



# A Novel Genetic Screen Identifies Modifiers of Age-Dependent Amyloid $\beta$ Toxicity in the *Drosophila* Brain

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The accumulation of amyloid  $\beta$  peptide (A $\beta$ ) in the brain of Alzheimer's disease (AD) patients begins many years before clinical onset. Such process has been proposed to be pathogenic through the toxicity of A $\beta$  soluble oligomers leading to synaptic dysfunction, phospho-tau aggregation and neuronal loss. Yet, a massive accumulation of A $\beta$  can be found in approximately 30% of aged individuals with preserved cognitive function. Therefore, within the frame of the "amyloid hypothesis", compensatory mechanisms and/or additional neurotoxic or protective factors need to be considered and investigated. Here we describe a modifier genetic screen in *Drosophila* designed to identify genes that modulate toxicity of A $\beta$ 42 in the CNS. The expression of A $\beta$ 42 led to its accumulation in the brain and a moderate impairment of negative geotaxis at 18 days post-eclosion (d.p.e) as compared with genetic or parental controls. These flies were mated with a collection of lines carrying chromosomal deletions and negative geotaxis was assessed at 5 and 18 d.p.e. Our screen is the first to take into account all of the following features, relevant to sporadic AD: (1) pan-neuronal expression of wild-type A $\beta$ 42; (2) a quantifiable complex behavior; (3) A $\beta$  neurotoxicity associated with progressive accumulation of the peptide; and (4) improvement or worsening of climbing ability only evident in aged animals. One hundred and ninety-nine deficiency (Df) lines accounting for ~6300 genes were analyzed. Six lines, including the deletion of 52 *Drosophila* genes with human orthologs, significantly modified A $\beta$ 42 neurotoxicity in 18-day-old flies. So far, we have validated CG11796 and identified CG17249 as a strong candidate (whose human orthologs are *HPD* and *PRCC*, respectively) by using RNAi or mutant hemizygous lines. *PRCC* encodes proline-rich protein PRCC (ppPRCC) of unknown function associated with papillary renal cell carcinoma. *HPD* encodes 4-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme in tyrosine degradation whose Df causes autosomal recessive Tyrosinemia type 3, characterized by mental retardation. Interestingly, lines with a partial Df of *HPD* ortholog showed increased

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**Abbreviations:** AD, Alzheimer's disease; A $\beta$ , amyloid  $\beta$  peptide; A $\beta$ 42, amyloid  $\beta$  peptide 1-42; CK, creatine kinases; Df, deficiency; DIOPT, *Drosophila* RNAi Screen Center Integrative Ortholog Prediction Tool; dpe, days post-eclosion; G4, Gal4; H&E, hematoxylin-eosin; HPPD, 4-hydroxy-phenylpyruvate dioxygenase; PBS, phosphate buffered saline; Ppil2, peptidylprolyl isomerase-like 2; ppPRCC, proline-rich protein PRCC; qRT-PCR, quantitative real-time PCR; RING, Rapid Iterative Negative Geotaxis; RM, repeated measures; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ThS, thioflavine S; UAS, upstream activating sequence.

intraneuronal accumulation of A $\beta$ 42 that coincided with geotaxis impairment. These previously undetected modifiers of A $\beta$ 42 neurotoxicity in *Drosophila* warrant further study to validate their possible role and significance in the pathogenesis of sporadic AD.

**Keywords:** amyloid  $\beta$ , Alzheimer's disease, neurodegeneration, genetic screen, *Drosophila*, dementia

## INTRODUCTION

Alzheimer's disease (AD) is the most prevalent form of dementia in the aged population worldwide and its impact is steadily growing due to the extension of life expectancy (Cacace et al., 2016; Scheltens et al., 2016). More than 95% of AD cases are sporadic, with age and the epsilon 4 allele of the apolipoprotein E gene as the major risk factors. Rare familial forms are associated with mutations in the amyloid precursor protein and presenilin 1–2 genes (Campion et al., 1995; Newman et al., 2007; Kandimalla et al., 2011, 2012; De Strooper and Karran, 2016).

AD brain is characterized by a pervasive synaptic loss and the accumulation of protein aggregates mostly composed of A $\beta$ 42 and microtubule-associated protein tau. Oligomeric species of A $\beta$ 42 have been proposed as early pathogenic molecules by inducing mitochondrial and endoplasmic reticulum stress, an increase in reactive oxygen species formation and action potential abnormalities (Karran et al., 2011; De Strooper and Karran, 2016). AD tau is excessively phosphorylated and aggregates intracellularly leading to microtubule instability and organelle failure (Khan and Bloom, 2016). However, the accumulation of A $\beta$  and phospho-tau is not sufficient for the development of AD. Large autopsy series show that about 30%–40% of individuals can sustain a normal or nearly normal cognitive function at a very old age despite extensive A $\beta$  and phospho-tau pathology (Bennett et al., 2006; Maarouf et al., 2011; Perez-Nievas et al., 2013). Several hypothesis have been put forward to explain such clinico-pathological dissociation, including differences in “cognitive/brain reserve” or the presence of compensatory mechanisms at a functional or molecular level (Maarouf et al., 2011; Steffener and Stern, 2012). In this context, the search for novel genetic and epigenetic factors that partake in neurotoxicity mechanisms related to A $\beta$  is of key importance for understanding the disease process.

*Drosophila* is widely used for genetic screens applied to study the molecular bases of neurodegenerative disorders including AD (Crowther et al., 2005; Moloney et al., 2010; Lenz et al., 2013; Prüßing et al., 2013; Shulman et al., 2014; Fernandez-Funez et al., 2015; Liu et al., 2015). Major advantages of this animal model include a complex CNS, the fact that about 70% of human genetic diseases have a *Drosophila* genetic counterpart (Jackson, 2008; Bouleau and Tricoire, 2015; Lim et al., 2016) and the availability of large collections of mutant and transgenic lines.

Forward genetic screens in *Drosophila* have been used to identify modifiers of A $\beta$  neurotoxicity. Cao et al. (2008) used a collection of transgenic lines carrying directionally inserted P elements and screened for enhancers or suppressors of a rough eye phenotype induced by A $\beta$ 42. In this way, they

identified candidate genes involved in cellular processes such as transcription regulation, proteolysis in the secretory pathway and cholesterol metabolism (Finelli et al., 2004; Cao et al., 2008). By screening a collection of chromosomal deletions, the same group found that the toll-NF $\kappa$ B pathway enhanced both A $\beta$ -induced rough eye and a negative effect upon life span (Tan et al., 2008). Rival et al. (2009) screened 3000 lines carrying P element inserts for modifiers of a shorter life span induced by the “Arctic” variant of A $\beta$ 42 (A $\beta$ E22G) associated with familial AD. Notably, they found that genes associated with redox or antioxidant activities were strong modifiers of A $\beta$ E22G neurotoxicity (Rival et al., 2009). By inducing misexpression of genes involved in specific developmental pathways, several modifiers of A $\beta$ 42 toxicity upon photoreceptors have been described (Moran et al., 2013). In addition to the eye phenotype and life span, the gravitaxis behavior (negative geotaxis) can be used for genetic screening. This test provides easily quantifiable data, explores a complex behavior of the *Drosophila* CNS and allows a rapid assessment of age-dependent A $\beta$  toxicity. Recently, Liu et al. (2015) developed an automatic device for the Rapid Iterative Negative Geotaxis (RING) assay and screened a collection of chromosomal deletions to find modifiers of A $\beta$ E22G neurotoxicity upon the giant fiber system neurons (Gargano et al., 2005; Liu et al., 2015).

