UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of PNKP in a Drosophila Model of Tauopathy

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by

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THESIS APPROVAL PAGE

The thesis of Bobby Nhan as it is listed on UC San Diego Academic Records is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

I would like to dedicate my thesis to my parents who have given me their unconditional support through all phases of my education and athletics. Without them, none of this would have even been remotely possible. I would also like to thank my friends who have understood that our studies limit our abilities to attend social gatherings and have stuck with us through tough times despite that. Lastly, I would like to thank my girlfriend for supporting me through all my endeavors. She is my support system, my motivation, and is by far the best lab partner I have ever had to this day. I want to thank her for believing in me even when I did not believe in myself.

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ABSTRACT OF THE THESIS

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Master of Science in Biology

University of California San Diego, 2022

Professor Xu Chen, Chair

Professor Enfu Hui, Co-Chair

Alzheimer's Disease (AD), along with several related neurodegenerative diseases called tauopathies, are characterized by neurofibrillary tangles (NFTs) consist of aggregated tau proteins

in the brain, leading to cognitive dysfunctions and neurodegeneration. Accumulation of DNA damage has been found in AD brains, which is thought to underlie neurodegeneration. Recent studies from our collaborators have shown that AD patient brains have decreased activity of a DNA repair enzyme – polynucleotide kinase 3'-phosphatase (PNKP) – compared to healthy brains. Dysfunction of PNKP has been implicated in the pathophysiology of other neurodegenerative diseases, including Huntington's Disease (HD) and spinocerebellar ataxia type 3 (SCA3). In a previous study on SCA3, increasing the expression of PNKP rescued DNA damage accumulation and neurodegenerative phenotypes in an animal model. However, the role of PNKP in context of tau-mediated neurodegeneration is unknown. In this study, we aimed to characterize the role of PNKP in tauopathies using a *Drosophila* model. We generated transgenic fly lines for wild-type *Drosophila* PNKP (dPNKP.WT) and several dPNKP mutants. Our results showed that overexpression of dPNKP.WT in human tau (hTau)-transgenic flies rescued the rough eye phenotype and motor dysfunction. These findings show that increasing PNKP expression ameliorates tau-mediated neurotoxicity in flies, suggesting that enhancing PNKP level or activity may be a therapeutic strategy for tauopathies including AD.

INTRODUCTION

Tauopathies are a class of diseases characterized by aggregation of the microtubule binding protein, tau. Under normal physiological conditions and levels, tau functions as a microtubule stabilizing protein and facilitates in axonal [4]. However, under pathological conditions, tau aggregates into neurofibrillary tangles (NFTs) in the brain, which correlate with cognitive dysfunctions and neurodegeneration. Tau aggregates are the pathological hallmark of Alzheimer's Disease (AD) and other neurodegenerative tauopathies [7]. Mutations to the microtubule associated protein tau (MAPT) gene, mostly within or near the microtubule-binding repeat domain, can cause non-AD tauopathies such as frontotemporal lobar degeneration.

Accumulation of DNA damage has been found to be associated with neurodegenerative disorders. Decreased DNA repair proteins and increases in double stranded DNA breaks (DSB) have been reported in the brains of AD patients [22]. Tau has been reported to localize to the nucleus and interact with nucleic acids, suggesting potential roles for tau in genomic stability [10]. Interestingly, mutations associated with the MAPT gene has been shown to affect the function of P53, a well described regulator of DNA repair, during DNA damage in neuroblastoma cells [20]. However, a direct link between tau and DNA damage in neurodegeneration is still poorly understood.

Functioning DNA repair mechanisms are most important during the developmental phases of the nervous systems in reducing the number of point mutations and DSBs in the genome. After exiting the developmental phases, cells will need to rely on non-homologous end joining (NHEJ) as the method of DNA repair since homologous recombination activity is reduced in cells that have exited the cell cycle [18]. One particular enzyme involved in DNA repair \ is Polynucleotide Kinase 3'-Phosphatase (PNKP). PNKP is a DNA processing enzyme with a catalytic C-terminal

domain containing the kinase and phosphate region, and an N-terminus forkhead-associated (FHA) domain that interacts with scaffolding proteins in order to assemble DNA repair machinery [12]. During DNA repair, PNKP functions by removing 3'-phosphate groups (3'-P) and catalyzing phosphorylation of the 5'-OH terminals. Given its function as both a kinase and phosphatase, PNKP is involved in both single-strand break (SSB) and DSB repair in DNA. [12].

Past studies have linked dysfunctions in DNA repair with the onset of neurodegenerative diseases [9][14][22]. Defective PNKP function has been implicated in the connection of DNA repair impairment with several neurodegenerative disease, including ocular motor apraxia (AOA) and microcephaly with seizures (MCSZ). AOA type-4 (AOA4), an autosomal-recessive disease characterized by muscular atrophy and neuropathy, is associated with a mutation in PNKP that results in a glutamine to glutamate (Q50E) amino acid change in the PNKP FHA domain []. In addition, missense mutations located in PNKP's FHA domain and kinase domains have been associated with the pathological phenotypes that are characteristic of MCSZ [12]. One known non-human case in which a PNKP mutation causes a pathological phenotype was observed in pigs. A glutamine to arginine (Q96R) missense mutation caused embryonic lethality in a pig model [11]. Besides PNKP, mutations of PNKP substrates also cause dysfunction in DNA repair.

