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Drosophila as a model to study neurodegenerative diseases

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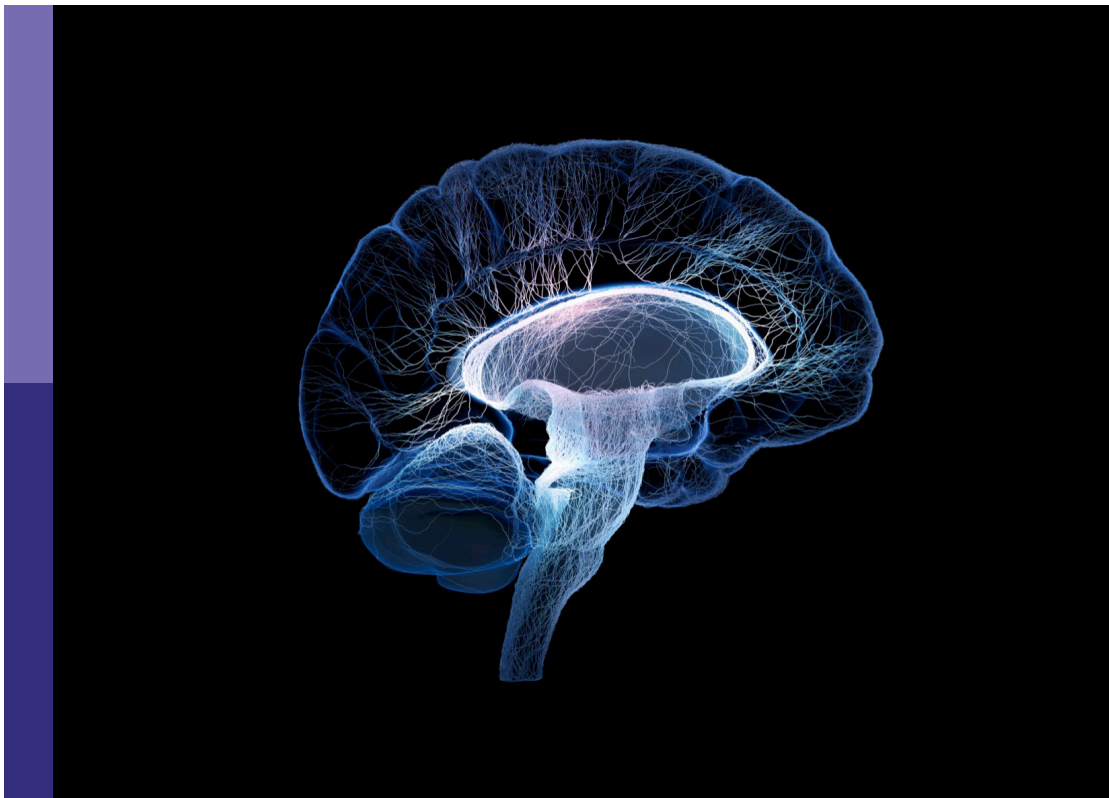
Published in
Frontiers in Neuroscience



ISSN 1664-8714
ISBN 978-2-8325-3436-6
DOI 10.3389/978-2-8325-3436-6

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Drosophila as a model to study neurodegenerative diseases

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Citation
Pandey, U., Digilio, F. A., Liguori, F., eds. (2023). *Drosophila as a model to study neurodegenerative diseases*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-3436-6

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frontiers | Frontiers in Neuroscience

TYPE Editorial
PUBLISHED 24 August 2023
DOI 10.3389/fnins.2023.1275253

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 09 August 2023
ACCEPTED 16 August 2023
PUBLISHED 24 August 2023

CITATION
Liguori F, Pandey UB and Digilio FA (2023) Editorial: *Drosophila* as a model to study neurodegenerative diseases.
Front. Neurosci. 17:1275253.
doi: 10.3389/fnins.2023.1275253

Editorial: *Drosophila* as a model to study neurodegenerative diseases

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KEYWORDS

***Drosophila melanogaster*, neurodegenerative diseases, human disease model, Amyotrophic Lateral Sclerosis, Alzheimer's disease, Parkinson's disease, Spinocerebellar Ataxia Type 3, proteinopathies**

Editorial on the Research Topic
Drosophila as a model to study neurodegenerative diseases

Neurodegenerative diseases (NDDs) are incurable and debilitating conditions characterized by progressive loss of selectively vulnerable populations of neurons in brain, spinal cord and peripheral nerves. This causes worsening motor (ataxia), cognitive (dementia) and autonomic dysfunction over time. NDDs impact many families and represent one of the greatest public health burdens worldwide. Most NDDs are due to a combination of genetic and environmental factors. The largest known risk factor is age, so NDDs are becoming more prevalent due to an aging global population, eventually becoming a globally unmanageable problem. There are currently no drugs available to prevent or treat NDDs, and multiple scientific approaches are needed to understand the etiology of these diseases and develop a therapeutic treatment.

An approach toward understanding diseases is to model mechanisms and identify disease-modifying pathways in less complex, but analogous, organisms. The fruit fly *Drosophila melanogaster* is a highly tractable animal for studying NDDs (Ugur et al., 2016). It shares many genes and biological pathways with humans and has a sophisticated nervous system. Beyond a number of experimental advantages, such as short lifespan, numerous progeny and no ethical concerns, *Drosophila* allows answering complicated biological questions through sophisticated genetic experiments, using many mutants and transgenic lines readily available. In fact, many basic and fundamental biological pathways (e.g., notch and circadian rhythm) were discovered in fly models. As a result, *Drosophila* is now being actively employed not only to study *in vivo* the functions of human NDD genes, but also to select and evaluate potential drugs for therapeutic research.

This Research Topic includes 10 original manuscripts which report specific examples or provide a general overview of the value of using *Drosophila* models to contribute to mechanistic and therapeutic studies of NDDs.

Koza et al. used a *Drosophila* model of Parkinson's Disease (PD) to investigate Sexual Dysfunction (SD) which, despite being one of the most common non-motor symptoms of PD, is still poorly understood and studied. PD is a NDD characterized by a series of motor impairments due to a reduction in the number of dopaminergic (DAergic) neurons in the substantia nigra. Leveraging *Drosophila* courtship and climbing behaviors, authors showed that SD precedes motor defects, as well as brain DAergic neurodegeneration and

alteration in dopamine metabolism. Interestingly, courtship-related traits could be used as early markers to identify the later onset of PD in the model *Drosophila*.

Rozich et al. presented an optimized protocol, based on the Gal80-DD tool, that allows finely controlled pan-neuronal expression in adult flies of NDD-associated genes, whose manipulation causes developmental lethality in *Drosophila*. Authors tested this method by examining the degenerative phenotypes caused by disruption in the adult brain of the *Vps13D* gene that, in human, causes the ataxia and whose functional inactivation leads to developmental lethality in *Drosophila*.

Prifti et al. used a Spinocerebellar Ataxia Type 3 (SCA3) *Drosophila* model to investigate how specific protein domains of Ataxin3 participate to the toxicity induced by the aberrant form of this protein; Authors reported that mutations in the UbS1 domain enhanced the *in vivo* toxicity of pathogenic Atxn3 through its role in ubiquitin processing and suggest to further explore this domain as a target for therapeutic interventions.

In their Brief Research Report, Borg et al. evaluated the contribution of the *DCTN1* gene in the pathology of Amyotrophic Lateral Sclerosis (ALS), constitutively or tissue-specifically silencing its fly ortholog. Interestingly, authors showed that *Dctn1* and its related paralog (*Dred*) are required for neuronal and muscular function in *Drosophila*.

In their Methods Article, Ayajuddin et al. proposed an easy and inexpensive fluorescence microscopy-based method to quantify neurodegeneration of dopaminergic neurons in a PD *Drosophila* model. The extent of DAergic neurodegeneration was correlated to the fluorescence intensity obtained from tyrosine hydroxylase immunostaining. Present method can also be used to characterize the extent of degeneration of different cell types with little modification and would be of interest to laboratories lacking confocal microscopy.

Varte et al. reviewed how *Drosophila* can contribute to understanding the molecular mechanisms linking mitochondrial dysfunction with Alzheimer's Disease (AD). Authors focused on specific mitochondrial insults caused by amyloid-β and tau in transgenic flies and on the available genetic tools and sensors to study these defects in flies.

Recently, several studies reported the potential benefits of hypertension drugs in AD patients, although their mechanisms of action in the context of AD is unclear. In AD *Drosophila* models, renin-angiotensin system (RAS) inhibitors can suppress neuronal cell death and memory defects even if RAS is not conserved in flies. Ghalayini and Boulianne summarized the studies of renin angiotensin inhibitors in AD and proposed to use *Drosophila* to further elucidate mechanisms underlying RAS system in AD.

Ye et al. summarized novel insights obtained through recent studies examining age and gender as contributing factors to trauma-mediated neurodegeneration in humans and preclinical models, including mammalian and *Drosophila* models. They discussed the central role played by *Drosophila*-based injury models, which offer a unique opportunity not only to study important risk factors associated with NDDs, particularly age and gender, but also to investigate mechanisms underlying head trauma-induced neurodegeneration and to identify therapeutic targets for treatment and recovery.

In their comprehensive review Pan et al. discussed the implications of sphingolipid (SL) metabolism which is affected in a surprisingly broad set of NDDs. These include some lysosomal storage diseases, Friedreich's ataxia, as well as some forms of ALS and PD. Many of these diseases have been modeled in *Drosophila* and are associated with elevated ceramide levels. Authors summarized the elegant research work done in fly models that has advanced our understanding of the nature of defects in SL metabolism, the organelles implicated and potential therapies for these diseases.

Santarelli et al. reported how the use of several *Drosophila* models that overexpress the human mutated genes that cause neuro-proteinopathies (PPs), such as Huntington's Disease (HD), PD, AD, crucially contributed to our understanding of the close relationship between PPs and autophagy. Authors highlighted the importance of these models for studying the function of several risk genes and the benefits of using specific genetic tools in *Drosophila* to generate additional models that will help to better understand the non-autonomous signals exchanged between glia and neuronal cells that could be responsible for some PPs.

Author contributions

FL: Writing—review and editing. UP: Writing—review and editing. FD: Writing—original draft, Writing—review and editing.

Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 17 January 2023
DOI 10.3389/fnins.2022.1112688

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 30 November 2022
ACCEPTED 21 December 2022
PUBLISHED 17 January 2023

CITATION
Prifti MV, Libohova K, Harris AL, Tsou W-L and Todi SV (2023) Ubiquitin-binding site 1 of pathogenic ataxin-3 regulates its toxicity in *Drosophila* models of Spinocerebellar Ataxia Type 3. *Front. Neurosci.* 16:1112688. doi: 10.3389/fnins.2022.1112688

Ubiquitin-binding site 1 of pathogenic ataxin-3 regulates its toxicity in *Drosophila* models of Spinocerebellar Ataxia Type 3

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Spinocerebellar Ataxia Type 3 (SCA3) is a member of the family of polyglutamine (polyQ) diseases that are caused by anomalous CAG triplet repeat expansions in several genes. SCA3 results from abnormal polyQ expansion in the deubiquitinase (DUB), ataxin-3 (Atxn3). To understand the role of the different domains of mutant Atxn3 on its pathogenicity, with the hope that they can be explored for therapeutic interventions, we have systematically studied their individual and collective effects on its toxicity. One such domain is ubiquitin-binding site 1 (UbS1) on the catalytic domain of Atxn3; UbS1 is necessary for the enzymatic activity of Atxn3. Here, we investigated the importance of UbS1 on the toxicity of pathogenic Atxn3. We generated transgenic *Drosophila melanogaster* lines that express polyQ-expanded Atxn3 with and without a functional UbS1. We found that mutating UbS1 markedly exacerbates the toxicity of pathogenic Atxn3. Additional studies indicated that UbS1 regulates the toxicity of Atxn3 not by affecting its aggregation or sub-cellular localization, but by impacting its role in ubiquitin processing. Our findings provide additional insights into the role of Atxn3's domains in the pathogenicity of SCA3.

KEYWORDS

ataxia, CAG triplet repeat, *Drosophila*, misfolding and aggregation, neurodegeneration, polyglutamine (polyQ), ubiquitin

Introduction

Polyglutamine (polyQ)-expansion diseases are inherited neurodegenerative disorders that result from the abnormal lengthening of a CAG triplet nucleotide repeat in specific genes, which is translated into a polyQ tract in corresponding proteins. Abnormal polyQ expansion leads to disease protein misfolding, formation of proteinaceous aggregates that include the offending protein, cellular malfunction, and death. The polyQ family consists of 9 clinically distinct disorders including Huntington's Disease (HD), Dentatorubral–pallidoluysian atrophy, Kennedy's Disease,

and Spinocerebellar Ataxia Type (SCA) 1, 2, 3, 6, 7, and 17 (Todi et al., 2007b; Johnson et al., 2022b).

Spinocerebellar Ataxia Type 3 (SCA3, which is also known as Machado-Joseph Disease) is a progressive, dominant ataxia. SCA3 patients experience coordination and balance issues accompanied by neurodegeneration in cerebellar pathways, substantia nigra, cranial motor nerve nuclei, dentate nuclei, and peripheral neurons. These symptoms typically appear in mid-adulthood; however, the time of their onset and severity is influenced by the length of the polyQ expansion within the disease protein, ataxin-3 (Atxn3). Atxn3 is a ubiquitously expressed deubiquitinating enzyme (DUB) that functions by binding poly-ubiquitin chains and cleaving them; it is involved in protein quality control, cytoskeletal regulation, DNA damage control, sensory organ development and function, and more (Matos et al., 2011, 2019; Costa Mdo and Paulson, 2012; Dantuma and Herzog, 2020; Toulis et al., 2020; Johnson et al., 2022b). In mice, Atxn3 is not essential (Schmitt et al., 2007; Switonski et al., 2011; Reina et al., 2012; Zeng et al., 2013).

Located on the N-terminal half of Atxn3 (**Figure 1A**) is the Josephin domain that contains the catalytic site and two ubiquitin-binding sites (UbS1, UbS2) that coordinate spatial interactions between the catalytic groove and ubiquitin (Nicastro et al., 2006, 2009, 2010). UbS2 additionally interacts with the proteasome shuttle protein, Rad23; this interaction controls ataxin-3 stability and toxicity (Nicastro et al., 2006, 2009, 2010; Blount et al., 2014; Tsou et al., 2015b; Sutton et al., 2017). Downstream the Josephin domain resides the C-terminal half of Atxn3, comprising ubiquitin-interacting motifs (UIMs), whose interaction with the chaperone, Hsc70-4 also regulates the toxicity of pathogenic Atxn3; and the VCP-binding domain (VBM), whose interaction with the AAATPase, VCP seeds the aggregation of polyQ-expanded Atxn3 (Boeddrich et al., 2006; Winborn et al., 2008; Ristic et al., 2018; Johnson et al., 2021). Nestled between the VBM and the terminal UIM is the polyQ repeat whose anomalous expansion causes SCA3 (Johnson et al., 2022b). There is also an isoform of Atxn3 that does not contain a terminal, third UIM but instead comprises a degron that expedites the host protein's degradation (Harris et al., 2010; Johnson et al., 2019; Blount et al., 2020). In this study, we utilize the 3-UIM version of Atxn3 as it is the dominant isoform (Harris et al., 2010).

Over the past several years, our group has dissected the individual and combined effects of the various Atxn3 protein domains to understand their role in SCA3 and to explore their utility as therapeutic targets: The catalytic site, UbS2, the VBM, and the UIMs (Tsou et al., 2013, 2015b; Blount et al., 2014, 2018, 2020; Ristic et al., 2018; Johnson et al., 2019, 2020, 2021, 2022a). The final domain in this series of studies, UbS1, is the focus of this report. Here, to explore the role of UbS1 in Atxn3 pathogenicity we again utilize *Drosophila melanogaster* as the model organism.

As a DUB, recombinant Atxn3 cleaves ubiquitin chains with preference for K63-linked species that are longer than 4 ubiquitin moieties long (Winborn et al., 2008). Detailed structural and biochemical studies of the isolated Josephin domain by the Pastore lab identified UbS1 and UbS2 as important regions for enzymatic activity (Nicastro et al., 2009). UbS1 was shown to be necessary for the activity of Atxn3 *in vitro* (Nicastro et al., 2006, 2009, 2010; Todi et al., 2010), but its importance has not been evaluated *in vivo*. Since mutating the catalytic cysteine renders pathogenic Atxn3 markedly more toxic (Warrick et al., 2005; Tsou et al., 2015b; Sutton et al., 2017), we hypothesize that UbS1 mutation similarly enhances toxicity in *Drosophila*. Indeed, we find that UbS1 mutation (denoted here as UbS1*) causes a marked increase in the toxicity of polyQ-expanded Atxn3 in all tissues and stages tested. However, unlike with the mutation of other domains, UbS1 mutation enhances Atxn3 toxicity without seemingly altering its overall protein levels or aggregation. Instead, based on additional genetics and biochemical evidence, we propose that UbS1 regulates the pathogenicity of Atxn3 by impacting the processing of endogenous ubiquitin species.

Materials and methods

Plasmid design

Atxn3(Q80) cDNA sequence was based on the human *ATXN3* sequences used in previous publications (Winborn et al., 2008; Todi et al., 2009, 2010; Harris et al., 2010; Nicastro et al., 2010; Blount et al., 2014, 2020; Tsou et al., 2015b; Costa et al., 2016; Sutton et al., 2017; Ristic et al., 2018; Johnson et al., 2019, 2020, 2021, 2022a). The mutagenesis of Atxn3(Q80)-UbS1*, Atxn3(Q80)-C14*-UbS1* and Atxn3(Q80)-UbS1*-UIM* were carried out by Genscript[1]. The transgenes were subcloned into pWalium-10.moe and fly lines were generated using phiC31 integrase-mediated insertion (Markstein et al., 2008) into attP2 site on chromosome 3 (Duke University Model Systems). The polyQ repeat of Atxn3 comprised an alternating CAGCAA sequence to minimize the possibility of additional toxicity effects from mRNA or unconventional translation processes (Johnson et al., 2022a).

Antibodies

Anti-HA (rabbit monoclonal C29F4, 1:1000, Cell Signaling Technology); anti-ataxin-3 (mouse monoclonal 1H9, MAB5360, 1:500–1000, Millipore-Sigma); anti-tubulin (mouse monoclonal T5168, 1:10,000, Millipore-Sigma); anti-lamin (mouse monoclonal ADL84.12-5, 1:1000, Developmental

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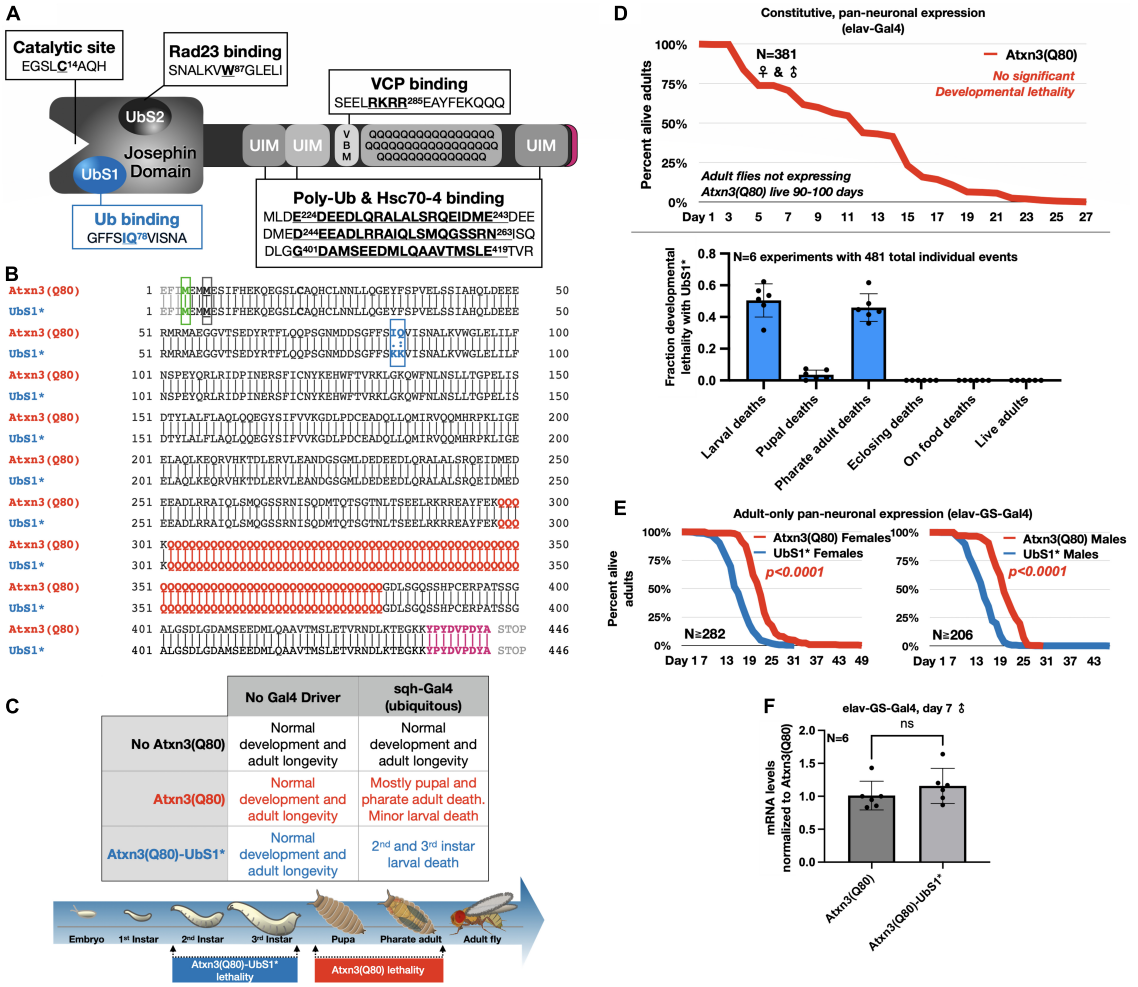


FIGURE 1

UbS1 mutation exacerbates Atxn3(Q80) phenotypes. **(A)** Diagram of Atxn3 domains. This deubiquitinase (DUB) comprises a catalytic domain known as the Josephin domain and a C-terminal portion that consists of various domains. UbS1, ubiquitin-binding site 1; UbS2, ubiquitin-binding site 2; UIM, ubiquitin-interacting motif; VBM, VCP-binding motif. Boxes associated with domains indicate residues important for interactions. **(B)** Amino acid sequence of Atxn3 proteins used in this study, highlighting first methionine (green); endogenous methionine (black); UbS1 mutation (blue); polyQ tract (red); HA epitope tag (purple). **(C)** Summary of results from crosses leading to the expression of Atxn3 in all tissues (sqh-Gal4). **(D)** Summary of results from crosses leading to the expression of Atxn3 in all neurons (elav-Gal4). **(E)** Summary of results from crosses leading to the expression of Atxn3 in all adult neurons (elav-GS-Gal4). Flies were reared in media without the inducer, RU486, then switched into media with RU486 on the day that they eclosed from their pupal cases. *P*-values are from log-rank tests. **(F)** Quantitative-PCR results from expression of Atxn3 versions in adult fly neurons for 7 days. "ns": not significant, from Wilcoxon test.

Studies Hybridoma Bank); anti-FLAG (rabbit monoclonal D6W5B, 1:500, Cell Signaling Technology); anti-ubiquitin (HRP-conjugated, mouse monoclonal A-5, sc-166553, 1:1000, Santa Cruz Biotechnology); peroxidase-conjugated secondary antibodies: Goat anti-mouse and goat anti-rabbit, 1:5000, were from Jackson ImmunoResearch.

Drosophila husbandry

GMR-Gal4 (#8605) was from the Bloomington *Drosophila* Stock Center. UAS-DnaJ-1 lines were from FlyORF (#002258) and Bloomington *Drosophila* Stock Center (#30553). The following stocks were gifts: sqh-Gal4 (Dr. Daniel P. Kiehart, Duke University), elav-GS-Gal4 (Dr. R. J. Wessells, Wayne State University), elav-Gal4 (Dr. Daniel F. Eberl, University of Iowa). All flies were heterozygous for driver and transgene(s). All transgenic Atxn3 lines used here were generated by us and were reported previously (Tsou et al., 2013, 2015b; Sutton et al., 2017; Johnson et al., 2019, 2020, 2022a; Blount et al., 2020) or are being reported here for the first time, as noted in the results section and figure legends. All crosses were conducted at 25°C in diurnal environments with 12 h light/dark cycles and on conventional cornmeal media. All resulting offspring were maintained in the

same conditions, except for crosses using elav-GS-Gal4, where offspring were switched into media containing RU486 soon after emerging as adults to induce the expression of Atxn3 transgenes.

Drosophila longevity

For experiments measuring lifespan, adults were collected daily and secluded into groups of the same size (10 adults per vial) and gender to record deaths, unless otherwise noted. Flies were flipped into new food every other day. The number of adults tracked (N) is noted in figures. In cases where death tracking was focused on the developmental stage, flies were observed from the embryo stage through eclosure or adult death, and deaths at each stage were recorded multiple times each week.

Western blots and quantification

Western blots were performed with 5 adult flies or 5 dissected fly heads per biological sample (N), depending on the experiment and driver being used. Samples were physically homogenized in boiling lysis buffer [50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol (DTT)], sonicated, boiled for 10 min, and centrifuged for 7 min at 13,300 rpm at room temperature. Samples were electrophoresed through pre-cast, 4–20% Tris/Glycine gels (Bio-Rad). ChemiDoc (Bio-Rad) was used to image Western blots, which were then quantified with ImageLab (Bio-Rad). Unless otherwise noted, quantification was done using the volume of whole lanes measuring the levels of each protein of interest and corrected for their respective background: Main band to top of gel for Atxn3 and whole lane for ubiquitin signal. Direct blue stains of total protein were performed by saturating the PVDF membrane for 10 min in 0.008% Direct Blue 71 (Sigma-Aldrich) in 40% ethanol and 10% acetic acid, and then rinsed with a solution of 40% ethanol/10% acetic acid before being air dried and imaged. Direct Blue signal was used as loading control, unless otherwise stated.

Filter-trap assays

Three whole adult flies were used per sample. Samples were homogenized thoroughly in 200 μL NETN lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40) to which protease inhibitor cocktail (PI; S-8820, Millipore-Sigma) was added. The lysates were then diluted with 200 μL 0.5% SDS in PBS. Diluted lysates were sonicated, centrifuged at 4,500 × *g* for 1 min at room temperature, and then diluted further by combining 100 μL lysate (supernatant only) with 400 μL PBS. 40 μL of this final lysate was added to a Bio-Dot apparatus (Bio-Rad) and vacuumed through a 0.45 μm nitrocellulose membrane (Schleicher and Schuell) that had been pre-incubated in 0.1% SDS in PBS. The membrane was then rinsed with 0.1% SDS/PBS twice, processed for Western blotting, and imaged with ChemiDoc.

Quantitative PCR

Total RNA was isolated from 5 whole flies or 30 fly heads per sample using TRIzol reagent (Thermo-Fisher Scientific) and treated with TURBO DNAse (Thermo-Fisher Scientific) to remove genomic DNA. cDNA was generated with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR reactions were carried out on a StepOnePlus system (Applied Biosystems) using Fast SYBR™ Green Master Mix (Applied Biosystems). The PCR cycle threshold (Ct) was used for calculation of the relative mRNA expression level. Rp49 was used as internal control. Primers were: Atxn3-F: 5′-GAATGGCAGAAGGAGGAGTTACTA-3′; Atxn3-R: 5′-GACCCGTCAAGAGAGAATTCAAGT-3′; DnaJ-1-F: 5′-GTACAAGGAGGAGAAGGTGCTG-3′; DnaJ-1-R: 5′-CAGACTGATCTGGGCTGTATACTT-3′; Rp49-F: 5′-AGATCGTGAAGAAGCGCACCAAG-3′; Rp49-R: 5′-CA CCAGGAACTTCTTGAATCCGG-3′.

