Miro, a Rho GTPase genetically interacts with Alzheimer’s disease-associated genes (\(\text{Tau}, \text{A}\beta_{42} \text{ and } \text{Appl}\)) in \textit{Drosophila melanogaster}

Komal Panchal and Anand Krishna Tiwari*

**ABSTRACT**

Miro (mitochondrial Rho GTPases), a mitochondrial outer membrane protein, facilitates mitochondrial axonal transport along the microtubules to facilitate neuronal function. It plays an important role in regulating mitochondrial dynamics (fusion and fission) and cellular energy generation. Thus, Miro might be associated with the key pathologies of several neurodegenerative diseases (NDs) including Alzheimer’s disease (AD). In the present manuscript, we have demonstrated the possible genetic interaction between Miro and AD-related genes such as \(\text{Tau}, \text{A}\beta_{42} \text{ and } \text{Appl}\) in \textit{Drosophila melanogaster}. Ectopic expression of \(\text{Tau}, \text{A}\beta_{42} \text{ and } \text{Appl}\) induced a rough eye phenotype, defects in phototaxis and climbing activity, and shortened lifespan in the flies. In our study, we have observed that overexpression of Miro improves the rough eye phenotype, behavioral activities (climbing and phototaxis) and ATP level in AD model flies. Further, the improvement examined in AD-related phenotypes was correlated with decreased oxidative stress, cell death and neurodegeneration in Miro overexpressing AD model flies. Thus, the obtained results suggested that Miro genetically interacts with AD-related genes in \textit{Drosophila} and has the potential to be used as a therapeutic target for the design of therapeutic strategies for NDs.

This article has an associated First Person interview with the first author of the paper.

**KEY WORDS:** Miro, \(\text{Tau}, \text{A}\beta_{42} \text{, Appl, Mitochondria, Alzheimer’s disease}\)**

**INTRODUCTION**

Mitochondrial Rho GTPase (Miro) is an evolutionary conserved mitochondrial outer membrane protein that plays a pivotal role in mitochondrial axonal transport and maintenance of mitochondrial dynamics (fusion and fission) (Kay et al., 2018; Lee and Lu, 2014; Reis et al., 2009; Tang, 2016). Miro forms a major protein complex with Milton (adaptor protein) and, kinesin and dynein (motor proteins) to facilitate mitochondrial bi-directional axonal transport such as anterograde (cell body to axon) and retrograde (axon to cell body) transport (Cai and Sheng, 2009; Panchal and Tiwari, 2018). The involvement of Miro in the impairment of mitochondrial axonal transport that ultimately leads to neurodegeneration has previously been reported (López–Doménech et al., 2018; Russo et al., 2009; Tang, 2016).

Alzheimer’s disease (AD) is the second most common neurodegenerative disorder characterized by the formation of extracellular \(\text{A}\beta_{42}\) plaques (amyloidogenic cleavage of \text{APPL} protein) by \(\beta\) and \(\gamma\)-secretase and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein (Binder et al., 2005; O’Brien and Wong, 2011). Various molecular changes have been reported in AD, which includes early metabolic changes, neuronal death, memory loss, cognitive decline, mitochondria dysfunction and defective mitochondrial axonal transport (Mosconi et al., 2014; Tan and Azzam, 2017). The fruit fly, \textit{Drosophila melanogaster}, is commonly used as a model organism to explore the molecular details of several neurological diseases including AD. In the \textit{Drosophila} model of AD, neuronal death results in rough eye phenotype, learning and memory loss, impaired climbing and phototaxis activity with reduced lifespan (Jahn et al., 2011; McGurk et al., 2015; Simon et al., 2012). Further, weight loss, an early metabolic change, associated with \(\text{A}\beta\)-mediated toxicity in hypothalamic neurons induces a reduction in body weight in AD model flies (Cova et al., 2016; Sergi et al., 2013). Moreover, several studies have suggested that overexpression of AD-related genes (\text{Appl}, \text{A}\beta_{42} \text{ and } \text{Tau}) in \textit{Drosophila} induced caspase-dependent cell death (apoptosis) via increasing cellular stress and mitochondrial dysfunction resulting in reduced ATP level and enhanced oxidative stress (Cai et al., 2005; Pérez et al., 2018; Park et al., 2013). Thus, studying the different parameters such as behavior, cell death, mitochondrial function including ATP level and oxidative stress would be very useful to study the molecular details of AD-related pathologies.

A study by Iijima-Ando et al. (2009) showed that overexpression of \(\text{A}\beta_{42}\) in AD model flies results in the reduction of mitochondria numbers in axons and dendrites, and increases mitochondria accumulation in somata of the neurons. This mitochondrial mislocalization exacerbated by Miro mutation ultimately enhances \(\text{A}\beta_{42}\)-induced behavioral deficits in \textit{Drosophila}. Moreover, knockdown of Miro in AD model flies has been reported to enhance the tau-induced neurodegeneration by increasing the tau phosphorylation in AD-related site S262 by PAR1 kinase (Iijima-Ando et al., 2012), suggesting that Miro might play an important role in the modulation of AD-related pathologies.

Interestingly, an axonal transport study in \textit{Drosophila} has revealed that \textit{Drosophila} Miro is functionally homologous to human Miro 1 and Miro 2 proteins (Tang, 2016; Kay et al., 2018). The \textit{Drosophila} mitochondrial axonal transport protein complex Miro/Milton/kinesin is also homologous to mammalian Miro/TRAK/KIF5 protein complex (Lee and Lu, 2014; Tang, 2016). These similarities between \textit{Drosophila} and human mitochondrial...
axonal transport proteins make Drosophila a powerful model organism to study the mitochondria dysfunction related pathologies in AD (Kay et al., 2018; Russo et al., 2009).

As mentioned above, Miro plays a key role in neurodegeneration by regulating the mitochondrial axonal transport, but the molecular details of how Miro interacts with AD-related genes (Tau, Aβ42 and Appl) are not well understood yet. Thus, in the current study, we studied the possible genetic interaction between the mitochondrial axonal transport gene Miro and AD-related genes (Tau, Aβ42 and Appl) in Drosophila.

RESULTS
Ectopic expression of AD-associated genes (Tau, Aβ42 and Appl) showed AD-related pathologies in Drosophila

Tau, Aβ42 and Appl (AD-related genes) are involved in the pathogenesis of AD and their ectopic expression results in several phenotypic/behavioral abnormalities such as rough eye phenotype, phototaxis and climbing defect, and reduced survival and body weight in Drosophila (Fernández-Moriano et al., 2015; Gistelinck et al., 2012; Iijima-Ando and Iijima, 2010; Peng et al., 2015; Roy and Jackson, 2014).

To examine the AD-related pathologies such as rough eye phenotype and behavioral changes (phototaxis and locomotor), AD model flies were used in the present study. We have expressed AD-related genes such as UAS-TauWT, UAS- Aβ42(Human) and UAS-ApplRNA4 in the eyes of flies using pan-retinal GMR-GAL4 [GMR-GAL4-UAS-TauWT/+, GMR-GAL4-UAS-Aβ42(Human)/+ and GMR-GAL4/+:UAS-ApplRNA4] and expressed other AD genes such as UAS-Aβ42E693G and UAS-APP.C99-UAS-MAPT in the neurons using pan-neuronal elav-Gal4155 (elav-Gal4155/+/;+;UAS-Aβ42E693G/+ and elav-Gal4155/++;+/UAS-APP.C99-UAS-MAPT/+).

It was observed that the ectopic expression of AD-related genes in the eye flies results in a degenerated eye phenotype in Drosophila (Fig. 1A–E,A’–E,a–e). Further, the magnified view of Drosophila eye images from the AD model showed retinal degeneration along with disarrangements of ommatidia and bristles in the eye (Fig. 1c–e) as compared to wild type (OregonR’) and experimental control (GMR-GAL4/+) flies (Fig. 1a,b).

Phototaxis activity (expressed as light preference index) was also decreased in 10-day-old AD model flies [GMR-GAL4-UAS-TauWT/+, GMR-GAL4-UAS-Aβ42(Human)/+ and GMR-GAL4/+:UAS-ApplRNA4] to 7.3, 10.3 and 10.0, respectively, as compared to GMR-GAL4/+ flies which had a light preference index of 17.8 (Fig. 1F).

The climbing assay (to examine climbing deficits) was performed in 10-, 20- and 30-day-old AD model flies (elav-Gal4155/++;+/; UAS-Aβ42E693G/+). The climbing activity was significantly decreased to 60.56%, 43.76% and 25.92% in 10-, 20- and 30-day-old AD model flies, respectively, as compared to 10-, 20- and 30-day-old experimental control flies (elav-Gal4155/++;+/; +/+), which had climbing activity of 96.22%, 87.46% and 81.08%, respectively (Fig. 1G).