During formation of the DNA repair machinery, PNKP complexes with several proteins including huntingtin protein (HTT) and the ataxin protein (ATXN). Mutations to HTT and ATXN3 have been linked to Huntington's Disease (HD) – characterized by chorea and neuropathy, and spinocerebellar ataxia type-3 (SCA3) – characterized by neuropathy with related ataxia, respectively [14]. The mutations of HTT and ATXN3 are tri-nucleotide repeats containing a polyglutamate (polyQ) expansion [19]. CAG repeats associated with the mutant huntingtin (mHTT) protein causes disruptions to the interactions with DNA repair components, including

PNKP, which leads to accumulation of DNA strand breaks and dysfunction in DNA repair [14]. Similar to HTT, the mutant ATXN3 protein responsible for SCA3 has polyQ expansion. ATXN3 functions normally as a deubiquitinating enzyme that enhances PNKP activity by preventing its degradation by ubiquitin proteasomal system [9]. Mutant ATXN3 causes a reduction in its ubiquitinase activity, which leads to degradation of components of the DNA repair machinery, ultimately causing a decrease in PNKP activity. A subsequent accumulation of DNA damage occurs. DNA damage accumulation triggers activation of the ataxia telangiectasia mutated (ATM)-p53 pathway resulting in apoptosis and neurodegeneration [9][14]. A previous study, using a SCA3 *Drosophila* model expressing mutant ATXN3 protein in retinal and motoneurons, found that overexpression of a *Drosophila* PNKP homolog gene (dPNKP) rescued the rough eye phenotype and locomotion deficits associated with ATXN3 pathology. Less DSBs were detected by subsequent anti-γH2A.v staining in the flies that overexpressed PNKP, indicating improved DSB repair activity [9].

More recently, PNKP activity has been found to be significantly decreased in human AD brains in comparison to healthy brains. Preliminary data from the Hazra lab, characterizing PNKP in AD patient brains, found a significant decrease in PNKP activity in AD patient brains and PS19 tau mice cortices by western blot analysis (Hazra lab data, unpublished). Taken together, mutations to PNKP and/or its binding partners lead to dysfunction of the DNA repair mechanism, resulting in DNA damage accumulation and neurodegeneration. Though DNA repair mechanisms have been well implicated in the stability of the genome and mitigation of neurodegenerative pathology in diseases such as HD and SCA3, the role of DNA repair in the context of tauopathies is still unclear.

To study the role of PNKP in the context of tauopathies, we used a *Drosophila* model of tauopathy. *Drosophila,* otherwise known as the fruit fly, has been previously used to model a number of neurodegenerative diseases including Parkinson's Disease (PD) and Alzheimer's Disease [21]. Since its in-depth genome sequencing during the Human Genome Project, it has been found that *Drosophila* share remarkable gene homology with humans [23]. Many human genes are conserved in *Drosophila* and share similarities in molecular pathways, allowing for use of *Drosophila* to model and dissect human disease mechanisms [21][23]. Perhaps the most important advantage of a *Drosophila* model is the extensive genetic toolkit that has been developed. A gene expression system called the UAS-GAL4 system has made it possible to express genes of interest in different tissues of *Drosophila*. This system works by using the yeast-derived gene for the transcriptional activator protein, GAL4, to drive expression of a cloned gene that is downstream of a GAL4 binding site, or the upstream activator sequence (UAS). Another strength of the UAS-GAL4 system is its ability to control gene expression in a cell-type specific manner, using tissue specific promoters driving the expression of GAL4 [6]. Previously established tauopathy models in *Drosophila* have characterized the phenotypes caused by the overexpression of wild-type (WT) human tau (hTau) using the UAS-GAL4 system. Flies expressing a frontotemporal dementia (FTD)-linked hTau mutant, $hTau^{P301L}$, have been shown to exhibit deficiencies and more neurodegeneration in comparison to overexpressed WT hTau [1][15][24]. These models have demonstrated the feasibility of over-expressing hTau, especially the FTD mutant $hTau^{P301L}$, in a fly model in which we can co-overexpress PNKP to study its modulating effects on the disease phenotype.

In this study, I aimed to determine if overexpression of PNKP could modulate the neurodegenerative phenotypes observed in a *Drosophila* model of tauopathy. Transgenic fly lines

carrying wild-type *Drosophila* PNKP, with an upstream activator sequence (UAS-dPNKP), and dPNKP mutants carrying the homologous mutation of human AOA4 mutation (dPNKP.Q61A, dPNKP.Q61E, dPNKP.Q61R) were created. RT-qPCR was performed to confirm the overexpression of dPNKP in the transgenic fly lines. These transgenic dPNKP fly lines were then crossed to different tissue-specific GAL4 fly lines for phenotypic characterization using several behavioral assays, including lifespan assays and adult fly climbing assays, to determine if overexpression of wild-type or mutant dPNKP causes any phenotype in an otherwise wild-type background. After confirming the viability and characterization of our transgenic dPNKP flies, UAS-dPNKP.WT and dPNKP mutants were crossed to two fly lines expressing $hTau^{P301L}$ or WT hTau under different GAL4 promotors, including motoneuron- and retina-specific GAL4. We found that overexpression of wild-type dPNKP rescued the rough eye phenotype and the locomotor deficits associated with tau toxicity. These findings suggest that increasing PNKP activity ameliorates tau induced neurotoxicity and implicate DNA repair as a potential therapeutic target.

