sex-matched brains of w^{1118} and flies expressing CGG_{60} repeats in neurons (elav/+; UAS-CGG_{60}-EGFP). We found that fragile X premutation rCGG repeats, but not SCA1, could cause increased expression of CG1583 in fly brains (Fig. 4B). These results together suggest that CG1583, a *Drosophila* ortholog of PLA_2, is a specific modulator of rCGG-mediated neurodegeneration.

DISCUSSION

FXTAS, a late age of onset neurodegenerative disorder uncoupled from the neurodevelopmental disorder, FXS, has been recognized in older males of FXS families. Several lines of evidence, including ours, have led to the proposal of an RNA-mediated gain-of-function toxicity model for FXTAS, in which rCGG repeat-binding proteins could become functionally limited by their sequestration to lengthy rCGG repeats (33–35). There is currently no effective therapeutic intervention available for FXTAS (36). In recent years, *Drosophila* has emerged as a premiere model system for the study of human neurodegenerative diseases, due to that flies and humans share many structurally and functionally related gene families (16–18,31,32). Development of fly disease models allows us to address specific hypotheses concerning disease progression, test candidate modifier genes or therapeutic drug compounds and potentially identify novel small molecules via unbiased chemical screens. We have previously established a *Drosophila* model and shown that fragile X premutation rCGG repeats are sufficient to cause neuronal toxicity (11). Here, we used this same model to conduct an unbiased chemical screen and identified the small molecules that can ameliorate the toxic effects of fragile X premutation rCGG repeats. Specifically, we found that inhibition of PLA_2 activity could suppress neuronal toxicity caused by fragile X premutation rCGG repeats. Our results reveal a previously unrecognized pathway and druggable target for FXTAS and highlight the general potential of using *Drosophila* for unbiased chemical screens in drug development.

The discovery of lead compounds that have the potential to elicit a pharmacological effect is often achieved via the screening of small molecule libraries for interaction with purified proteins, or for an ability to induce a desired physiological response in cultured cells. Since complex biological processes cannot be recapitulated in cell or organ culture, efforts are now underway to perform chemical screens on whole animals. One of the most commonly used animal models in biology is mouse; however, its cost limits its use in large-scale therapeutic screening. Fortunately, extensive research during the past two decades, coupled with the elucidation of annotated genome sequences for human and other eukaryotic organisms, has revealed a remarkable degree of conservation of biochemical pathways. This conservation has made it feasible to consider using invertebrate models as tools for drug discovery and validation (37). Indeed, fly models of human neurodegenerative disorders, such as Huntington's disease, Parkinson's disease (PD) and Alzheimer's disease (AD), have been used to validate candidate compounds for therapeutic interventions (17). However, whether fly models could be used for unbiased chemical screens or to reveal new biological pathway(s) has not been explored. Our results here indicate that a fly model of a human neurological disorder could indeed be used for an unbiased chemical screen. Through our screen, we revealed altered PLA_2 activity in the presence of fragile X premutation rCGG repeats, which is particularly interesting, since many PLA_2 inhibitors have been or are being developed for therapeutic use.

The biological functions of the compounds identified through our initial screen implicate various mechanisms of action (Fig. 1B). In particular, our results suggest that PLA_2 inhibitors could be potent suppressors of rCGG-mediated neuronal toxicity. Indeed, altered PLA_2 activity has been linked to several neurodegenerative diseases and brain trauma. A number of neurodegenerative disorders, including AD, PD and multiple sclerosis, as well as acute neural trauma, such as ischemia, spinal cord injury and head injury, have been characterized by inflammatory reactions, oxidative stress, altered phospholipid metabolism, accumulation of lipid peroxides and increased phospholipase A (21,22,27,30). Interestingly, several PLA_2 inhibitors active in our assays are known to exert their neurodegeneration-preventive effects by suppressing or lowering transcription of genes for PLA_2 isozymes (22). In our FXTAS fly model, we found that CG1583, a PLA_2 ortholog, could modulate the neuronal toxicity caused by fragile X premutation rCGG repeats. Furthermore, the total steady-state level of CG1583 mRNA was also elevated in the fly brain expressing fragile X rCGG repeats. These findings suggest that the altered PLA_2 activity could directly or indirectly contribute to the molecular pathogenesis of FXTAS, which could make an important therapeutic target for FXTAS.

The remaining compounds identified through our screen may also be viable candidate compounds for drug

development. In particular, acetylcarnitine, a cholinergic drug, has been used to prevent neuronal cell death. It has been reported as neuroprotective in instances of cerebral ischemia in rats and may be useful in treating peripheral nerve injury; it may also be of benefit in the treatment of PD (38).