The aim of the present study was to develop a modifier screen designed to study the effect of chromosomal deletions upon neuronal toxicity mediated by pan-neural expression of wild-type A $\beta$ 42 in the CNS (the major isoform that accumulates in the brain of sporadic AD patients). Fly lines with defined genomic deletions were found to exert a dominant effect under the presence of A $\beta$ 42. Deficiency (Df) lines that significantly enhanced age-dependent A $\beta$ 42 toxicity included *CG17249* and *CG11796* whose human orthologs are *PRCC* and *HPD*, respectively. *PRCC* encodes proline-rich protein PRCC (ppPRCC), a protein of unknown function associated with renal cell carcinomas. *HPD* encodes 4-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme in tyrosine degradation.

## MATERIALS AND METHODS

### Fly Stocks

Flies were raised at 25°C in a standard corn meal with a light:dark cycle of 12 h:12 h. The line expressing A $\beta$ 1–42 fused with the rat pre-proenkephalin signal peptide was kindly provided by Dr. Mary Konsolaki (Rutgers University). The upstream activating sequence (UAS)-A $\beta$ 42 construct is inserted in the 2nd chromosome. Lines *w*<sup>1118</sup> #5905 (+), *elav*<sup>c155</sup> [Gal4] #458 (G4), lines from the Df kit and the mutants for *CG11796* #51528 and

CG17249 #16098 were obtained from Bloomington *Drosophila* Stock Center (NIH P00D018537). The CG11796 RNAi line #103482 was obtained from VDRC Stock Center. The *elav* [Gal4]; [UAS] A $\beta$ 42/Cyo line (G4 > A $\beta$ 42) was generated for the screen.

## RING Assay

Groups of 30–40 male flies were raised at 25°C in 4-inch glass vials with food replacement every 2–3 days. The geotaxis behavior was tested using the RING assay as described (Gargano et al., 2005). The day before the test, 10 flies were shortly anesthetized with CO<sub>2</sub> and placed into a fresh vial. They were let to recover overnight at 25°C, transferred to clear glass vials and placed them in the negative geotaxis device. The device was tapped three times in rapid succession to initiate the response and climbing was recorded for 10 s. The climbed distance in cm was measured for each fly and the average height from five technical replicates per genotype was calculated using the Scion Image software.

## SDS-PAGE and Western Blots

Forty heads from 5 to 18-day-old flies were homogenized in 60  $\mu$ l of RIPA buffer, pH 7.4, containing 1% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 0.5  $\mu$ g/ml leupeptin, 0.5  $\mu$ g/ml aprotinin, 1 mg/ml pepstatin and 50 mM NaF. Homogenates were centrifuged at 10,000 $\times$  g for 1 h at 4°C. Twenty  $\mu$ l of the supernatant containing  $\sim$ 150  $\mu$ g of total proteins, were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% Tris-tricine gel. After transfer to polyvinylidene fluoride membranes, proteins were analyzed by Western blot. A $\beta$ 42 was detected with anti-A $\beta$  monoclonal 6E10 (Biolegend Co.) used at 1:1000. Actin was detected with rabbit polyclonal anti-actin (Sigma) at 1:1000. After washing with PBS-T, membranes were incubated anti-rabbit or anti-mouse horseradish peroxidase-labeled IgGs (Dako, Denmark) at 1:10000. Immunoreactivity was visualized by chemiluminescence with ECL Prime (GE Bioscience, Piscataway, NJ, USA) and scanned with an Image Quant LAS 4000 apparatus (GE Bioscience, Piscataway, NJ, USA). For relative quantitation, optical densities from each lane were obtained and analyzed with the ImageJ software. Synthetic A $\beta$ 1-42 was obtained from American Peptide Co.

## Immunohistochemistry and Thioflavin S Staining

Adult heads were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 45 min at room temperature (RT). Fly brains were dissected in PBS containing 0.1% Triton X-100 (PT). Brains were blocked in 10% normal goat serum for 1 h in PT and incubated with antibody 6E10 at 4°C overnight. After incubation with Cy3-labeled anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at RT, brain tissue was stained with DAPI, washed with PBS and mounted in PBS containing 80% glycerol. For amyloid fibril staining, brains were incubated in 50% ethanol containing 1% thioflavine S (ThS; Sigma, St.Louis, MO, USA) overnight

at 4°C. Samples were washed with PBS containing 50% ethanol and mounted in 80% glycerol. Brain samples from a transgenic mouse carrying the “Swedish” mutation of amyloid precursor protein (Tg2576) were used as positive controls. Images were captured with a Zeiss LSM 510 Meta Confocal microscope.

## Histology and Vacuolization Assessment

Fly heads were fixed overnight in Carnoy solution (60% ethanol, 30% chloroform, 10% acetic acid) at 4°C and dehydrated in increasing concentrations of ethanol. Then, they were treated with butanol:ethanol (1:1), butanol:toluene (1:1) and toluene 30 min each, and finally soaked in toluene:paraffin (1:1) for 30 min at 65°C. After a 2-h incubation at 65°C in pure paraffin, heads were embedded and cut in 8  $\mu$ m serial frontal sections. After H&E staining, images were captured using an OLYMPUS B  $\times$  50 Microscope and analyzed with the ImageJ software. Brain tissue loss was quantified as described (Sarantseva et al., 2009). The area occupied by vacuoles with a diameter of at least 3  $\mu$ m was divided by the total area of the section and expressed as percentage of area loss. At least eight brains per genotype were analyzed.

## Genetic Screen

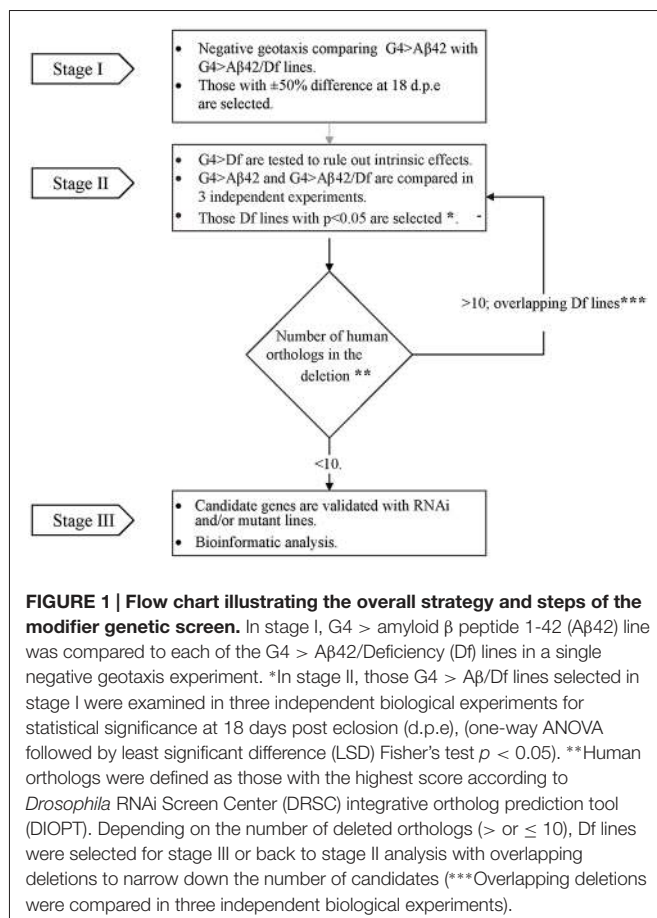
To perform the genetic screen, the G4 > A $\beta$ 42 line was mated with Df lines from the Bloomington Df kit (Cook et al., 2012; Cook, 2016) to generate *elav*<sup>c155</sup> [Gal4]; [UAS] A $\beta$ 42/+> Df/+ (G4 > A $\beta$ 42/Df). The experimental design consisted of three stages (Figure 1). In stage I, G4 > A $\beta$ 42/Df lines were analyzed at 5 and 18 days post-eclosion (d.p.e) to find a modified phenotype as compared to G4 > A $\beta$ 42. Genetic controls included G4>+ and +>A $\beta$ 42. Those Df lines that showed a difference of at least 50% in negative geotaxis only at 18 d.p.e in a single biological experiment were selected. In stage II, each chromosomal deletion; *elav*<sup>c155</sup> [Gal4]; Df/+ (G4 > Df) was assessed to rule out that it did not affect negative geotaxis in the absence of A $\beta$ 42 expression. Three independent biological experiments were performed comparing G4 > A $\beta$ 42 with G4 > A $\beta$ 42/Df to select the Df lines that reached statistical significance. Deleted genes were queried for the identification of human orthologs with expression in the adult CNS (see below). If the deletion was large and included more than 10 human orthologs, overlapping deletions were analyzed as in stage II to reduce the number of candidates. Deletions with less than 10 human orthologs were selected for analysis with RNAi or mutant lines in stage III.