Co-immunopurification assays

Ten adult flies or 30 dissected adult fly heads, depending on experiment, were homogenized in 800 μL NETN or 1:1 NETN/PBS lysis buffer supplemented with PI. Samples were incubated at 4°C with agitation for 25 min. Following centrifugation of the incubated samples (5,000 × *g*, 4°C, 10 min), supernatant was combined with bead-bound antibodies and tumbled at 4°C for 4 h. Beads were rinsed five times each with lysis buffer, and bead-bound complexes were eluted by adding Laemmli buffer (Bio-Rad) and boiling.

Cytoplasmic/nuclear fractionations

Fractionation was performed using the ReadyPrep Protein Extraction Kit (1632089, Bio-Rad) using seven whole flies per group that were lysed in cytoplasmic extraction buffer (Bio-Rad) and processes as delineated by the supplier's protocols.

Statistics

Statistical tests were conducted using Prism 9 (GraphPad) and are specified in figure legends. The number of biological replicates is noted on figures and corresponding legends.

Results

UbS1 mutation enhances the toxicity of pathogenic Atxn3 in *Drosophila*

To investigate the impact of UbS1 on the pathogenicity of Atxn3, we generated transgenic flies that express pathogenic, full-length, human Atxn3 with 80 polyQ repeats [within human disease range (Johnson et al., 2022b)] with an intact UbS1 domain, as well as one harboring mutations that inactivate it by precluding binding to ubiquitin (Nicastro et al., 2005, 2006, 2009, 2010; **Figure 1B**). Each transgene is inserted into the same "safe harbor" site on chromosome 3 of *Drosophila*, attP2. A single copy of the transgene is inserted in each case, in the same orientation. The expression system that we utilized to examine the effect of UbS1 mutation is the binary Gal4-UAS system, which enables the expression of targeted transgenes in a specific temporal and spatial manner (Brand and Perrimon, 1993).

Atxn3 is expressed ubiquitously in mammals (Johnson et al., 2022b). We therefore began our studies by examining the effect of pathogenic Atxn3 expression in all fly tissues. We found that ubiquitous expression of pathogenic Atxn3(Q80) with intact UbS1 leads to developmental death in pupal and pharate adult phases; no adults eclose successfully from their pupal cases (**Figure 1C**). Mutation of UbS1 (UbS1*) markedly enhances this phenotype: Developing flies expressing pathogenic Atxn3(Q80)-UbS1* die as 2nd and 3rd instar larvae (**Figure 1C**). This enhancement of toxicity was also observed when pathogenic Atxn3 was expressed selectively in neuronal cells, the type of tissue most impacted in SCA3, throughout development. Whereas pathogenic Atxn3 expression pan-neuronally leads to early adult lethality, mutating UbS1 leads to developmental lethality (**Figure 1D**), enhancing the phenotype. Lastly, since SCA3 is adult-onset, we examined whether expression of pathogenic Atxn3 with an intact or mutated UbS1 is similarly toxic in adult neurons. We found that UbS1 mutation again renders pathogenic Atxn3 more toxic in both male and female adults (**Figure 1E**); this difference in toxicity is not due to variation in mRNA levels, as the two transgenes are expressed similarly in adult neurons (**Figure 1F**). We conclude that UbS1 mutation enhances the toxicity of pathogenic Atxn3 in flies.

UbS1 mutation does not impact Atxn3 aggregation or its sub-cellular distribution

In our prior investigations into the toxicity of polyQ-expanded Atxn3, we found that increased pathogenicity often correlates with higher aggregation of the SCA3 protein (Tsou et al., 2013, 2015b; Blount et al., 2014; Sutton et al., 2017; Ristic et al., 2018; Johnson et al., 2019, 2020, 2021). Therefore, we examined whether UbS1 mutation impacts the aggregation of Atxn3 with an expanded polyQ repeat. As shown in **Figure 2**, temporal examination of SDS-resistant species from flies expressing Atxn3(Q80) only in adult neurons does not lead to consistent differences between transgenes with an intact or mutated UbS1, and the overall levels of Atxn3 protein are also comparable between the two versions (**Figure 2A**; additional examples are in **Figures 4–6**). Filter-trap assays, used to examine larger aggregates that do not enter SDS-PAGE gels, do not indicate that UbS1-mutated pathogenic Atxn3 is more or less aggregation prone in fly neurons (**Figure 2B**). Because pathogenic Atxn3 is most toxic when it localizes to the nucleus (Bichelmeier et al., 2007), we also examined whether UbS1 mutation impacts sub-cellular distribution. Based on biochemical cytoplasmic/nuclear separation, this was not the case (**Figure 2C**). We conclude that UbS1 does not impact the levels, localization or aggregation of Atxn3(Q80) in a manner that correlates with the markedly increased toxicity. We next turned our focus to activities of Atxn3 that may be impacted by this binding domain.

UbS1 mutation enhances Atxn3 toxicity in fly eyes, revealing potential mechanism of action

Expression of pathogenic Atxn3 with an intact UbS1 in fly eyes does not lead to detectable structural anomalies on day 1 adults, as we showed before (Johnson et al., 2021) and as pictured in **Figure 3A**; eye degeneration does ensue over time (Johnson et al., 2020, 2021). However, expression of pathogenic Atxn3(Q80)-UbS1* is markedly toxic to fly eyes from day 1 in adults, regardless of sex (**Figure 3A**), consistent with the increased toxicity that we observed in other tissues because of UbS1 mutation (**Figure 1**).

To explore which aspects of Atxn3 functions in flies may account for the increased toxicity of UbS1*, we continued using fly eyes (Bonini and Fortini, 2003; Zhang et al., 2010; Casci and Pandey, 2015; Perrimon et al., 2016). Since we did not see a detectable difference between males and females in this phenotype, for the rest of eye-based studies we used males and females interchangeably. We previously reported that wild-type Atxn3 leads to higher levels of the co-chaperone, DnaJ-1 in *Drosophila* at transcript and protein levels in a catalytic activity-dependent manner (Tsou et al., 2015b). This increase in DnaJ-1 levels is neuroprotective against SCA3 and other polyQ models (Tsou et al., 2015a,b). As shown in **Figure 3B**, over-expression of DnaJ-1 from two different transgenic lines suppresses the UbS1* eye phenotype.

At least in part by increasing DnaJ-1 levels, Atxn3 protects against other polyQ disease proteins in the fly in a catalytic activity-dependent manner (Tsou et al., 2015a,b). As shown in **Figure 3C**, expression of various forms of Atxn3 that

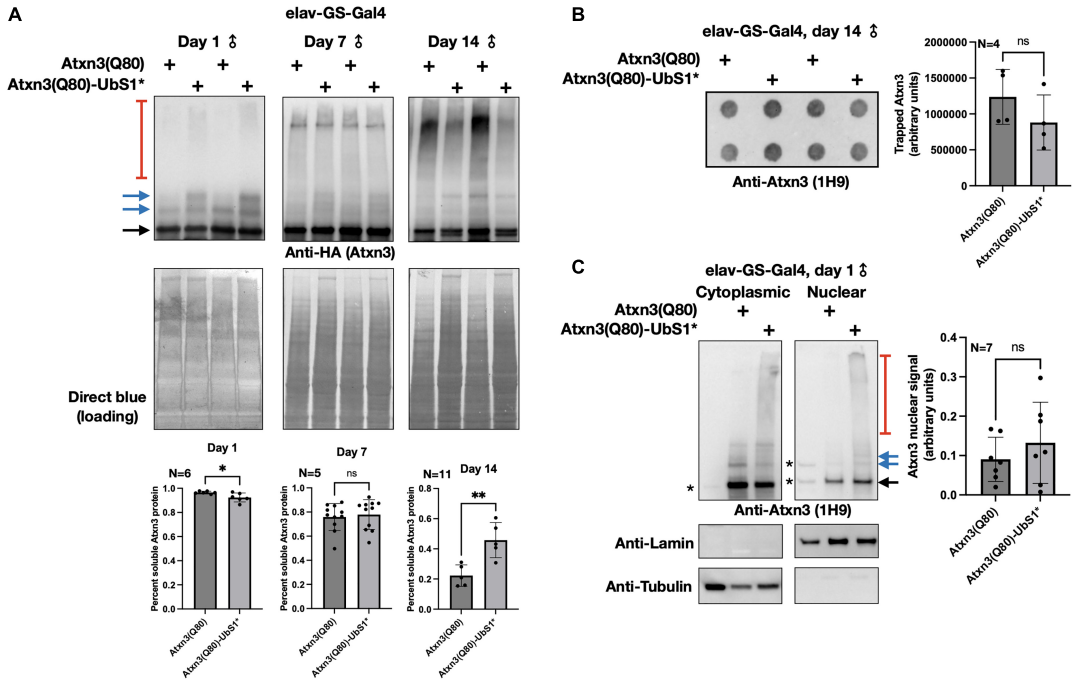


FIGURE 2
Aggregation of Atxn3(Q80) with intact or mutated UbS1. **(A)** Top: Western blots of Atxn3 versions noted in the figure. Black arrow, main Atxn3 band. Blue arrows, ubiquitinated Atxn3. Red bracketed line, SDS-resistant species of Atxn3. Each lane is an independent repeat. Bottom: Quantification of signal from blots above and other independent repeats. $^{*}p < 0.05$, $^{**}p < 0.01$, "ns", not significant. Statistics: *t*-tests. For quantifications, SDS-soluble species (black and blue arrows) were quantified separately from the SDS-resistant ones (red bracketed lines). **(B)** Left: Filter-trap assay using flies that express Atxn3(Q80) with an intact or mutated UbS1 in adult neurons for 14 days. Right: Quantification of results from the left as well as other, independent repeats. "ns", not significant. Statistics: *t*-test. **(C)** Left: Results from biochemical separation of Atxn3 protein fractions in cytoplasmic and nuclear compartments when Atxn3 was expressed in adult fly neurons (1 day in RU486). Asterisks, non-specific signal. Black arrow, main Atxn3 band. Blue arrows, ubiquitinated Atxn3. Red bracketed line, SDS-resistant Atxn3. Right: Quantification of the signal from the left and other, independent repeats. "ns", not significant from *t*-test. The entire Atxn3 signal in each lane was used for quantifications. Tubulin and lamin served as loading controls for their respective fractions.

are catalytically active (wild-type, polyQ-expanded, or polyQ-expanded but with mutated UIMs) markedly corrects the UbS1* phenotype. However, catalytically inactive Atxn3 is either unable to suppress UbS1* toxicity [Atxn3 with normal polyQ and mutated catalytic cysteine; C14*(Winborn et al., 2008)] or enhances UbS1* toxicity (polyQ-expanded Atxn3 with mutated C14). These results suggest that a key component of UbS1*-related toxicity is the catalytic activity of Atxn3. This is not entirely surprising, because mutating UbS1 abrogates the DUB activity of Atxn3 *in vitro* (Nicastro et al., 2010). We next explored the role of DnaJ-1 in UbS1*-dependent phenotypes in flies.

UbS1* leads to lower DnaJ-1 levels, whose overexpression suppresses neuronal toxicity

We began by examining the mRNA levels of DnaJ-1 in flies expressing UbS1-mutated Atxn3. We observed that DnaJ-1 mRNA levels are significantly lower in the presence of Atxn3(Q80)-UbS1* (Figure 4A), similarly to what we reported before for inactive Atxn3 (C14*) with a normal polyQ repeat (Tsou et al., 2015b). (Due to the unavailability of antibodies that reliably detect fly DnaJ-1, we were unable to investigate the endogenous protein for this study). Next, we confirmed that exogenous DnaJ-1 is protective against UbS1-mutated, pathogenic Atxn3 in tissues other than eyes. As shown in Figure 4B, co-expression of DnaJ-1 in adult fly neurons reduces the toxicity of both Atxn3 with a functional UbS1 and with UbS1*, independently of sex (Figure 4B). Concomitant with reduced toxicity, we observed that exogenous DnaJ-1 leads to higher levels of SDS-soluble Atxn3, but does not visibly impact the SDS-soluble/insoluble levels of UbS1-mutated Atxn3 (Figure 4C). Lastly, according to co-immunoprecipitation (co-IP) assays, pathogenic Atxn3 with mutated UbS1 interacts more strongly with exogenous, FLAG-tagged DnaJ-1 compared to pathogenic Atxn3 with intact UbS1. We interpret these data to suggest that UbS1 mutation enhances Atxn3(Q80) toxicity at least in part by compromising endogenous DnaJ-1 expression as well as its downstream functions because of a tighter interaction with the disease protein.

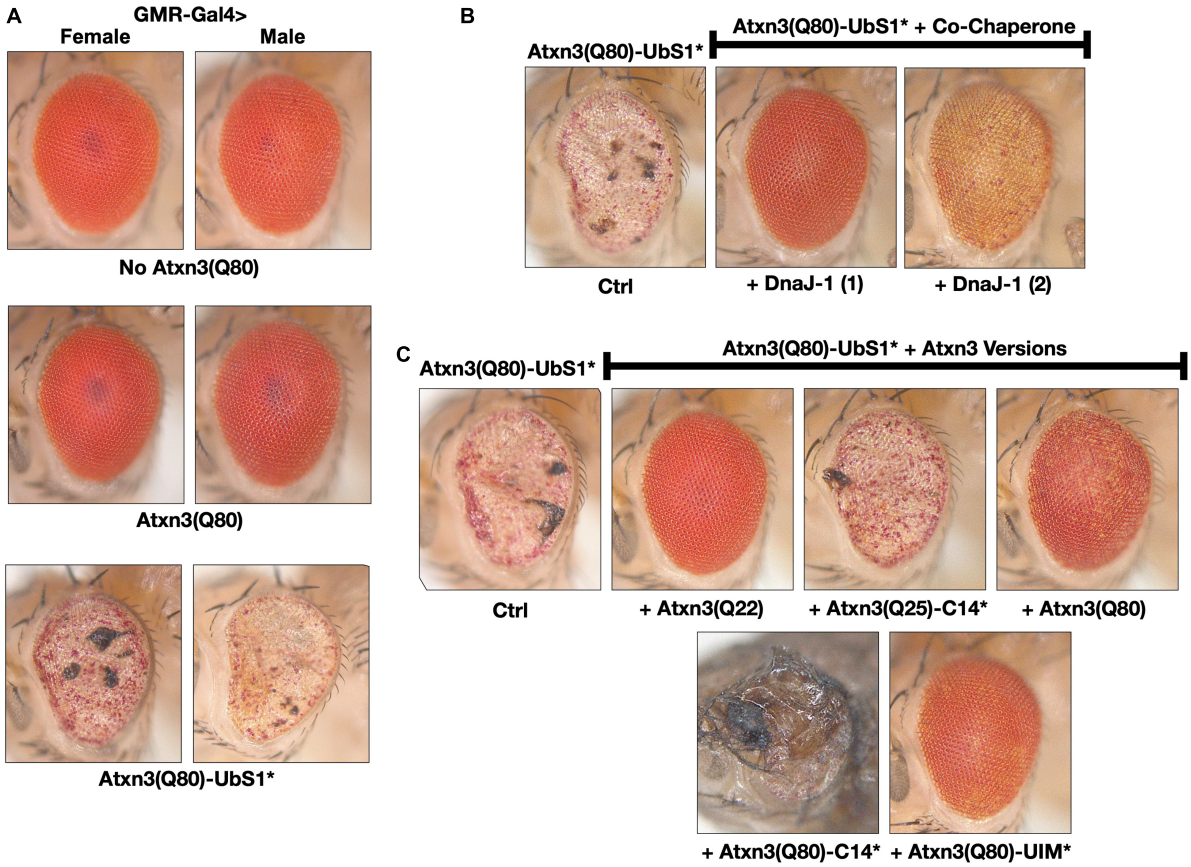


FIGURE 3
Toxicity from Atxn3(Q80)-UbS1* in fly eyes is modulated by DnaJ-1 and catalytically active Atxn3. **(A–C)** Images of external fly eyes expressing the noted transgenes. All flies were 1 day old. C14*: Mutated catalytic cysteine at position 14 into alanine to render Atxn3 catalytically inactive (Winborn et al., 2008). UIM*: Mutations in each of the ubiquitin-interacting motifs of Atxn3 to disable their interaction with ubiquitin chains (Winborn et al., 2008; Todi et al., 2009). Q(n): Length of the polyQ, where 22 and 25 denote wild-type range and 80 denotes disease range.

The role of Atxn3 ubiquitin binding and cleavage on UbS1*-dependent toxicity

As a result of: (1) previously published results that UbS1* renders Atxn3 catalytically dead (Nicastro et al., 2009, 2010), and (2) the current observations that any version of Atxn3 that is catalytically active suppresses toxicity from Atxn3(Q80)-UbS1* (**Figure 3C**), we examined genetically the impact of the catalytic site and of the UIMs on UbS1*-dependent toxicity. The point we sought to explore was whether Atxn3(Q80)-UbS1* is more toxic because it can bind ubiquitin chains, but it is unable to cleave them thus potentially sequestering various ubiquitinated species and perturbing homeostasis. If that is the case, rendering the catalytic site inactive by mutating the cysteine at position 14 into alanine should not impact the UbS1* phenotype, whereas mutating the UIMs, thus precluding the binding of ubiquitin chains by Atxn3, should ameliorate it.

To explore these possibilities, we generated additional, transgenic fly lines that express Atxn3(Q80)-UbS1* either with a catalytic cysteine mutation (C14*), or with mutated UIMs [(Winborn et al., 2008; Todi et al., 2009) UIM*; **Figure 5A**]. We confirmed that each transgenic line expresses pathogenic Atxn3 and that the overall protein levels are comparable (**Figure 5B**; mutation of the UIMs of Atxn3 can reduce the extent of its own ubiquitination in some circumstances Berke et al., 2005; Todi et al., 2007a). With these new lines on hand, we examined the effect of expressing the transgenes everywhere, only in eyes, or only in neuronal tissue.

Ubiquitous expression leads to larval death in every line expressing Atxn3(Q80)-UbS1*, regardless of additional mutations (**Figure 5C**); we did not observe changes in phenotype with this expression pattern. Expression in fly eyes allowed us to detect clear variation from the toxicity of UbS1* with additional mutations. Mutating C14 in addition to UbS1 leads to an external eye phenotype that resembles UbS1*, whereas mutating the UIMs improves the phenotypes, but does not suppress it to the level of pathogenic Atxn3 without UbS1 mutations (**Figures 5D,E** shows the relative expression levels of the transgenes in **Figure 5D**).

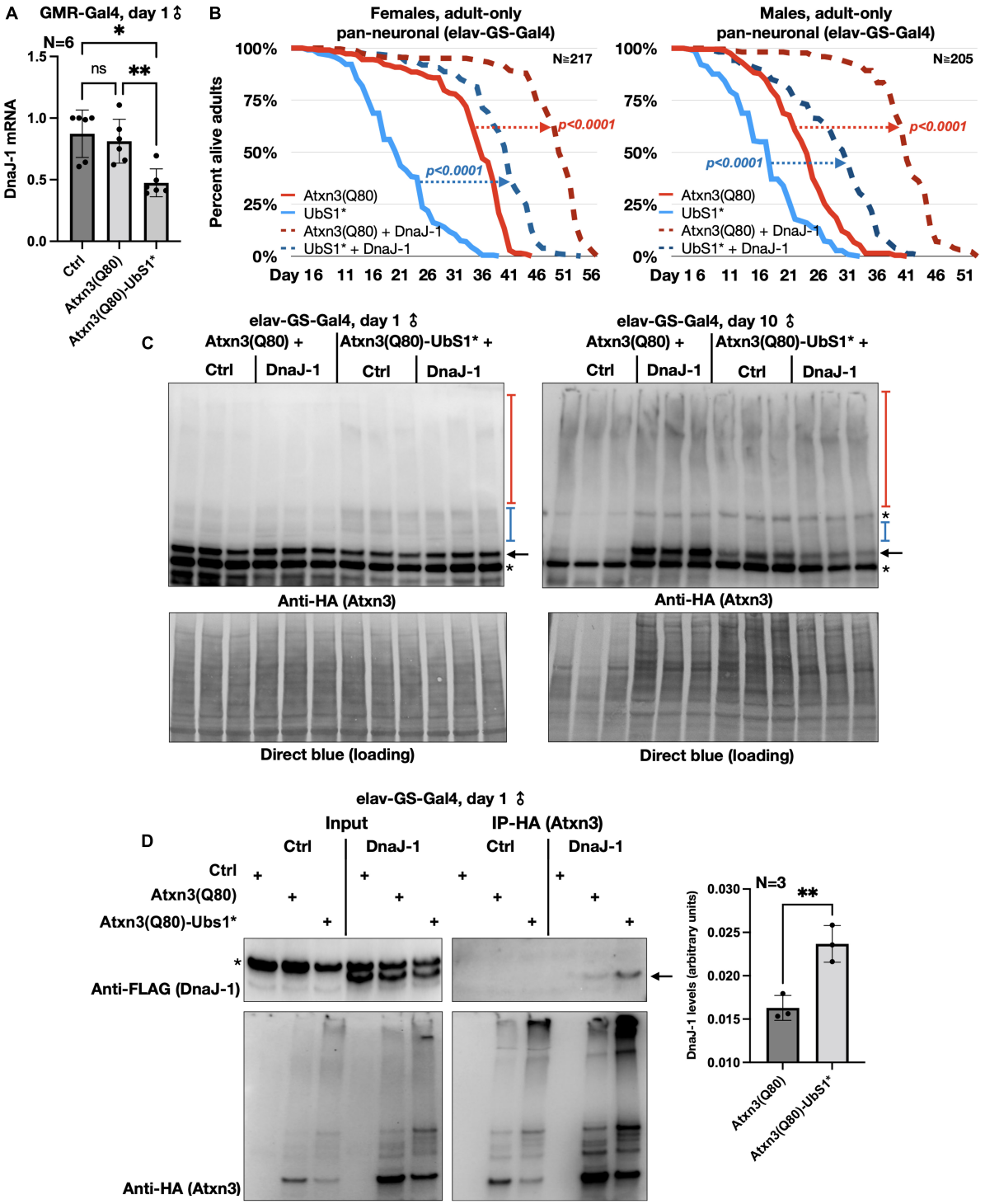


FIGURE 4
DnaJ-1 suppresses neuronal toxicity from UbS1-mutated Atxn3 and binds it more strongly than Atxn3(Q80) with intact UbS1. **(A)** qRT-PCR from dissected fly heads expressing the noted versions of Atxn3. Ctrl: Gal4 driver on the background line of Atxn3 transgenic flies. *$p < 0.05$, **$p < 0.01$, "ns", not significant. Statistics: One-way ANOVA with Tukey's *post hoc*. **(B)** Adult fly longevities when Atxn3(Q80) with intact or mutated UbS1 was expressed in adult neurons in the absence or presence of exogenous DnaJ-1. *P*-values are from log-rank tests. **(C)** Western blots from whole flies expressing the noted transgenes in adult neurons for the indicated amounts of time. Black arrow, unmodified Atxn3 band. Blue bracketed bar, ubiquitinated Atxn3 species. Red bracketed bar, SDS-resistant Atxn3. Asterisks, non-specific signal. Ctrl: Flies expressing Atxn3 in the absence of exogenous DnaJ-1. **(D)** Left: Western blots of input and HA-IPs. Black arrow, DnaJ-1 band. Asterisk, non-specific signal. Right: Quantification of blots from the left and other, independent repeats. **$p < 0.01$ from *t*-test.

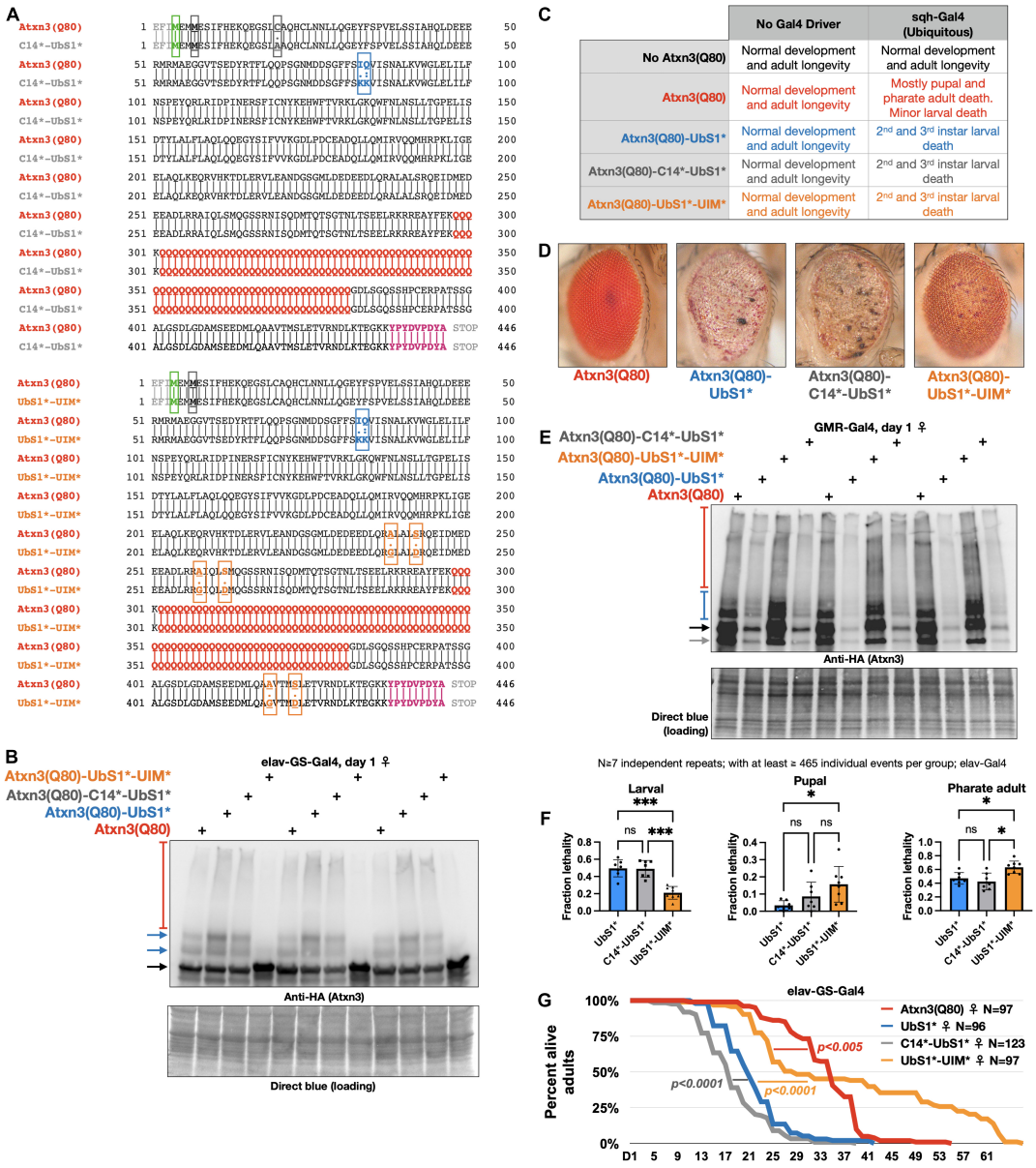
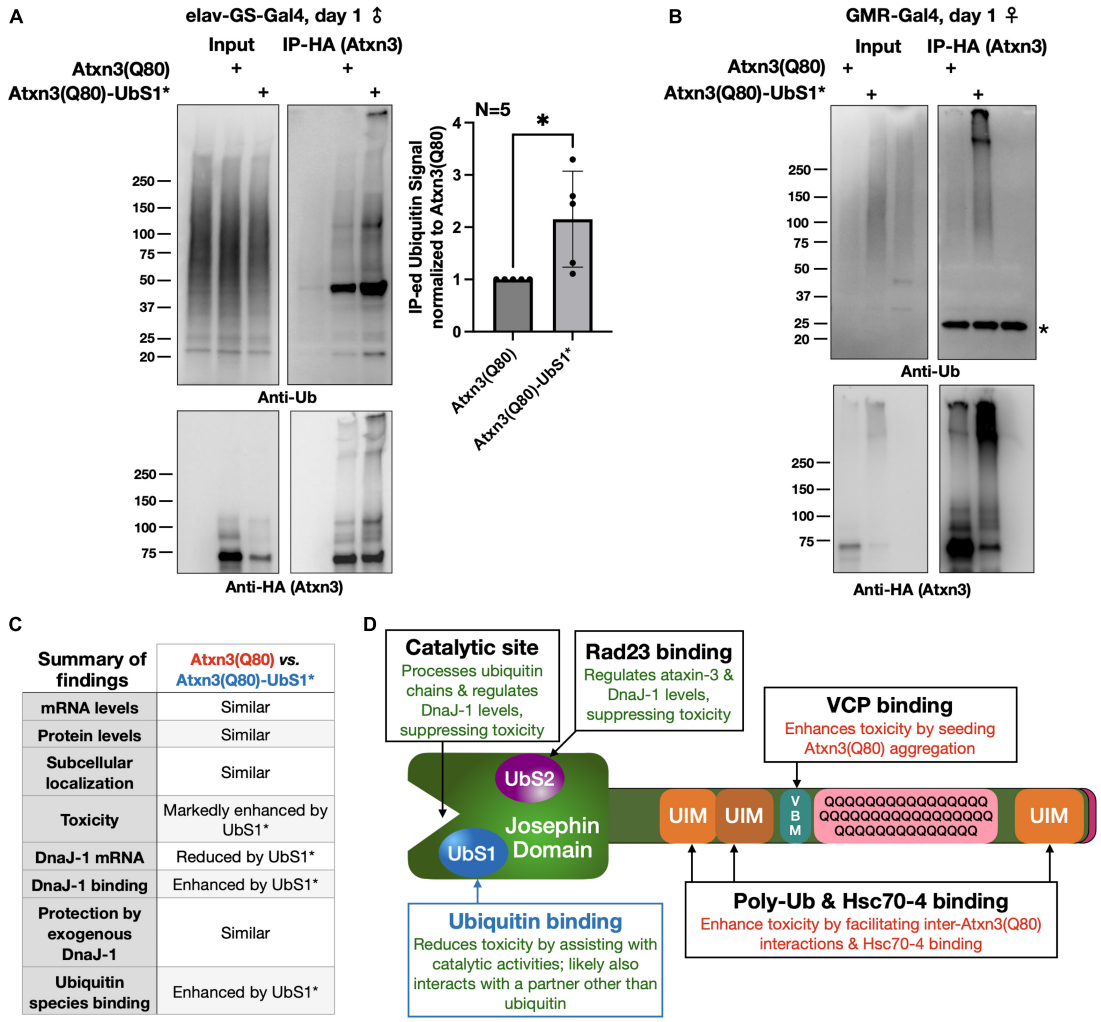