In summary, we have conducted an unbiased chemical screen using the FXTAS fly model that we developed previously. Through this screen, we identified the small molecules that can ameliorate the toxic effects of fragile X premutation rCGG repeats. In particular, we show that specific inhibition of PLA_2 activity could indeed suppress neuronal toxicity caused by fragile X premutation rCGG repeats. Our results reveal a previously unrecognized pathway and druggable target for FXTAS and highlight the general potential of using *Drosophila* for unbiased chemical screens in drug development.

MATERIALS AND METHODS

Drosophila genetics

Transgenic flies expressing $rCGG_{90}$ and $rCGG_{60}$ repeats were described previously. The *Cyo:hs-hid, elav*-Gal4, *TM2* chromosome balancer fly line and $CG1583^{EP1516}$ insertions were obtained from Bloomington Stock Centre (Bloomington, IN, USA). Flies of other neurological disease models, including UAS-HD^{128G}/+, UAS-CUG_{480}/+ and UAS-SCA1 82Q/+, were obtained from Juan Botas at Baylor College of Medicine. Other lesions used for genetic interactions as depicted in Table 1 were obtained from either Bloomington or Vienna Drosophila RNAi Center, Austria. All crosses were grown on standard medium at 25°C in 50–70% humidity.

Small molecule screen and validation test

Flies carrying the UAS-CGG_{90}-EGFP transgene over a heat shock-inducible *hid*-balancer chromosome (*Cyo:hs-hid, TM2*) were generated for viability assay. Progeny embryos from the crosses between UAS-CGG_{90}-EGFP/Cyo:hs-hid and homozygous pan-neuronal *elav*-Gal4 driver were subjected to heat shock to ensure *hid*-induced lethality. The Spectrum Collection compound library was obtained from MicroSource Discovery Systems, Inc. In the initial screen, crosses were performed in small vials with 40 μM of individual drugs. Crosses were kept at 25°C, and the percentages of pupae and adults recovered were scored. Drugs used in larger quantities for confirmation and subsequent analyses were obtained from Sigma. Drugs supplemented with Jazz-Mix commercial food (CF) for the screen were added to the final concentration of 40 μM at 50°C for uniform drug dispersion in the food. For the confirmation of the 11 compounds from the initial screen, viability assays were performed on a larger scale using 5 μM and 40 μM doses of the drug.

Locomotion assay

UAS-CGG_{90}-EGFP/Cyo transgenic flies were crossed with *nervana*-Gal4/Cyo in a CF and in CF plus drug from the top 11 compounds. Drugs were used at their optimum dose and mixed with CF at 50°C for homogenous distribution. Homozygous progeny females (UAS-CGG_{90}-EGFP/*nervana*-Gal4) 48 h of age were collected for locomotor activity assays. To continuously monitor the locomotion, a DAM system (TriKinetics) was used. For each individual compound, we simultaneously monitored the locomotion of 32 flies that were treated. The readings were recorded each time the fly crossed the infrared beam. These interruptions were collected as a bin of 1 h. The activity of flies was calculated as the average number of movements in a given time recorded together with control flies. Throughout the assay, UAS-CGG_{90}-EGFP/*nervana*-Gal4 females were maintained in a drug in which they were initially raised. Since behavior is often sexually dimorphic, only 48 h old virgin female flies were used in the assay. Data were collected and analyzed for locomotor activity.

RNA isolation and quantitative RT–PCR

For the RNA isolation, adult heads of the required genotype were mashed in Trizol (Gibco BRL Life Technologies) using plastic kontes. After precipitation, RNA was further cleaned with an RNeasy Kit (QIAGEN). For RT–PCR, RNA was reverse-transcribed with random primers using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed with commercial TaqMan probes obtained against CG1583, and TaqMan Master Mix (Applied Biosystems) using a 7500 Fast Real-Time PCR system (Applied Biosystems). TaqMan probes Dm_RpL32 (Applied Biosystems) were used as internal control.

Statistical methods

We used DAM Scan software (TriKinetics) to detect any outliers in the readings obtained. The readings obtained in a bin of 1 h were averaged over 24 h. Recordings for a period of 7 days for each genotype per drug were plotted. Analyses to show significant differences between genotypes/treatments were performed using analysis of variance, *post hoc t*-tests (two sample assuming equal variances). All data are shown as mean with standard error of mean.

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Conflict of Interest statement. None declared.

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