Furthermore, we performed the survival assays in AD model flies to check the lifespan (Fig. 1H). It was observed that the median lifespans of elav-Gal4155/++;+/; UAS-Aβ42E693G/+ and elav-Gal4155/++;+/; UAS-APP.C99-UAS-MAPT/+ flies were significantly decreased to 32 and 34 days as compared to elav-Gal4155/++;+/; +/+ flies, which had a median lifespan of 64 days (Fig. 1H).

The body weight analysis of 10-, 20- and 30-day-old AD model flies (elav-Gal4155/++;+/; UAS-Aβ42E693G/+ and elav-Gal4155/++;+/; UAS-APP.C99-UAS-MAPT) was also performed. It was observed that the body weights of 10-, 20- and 30-day-old (elav-Gal4155/++;+/; UAS-Aβ42E693G/+ slices were significantly decreased to 11.12 mg, 10.72 mg and 9.25 mg, respectively, as compared to similar age (elav-Gal4155/++;+/; +/+ flies, which had body weights of 13.5 mg, 12.85 mg and 12.02 mg, respectively (Fig. 1I)). In the case of 10-, 20- and 30-day-old (elav-Gal4155/++;+/; UAS-APP.C99-UAS-MAPT) flies, body weight was significantly decreased to 10.07 mg, 9.22 mg and 8.66 mg, respectively, as compared to similar age (elav-Gal4155/++;+/; +/+ flies, which had body weights of 13.5 mg, 12.85 mg and 12.02 mg, respectively (Fig. 1I)).

Together, these results suggest that Drosophila models of AD used in the present study show AD-related pathologies (Fig. 1).

Overexpression/knockdown of Miro alters the AD-related pathologies in Drosophila

As mentioned above, Miro plays a key role in mitochondrial axonal transport and dynamics (Guo et al., 2005; Saxton and Hollenbeck, 2012). The defect in mitochondrial axonal transport and dynamics are one of the key pathologies associated with AD. Thus, to find out the participation of Miro in AD, we have performed a genetic interaction study between the mitochondrial axonal transport gene Miro and the AD-associated genes (Tau, Aβ42 and Appl) in Drosophila. The genetic interaction study was performed by crossing the AD model flies with Miro overexpressing/knockdown strains and examining the offspring for any phenotypic manifestation. Any alteration in the phenotype will suggest the possible genetic interaction between Miro and AD-associated genes in Drosophila. The genetic interaction study by enhancer and suppressor analysis is a key method for finding out the functional relationships between genes and pathways, and gives indispensable information regarding gene functions (Michaut and Bader, 2012; Thibault, 2011).

Drosophila Miro gene was overexpressed and knocked down in AD model flies genetic background using UAS-Miro and UAS-MiroRNA4 fly lines, respectively. We did not observe any changes in the eye phenotype in flies overexpressing Miro alone (GMR-GAL4/ UAS-Miro) (Fig. 2A,A’) as compared to control GMR-GAL4/+ flies (Fig. 1B,B’ and b) while there was a small extent of disarrangement of ommatidia and bristles observed in the Miro knockdown flies alone (GMR-GAL4/+: UAS-MiroRNA4/+) (Fig. 2E,E’).

It was discovered that the rough eye phenotype as well as ommatidial and bristles arrangements associated with AD model flies were significantly improved by Miro overexpression [GMR-GAL4-UAS-TauWT/+, GMR-GAL4-UAS-Aβ42(Human)/+ and GMR-GAL4/+:UAS-ApplRNA4] (Fig. 2B–D,B’–D’). Knockdown of Miro in AD model flies genetic background [GMR-GAL4-UAS-TauWT/+, UAS-MiroRNA4/+, GMR-GAL4-UAS-Aβ42(Human)/+ and GMR-GAL4/+:UAS-ApplRNA4] showed enhanced rough eye phenotype as well as ommatidial and bristles disarrangements (Fig. 2F–H,F’–H’).

The fly eye size analysis showed that the eye length and width of AD model flies (GMR-GAL4-UAS-TauWT/) was significantly decreased to 426.98 µm and 269.59 µm, respectively, as compared to GMR-GAL4/+ flies, which had an eye length of 496.35 µm and eye width of 338.54 µm (Fig. 2I). The knockdown of Miro in the AD model flies genetic background (GMR-GAL4-UAS-TauWT/+, UAS-MiroRNA4/+) significantly decreased the fly eye width to 182.12 µm as compared to the AD model flies having an eye width of 269.59 µm (Fig. 2I). This result indicated that knockdown of Miro enhanced the eye degeneration associated with AD model flies.

As mentioned above, the learning and memory defect directly affects the behavioral activities in the AD model flies (Chakraborty et al., 2012; Fernández-Moriano et al., 2015; Gistelinck et al., 2012; Iijima-Ando and Iijima, 2010; Peng et al., 2015; Roy and Jackson, 2014).
et al., 2011; Moloney et al., 2010; Nichols et al., 2012). Thus, we examined the effect of Miro overexpression/knockdown on the phototaxis activity of 10-day-old AD model flies by performing the phototaxis assay (Fig. 2J).

As shown in Fig. 2J, flies overexpressing Miro alone (GMR-GAL4/UAS-Miro) did not show any changes in light preference index while knockdown of Miro (GMR-GAL4/UAS-MiroRNAi) showed a significantly decreased light preference index of 7.0 as compared to GMR-GAL4 flies, which had a light preference index of 17.8 (Fig. 2J).

We observed that light preference index was significantly decreased to 7.25 and 10.25 in AD model flies [GMR-GAL4-UAS-TauWT/+] and [GMR-GAL4-UAS-TauE693G/+] respectively (Fig. 2J).

Fig. 1. AD related pathologies in Drosophila. (A–E) Light microscopic and (A′–E′, a–e) SEM images of eyes of 10-day-old adult flies from OregonR′ (wild-type control) (A,A′,a), GMR-GAL4/+ (experimental control) (B,B′,b), GMR-GAL4-UAS-TauWT/+ (C,C′,c), GMR-GAL4-UAS-TauE693G/+ (D,D′,d) and GMR-GAL4+/+; UAS-AppleRNAi/+ (E,E′,e). (a–e) are magnified images of SEM. Scale bar: 100 μm (A–E,A′–E′) and 20 μm (a–e). The yellow marked area shows degenerated part of eyes (C–E,C′–E′). n=50. (F) Phototaxis activity of 10-day-old control (OregonR′, GMR-GAL4/+ and AD model flies GMR-GAL4-UAS-TauWT/+, GMR-GAL4-UAS-TauE693G/+ and GMR-GAL4+/+; UAS-AppleRNAi/+). Phototaxis activity presented as a light preference index. n=100. In the box and whisker plot, the box outlines show the upper and lower quartiles.

(G) Histogram showing climbing activity [expressed as % climbing (8 cm 10 s⁻¹)] of 10-, 20- and 30-day-old adult flies of elav-Gal4C155/+;+/+;+/+ and elav-Gal4C155/+;+/+;UAS-Aβ42E693G/+; n=100. (H) Survival assay of elav-Gal4C155/+;+/+;+/+ (yellow line), elav-Gal4C155/+;+/+;UAS-Aβ42E693G/+ (blue line) and elav-Gal4C155/+;+/+;UAS-AppleC99-UAS-MAPT/+ (pink line). n=100. The Kaplan–Meier survival test was performed and significance was determined by Montel-Cox log-rank test. A table indicating data comparison between control flies versus AD model flies with median lifespan (days), Chi-Square test ($\chi^2$) and P-value (Montel-Cox log-rank test). Data comparison: life span of AD model flies such as elav-Gal4C155/+;UAS-Miro/+; UAS-Aβ42E693G/+ (P<0.0001) and elav-Gal4C155/+;UAS-Miro+/+;UAS-AppleC99-UAS-MAPT/+ compared with control flies (elav-Gal4C155/+;+/+;+/+) (P=0.0001). (I) Body weight analysis of 10-, 20- and 30-day-old flies of elav-Gal4C155/+;+/+;+/+, elav-Gal4C155/+;+/+;UAS-Aβ42E693G/+ and elav-Gal4C155/+;+/+;UAS-AppleC99-UAS-MAPT/+. n=100. Error bars represent means±s.e.m. Data significance was calculated by one-way ANOVA analysis with Tukey’s test using GraphPad Prism 5.0 and is indicated as ***P<0.0001.
GMR-GAL4/US-Aβ42(Human)/+, respectively, as compared to GMR-GAL4/+ flies, which had a light preference index of 17 (Fig. 2). The light preference index was (7.25) of GMR-GAL4/US-TauWT/+ flies was restored to 16.0 by Miro overexpression (GMR-GAL4/US-TauWT/+; UAS-Miro) while knockdown of Miro (GMR-GAL4/US-TauWT/+; UAS-MiroRNAi) decreased the light preference index to 4.0 (Fig. 2).