## Bioinformatic Analysis of Deficiency Lines

Genomic deletions were queried in Bloomington Stock web page<sup>1</sup>. The corresponding gene list was obtained from FlyBase<sup>2</sup> (Attrill et al., 2016) using the GBrowse function and the Hit List tool. Each gene was searched for its human ortholog with the highest weighted score using the *Drosophila* RNAi Screen Center (DRSC) Integrative Ortholog Prediction Tool (DIOPT)

<sup>1</sup><http://www.flystocks.bio.indiana.edu/Browse/df/dfkit.php>

<sup>2</sup><http://www.flybase.org>



from the DRSC<sup>3</sup>. Fly gene expression was searched in NCBI web page<sup>4</sup> and the RNA-seq Profile provided by FlyBase on the gene query subtitle expression data<sup>5</sup>. Gene products, their known functions, patterns of expression in humans, protein-protein interactions and association with human diseases were obtained from UNIPROT<sup>6</sup>, Genecards<sup>7</sup> and OMIM<sup>8</sup> databases.

## Preparation of cDNA Samples and Quantitative Real-Time PCR

RNA from 35 fly heads was extracted with the TriZol reagent (Invitrogen) according to manufacturer's instructions. cDNA was generated from 3  $\mu$ g of RNA, previously treated with DNase (Promega) using the SuperScript III system (Invitrogen). SYBR-Green quantitative real-time PCR (qRT-PCR) was performed using KAPA SYBR\_FAST Universal 2X qPCR Master Mix. Reactions were run in a Stratagene Mx3005P cycler (Agilent Technologies) and analyzed by the calibration curve method. For *CG11796* primers 5'AAAGGAACCAAACCTGAA GC 3'

<sup>3</sup>[http://www.flyrnai.org/cgi-bin/DRSC\\_orthologs.pl](http://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl)

<sup>4</sup><http://www.ncbi.nlm.nih.gov/gene>

<sup>5</sup><http://flybase.org/reports/FBgn0036992.html>

<sup>6</sup><http://www.uniprot.org/>

<sup>7</sup><http://www.genecards.org/>

<sup>8</sup><http://www.omim.org/>

(forward) and 5'ATCCCTGATAGCCAAGTGGT 3' (reverse) were used. *RPL32* was amplified for normalization using the following primers: 5'ATGCTAAGCTGTCGCACA AATG 3' (forward) and 5'GTTTCGATCCGTAACCGATGT 3' (reverse).

## Statistical Analysis

Results are presented as the mean  $\pm$  SEM of at least three independent biological experiments unless otherwise stated. Data were analyzed by repeated measures (RM) two-way ANOVA with *post hoc* Bonferroni's test, RM one-way ANOVA followed by Least Significant Difference (LSD) Fisher's test or Student's *t* test using the Prism<sup>®</sup> Graphpad 6 software. Wilcoxon non-parametric test were used when indicated. The level of significance was set at  $p < 0.05$ .

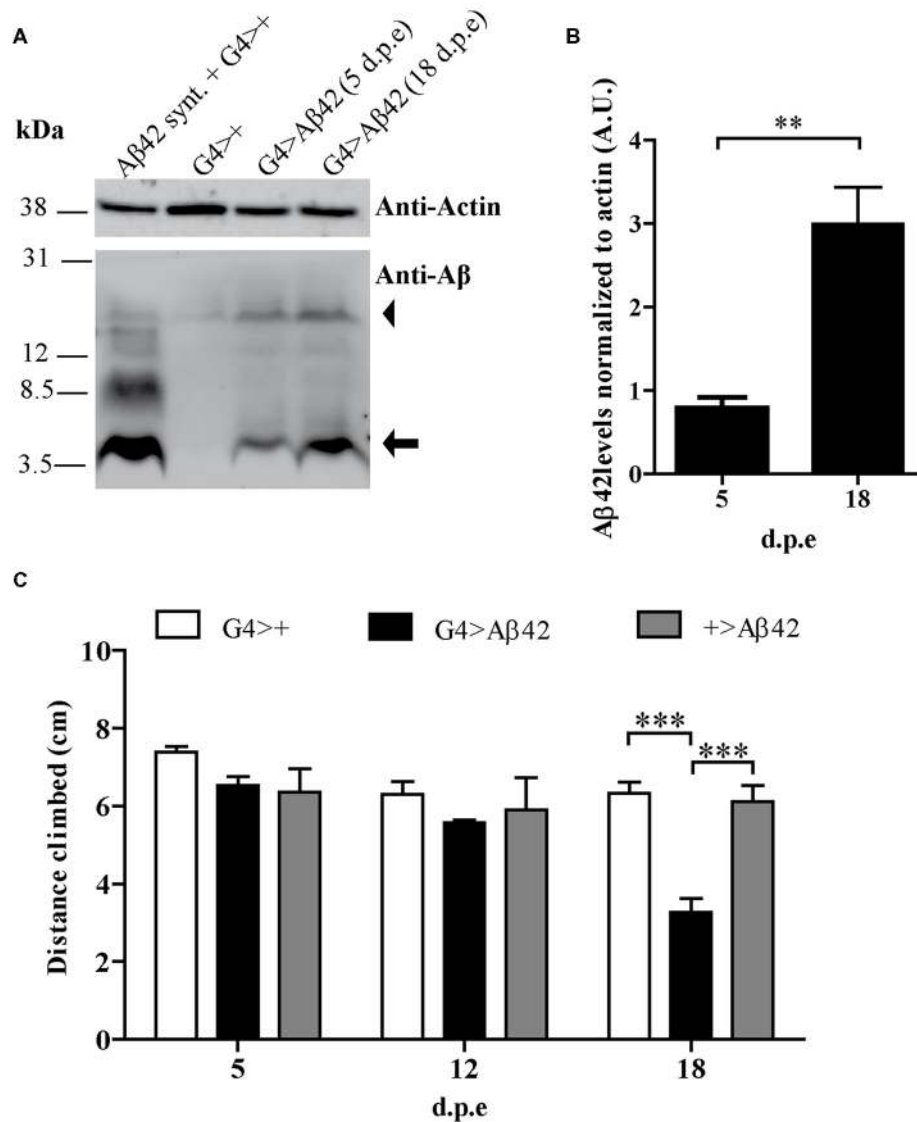
## RESULTS

### G4 > A $\beta$ 42 Line Shows a Moderate and Age-Dependent Toxic Phenotype

A transgenic line with constitutive, pan-neuronal expression of A $\beta$ 42 maintained at 25°C was examined as a candidate for the screen. Western blots of fly head homogenates showed a  $\sim$ 4.5 kDa band consistent with detergent-soluble A $\beta$ 42 correctly targeted and cleaved in the secretory pathway. A minor band consistent with SDS-resistant A $\beta$ 42 oligomers was also seen. Between 5 and 18 d.p.e there was a robust 3-fold increase of A $\beta$ 42 levels (Figures 2A,B). Negative geotaxis was not impaired in 5-day-old flies as compared with controls, strongly suggesting that there were no developmental effects upon the CNS due to A $\beta$ 42 expression. In 18-day-old flies, a significant decrease in climbing ability ( $\sim$ 50%) was apparent only in A $\beta$ 42-expressing animals as compared to genetic controls, G4>+ and +>A $\beta$ 42 (Figure 2C). Microscopic examination of the brains of affected flies revealed very mild vacuolization and negative ThS staining (see below). Therefore, this line showed age-dependent A $\beta$ 42 accumulation and CNS neurotoxicity, and the magnitude of the functional decline was optimal for the search of enhancers and suppressors. In addition, the accretion of non-fibrillar A $\beta$ 42 suggests that toxicity was induced by soluble oligomers, as proposed for AD. Taken together, these features and experimental conditions made this A $\beta$ 42 transgenic line highly suitable for a forward genetic screen.