FIGURE 5
Effect of catalytic cysteine mutation and UIM mutations on toxicity from UbS1*. **(A)** Amino acid sequences of the transgenic Atxn3 expressed in the new lines that we generated. Highlighted: First methionine (green), endogenous methionine (black); C14 mutation (gray); UbS1 mutation (blue); polyQ tract (red); UIM mutations (orange); HA epitope tag (purple). **(B)** Western blots of flies expressing the noted versions of Atxn3 in adult neurons for 1 day. Each lane is an independent repeat. Black arrow, main Atxn3 band. Blue arrows, ubiquitinated Atxn3. Red bracketed bar, SDS-resistant Atxn3. **(C)** Summary of results when the noted versions of Atxn3 are expressed in all tissues. **(D)** Representative photographs of external eyes expressing the noted versions of Atxn3. Flies were 1 day old. **(E)** Western blots from expression of the noted versions of Atxn3 in fly eyes. Flies were 1 day old. Each lane is an independent repeat. Black arrow, main Atxn3 band. Blue bracketed bar, ubiquitinated Atxn3. Red bracketed bar: SDS-resistant species of Atxn3. Gray arrow: Proteolytic fragment we observe sometimes. Reduced Atxn3 signal intensity in the lanes with UbS1* and C14*-UbS1* mutations are from loss of eye mass due to degeneration. **(F)** Summary of results when the noted versions of Atxn3 are expressed in all neurons, throughout development (elav-Gal4). "ns", not significant. $*p < 0.05$, $***p < 0.001$. Statistics: Brown-Forsythe and Welch ANOVA tests. **(G)** Longevity of flies expressing the noted versions of Atxn3(Q80) in all adult neurons (elav-GS-Gal4). *P*-values: log-rank tests.

We then examined whether the C14 and UIM mutations impact the UbS1* phenotype in all neurons. As we showed in Figure 1, pan-neuronal expression of Atxn3(Q80)-UbS1* during development leads to lethality at larval, pupal, and pharate adult stages. Expression of Atxn3(Q80)-UbS1*-UIM* in all neurons suppresses toxicity compared to the version with intact UIMs (Figure 5F), but this improvement falls short of what we observe with Atxn3(Q80) without



Summary of findings	Atxn3(Q80) *vs.* Atxn3(Q80)-UbS1*
mRNA levels	Similar
Protein levels	Similar
Subcellular localization	Similar
Toxicity	Markedly enhanced by UbS1*
DnaJ-1 mRNA	Reduced by UbS1*
DnaJ-1 binding	Enhanced by UbS1*
Protection by exogenous DnaJ-1	Similar
Ubiquitin species binding	Enhanced by UbS1*

FIGURE 6
Atxn3(Q80)-UbS1* co-precipitates more ubiquitinated species than Atxn3(Q80). **(A,B)** Western blots of Atxn3(Q80)-HA co-IPs. Ub, ubiquitin; **(A)** Adult neuronal expression; **(B)** Eye expression. Graph: Quantification of results on the left and additional, independent repeats. $^*p < 0.05$, Wilcoxon test. Some of the ubiquitinated species on the anti-ubiquitin blot may include ubiquitinated Atxn3 itself, which is ubiquitinated and interacts with other Atxn3 proteins (Todi et al., 2007a, 2009, 2010; Johnson et al., 2021). **(C)** Summary of the findings presented in this study. **(D)** Graphical summary of the role of various Atxn3 domains, based on our studies in *Drosophila* models of SCA3; references are in main text.

UbS1 mutation, which leads to adult flies coming out (Figure 1). Mutating the catalytic cysteine in addition to UbS1 leads to a phenotype similar to the UbS1 mutation alone (Figure 5F).

Lastly, we compared the longevity of flies that expressed UbS1*-containing Atxn3(Q80) without additional mutations, or in addition to C14* or UIM*, only in adult neurons. We observed that mutating the catalytic cysteine slightly but significantly worsens the UbS1* phenotype (Figure 5G). However, UIM* mutations markedly improve the UbS1* longevity phenotype to an extent that is significantly better even when compared to the longevity of adults expressing Atxn3(Q80) without UbS1 mutation (Figure 5G). Collectively, these results indicate that ubiquitin binding and processing both contribute to the toxicity of Atxn3(Q80)-UbS1*: C14 mutation either does not impact phenotype or can worsen it under some circumstances, whereas the UIM* mutations can improve it to varying degrees, depending on the type of tissue and stage of expression.

Pathogenic Atxn3 with mutated UbS1 interacts more strongly with ubiquitinated species

The reduction in toxicity that we observed when the UIMs of Atxn3(Q80)-UbS1* are disrupted led us to examine whether UbS1-mutated Atxn3(Q80) interacts more strongly with ubiquitinated species in flies. We expressed Atxn3(Q80)

with intact or mutated UbS1 in adult fly neurons for 24 h, IP-ed Atxn3, and then examined the levels of ubiquitin species that co-precipitate with it. We observed that UbS1 mutation leads to higher amounts of ubiquitinated species co-IP-ing with Atxn3(Q80)-UbS1* compared to Atxn3(Q80) (**Figure 6A**). We observed the same pattern when utilizing fly eyes (**Figure 6B**). We conclude that UbS1 mutation increases the association of pathogenic Atxn3 with ubiquitinated species in *Drosophila*. Alongside the other data presented here (**Figure 6C**), we propose that UbS1 mutation enhances the toxicity of pathogenic Atxn3 at least in part because of its impaired functions as a DUB (**Figure 6D**). But, as discussed below, this need not be the only route of action.

Discussion

Protein-protein interactions play critical roles in the precise symptomatology of polyQ diseases (Johnson et al., 2022b). Toward understanding the role of non-polyQ domains on the toxicity of the SCA3 protein, over the recent years we embarked on systematic studies to dissect the manner in which each of its domains impacts polyQ toxicity (our findings from various publications are summarized in **Figure 6D**; Blount et al., 2014; Tsou et al., 2015b; Sutton et al., 2017; Ristic et al., 2018; Johnson et al., 2019, 2020, 2021, 2022a). In this study, we investigated UbS1 thereby completing our series of Atxn3 domain examinations from the perspective of *Drosophila melanogaster*. These investigations have collectively increased the understanding of the disease-causing properties of Atxn3 and have identified potential therapeutic points of intervention.

Here, we determined that UbS1 is a key player in the toxicity of pathogenic Atxn3, not dissimilar from the effect of the catalytic site of this DUB—mutation of each domain markedly increases the toxicity of pathogenic Atxn3 in the fruit fly [the data in this report and references (Warrick et al., 2005; Tsou et al., 2013, 2015b; Sutton et al., 2017)]. This outcome was expected, because: (1) we previously demonstrated that UbS1 is important for the catalytic activities of Atxn3 *in vitro* (Nicastro et al., 2010); (2) we and others showed that mutating the catalytic site of Atxn3 renders the pathogenic protein markedly more toxic (Warrick et al., 2005; Tsou et al., 2015b; Sutton et al., 2017); (3) studies from others and us showed that Atxn3 has protective properties in *Drosophila*, which depend on its catalytic activity (Warrick et al., 2005; Tsou et al., 2015b; Sutton et al., 2017); and (4) even the pathogenic version of Atxn3 has protective properties against polyQ-dependent degeneration, as long as it is catalytically active (**Figure 3C**). Thus, we originally hypothesized that mutating UbS1 would act similarly to mutating the catalytic site, enhancing Atxn3 pathogenicity. Our results in flies generally support this hypothesis.

Why does UbS1 mutation render pathogenic Atxn3 more toxic in *Drosophila*? The mechanism of this enhanced toxicity remains to be determined and will require a deeper understanding of the biological functions of wild-type Atxn3 and how those functions are impacted by polyQ expansions. Those examinations are outside the scope of this study and are confounded by the fact that the precise role of Atxn3 in intact organisms remains unclear. While various studies have provided significant evidence that this DUB is involved in endoplasmic reticulum-associated degradation and other types of protein quality control, DNA damage repair, response to heat stress, transcriptional regulation, sensory organ development and function, synaptic communication, etc. (Costa Mdo and Paulson, 2012; Matos et al., 2019; Herzog et al., 2020; Toulis et al., 2020, 2022), it is also true that mice that lack Atxn3 are generally fine (Schmitt et al., 2007; Reina et al., 2010; Switonski et al., 2011; Zeng et al., 2013). As with other diseases in the polyQ family, more remains to be learned about the SCA3 biology of disease. We hope that the studies that we conducted, including with UbS1, will provide new clues.

What we propose based on this study and the investigations of other domains of Atxn3 (Blount et al., 2014; Tsou et al., 2015b; Sutton et al., 2017; Ristic et al., 2018; Johnson et al., 2019, 2020, 2021, 2022a) is that mutations in UbS1 render this DUB unable to process ubiquitin species—which it binds through the UIMs—due to compromised catalytic activity. As noted above, UbS1 coordinates ubiquitin to allow for proper "attack" of isopeptide bonds by the catalytic site (Nicastro et al., 2009, 2010). In the absence of this interaction, Atxn3 binds but cannot process ubiquitinated species thereby, we posit, sequestrating them. Consequently, UIM mutations alleviate the increased toxicity of UbS1*. We propose that these ubiquitinated species—that have been sequestered—include proteins necessary for cellular biology. Their identity remains unknown at this time and will be the focus of future investigations, but may involve factors important in the transcription of the co-chaperone, DnaJ-1, whose levels are reduced in the presence of UbS1* pathogenic Atxn3 in fly eyes. Additionally, considering: (1) that we observe a stronger interaction of UbS1-mutated Atxn3 with DnaJ-1 compared to Atxn3 with an intact UbS1; and (2) that exogenous DnaJ-1 improves UbS1* pathology, DnaJ-1 may be one factor sequestered by Atxn3(Q80)-UbS1*. (As noted below, future studies will shed light on the interactions and functions of UbS1 by itself and in concert with other ubiquitin binding and cleaving domains of Atxn3). Furthermore, as we reported recently (Johnson et al., 2020), the UIMs of Atxn3 also help it interact with the heat shock protein, Hsc70-4 in *Drosophila* and also help mediate Atxn3-Atxn3 interactions in the fly, which also occur in mammalian cells (Todi et al., 2007a). Thus, some potential substrates of Atxn3 could include heat shock proteins beyond DnaJ-1 as well as other Atxn3 proteins. We predict that complex interactions are at play that

collectively lead to the enhanced toxicity of UbS1*, potentially in a tissue-dependent manner.

However, the above is likely not the only mechanism through which UbS1-mutated, pathogenic Atxn3 becomes more toxic. After all, UIM mutations do not fully reverse UbS1*-dependent toxicity in eyes and in developing neurons, and have no detectable modifying effect on UbS1-mutated Atxn3 expressed everywhere. Also, mutation of the catalytic cysteine of Atxn3 enhances UbS1*-dependent toxicity in adult neurons—although it does not noticeably impact it when tested in fly eyes or during development. Perhaps UbS1 also directly binds proteins other than ubiquitin and these interactions further impact Atxn3 toxicity. This idea is supported by the fact that the other ubiquitin-binding site on Atxn3's catalytic domain, UbS2 directly interacts with both ubiquitin and the proteasome shuttle protein, Rad23; the latter interaction has critical outcomes for the degradation and pathogenicity of Atxn3 (Nicastro et al., 2005, 2009, 2010; Blount et al., 2014; Sutton et al., 2017). This proposed, novel UbS1 interaction may also be tissue-specific and could explain differences in the modulation of toxicity of UbS1* in all tissues vs. eyes vs. neurons by catalytic cysteine and UIM mutations. We will pursue the possibility of UbS1 interactions with non-ubiquitin proteins in the future, with the added hope that this potential interaction may unveil entry points for SCA3 therapy.

To conclude, UbS1 mutations exacerbate the toxicity of Atxn3 in *Drosophila* (**Figure 6D**), at least in part due to compromised ubiquitin processing by this DUB. Continued interrogation of this disease protein is necessary to understand the biology of SCA3 and to devise therapeutics for it. We believe that this investigation into the pathophysiological properties of polyQ-expanded Atxn3 will prove impactful toward such efforts.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MP: conceptualization, data curation, software, formal analysis, funding acquisition, validation, investigation, visualization, methodology, and writing and editing. KL: data curation, formal analysis, supervision, validation, investigation, and visualization. AH: data curation, formal analysis, validation, and visualization. W-LT: conceptualization, data curation, software, validation, investigation, visualization, methodology, and writing and editing. ST: conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, and writing and editing. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Wayne State University Graduate School Dean's Diversity Fellowship (MP), NIGMS T34GM140932 (AH), and NINDS R01NS086778 (ST).

Conflict of interest

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

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frontiers | Frontiers in Neuroscience

TYPE Review
PUBLISHED 16 February 2023
DOI 10.3389/fnins.2023.1137893

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 05 January 2023
ACCEPTED 27 January 2023
PUBLISHED 16 February 2023

CITATION
Pan X, Dutta D, Lu S and Bellen HJ (2023) Sphingolipids in neurodegenerative diseases. *Front. Neurosci.* 17:1137893. doi: 10.3389/fnins.2023.1137893

Sphingolipids in neurodegenerative diseases

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Neurodegenerative Diseases (NDDs) are a group of disorders that cause progressive deficits of neuronal function. Recent evidence argues that sphingolipid metabolism is affected in a surprisingly broad set of NDDs. These include some lysosomal storage diseases (LSDs), hereditary sensory and autonomous neuropathy (HSAN), hereditary spastic paraplegia (HSP), infantile neuroaxonal dystrophy (INAD), Friedreich's ataxia (FRDA), as well as some forms of amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). Many of these diseases have been modeled in *Drosophila* melanogaster and are associated with elevated levels of ceramides. Similar changes have also been reported in vertebrate cells and mouse models. Here, we summarize studies using fly models and/or patient samples which demonstrate the nature of the defects in sphingolipid metabolism, the organelles that are implicated, the cell types that are initially affected, and potential therapeutics for these diseases.

KEYWORDS

***Drosophila*, neurodegeneration, sphingolipids, ceramides, mitochondria, lysosome, Parkinson's disease**

Introduction

Neurodegenerative diseases (NDDs) are a group of progressive disorders that cause physiological deficits in the nervous system. Depending on the nature of the NDD, specific brain areas and neuronal subtypes are affected first and other neuronal subtypes often become affected during disease progression. Symptoms, such as decline in motor and cognitive functions, often also worsen with time. However, for many NDDs the initial pathological changes are insidious and observable symptoms do not arise until the disease is at a more advanced stage. For example, in Parkinson's disease (PD), a considerable loss of dopaminergic neurons occurs before canonical PD-associated symptoms are evident (Fearnley and Lees, 1991; Greffard et al., 2006). An early diagnosis of NDDs requires an in-depth understanding of the underlying mechanisms as well as the identification of useful biomarkers that are detectable during the latent period of the diseases.

Most of our knowledge of the pathogenesis of NDDs are derived from studying the function of genes that are associated with these diseases. Many NDDs with low prevalence have a monogenic etiology. Even for more common NDDs, such as PD and amyotrophic lateral sclerosis (ALS), where the majority of cases are sporadic, the understanding of the disease mechanism heavily relies on studies of familial cases and disease-causing genes/variants. Interestingly, some rare NDD genes are also identified as risk factors for common NDDs, suggesting that the pathogenesis of different NDDs share similar molecular mechanisms (Ma et al., 2022). This is corroborated by similar pathological defects observed in various NDDs, including the presence of aberrant protein deposits and aggregates as well as deficits in lysosomal and mitochondrial functions (Soto and Pritzkow, 2018; Wallings et al., 2019;

Monzio Compagnoni et al., 2020; Nixon, 2020). Recent studies indicate that sphingolipids (SLs) are key players in a surprisingly broad set of NDDs. The importance of SLs in NDDs is supported by the association of genes that play a role in SL metabolism and the changes in SL profile in patients and disease models.

Fruit flies are a premier model organism for genetic studies (Bellen et al., 2010). The conservation between fly and human genes and the sophisticated nervous system of flies allows for the study of NDDs in flies (Bellen et al., 2019; Ma et al., 2022). A forward genetic screen on the *Drosophila* X chromosome identified 165 genes that are required for the development, function, and maintenance of the nervous system (Yamamoto et al., 2014). More than 90% of these genes are conserved in humans and currently, more than 65% of the identified genes have been associated with diseases based on the Online Mendelian Inheritance in Man (OMIM) (Amberger et al., 2019). Recently powerful genetic tools have been developed in flies that allow for in-depth analysis of disease-associated genes. This includes the T2A-GAL4 gene trap technology which permits the determination of gene expression patterns and protein subcellular localization, assessment of loss-of-function phenotypes, humanization of the fly models, assessment of impacts of human variants on gene function, tissue-specific and reversible removal of proteins, and identification of interacting proteins *in vivo* (Diao et al., 2015; Nagarkar-Jaiswal et al., 2015; Lee et al., 2018; Li-Kroeger et al., 2018; Bellen et al., 2019; Kanca et al., 2019, 2022).

In this review, we will focus on the NDDs that are associated with changes in SL metabolism. We will first summarize the SL metabolic pathway. We then introduce SL-related NDDs and the models generated in flies. We summarize the symptoms observed in patients and phenotypes associated with disease models with a focus on changes in SL metabolism. We explore how elevated SLs are a common pathogenic factor during the progression of NDDs and discuss future issues.

Overview of sphingolipid metabolism

Sphingolipid refers to a class of lipids that contain a sphingoid base, a long chain amino alcohol moiety (red in **Figure 1**). The majority of SLs also have an N-linked acyl chain (green in **Figure 1**), and a hydrophilic head group (blue in **Figure 1**). The metabolism of SLs involves a complex biochemical network that occurs in different cellular compartments (**Figure 2**). Each pathway is tightly controlled by metabolic enzymes (**Figure 2** and **Table 1**). Here we briefly introduce these pathways, the genes and enzymes involved in the pathways, and compare SL metabolism in human and flies.

SL *de novo* synthesis

The *de novo* synthesis of SLs occurs in the endoplasmic reticulum (ER) (**Figure 2**, top left). The first, and rate limiting, reaction in the pathway is the condensation of L-serine and acyl-CoA to produce 3-keto-dihydrosphingosine. This reaction is catalyzed by serine palmitoyltransferase (SPT), an enzyme complex containing three subunits SPTLC1, SPTLC2/3, and SPTSSA/B. Next, 3-keto-dihydrosphingosine is processed into ceramide, the central molecule in the SL metabolic network, by three sequential reactions. The activity of the pathway is regulated by the ORMDL protein. ORMDL interacts with SPT and suppresses SPT activity in the presence of excessive ceramide, forming a negative feedback loop (Hjelmqvist et al., 2002; Siow and Wattenberg, 2012; Gupta et al., 2015). Flies have orthologs of all the genes that encode the SL *de novo* synthesis enzymes. *Schlank*, the fly gene encoding ceramide synthase, is the only ortholog of six human genes (*CERS1-6*) (Bauer et al., 2009).

Complex SL synthesis

Ceramide is transported from the ER to the Golgi by a specific transporter CERT (Hanada et al., 2003; Rao et al., 2007). Complex SLs, which refer to the SLs that have an O-linked head group, are synthesized in the Golgi apparatus (**Figure 2**, bottom left). Complex SLs include glycosphingolipids (GSLs, carbohydrate head groups), sphingomyelin (SM, phosphocholine headgroup) and ceramide phosphoethanolamine [CPE, phosphoethanolamine (PE) headgroup]. Unlike SM and CPE which have unique headgroup, GSLs are very diverse in their headgroups. The simplest GSLs are glucosylceramide (GlcCer) and galactosylceramide (GalCer), which have a single sugar headgroup. All higher order GSLs (GSLs with extended sugar chain) are derived from GlcCer by adding a galactose first (lactosylceramide, LacCer) followed by branched saccharide chains of different length and sugar composition (D'Angelo et al., 2013). Upon their synthesis, the complex SLs are transferred to the plasma membrane through vesicular transport.

Flies have an ortholog of ceramide glucosyltransferase (*GlcT*), which mediates GlcCer synthesis. However, flies do not produce SM but use CPE as the major SL component in the plasma membrane (Rietveld et al., 1999). CPE is also utilized in flies to perform similar roles of GalCer in the glial cells ensheathing the peripheral axons (Schwann cells in human and wrapping glia in flies) (Ghosh et al., 2013). The higher order GSLs in flies also differ from the human counterparts in that they are built on mannosyl glucosylceramide (MacCer) rather than LacCer. However, expression of human *B4GALT6* gene, which encodes a LacCer synthase, rescues the loss of the fly MacCer synthase gene *egh*, arguing that the functions of the GSLs in human and flies are comparable (Wandall et al., 2003, 2005).

SL catabolism—SM pathway and salvage pathway

Besides *de novo* synthesis, Cer can also be produced by the hydrolysis of complex SLs. The catabolic pathways occur at two locations, the plasma membrane and the lysosomes. On the plasma membrane, some complex SLs, such as SM, can be hydrolyzed into Cer by neutral hydrolases (also known as SM pathway) (**Figure 2**, bottom right). Alternatively, sections of the plasma membrane can be endocytosed *via* the endolysosomal pathway, and the complex SLs in the membrane are hydrolyzed in the lysosomes by the acidic enzymes (also known as salvage pathway) (**Figure 2**, top right). Among the complex SLs, SM and GlcCer can be transformed into Cer by both pathways, while the hydrolysis of CPE, GalCer and higher order GSLs occurs exclusively in lysosomes. At both locations, Cer can be further hydrolyzed into Sph by ceramidase, which can feed into the SL degradation pathway (described below).

In flies, GlcCer can be hydrolyzed by both acidic and neutral glucosylceramidase. The pathway mediating CPE hydrolysis,

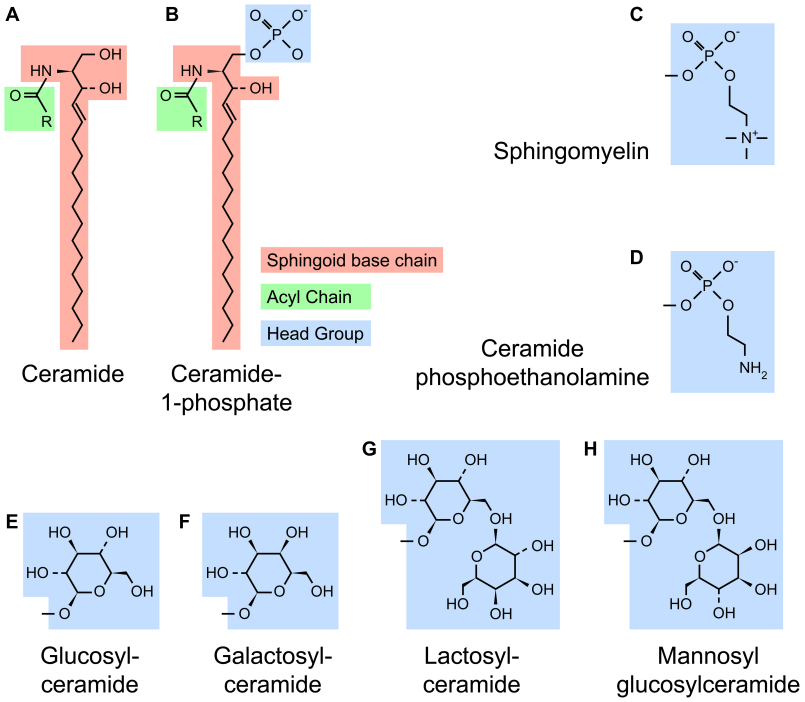

FIGURE 1
Structure of sphingolipids. **(A)** A typical SL [such as ceramide (Cer)] contains a sphingoid base (red) and a N-linked acyl chain (green). **(B)** Complex SLs have an O-linked head group (blue) on the ceramide backbone [ceramide-1-phosphate (C1P) with a phosphate headgroup as shown]. **(C–H)** Several examples of SL headgroups observed in human and flies are shown. Humans produce all SL species except mannosyl glucosylceramide (MacCer), while flies produce ceramide phosphoethanolamine (CPE), glucosylceramide (GlcCer) and MacCer. All higher order glycosphingolipids (GSLs) are synthesized from lactosylceramide (LacCer) in human or MacCer in flies.

however, is not well studied. Mammals only produce trace amount of CPE (Bickert et al., 2015). While no specific CPE hydrolase has been identified in humans, human SMPD1 (acidic sphingomyelinase) is able to use CPE as an alternative substrate (Breiden and Sandhoff, 2021). Flies have three predicted orthologs of *SMPD1* and one ortholog of *SMPD2* (neutral sphingomyelinase), but the activity of these enzymes has not been studied. For Cer hydrolysis, flies have an ortholog of neutral ceramidase (*ASAH2*) but not acidic ceramidase (*ASAH1*), indicating that the hydrolysis of Cer primarily occurs on the plasma membrane in flies.

S1P-mediated SL degradation

Cer can be converted to Sph upon hydrolysis, as mentioned above. Sph is then phosphorylated to make Sph-1-phosphate (S1P) and degraded into PE and fatty aldehyde by the S1P lyase. S1P can be hydrolyzed back into Sph by S1P phosphatases (Mandala, 2001), while the degradation of S1P by S1P lyase is irreversible. The S1P-mediated degradation pathway is highly conserved between human and flies.