RESULTS

Generation and characterization of UAS-dPNKP transgenic flies

New transgenic lines of UAS-dPNKP flies were generated by using the phiC31 integrase system to insert the dPNKP constructs, containing pACUH-attB (cloned by the Hazra lab), into the *Drosophila* genome. The UAS-dPNKP cassettes were injected into *Drosophila* embryos carrying attP sites on specific locations of selected chromosomes. Four different lines of flies carrying wild-type and mutant dPNKP that were generated, including wild-type dPNKP (dPNKP.WT), dPNKP.Q61A, dPNKP.Q61E, and dPNKP.Q61R. These point mutations are homologous to mutations found in previous studies to be associated with neurodegenerative diseases including AOA4 and the mutation found to cause embryonic lethality in a pig model [8][11].

Since these new fly lines have not been previously characterized, I first determined if overexpression of dPNKP in an otherwise wild-type background causes any aberrant phenotype, the UAS-dPNKP flies were crossed to the C155-GAL4, carrying the C155 promoter that drives pan-neuronal expression of GAL4. GAL4-C155 driven (C155>dPNKP) expression showed robust overexpression of dPNKP in the heads of flies, as shown by the RT-qPCR (**Fig. 1**).

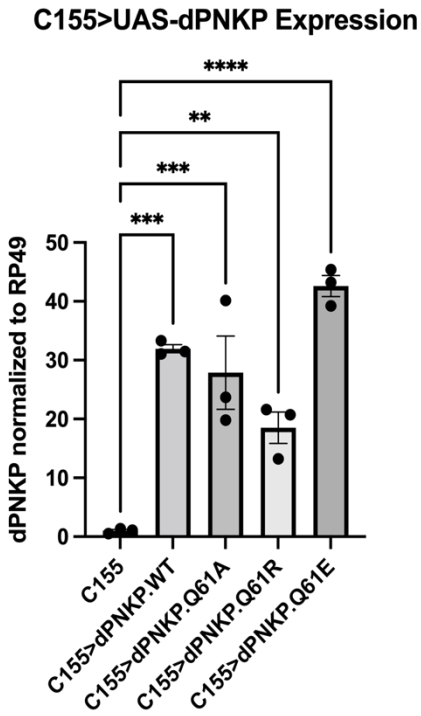

Figure 1. mRNA levels of flies with pan-neuronal overexpression of transgenic PNKP. Transgenic UAS-dPNKP flies were crossed with C155-GAL4 flies. RT-qPCR was performed to quantify mRNA levels of dPNKP variants in the fly heads and normalized to the RP49 housekeeping gene. Genotypes: C155-GAL4/+ (control). C155/+; UAS-dPNKP.WT/+ (C155>dPNKP.WT). C155/+; UAS-dPNPK.Q61A/+ (C155>dPNKP.Q61A). C155/+; UAS-dPNPK.Q61R/+ (C155>dPNKP.Q61R). C155/+; UAS-dPNPK.Q61E/+ (C155>dPNKP.Q61E). Ordinary one-way ANOVA; *P<0.05, **P<0.01, ***P<0.005, ****P<0.001. n=40 per genotype.

Next, I performed lifespan assays to determine if pan-neuronal overexpression of the transgenic wild-type and mutant dPNKP cause any changes in lifespan in comparison to control flies that are not overexpressing dPNKP. Overexpression of genes having a dominant-negative effect using a pan-neuronal driver (C155-Gal4) may result in compromised lifespan in *Drosophila*, providing a measurement for neurotoxicity [1]. The progeny of the crosses between C155-GAL4 and transgenic dPNKP flies were separated by sex. Their lifespans were then tabulated by counting the survival of flies in comparison to the controls (C155-Gal4 driver only). We observed that average fly lifespan is approximately 60 to 80 days with females having a longer lifespan than that of males, as previously reported [3]. We did not observe significant

deficiency in lifespan in the C155>UAS-dPNKP.WT and the C155>UAS-dPNKP.Q61A flies in either males or females when compared to the controls (**Fig. 2A, D**). Slight lifespan deficiency was observed in C155>UAS-dPNKP.Q61E and C155>UAS-dPNKP.Q61R male flies, although their 50% probability of survival was similar to the controls (**Fig. 2B, C**). These results suggest that overexpression of dPNKP.WT and mutant dPNKP.Q61A does not change lifespan, whereas overexpression of dPNKP.Q61R and dPNKP.Q61E mutants slightly reduced lifespan in males.