### Identification of Df Lines that Modify Age-Dependent A $\beta$ 42 Toxicity

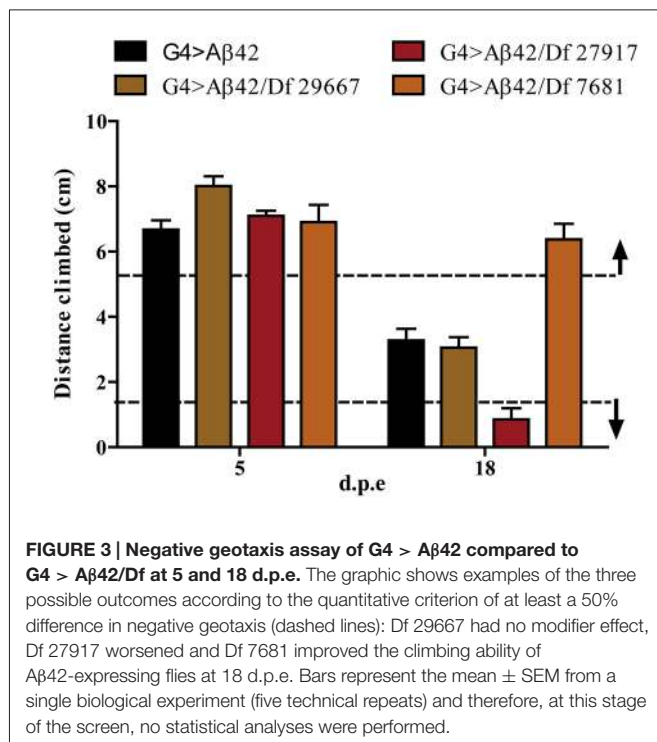
One hundred and ninety-nine lines with defined deletions from the 2nd, 3rd and 4th chromosomes, accounting for approximately 6300 genes, were tested in the first stage of the screen. Negative geotaxis of G4 > A $\beta$ 42 line was compared to G4 > A $\beta$ 42/Df lines at 5 and 18 d.p.e. Figure 3 shows actual examples of the three possible outcomes: Df 29667 had no modifying effect, Df 27917 worsened and Df 7681 rescued A $\beta$ 42-induced climbing dysfunction. At this stage, 73 G4 > A $\beta$ 42/Df lines showed a difference in climbing ability of at least 50% when compared to G4 > A $\beta$ 42 and



**FIGURE 2 | (A)** Representative Western blot of fly brain homogenates at 5 and 18 d.p.e showing A $\beta$ 42 expression detected with anti-A $\beta$  monoclonal antibody 6E10. The 4.5 kDa band (arrow) indicates A $\beta$ 42 correctly processed in the secretory pathway. The arrowhead indicates a band consistent with sodium dodecyl sulfate (SDS)-resistant A $\beta$ 42 oligomers. A G4>+ brain homogenate was spiked with synthetic A $\beta$ 1-42 (A $\beta$ 42 Synt.) for electrophoretic mobility control. Membrane was cut above the 31 kDa marker and probed with anti-actin for normalization. **(B)** Quantification of A $\beta$ 42 levels relative to actin in arbitrary units (A.U.). Bars represent the mean  $\pm$  SEM from three independent experiments; \*\* $p$  < 0.01 (Student's  $t$ -test). **(C)** Pan-neuronal A $\beta$ 42-expressing flies (G4 > A $\beta$ 42) showed climbing impairment at 18 d.p.e as compared with genetic controls (G4>+ and +>A $\beta$ 42). Bars represent the mean  $\pm$  SEM from at least three independent biological experiments; \*\*\* $p$  < 0.001 (repeated measures [RM] two-way ANOVA followed by Bonferroni's *post hoc* test).

such differences were only seen in aged animals. These lines were selected and analyzed in stage II and six lines met statistical criteria to be considered as positive hits. Df lines 24392, 27369, 27372, 27404 and 27917 worsened negative geotaxis while line 7681 reduced A $\beta$ 42 toxicity to a full rescue of the phenotype (Figure 4). In the absence of A $\beta$ 42 expression, Df lines showed no intrinsic effect and none of the enhancer Df lines induced climbing impairment in G4 > A $\beta$ 42 line at 5 d.p.e, ruling out a possible acceleration

of A $\beta$ 42 toxicity (not shown). Within these six Df lines, 36 *Drosophila* genes with human orthologs remain to be tested to identify enhancers and 14 genes to pin point suppressors of A $\beta$ 42 neurotoxicity. Interestingly, 14 out of 15 enhancer and six out of seven suppressor Df lines described in a previous screen based on negative geotaxis (Liu et al., 2015) were selected in stage I of our screen but did not reach statistical significance in stage II and were not further analyzed.



### Specific Reduction of *CG11796* Expression Enhances A $\beta$ 42 Toxicity

Thus far, three out of the five enhancer Df lines that passed stage II have been partially analyzed in stage III. The enhancer Df line 27372 included the deletion of *CG17249* whose human ortholog is *PRCC*. We used a line carrying a Piggy Bac transposon in the 3' region of *CG17249* to assess toxicity. A significant enhancement in A $\beta$ 42 neurotoxicity was observed in mutant hemizygous flies (Figure 5A). Although unlikely, the 3' insertion may compromise the expression of neighboring genes and therefore, RNAi experiments are required to validate *CG17249*. Df lines 27917 and 27369 also worsened negative geotaxis in the presence of A $\beta$ 42 and the overlapping chromosomal segment included *CG11796* whose human ortholog is *HPD* encoding HPPD, a key enzyme involved in tyrosine catabolism. To determine if a reduced expression of *CG11796* was capable of enhancing A $\beta$ 42 toxicity, we used two independent approaches: a mutant line in which a Mi[Mic] transposon was inserted in the *CG11796* gene and a specific RNAi with pan-neuronal expression using the *elav* promoter. These lines had no impairment in negative geotaxis as compared with control flies despite the reduction of *CG11796* mRNA. Yet, in the presence of pan-neuronal A $\beta$ 42 expression, *CG11796* downregulation in both the RNAi and mutant lines induced a significant enhancement of A $\beta$ 42 toxicity, similar to the overall effect of the chromosomal deletions detected at stages I-II of the screen (Figures 5B,C). The specificity of the RNAi was assessed by qRT-PCR from fly heads, which showed a strong reduction of *CG11796* mRNA of approximately 85% in G4 > *CG11796*<sup>RNAi</sup> and 55% in *CG11796*<sup>Mut</sup>, similar to the expected ~50% mRNA reduction in Df line 27917 (Figure 5D).