Modeling neurodegenerative diseases using *Drosophila*

Fruit flies have a sophisticated nervous system which has numerous similarities to the vertebrate nervous system. Its sensory modalities include vision (Joly et al., 2016; de Andres-Bragado and Sprecher, 2019), hearing (Albert and Gopfert, 2015), olfaction (Ramdya and Benton, 2010), taste (Yarmolinsky et al., 2009), thermosensation (Barbagallo and Garrity, 2015), mechanosensation (Tuthill and Wilson, 2016), proprioception (Agrawal and Tuthill, 2022), and nociception (Im and Galko, 2012; Gibbons et al., 2022). The motor system is also sophisticated and allows fine control of motor activities including gait and flight (Clark et al., 2018; DeAngelis et al., 2019; Zarin et al., 2019; Phelps et al., 2021). The fly nervous system also has complex sensory-motor circuits and the neuronal networks permit high order functions including complex behavioral outputs, including learning, and memory (Frye, 2010; Pool and Scott, 2014; Auer and Benton, 2016; Cognigni et al., 2018; Modi et al., 2020). At the cellular level, flies contain many neuron subtypes that use 10 out of 11 classes of neurotransmitters present in human, including -aminobutyric acid (GABA), glutamate, acetylcholine, and dopamine. Moreover, compelling data argue that glial cells in flies are functionally analogous to human astrocytes, oligodendrocytes, and Schwann cells (Freeman and Doherty, 2006; Deng et al., 2019). Recent single cell transcriptomics studies identified ~90 distinct neuronal cell types in fly heads, again implying a high level of complexity of the fly nervous system (Li et al., 2022; Lu et al., 2022).

The similarities at the molecular, cellular, and functional levels between the human and fly nervous system empower the study of gene function in flies and permit comparisons with vertebrate nervous systems (Ugur et al., 2016; Ma et al., 2022). At the subcellular level, defects in organelles like ER, mitochondria and lysosomes are commonly observed in NDDs, which are readily detected in flies using molecular markers or transmission electron microscopy

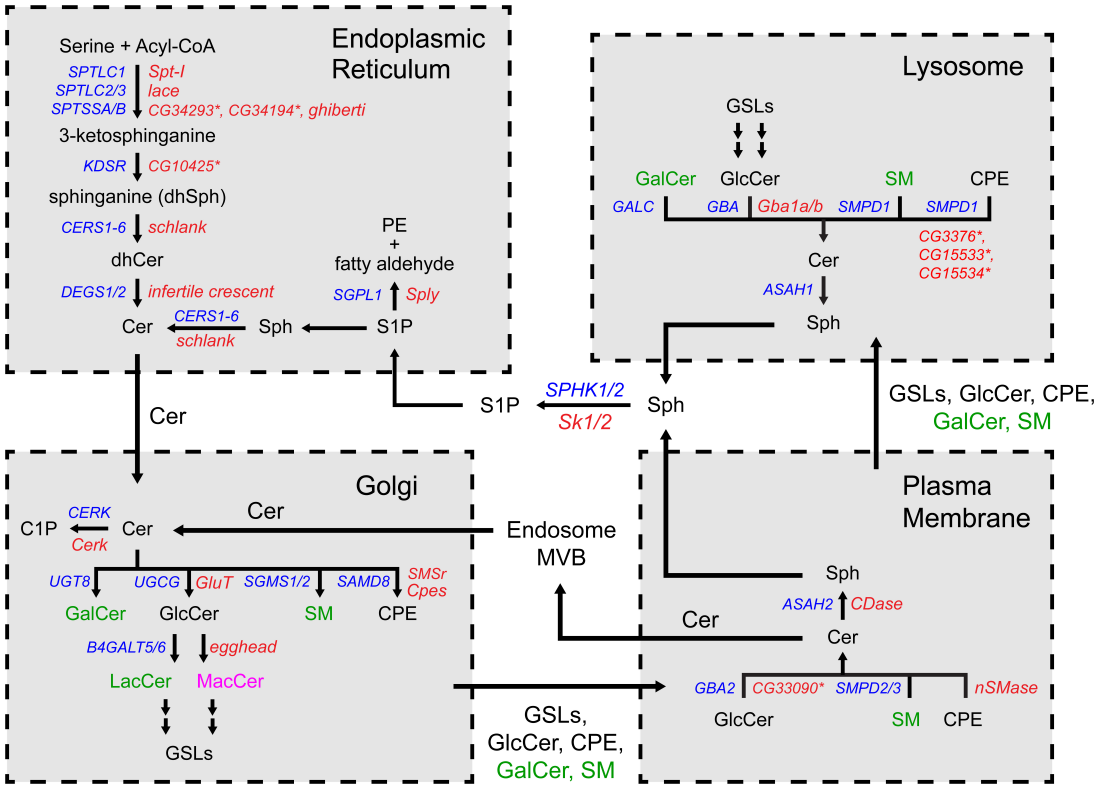

FIGURE 2
The sphingolipid metabolism network. The biochemical reactions are grouped by the organelles in which they occur. The substrates and products are indicated in three colors depending on their presence in human and flies: black (both human and fly), green (human) and magenta (fly). The genes encoding the enzymes are also indicated in colors: blue (human) and red (fly). Asterisks indicate predicted orthologs in flies. Cer, ceramide; dhCer, dihydroceramide; C1P, ceramide-1-phosphate; GalCer, galactosylceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; MacCer, mannosyl glucosylceramide; PE, phosphoethanolamine; CPE, ceramide phosphoethanolamine; SM, sphingomyelin; Sph, sphingosine; dhSph, dihydrosphingosine; S1P, sphingosine-1-phosphate; GSL, glycosphingolipid.

(TEM). These changes, as well as the morphological changes in neurons/glia, can be easily assessed in numerous neuronal tissues (Table 2). At the organismal level, a battery of fly behavioral assays enables the assessment of defects associated with neuronal dysfunction. These include shortened lifespan, defects in climbing and flight, susceptibility to seizures induced by mechanical and heat stress, aberrant circadian rhythms and sleep, impaired vision, olfaction, learning and memory, as well as defects that can be assayed with numerous other behavioral paradigms that are used less frequently (Table 2).

Of note, the fly eye has been a very valuable model to study the molecular and cellular mechanisms underlying numerous neurological disorders. The compound eye consists of ~800 independent photosensing units named ommatidia. Every ommatidium is comprised of photoreceptor neurons, glia, and other cell types organized in a highly stereotyped pattern, allowing identification of all the different cell types (Perry et al., 2017). Second, one can induce small to large homozygous mutant clones in the eye and hence often avoid issues associated with lethality caused by the loss of essential genes (Wang et al., 2014; Yamamoto et al., 2014; Chen et al., 2016b). Third, the repetitive pattern of ommatidia allows detection of subtle phenotypes and comparison between neighboring wildtype and mutant ommatidia. Fourth, neuronal activity in the eye can be tightly controlled by modulating light intensity, which is a unique and important feature as it is difficult to continuously control neuronal activity in other neurons (Wang et al., 2014, 2022c). Fifth, neuronal activity can be easily and precisely measured using electroretinography (ERG). Sixth, the detailed cellular morphology can easily be visualized by TEM to detect cellular and especially organellar defect (Lin et al., 2018; Wang et al., 2022c). Finally, genetic approaches allow manipulations of different neuronal and glial cells which facilitates the study the function of genes in different cells as well as neuron-glia interactions (Liu et al., 2015, 2017; Moulton et al., 2021; Wang et al., 2022c). The unique anatomical and physiological characteristics of the fly eye combined with numerous tools allow in depth studies that cannot be easily achieved in most models. Although the fly eye does not recapitulate all the features of other neurons and glia, the wealth of knowledge obtained in the fly eye has very often proved true in other areas of the nervous system, especially with respect to the study of the mechanism of neurodegeneration.

Inherited peripheral neuropathies

Neurodegenerative diseases of the peripheral nervous system (PNS) can be classified into motor neuropathies, motor and sensory neuropathies, and sensory and autonomous neuropathies based on the nature of the affected neurons. Among these many are based on a monogenic inheritance pattern, and recent findings established an intriguing link between the genes encoding subunits of SPT, the rate limiting enzyme in the SL *de novo* synthesis pathway, and three inherited peripheral neuropathies.

Hereditary sensory and autonomous neuropathy 1

Hereditary sensory and autonomous neuropathy (HSAN) refers to a group of neurodegenerative disorders with heterogenous clinical features with sensory, autonomous and minor motor neuron involvement. HSAN1 is one of five HSAN subtypes. It is inherited in an autosomal dominant (AD) fashion by mutations in one of five genes: *SPTLC1* (Dawkins et al., 2001; Bode et al., 2016; Gantner et al., 2019), *SPTLC2* (Rotthier et al., 2010; Bode et al., 2016; Gantner et al., 2019), *ATL1* (Guelly et al., 2011), *ATL3* (Kornak et al., 2014), and *DNMT1* (Klein et al., 2011). The symptoms include loss of pain and temperature sensation that initiate in the distal section of the lower limbs and progressively spread proximally. In addition, variable motor and autonomous symptoms present in some patients.

The clinical features of *SPTLC1*- (OMIM# 162400) and *SPTLC2*-associated HSAN1 (OMIM# 613640) are very similar, indicating a common disease-causing mechanism. Variants in both *SPTLC1* and *SPTLC2* disrupt the substrate selectivity for L-serine vs. L-alanine. This causes an aberrant reaction with palmitoyl-CoA. The product of the L-alanine/palmitoyl-CoA condensation is 1-deoxysphinganine (doxSA), which cannot be further processed into complex SLs by the downstream enzymes. Therefore, doxSA accumulates in cells and causes neurotoxicity (Gable et al., 2010; Penno et al., 2010). Based on

TABLE 1 List of human SL metabolism genes and the fly orthologs.

Human genes	Fly genes	DIOPT score† (out of 16)	Activity	References††
Sphingolipid *de novo* synthesis pathway				
SPTLC1	*Spt-I*	15	Serine palmitoyltransferase, serine + acyl-CoA → 3-ketosphinganine	Oswald et al., 2015
SPTLC2/3	*lace*	15 (*SPTLC2*)		Adachi-Yamada et al., 1999
SPTSSA/B	*CG34293*/ CG34194*/ghi*	8 (*SPTSSA-CG34293*)		Guan et al., 2013
ORMDL1/2/3	*Ormdl*	14 (*ORMDL3*)	SPT activity suppression	Hjelmqvist et al., 2002
KDSR	*CG10425**	15	3-keto-dhSph reductase, 3-ketosphinganine → dhSph	
CERS 1/2/3/4/5/6	*schlank*	13 (*CERS5/6*)	Ceramide synthase, dhSph + acyl-CoA → dhCer Sph + acyl-CoA → Cer	Bauer et al., 2009
DEGS1/2	*ifc*	15 (*DEGS2*)	SL delta(4)-desaturase, dhCer → Cer	Ternes et al., 2002
SM and CPE synthesis				
CERT1	*Cert*	14	ER–Golgi Cer trafficking	Rao et al., 2007
SGMS1/2	No fly ortholog	N/A	SM synthase, Cer + phosphatidylcholine → SM + DG	
SAMD8	*SMSr*	13	CPE synthase, Cer + phosphatidylethanolamine → CPE + DG	Vacaru et al., 2013
No human ortholog	*Cpes*	N/A	CPE synthase, Cer + CDP-ethanolamine → CPE + CMP	
GSL synthesis (mono- and di- glycosyl ceramide)				
UGCG	*GlcT*	15	Cer glucosyltransferase, Cer + UDP-glucose → GlcCer + UDP	Kohyama-Koganeya et al., 2004
UGT8	*Ugt50B3**	10	Cer galactosyltransferase, Cer + UDP-galactose → GalCer + UDP	
B4GALT5/6	No fly ortholog	N/A	GlcCer beta-1,4-galactosyltransferase, GlcCer + UDP-galactose → LacCer + UDP	
No human ortholog	*egh*	N/A	GlcCer glycosyltransferase, GlcCer + GDP-mannose → MacCer + GDP	Wandall et al., 2003
SM and salvage pathways				
PSAP	*Sap-r*	12	Facilitate GSL hydrolysis	Hindle et al., 2017; Sellin et al., 2017
SMPD1	*CG3376*/ CG15533*/ CG15534**	15 (*CG3376*)	Acidic sphingomyelinase, SM → Cer + PC (human)	
SMPD2	*nSMase*	15	Neutral sphingomyelinase, SM → Cer + PC (human) CPE → Cer + PE (fly)	Chen et al., 2022
SMPD3	No fly ortholog	N/A	Neutral sphingomyelinase, SM → Cer + PC (human)	
SMPD4	*CG6962**	15		
SMPD5	No fly ortholog	N/A		
ENPP7	No fly ortholog	N/A	Alkaline sphingomyelinase, SM → Cer + PC (human)	
GBA	*Gba1a/b*	14 (*Gba1a/b*)	Acidic glucosylceramidase, GlcCer → Cer + Glucose	Davis et al., 2016
GBA2	*CG33090**	15	Neutral glucosylceramidase, GlcCer → Cer + Glucose	
GALC	No fly ortholog	N/A	Acidic galactosylceramidase, GalCer → Cer + Galactose	
ASAH1	No fly ortholog	N/A	Acidic ceramidase, Cer → Sph + fatty acid	
ASAH2	*Cdase*	9	Neutral ceramidase, Cer → Sph + fatty acid#	Yuan et al., 2011
ACER1/2/3	*bwa*	14 (*ACER2*)		
SGPP1/2	No fly ortholog	N/A	S1P phosphatase, S1P → Sph	

(Continued)

TABLE 1 (Continued)

Human genes	Fly genes	DIOPT score† (out of 16)	Activity	References††
S1P synthesis and catabolism				
SPHK1/2	*Sk1/2*	13 (*SPHK1-Sk2*)	Sph kinase, Sph → S1P	Herr et al., 2004
SGPL1	*Sply*	15	S1P lyase, S1P → PE + fatty aldehyde	Herr et al., 2003
C1P synthesis				
CERK	*Cerk*	14	Ceramide kinase, Cer → C1P	Dasgupta et al., 2009

†DRSC integrative ortholog prediction tool (DIOPT) score indicates the level of homology between human and fly orthologs (Hu et al., 2011). The score is evaluated for each pair of human-fly orthologs. A high score indicates a high level of homology between orthologs, and the maximum score is 16. Only the highest score is listed in the table, and the gene symbol indicates the ortholog or the human-fly ortholog pairs that receive the score.
††The references indicate the studies on the fly orthologs.
*Predicted orthologs in flies.
#Fly Bwa does not have ceramidase activity.
SPT, serine palmitoyltransferase; Sph, sphingosine; dhSph, dihydrosphingosine; Cer, ceramide; dhCer, dihydroceramide; SM, sphingomyelin; PC, phosphocholine; CPE, ceramide phosphoethanolamine; PE, phosphoethanolamine; GlcCer, glucosylceramide; GalCer, galactosylceramide; LacCer, lactosylceramide; MacCer, mannosyl glucosylceramide; GSL, glycosphingolipid; S1P, sphingosine-1-phosphate; C1P, ceramide-1-phosphate.

TABLE 2 Assays to study neurodegeneration in *Drosophila*.

Assays	Descriptions	Related defects in human	References
Lifespan	Measurement of the lifespan of flies from eclosion to death	Reduced lifespan	Lin et al., 2018; Lu et al., 2022; Srivastava et al., 2023
Climbing and flight activity	Measurement of the anti-geotaxis climbing and flying activities of adult flies	Motor defects	
Bang and heat sensitivity	Measurement of the time to recover from a vortex induced shaking or 42°C heat shock	Seizures	
Circadian rhythm and sleep	Evaluation of the circadian rhythm and sleep by continuous monitoring of locomotion activity using a *Drosophila* activity monitoring system	Defects in circadian rhythm and sleep	Pfeiffenberger et al., 2010; Wangler et al., 2017
Vision and olfaction	Evaluation of the vision and olfaction of flies using photo-taxis and odor-taxis assays	Vision or olfactory loss	Ali et al., 2011; Kahsai and Zars, 2011; Mariano et al., 2020
Learning and memory	Evaluation of the learning and memory capacity of odors, visual patterns, and spatial cues using conditioning assays	Learning disability, memory loss	
Neuromuscular junction (NMJ) (Morphology and electrophysiology)	Measurement of the number, distribution, size, and morphology of synaptic boutons at the NMJs, and precise measurement of the physiological properties of synaptic transmission	Neuromuscular defects	Oswald et al., 2015
Motor neuron axons (Morphology)	Measurement of the structure integrity and axonal transport of the long axon extended from the ventral nerve cord to the muscles	Axonal defects in motor neurons	Yalcin et al., 2017
Wing margin nerves (Morphology)	Measurement of the degeneration of axons and wrapping glia	Axonal defects and demyelination	Chung et al., 2020
Compound eye (ERG)	Measurement of the neuronal activity triggered by light stimulation	Functional abnormalities in retina and other neurological defects	Wang et al., 2014, 2022c; Ye et al., 2020
Compound eye (Histology)	Observation of the pathological changes in ommatidia using histological staining, fluorescent markers and immunolabeling	Cellular and subcellular neurodegenerative changes	
Compound eye (TEM)	Observation of the ultrastructural and organelle changes in ommatidia		

cryo-EM 3D structure of the SPT enzyme complex, all the HSAN1 variants map to the active site of the enzyme complex surrounding the SPTLC1-SPTLC2 interacting interface, supporting that this is a critical disease mechanism (Li et al., 2021; Wang et al., 2021).

SPTLC1/2-associated HSAN1 has been modeled in mammals and flies. In a mouse model, a disease-causing $Sptlc1^{C133W}$ variant was overexpressed ubiquitously and shown to cause doxSA production. However, the mice display no motor phenotype and very mild, age-dependent sensory phenotypes at 8–10 months. Despite this mild phenotype, the ectopic expression of $Sptlc1^{C133W}$ causes demyelination of axons and axonal damage in the PNS (McCampbell et al., 2005). Recently, a novel $Sptlc1^{C133W}$ knock-in mice model was generated. The 12-month-old $Sptlc1^{C133W/+}$ mice display aberrant doxSA production, mild sensory and motor defects, and no sign of axon degeneration (Hines et al., 2022).

Spt-I is the fly ortholog of *SPTLC1*. A fly HSAN1 model was generated by overexpressing a *Spt-1* variant analogous to human $SPTLC1^{C133W}$ variant ($Spt\text{-}I^{C129W}$) under control of the GAL4/UAS system (Oswald et al., 2015). UAS-$Spt\text{-}I^{C129W}$ was expressed ubiquitously (*tub-Gal4*) or specifically in a subset of peripheral sensory neurons (*ppk-Gal4*). $Spt\text{-}I^{C129W}$ expression in the sensory neurons induces a sensory deficit in a heat avoidance

assay, which is obvious in larvae. Ubiquitous *Spt-I*^{C129W} expression compromises viability to adulthood but only causes a slight, non-significant overproduction of deoxysphingoid bases. Importantly, feeding flies with a dietary L-alanine supplement significantly elevates the deoxysphingoid base production and exacerbates the toxicity of the transgene. In contrast, dietary L-serine alleviates the defects caused by the transgene, indicating a possible therapeutic strategy (Oswald et al., 2015). Dietary L-serine supplement was also tested in the *Sptlc1*^{C133W} overexpression mouse model as well as in patients with the *SPTLC1*^{C133Y} variant. The treatment significantly lowers the level of doxSA in mice tissues and patient plasma. The symptoms in mice are significantly improved by L-serine treatment (Garofalo et al., 2011). Following this study, a clinical trial was carried out which shows that oral L-serine supplementation appears safe in patients and is potentially effective at slowing disease progression (Fridman et al., 2019).

Juvenile-onset amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is a motor neuron disease with an incidence between 0.6 and 3.8 per 100,000 person per year (Longinetti and Fang, 2019). The disease is characterized by progressive degeneration of both upper and lower motor neurons (UMNs and LMNs). While most of the sporadic cases show a late onset (between 51 and 66 years in average) (Longinetti and Fang, 2019), patients with familial inherited ALS can present with symptoms earlier. The initial clinical presentations of ALS vary among individuals. In some individuals the disease onset is in the limbs, characterized by a combination of UMN (spasticity, weakness, and increased deep tendon reflexes) and LMN signs (muscle twitching, wasting, weakness). Others have a bulbar onset, with speech issues (dysarthria) and swallowing difficulties (dysphagia). In either case, the disease is relentlessly progressive, and most cases ultimately develop severe dysphagia and respiratory insufficiency which cause malnutrition and ultimately death. Currently the management of the disease largely involves symptomatic treatments. Although the majority of the ALS cases do not have discernible family history, a number of monogenic subtypes of ALS have been identified (Goutman et al., 2022) and many of the ALS disease-causing genes have been modeled in organisms including fruit flies (Azuma et al., 2018).

Changes in SL levels have been assessed in ALS patient samples and mice models, but results vary depending on the tissue type and animal models tested. Elevated levels of Cer, SM, and GSLs are observed in patient spinal cord samples, indicating an association between SL and ALS (Cutler et al., 2002; Dodge et al., 2015). However, SL data of serum and CSF in different studies are not consistent (Blasco et al., 2017; FernAndez-Eulate et al., 2020; Goutman et al., 2020; Area-Gomez et al., 2021; Sol et al., 2021). Interestingly, *wobbler* mice which are considered a model for ALS and carry a partial loss-of-function mutation of *Vps54* (Moser et al., 2013), have elevated SL levels in embryonic fibroblasts and increased Sph levels in the spinal cord. Treatment with myriocin, an inhibitor of the SPT complex, improves motor activity and neuropathological changes, suggesting that an accumulation of SL contributes to the progression of disease (Petit et al., 2020).

Recently, two independent studies reported patients with *SPTLC1* variants who present with a juvenile-onset ALS (Johnson et al., 2021; Mohassel et al., 2021), providing a direct link between sphingolipid metabolic dysfunction and the pathogenesis of ALS. Unlike the *SPTLC1* variants associated with HSAN1, the ALS *SPTLC1* variants map to the ER transmembrane domain of SPTLC1 where it interacts with ORMDL protein (Li et al., 2021; Wang et al., 2021). ORMDL suppresses SPT activity in the presence of excessive ceramide in the ER (Siow and Wattenberg, 2012; Gupta et al., 2015; Davis et al., 2019). The variants dominantly disrupt the interaction between SPTLC1 and ORMDL protein and cause an increase in SL *de novo* synthesis (Johnson et al., 2021; Mohassel et al., 2021). Furthermore, Mohassel et al. (2021) showed that allelic-specific siRNA effectively reduced the mutant mRNA levels and the SL levels in patient derived fibroblasts. So far, no animal model of the *SPTLC1*-associated ALS has been reported and future efforts are needed to elucidate the mechanism by which elevated SL synthesis causes degeneration of the motor neurons.

Hereditary spastic paraplegia

Hereditary spastic paraplegia (HSP) refers to a group of motor neuron disease that are characterized by the progressive spasticity and weakness in the lower extremities. Unlike ALS which affects both UMNs and LMNs, HSP only involves the degeneration of LMNs. Clinically the disease can be classified into a pure form and a complex form, depending on the existence of complications other than the LMN signs. Nearly all the HSP subtypes show monogenic inheritance and more than 80 genes have been associated with the disease (Panza et al., 2022). Depending on the affected gene, the disease can be inherited in AD, autosomal recessive (AR), X-linked recessive (XR), or *via* a mitochondrial inheritance pattern. The age of disease onset also varies among different disease subtypes.

The involvement of sphingolipid metabolism in the progression of HSP recently surfaced. Variants in *SPTSSA* cause an early onset and complex form of HSP. Individuals with *SPTSSA* variants were reported to develop spasticity and weakness in lower limbs as well as epilepsy, axial hypotonia and sensorineural hearing loss. SPTSSA is an activating subunit in the SPT complex. The disease-causing variants affect the C-terminus of SPTSSA protein, where it interacts with the ORMDL protein. Similar to the ALS variants in the *SPTLC1* gene, the *SPTSSA* variants disrupt the interaction between SPTSSA and ORMDL and lead to increased SPT activity and SL *de novo* synthesis in human cells (Srivastava et al., 2023).

To study the phenotypes associated with increased SL synthesis *in vivo*, a fruit fly model was established by overexpressing the three human SPT subunits as a fusion protein. Expression of human SPT in flies leads to excessive SL synthesis and causes severe motor defects as well as a shortened lifespan. However, co-expressing human ORMDL3 fully rescues the defects caused by the reference SPT showing that the human enzyme complex is functional and properly regulated in flies. The SPT fusion protein with the *SPTSSA*^{T51I} variant causes similar defects as expression of the reference SPT, showing that the missense mutation does not alter enzymatic activity. However, *SPTSSA*^{T51I} activity is only partially suppressed by expression of ORMDL3 leading to elevated levels of Cers, motor defects, and shorter lifespan (Srivastava et al., 2023). The model was also used to test a mice *Sptssb*^{H56L} variant which causes increased SL *de novo* synthesis and neurodegeneration in *Stellar* mice (Zhao et al., 2015). Similar to the *SPTSSA*^{T51I} variant, the *SPTSSA*^{H59L} (*Sptssb*^{H56L} analog) also disrupts the SPTSSA-ORMDL

interaction and causes neurological phenotypes in flies (Srivastava et al., 2023). These data establish that the SPTSSA variants found in three individuals cause elevated Cer levels because they fail to be properly regulated by ORMDL.

Lysosomal storage diseases

Lysosomal storage diseases (LSDs) are a group of inherited metabolic disorders that affect lysosomal functions [for a comprehensive review of LSDs, see (Platt et al., 2018)]. Most LSDs are caused by variants in genes that encode lysosomal proteins required for lysosomal catabolism. The loss-of-function of the affected protein results in the accumulation of substrates in lysosomes and ultimately causes a severe cell dysfunction. LSDs are clinically heterogeneous, but most LSDs present with early onset neurodegenerative features. Interestingly, many LSD genes have been implicated as risk factors in PD (Robak et al., 2017; Blauwendraat et al., 2020a).

Accumulation of SL species (sphingolipidosis) has been documented for several LSDs (**Table 3**), including those associated with genes encoding SL salvage pathway enzymes as well as the *PSAP* gene. *PSAP* encodes the prosaposin protein, the precursor of four saposin proteins (saposins A–D) which facilitate the hydrolysis of GSLs in lysosomes (O'Brien and Kishimoto, 1991). Besides the SL-related LSDs, sphingolipidosis is also observed in other LSDs that are not directly associated with SL-related genes. Niemann-Pick Disease type C (NPC, OMIM# 257220, 607625) is associated with *NPC1* and *NPC2* genes that encode lysosomal cholesterol transporter proteins. Variants in these genes cause a lysosomal accumulation of cholesterol as well as various SL species including Sph, SM and GSLs (Newton et al., 2018). Secondary accumulations of gangliosides (one subtype of GSL) are observed in a wide spectrum of LSDs, and are not limited to those involving primary defects in ganglioside degradation (Walkley, 2004).

Limited by the scope of this review, we will only discuss two LSDs that are well-modeled in fruit flies: Gaucher disease (GD) and combined saposin deficiency. Though Niemann-Pick Disease type C has been modeled in fruit flies, the changes in SL metabolism were not assessed and therefore will not be discuss here (Huang et al., 2007; Phillips et al., 2008).

Gaucher disease

Gaucher disease is a rare, AR disease caused by variants in *GBA* (Mistry et al., 2017; Grabowski et al., 2021). *GBA* encodes a lysosomal glucosylceramidase (GCase, also called glucocerebrosidase) which facilitates hydrolysis of GlcCer into Cer. Deficiency of GCase activity results in accumulation of the substrate GlcCer. Classically, the disease is classified into three subtypes: type I (non-neuronopathic; OMIM# 230800), type II (acute neuronopathic; OMIM# 230900), and type III (subacute neuronopathic; OMIM# 231000, 231005), depending on the presence of neurological symptoms and the age of onset. The clinical presentations among subtypes are highly variable and form a continuum. Type II GD patients present in infancy with progressive brainstem dysfunction, seizures and other neurological deficits. Type III GD patients present with similar manifestations but have a later onset (from childhood to adult) and the disease progresses more slowly (Roshan Lal and Sidransky, 2017). Although type I GD is classified as non-neuronopathic, neurological symptoms have been reported in a fraction of type I GD patients as the disease progresses (Biegstraaten et al., 2008; Capablo et al., 2008). In addition to the direct association with GD, *GBA* has also been identified as a risk factor for PD (see section "Parkinson's disease and parkinsonism").