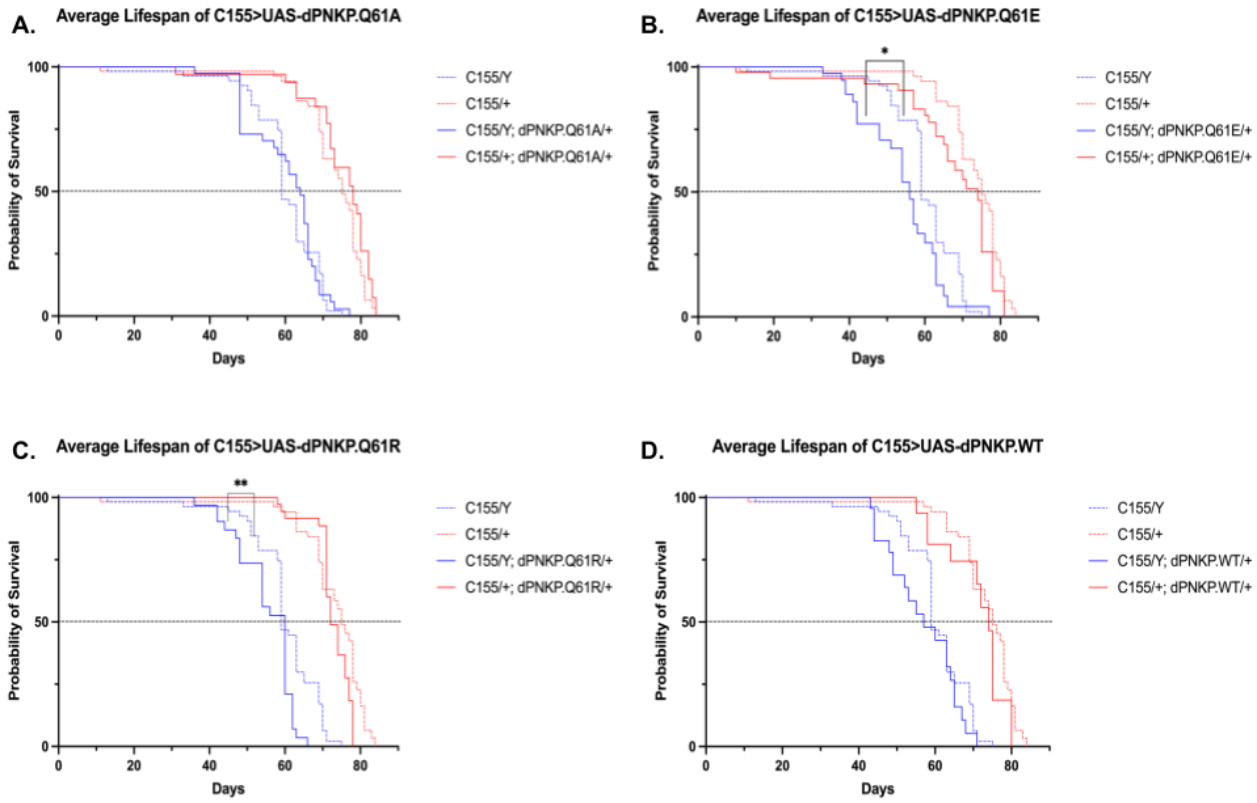

Figure 2. Average lifespan of flies overexpressing wild-type and mutant dPNKP pan-neuronally. The average lifespan of adult flies with C155-Gal4 driving overexpression wild-type and mutant UAS-dPNKP were record and analyzed using the Kaplan-Meier curve. Comparisons were made between flies overexpressing the different UAS-dPNKP genes and the driver-only controls of the respective sexes. Analysis of lifespan was performed using the log-rank test. Male flies are represented by blue lines in the graphs. Female flies are represented in red in the graphs. Driver: C155-Gal4 (pan-neuronal) Log-rank test; *P<0.05, **P<0.01. n=20 per genotype.

Impaired locomotor function of flies is often observed in aging and neurodegenerative diseases models including tauopathies, which can be measured by fly climbing assays [13]. I performed an adult fly climbing assay to determine whether the overexpression of the transgenic dPNKP variants elicit any neurotoxic effects. The transgenic dPNKP flies were crossed to a fly line carrying the OK6-GAL4 motoneuron driver to induce overexpression of transgenic dPNKP in motoneurons. Flies have a geotactic response that will trigger an instinctive reaction to fly or climb away from the ground when they are knocked down to the bottom of a container. Deficits to their motor function hinder this innate response [13]. Our adult fly climbing assay uses a graduated cylinder to measure the average score of each fly overexpressing the different transgenic dPNKP genes. In both females and males, overexpression of the transgenic dPNKP genes did not cause any significant changes in climbing ability compared to the controls of their respective sex and age groups **(Fig.3A, B)**. Although there was a slight decrease in climbing performance observed in 28 day old-dPNKP.Q61A females when compared to wild-type dPNKP, the difference was not significant (**Fig. 3A**). These findings indicate that there are no deficits in locomotor ability when the transgenic dPNKP genes are overexpressed in motoneurons in an otherwise wild-type background.

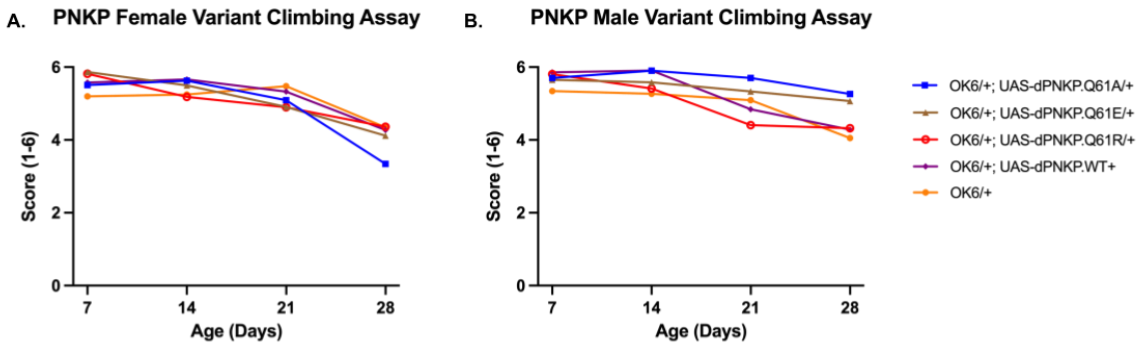

Figure 3. Adult climbing ability is normal in flies with motoneuron-specific expression of transgenic dPNKP. Adult fly climbing assays were performed to assess locomotor function of flies overexpressing wild-type and mutant dPNKP in motoneurons. Driver: OK6-Gal4 (motoneuron). All flies were aged to 7 days old and assessed every week until 28 days old. Mixed-effect analysis. n=15 per genotype.