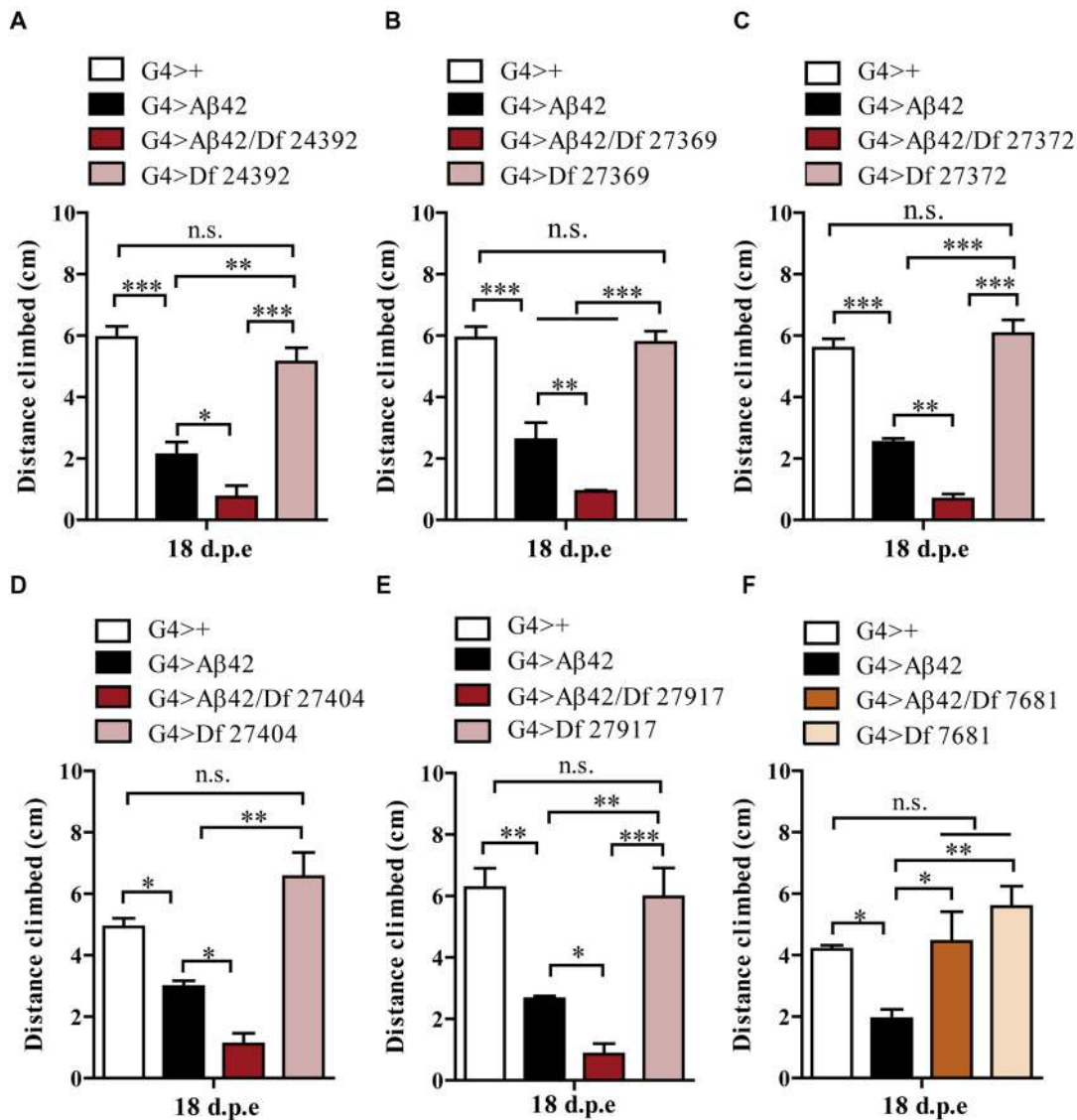
### Reduction of *CG11796* Expression Promotes the Accumulation of Non-Fibrillar A $\beta$ 42

A $\beta$ 42 levels were analyzed in the brains of flies with partial Df of *CG11796* at 18 days of age, when the toxic phenotype was detected. Confocal immunofluorescence showed extensive intraneuronal perinuclear A $\beta$  accumulation which was ~2-fold higher in both *CG11796* hemizygous mutant and *CG11796*<sup>RNAi</sup> as compared with flies expressing A $\beta$ 42 alone (Figure 6). Western blots of head homogenates showed a 70%–80% increase in the A $\beta$  monomer band in *CG11796* mutant and RNAi lines, consistent with the immunofluorescence results (Figure 7). The increment of A $\beta$  abundance was not accompanied by ThS staining, indicating that a partial Df of *CG11796* expression promoted the accumulation of non-fibrillar A $\beta$  species (Figure 8). Instead, the pattern of immunostaining and detergent solubility suggest the accretion of intraneuronal oligomeric A $\beta$  which concurs with a higher neurotoxicity in *CG11796*<sup>Mut</sup> and *CG11796*<sup>RNAi</sup> flies. To assess neurodegeneration further, the extent of vacuolization in the brain was determined for each genotype. As mentioned above, there was a mild though significant increase of vacuolization in flies expressing A $\beta$ 42 as compared with their genetic controls. Yet, tissue loss did not increase in A $\beta$ 42-transgenic flies expressing *CG11796* mutant or RNAi (Figure 9). Together, these results strongly suggest that a partial Df of the HPD ortholog promotes the accumulation of toxic A $\beta$ 42 oligomers in the CNS leading to cellular dysfunction without histologically detectable neuronal loss.

## DISCUSSION

The finding of proteins that modulate A $\beta$  neurotoxicity in animals with a complex CNS such as *Drosophila* may impact on AD research in several ways. First, by providing novel players in the cellular mechanisms by which A $\beta$  promotes synaptic dysfunction and neuronal death. Second, changes in the levels or activity of those proteins may be validated in human samples including post-mortem tissue and, more relevant, in biological fluids as potential biomarkers. Third, in the long range, it may open therapeutic strategies alternative to the current ones mostly aimed at A $\beta$  and tau. Previous modifier screens in the fly have yielded interesting candidates that modulate wild-type A $\beta$  toxicity in the eye, upon life span, or negative geotaxis induced by an aggressive A $\beta$  mutant (Cao et al., 2008; Tan et al., 2008; Rival et al., 2009; Liu et al., 2015). Our screen was designed to search for modifiers in a context of neurotoxicity more related to what may occur in sporadic AD, including pan-neuronal expression of wild-type A $\beta$ 42 and age-dependent accumulation with no detectable behavioral impairment in young animals. Moreover, the A $\beta$ 42 transgenic line had a rather mild phenotype at ~3 weeks of age, with little neuronal loss and the accumulation of detergent-soluble, non-fibrillar species of A $\beta$ , avoiding features that are found in late stages of AD.

The discrepancies between our results and those reported by Liu et al. (2015) may be due to criteria for defining positive hits and the use in their study of A $\beta$ E22G driven to