In the case of GMR-GAL4/US-Aβ42(Human)/+ flies, they had a light preference index of 10.25, which was restored to 12.75 by Miro overexpression [GMR-GAL4/US-Aβ42(Human)/+; UAS-Miro]. Knockdown of Miro in AD flies genetic backgrounds (GMR-GAL4/US-Aβ42(Human)/+; UAS-MiroRNAi) decreased the light preference index to 0.3 (Fig. 2).

Further, we checked the effect of Miro on the climbing activity associated with AD model flies. As shown in Fig. 2K, the climbing activity of 10-, 20- and 30-day-old Miro overexpressing flies (elav-Gal4C155/+; UAS-Miro/+; +) was significantly decreased to 87%, 78% and 61%, respectively, as compared to similar age elav-Gal4C155/+; +/+ flies, which had climbing activity of 96.22%, 87.46% and 81.08%, respectively (Fig. 2K).

The climbing activity of 10-, 20- and 30-day-old AD model flies (elav-Gal4C155/+; +/+; UAS-Aβ42E693G/+; UAS-Miro/+; UAS-Aβ42E693G/+; UAS-Aβ42E693G/+; UAS-Miro) significantly increased the climbing activity to 83.18%, 67.84% and 49.84%, respectively, as compared to same aged elav-Gal4C155/+; +/+; UAS-Aβ42E693G/+; UAS-Miro flies (Fig. 2K).

We also examined the effect of Miro overexpression on the body weight of AD model flies. As shown in Fig. 2L, the bodyweight of 10-, 20- and 30-day-old flies overexpressing Miro alone (elav-Gal4C155/+; UAS-Miro/) was significantly decreased to 12.5, 11.47 mg and 10.27 mg, respectively, as compared to control elav-Gal4C155/+; +/+ flies, which had body weight of 13.5 mg, 12.9 mg and 12.02 mg, respectively (Fig. 2L).

The body weight of 10-, 20- and 30-day-old elav-Gal4C155/+; +/++; UAS-Aβ42E693G/+; UAS-Aβ42E693G/+; UAS-Miro flies was significantly decreased to 11.2 mg, 10.72 mg and 9.25 mg, respectively, which increased to 12.56 mg, 11.98 mg and 11.26 mg, respectively, in a Miro overexpressing genetic background (elav-Gal4C155/+; UAS-Miro/; UAS-Aβ42E693G/+; UAS-Miro) (Fig. 2L).

In the case of 10-, 20- and 30-day-old elav-Gal4C155/+; +/++; UAS-Aβ42E693G/+; UAS-APP.C99-UAS-MAPT/+ AD model flies, the body weight was significantly decreased to 10.07 mg, 9.22 mg and 8.66 mg, respectively, as compared to same aged elav-Gal4C155/+; +/++; UAS-Aβ42E693G/+; UAS-APP.C99-UAS-MAPT/+ flies. While overexpression of Miro in an AD model fly genetic background (elav-Gal4C155/+; UAS-Miro/+; UAS-APP.C99-UAS-MAPT) significantly increased the body weight to 12.39 mg, 11.15 mg and 10.59 mg, respectively (Fig. 2L).
**Overexpression of Miro increases the lifespan of AD model flies**

As we have shown in Fig. 1H, AD model flies have a shortened lifespan as compared to control flies. Thus, we checked the effect of *Miro* overexpression on the median lifespan of AD model flies. We observed that median lifespan of *Miro* overexpressing flies (elav-Gal4C155/+; UAS-Miro+/+;+/+) was significantly decreased as compared to elav-Gal4C155/+;+/+;+/+ flies, which had a median lifespan of 64 days (Fig. 3). Further, we observed that the median lifespans of *Miro* overexpressing AD model flies (elav-Gal4C155/+; UAS-Miro+/+; UAS-βGAL4E693G/+ and elav-Gal4C155/+; UAS-Miro+/+; UAS-APP.C99-UAS-MAPT/+) were significantly extended to 48 days and 44 days, respectively, as compared to AD model flies (elav-Gal4C155/+; UAS-Miro+/+; UAS-βGAL4E693G/+ and elav-Gal4C155/+; UAS-Miro+/+; UAS-APP.C99-UAS-MAPT/+), which had median lifespans of 32 days and 34 days, respectively (Fig. 3).

**Overexpression of Miro modulates the cell death in eye imaginal discs of AD model flies**

As shown in the above Fig. 2A–H, overexpression of *Miro* improved while knockdown of *Miro* potentiated the rough eye phenotype associated with AD model flies. Thus, to find out whether the rough eye phenotype was associated with ectopic cell death in eyes, we performed Acridine Orange (AO) staining in third instar larval eye imaginal discs (Fig. 4A–I) of experimental control (GMR-GAL4/+, *Miro* overexpressing (GMR-GAL4/UAS-Miro) and knockdown (GMR-GAL4/+; UAS-MiroRNAi/+) flies, AD model flies (GMR-GAL4-UAS-TauWT/+ and GMR-GAL4-UAS-ABβ2,Human/+ and GMR-GAL4-UAS-ABβ2,Human) and AD model flies with *Miro* overexpression (GMR-GAL4-UAS-TauWT/UAS-Miro and GMR-GAL4-UAS-ABβ2,Human/UAS-Miro) and *Miro* knockdown (GMR-GAL4-UAS-TauWT/UAS-MiroRNAi/+) and GMR-GAL4-UAS-ABβ2,Human/UAS-MiroRNAi/+.

We observed that AD model flies showed excessive cell death (AO positive cells) posterior to the morphogenetic furrow (MF) in larval eyes (Fig. 4D,G,J) as compared to the GMR-GAL4/+ flies showing few apoptotic cells (Fig. 4A,J). Overexpression of *Miro* in AD model flies (Fig. 4E,H,J) showed a significant reduction in cell death, while knockdown of *Miro* in AD model flies genetic background (Fig. 4F,II) did not show any significant changes in apoptotic cells as compared to the respective AD model flies. As shown in Fig. 4B and C, where *Miro* alone was overexpressed or knocked down did not show any changes in apoptotic cells as compared to GMR-GAL4/+ flies. This result clearly suggests that overexpression of *Miro* modulates apoptosis in AD model flies.

As seen in the above results, overexpression of *Miro* modulates AD-related pathologies. Thus, we have checked Miro gene expression level in 30-day-old AD model flies by performing quantitative real-time PCR analysis. We observed that relative expression of the *Miro* gene was significantly increased in AD model flies [GMR-GAL4-UAS-TauWT/+; GMR-GAL4-UAS-ABβ2,Human/+ and GMR-GAL4/+; UAS-ApppRNAi/+] to 1.7, 2.2- and twofold, respectively, as compared to GMR-GAL4/+ flies (Fig. 4K). This result indicated that the expression of *Miro* genes significantly upregulates the *Miro* gene in *Drosophila*. It suggests that the function of *Miro* in AD-related pathologies is conserved in *Drosophila*.

**Overexpression of Miro decreases mitochondrial and cellular oxidative stress in AD model flies**

It is well known that oxidative stress and altered mitochondrial dynamics play a key role in AD pathogenesis (Wang et al., 2009; Zhu et al., 2013). Thus, to examine the status of mitochondria-mediated oxidative stress and the effect of overexpression/knockdown of *Miro* in AD model flies, we have performed MitoSOX Red staining to measure the mitochondrial ROS, MitoTracker Green to labelled mitochondria and Hoechst 33342 staining to visualize the nucleus in third instar larval brain of control and experimental group flies (Fig. 5A–X).

**AD model flies** (elav-Gal4C155/+;+/+; UAS-ABβ2E693G/+ and elav-Gal4C155/+;+/+; UAS-APP.C99-UAS-MAPT/+) showed a significant induction of MitoSOX Red fluorescence and increased co-localization (yellow) with MitoTracker Green staining suggesting a higher level of mitochondrial ROS production in both the AD model flies (Fig. 5I–L,Q–T,a), as compared to elav-Gal4C155/+;+/+;+/+ flies (Fig. 5A–D,a). Overexpression of *Miro* in AD model flies such as elav-Gal4C155/+; UAS-Miro/+; UAS-ABβ2E693G/+ and elav-Gal4C155/+; UAS-Miro/+; UAS-APP.C99-UAS-MAPT/+ showed a significant reduction of MitoSOX Red fluorescence suggesting that *Miro* overexpression helps in the reduction of mitochondrial ROS level in AD model flies.