Overexpressing dPNKP.WT rescues rough eye phenotype caused by retinal expression of hTau

After our initial characterization study, I observed no gross aberrant phenotypes in flies when wild-type dPNKP and the dPNKP mutants were overexpressed in normal flies. Next, I performed rescue experiments with dPNKP overexpression in tauopathy fly models. First, I tested if overexpression of wild-type dPNKP rescues the rough eye phenotype observed in tau flies with retinal overexpression of human Tau. In wild-type flies, the complex eye normally consists of approximately 800 organized ommatidia units as well as an organized array of mechanosensory bristles. Each of these ommatidia units contain eight photoreceptor neurons and form complexes with adjacent ommatidia and mechanosensory bristles to compose the ommatidia array in the highly conserved fly eye [17]. Previous studies showed that overexpression of human tau in the retina causes disruption to the ommatidial arrangement, a decrease in eye size, and loss of mechanosensory bristle array [23]. When observed under light microscopy, tau shows show a rough texture to the eye surface. I aimed to determine if overexpression of wild-type dPNKP could rescue the rough eye phenotype associated with tau pathology in the retina. UAS-dPNKP.WT flies were crossed to a fly line carrying the C155-Gal4 driver as well as a

constitutively expressed wild-type 2N4R hTau (gl-hTau1.1) driven by the GMR promoter. It is important to note that carrying the C155 driver and GMR promoter does not cause double the level of expression of hTau, because hTau1.1 does not respond to UAS/Gal4. The GMR promoter directly drives the expression of hTau1.1 whereas the C155 drives the overexpression of wild-type dPNKP. As expected, flies overexpressing hTau1.1 in the retina exhibited rough eye phenotype, loss of ommatidia organization, and loss of mechanosensory bristles (**Fig. 4B, E**). Remarkably, tau flies overexpressing dPNKP.WT significantly rescued the rough eye phenotype (**Fig. 4C**). Closer inspection of the imprinting of the surface of the complex eyes shows recovery of ommatidia and mechanosensory bristle organization in gl-hTau1.1 flies with transgenic dPNKP.WT overexpression (**Fig. 4C, F**). In addition, the size of the ommatidia have been recovered as well (**Fig. 4D, F**). Across the control flies, gl-hTau1.1 flies, and gl-hTau1.1 flies with overexpression of dPNKP, no significant decrease in eye diameter was observed, although there is a trend of reduced eye size in the hTau1.1 flies which is rescued by dPNKP.WT overexpression (**Fig. 4G**). In my previous experiment characterizing C155 driven UAS-dPNKP overexpression in a wild-type background, I did not observe any abnormality in the eyes. Light imaging and imprinting of the eyes of this additional control line is underway. These results suggest that increasing wild-type dPNKP expression or activity can ameliorate the rough eye phenotype associated with tau-mediated neurotoxicity in flies.