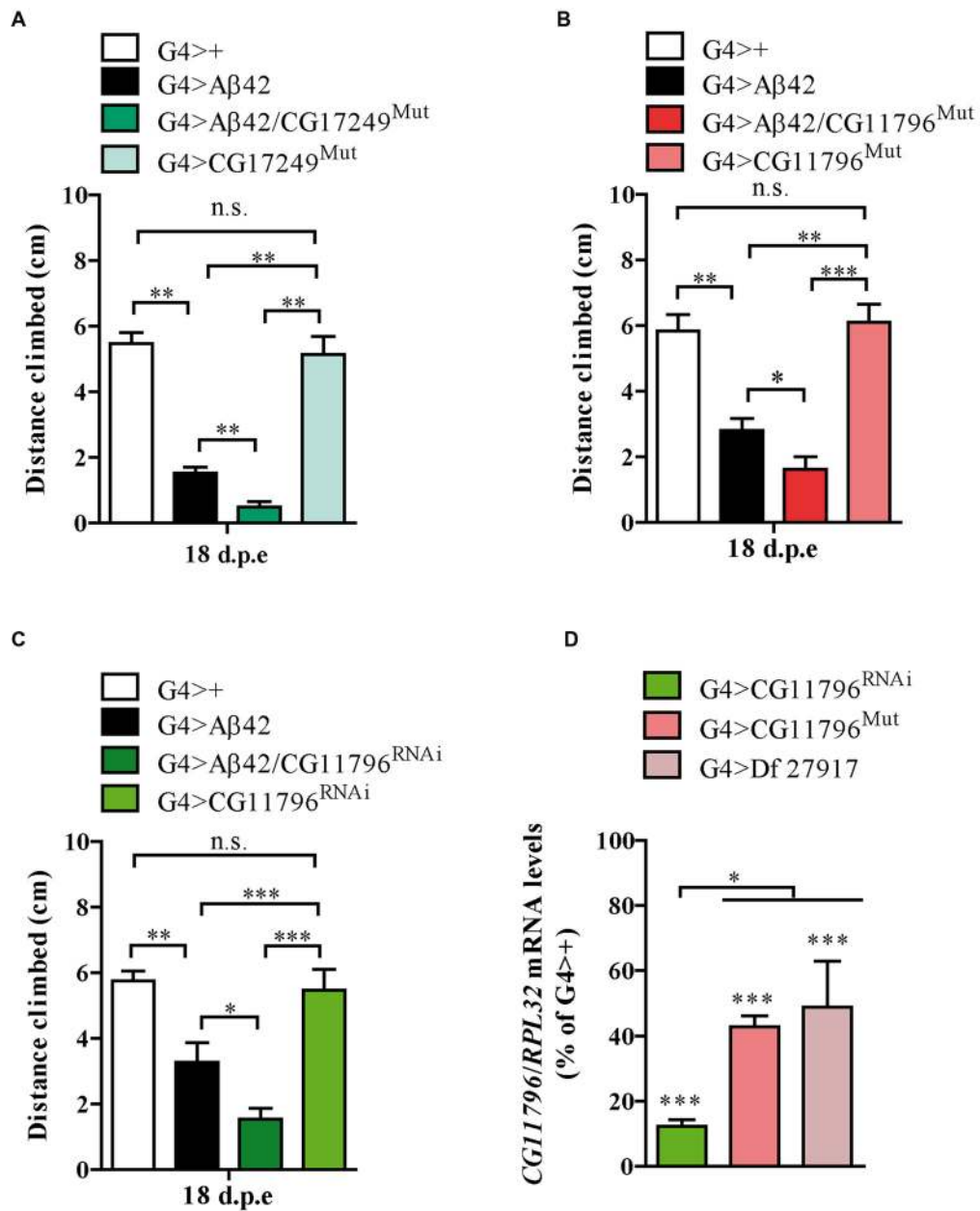


**FIGURE 4 | Negative geotaxis assay of G4>+, G4 > A $\beta$ 42, G4 > A $\beta$ 42/Df and G4 > Df at 18 d.p.e. (A) Df 24392; (B) Df 27369; (C) Df 27372; (D) Df 27404; and (E) Df 27917, worsened the A $\beta$ 42-induced phenotype. (F) Df 7681 improved the climbing ability of A $\beta$ 42-expressing flies. Df lines had no effect in the absence of A $\beta$  expression. Bars represent the mean  $\pm$  SEM from at least three independent biological experiments; \* $p$  < 0.5, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 (RM one-way ANOVA followed by LSD Fisher's test).**

specific interneurons that relay to thoracic muscles instead of pan-neuronal wild-type A $\beta$ 42. Noteworthy, in both studies Df line 7681 was a strong suppressor, suggesting that one or more genes in homozygosity within this deletion are necessary for A $\beta$  to impair geotaxis behavior, independent of A $\beta$  species and type of neurons involved.

A limitation of our study was its restriction to the effect of gene deletions and therefore, likely dependent on lower than physiological levels of the encoded proteins. Those genes that modulate A $\beta$ 42 toxicity through overexpression would be missed with our strategy.

So far, two genes have passed stage III of our screen whose human orthologs are *PRCC* and *HPD*. While *PRCC* requires a final validation step with RNAi, *HPD* was unambiguously identified. The function of ppPRCC is largely unknown although early studies suggest that it may have a role in pre-mRNA splicing (Skalsky et al., 2001). A search for ppPRCC protein-protein interactions revealed association with peptidylprolyl isomerase-like 2 (Ppil2), a chaperone with putative ubiquitin ligase activity (Hatakeyama et al., 2001; Pushkarsky et al., 2005; Hegele et al., 2012). Thus, a possible role of a ppPRCC-Ppil-2 complex in protein folding, transport and degradation warrants

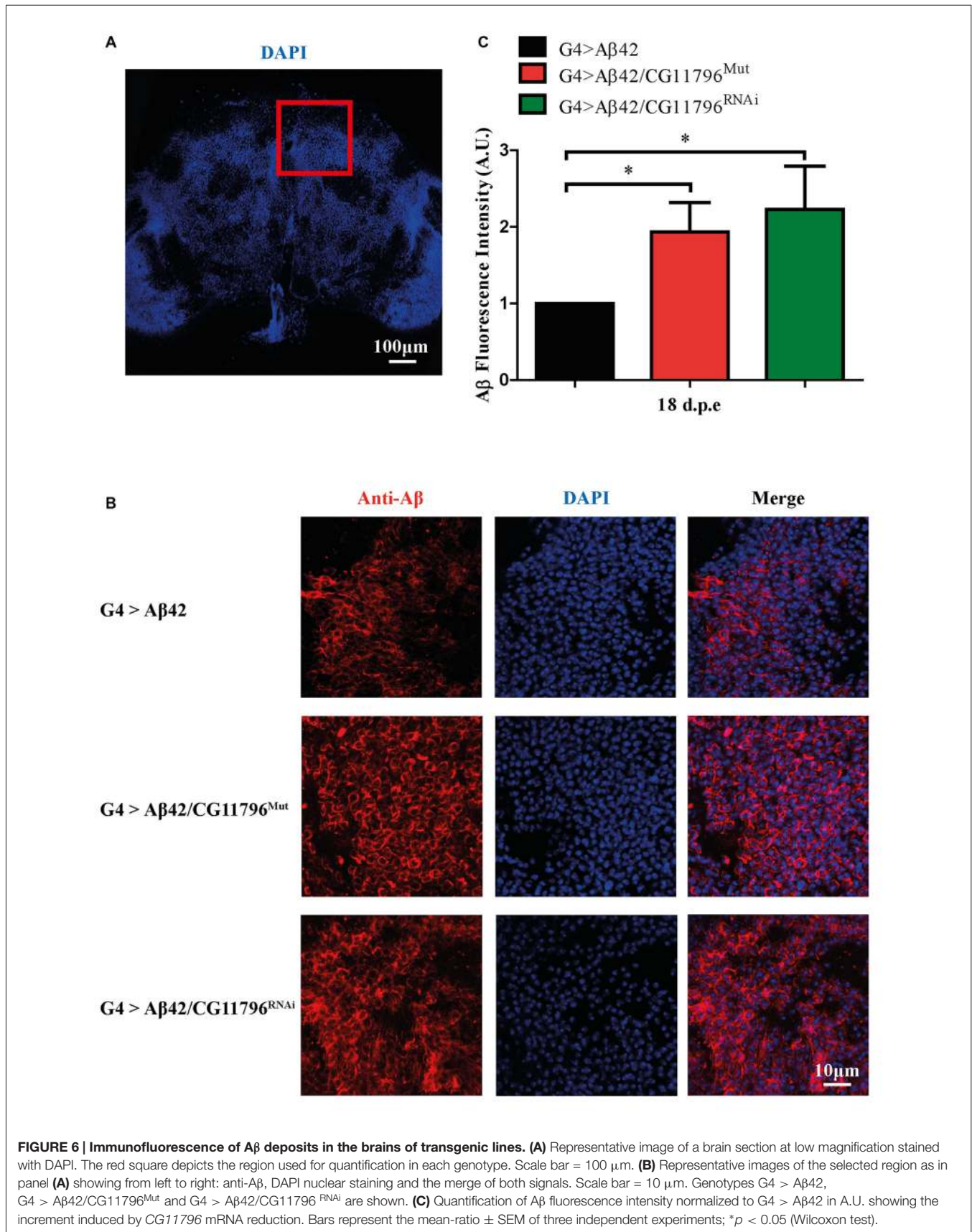


**FIGURE 5 | Mutants and RNAi of candidate genes enhance A $\beta$  toxicity. (A)** *CG17249* hemizygous mutant (human ortholog, *PRCC*); **(B)** *CG11796* hemizygous mutant (human ortholog, *HPD*) and **(C)** *CG11796* RNAi. Mutant and RNAi lines had no effect in the absence of A $\beta$ 42 expression. Bars represent mean  $\pm$  SEM from at least three independent biological experiments; \* $p$  < 0.5; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001 (one-way ANOVA followed by LSD Fisher's test). **(D)** Quantification of *CG11796* endogenous mRNA showed a ~40%–50% reduction in *Df 27917* and *CG11796*<sup>Mut</sup> lines, while for *CG11796*<sup>RNAi</sup> a ~85% reduction was observed. Brain samples were taken from 5 day-old flies and *RPL32* mRNA was used for normalization in each quantitative real-time PCR (qRT-PCR) assay. \*\*\* $p$  < 0.001 for *Df 27917*, *CG11796*<sup>Mut</sup> and *CG11796*<sup>RNAi</sup> as compared to *G4>+*. \* $p$  < 0.05 (RM one-way ANOVA followed by LSD Fisher's test from three independent biological experiments).