We have also checked the cellular (cytosolic) ROS level in control and AD model flies alone and in the *Miro* overexpressing AD model flies genetic background using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) dye. In the presence of cellular ROS, non-fluorescent H2DCFDA was oxidized and converted into highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Kalyanaraman et al., 2012; Tetz et al., 2013). As shown in Fig. 5b, AD model flies (elav-Gal4C155/+;+/+; UAS-ABβ2E693G/+ and elav-Gal4C155/+;+/+; UAS-APP.C99-UAS-MAPT/+) showed strong fluorescence of DCF, suggesting higher cellular ROS production (Fig. 5b). While overexpression of *Miro* in AD model flies (elav-Gal4C155/+; UAS-Miro/+; UAS-ABβ2E693G/+ and elav-Gal4C155/+; UAS-Miro/+; UAS-APP.C99-UAS-MAPT/+) significantly decreased the DCF fluorescence in adult brain as compared to the control (Fig. 5b). The DCF fluorescence was not affected by overexpression of *Miro* alone (elav-Gal4C155/+; UAS-Miro/+;+/+) as compared to elav-Gal4C155/+;+/+;+/+ flies (Fig. 5b).

To further validate the above observations, we have examined the effect of *Miro* overexpression on the anti-oxidant enzymes genes expression levels such as Manganese Superoxide dismutase (*Mn-SOD*) and Catalase (*CAT*) in 30-day-old AD model flies by performing quantitative real-time PCR analysis (Fig. 5c). It is known that the cooperative function of SOD and CAT helps in the protection against oxidative stress (Ighodaro and Akinloye, 2018; Luangwattanam et al., 2016). Mitochondrial *Mn-SOD* is also known as *Drosophila* SOD2, which cleans mitochondrial ROS via eliminating the superoxide radical (Candas and Li, 2014). *Mn-SOD* cleaved superoxide radical and produce H₂O₂, which is further degraded into H₂O and O₂ by CAT enzyme (Candas and Li, 2014; Tetz et al., 2013). As shown in Fig. 5c, the expression level of *Mn-SOD* and *CAT* genes was significantly increased in AD model flies (elav-Gal4C155/+;+/+; UAS-ABβ2E693G/+ and elav-Gal4C155/+;+/+; UAS-APP.C99-UAS-MAPT/+) to 1.8 and 1.7-fold, respectively, as compared to elav-Gal4C155/+;+/+;+/+ flies (Fig. 5c). This higher level of *Mn-SOD* gene expression was significantly decreased to 0.8- and 0.6-fold in *Miro* overexpressing AD model flies such as elav-Gal4C155/+; UAS-Miro/+; UAS-ABβ2E693G/+ and elav-Gal4C155/+; UAS-Miro/+; UAS-APP.C99-UAS-MAPT/+, respectively (Fig. 5c).
In the case of the CAT gene, the relative expression level of CAT in AD model flies (elav-Gal4C155/+;+/+;+/+;UAS-APP.C99-UAS-MAPT/+, UAS-β42E693G/+;elav-Gal4C155/+;+/+;UAS-APP.C99-UAS-MAPT/) was significantly increased to 2.6- and 2.8-fold, respectively, as compared to elav-Gal4C155/+;+/+;+/+ flies (Fig. 5c). While overexpression of Miro in AD model flies (elav-Gal4C155/+;+/+;UAS-APP.C99-UAS-MAPT/+, UAS-β42E693G/+;elav-Gal4C155/+;+/+;UAS-APP.C99-UAS-MAPT) significantly decreased the relative CAT gene expression level to 2.1- and twofold, respectively, as compared to the respective AD model flies (Fig. 5c).

As shown in Fig. 5c, overexpression of Miro alone did not affect the expression of any of the antioxidant enzymes.

The above results confirmed that overexpression of Miro decreases expression of the antioxidant Mn-SOD and CAT enzymes via reducing mitochondrial and cellular ROS in AD model flies. CAT expression level in Miro expressing AD model flies was still higher than in control flies. This suggests that the overexpression of Miro may affect mitochondrial ROS more than cellular ROS.

Overexpression of Miro altered mitochondrial dynamics in AD model flies

It has been shown that overexpression of Miro increased average length of mitochondria by increasing mitochondrial fusion (Kay et al., 2018; Tang, 2016). Thus, to examine the effect of Miro on mitochondrial dynamics in AD model flies, we have checked the average length of mitochondria using GMR-GAL4-UAS-Mito-GFP flies (Fig. 6A–G). As shown in Fig. 6, the average length of mitochondria was significantly increased in flies overexpressing Miro alone (GMR-GAL4-UAS-Mito-GFP/+;elav-Gal4C155/+;+/+;UAS-β42E693G/) to 2.6- and 2.8-fold, respectively, as compared to control GMR-GAL4-UAS-Mito-GFP/+ flies (Fig. 6A,G). As shown in Fig. 6, the average length of mitochondria was significantly increased in flies overexpressing Miro alone (GMR-GAL4-UAS-Mito-GFP/+;elav-Gal4C155/+;+/+;UAS-β42E693G/) to 2.6- and 2.8-fold, respectively, as compared to control GMR-GAL4-UAS-Mito-GFP/+ flies (Fig. 6A,G). Furthermore, the average length of mitochondria was decreased to 1.3 µm and 1.8 µm in AD model flies (GMR-GAL4-
UAS-Mito-GFP/+; UAS-β42E693G/+ and GMR-GAL4-UAS-Mito-GFP/+; UAS-APP.C99-UAS-MAPT/+, respectively, as compared to control flies (Fig. 6C,E and G). The average length of mitochondria was significantly increased to 13.8 µm and 14.3 µm in Miro overexpressing AD model flies (elav-Gal4C155/+; UAS-Miro; UAS-β42E693G/+ and elav-Gal4C155/+; UAS-MiroRNAi/+; UAS-β42E693G/+), respectively (Fig. 6D,F,G).

Further, to examine whether increased mitochondrial average length is associated with mitochondrial fusion, we checked the expression level of the mitochondrial fusion gene, Mitofusin (Mfn) by performing quantitative real-time PCR analysis. As shown in Fig. 6H the relative expression level of the Mfn gene in AD model flies (elav-Gal4C155/+; UAS-β42E693G/+ and elav-Gal4C155/+; UAS-APP.C99-UAS-MAPT/) was significantly decreased to 1.7- and 0.7-fold, respectively (Fig. 6H), while overexpression of Miro in AD model flies (elav-Gal4C155/+; UAS-Miro/+; UAS-β42E693G/+ and elav-Gal4C155/+; UAS-Miro/+; UAS-APP.C99-UAS-MAPT/) showed significantly increased mitochondrial average length to 5.2- and 4.8-fold, respectively (Fig. 6H). This result suggests that overexpression of Miro modulates mitochondrial dynamics via increasing the average length of mitochondria and altering the Mfn gene expression level in AD model flies.

**Overexpression of Miro increases the ATP level in AD model flies**

As discussed above, overexpression of Miro increased mitochondrial fusion via increasing mitochondrial average length and the Mitofusin gene expression in AD model flies (Fig. 6). It has been reported that the fusion of mitochondria might result in an increased level of ATP production (Rambold et al., 2011; Song and Hwang, 2019). Thus, we examined the effect of Miro on ATP level in 30-day-old AD model flies (Fig. 7A). As shown in Fig. 7A, the
ATP level in flies overexpressing Miro alone (elav-Gal4C155+/; UAS-Miro+/;+/+) was $4.2 \times 10^5 \mu M \mu g^{-1}$ of protein, which was similar to the control flies (elav-Gal4C155+/;+/;+/+) that had $4.6 \times 10^5 \mu M \mu g^{-1}$ of protein (Fig. 7A). In the case of AD model flies (elav-Gal4C155+/;+/;UAS-AB42E693G+/ and elav-Gal4C155+/;+/UAS-APP.C99-UAS-MAPT+/), the ATP was significantly decreased to $2.4 \times 10^5$ and $3.6 \times 10^5 \mu M \mu g^{-1}$ of protein as compared to control flies (Fig. 7A). This decreased ATP in AD model flies was significantly increased to $4.3 \times 10^5$ and $4.5 \times 10^5 \mu M \mu g^{-1}$ of protein in Miro overexpressing AD model flies such as elav-Gal4C155+/;UAS-Miro+/;UAS-AB42E693G+/ and elav-Gal4C155+/;UAS-Miro+/;UAS-APP.C99-UAS-MAPT+/, respectively (Fig. 7A).