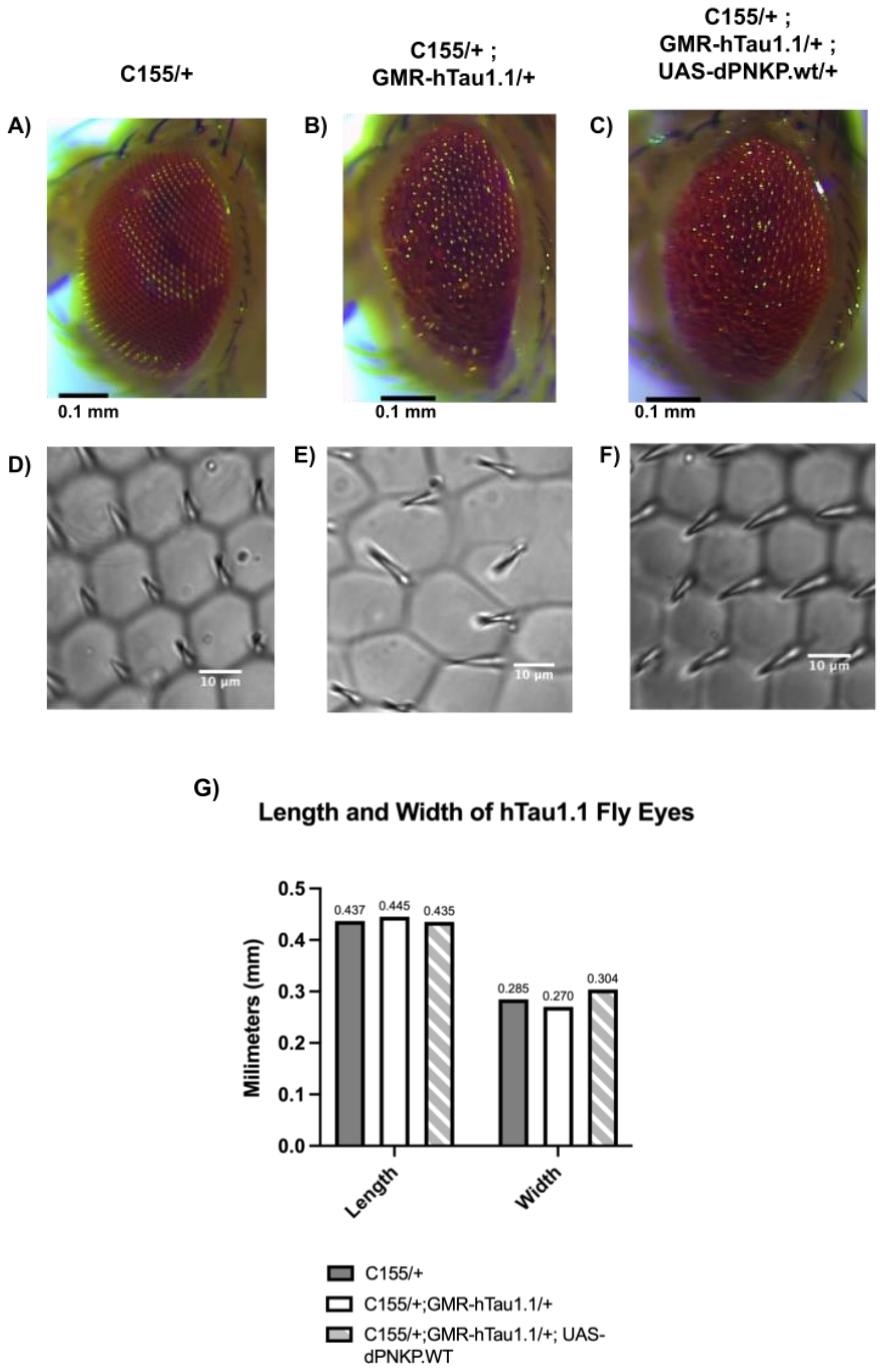

Figure 4. Overexpression of dPNKP in GMR-hTau1.1 fly retinal neurons rescues rough eye phenotype. *(A-C)* Adult flies expressing the C155 driver only, C155; GMR-hTau1.1 (gl-hTau1.1), and C155; GMR-hTau with UAS-dPNKP.WT were imaged using light microscopy. *(D-F)* Eye imprinting using nail polish was performed on the same flies. *(G)* Diameters of the length and width of the eyes using in imaging (A-C) were measured using Fiji ImageJ. Genotypes: C155/+ (control), C155/+; GMR-hTau1.1/+, C155/+; GMR-hTau1.1/+; UAS-dPNKP.WT/+.

Overexpression of dPNKP.WT and dPNKP.Q61E rescue motor deficits caused by motoneuron expression of mutant hTau^{P301L}

As with adults, the locomotor function of larvae measured by their crawling ability can also be used to characterize neuropathological phenotypes caused by hTau overexpression [1]. To determine if overexpression of wild-type and mutant transgenic dPNKP could rescue motor dysfunction, I performed a larval crawling assay. In this experiment, we created a recombinant fly line expressing the mutant hTau^{P301} carrying a mutation of frontotemporal dementia with parkinsonism (FTDP) [16], in the motoneurons using the D42 driver (D42-P301L). The D42 driver also drove overexpression of transgenic dPNKP in the rescue flies. Larval motor function was measured by the average distance traveled in one minute for each larva (**Fig.5A, B**). D42-P301L larvae exhibit a significant reduction of crawling distance compared to the control larvae (D42/+). We observed a significant improvement in crawling ability when transgenic dPNKP.WT and dPNKP.Q61E were overexpressed in D42-P301L larvae (**Fig. 5C**). Overexpression of the mutant dPNKP.Q61R and dPNKP.Q61A genes did not significantly rescue the crawling ability of D42-P301L larvae (**Fig. 5C**). These results indicate that overexpression of both dPNKP.WT and dPNKP.Q61E were capable of rescuing the motor deficits associated with motoneuron expression of mutant hTau^{P301L}. Together, the rescue experiments suggest that overexpression of dPNKP can ameliorate tau-mediated neurotoxicity in *Drosophila*.