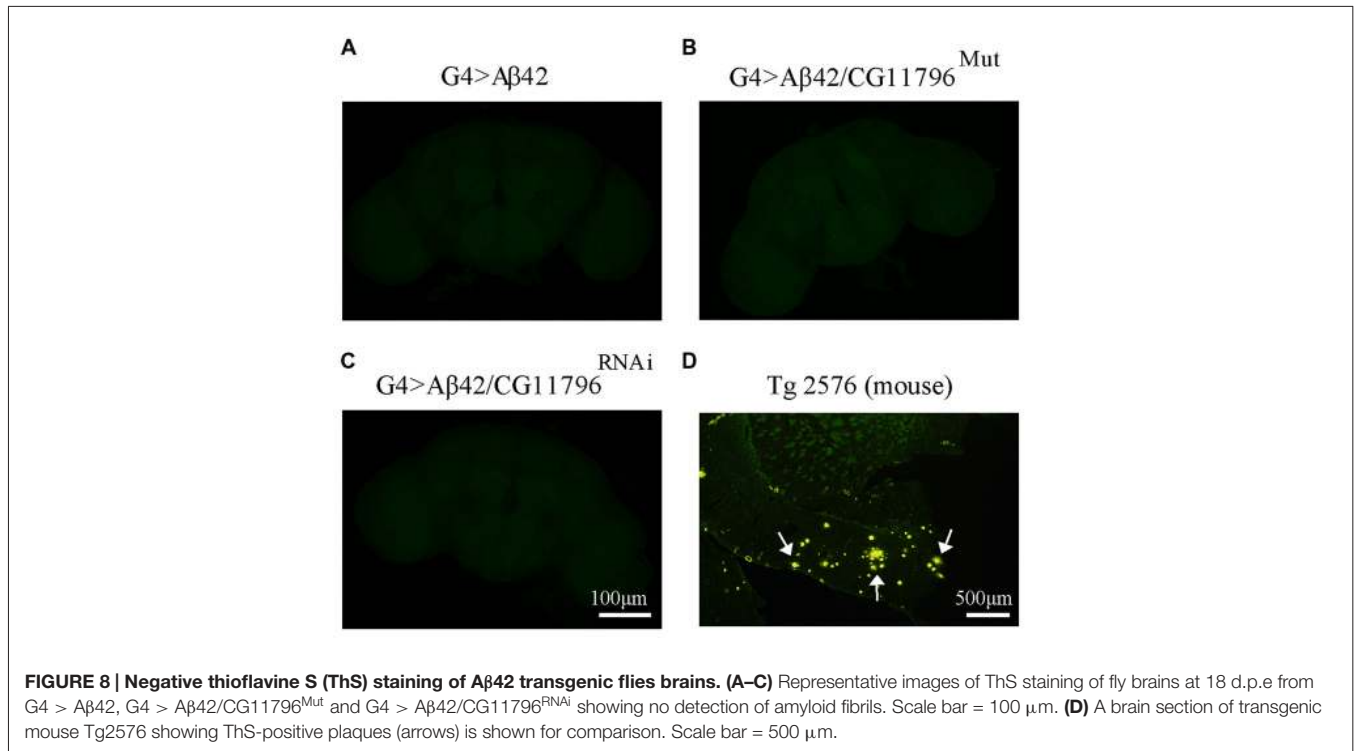
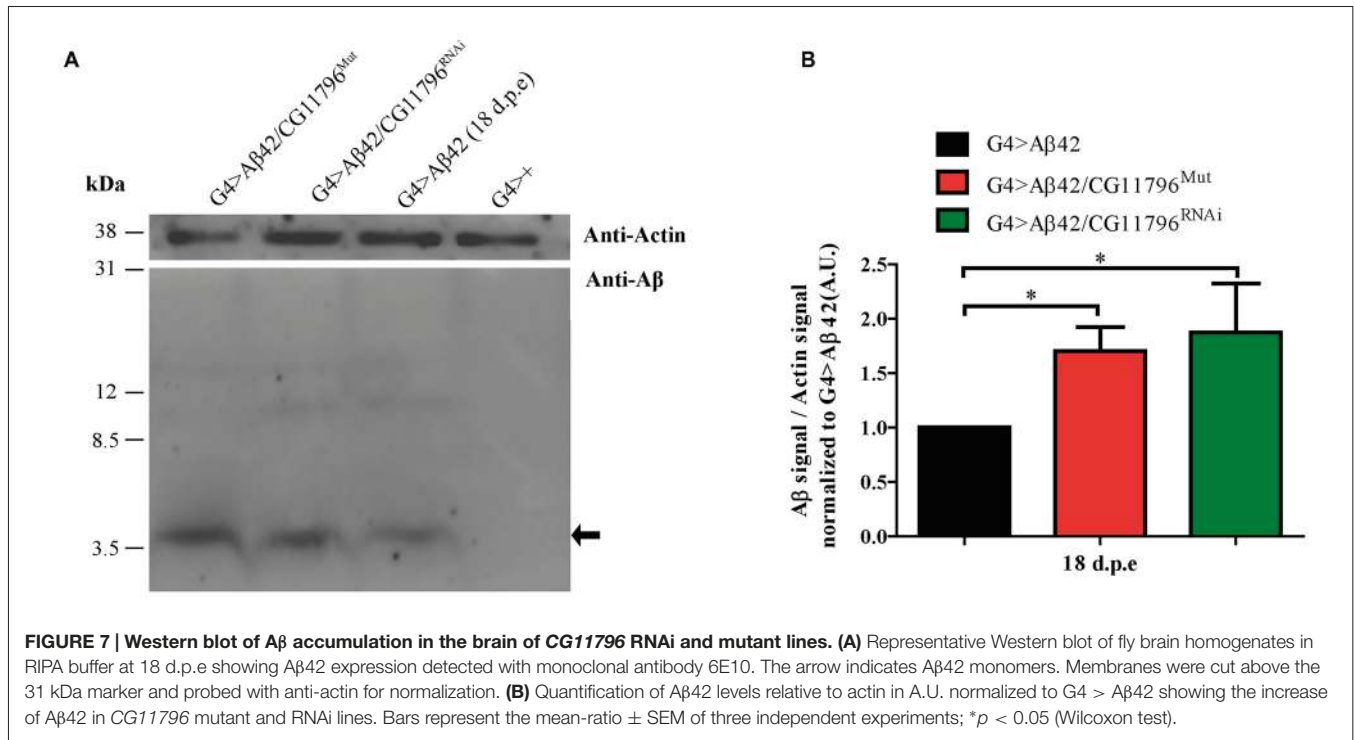
further study in the context of A $\beta$  neurotoxicity. *HPD* encodes a highly conserved protein that catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate, the second step in the tyrosine degradation pathway. Mutations in *HPD* cause the

rare diseases Tyrosinemia type 3 and Hawkinsiuria. Tyrosinemia type 3 is autosomal recessive; patients show mental retardation and elevated levels of tyrosine and its derivatives in blood and urine due to HPPD *Df* (reviewed in Scott, 2006). Hawkinsiuria