To further confirm this, we have checked the expression level of ATP synthase beta gene by performing quantitative real-time PCR in 30-day-old adult fly heads of control and experimental group flies. As shown in Fig. 7B, we did not find any change in relative expression of ATP synthase beta in the flies overexpressing...
Miro alone (elav-Gal4C155/+;UAS-Miro/+;+/+) as compared to elav-Gal4C155/+;+/+;+/+ control flies (Fig. 7B). Further, we observed the relative expression of ATP synthase beta was decreased to 0.4- and 0.5-fold in AD model flies such as elav-Gal4C155/+;+/+;UAS-Aβ42E693G/+ and elav-Gal4C155/+;+/+;UAS-APP.C99-UAS-MAPT/+ respectively, as compared to the control flies (Fig. 7B). The decreased relative expression of the ATP synthase gene was increased to 2.2- and 1.8-fold in Miro overexpressing AD model flies such as elav-Gal4C155/+;UAS-Miro/+;UAS-Aβ42E693G/+ and elav-Gal4C155/+;UAS-Miro/+;UAS-APP.C99-UAS-MAPT/+ respectively (Fig. 7B).

Together, these results suggest that overexpression of Miro increases the energy level in the form of ATP in AD model flies.

Overexpression of Miro decreases cell death and neurodegeneration in the brain of AD model flies

We further examined the effect of Miro overexpression on cell death in AD model flies by staining third instar larval brain with AO, an apoptosis marker (Fig. 8A-F). As shown in Fig. 8B, we did not observe any changes in apoptosis in flies overexpressing Miro alone (elav-Gal4C155/+;UAS-Miro/+;+/+) (Fig. 8B, G), while cell death was significantly increased in elav-Gal4C155/+;+/+;UAS-
Aβ42E693G/+ and elav-Gal4C155/+;+/+;UAS-APP.C99-UAS-MAPT/+ flies (Fig. 8C,E,G) as compared to elav-Gal4C155/+;+/+;+/+ control flies (Fig. 8A,G). Overexpression of Miro in AD model flies (elav-Gal4C155/+;UAS-Miro/+;+/+;UAS-APP.C99-UAS-MAPT/+ and elav-Gal4C155/+;UAS-Miro/+;UAS-APP.C99-UAS-MAPT/+ flies) significantly decreased cell death (Fig. 8D,F,G) in the larval brain.

To further confirm the above result, we performed anti-cleaved caspase-3 staining in third instar larval brains of Miro overexpressing AD model flies. Caspase-3 is a typical cell death marker and an important mediator of programmed cell death (apoptosis) (Kumar and Tiwari, 2018; Porter and Jänicke, 1999). As shown in Fig. 8B,b’ and G’ cleaved caspase-3 fluorescence intensity in flies overexpressing Miro alone (elav-Gal4C155/+;UAS-Miro/+;+/+) was similar to the elav-Gal4C155/+;+/+;+/+ flies (Fig. 8A,a’,G’). We observed a significant increase in cleaved caspase-3 fluorescence intensity in AD model flies (elav-Gal4C155/+;+/+;UAS-APP.C99-UAS-MAPT/+ flies, RP49 used as an endogenous control. Error bar represents mean ±s.e.m. Data significance was calculated by one-way ANOVA analysis with Tukey’s test using GraphPad Prism 5.0 software and is indicated as: ns, non-significant; *P<0.05, **P<0.01, ***P<0.0001.
compared to fluorescence intensity was measured by using ImageJ software, NIH, USA. (A) brains of overexpression such as elav-Gal4C155/+;UAS-Miro/+;UAS-APP.C99-UAS-MAPT/+ model flies was significantly decreased to 7.8 and 11.6 by was significantly increased in flies. We observed that the number of vacuoles (neurodegeneration) Eosin (H&E) staining in the histological sections of overexpressing AD model flies, we performed Hematoxylin and Scale bar: 20 µm (A′). (A″) Confocal images showing anti-cleaved-caspase-3 staining in third instar larval brain of elav-Gal4C155/+;+/+;+/+;+/+;+/+;+/+;+/+;UAS-Miro/+;UAS-APP.C99-UAS-MAPT/+ flies. White arrowheads indicate AO positive cells in optic lobes (OL) of third instar larval brains. Scale bar: 10 µm (A′). (A″) Magnified confocal images of (A′). Scale bar: 20 µm (a–f), n=20. White arrowheads indicate caspase positive cells (A′–f, a–f). (G) Histogram showing average fluorescence intensity of cleaved caspase-3 in third instar larval brains of each genotype. Cleaved caspase-3 fluorescence intensity was measured by using ImageJ software, NIH, USA. (A′–f′) Gray scale images of H&E stained paraffin sections of 30-day-old flies brains of elav-Gal4C155/+;+/+;+/+(A), elav-Gal4C155/+;AD;UAS-Miro/+;+/+;+/+(B), elav-Gal4C155/+;+/+;+/+(C), elav-Gal4C155/+;+/+;+/+(D), elav-Gal4C155/+;+/+;+/+(E), elav-Gal4C155/+;+/+;+/+(F). Scale bar: 50 µm (A′–f′). (a–f′) Magnified images of central region of the adult fly brains (A′–f′). Red-colored round shape indicates vacuoles (neurodegeneration) (a–f′). Scale bar: 20 µm (a–f′), n=10. (G′) The histogram shows an average number of vacuoles in each genotype of adult brains. Quantification of AO positive cells and the number of vacuoles was done by using ImageJ software (NIH, USA). Error bar represents mean±s.e.m. Data significance was calculated by one-way ANOVA analysis with Tukey’s test using GraphPad Prism 5.0 and is indicated as: ns, non-significant, and ***P<0.0001.

significantly involved in the reduction in apoptosis (caspase-dependent) in AD model flies. These results suggest that Miro is notably involved in modulating the AD-related apoptosis in Drosophila.

Further, to check the neurodegeneration status in the Miro overexpressing AD model flies, we performed Hematoxylin and Eosin (H&E) staining in the histological sections of Miro overexpressing AD model flies and counted the number of vacuoles in the adult brain of control and experimental group flies. We observed that the number of vacuoles (neurodegeneration) was significantly increased in elav-Gal4C155/+;+/+;UAS-Aβ42E693G/+/+;+/+;+/+;UAS-APP.C99-UAS-MAPT/+ to 83.7 and 96.9, respectively (Fig. 8C′,c′,e′,e′,G′), as compared to elav-Gal4C155/+;+/+;+/+;+/+;+/+;+/+;+/+;UAS-APP.C99-UAS-MAPT/+ flies, which had 4.9 number of vacuoles (Fig. 8A′,a′,G′). The increased number of vacuoles in AD model flies was significantly decreased to 7.8 and 11.6 by Miro overexpression such as elav-Gal4C155/+;UAS-Miro/+/+;UAS-Aβ42E693G/+/+;+/+;UAS-APP.C99-UAS-MAPT/+ (Fig. 8D′,d′,G′) and elav-Gal4C155/+;+/+;UAS-APP.C99-UAS-MAPT/+, respectively (Fig. 8F′,f′,G′).

**DISCUSSION**

Mitochondrial dynamics and mitochondrial axonal transport play a crucial role in neuronal growth and survival (Lovas and Wang, 2013; Mandal and Drerup, 2019). Several studies have suggested that altered mitochondrial dynamics and improper axonal transport are the early events of the onset of many neurodegenerative diseases (Guo et al., 2020; Kay et al., 2018). Miro is the sole protein that plays a vital role in the bi-directional mitochondrial axonal transport such as anterograde transport (from cell body to axon) and retrograde transport (from axon to cell body) via forming a major protein complex with Milton (adapter protein), kinesin and dynein (motor proteins) (Russo et al., 2009; Cai and Sheng, 2009; Panchal and Tiwari, 2018). Miro provides ATP in the axons via facilitating the mitochondrial anterograde transport and promotes neuronal survival, while retrograde transport of mitochondria helps in the elimination of damaged mitochondria via mitophagy (Russo et al., 2009; Guo et al., 2005). The various roles of Miro in synapses are to fulfill the ATP demand, maintaining the Ca2+ buffering and bioenergetics facilitated by mitochondrial axonal transport, which helps in the proper neurotransmission and neuronal survival (Lee and Lu, 2014). Thus, any alteration in Miro function may be associated with neurodegenerative disease conditions including AD (Wang et al., 2011; Lee and Lu, 2014). It has been demonstrated that knockdown of Miro induces mislocalization of mitochondria in the neurons that results in the accumulation of mitochondria in cell body of neurons (Iijima-Ando et al., 2009). Apart from this, Miro also maintains mitochondria structure via regulating mitochondrial dynamics (fusion and fission) (Lee and Lu, 2014). Thus, it was inferred that Miro might play an important role in the modulation of AD-related pathologies (Berndt and Holzhütter, 2013; Iijima-Ando
and Iijima, 2010; Kay et al., 2018; Lee and Lu, 2014; López–Doménech et al., 2018). In the present study, we used transgenic AD fly models expressing AD-related genes such as Tau, Aβ42, and Appl, and demonstrated their possible genetic interaction with the Drosophila Miro gene. The genetic interaction study gives a new insight into understanding the complex mechanisms of AD as well as possible interactors of AD genes.