Drosophila has two orthologs of *GBA*, *Gba1a*, and *Gba1b*. *Gba1a* is expressed in the fly gut, while *Gba1b* is more broadly expressed (Davis et al., 2016). Several *Gba1b* mutant fly models have been established to study neuronopathic GD (Davis et al., 2016; Kinghorn et al., 2016; Thomas et al., 2018; Wang et al., 2022c). The *Gba1b* null mutants exhibit a 16-fold GlcCer accumulation in the head and have a short lifespan, progressive motor defects, and memory defects when compared to controls. In the fly CNS, synaptic loss and neurodegeneration is obvious. At the cellular level, the loss of *Gba1b* results in the accumulation of p62/Ref(2)P-containing protein aggregates, lysosomal expansion and disrupted ATP production in mitochondria (Davis et al., 2016; Kinghorn et al., 2016). Importantly, a recent study found that Gba1b is enriched in glial cells, but not in neurons. In flies increased neuronal activity induces GlcCer production in neurons. The GlcCer is secreted by neurons *via* exosomes, taken up by glia where it is degraded in lysosomes. This process is triggered by glial-derived TGF-β/BMP signal (Wang et al., 2022c). Intercellular GlcCer transport also occurs in mammalian cells and mammalian glial cells express a significantly higher level of *GBA* than do neurons, providing evidence for the conservation of the GlcCer transfer from neurons to glia in mammals (Wang et al., 2022c). The findings support an important role for glial cells in the progression of neuronopathic GD.

Saposin deficiencies

Saposins are a group of proteins that facilitate GSL hydrolysis in the lysosomes [for review, see (O'Brien and Kishimoto, 1991)]. A single gene, *PSAP*, encodes the precursor of saposins (prosaposin), which is further processed in endosomes into four proteins (Saposin A–D). All saposin proteins are non-enzymatic activators of lysosomal GSL hydrolases (O'Brien and Kishimoto, 1991; Azuma et al., 1994). Saposin-A and C activate glucosylceramidase and galactosylceramidase. Saposin-B activates arylsulfatase A, α-galactosidase and β-galactosidase. Last, Saposin-D activates acid sphingomyelinase and ceramidase. Variants in the *PSAP* gene cause four distinct disorders depending on the domain(s) of the prosaposin protein affected. Deficiency in one single saposin protein causes Krabbe- (Saposin-A), metachromatic leukodystrophy- (Saposin-B) or Gaucher-like phenotypes (Saposin-C). Variants affecting multiple saposins cause a combined saposin deficiency which causes a sphingolipidosis with very early onset and severe neurological deficits (Harzer et al., 1989; Hulkova et al., 2001; Kuchar et al., 2009). One study reported that variants affecting Saposin-D cause an AD form of parkinsonism (PARK24, OMIM# 619491) in three families and that *PSAP* intronic variants near the Saposin-D domain-coding exons are a risk factor for sporadic PD (Oji et al., 2020b). Unexpectedly, no significant loss of SL hydrolase activity was observed in the patient-derived cells (Oji et al., 2020b) and follow-up studies reported that *PSAP* variants are rarely identified in large PD cohorts

TABLE 3 Lysosomal storage disorders associated with lysosomal sphingolipid accumulation.

Disease genes	Protein	Substrate accumulated	Disease	OMIM#
ASAH1	Ceramidase	Cer	Farber disease	228000
GBA	Glucosylceramidase	GlcCer	Gaucher disease	230800, 230900, 231000, 231005, 608013
GALC	Galactosylceramidase	GalCer	Krabbe disease	245200
SMPD1	Sphingomyelinase	SM	Niemann-pick disease A/B	257200, 607616
ASRA	Arylsulfatase A	Sulfatide	Metachromatic leukodystrophy	250100
GLA	α-galactosidase	Gb3 globosides	Fabry disease	301500
GLB1	β-galactosidase	GM1 gangliosides	GM1-gangliosidosis	230500, 230600, 230650
HEXA	β-hexosaminidase	GM2 gangliosides	Tay-sachs disease	272800
HEXB	β-hexosaminidase	GM2 gangliosides	Sandhoff disease	268800
PSAP	Saposin A-D	Multiple GSLs	Combined SAP deficiency	611721
	Saposin A	GalCer	Krabbe disease, atypical	611722
	Saposin B	Sulfatide	Metachromatic leukodystrophy	249900
	Saposin C	GlcCer	Gaucher disease, atypical	610539
NPC1	NPC1	Cholesterol, Sph, SM, GSLs, etc.	Niemann-pick disease C	257220
NPC2	NPC2			607625

(Facchi et al., 2020; Oji et al., 2020a, 2021a,b,c; Sosero et al., 2020; Chao et al., 2021; Lin et al., 2021; Zhao et al., 2021).

The combined saposin deficiency has been modeled using fruit flies. The fruit fly has one ortholog of *PSAP*, *Sap-r*. *Sap-r* mutants have a short lifespan, a progressive decline in locomotor activity and a progressive decline in neuronal activity in photoreceptors. *Sap-r* loss causes an age-dependent vacuole formation and neurodegeneration in the adult brain. It also induces autophagy, lysosome expansion and mitochondrial dysfunction in multiple tissues based on transmission electron microscopy. Lipidomic profiling revealed a progressive sphingolipidosis including Cer, GlcCer, CPE and higher order GSLs (Hindle et al., 2017; Sellin et al., 2017). Similar complex sphingolipidosis are observed in combined saposin deficiency patients and *SAP* knockout mice models (Bradova et al., 1993; Fujita et al., 1996; Oya et al., 1998; Hulkova et al., 2001; Kuchar et al., 2009).

Friedreich's ataxia

Friedreich's ataxia (FRDA, OMIM# 229300) is an AR neurodegenerative disorder that affects 1 in every 40–50,000 children worldwide. Typically, the symptoms start to develop between the ages of 10–15, and patients present with gait issues and ataxia, dysarthria, muscle weakness, and cardiomyopathy (Cook and Giunti, 2017; Indelicato et al., 2020). Patients also suffer from diabetes mellitus, scoliosis, and late-onset optic neuropathy (Fortuna et al., 2009; Noval et al., 2012; Cook and Giunti, 2017). More than 95% of FRDA are caused by a homozygous triplet repeat expansion of GAA in the first intron of the *FXN* gene. *FXN* encodes a mitochondrial protein Frataxin. Less than 5% of the FRDA patients are compound heterozygous for *FXN*, where one allele contains a point mutation and the other the repeat expansion in the first intron. Notably, the age of onset is directly correlated with the GAA repeat numbers and protein levels (Indelicato et al., 2020). In healthy individuals, the first intron of *FXN* contains <40 GAA repeats whereas the repeat number increases up to >1000 in individuals with FRDA (Cook and Giunti, 2017; Kelekci et al., 2022). Increased GAA repeat numbers in *FXN* reduces the protein levels to 4–29% of normal levels, providing supporting evidence that loss of Frataxin is the cause for the disease (Campuzano et al., 1997). Frataxin is required for iron-sulfur (Fe-S) cluster assembly in mitochondria. Fe-S clusters act as cofactors for aconitase and functions together with the enzymes of the electron transport chain (ETC) in mitochondria as well as in regulation of iron metabolism (Maio and Rouault, 2020). Indeed, loss of *FXN* leads to iron deposition in the brain and cardiomyocytes in FRDA patients (Lamarche et al., 1980; Waldvogel et al., 1999; Michael et al., 2006; Ward et al., 2019).

Similar phenotypes including mitochondrial abnormalities, iron accumulation and progressive neurodegeneration have been noted in fruit fly FRDA models. Ubiquitous knockdown of fruit fly *frataxin* (*fh*) causes developmental lethality whereas neuronal or glial knockdown reduces lifespan and impairs motor activity (Anderson et al., 2005; Navarro et al., 2010; Monnier et al., 2018). In addition, loss of glial *fh* leads to glial lipid droplet accumulation and ubiquitous knockdown leads to elevated levels of fatty acids (Navarro et al., 2010). Chen et al. (2016a),b characterized the first fly *fh* mutant and discovered an increase in ceramide levels in flies as well as in FRDA patient-derived cardiac samples. Both in *fh* mutants as well as in patient heart samples, the levels of dhSph, Sph, dhCer, Cer were increased and a recent study also reported elevated ceramide levels in FRDA patient-derived fibroblasts (Wang et al., 2022b). These observations point to a connection between mitochondrial dysfunction, elevated iron levels and elevated Cer levels. However, how impaired mitochondrial function and/or iron metabolism elevates Cer levels in FRDA patients has not yet been explored. Interestingly, some of the SL metabolic enzymes, namely ceramide synthase, ceramidase, sphingomyelinase and SPTLC2, have been reported to be present in mitochondria (Roszczyc-Owsiejczuk and Zabielski, 2021; Aaltonen et al., 2022). Further studies are required to determine how mitochondrial iron dyshomeostasis increases the levels and/or activity of these enzymes and contribute to the elevated ceramide levels in FRDA.

S1P-associated disorders

Acyl-CoA oxidase deficiency

Peroxisomes are subcellular organelles that are involved in metabolic processes including the β-oxidation of very long chain fatty acids (VLCFA) (Singh et al., 1984). Acyl-CoA oxidase 1 (ACOX1) is the first and rate-limiting enzyme in the fatty acid β-oxidation of VLCFA (Fournier et al., 1994). The oxidase activity of ACOX1 produces hydrogen peroxide (H_2O_2) as a byproduct (Schrader and Fahimi, 2006). AR mutations in *ACOX1* are associated with acyl-CoA oxidase deficiency (OMIM# 264470). Patients with acyl-CoA oxidase deficiency are reported to develop a rapid and severe loss of function in the nervous system, characterized by hypotonia, seizures, visual system failure, white matter abnormalities, inflammatory responses and loss of motor achievements (Ferdinandusse et al., 2007).

Drosophila dACOX1, the ortholog of human *ACOX1*, is expressed mostly in glia and absent in neurons of the central nervous system (Chung et al., 2020). Loss of *dACOX1* leads to increased VLCFA levels, glial cell death, reduced neuronal survival, and shortened lifespan in flies (Chung et al., 2020). In addition, lack of *dACOX1* specifically in glia leads to elevated levels of Cers with VLCFA which are highly enriched in membranes of cells that wrap around axons, similar to Schwann cells in vertebrates and wrapping glia in flies (Chung et al., 2022). Interestingly, elevated levels of VLCFA-Cers in glia are not toxic in flies but they lead to the production of elevated levels of S1P in glia, which is toxic (Chung et al., 2022). S1P regulates diverse cellular processes and induces immune responses and inflammation (Spiegel and Milstien, 2011). Moreover, S1P is produced and released from glia and taken up by neurons in flies and elevated levels of S1P causes an activation of the immune deficiency pathway (IMD) and an invasion of immune cells in the brain. Neuronal expression of either the fly gene (*sply*) or the human gene (*SGPL1*) encoding S1P lyase significantly rescues the motor defects caused by glial overproduction of S1P, showing that S1P is toxic in neurons (Chung et al., 2022). Other studies have shown that S1P prevents ceramide-induced apoptosis in non-neuronal, non-glial cells (Cuvillier et al., 1996; Osawa et al., 2001; Castillo and Teegarden, 2003), indicating cell-type specificity of S1P activity. Interestingly, drugs that lower VLCFA synthesis or inhibit the action of S1P are highly beneficial in flies that lack *ACOX1* as well as in mouse model for Multiple Sclerosis (Chung et al., 2022). Hence, a specific population of ceramides with VLCFA may be at the root of elevated S1P synthesis when Schwann cells or oligodendrocytes are affected in some NDDs.

SGPL1-associated Charcot-Marie-Tooth disease

Sphingosine-1-phosphate lyase 1 (SGPL1), an ER-localized enzyme encoded by the *SGPL1* gene, catalyzes the final step of S1P breakdown (Prasad et al., 2017). SGPL1 deficiency causes an AR, axonal form of Charcot-Marie-Tooth disease (CMT). CMT represents a heterogeneous group of peripheral motor and sensory neuropathies. Only two probands with *SGPL1*-associated CMT have been identified thus far. Probands present with a juvenile-onset muscle weakness, muscle wasting, and a progressive decrease of motor neuron conduction velocity. A mild sensory deficit is observed in one of the two probands. Increased levels of S1P, as well as Sph/dhSph, are observed in the plasma of both probands (Atkinson et al., 2017). Although the toxicity of S1P accumulation is not directly demonstrated, the case report is suggestive of a role of S1P accumulation in neurodegeneration.

The *Drosophila* ortholog of *SGPL1* is *Sply*. Loss of *Sply* causes semi-lethality and increased apoptosis in developing embryos. The surviving flies display an abnormal flight muscle morphology (Herr et al., 2003). Blocking the S1P synthesis by a sphingosine kinase inhibitor, D,L-threo-DHS, partially rescues the phenotypes of *Sply* mutants, suggesting that the muscle phenotype of *Sply* mutants is caused by S1P accumulation (Herr et al., 2003). Specific knockdown of *Sply* in neurons caused impaired arborization and reduced synaptic bouton number at the neuromuscular junction (NMJ) as well as degeneration of the sensory neurons in the wing blades, further indicating the toxicity of S1P accumulation in neurons (Atkinson et al., 2017).

Parkinson's disease and parkinsonism

Parkinson's disease is a common neurodegenerative disease with an increasing prevalence in the aging population (de Lau and Breteler, 2006). The disease is primarily defined by core motor symptoms including bradykinesia, rest tremor and rigidity. It can also cause a wide spectrum of non-motor symptoms, such as cognitive impairment, autonomic dysfunction and sleep disorders (Schapira et al., 2017). The loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* is commonly observed in patients, leading to disruption of nigrostriatal pathway and progression of motor dysfunctions (Giguere et al., 2018). A pathological hallmark of PD is the accumulation of α-synuclein (α-Syn) that results in the formation of proteinaceous cytoplasmic inclusions known as Lewy bodies and Lewy neurites (Sulzer and Edwards, 2019). The existing treatments for PD motor symptoms are primarily dopamine based, which decelerate the disease progression but do not modify the pathogenesis (Armstrong and Okun, 2020). Disease-modifying α-Syn targeting therapies have also been proposed (Fields et al., 2019; Fleming et al., 2022), but none has been approved by the FDA in the US. So far, there is no cure for PD.

Historically, PD was considered a sporadic disease until the identification of *SNCA* variants that caused monogenic inheritance of PD (Polymeropoulos et al., 1997). In the past 2.5 decades, our understanding of the genetics of PD has vastly improved (Vazquez-Velez and Zoghbi, 2021; Ye et al., 2022). Numerous Mendelian inherited PD subtypes and the identification of ∼100 risk genes/loci *via* genome-wide association studies (GWAS) have provided potential clues as to what triggers PD in some individuals (Nalls et al., 2019; Blauwendraat et al., 2020a; Guadagnolo et al., 2021). Highly penetrant, rare variants of the known PD-causing genes account for 10–15% of all the PD cases (Verstraeten et al., 2015). In other cases, the disease is associated with disease-causing variants with incomplete penetrance, such as those in the *LRRK2* and *GBA* genes (Healy et al., 2008; Sidransky et al., 2009; Milenkovic et al., 2022; Rocha et al., 2022). New insights have also been driven by the discovery of genetic modifiers and oligogenic etiology of PD (Lubbe et al., 2016; Robak et al., 2017; Rousseaux et al., 2018; Bandres-Ciga et al., 2020; Blauwendraat et al., 2020b; Iwaki et al., 2020; Ren et al., 2022; Straniero et al., 2022).

The linkage between SL dysmetabolism and PD was first implicated by the increased incidence of PD in GD patients and individuals carrying a single *GBA* variant. Patients with type I GD have a 20-fold increased risk of developing PD when compared to the general population (Bultron et al., 2010). Further, the presence of a single variant of *GBA* increases the PD risk by a factor of 5 (Sidransky et al., 2009). In addition to *GBA*, at least three other SL-related LSD genes have been identified as PD risk factors, including *SMPD1*, *ASAH1*, and *PSAP* (Robak et al., 2017; Oji et al., 2020b). In non-*GBA* PD cohorts or cohorts without genotype information, lipidomics data have not revealed consistent SL level changes in PD patients vs. healthy controls in postmortem brain tissue, serum and CSF (Custodia et al., 2021; Esfandiary et al., 2022). However, in one *GBA*-PD cohort mild increases in levels of Cer, hexosylceramide (GlcCer and GalCer) and LacCer were observed in serum samples (Guedes et al., 2017). The lack of compelling evidence may be due to the nature of the samples that were studied or the dynamic flux in SLs (Lansbury, 2022). However, a role for SLs in the progression of PD is supported by studies in animal models, mostly in fruit flies. Here, we will mainly focus on the fruit fly orthologs of human genes that have been shown to cause PD or parkinsonism and affect SL metabolism. For other PD models in fruit flies, see the following reviews (Hewitt and Whitworth, 2017; Dung and Thao, 2018; Aryal and Lee, 2019).

SNCA and α-synuclein

Missense and copy number variants of the *SNCA* gene cause AD forms of PD, PARK1 (OMIM# 168601) and PARK4 (OMIM# 605543) respectively. Non-coding *SNCA* variants also increase the susceptibility of PD development (Pihlstrom and Toft, 2011; Deng and Yuan, 2014). The pathogenicity of the *SNCA* copy number variants and the presence of α-Syn containing Lewy Bodies in PD patients lead to multiple efforts to generate α-Syn overexpression transgenic animal models [for review, see (Deng and Yuan, 2014)]. *Drosophila* does not have a *SNCA* ortholog. However, expressing human α-Syn in neurons, either the wildtype protein or the p.A30P and p.A53T pathogenic variants, causes loss of DA neurons and the appearance of α-synuclein positive inclusion bodies in fly brain. This correlates with an age-dependent locomotor dysfunction, however, whether the locomotor defects are caused by DA neuron loss is not determined (Feany and Bender, 2000). Follow up studies further show that DA neuron loss can be induced by specific expression of α-Syn in DA neurons (Auluck et al., 2002; Trinh et al., 2008). Adult specific α-Syn overexpression in fly retina causes marked vacuolization, progressive photoreceptor cell death, and late-onset electroretinogram (ERG) defects (Chouhan et al., 2016). Interestingly, treatment with myriocin, a drug that suppresses SL *de novo* synthesis, suppresses neurodegeneration, indicating that the α-Syn toxicity is, at least partially, mediated by the accumulation of SLs in fly neurons. This is corroborated by a very significant increase in levels of SLs observed in cultured human neurons expressing α-Syn (Lin et al., 2018).

GBA

The association between *GBA* and PD has been intensely studied in *Drosophila* models. Flies double heterozygous for mutations in *Gba1a* and *Gba1b*, the two orthologs of *GBA*, cause loss of DA neurons, locomotor defects, and a shorter lifespan. These data suggest that GBA heterozygosity may promote PD development, although the direct association between DA neuron loss and other phenotypes is not addressed (Maor et al., 2016). Mutations in *Gba1b* alone does not cause loss of DA neurons in adult fly brains, but the GCase deficiency causes p62/Ref2P-containing protein aggregates that accumulate both in the head and the body of adult mutant flies. GCase deficiency also enhances α-Syn aggregation when α-Syn is expressed in mutant flies, but it does not seem to modify the toxicity of α-Syn (Davis et al., 2016). Yet, Lewy body pathology is commonly observed in GD patients, suggesting an important role of GCase and its substrate GlcCer in the progression of synucleinopathies (Furderer et al., 2022).

The toxicity of α-Syn is due to multiple factors including its misfolding, aggregation, and its propagation across the nervous system (Steiner et al., 2011). Exosome trafficking has been implicated in α-Syn propagation (Danzer et al., 2012; Han et al., 2019). Interestingly, exosomes also mediate the neuron-to-glia trafficking of GlcCer in both flies and human cells (Wang et al., 2022c) and GlcCer facilitates the aggregation of α-Syn (Mazzulli et al., 2011). Collectively, the evidence points to a mechanism by which GCase deficiency promotes PD progression. In this model, accumulated GlcCer promotes α-Syn aggregation which co-propagates with the aggregates *via* exosomes. *Gba1b* mutant flies exhibit a marked increase in exosomes (Thomas et al., 2018), which may exacerbate the pathology. This hypothesis is supported by the findings that pharmaceutical inhibition of GCase leads to an increased number of exosomes containing α-Syn oligomers in mice (Papadopoulos et al., 2018). However, further evidence is required to support this model.

VPS35

Variants in *VPS35* cause an AD form of PD (PARK17, OMIM# 614203). *VPS35* encodes a core component of the retromer, a complex of three proteins VPS26, VPS29, and VPS35 that mediate the recycling of cargoes from the endosome to the *trans*-Golgi network and the plasma membrane (Small and Petsko, 2015). One of the confirmed disease-causing PD variants, $VPS35^{D620N}$ (Vilarino-Guell et al., 2011), is a partial loss-of-function variant in various models, indicating that the disease is due to lack of retromer function (Follett et al., 2014; Malik et al., 2015; Ishizu et al., 2016). Single copy loss of *Vps35* in flies does not cause obvious phenotypes, but *Vps35* null mutants are lethal at late larval or prepupal stages (Korolchuk et al., 2007). The loss of *Vps35* in mutant larvae causes structural and functional defects at the NMJ including excessive formation of synaptic terminals, irregular number and size of synaptic vesicles, as well as disrupted neurotransmitter release (Korolchuk et al., 2007; Inoshita et al., 2017). In clones of fly photoreceptors, loss of either *Vps26* or *Vps35* affects the recycling of Rhodopsin upon light exposure, a dramatic increase in late endosomes and lysosomes, and ultimately the degeneration of photoreceptors (Wang et al., 2014). A significant increase in GlcCer in photoreceptors was also observed in mutant *Vps26* and *Vps35* photoreceptors (Lin et al., 2018), indicating a role of GlcCer in the neurodegeneration caused by retromer dysfunction. In contrast, loss of *Vps29* does not cause developmental lethality. However, *Vps29* mutants phenocopy the NMJ defects of *Vps35* mutant larvae and have an activity-dependent neurodegeneration in adult photoreceptors (Wang et al., 2014; Inoshita et al., 2017; Ye et al., 2020). In addition, *Vps29* mutant flies

exhibit shortened lifespan and age-dependent locomotor defects (Ye et al., 2020).

Loss of *Vps35* also impairs α-Syn degradation and exacerbates its neurotoxicity in flies (Miura et al., 2014). This increase in α-Syn toxicity was attributed to a disruption in trafficking of lysosomal proteases (Miura et al., 2014). However, as mentioned, an accumulation of Cer/GlcCer was observed in *Vps35* mutant clones, suggesting that SL dysmetabolism may also be involved (Lin et al., 2018). If GlcCer facilitates the aggregation of α-Syn, the *Vps35* deficiency may promote its toxicity. Note that *Vps35*- and *Gba1b*-associated neurodegeneration in photoreceptors are both activity dependent, supporting a role for GlcCer-mediated α-Syn neurotoxicity when *Vps35* is lost or reduced (Wang et al., 2014, 2022c).

PLA2G6

Biallelic variants of *PLA2G6* cause three neurological disorders: early adulthood-onset dystonia-parkinsonism (PARK14, OMIM# 612953), early childhood to juvenile-onset atypical neuroaxonal dystrophy (OMIM# 610217), and infantile neuroaxonal dystrophy (INAD, OMIM# 256600). *PLA2G6* encodes a phospholipase. Interestingly, a fly model of *PLA2G6* deficiency uncovered that neuronal accumulation of Cer/GlcCer is a major contributor to the development of the disease (Lin et al., 2018). The fly ortholog of *PLA2G6* is *iPLA2-VIA*. Loss of *iPLA2-VIA* in flies causes a short lifespan and neurodegeneration. Lipidomics studies revealed that the levels of many SL species, but not phospholipids, are elevated in the *iPLA2-VIA* mutant flies. Biochemical assays showed that iPLA2-VIA binds to Vps26 and Vps35, independent of its phospholipase activity and that an enzyme-dead protein is able to rescue the mutant phenotypes (Lin et al., 2018). iPLA2-VIA deficiency causes a significant reduction of both Vps26 and Vps35 and an impairment of retromer function, leading to an imbalance in the routing of endosomal components, including Cer/GlcCer, to lysosomes. Hence, lysosomal trafficking is disrupted and Cer/GlcCer accumulates in lysosomes, both of which lead to lysosome expansion and dysfunction, ultimately causing the demise of neurons. In attempt to rescue phenotypes, Cer synthesis was suppressed in three ways: knocking down fly SPT subunit *lace*, treatment with SPT inhibitor myriocin, and with the sphingomyelinase inhibitor, desipramine. In all three ways the cellular and the neurological defects of the mutant flies are rescued, showing that Cer accumulation accounts for many of the neurological defects caused by *iPLA2-VIA* deficiency (Lin et al., 2018).

In a follow up study, Cer accumulation and lysosomal expansion phenotypes were observed in INAD patient cells. Moreover, an accumulation of Cer in Purkinje cells and DA neurons was also observed in *PLA2G6* mutant mice, arguing that the neuropathological mechanisms are evolutionary conserved. Drugs targeting the endolysosomal pathway suppress some of the phenotypes and alleviate lysosomal stress in human cells and in flies (Lin et al., 2023).

PINK1

Variants in *PINK1* cause an AR, early onset form of parkinsonism (PARK6, OMIM# 605909). *PINK1* encodes a serine/threonine kinase PINK1, which localizes to mitochondria and performs important roles in mitochondrial homeostasis together with Parkin, another PD risk factor encoded by the *PRKN* gene (McWilliams and Muqit, 2017). In contrast to mice models for *Pink1*, which do not exhibit gross physiological, neurological or behavioral phenotype (Paul and Pickrell, 2021), fly *Pink1* mutants exhibit obvious phenotypes, including shortened lifespan and early onset locomotor defects. Mutant flies also exhibit mitochondrial dysfunction in muscles and DA neurons, which leads to the degeneration of myocytes and DA neurons in aged flies (Clark et al., 2006; Park et al., 2006). Similar phenotypic and pathological changes were observed in *Pink1* knockdown fly models (Wang et al., 2006; Yang et al., 2006). Loss of *Pink1* affects mitochondrial homeostasis by modulating the mitochondrial fission/fusion machinery and mitophagy, a selective autophagic process targeting damaged or dysfunctional mitochondria (Deng et al., 2008; Poole et al., 2008; Yang et al., 2008; Cornelissen et al., 2018; Kim et al., 2019). A recent study uncovered that the *Pink1*-associated mitochondrial defects are, at least partially, mediated by Cer accumulation (Vos et al., 2021). Increased Cer levels were also observed in *Pink1*$^{-/-}$ mouse embryonic fibroblasts, *Pink1* mutant fly muscles and *PINK1*-PD patient fibroblasts. The defects in ATP levels and mitochondrial morphology in *Pink1* mutant flies were effectively rescued by either knocking down fly ceramide synthase *schlank* or by treating flies with the SPT inhibitor myriocin, again providing evidence for a role for Cer accumulation in the pathogenesis of the disease. Further, Cer accumulation induces mitophagy, which the authors proposed facilitates the clearance of damaged mitochondria in *Pink1* mutant animals (Vos et al., 2021). How *Pink1* loss induces changes in Cer homeostasis remains to be determined.

Future directions

Which SL species are toxic?