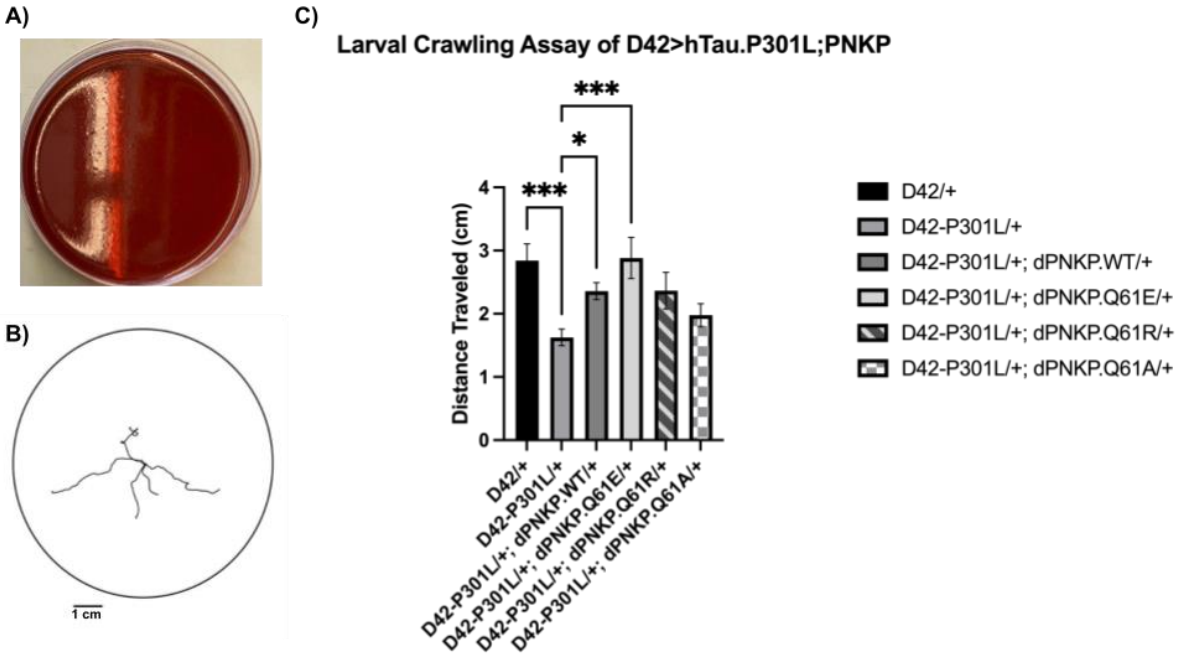

Figure 5. Overexpression of dPNKP in hTau^{P301L} fly motoneurons improves larval crawling performance. *(A)* Larval crawling assays were performed on a 1.5% grape juice agar plate. *(B)* Representative crawling trace of five larvae in one minute. The distances traveled by 10-15 larvae from each genotype were measured by Fiji. *(C)* Tau larvae expressing different transgenic UAS-dPNKP genes were compared to driver-only controls and to tau larvae not expressing any dPNKP transgene. Genotypes: D42-GAL4/+ (control), D42- hTau^{P301L}/+, D42- hTau^{P301L}/+; UAS-dPNKP.WT/+, D42- hTau^{P301L}/+; UAS-dPNPK.Q61A/+, D42- hTau^{P301L}/+; UAS-dPNPK.Q61R/+, D42- hTau^{P301L}/+; UAS-dPNPK.Q61E/+. Ordinary one-way ANOVA; *P<0.05, **P<0.01, ***P<0.005. n=10-15 per genotype.

DISCUSSION

Previous studies have found increased levels of DNA damage in the brains of AD patients as well as colocalization of DSB and aggregated tau [2][10]. Previous studies have found that dysfunction of DNA repair mechanisms is critically involved in other neurodegenerative diseases such as SCA3 and HD [9][14]. Recent studies from the Hazra lab have found decreased activity of PNKP – a key DNA repair enzyme – in the cortex of AD patient brains, suggesting PNKP dysfunction as a contributing factor in the DNA damage observed in AD. As the role of PNKP in tauopathies is still unknown, our study looks to determine the effect of PNKP overexpression in the context of tauopathy using a tau fly model.

We first began by creating transgenic flies carrying wild-type *Drosophila* PNKP (dPNKP.WT) and several dPNKP mutants. Our system for overexpressing UAS-dPNKP.WT and the UAS-dPNKP mutants using the GAL4-UAS was confirmed through RT-qPCR **(Fig.1)** Subsequent characterization of the transgenic dPNKP flies showed no aberrant phenotypes in flies overexpressing dPNKP.WT and dPNKP mutants in an otherwise wild-type background, aside from slight decreases in lifespan observed in dPNKP.Q61E and dPNKP.Q61R male flies **(Fig.2).**

Tau-induced neurotoxicity can manifest itself in several phenotypes in *Drosophila*, including a rough eye phenotype and locomotor dysfunction. We found that overexpression of wild-type dPNKP in gl-hTau1.1 flies rescued the rough eye phenotype associated with tau toxicity in the fly retina (**Fig.4**). In the D42-hTau^{P301L} flies where hTauP301L is overexpressed in motoneurons, larvae overexpressing dPNKP.WT and dPNKP.Q61E showed a rescue of the crawling deficit (**Fig.5**).

Results from this study provide insight on the role of DNA repair in tauopathies. The rescue of hTau flies by overexpression of dPNKP in this present study is similar to the rescue of SCA3 flies in that both rescue experiments focus on increasing PNKP activity through the increase of PNKP levels by overexpression [9]. These findings offer a potential strategy of restoring or supplementing PNKP activity in order to increase the DNA repair activity via gene therapy. Recent studies characterizing PNKP in AD brains, performed by the Hazra lab, showed that AD brains exhibit a decrease in PNKP activity. Interestingly, they found that supplementing AD brains with a metabolite, fructose-2,6-bisphosphate, increased PNKP activity (Hazra lab, unpublished). These preliminary findings suggest another potential strategy for increasing PNKP activity.