**Preliminary mechanisms involved in Tau, Aβ42 and Appl induced toxicity in Drosophila models of AD**

As shown above, the Drosophila models of AD mimic various AD-related pathologies. The rough eye phenotype (Fig. 1A–E,A′–E,a–e) and defective phototaxis activity (Fig. 1F) shown by AD model flies were reported by previous studies showing that expression of AD associated genes results in accumulation of Aβ42 plaques and neurofibrillary tangles which cause excessive cell death in fly retina and results in the degeneration of photoreceptor cells (Ferreiro et al., 2018; Higham et al., 2019; Iijima-Ando and Iijima, 2010; Pak, 2010). Further, we observed defective climbing activity, reduced median lifespan and decreased body weight in AD model flies (Fig. 1G–I). This might be due to the excessive cell death occurring in the AD model flies as seen in AO stained third instar larval eye imaginal discs (Fig. 4D,G,J). Further, the phenotypic manifestation seen in AD model flies was correlated with increased mitochondrial/cellular ROS (Fig. 5I–L,G–T,a) and an increased expression of anti-oxidant enzymes (Mn-SOD and CAT) in AD as a result of the compensatory mechanism against the increased ROS level (Fig. 5c) (Flynn and Melov, 2013; Niedzielska et al., 2016). Several studies have also suggested that increased ROS levels in AD is associated with mitochondrial damage, altered mitochondrial dynamics, and reduced ATP level (Castellani et al., 2002; Huang et al., 2016; Manoharan et al., 2016; Panchal and Tiwari, 2018). Thus, we examined the mitochondrial dynamics and observed fragmented mitochondria (reduced mitochondrial average length) (Fig. 6C,E,G) along with decreased expression of mitochondrial fusion related gene Mitofusin (Mfn) (Fig. 6H) in the AD model flies that ultimately results in reduced ATP level in AD model flies (Fig. 7A). This is in accordance with studies suggesting that expression of AD-related genes in Drosophila induced excessive cell death, higher oxidative stress and ATP deficiency that results in altered climbing activity, reduced body weight and reduced median lifespan (Keating, 2008; Lee et al., 2016; Ray et al., 2017; Winklhofer and Haass, 2010).

Further, the increased apoptosis seen in the larval brain of AD model flies (Fig. 8A–G,A′–G′) might be due to the higher oxidative stress and increased neurodegeneration. This was supported by the previous study by Wu et al. (2017) showing that increased apoptosis induced neurodegeneration in fly brain. Together these results suggest that expression of AD-associated genes (Tau, Aβ42, and Appl) induced AD-related pathologies such as rough eye phenotype, defective behaviors (phototaxis and climbing), increased cell death, oxidative stress and neurodegeneration in Drosophila.

**Possible mechanisms underlying Tau, Aβ42 and Appl induced toxicity modulated by overexpression of Miro in AD model flies**

There was improvement seen in the AD-related pathologies such as rough eye phenotype and phototaxis activity in Miro overexpressing genetic background (Fig. 2A–D,A′–D′,J) and increased pathology and behavioral deficits in Miro knockout flies (Fig. 2E–H,E′–H′, J). These results are supported by a previous study by Iijima-Ando et al. (2012) that demonstrated that the knockdown of Miro increases Tau mediated toxicity via increasing the accumulation of hyperphosphorylated Tau via PAR1 kinase activation. Thus, toxicity induced by the abnormal accumulation of Tau in AD model flies might lead to an increase in rough eye phenotype along with defective phototaxis activity in Miro knockout flies. These improvements might be due to the overexpression of Miro that reduced cell death in eye imaginal discs (Fig. 5E,H,J). Thus, overexpression of Miro might help in the reduction of neurodegeneration of photoreceptor neurons, improvement in the rough eye phenotype and phototactic behavior (Cutler et al., 2015; Gistelinck et al., 2012; Prüßing et al., 2013; Wang and Montell, 2007).

The overexpression of Miro also improved the climbing activity (Fig. 2K), increased the body weight (Fig. 2L) and median lifespan (Fig. 3) associated with AD model flies. This might be due to the cumulative effect of reduced Aβ42 induced toxicity, and reduction in cell death and oxidative stress due to the overexpression of Miro in AD flies (Gorman, 2008; Niikura et al., 2006).

Further, decreased mitochondrial (Fig. 5M–P,U–X) and cellular (Fig. 5b) ROS level examined in the Miro overexpressing AD model flies might be associated with a regulatory role of Miro in the maintenance of mitochondrial structural integrity by reducing the toxicity associated with expression of AD-related genes in Drosophila. This result was supported by the previous study showing that inhibition of abnormal mitochondrial fission and mitochondrial dysfunction could significantly reduce ROS level (Tönnes and Trushina, 2017; Wang et al., 2014). It is suggested that Miro might help in the maintenance of mitochondrial dynamics and their proper function. Further, we have checked the effect of Miro overexpression on mitochondrial dynamics in AD model flies. We observed that overexpression of Miro increased mitochondrial average length (Fig. 6D,F) that was associated with increased expression of the mitochondrial fusion gene, Mitofusin (Mfn) in AD model flies (Fig. 6H). The increased mitochondrial length in Miro overexpressing flies was correlated with increased mitochondrial fusion related protein ‘Mitofusin’ (Lin and Sheng, 2015; Panchal and Tiwari, 2018). Thus, it was inferred that overexpression of Miro increases the mitochondrial length and improves the mitochondrial dynamics with decreased ROS level in AD model flies.

Moreover, the increased ATP level seen in Miro overexpressing AD model flies (Fig. 7A) is supported by the previous studies showing that mitochondria fusion is involved in increasing ATP production (Mitra et al., 2012; Song and Hwang, 2019). Thus, the increased ATP level along with reduced ROS subsequently improves the motor function, climbing activity, body weight and lifespan of AD model flies.

Moreover, the reduced number of apoptotic cells seen in third instar larval brain of Miro overexpressing AD model flies (Fig. 8A–F,A′–F′) might be due to the decreased oxidative stress and increased ATP level resulting from Miro overexpression in AD model flies (Fernández-Moriano et al., 2015; Santos et al., 2010). Furthermore, the histological analysis of adult fly brains suggests that neurodegeneration in Miro overexpressing AD model flies was significantly decreased (Fig. 8A′–F′). This result was supported by the previous study demonstrating that reduced cell death decreases the neurodegeneration in the fly brain (Cai and Tammineni, 2017; Pathak et al., 2013).

**Conclusion**

We demonstrated that overexpression of Miro modulates the AD-related pathologies in fly models of AD by decreasing the rough eye phenotype, improving the behavior defects such as phototaxis and climbing activity along with reducing apoptosis, increasing the ATP
level and decreasing neurodegeneration in the AD model flies. Based on these observations, we conclude that the mitochondrial axonal transport gene Miro genetically interacts with AD-associated genes (Tau, Aβ42 and Appl) in Drosophila and is a potential target for therapeutic intervention for neurodegenerative diseases.

**MATERIALS AND METHODS**

**Fly stocks and genetics**

OregonR is a wild-type strain of D. melanogaster. GAL4 fly stocks: Panretinal GMR-GAL4 (Chromosome (ChrII)) drives the expression of the genes in all cells posterior to the morphogenetic furrow (MF) in the developing eye and later on it becomes active throughout most of the pupal eye (Ellis et al., 1993; Freeman, 1996), Pan-neuronal elav-Gal4C155 (Chrx) [Bloomington number (BL# 458)] drives the expression of genes in the neurons of the fly brain under elav control. Both of these flies (GMR-GAL4 and elav-Gal4C155) were used as an experimental control.