Sphingolipids correspond to a large collection of lipids, and an obvious question is: which SL species accumulate and facilitate neurotoxicity? In the diseases discussed herein, an accumulation of Cer/GlcCer is most commonly observed. However, in no case was an accumulation of only one SL species observed. In the INAD fly model, loss of *iPLA2-VIA* causes elevated level of Sph, Cer and GlcCer. The mutant phenotypes are alleviated by suppression of either *de novo* synthesis or SM hydrolysis, suggesting that Cers may be the toxic SL species. However, inhibiting GlcCer synthesis with Miglustat does not improve the phenotypes, suggesting that GlcCer is not a major contributor to pathogenesis (Lin et al., 2023). However, in GD GlcCer is the major SL that accumulates in cells and in the *Gba1b* mutant fly model a 16-fold increase of GlcCer is observed in fly heads (Kinghorn et al., 2016; Wang et al., 2022c). This indicates that the GlcCer induced toxicity is due to its very elevated level. In the *SPTLC1*-ALS and *SPTSSA*-HSP cases, elevated *de novo* synthesis induces increased levels of dhSph, Cer as well as downstream products such as GlcCer and SM (Johnson et al., 2021; Mohassel et al., 2021; Srivastava et al., 2023). In summary, the complexity of SL metabolism makes it difficult to determine which SL species cause toxicity. However, simultaneously lowering many SLs by suppressing *de novo* synthesis suppress phenotypes in several fly models, including INAD, FRDA, and *PINK1*-PD, suggesting that suppressing *de novo* synthesis may be

a viable therapeutic strategy for many diseases (Chen et al., 2016b; Lin et al., 2018; Vos et al., 2021).

How to accurately detect SL accumulation?

Lipids are commonly detected using mass spectrometry-dependent approaches. However, the extreme structural diversity of SLs prevents the measurement of all SLs, or even all Cers, in a cost-effective manner (Pruett et al., 2008). Discriminating between GlcCer and GalCer by mass spectrometry requires additional efforts (Boutin et al., 2016). Hence, the two species are often labeled collectively as hexosylceramide (HexCer). Currently, the common choice for measuring SL is either a targeted approach for a specific SL subclass (Cer as the most common choice) or an unbiased large-scale lipidomic approach that encompasses both SLs and other lipids. An unbiased "sphingolipidomic" assay should be developed to facilitate a time- and cost-efficient way of measuring SLs.

The choice of sample types is also key to provide a precise landscape of SL changes in diseases. Limited by sample accessibility, blood cells and plasma/serum are the most commonly used samples. Most SLs including Cers are incorporated in membranes in cells and are not soluble in an aqueous environment such as cytosol and plasma. SLs are transported to the plasma membrane, extruded, and captured by lipoproteins in plasma (Iqbal et al., 2017). When cells become dysfunctional, SL species may not be efficiently transported to the plasma membrane, which may affect their distribution in lipoproteins (Wang et al., 2018). Hence, SL levels in plasma may not reflect what is happening in neurons and glia. In apolipoprotein bound SL in plasma, SM is the dominant species (~87%) while Cer and HexCer correspond to only ~6% (Hammad et al., 2010). Some metabolic disorders also affect plasma SL levels, which may mask the plasma SL profile changes caused by the SL dysmetabolism in the nervous system. For example, increased levels of multiple SLs including SM, Cer and GSLs are observed in individuals with diabetes (Russo et al., 2013). For some neurological diseases, blood cells or fibroblasts may reflect the status of SL metabolism in the nervous system better than what is observed in plasma. For example, elevated levels of Cers are observed in FRDA patient skin fibroblasts (Wang et al., 2022b), consistent with the observations in *fh* mutant flies and patient heart samples (Chen et al., 2016a,b). However, in patient plasma samples levels of Cers with C16-C18 acyl chain do not significantly change, while levels of VLCFA-Cers decrease (Wang et al., 2022a). Given that sampling neurons of patients is challenging, neuronal cells derived from induced pluripotent stem cells (iPSCs) or transdifferentiated from blood cells or fibroblasts can be used as surrogates and may provide the best source for SL profiling (Engle et al., 2018; Mollinari et al., 2018). In sum, a systematic lipidomics-based analysis of various cell types (blood cells, fibroblasts and induced neurons) and body fluids (serum and CSF) may provide a more precise read-out of SL changes in the nervous system.

How are SL levels altered?

The accumulation of SLs such as Cer could be a consequence of either increased synthesis or decreased degradation. The *SPTLC1*- and *SPTSSA*-associated disorders represent the former case, while Farber Disease (deficiency of ceramidase encoded by *ASAH1*) corresponds to an example of the latter case. Increased anabolism as well as reduced catabolism can also occur in the same disease. For example, in the case of fly INAD model, the disruption of retromer function causes an increase in endolysosomal trafficking and an accumulation of SLs. However, through an unknown process, the *de novo* synthesis pathway is also activated, given that the dhSph level is elevated. Moreover, inhibition of *de novo* synthesis by *lace* knockdown or by myriocin treatment, or knockdown of the salvage pathway using the SM inhibitor, desipramine, both effectively rescue the defects in the disease model, showing that both pathways contributed to disease progression (Lin et al., 2018).

The source of SL accumulation may not be obvious when the disease is not directly caused by defects in anabolism or catabolism of SLs, as for example in FRDA and *PINK1*-PD. In models of both of these diseases, iron accumulation and mitochondrial defects are observed (Esposito et al., 2013; Chen et al., 2016b). How these changes lead to Cer accumulation and whether the two diseases share a similar mechanism is not yet established.

Bridging the defects: Mitochondria and lysosomes

Mitochondria and lysosome are the two most important organelles that are often affected in PD (Haelterman et al., 2014; Vazquez-Velez and Zoghbi, 2021; Ye et al., 2022). The driver of the disease pathogenesis may be either one or both of these organelles. For example, variants in the *PINK1* and *PRKN* genes mainly cause mitochondrial dysfunction, while *VPS35* and *PLA2G6* deficiency mainly result in lysosomal deficits. However, both organelles seem to be affected as the diseases progress. Similar inter-organelle influences can also be observed in rare NDDs when the defects seem to originate from one organelle, such as in LSDs (Stepien et al., 2020).

In NDDs that primarily affect mitochondria or lysosomes, an accumulation of SLs (especially Cers) has been observed. Hence, Cer accumulation may bridge the defects in both organelles and underlie a synergy between the two sources to promote neurodegeneration. Increased levels of Cers in membranous structures stiffen membranes and impair vesicular trafficking (Huttner and Zimmerberg, 2001; Castro et al., 2014). Hence, an increase in Cer levels caused by mitochondrial dysfunction, such as that in *Pink1*/*PINK1* models (Vos et al., 2021), may disrupt endolysosomal trafficking and cause lysosomal defects. In contrast, an increased production of Cer in lysosomes may also disrupt mitochondrial homeostasis, as observed in the *PLA2G6* models (Lin et al., 2018, 2023).

Origin of the defects: Neurons versus glia

The cellular origin of the defects that cause NDDs is not always obvious. Studies in flies have revealed that defects in SL metabolism originate in glial cells when *dACOX1* or *Gba1b* is lost (Chung et al., 2022; Wang et al., 2022c). Expression of both *dACOX1* and *Gba1b* are highly enriched in glial cells. In the *dACOX1* model, loss of *dACOX1* causes increased levels of VLCFA-Cer and S1P in glial cells, while the glia-derived S1P is transferred to neurons where it induces neurotoxicity (Chung et al., 2022). In the *Gba1b* case, GlcCer is produced in neurons. However, it is transferred to glia where it

accumulates, causing glia to be affected before neurons (Wang et al., 2022c).

In contrast to *Gba1b*, *iPLA2-VIA* is mainly expressed in neurons. In both *Gba1b* and *iPLA2-VIA* mutants an elevation of Cer/GlcCer is observed. However, the accumulation of GlcCer occurs only in neurons when *iPLA2-VIA* is lost, whereas GlcCer accumulation occurs first in glia, then in neurons, when *Gba1b* is lost (Lin et al., 2018; Wang et al., 2022c). Interestingly, both *PLA2G6* and *GBA* genes are associated with PD/parkinsonism and the fly models share many similar phenotypes (Lin et al., 2018; Wang et al., 2022c) yet the data clearly show that the cellular origin of the phenotypes is quite different.

Sphingolipids as potential drug targets

The idea of modulating cellular SL levels is not novel. Miglustat, an inhibitor of ceramide glucosyltransferase, has been approved by the FDA to be used to treat type I GD. Treatment with Miglustat effectively alleviates the symptoms in type I GD patients such as hepatosplenomegaly and anemia (Ficicioglu, 2008). However, a clinical trial with Miglustat failed to show an improvement in neurological defects in type III GD patients (Schiffmann et al., 2008). In addition to GD, Miglustat has been shown to provide beneficial effects in NPC (Patterson et al., 2007) and Sandhoff Disease (OMIM# 268800) (Tallaksen and Berg, 2009; Masciullo et al., 2010). In both diseases, accumulation of GSLs derived from GlcCer is observed (Newton et al., 2018; Breiden and Sandhoff, 2019). However, due to its specificity to downregulate GlcCer synthesis and its derivatives, Miglustat was not tested in other types of sphingolipidosis, such as Krabbe Disease (GalCer) and Niemann-Pick A/B (SM).

The disease-causing variants in *SPTLC1* and *SPTSSA* lead to an elevation of the *de novo* synthesis (Johnson et al., 2021; Mohassel et al., 2021; Srivastava et al., 2023), suggesting that inhibiting the SPT complex should suppress the associated phenotypes. Suppression of the *de novo* synthesis should lower levels of many, if not most, of the downstream SL species. SPT inhibition, either genetically or pharmacologically, has been shown to alleviate the neurological phenotypes in disease models for FRDA, INAD, and *PINK1*-PD, suggesting a strategy for treatment (Chen et al., 2016b; Lin et al., 2018; Vos et al., 2021). In model organisms, myriocin is a commonly used SPT inhibitor. However, oral administration of myriocin has been shown to cause strong intestinal toxicity and the ability of myriocin to cross the BBB is not established (Osuchowski et al., 2004). Hence, novel SPT inhibitors, which have low toxicity and the ability to cross the BBB, are desirable.

Author contributions

HB and XP contributed to the conception of the review. XP wrote the first draft of the manuscript. XP, DD, SL, and HB wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This work was supported by grants from the Huffington Foundation, the INADcure Foundation, the NIH (R24OD022005 and R24OD031447), and the Chair in Neurogenetics of the Jan and Dan Duncan Neurological Research Institute at Texas Children's Hospital to HB, the Shan and Lee-Jun Wong fellowship from Baylor College of Medicine to DD. This work was also supported in part by the Baylor College of Medicine IDDRC (P50HD103555) from the Eunice Kennedy Shriver National Institute of Child Health and Human Development for use of the Microscopy Core Facilities.

Acknowledgments

We thank Drs. Guang Lin, Lindsey D. Goodman, Matt J. Moulton, and Oguz Kanca for their insight and feedback on this review.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 21 March 2023
DOI 10.3389/fnins.2023.1143793

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 13 January 2023
ACCEPTED 06 March 2023
PUBLISHED 21 March 2023

CITATION
Koza Z, Ayajuddin M, Das A, Chaurasia R, Phom L and Yenisetti SC (2023) Sexual dysfunction precedes motor defects, dopaminergic neuronal degeneration, and impaired dopamine metabolism: Insights from *Drosophila* model of Parkinson's disease.
Front. Neurosci. 17:1143793.
doi: 10.3389/fnins.2023.1143793

Sexual dysfunction precedes motor defects, dopaminergic neuronal degeneration, and impaired dopamine metabolism: Insights from *Drosophila* model of Parkinson's disease

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Sexual dysfunction (SD) is one of the most common non-motor symptoms of Parkinson's disease (PD) and remains the most neglected, under-reported, and under-recognized aspect of PD. Studies have shown that Dopamine (DA) in the hypothalamus plays a role in regulating sexual behavior. But the detailed mechanism of SD in PD is not known. *Drosophila melanogaster* shares several genes and signaling pathways with humans which makes it an ideal model for the study of a neurodegenerative disorder such as PD. Courtship behavior of *Drosophila* is one such behavior that is closely related to human sexual behavior and so plays an important role in understanding sexual behavior in diseased conditions as well. In the present study, a sporadic SD model of PD using *Drosophila* was developed and SD phenotype was observed based on abnormalities in courtship behavior markers. The *Drosophila* SD model was developed in such a way that at the window of neurotoxin paraquat (PQ) treatment [PQ is considered a crucial risk factor for PD due to its structural similarity with 1-methyl-4-phenyl pyridinium (MPP+), the active form of PD-inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)], it does not exhibit mobility defects but shows SD. The whole brain tyrosine hydroxylase immunostaining showed no observable dopaminergic (DAergic) degeneration (number of DA neurons and fluorescence intensity of fluorescently labeled secondary antibodies that target anti-TH primary antibody) of the SD model. Similarly, there was no significant depletion of brain DA and its metabolite levels (HVA and DOPAC) as determined using HPLC-ECD (High-Performance Liquid Chromatography using Electrochemical Detector). The present study illustrates that the traits associated with courtship and sexual activity provide sensitive markers at the earlier stage of PD onset. This PQ-induced SD fly model throws an opportunity

to decipher the molecular basis of SD under PD conditions and to screen nutraceuticals/potential therapeutic molecules to rescue SD phenotype and further to DAergic neuroprotection.

KEYWORDS

Parkinson's disease, *Drosophila*, courtship behavior, dopamine, sexual dysfunction

Introduction

Parkinson's disease (PD) is the second most dominant neurodegenerative disorder that affects about 1% of the population over age 50 (Modi et al., 2016). Sexual dysfunction (SD) is one of the most common non-motor disorders affecting Parkinsonian patients and remains the most neglected, underreported, and underrecognized aspect of PD (Jitkritsadakul et al., 2015; Bhattacharyya and Rosa-Grilo, 2017; Vela-Desojo et al., 2020; Benigno et al., 2022; Bronner et al., 2022; Elshamy et al., 2022). The most frequently reported sexual malfunctions in PD men are a decline in libido/loss of sexual interest, decline in sexual intercourse, diminution in orgasm/inability to experience orgasm/orgasmic dissatisfaction, decline in erection, erectile dysfunction, diminution in ejaculation, and premature ejaculation (Koller et al., 1990; Wermuth and Stenager, 1995; Lucon et al., 2001; Sakakibara et al., 2001; Hobson et al., 2003; Jitkritsadakul et al., 2015; Bhattacharyya and Rosa-Grilo, 2017).

Neurotransmitter Dopamine (DA) which is highly conserved throughout evolution has been suggested to play an important role in normal sexual function (Hull et al., 2004; Andersson, 2011) and any disruption in the levels of DA leads to abnormal sexual behavior (Zahran et al., 2001; Shaltiel-Karyo et al., 2012). Studies have shown that DA in the hypothalamus plays a role in regulating sexual behaviors, however, the detailed mechanism of SD in PD is not known (Sakakibara et al., 2011). A study reported the relationship between the severity of SD and specific patterns of nigrostriatal dopaminergic (DAergic) denervation (especially involving both putamina) in newly diagnosed drug-naïve PD patients (Contaldi et al., 2022). The understanding of the function and regulation of the behavioral circuits involved in mating will have potential implications for the medical treatment of SD in humans (Emmons and Lipton, 2003).

Drosophila melanogaster has been used widely as a model organism to understand the pathophysiology of human disease (s) specifically neurodegenerative disorders (NDDs) like PD as several disease-causing genes and signaling pathways are conserved between fly and humans (Reiter et al., 2001; Muñoz-Soriano and Paricio, 2011; Varga et al., 2014; Rahul and Siddique, 2022; Suzuki et al., 2022). The shorter life cycle (10–12 days at 25°C), a large number of progenies, established genetic methods, and molecular biology tools to manipulate genome and generate mutants and further perform loss and gain of function analysis make *Drosophila* an effective model system in biomedical research (Ayajuddin et al., 2018). *Drosophila* models a network of behaviors that are closely connected to humans, such as courtship (Dickson, 2008) which is believed to be one of the finest behaviors displayed by the fruit fly, and grooming (Tauber et al., 2011). The courtship ritual of a normal fly consists of fixed action patterns which are followed by several discrete steps such as the orientation of the male toward the female, tracking or following of the female, approaching and tapping the female, enthralling the female with a species-specific love song, licking the female's genitalia, attempt to copulate, rejection or acceptance of the male by the female, mounting and copulation by the male upon female's acceptance, and disconnection of the genitalia followed *via* dismounting by the male (Spieth, 1974; Koza et al., 2021). Therefore, courtship behavior is very important in determining the sexual status of male and female flies.

The purpose of the present study is to characterize SD phenotype in the *Drosophila* model of PD, by taking advantage of fly courtship behavior markers and to understand if SD sets in before the onset of motor defects. As DA plays an important role in sexual function, levels of DAergic neurodegeneration in the fly brain were checked using whole-brain immunostaining through fluorescence microscopy with anti-tyrosine hydroxylase (TH) (a rate-limiting enzyme in the synthesis of DA) antibody labeling and quantification of brain-specific DA and its metabolites DOPAC (3,4-Dihydroxyphenyl acetic acid) and HVA (Homovanillic acid) levels were checked using High-Performance Liquid Chromatography with an Electrochemical Detector (HPLC-ECD). Here we characterized the courtship dysfunctions in a *Drosophila* model and showed that the male fly showed courtship abnormality although there were no visible motor defects, no observable DAergic neurodegeneration; no variation in the level of synthesis of DA, HVA, DOPAC, and their turnover rate. This model illustrates that SD precedes motor defects in PD, hence it is of great value in understanding the progression of PD before the DAergic degeneration sets in. Therefore, this model will further throw an opportunity to screen nutraceuticals/potential therapeutic molecules to assess their efficacy to rescue SD and for possible DAerigc neuroprotective efficiency.

Materials and methods

Drosophila stock and husbandry: A collection of virgin and bachelor flies

Oregon K (OK) flies of *Drosophila melanogaster* used in this study were obtained from the National Drosophila Stock Centre (Department of Biotechnology, India supported) of the University of Mysore, Mysuru, Karnataka, India. Flies were maintained under standard laboratory conditions of 22 ± 2°C with a 12:12 h light and dark cycle (*Drosophila* environmental chambers from Percival, USA). The adult flies were propagated in media containing sucrose,

yeast, agar-agar, and propionic acid in a definite standardized proportion (Phom et al., 2014).

For collecting unmated male and female flies, the adult flies were cleaned off from the culture vials and the newly emerged flies were collected within 2 h of emergence. For collecting the flies, they were mildly anesthetized with a few drops of diethyl ether. Male and female flies were separated and aged in same-sex groups of 25 in each vial. The collected flies were transferred to a fresh media vial every other day till they reached the age of 5 days old.

Chemicals

The required chemicals viz., Methyl viologen dichloride hydrate/Paraquat (PQ) (Sigma-Aldrich; St. Louis, MO, USA, Cat. No. 856177), Sucrose (SRL, Maharashtra, India, Cat. No. 1947139), DMSO (Sigma-Aldrich; St. Louis, MO, USA, Cat. No. D8418). Phosphate buffered Saline (PBS; HiMedia, Maharashtra, India, Cat. No. ML023), Trichloro Acetic Acid (TCA; SRL, Maharashtra, India, Cat. No. 204842), Dopamine (DA; Sigma-Aldrich, St. Louis, MO, USA, Cat. No. H8502); 3,4-Dihydroxyphenylacetic acid (DOPAC; Sigma-Aldrich, St. Louis, MO, USA, Cat. No. 11569); Homovanillic acid (HVA; Sigma-Aldrich, St. Louis, MO, USA, Cat. No. 69673) were used for quantifying DA and metabolites. Paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. I58127), Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. T8787), Triton X-100 (Sigma, St. Louis, MO, USA), Normal goat serum (NGS, Vector labs, CA, USA), VECTASHIELD mounting medium (Vector Labs, CA, USA, Cat: H1000), Rabbit anti-Tyrosine hydroxylase (anti-TH) polyclonal primary antibody (Millipore, MA, USA, Cat: Ab152) and Goat anti-rabbit IgG H&L (TRITC labeled) polyclonal secondary antibody (Abcam, MA, USA, Cat: Ab6718) were used for immunostaining. Whatman filter paper no. 1 disk was used as a feeding medium in the experiment in a 30 × 100 mm glass vial.

Fly treatment

Ten mM PQ solution (treated) was prepared in 5% sucrose and a volume of 275 μL of treated and 5% sucrose (control) was pipetted on a filter disk placed on a 30 × 100 mm glass vial. Unmated male flies (5–6 days old) were treated with freshly prepared 5% sucrose (control) and 10 mM PQ solution (treated) for 2/3/4/5/6 h. Unmated female flies (5–6 days old) were used to study the PQ-induced male SD.

Negative geotaxis assay

The motor ability of the flies was assessed through a negative geotaxis assay as described by Botella et al. (2004) and Phom et al. (2021). In brief, a single male was aspirated out from the vial using an aspirator and put in a plastic tube 26 cm long and 1 cm in diameter. The fly was then gently tapped to the bottom of the tube to acclimatize for 2 min. After 2 min, the fly was tapped gently to the bottom, and the height it could climb in 12 s was noted. This was repeated thrice for each fly and a minimum of 15 flies were observed for each group.

Immunostaining to visualize DAergic neurons in the whole fly brain

Immunostaining assay was performed following the protocol of Ayajuddin et al. (2022). In brief, 5 days old male OK flies were fixed in 4% paraformaldehyde (PFA) containing 0.5% TritonX-100, at room temperature for 2 h. The brains were then washed 5 times for 15 min duration each (75 min) at room temperature with phosphate-buffered saline (PBS) containing 0.1% Triton-X 100 (PBST). Blocking was done using 5% Normal Goat Serum (NGS) in 0.5% PBST for 2 h at room temperature. Then, the brains were incubated for 72 h at 4°C with primary antibody (anti-tyrosine hydroxylase anti-TH, 1:250 dilution). The excess primary antibody was removed by washing brains in PBST [5 times with 15 min duration each (75 min)]. Brains were then incubated with secondary antibody (TRITC labeled, 1:250 dilution) for 24 h under the dark condition at room temperature, followed by a thorough wash in PBST [5 times with 15 min duration each (75 min)]. Then the brains were embedded in VECTASHIELD® mounting medium and proceeded for image acquisition on the same day. Mounting of the whole fly brain for fluorescence microscopy (Carl Zeiss, Axio Imager M2 with ZEN 2012 SP2 software, Germany) was done as described in Ayajuddin et al. (2022).

The quantification of anti-TH signals was also performed following the protocol described in Ayajuddin et al. (2022). In brief, prepared/stained brains (3–5 brains for each group) were viewed under a fluorescence microscope at a 40 × magnification. A Rhodamine filter was used for image scanning. For image acquisition at 40 ×, a red dot test [for visibility of neuron (s) and assessing the signal saturation] was done for all the brains. Z-stack programming with constant intervals was performed. For image processing, on the method column, image subset and maximum intensity projection (MIP) with X-Y Plane were created. From 3D images of Z- stack, PAL, PPL1, PPL2, PPM1/2, and PPM3 (PAL, protocerebral anterior lateral; PPL, protocerebral posterior lateral; PPM, protocerebral posterior medial) brain regions were selected. The images were enlarged to see clear neuritis and a line was drawn around the neuron using draw spline contour from graphics tools and the intensity sum was created in .xml format. The procedure was repeated for all the neurons in different clusters. Care was taken to select the fly brains with the same orientation.

Quantification of DA and its metabolites (DOPAC and HVA) using HPLC-ECD

Brain-specific DA and its metabolites were quantified using HPLC-ECD (HPLC-Thermo Scientific, Dionex Ultimate 3000) following the protocol described by Ayajuddin et al. (2021). Briefly, post-exposure control and PQ-fed groups of flies were quickly frozen. Followed by the procedure with a sharp scalpel 15 fly heads were decapitated on ice to prevent the thawing of tissue and degradation of biomolecules. Head tissues were homogenized in chilled 300 μL of PBS. The homogenate is subjected to sonication (for 20 s with 5 s interval at 30 percent amplitude) followed by centrifugation at 4°C for 10 min at 6,000 rpm. The supernatant collected after centrifugation was mixed with 5% TCA in a ratio of 1:1. 50 μL of the supernatant was kept aside for protein

quantification before mixing with TCA. A composite standard mix comprising standard DA, DOPAC, HIAA, 5-HT, and HVA was prepared in PBS, each having a final concentration of 300 ng/mL. The solvent was mixed with 5% TCA in a ratio of 1:1 and kept chilled to avoid catecholamine degradation. A total of 20 μL of composite standard and 50 μL of the sample were loaded onto HPLC for quantification. MCM 15 cm 4.6 mm, 5 m C–18 packed column (Thermo Scientific, Cat: 70–0340) was used as the stationary phase for the elution of the catecholamines, while MD-TM from Thermo Scientific (Cat: 701332) was utilized as the mobile phase. A range of -175 to +225 mV was used for the reduction and oxidation potentials inside the primary ECD, which contains two cells, to detect the catecholamines. To cut down on background noise, Omnicell's third cell, which serves as the secondary ECD module, was tuned to +500 mV. It was decided to use a 5 Hz data collection rate. Chromeleon®7 from Thermo Scientific, USA, was used to analyze chromatograms. The retention time of a catecholamine was compared between standard and sample chromatograms. To accurately pinpoint the DA, DOPAC, and HVA peak in the sample, 10 μL of the composite standard was mixed and the sample was run again in HPLC. The peaks that spiked according to the detection sequence were identified as the monoamines of interest.

Quantification and normalization of peaks were done as described in Ayajuddin et al. (2021).

In brief, (1). The concentration of a catecholamine of interest is: C_{Std} (ng/mL), (2). The area of the catecholamine in the standard chromatogram is: A_{Std} and the injection volume of the standard solution is: I_{Std} (μL), (3). Similarly, the area of the catecholamine in the sample chromatogram is: A_{Samp} and the sample injection volume is I_{Samp} (μL), (4). The total protein concentration of the brain extract is: P_{Samp} (μg/μL).

Calculation:

1. Concentration of the standard catecholamine in I_{Std} (μl) injection volume: (C_{Std} X I_{Std})/1,000 = V1 (ng).
2. Concentration of catecholamines in brain tissue extract sample: (A_{Samp} X V1)/A_{Std} = V2 (ng).
3. Total protein in I_{Samp} (μL) injection volume: (P_{Samp} X I_{Samp}) = V3 (μg).
4. Determining the catecholamine concentration in total injected protein and normalizing for 1 mg (1,000 μg) of protein = (V2 × 1,000)/V3 = V4 (ng).
5. Since injected brain tissue extract and standard solution contain 5% TCA in a 1:1 ratio, the actual catecholamine concentration in brain tissue extract is (V4/2) = V5 (ng) in 1 mg of total protein.

The actual concentration values of each catecholamine of each experimental group were normalized to their respective control. The relative values of all runs were used to present the data and analyze the significance of the trend.

Courtship assay

For each observation of courtship behavior, a single unmated and un-etherized male and female was transferred into a mating chamber (4 × 4 cm glass cavity block with an inner circular diameter of 3.3 cm) with the help of a mouth aspirator and allowed to acclimatize for 1 min and if the pair did not mate within 15 min, they were recorded as not mated and replaced by a fresh pair. After each observation, the mating chamber was cleaned thoroughly with 70% alcohol and air-dried. Courtship assay was conducted in 25 successful matings. All the observations were made from 8:00 to 15:00 h.

Below are male courtship activities that were observed (Spieth, 1974; Hegde and Krishna, 1997; Shaltiel-Karyo et al., 2012; Koza et al., 2021).

- NON-SEXUAL ENCOUNTER (NSE): Male and female come to cross each other but there is no sexual encounter.
- COURTSHIP LATENCY (CL): Time between the introduction of male and female together into the mating chamber until the orientation of the male toward the female.
- MATING LATENCY (ML): Time between the introduction of male and female together into the mating chamber until initiation of copulation of each pair.
- ATTEMPTED COPULATION (AC): The number of times the male attempt to copulate or mount the female.
- VIBRATION: Movement of the wings involves expanding them laterally from the resting position and then rapidly moving up and down.
- SCISSORING: The male repeatedly and rapidly extends both wings horizontally outward and back to the resting position.
- LICKING: Male opens labellar lobes, extends proboscis, and licks female genitalia.
- CHASING: The male fly chases the female for a few seconds or minutes until he is able to mount on the female.
- TAPPING: Male lifts, straightens foreleg, and strikes downward against the female; almost invariably occurs at the start of courtship.
- CIRCLING: Male periodically circles around the female, facing her as he moves, often from rear to front and back, sometimes completely about the female.

Videography.

All courtship assays were performed in the courtship chamber. The courtship behavior was recorded using a Microsoft 1080 HD sensor and video editing was done with WondershareFilmora v8.3 software.