Though these are promising potential methods of treatment in tauopathies, more investigation is needed to elucidate the molecular mechanism by which PNKP modulates tau pathophysiology. As the next step, we will determine the level of DNA damage with and without PNKP overexpression in tauopathy fly models, by immunohistochemistry staining with H2A.v antibody in the brains, and by long-amplification PCR to detect DNA breakage. In addition, identification of the underlying molecular changes that cause the changes in PNKP activity still need to be further explored. In summary, findings from this study show that the neurotoxicity in fly tauopathy models, brought on by the dysfunction of DNA repair, may be ameliorated through the increase of PNKP activity. Further investigation is needed to elucidate the underlying mechanism, and to devise therapeutic strategies for intervention in tauopathies and other neurodegenerative diseases.

MATERIALS AND METHODS

Drosophila genetics

Generation of transgenic UAS-dPNKP fly lines

Transgenic dPNKP fly lines were generated using the phiC31 integrase system. The phiC31 integrase mediates the recombination between two attachment sites, attB and attP. The attB site is carried in the plasmid construct and the attP site is located in the drosophila genome [5]. The DNA constructs were cloned in the pACUH backbone, with UAS-dPNKP cDNA inserted. The plasmids were injected into fly embryos carrying the attP site in their genome on the second chromosome in a position that would not interfere with the endogenous PNKP gene. The resulting transgenic flies were crossed with the second chromosome balancer carrying CyO, and self-crossed to generate a homozygous stock of UAS-dPNKP flies.

Creation of D42-UAS-hTau.P301L flies

The recombinant D42-UAS-hTau.P301L was generated by crossing D42-Gal4 (Chromosome III) flies to UAS-hTau.P301L (Chromosome III) flies. The F1 female progeny were crossed with chromosome III balancer TM3/TM6. Single flies of the F2 progeny were crossed with TM3, Sb/TM6, Tb balancer line to establish 25 individual lines to isolate any recombination events between D42-Gal4 and UAS-hTau.P301L. Homozygous flies from each of the resulting stocks were collected and screened by genotyping to confirm the successful recombination event resulting in the presence of both D42-Gal4 and UAS-hTau.P301L on chromosome III.

Fly lines used:

Obtained from Bloomington Drosophila Stock Center (Indiana University):

1. C155; GMR-hTau1.1 (BDSC Stock #51360)
2. Sna(Sco)/CyO, actGFP (BDSC Stock #9325)
3. C155-Gal4 (BDSC Stock #458)
4. D42-Gal4 (BDSC Stock #8816)
5. OK6-Gal4 (BDSC Stock #64199)

Drosophila Lifespan Assay

Transgenic flies carrying the UAS-dPNKP.WT and UAS-dPNKP variant (Q61E, Q61R, Q61A) genes were used in the characterization. The flies carrying only the respective UAS-dPNKP genes were collected within three days of hatching and pooled together according to their respective sex. Each pool of male and female progeny consisted of 20 flies with three biological repeats. Fly survival was scored daily based on how many flies had died on a given day until all flies in a single vial had all died. Subsequent visualization of lifespan data was generated using a Kaplan-Meier lifespan curve in GraphPad Prism and analyzed using the log-rank test.

Drosophila Larval Crawling Assay

Crawling assays were performed on 1.5% agarose plates made with a 2.3:1 combination of grape juice and water. A sample size of 10 to 15 larvae were selected for each genotype and assays were done using larvae in the third instar state. The larvae were first removed from vials and gently placed into a petri dish containing deionized water to allow for residual food to be washed off the body. After 15 seconds, the larvae were transferred to a petri dish containing the 1.5% agarose mixture and were given one minute to rest. They were then transferred to a second dish filled with the 1.5% agarose mixture and timed immediately for one minute, during which their crawling performance was measured. A transparent plastic lid was placed on top of the

plates and the crawling path of the larvae were traced. Observations of the crawling activity were done under a light microscope. The brightness and distance of the light source above the plates were kept constant across all trials and genotypes. The crawling paths of the larvae were measured using FIJI ImageJ and the average distance traveled was taken for each genotype.

Drosophila Climbing Assay

Adult fly progeny was collected and aged to seven days old before performing the first assessment. At the day of assay, flies were transferred from their respective vials to a graduated cylinder with equidistant zones arbitrarily numbered from one to six, with one being the bottom of the cylinder and six being the top. After flies were transferred to the assaying graduated cylinder and the cylinder was capped with a cotton plug, they were given one minute to rest. The cylinder was gently tapped on a padded surface to bring all the flies to the bottom of the cylinder, at which a timer was started for 10 seconds (t=0). An image was taken after 10 seconds (t=10) and the number of flies in each of the numbered sections of the cylinder was recorded. Flies were assayed again after one minute of rest. Five trials were performed on each biological repeat. Averages were taken from each biological repeat and averages of the three biological repeats were taken per sex for each genotype.

Drosophila Eye Imaging/Imprinting

Eye imprinting

Eye imprinting was performed on adult flies using a layer of clear nail polish. Clear nail polish was applied onto the surface of the eye of anesthetized flies using a brush applicator. After letting the nail polish set and solidify, fine-tipped tweezers were used to carefully remove the

layer of nail polish from the eye without touching the area of the imprint that was in contact with the eye surface. The imprint was placed into drop of water on top of a microscope slide and covered with a glass slide cover slip. Subsequent imaging of the eye imprint was performed using a compound light microscope.

Eye Imaging

Flies were briefly anesthetized using ether and transferred to the light microscope platform. A microscope camera was placed over the lens and connected to a computer to provide a real time image of the flies. A bilateral image of the fly was then taken with the eye facing up.

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