In this study, AD genes were crossed with elavC155;GAL4 to express AD causing genes in the neurons, which induces degenerative phenotypes, such as pathological morphologies and behavioral changes. AD-associated genes were also expressed in the fly eye using the GMR-GAL4 driver, which induced retinal degeneration that is indicated as rough eye phenotype. Miro overexpressing/knockdown transgenic fly stocks: UAS-Miro (ChrlI) (BL# 51646) overexpresses the Miro gene (Russo et al., 2009) and UAS-MiroRNA (ChrlI) (BL# 43973) is an RNA interference (RNAi) line of Miro gene knockdown. Transgenic fly stocks overexpressing or knocking down AD-related genes: UAS-TauGFP (ChrlI) (BL# 51362) expresses wild-type Tau under the control of UAS, UAS-Aβ42(Human)/CyO (ChrlI), expressed human Aβ42 gene under the control of UAS, UAS-AppRNAi (ChrlI) (BL# 28043) is an RNAi line of Appl gene, w*;GMR-GAL4-UAS-TAU+GFP/CyO;+/+ is a recombinant fly stock of GMR-GAL4 with UAS-TAU+GFP/CyO, w*;GMR-GAL4-UAS-Aβ42(Human)/CyO;+/+ is a recombinant fly stock of GMR-GAL4 with UAS-Aβ42 (Human)/CyO. UAS-Aβ42;E693G (ChrlI) (BL# 33774) expressed the human Abeta42 fragment of APP carrying the familial Alzheimer’s ‘Aric’tic’ mutation (E693G - amino acid numbering based on APP sequence) under the control of UAS, UAS-APP.C99-UAS-MAPT (ChrlI) (BL# 33803) expresses the C99 fragment of APP with the human APP signal peptide and human MAPT (tau) under the control of UAS.

We used the UAS-Aβ42;E693G and UAS-APP.C99-UAS-MAPT fly strain for climbing and survival assays, body weight, ROS and ATP level measurement, mitochondrial dynamics, cell death and neurodegeneration analysis because the other AD transgenic flies such as UAS-TauGFP, UAS-Aβ42(Human)/CyO and UAS-Miro flies are located on the second chromosome. Therefore, it was not possible to cross each AD-related gene and UAS-Miro with elavC155;GAL4.

GFP tagged mitochondria fly stock: UAS-Mito-GFP/CyO (ChrlI) is a transgenic fly line expressing a GFP tagged N-terminal mitochondrial localization signal (Wang and Schwarz, 2009). w*;GMR-GAL4-UAS-Mito-GFP/CyO;+/+ is a recombinant fly stock of UAS-Mito-GFP with GMR-GAL4.

All flies were maintained at 22±1°C in a BOD incubator on standard Drosophila food media containing agar–cornmeal–sugar–yeast, nepagin (anti-fungal agent) and propionic acid (anti-fungal agent).

**Light microscopic imaging**

For light microscopic imaging of the eyes of flies, 10-day-old adult flies from control and AD model flies were taken. Flies were anesthetized and eye images were captured at 51.2X magnification using a Carl Zeiss Stemi™ DV4 stereo binocular microscope with TStview7 software (version 7.1.3.7), which is expressed in micrometers. A total of 50 flies from each genotype were used for light microscopic imaging.

**Scanning electron microscopy (SEM)**

SEM was performed to examine the detailed external morphology of Drosophila eyes as described by Iyer et al. (2016) with slight modifications. Briefly, 10-day-old flies of desired genotypes were decapitated and fixed in 2.5% glutaraldehyde (cat# G5882, Sigma-Aldrich, USA) prepared in 0.2 M sodium cacodylate (cat# C0250, Sigma-Aldrich, USA) buffered overnight at 4°C followed by three washes with 0.1 M PBS, 15 min each at room temperature (RT). Samples were immediately dehydrated in a graded series of ethanol (50%, 70%, 80% and 100%) and freeze-dried using lyophilizer (FreeZone, Labconco, USA). The dried samples were mounted on carbon taped SEM stubs and sputter coated with platinum for 90 s. Images were taken using a SEM (Jeol-JSM-7600F, Japan). A total 50 flies from each genotype were used for SEM study.

**Phototaxis assay**

The Phototaxis assays were performed as described by Panchal and Tiwari (2017). For this, 10-day-old flies of desired genotypes were added to a Y-maze tube (Y-maze tube possesses one light arm and one dark arm) and allowed to acclimatize for 2 min in the tube. Flies were tapped gently to the bottom of the tube and allowed to move through the Y-maze tube for 20 s and the number of flies moving along the light and dark paths were counted. 20 flies of each genotype were placed in the Y-maze tube at a time and the experiment was repeated five times. A total of 100 flies from each genotype were used for phototaxis assay. The assay was performed under standard lighting conditions (~500 Lux) and the phototaxis activity was presented as a light preference index= (number of flies that travelled along the light path - number of flies that travelled along the dark path/ total number of flies).

**Climbing assay**

The climbing assay was performed as mentioned in Panchal and Tiwari (2017). For this, 10-, 20- and 30-day-old flies were placed in a vertical glass tube (3 cm long x 1.5 cm wide) and allowed to acclimatize for 2 min. Flies were tapped gently to the bottom of the vial and the number of the flies crossing 8 cm 10 s⁻¹ was counted. 20 flies of each genotype were placed in the glass tube at a time and the experiment was repeated five times. A total of 100 flies from each genotype were used. The assay was performed under standard lighting conditions. The climbing assay was expressed as % climbing 8 cm 10 s⁻¹.

**Survival assay**

The survival assay was performed as mentioned in Kumar et al. (2017). Briefly, the survival of adult flies was measured from the day of eclosion. Each vial of flies was transferred to fresh medium on every alternate day and the number of dead flies was counted until all flies were dead. A total of 100 flies were taken (20 flies/vial) for all genotypes. The median lifespan was calculated using the Kaplan–Meier method (Kaplan and Meier, 1958) and displayed as survival curves by using GraphPad Prism 5.0 software. The statistical significance in the median lifespan between genotypes was assessed using Mantel-Cox (Mantel, 1966) log-rank test. The statistical data analysis was performed using GraphPad Prism 5.0 software.

**Body weight analysis**

The body weight analysis was performed as mentioned in Panchal and Tiwari (2017) with little modification. For body weight analysis, 10-, 20- and 30-day-old flies were used for each genotype. The body weight of flies was measured by weighing 20 flies at a time using a weighing balance (Sartorius, Germany). The experiment was repeated five times. A total of 100 flies were taken for each genotype. Body weight of flies were measured in milligrams (mg).

**Quantitative real time PCR (RT-qPCR)**

RT-qPCR was performed as described by Hwang et al. (2019) with slight modification. Briefly, miRNA from 30-day-old flies’ heads were isolated using TRizol reagent (cat. #15596026, Invitrogen, USA). cDNA was synthesized by using Verso cDNA Synthesis Kit (cat. #AB-1453/B, Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. cDNAs were amplified using the desired gene specific primers. A total of 20 µl of reaction mixture was prepared by adding cDNA, primers and PowerUp™ SYBR™ Green Master Mix (cat. #A25742, Applied Biosystems, Thermo Fisher Scientific, USA). Step one plus system (Applied Biosystems, USA) was used for RT-qPCR. Relative quantification was performed using the ‘delta-delta Ct’ method to normalize with the RP49 endogenous gene. Data are presented as Mean±s.d. (in the case of the Miro
gene) and Mean±s.e.m. Relative levels of mRNA were analyzed by one-way ANOVA and analysis with Tukey’s test was performed using GraphPad Prism 5.0 software.

The following primers were used: Miro (F): 5′-GGACGATGACGACA-CTTTGGA-3′, (R): 5′-CCAGGGAGGTTGACCT-3′; Mitofusin (Mfn) (F): 5′-TCTCAGAGTGTCTGTAAGA-3′, (R): 5′-CATGTACCCGAAACATCCTT-3′; Mn-SOD (F): 5′-CCAGCATTCAACAAATC-3′, (R): 5′-GATGCCTCTTCAGATCACT-3′; CAT (F): ACCAGGCGAT-CAGAATCTG-3′, (R): 5′-AACCTTTGCGCTGCTGA-3′. ATP synthase beta (F): 5′-TCGGTTTGTGGTGTCTGA-3′, (R): 5′-CATGTCCGGTACGGACCA-3′.