Link to video on courtship behavior:
drive.google.com/file/d/1EapWLpCdxeqtcTK3WMX9Iyw79mtoZt-p/view?usp=sharing.

Statistical analysis

Graph Pad Prism 5 software was used for statistical analysis. Significant differences between the two groups were analyzed using a two-tailed Student's *t*-test. For more than two groups, one-way ANOVA (with Tukey's post-hoc correction for multiple comparisons) was used. The error bar represents the standard error of the mean (SEM). Sexual activity of PQ-treated flies was represented in percentage (%) with reference to control fly performance (PQ-treated/control × 100).

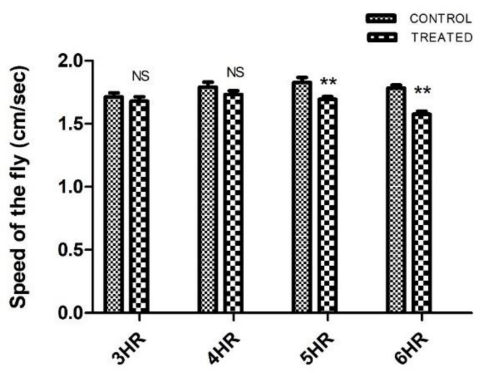

FIGURE 1
Characterization of paraquat (PQ) exposure window at which fly does not exhibit motor dysfunctions as determined by negative geotaxis assay: *Drosophila* male was exposed to 10 mM PQ and 5% sucrose (control) at different time points. A climbing assay was performed to check the motor ability of the fly. Fly started showing motor defects from 5 h of treatment onward. However, there was no significant difference in the motor ability between control and treated flies up to 4 h of treatment, providing an opportunity to understand SD well before the onset of mobility defects. (** signifies $P < 0.01$; NS: not significant).

Results

Characterization of PQ treatment window at which fly does not exhibit motor dysfunctions

Paraquat-induced motor dysfunction was assessed by exposing the male flies to PQ and subjecting them to a negative geotaxis assay. Male flies of age 5–6 days (the age of flies tested in the courtship assay) were exposed to PQ at different time points and were assessed to check their mobility efficiency. Idea is to select a time point of PQ exposure where the fly does not exhibit any mobility defects. Flies showed no difference in climbing speed at 4 h of PQ exposure as compared to the control flies which were on 5% sucrose for 4 h. Further exposure i.e., 5 h onward, the fly shows climbing defects as compared to the control (Figure 1). Thus, our observation at 4 h of PQ exposure where there were no mobility defects (compared to the control fly) provides an opportunity to screen for SD, if any, and will help further to characterize abnormalities in courtship behavior before the onset of mobility defects.

Whole brain immunostaining illustrates no difference in DA neuronal number and TH synthesis levels in PQ-induced SD fly

The adult *Drosophila* brain comprises six quantifiable DA neuronal clusters in each brain hemisphere (Figures 2A, B). To understand the DA neuronal dysfunction in the whole brain, 5 days old male fly model was immuno-stained for TH (the rate-limiting enzyme in the synthesis of dopamine) (Figure 3A). Upon visualizing the number of DA neurons, no significant difference was observed in all the clusters analyzed between the control and PQ-induced SD fly group (Figure 3B). Subsequently, to decipher if there is any change in the level of synthesis of TH, we quantified the fluorescence intensity of the DA neurons (fluorescently labeled secondary antibody targets the primary anti-TH antibody, and hence fluorescence intensity is correlated to the level of TH protein synthesis). Upon quantifying the fluorescence intensity of DA neurons, results illustrate that there is no difference in the fluorescence intensity in all the neuronal clusters (Figure 3C) and *in toto* (Figure 3D) between the control and the PQ treatment group. This confirms that there is no change in the levels of the rate-limiting enzyme of DA synthesis.

HPLC-ECD data revealed that there is no difference either in DA or in its metabolites in the PQ-induced SD fly model

Further to confirm whether there is DA synthesis depletion in PQ-induced SD fly, quantification of brain-specific DA and its metabolites was performed in tissue extract from the fly heads using the HPLC-ECD method (Figures 4A, B). The result shows that the SD model does not lead to any significant depletion of the DA level (Figure 4C). Further analysis revealed that there is no difference in the levels of DA metabolites i.e., DOPAC and HVA (Figure 4C). Further, there is no difference in the DA turnover which signifies no alteration in the DA catabolism (Figure 4D).

Characterization of sexual behavior illustrates courtship behavior anomalies before the onset of motor defects

The courtship activity was normalized to consider control male flies as ideal (100% sexual activity). Males with PQ treatment show significant courtship disparity when compared to control males (Figure 5). Courtship markers like attempted copulation, NSEs, and circling were increased by 200, 140, and 200%, respectively, in PQ treatment males as compared to the control males. Courtship latency and mating latency were increased by 70 and 60%,respectively, as compared to the control group; however, courtship behaviors like scissoring and licking were decreased by 60% in PQ-treatment males as compared to control. There was no difference between control and treated males in courtship activities like tapping and vibration (Figure 5). The duration of copulation between the males and the females showed no difference in control and treated males, but there was a significant difference in the percentage of successful copulation as treated males showed 60% less in successful copulation compared to their control counterparts (Figure 6).

Discussion

Drosophila has been widely used as a model animal to study NDDs like PD as they share the same types of

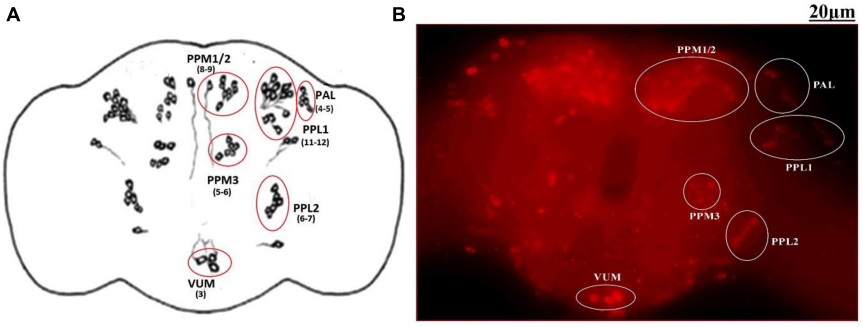

FIGURE 2
Quantifiable dopaminergic neuronal clusters in whole brain of *Drosophila*: cartoon of *Drosophila melanogaster* brain illustrating the position of quantifiable DAergic neurons **(A)** and image of the whole-brain mount of 5 days old male *Drosophila* captured by *ZEN software* of Carl Zeiss Fluorescence Microscope using fluorescently labeled secondary antibody targeted against the primary anti-TH antibody **(B)**. There are around 141 dopaminergic neurons (including ~100 neurons of the PAM cluster which cannot be quantified) arranged in different clusters in each hemisphere. (PAL, proto-cerebral anterior lateral; PAM, proto-cerebral anterior medial; PPL, proto-cerebral posterior lateral; PPM, proto-cerebral posterior medial; VUM, ventral unpaired medial).

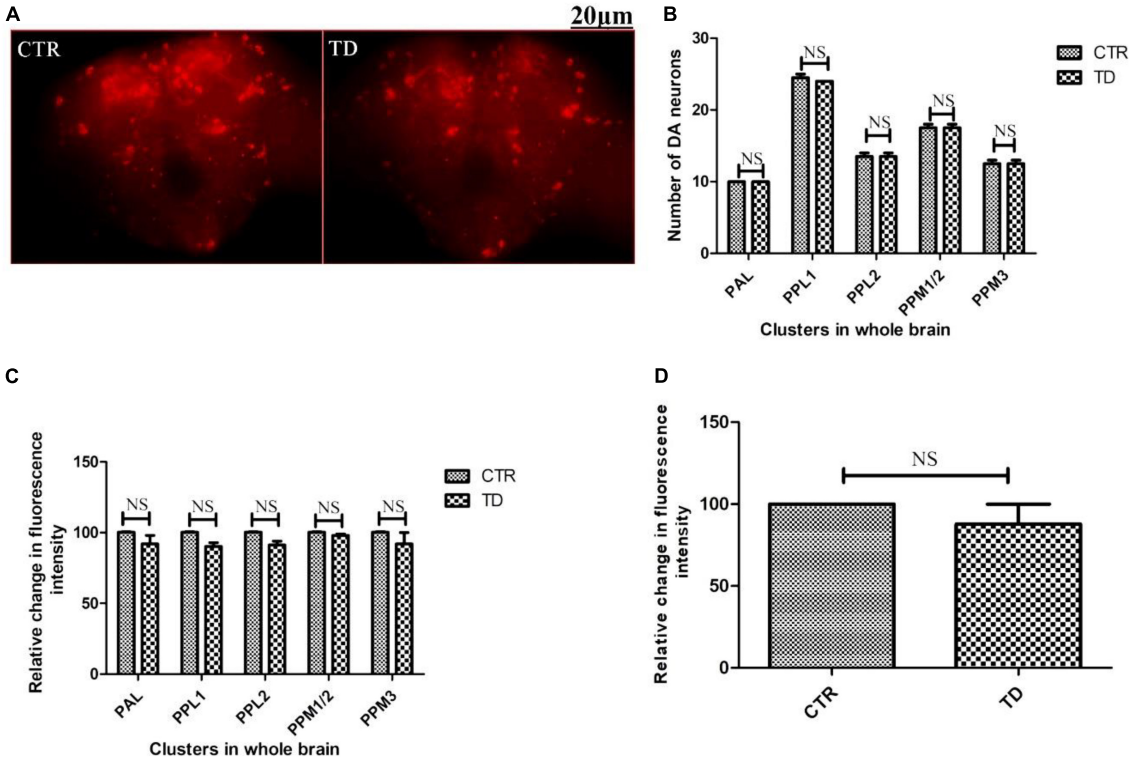

FIGURE 3
Characterization of DAergic neurodegeneration in the whole fly brain of control and paraquat (PQ)-induced sexual dysfunction (SD) fly **(A)** through anti-TH antibody immunostaining reveals that there is no loss in the number of DA neurons *per se* **(B)** and no change in the level of TH synthesis in all the clusters **(C)**, and *in toto* **(D)** between the control and treated group as analyzed by quantification of fluorescence intensity of fluorescently labeled secondary antibody that targets the anti-tyrosine hydroxylase (TH) primary antibody. (CTR, control; TD, treated with 10 mM PQ; Represented images are "merged" Z-stacking images; however, the quantification of DA neuronal number and fluorescence intensity is performed in 3D Z-stack images; PAL, protocerebral anterior lateral; PPL, protocerebral posterior lateral; PPM, protocerebral posterior medial). The scale bar of the brain images in the panel is 20 µm. Statistical analysis was performed using a *t*-test (compared to the control, NS, not-significant).

neurotransmitter systems like gamma-aminobutyric acid (GABA), glutamate, DA, serotonin, and acetylcholine, and can perform complex behaviors viz., sexual display, social behavior, and learning (Botella et al., 2009). SD in PD seemed to be multifactorial with no single cause identified (Brown et al., 1990) but the involvement of DA remains one of the most important factors (Heaton, 2000; Contaldi et al., 2022). However, very little is known about the SD mechanisms involved in sporadic PD. And very few studies have been done to understand the effects of PQ that led to neurodegenerative disorders such as PD and their effects on sexual functions.

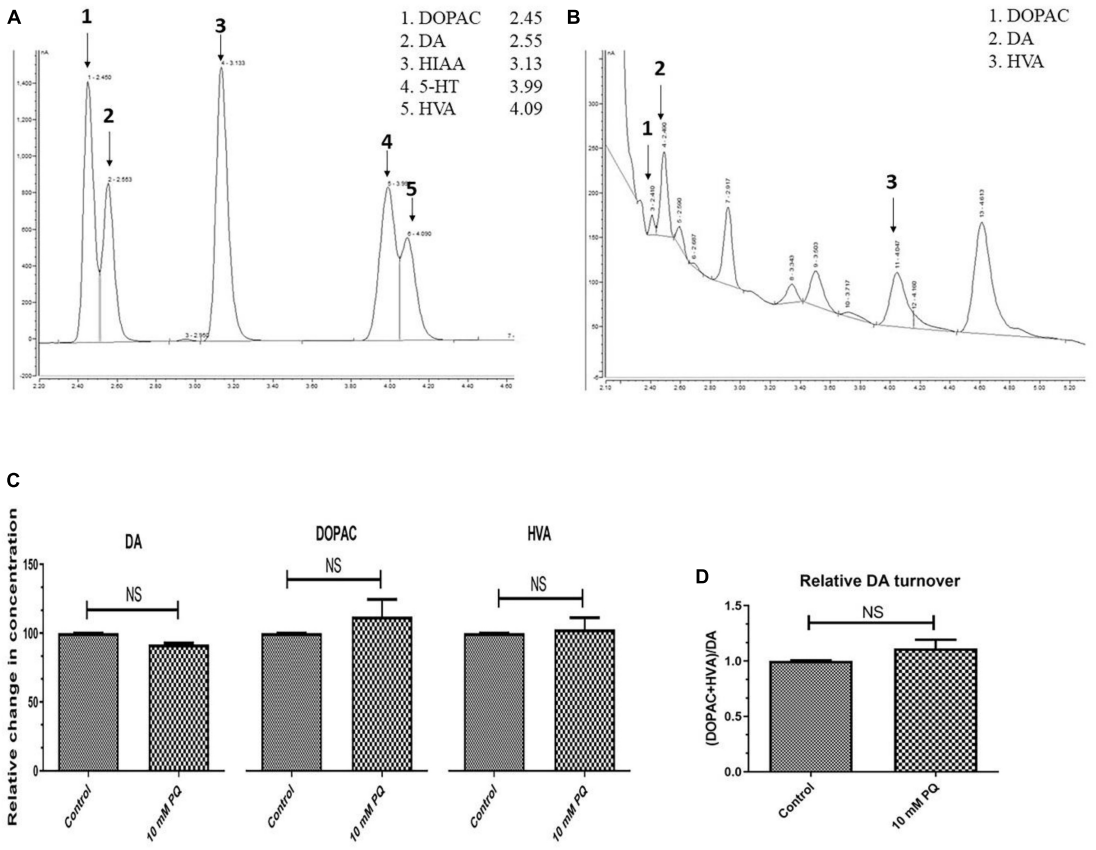

FIGURE 4
Quantification Dopamine (DA) and its metabolites- 3,4-Dihydroxyphenyl acetic acid (DOPAC) and Homovanillic acid (HVA) using High-Performance Liquid Chromatography (HPLC) in fly brain homogenate: The retention time of standard DA, DOPAC, and HVA is shown in the chromatogram **(A)** and chromatogram for the fly brain homogenate shows the detected monoamines **(B)**. The relative level of DA and its metabolites (DOPAC and HVA) shows that there is no significant difference between the control and PQ-treated groups **(C)**. Results also revealed that in PQ treated group compared to the control, there is no alteration of DA catabolism to DOPAC and HVA, as represented by unaltered relative DA turnover **(D)**. Statistical analysis was performed using a *t*-test (compared to the control), NS, not-significant.

In the present study, we report courtship/sexual behavioral deficits in the male fly even before there were any mobility defects. Epidemiological studies have also shown that most PD patients had experienced SD before being diagnosed with PD (Bronner et al., 2022), A decline in climbing ability is a convenient behavioral measure of degeneration of DAergic neurons/neurological damage in *Drosophila* (Feany and Bender, 2000). Previously our laboratory demonstrated that exposure to young flies (5–6 days old) with 10 mM PQ for 24 h shows a 30% decline in climbing speed, imitating PD-like symptoms of resting tremor, bradykinesia, and depleted brain DA levels (Phom et al., 2014). In human PD patients, tremors and other motor signs are diagnosed only when 50–60% of DAergic neurons degenerate leading to a 70–80% depletion of DA levels in the dorsal striatum where these neurons project (Fearnley and Lees, 1991). This is the prime reason to study courtship dysfunction (s) before the onset of motor dysfunction. We examined eleven components of male courtship behavior and in eight traits the PQ-treated flies exhibit defective behavior, suggesting PQ treatment though did not cause mobility defects in male flies, it induced courtship dysfunctions.

In the present study of the sporadic PD model, male exhibits the following courtship disparity when compared to control males, (1) decreased scissoring behavior (60%), (2) decreased licking behavior (60%) besides a significant decrease in the percentage of successful copulation (60%). But an increase/enhancement in the following behavior was observed: (1) CL (70%), (2) ML (60%), (3) AC (150%), (4) NSEs (140%), and (5) circling behavior (200%). However, there was no difference in behaviors such as tapping, vibration, and copulation duration between the control and treated group, which can be attributed to the differential genetic/molecular basis of regulation of different courtship markers. Chauhan et al. (2017) in their study using *D. melanogaster* as a model animal showed that after exposure to methylmercury (MeHg, a neurotoxic heavy metal), the male fly showed decreased wing-flapping behavior and failure to copulate with the female. The sporadic PD fly model showed an increase in CL after exposure to PQ. A similar observation of the increased CL was reported by Jantrapirom et al. (2020) in the ubiquilins (UBQLNs) depleted fly model. UBQLNs is an important group of proteins involved in proteostasis, which is also associated with pathological inclusions of Lewy bodies (intracytoplasmic proteinaceous inclusions) in the PD brain, the UBQLNs depleted flies show a reduction in DA and serotonin levels and when paired for courtship, they showed a longer CL of nearly double that of control pairs. An alteration of either

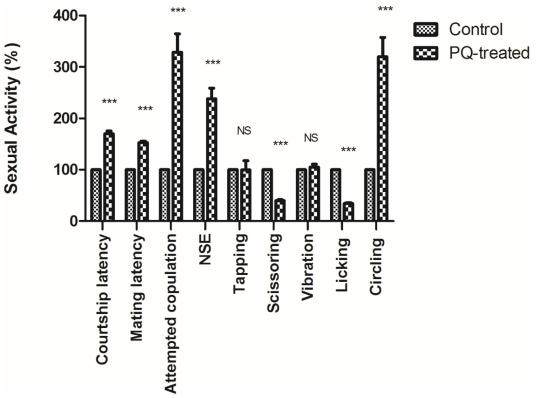

FIGURE 5
Sexual dysfunction precedes motor dysfunctions in the *Drosophila* model of Parkinson's disease (PD). Paraquat (PQ) treated males exhibited following courtship anomalies when compared to control males, such as increased courtship latency, mating latency, attempted copulation, NSEs, and circling behavior but decreased scissoring and licking behavior. However, there was no difference between control and treated in behaviors such as tapping and vibration. A Statistical analysis was performed using a *t*-test (compared to control). ***$p < 0.0001$. NS, not-significant.

copulation processes such as an extension of CL or a shortening of the copulation period might reveal some defects in courtship behaviors (Jantrapirom et al., 2020).

Shaltiel-Karyo et al. (2012) in their study using alpha-synuclein (α-syn) A30P mediated PD fly model reported impairment of courtship traits such as orientation, vibration, licking, AC, NSEs, and copulation when compared to control. Observed similarities of courtship behavioral markers between genetic and sporadic PD fly models, illustrate the involvement of DAergic pathways in the distorted courtship behavior.

It would be interesting to study further and figure out the interaction between genes and the environment in PD, for which the present model would be of immense help. It will further help to screen potential therapeutic molecules for PD.

Feany and Bender (2000) first reported the *Drosophila* model of PD by expressing normal and mutant forms of α-syn and showed the adult-onset loss of DAergic neurons. Several studies in PD models have shown the varying level of DAergic cell loss in various brain DA clusters (Auluck et al., 2002; Chen and Feany, 2005; Cooper et al., 2006; Trinh et al., 2008, 2010; Barone et al., 2011). However, in the present study, the SD model does not exhibit variation in DA neuronal number i.e., there was no observable DA degeneration (both in the number of DA neurons and fluorescence intensity of fluorescently labeled secondary antibodies that target anti-TH primary antibody) in the brain. There was also no variation in DA and its metabolite levels (DOPAC and HVA) in the PQ-induced SD model compared to the control. Further, the result demonstrated no ques that would lead to the postulate that there is enhanced DA degradation in the PQ-induced SD model compared to the control. Loss of DA neurons in the fly models of PD has been an issue of controversy. Navarro et al. (2014) in their study using three different flies PD model systems, viz. genetic (α-syn, Pink1, parkin) and two toxins based (rotenone and PQ) models of PD also reported the absence of DAergic neuronal loss in all models tested. The possible explanation for the observation (absence of variation in the levels of DA and its metabolites) in this PQ-induced SD fly, could be due to the following reasons:

a. At the selected window of PQ exposure, there may be no alteration in the levels of TH, DA, DOPAC, and HVA.
b. There lies a possibility for cell type-specific variation of DA and its metabolites (in the present study quantification is performed in whole brain tissue).
c. If the minute variation were to exist in a cell type-specific fashion that may not be possible to detect using the present method due to the limitation of sensitivity levels.
d. Further, other than catecholamines, genes like *fruitless* (Ryner et al., 1996) and certain neuroendocrine secretions such as juvenile hormone (JH) regulate male courtship behavior (Zhang et al., 2021). It will be interesting to probe further the biological regulation of *fruitless* and JH under induced PD condition.

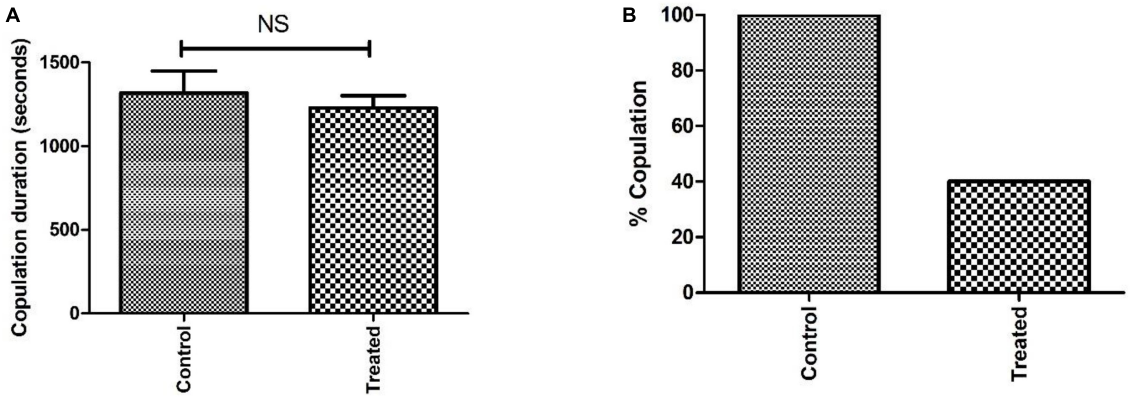

FIGURE 6
Analysis of sexual behavior before the onset of mobility defects showed that the difference in copulation duration between control and treatment is not significant (NS) **(A)**. However, the difference in percent copulation is about 60% less in male-treated mating (20 males mated successfully/25 in the control group and 9/25 in the treated group) when compared to control mating **(B)**.

Conclusion

The present study demonstrates that exposure to the neurotoxicant PQ leads to SD as characterized by male fly courtship behavior, which precedes motor defects and brain DAergic neurodegeneration and alteration in DA metabolism. Therefore, traits associated with courtship and sexual activity will help as sensitive early-stage markers to identify the later-onset of PD in the *Drosophila* model. As by the time motor defects set in, a significant amount of brain DAergic neurodegeneration already occurred, the present model will provide an opportunity to understand the progression of the incipient pathophysiology of PD. Further, this model will support the development of biological markers for PD. Further, it will be interesting to investigate whether the courtship dysfunction (s) will have any influence on the reproductive fitness of the parents, further on the development of the progeny. By taking advantage of the power of fly genetics, it will be possible to decipher the genetic basis of SD, knowledge of which may contribute to developing therapeutic strategies for PD in humans and to identify interacting partners of disease-causing genes; understanding which is critical to developing and screen novel therapeutic molecules for late-onset NDD such as PD which has few therapeutic options.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

ZK performed the experiments, data acquisition, and analysis of the data. MA participated in the experiments relating to HPLC and fluorescence microscopy. AD participated in the experiments relating to brain dopamine metabolism and analysis of the data. RC participated in the experiments relating to whole brain immunostaining and analysis of the data. LP participated in the experiments relating to developing PQ-induced SD fly. ZK, AD, and RC drafted the manuscript. SY contributed to the conception and design of the study, interpreted the data, revised the manuscript, obtained funding, and supervised the study. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the Department of Biotechnology (DBT), India (R&D grant nos. BT/405/NE/U-Excel/2013, BT/PR16868/NER/95/328/2015, and BT/COE/34/SP28408/2018) and the Science and Engineering Research Board (SERB) of the Department of Science and Technology (DST) India (R&D grant no. EMR/2016/002375, 27-3-2017). Part of the work was presented at the 9th IBRO (International Brain Research Organization) World Congress on Neuroscience- July, 7–11 2015, Rio de Janeiro, Brazil for which SY was awarded SERB (Science and Engineering Research Board) of DST (Department of Science and Technology), India's ITS (International Travel Support) Fellowship and at 4th International conference on Nutraceutical and Chronic Diseases (INCD-2019) held at Indian Institute of Technology (IIT), Guwahati, Assam, India in which ZK received Best Poster Award for the work. ZK received MANF (Maulana Azad National Fellowship) of the Ministry of Minority Affairs, India, MA, AD, RC, and LP received DBT-JRF (junior research fellowship), and AD, RC, and LP received DBT-SRF (senior research fellowship).

Acknowledgments

We thank the reviewers for the critical comments and suggestions.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1143793/full#supplementary-material

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frontiers | Frontiers in Neuroscience

TYPE Review
PUBLISHED 03 April 2023
DOI 10.3389/fnins.2023.1150694

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 24 January 2023
ACCEPTED 09 March 2023
PUBLISHED 03 April 2023

CITATION
Ye C, Behnke JA, Hardin KR and Zheng JQ (2023) *Drosophila melanogaster* as a model to study age and sex differences in brain injury and neurodegeneration after mild head trauma.
Front. Neurosci. 17:1150694.
doi: 10.3389/fnins.2023.1150694

Drosophila melanogaster as a model to study age and sex differences in brain injury and neurodegeneration after mild head trauma

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Repetitive physical insults to the head, including those that elicit mild traumatic brain injury (mTBI), are a known risk factor for a variety of neurodegenerative conditions including Alzheimer's disease (AD), Parkinson's disease (PD), and chronic traumatic encephalopathy (CTE). Although most individuals who sustain mTBI typically achieve a seemingly full recovery within a few weeks, a subset experience delayed-onset symptoms later in life. As most mTBI research has focused on the acute phase of injury, there is an incomplete understanding of mechanisms related to the late-life emergence of neurodegeneration after early exposure to mild head trauma. The recent adoption of *Drosophila*-based brain injury models provides several unique advantages over existing preclinical animal models, including a tractable framework amenable to high-throughput assays and short relative lifespan conducive to lifelong mechanistic investigation. The use of flies also provides an opportunity to investigate important risk factors associated with neurodegenerative conditions, specifically age and sex. In this review, we survey current literature that examines age and sex as contributing factors to head trauma-mediated neurodegeneration in humans and preclinical models, including mammalian and *Drosophila* models. We discuss similarities and disparities between human and fly in aging, sex differences, and pathophysiology. Finally, we highlight *Drosophila* as an effective tool for investigating mechanisms underlying head trauma-induced neurodegeneration and for identifying therapeutic targets for treatment and recovery.