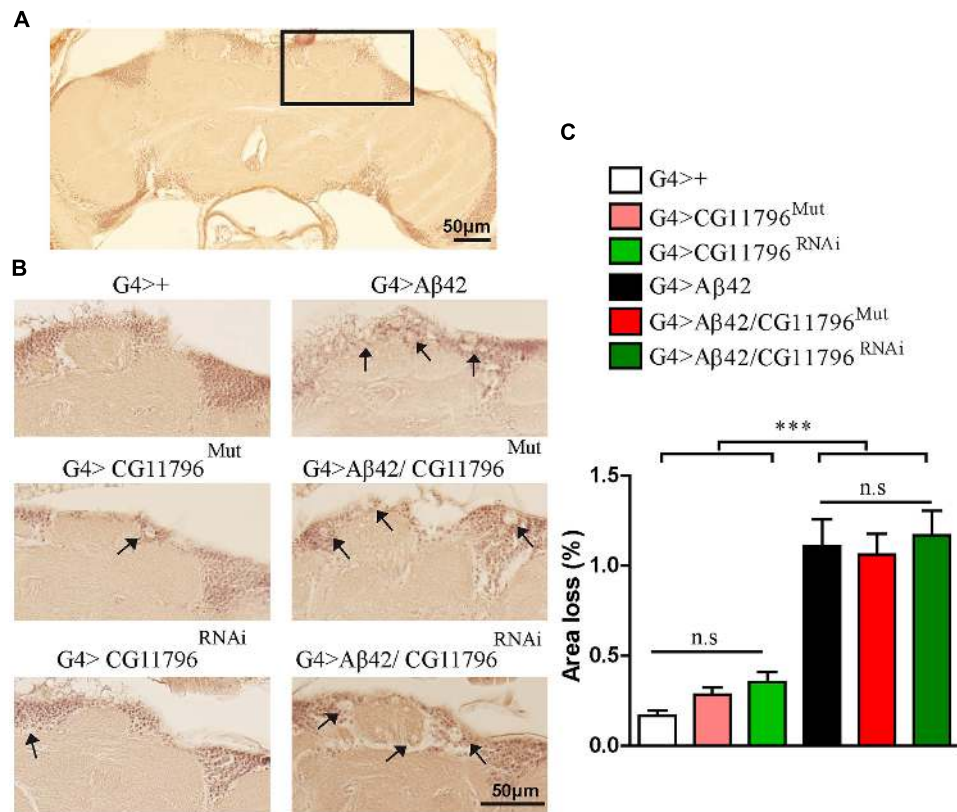


**FIGURE 6 | Immunofluorescence of A $\beta$  deposits in the brains of transgenic lines. (A)** Representative image of a brain section at low magnification stained with DAPI. The red square depicts the region used for quantification in each genotype. Scale bar = 100  $\mu$ m. **(B)** Representative images of the selected region as in panel **(A)** showing from left to right: anti-A $\beta$ , DAPI nuclear staining and the merge of both signals. Scale bar = 10  $\mu$ m. Genotypes G4 > A $\beta$ 42, G4 > A $\beta$ 42/CG11796<sup>Mut</sup> and G4 > A $\beta$ 42/CG11796<sup>RNAi</sup> are shown. **(C)** Quantification of A $\beta$  fluorescence intensity normalized to G4 > A $\beta$ 42 in A.U. showing the increment induced by CG11796 mRNA reduction. Bars represent the mean-ratio  $\pm$  SEM of three independent experiments; \* $p$  < 0.05 (Wilcoxon test).



is autosomal dominant and characterized by metabolic acidosis and urinary excretion of “hawkinsin”, a cyclic amino acid derived from quinolacetic acid produced by mutant HPPD (Brownlee et al., 2010). The mechanisms underlying mental

retardation in Tyrosinemia are not known, yet an increase of acetylcholinesterase activity and energy metabolic impairment have been postulated (Ferreira et al., 2012, 2015). In addition, high tyrosine levels may reduce the activity of thiol-dependent



**FIGURE 9 | Brain vacuolization in A $\beta$ -expressing lines alone and in a background of CG11796 Df. (A)** Representative whole brain section of G4>+ stained with H&E used for tissue loss analysis by bright-field microscopy. The rectangle demarcates a typical area with a high number of neuronal bodies. **(B)** The region depicted in **(A)** is shown for each genotype. Arrows indicate vacuoles with a diameter of at least 3  $\mu$ m. Scale bar = 50  $\mu$ m. **(C)** Quantification of tissue loss in hemi-brains was calculated as the percentage of the section area occupied by vacuoles. Flies expressing A $\beta$ 42 showed increased vacuolization as compared to control flies G4>+. No differences were found in G4 > A $\beta$ 42/CG11796<sup>Mut</sup> and G4 > A $\beta$ 42/CG11796<sup>RNAi</sup> compared with G4 > A $\beta$ 42. \*\*\* $p$  < 0.001 (one way ANOVA followed by Tukey's *post hoc* test).

creatine kinases (CK) leading to misbalance of a key ATP buffering and shuttling system (Wallimann et al., 2011; de Andrade et al., 2012). Interestingly, CK activity is reduced in AD brains as compared to age-matched controls and A $\beta$  induces a reduction of CK activity in cultured neurons (Aksenov et al., 1998, 2000; David et al., 1998). Consistent with these findings, creatine accumulates in old transgenic mice expressing a mutant APP and in the hippocampus of AD patients (Gallant et al., 2006). Our finding that the partial Df of *HPPD* ortholog promoted the accumulation of oligomeric A $\beta$ 42 provides a likely explanation for the worsening of age-dependent geotaxis performance. Yet, such degree of A $\beta$  accumulation seems to be sufficient to impact negatively upon neuronal function without inducing gross neuropathological changes up to 18 d.p.e. With regard to possible mechanisms for A $\beta$  accretion in the context of lower HPPD expression, the reduction in CK activity as a consequence of high tyrosine levels may accelerate A $\beta$  aggregation or impair its clearance due to lower ATP availability and oxidative stress (Meyer et al., 2006). Moreover, A $\beta$ 42 oligomers induce oxidative stress (Butterfield et al., 2013) leading to a vicious cycle in disease progression. Alternatively, the possibility that a partial Df of

HPPD is more directly involved in A $\beta$  accumulation deserves further investigation. Inhibitors of HPPD such as nitisinone are used to treat patients with hereditary Tyrosinemia type 1 in which downstream metabolites of HPPD activity accumulate and are highly toxic to the kidney and liver (Mayorandan et al., 2014; Zeybek et al., 2015). Long-term outcome of patients under nitisinone treatment show a high frequency of progressive cognitive impairment that has been related with chronically elevated tyrosine levels (Masarel-Paulet et al., 2008; Thimm et al., 2012). Early reports on tyrosine levels in the cerebrospinal fluid of AD as compared with controls remain controversial (Degrell et al., 1989; Martinez et al., 1993) and there are no studies on the levels and/or activity of HPPD in AD. In light of our results regarding A $\beta$  accumulation, such studies may be relevant to better understand the complex pathogenesis of AD.

In summary, our work describes the first genetic screen to search for modifiers of wild-type A $\beta$ 42 neurotoxicity in the CNS of *Drosophila* by exploring age-dependent alterations in a complex behavior. So far, this strategy has led us to identify candidate genes that warrant further research to determine their significance in sporadic AD.

## AUTHOR CONTRIBUTIONS

LFB-C, MSM and NIB performed the experiments, analyzed results, drafted and revised the manuscript; MFC and LM designed the work, analyzed data, interpreted the results and revised the manuscript. EMC designed the work, analyzed data, interpreted the results and wrote the article.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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