**AO staining**

AO staining was performed to examine the apoptotic cells as described by Kumar and Tivvari (2018) with slight modification. Briefly, third instar larval eye imaginal discs and third instar larval brain brains were dissected out in 1×phosphate-buffered saline (PBS) and incubated in 1 μg ml⁻¹ AO solution (cat. #877529, Invitrogen, USA) prepared in 1X PBS for 2 min. After a brief wash in 1X PBS, the tissue was mounted in 1X PBS and immediately observed under the laser scanning confocal microscope (TCS SP5II, Leica Microsystems, Wetzlar, Germany). A total of 20 third instar larval eye imaginal discs and larval brains were taken for each genotype. Quantification of AO positive cells was measured by using ImageJ 5.0 software (NIH, USA).

**Measurement of mitochondrial and cellular ROS**

Mitochondria superoxide (ROS) was measured using the ROS-sensitive MitoSOX™ Red staining (cat. #M36008, Invitrogen, USA) as described in Liu et al. (2013). MitoSOX is a DHE derivative that possesses a cationic triphenylphosphonium group (TPP+), which helps in the transport to the mitochondrial matrix (Fuentes-Retamal et al., 2020; Roelofs et al., 2015). In the presence of ROS, MitoSOX gets oxidized and emits red fluorescence which was used to measure the ROS production (Forkink et al., 2010; Sarmiento-Salinas et al., 2019). For this, third instar larval brains from desired genotypes were dissected in cold Hanks’ Balanced Salt Solution (HBSS) and incubated in 5 μM MitoSOX Red and 1 μM MitoTracker Green FM (cat. #M7514, Invitrogen, USA) for 20 min at 37°C. After removing MitoSOX Red and MitoTracker Green solutions, brains were washed with HBSS twice and mounted in 1 X PBS. The images were captured using laser scanning confocal microscope (TCS SP5II, Leica Microsystems, Wetzlar, Germany). The detection of the colocalization of MitoSOX Red and MitoTracker Green was done by observing the yellow fluorescence in the overlay images. A total of 20 larval brains were examined for each genotype. All brains used for immunofluorescence were examined using a laser scanning confocal microscope (TCS SP5II, Leica Microsystems, Wetzlar, Germany) and MitoSOX Red fluorescence intensity was measured by using ImageJ 5.0 software (NIH, USA). Cellular (cytosolic) ROS was measured using redox sensitive fluorescephore DCF-DA dye (cat. #D99, Thermo Fisher Scientific, USA) as described in Westfall et al. (2018). Briefly, the fresh-pooled Drosophila homogenates were prepared from 30 heads of 30-day-old flies of desired genotype flies in Tris-EDTA-TritionX-100 buffer (pH 7.4) with a pellet pestle on ice. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected for quantification of 2,7-dichlorofluorescein (DCF) fluorescence and 20 μl of fly homogenate with 170 μl of Locke’s buffer and 10 μl of 1 mM DCF-DA solution were added in each well of a 96-well plate and incubated for 15 min at RT. The DCF-DA fluorescent signal was analyzed by 488nm/527nm excitation/emission in a multimode microplate reader (SpectraMax®M2e, Molecular Devices, USA). The assay was performed in triplicates. Quantification was normalized to the amount of protein in each sample. The concentration of protein from the samples was also determined using the Bradford reagent (cat. #B6916, Sigma-Aldrich, USA). ATP Quantification

ATP quantification was performed as described in Tennessen et al. (2014). Briefly, the fresh pooled Drosophila homogenates were prepared from 30 heads of 30-day-old flies of desired genotypes in 100 μl of homogenization buffer [6 M guanidine HCl, 100 mM Tris (pH 7.8), 4 mM EDTA] with a pellet pestle on ice. Samples were centrifuged at 12,000 rpm for 15 min to remove the debris, and the supernatant was diluted (1:750) in dilution buffer [25 mM Tris (pH 7.8), 100 μM EDTA]. The diluted homogenate was centrifuged at 12,000 rpm and 10 μl of the supernatant was transferred to individual wells of a white, opaque 96 well plate (cat. #3362, Corning, USA). A series of ATP standards were prepared by diluting the 5 mM ATP stock solution provided with an ATP bioluminescence assay kit (cat. #A22066, Invitrogen, USA) with ddH₂O (0.01, 0.05, 0.1, 0.5, 1 μM). 10 μl of each ATP standard solution used for the standard curve. The assay was started by adding 100 μl of the luciferase reaction mix and measuring the luminescence at 560 nm with a plate reader (Centro LB 960, Berthold Technologies, Germany). The assay was performed in triplicates. The concentration of protein from the samples was also determined using the Bradford reagent (cat. #B6916, Sigma-Aldrich, USA) and the ATP level was normalized to the protein content.

**Histological analysis**

Histological analysis was performed as described in lijima-Ando et al. (2012) with little modification. Briefly, to analyze the neurodegeneration, heads of 30-day-old flies were fixed in Bouin’s fixative for 48 h at RT and incubated in 50 mM Tris/150 mM NaCl for 24 h. The tissues were processed in 10% formalin, ascending concentration of IPA (70%, 80%, 90% and 100%), xylene and infiltrated with paraffin wax at 65°C. Then tissues were subsequently embedded in paraffin wax. Serial sections (4 μm thickness) through the entire heads were taken on poly-L-lysine (cat. #P8920, Sigma-Aldrich, USA) coated glass slides using a microtome (HistoCore AUTOCUT, Leica, Germany). The tissues were stained with Hematoxylin (nucleus) and Eosin (cytoplasm) and examined under laser scanning confocal microscope (TCS SP5II, Leica Microsystems, Wetzlar, Germany). A total of ten adult flies brains were taken for each genotype. The numbers of vacuoles were counted to see the extent of neurodegeneration using ImageJ 5.0 software (NIH, USA).

**Immunostaining of larval brains**

The immunostaining of larval brain was performed by selecting third instar larval brains from desired genotypes and dissecting them in 1X PBS followed by fixation in 4% PFA for 30 min at RT. The brains were washed in 1% PBST (1X PBS, 1% Triton X-100) three times, 15 min each and blocked in blocking solution [4% bovine serum albumin (BSA) solution in 1X PBS] for 2 h at RT followed by incubation in primary antibody rabbit anti-cleaved caspase-3 (Asp175) (5A1E) (1:150, cat. #9664, Cell Signaling Technology, USA) blocked in blocking solution for 48 h at RT and incubated in 100 mM Tris (pH 7.8), 4 mM EDTA) with a pellet pestle on ice. The eye discs were mounted in 1, 4-Diazabicyclo[2.2.2] octane (DABCO, Sigma-Aldrich, USA), an antifade mounting medium and observed under the laser scanning confocal microscope (TCS SP5II, Leica Microsystems, Wetzlar, Germany). A total of 20 third instar larval eye imaginal discs were taken for each genotype.

**Statistical analysis**

All data are represented as Mean±s.e.m. except Fig. 4K RT-qPCR data, which are shown as Mean±s.d. For survival assays, the Kaplan–Meier
method and Mantel-Cox tests were performed using GraphPad Prism 5.0 Software (San Diego, CA, USA). The biological replicates are shown as n. Significance between genotypes for all experiments was analyzed by one-way ANOVA analysis with Tukey’s test using GraphPad Prism 5.0 for all data except survival assays. All images were assembled using Adobe Photoshop 7.0. The histograms for all data were prepared using GraphPad Prism 5.0 software and significance indicates as: ns, non-significant; *p<0.05, **p<0.01, ***p<0.001.

Acknowledgements
We thank Dr. Thomas Schwarz (Children’s Hospital Boston, Boston, MA, USA) for UAS-Mito-GFP/CyoFly Daikia (ISER, Pune, India) and Bloomington Drosophila Stock Center (BDSC) (Indiana University, 1001 E. Third Street, Bloomington, USA) for fly stocks, Central Instrument Facility (CIF), Indian Institute of Technology (IIT) Gandhinagar, India for SEM analysis of Drosophila eye samples. The Laser Scanning Confocal Microscope facility supported by Department of Biotechnology (DBT), India, financial support from DST-Innovation in Science Pursuit for Inspired Research (INSPIRE), New Delhi to K.P. [IF140990] is duly acknowledged. The authors are also thankful to the Puri Foundation for Education in India for Infrastructure support.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.P., A.K.T.; Methodology: K.P., A.K.T.; Software: K.P., A.K.T.; Validation: K.P., A.K.T.; Formal analysis: K.P., A.K.T.; Investigation: K.P., A.K.T.; Resources: A.K.T.; Data curation: K.P., A.K.T.; Writing - original draft: K.P., A.K.T.; Writing - review & editing: K.P., A.K.T.; Visualization: K.P., A.K.T.; Supervision: A.K.T.; Project administration: A.K.T.; Funding acquisition: A.K.T.

Funding
The work was supported by Science and Engineering Research Board (SERB), New Delhi, India [no. EMR/2016/006911/HS].

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