KEYWORDS

traumatic brain injury, neurodegeneration, sex difference, aging, risk factors

Introduction

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and frontotemporal dementia (FTD), involve progressive disruption of brain function and subsequent neuronal loss that are more prevalent in aged populations. While an increasing number of risk genes and mutations have been identified, these genetic factors only account for a small portion of neurodegenerative cases (Bertram and Tanzi, 2005; Hardy and Orr, 2006; Pihlstrøm et al., 2018; Fu and Ip, 2023). Aging is the main risk factor for

many neurodegenerative disorders and accelerated unhealthy aging likely plays a key role in the development of neurodegenerative conditions (Azam et al., 2021; Saikumar and Bonini, 2021). Other significant risk factors include environmental insults such as exposure to trauma, drugs, or toxins which can have harsh consequences later in life, including the development of dementia (Brown et al., 2005; Gupta and Sen, 2016; Schaefers and Teuchert-Noodt, 2016). In fact, head trauma induced by physical impacts is one of the greatest external risks for brain injury and disability, often inflicting a heterogeneous set of physical, cognitive, and emotional symptoms affecting millions of individuals each year worldwide (Roozenbeek et al., 2013; Heinzelmann et al., 2014; Ling et al., 2015; Stein et al., 2015). In particular, closed-head traumatic brain injury (TBI) represents one of the most common head injuries associated with contact sports, automobile accidents, and falls. The majority of TBI cases can be classified as mild TBI (mTBI) as they involve limited loss of consciousness and memory without diagnosable structural brain alterations (CDC, 2003; Cassidy et al., 2004). Mild head trauma can cause long-term disability and/or lead to late-life brain degeneration, such as chronic traumatic encephalopathy (CTE), AD, and other progressive neurodegenerative conditions (Ojo et al., 2016; Asken et al., 2017; Mez et al., 2017; Graham and Sharp, 2019; Brett et al., 2022). Even sub-concussive head impacts, especially when exposed repetitively, can cause significant cognitive impairment later in life (Ntikas et al., 2022). Previous preclinical TBI studies have implicated the involvement of neuronal excitotoxicity, increases in intracellular calcium, free radical production, mitochondrial dysfunction, and inflammatory mediators (Globus et al., 1995; Werner and Engelhard, 2007; Hawryluk and Manley, 2015) within the injury response cascade, as well as the disruption of blood brain barrier integrity (Shlosberg et al., 2010), activation of glia (Myer et al., 2006), and inhibition of regeneration (McGraw et al., 2001; di Giovanni et al., 2005; Itoh et al., 2007). Within these pathways, mitochondrial dysfunction and chronic inflammation have been suggested to be involved in long-term processes leading to neurodegenerative conditions (Wang et al., 2019, 2020; Gao et al., 2022). However, the study of many of these processes in preclinical models of mTBI has been limited to the acute phase of injury and it remains to be determined if any of these processes are responsible for the emergence of neurodegeneration years after injury exposure.

Age and sex are two important biological factors that affect outcomes following mild head trauma. Both clinical and preclinical studies show that aging is associated with worse outcomes following injury (Cheng et al., 2014; Abdulle et al., 2018; Ritzel et al., 2019). Elderly individuals are more prone to developing severe and long-lasting behavioral symptoms than younger adults, but the underlying causes remain to be fully elucidated. Sex-related differences have been documented in many medical conditions, including neurodegenerative disorders (Li and Singh, 2014; Podcasy and Epperson, 2016; Au et al., 2017; Nebel et al., 2018; Cerri et al., 2019; Mauvais-Jarvis et al., 2020; Vegeto et al., 2020) and neurotrauma of various severities (Marar et al., 2012; Berz et al., 2013; Dollé et al., 2018; Gupte et al., 2019; Yue et al., 2019; Rauen et al., 2021). Sex differences and sex-related factors, including anatomical differences (Mansell et al., 2005; Dollé et al., 2018), gonadal hormone differences (Stein, 2001), reproductive status (Isaac et al., 2010; Garbe et al., 2016; Dove et al., 2017), and immunological responses (Ertürk et al., 2016; Jassam et al., 2017; Späni et al., 2018), presumably affect vulnerability to neurodegeneration. How sex-related factors impact short-term responses to mTBI and the subsequent development and progression of neurodegenerative conditions are poorly understood. Interestingly, aging progression is known to be different between male and female (Hagg and Jylhava, 2021), indicating the complex interplay between these two factors in neurodegeneration. Clearly, further development and utilization of preclinical animal models with short lifespans can greatly accelerate the research focused on the understanding of molecular and cellular mechanisms underlying sex differences and age-dependence in trauma-elicited neurodegenerative conditions that emerge late in life. In this review, we first outline the pathology of mTBI and subsequent neurodegenerative conditions. We then discuss current understanding of age and sex in the progression of trauma-mediated neurodegeneration from human and mammalian models. Finally, we highlight *Drosophila* models of mTBI and their potential in studying head trauma-induced neurodegeneration and the role of age and sex within the injury response.

Traumatic brain injury and neurodegeneration

Traumatic brain injury is, "an alteration in brain function, or other evidence of brain pathology, caused by an external force" (Menon et al., 2010), that presents as a heterogeneous set of physical signs, and cognitive and emotional symptoms (Heinzelmann et al., 2014; Ling et al., 2015; Stein et al., 2015). The Centers for Disease Control and Prevention estimates an annual TBI incidence of 2.8 million individuals in the US, including 50,000 TBI-related fatalities (CDC, 2015). Over five million Americans are currently living with long-term TBI-related disability (CDC, 2015). Closed-head injuries, most commonly a result of falls and automobile accidents, are the most prevalent type of TBI, of which mild TBI, also known as *concussion*, account for ~75% of total TBI incidence (CDC, 2003). This is likely an underestimate as mTBI often goes undiagnosed, or even unrecognized in cases where medical care is not pursued. mTBI may present with subtle or even no obvious indications following injury, yet 11–38% of affected individuals experience symptoms 6-months after injury (Voormolen et al., 2018), indicating that mTBI may have long-lasting neurological effects (Nelson et al., 2019). Studies that compared asymptomatic athletes who play contact sports to athletes who play non-contact sports revealed long-term brain changes (Slobounov et al., 2017; Manning et al., 2019) and cognitive deficits (Killam et al., 2005; McAllister et al., 2012; Talavage et al., 2014), suggesting that even seemingly innocuous head impacts regardless of symptoms can lead to deleterious effects later in life. Furthermore, a history of repetitive mTBI, especially in high-risk individuals such as military personnel (Warden, 2006; Barnes et al., 2018) and contact athletes is associated with the insidious progressive neurodegenerative disease CTE, where symptom onset occurs years after exposure (Martland, 1928; Blennow et al., 2012; DeKosky et al., 2013; Baugh et al., 2014; Ling et al., 2015; Stein et al., 2015; McKee et al., 2016; Mez et al., 2017; Mackay et al., 2019). A prior history of head trauma is also associated with the development of age-related neurodegenerative diseases (Little et al., 2014; Esopenko and Levine, 2015; Perry et al., 2016; Asken et al., 2017; DeKosky and Asken, 2017; Mez et al., 2017; Erkkinen et al.,

2018), including AD (Mortimer et al., 1991; Barnes et al., 2018; Katsumoto et al., 2019), PD (DeKosky et al., 2013), and amyotrophic lateral sclerosis (ALS; Little et al., 2014), which collectively represent a leading cause of long-term morbidity and mortality worldwide (Erkkinen et al., 2018). Despite the critical concern related to repetitive head trauma exposure, its underlying mechanisms leading to neurodegeneration and long-term complications remain poorly understood (Asken et al., 2017; Fehily and Fitzgerald, 2017; Wojnarowicz et al., 2017).

Traumatic brain injury can be broken down into two injury components that are sustained in tandem: the primary injury, which is the initial immediate mechanical impact/insult sustained by the brain that results from linear and/or rotational head movement, followed by the secondary injury, which involves the subsequent myriad of downstream pathophysiological processes (Werner and Engelhard, 2007). Secondary injury consists of excitotoxicity, free radical production, mitochondrial dysfunction, and inflammatory mediators (Werner and Engelhard, 2007; Hawryluk and Manley, 2015) and can sustain for days, weeks, months and even longer depending on the nature of the injury. Axons are thought to be the most vulnerable portion of the neuron to mechanical trauma, given their large surface area to volume ratio (McKee and Daneshvar, 2015) and high degree of anisotropic organization of cytoskeletal elements (Johnson et al., 2013), principally composed of microtubules (Conde and Caceres, 2009). Shear stress and strain forces elicited by mechanical head trauma can cause rapid stretching of axons, followed by an unregulated influx of cations (Na^+ and Ca^{2+}) through sodium and calcium channels along the axolemma, resulting in indiscriminate depolarization and release of excitatory neurotransmitters (Lyeth et al., 1993; Palmer et al., 1993; Globus et al., 1995; Wolf et al., 2001; Yi and Hazell, 2006; Weber, 2012; Fehily and Fitzgerald, 2017). Persistent neuronal hyperexcitability can overburden Na^+/K^+ pumps needed to restore proper ionic homeostasis, and can last into the chronic phase of injury (Lima et al., 2008). Elevated ion pump activity can also overwhelm mitochondrial buffering of calcium and eventually result in metabolic dysfunction (Barkhoudarian et al., 2016). In addition to ionic disturbances, structural abnormalities are seen immediately following brain injury, including the breaking of microtubules directly in response to physical trauma (Tang-Schomer et al., 2010). Concurrently, elevated calcium can activate calcium-sensitive proteases, such as calpain, which cleave cytoskeletal components, such as microtubules within the axon (Saatman et al., 1996a,b, 1998). Microtubule degradation is evident in other parts of the neuron as well. Hippocampal microtubule-associated protein 2 (MAP2), which is localized mainly to the soma and dendrite, is also reduced following injury (Taft et al., 1992). Inhibiting calpain activation shortly after injury mitigates behavioral deficits and cytoskeletal breakdown in preclinical animal models of injury (Saatman et al., 1996b; Posmantur et al., 1997). Subsequent processes include axonal transport deficits (Ma et al., 2016), swelling (Smith et al., 2003), retraction (Johnson et al., 2013), cellular inflammation (Giza and Hovda, 2014) and eventual cell death (Giza and Hovda, 2014). The culmination of these processes is theorized to give rise to neurodegeneration, such as CTE pathology found in post-mortem brains from individuals with a history of repetitive trauma (McKee et al., 2016). CTE pathology is characterized by the presence of hyperphosphorylated tau accumulated within neurons and glia surrounding small blood vessels (perivascular) at sulci depths in a pattern distinct from AD (McKee et al., 2016; Mez et al., 2017). Other supportive pathological features include abnormal TDP-43 accumulation (McKee et al., 2013, 2016; Mez et al., 2017), and may include beta-amyloid (Aβ) deposition (McKee et al., 2016). The subsequent accumulation of toxic protein aggregates is thought to result in chronic neurodegeneration (Little et al., 2014; DeKosky and Asken, 2017; Gilmore et al., 2020). In addition to parenchymal brain loss, neurodegeneration is seen in other nerve fibers, including the progressive response that is evident in retired veterans who experience a decrease in longitudinal retinal nerve fiber layer (RNFL) thickness (Gilmore et al., 2020).

The mechanisms that connect primary and secondary injury sequelae to long-term neurodegeneration remain undefined. It is also unclear whether concussion and sub-concussive injuries represent distinct pathophysiological processes. Given that even mild injury exposure carries long-term risks, it is imperative to understand the injury cascades elicited during the latent or asymptomatic stage of injury that may potentiate late-life neurodegeneration. To date, there are no FDA-approved drugs explicitly developed for treating mTBI. This dearth of therapeutics leaves the treatment of moderate and severe cases of trauma with limited options to promote functional recovery aside from supportive and life-saving hemodynamic strategies. With limited therapeutics at our disposal for the treatment of head trauma, the identification of risk factors associated with worse outcomes following mild head trauma is an important component for mitigating risk in vulnerable populations. Here, we will discuss biological sex and age as two important risk factors in mTBI and long-term brain deficits.

Age affects mTBI outcome and subsequent neurodegeneration

Age is one of the strongest outcome predictors for complications following head trauma, including mild trauma (Jacobs et al., 2010; Moretti et al., 2012; Bittencourt-Villalpando et al., 2020). The cause of injury varies by age, with motor vehicle accidents being the greatest source of injury in young adults and falls being the greatest contributor to injury in elderly populations (Peterson et al., 2019). Although the cause of injury differs, older age is consistently associated with an increased incidence of head trauma, a slower overall recovery process, as well as greater morbidity and mortality following injury (Abdulle et al., 2018). Compared to the high rates of head trauma in the elderly, studies on the intersection of aging and mTBI-related neurodegeneration are surprisingly scarce. Functional outcome 6-months following injury reveals a slower recovery in affected older individuals (aged 60+ years of age) who initially present with the same degree of mild injury severity compared to younger adults (Mosenthal et al., 2004). Another study with mildly injured individuals 65+ years of age revealed that the level of one's education positively affected the chances for reaching a full recovery 6-months following injury (van der Naalt et al., 2017). Together, these findings lend support to the concept of cognitive reserve, which decreases with age and is associated with the onset of age-related neurodegenerative disorders such as AD (Stern, 2012), as a potential protective factor against the effects of mild head trauma. This is further supported by the finding that cognitive reserve is associated with improvements in multiple tested cognitive domains (memory, verbal fluency, and executive

function) across all injury severities of head trauma 1-year following injury in an adult cohort ranging from 19 to 79 years of age (Steward et al., 2018). At the cellular level, aging neurons in both the central and peripheral nervous system lose regenerative potential (Verdú et al., 2000; Nicaise et al., 2020), which may contribute to the increased vulnerability of aged individuals to developing brain deficits after exposure to mTBI. At this moment, it is difficult to disentangle the added effect of comorbidities and increasing frailty associated with aging, but pre-clinical work has begun dissecting these potential mechanisms which will then inform further clinical investigation.

There is generally an underrepresentation of young and aged cohorts in mTBI studies, accounting for less than 5% of rodent mTBI studies (Bodnar et al., 2019). Models of mTBI which more closely mimic clinical conditions, such as closed-head injuries, are very rarely conducted in aged animals (Iboaya et al., 2019). This is not to say that there is no evidence of the effects of age on mTBI-induced changes in the brain. Two studies have highlighted that age-at-injury affects long-term behavioral outcomes (Rowe et al., 2016) and neuropathology (Doust et al., 2021). Both studies subjected male rats to a single mild fluid percussion injury at post-natal day 17, day 35, 2-, 4-, or 6-months-old, the oldest time point roughly corresponding to a young adult human. Rats injured at earlier developmental timepoints were more vulnerable to developing motor and cognitive deficits shortly after injury. Rats injured in adulthood showed increased anxiety-like behavior compared to sham controls. However, when assessed at 10 months of age, injured rats seemed to have recovered and behave like naïve control rats (Rowe et al., 2016). This behavioral recovery did not extend to neuropathology, as immunohistochemistry revealed dendritic and axonal damage as well as glial activation in specific regions of the brain, regardless of age-at-injury (Doust et al., 2021). Furthermore, rats injured in adulthood had greater dendritic neuropathology in cortical grey matter and higher extent of microglial activation in the hippocampus. Though further chronic timepoints were not examined, it is surmisable that continuous and irreversible damage caused by the mild injury that is already visible a few months following injury, accelerates neurodegeneration. Another study gave 18-month-old human tau transgenic mice a single or repeated (five impacts over 9 days) closed-head mild injury that did not cause direct tissue damage (Ojo et al., 2013). Three weeks after the injuries, the study found a significant increase in phosphorylated tau in the repeated injury group when compared to the sham and single injury group, and notably consistent astrocyte and microglial activation in the injured regions. This study suggests that repetitive injury augments already-present neurodegenerative tau pathology in aged animals. However, the study did not compare the aged cohort to a younger cohort, so it is unclear whether tau abnormalities in young adult mice affect the injury response and whether aging mechanisms interact with injury mechanisms.

What are the specific mechanisms of aging that interact and compound with mechanisms of head injury? How may that inform future treatment options targeting head injury-induced neurodegeneration in the vulnerable aging population? Research in aging revealed that mitochondrial function (Cui et al., 2012; Chistiakov et al., 2014) and immune function (Weyand and Goronzy, 2016; Haynes, 2020) decline with aging. Given that mitochondrial dysfunction (Cheng et al., 2012; Fischer et al., 2016; Kim et al., 2017) and compromised immune responses (Needham et al., 2019; Verboon et al., 2021) have been implicated in TBI-induced brain dysfunction and degeneration, they may underlie or contribute to aging-increased vulnerability to brain injury and development of neurodegeneration in response to head trauma. Nonetheless, current limitations in human and rodent studies include small sample sizes and inadequate sampling, long experimental timelines, and heterogeneity of behavioral pathology that may result from different experimental manipulations. As we will discuss later, fruit flies, with their relatively simple nervous system and short lifespan, are uniquely poised to answer these questions.

Sex differences in mTBI and neurodegeneration

Sex-related differences have been documented in many medical conditions, including neurodegenerative disorders (Li and Singh, 2014; Podcasy and Epperson, 2016; Au et al., 2017; Nebel et al., 2018; Cerri et al., 2019; Mauvais-Jarvis et al., 2020; Vegeto et al., 2020) and neurotrauma of various severities (Marar et al., 2012; Berz et al., 2013; Dollé et al., 2018; Gupte et al., 2019; Yue et al., 2019; Rauen et al., 2021). The use of both sexes within preclinical TBI research has garnered greater attention recently, following a period of time that incorporated limited female representation in studies (Späni et al., 2018), and has become a greater point of emphasis since the NIH mandated that sex be considered a biological variable in 2016 (Späni et al., 2018; Woitowich and Woodruff, 2019). A growing body of evidence demonstrates that there exists a sex-dependent effect related to incidence and recovery from neurotrauma (Marar et al., 2012; Berz et al., 2013; Dollé et al., 2018; Yue et al., 2019; Rauen et al., 2021). This should come as little surprise given that there are sex differences in anatomy (Mansell et al., 2005; Dollé et al., 2018), sex gonadal hormones (Stein, 2001), and immunological responses (Ertürk et al., 2016; Jassam et al., 2017; Späni et al., 2018), which presumably all affect vulnerability to injury. There is an interesting disparity between clinical and pre-clinical sex-dependent findings, where female sex is associated with worse outcomes in human studies and better outcomes in preclinical studies (Gupte et al., 2019). Part of this disparity in findings is attributed to differences in injury severity and animal model (Gupte et al., 2019).

In a large literature review, human studies involving mild brain injury showed that the female sex was associated with worse outcome measures (Gupte et al., 2019). Human data from the TRACK-TBI study demonstrated that the female sex was associated with decreased six-month functional outcome measured using the Glasgow Outcome Scale-Extended (GOSE) following mild TBI (Yue et al., 2019). A recent cross-sectional human study revealed that females were more likely to report a less favorable health-related quality of life (HRQoL) during the chronic stage of mild TBI (10 years post-injury; Rauen et al., 2021). Additional evidence demonstrated that young female athletes may take longer to become symptom free following sports-related concussion (Berz et al., 2013). In a study conducted in 10-35-year-old patients, females report more sleep disturbances only after a single concussion (Oyegbile et al., 2017). In patients that reported more than one incidence of injury, there were no major differences. When the data was stratified by age, the study found sex differences in sleep disturbances but only in post-pubescent ages (>15), suggesting possible hormone interactions. It should be emphasized that women remain significantly underrepresented within sport and exercise

science research (Cowley et al., 2021). The sex data gap needs to be addressed by future studies that focus on the gender and sex differences in the risk and outcome of brain injury of various severities and the subsequent treatment and care.

Preclinical studies that consider sex as a variable while studying mild head trauma reliably find trends of differing trauma responses between sexes. A recent study using a mild closed head injury model in adult rats found that females showed greater deficits in recovery and locomotive behaviors after either repetitive sub-concussive impacts or one single concussive impact, whereas males exhibited increased anxiety and depressive-like behaviors (Wilson et al., 2023). Similarly, another study found worse recovery and higher deficits in behavioral tasks such as spatial memory in female rats (Wirth et al., 2017). However, in more severe injury models, findings are less consistent. Many studies find that female animals exhibit more favorable outcomes than males (O'Connor et al., 2003; Shahrokhi et al., 2010, 2012; Sarkaki et al., 2013), suggesting that female sex hormones are neuroprotective and anti-inflammatory, while others find female animals exhibit greater deficits and neurodegeneration or have produced mixed results (Mollayeva et al., 2018). Detailed discussion on sex differences in TBI and the contributing factors can be found in several reviews (Gupte et al., 2019; Ma et al., 2019; Rubin and Lipton, 2019; Chaychi et al., 2022). It is important to note that preclinical data showing the better outcomes of the female sex in severe injury models were the foundation for testing the neuroprotective effects of progesterone in the ProTECT (Progesterone for Traumatic Brain Injury, Experimental Clinical Treatment) human clinical trials for severe neurotrauma (Wright et al., 2014). Despite preclinical evidence demonstrating neuroprotection, exogenous progesterone failed to meet the clinical end point (a 10% improvement in the Extended Glasgow Outcome Scale 6-months post-injury; Wright et al., 2014). The paradoxical finding that exogenous progesterone exhibits neuroprotective properties in preclinical models, yet females experience a worse prognosis may be attributed to the cyclical nature of progesterone, and forms the basis of what is known as the "withdrawal hypothesis" (Wunderle et al., 2014; Valera et al., 2021). The "withdrawal hypothesis" posits that the relative abundance of progesterone dictates injury vulnerability. Females who sustain a mild TBI during the luteal phase of menstruation (when progesterone is high) report a lower quality of life (EuroQoL/EQ5D) and worse self-reported outcome measures (Rivermead Post Concussion Questionnaire) 1 month following injury compared to injured females on birth control, who exhibit elevated levels of progestins (Wunderle et al., 2014). A similar trend is seen across age-groups within females: pre-menarche and post-menopausal women report lower 3-month post-concussive symptoms compared to women of child-bearing years (Bazarian et al., 2010), indicating that injury vulnerability may be related to the disruption or natural cycling of female gonadal hormones. Taken together, both clinical and preclinical data reveal sex differences in the response to TBI of varying severities, the recovery, and short- and long-term outcomes. These complex and often conflicting outcomes associated with different sexes further emphasize the need to include both sexes in both clinical and preclinical studies and demand better sampling and controlling of experimental subjects for hormonal cycles, aging, and social/psychiatric factors.

While the mechanisms underlying the sex differences in brain injury responses and development of neurodegenerative conditions remain to be elucidated, alterations in mitochondrial functions and immune responses are considered the key candidates. Mitochondria dysfunction is thought to be involved in the pathogenesis of many neurodegenerative diseases (Lin and Beal, 2006; Wang et al., 2019, 2020; Gao et al., 2022). Mitochondria play an important role in cell death *via* their release of pro-apoptotic factors after undergoing outer membrane permeabilization, which commonly occurs after TBI (Cheng et al., 2012; Fischer et al., 2016). Sex differences exhibited in mitochondrial function could potentially result in disparate sex responses to TBI. Females of reproductive age typically display better mitochondrial function, lower levels of reactive oxygen species, and have higher levels of antioxidant enzymes compared to males (Silaidos et al., 2018). This trend is reversed however when females undergo menopause, which could potentially contribute to the higher rates of AD observed in females (Silaidos et al., 2018) as well as post-TBI brain degeneration.

Sex differences in the immune system likely affects neuroinflammation and neurodegeneration after mTBI, which has been reviewed in great detail by Klein and Flanagan (2016). Females mount higher innate and adaptive responses across all species, which is believed to be somewhat of an evolutionary tradeoff between survival strategies and reproduction. Most sex differences in the innate immune response, such as expression and activation of Toll-like receptors, are encoded in the germline and directly result from sex chromosomes. Though not very well understood, sex differences in microglia may play a role in a variety of neurodegenerative conditions (Lopez-Lee et al., 2022). Microglia are the main players in the innate immune response in the brain (Mason and McGavern, 2022). Following brain injury, physical damage to the blood–brain barrier and astrocytes elicits microglial activation for repair. However, microglia may fail to return to a naïve state, particularly when microglia are continuously activated by repeated injuries, which can lead to aberrant deposition of proteins such as Tau. The adaptive immune response, such as T and B cell activity and infiltrating myelomonocytic cells, is also stronger in females regardless of age and may be involved in both acute and chronic stages of TBI (Klein and Flanagan, 2016). Sex hormones are believed to affect neurodegeneration by their regulation of immune responses (Mollayeva et al., 2021), and environmental factors such as nutrition or microbiome can also affect immunity. Finally, the age and reproductive status of an individual are also important determinants of sex differences in immune responses. It is clear that animal models with short lifespan, available molecular and genetic toolboxes, and better control of sex-related changes are needed to detangle the complex interplays of age- and sex-related differences and to elucidate the molecular, genetic, and cellular mechanisms underlying age- and sex-dependent late-life development of neurodegenerative conditions after environmental insults. As we discuss in the next section, fruit flies represent one of such animal models.

Drosophila melanogaster as a model for studying neurodegenerative diseases and TBI

Drosophila have served as a powerfully tractable model organism to investigate fundamental neurobiological processes and mechanisms of neurodegeneration (Wittmann et al., 2001; Dias-Santagata et al., 2007; Chatterjee et al., 2009; Ali et al., 2011; Ling and Salvaterra, 2011;

Burnouf et al., 2016; Sunderhaus and Kretzschmar, 2016; Coelho et al., 2018). This is made possible due to conserved neurobiology that exists between both *Drosophila* and mammalian species, including Na^+/K^+-based action potentials and inhibitory and excitatory neurotransmitters with shared neurosecretory-released mechanisms (Freeman, 2015). Like that of the mammalian brain, the fly brain consists of an organized arrangement of discrete neuronal structures and circuitry, but exists within a much smaller, more easily dissectible brain size that enables the study of individual neurons and their corresponding functional roles (Scheffer et al., 2020). The brain of *Drosophila melanogaster* is comprised of ~100,000 neurons (Kremer et al., 2017; Scheffer et al., 2020) and 10,000 glia (Freeman, 2015; Kremer et al., 2017), which makes it several orders of magnitude smaller than the human brain [86 billion neurons (Scheffer et al., 2020), with an equal number of glia (von Bartheld et al., 2016)]. Specifically, the fly brain consists of a central brain and two large optic lobes, each with an outer cortex that contains cell bodies and a synaptically-dense inner neuropil (Buchanan and Benzer, 1993; Ito et al., 2014). This is in contrast to the vertebrate brain, which features a synaptically-dense outer region within the superficial cortical layers (Subramanian et al., 2020). The outer cortex of the fly brain is surrounded by a perineurium that serves a functional equivalent to the blood–brain barrier within vertebrates (Buchanan and Benzer, 1993). The ventral nerve cord is the invertebrate equivalent of the mammalian spinal cord, which extends into the thorax where it relays motor-sensory information (Court et al., 2020). At the cellular level, *Drosophila* neurons share the same three subcellular compartments as mammalian neurons (axon, soma, dendrite), which make up a mix of unipolar and multipolar neurons (Rolls, 2011). However, unlike mammalian dendrites, *Drosophila* dendrites do not possess clearly demarcated spines that occupy post-synaptic sites (Rolls, 2011). Furthermore, the mammalian brain is a highly vascularized structure that is suspended and cushioned in cerebral spinal fluid (CSF), and contained within a boney skull, whereas the *Drosophila* brain is surrounded by trachea and air sacs that distribute nutrients and oxygen like the mammalian circulatory system and is enclosed within a chitinous exoskeleton. Although the *Drosophila* brain is not surrounded by CSF, the surrounding air sacs serve as a fluidic equivalent in being able to suspend the brain and serve as a buffer between the brain and outer enclosure.

Several key characteristics of *Drosophila melanogaster* make it an ideal model organism for the study of neurological diseases. 75% of disease-related genes in humans have corresponding fly orthologs (Pandey and Nichols, 2011). They have a short reproductive and life cycle, which enables lifelong processes such as those related to neurodegeneration and aging in a much more considerable time frame (Wittmann et al., 2001; Duffy, 2002; Jackson et al., 2002; Pandey and Nichols, 2011; Wang and Jin, 2011; He and Jasper, 2014; Coelho et al., 2018). Adult flies exhibit complex behaviors stemming from their organized central nervous systems, all while sharing conserved neural mechanisms with that of humans (Pandey and Nichols, 2011). Like humans, wildtype flies exhibit neurodegeneration associated with age. Reliable histological and behavioral assays have been developed to investigate aspects of neurodegeneration, such as examination of eye and retinal structures, scoring of brain vacuolization and abnormal protein deposits *via* immunohistochemistry, analyses of lifespan, measuring of sensorimotor functions using negative geotaxis assays (NGA), and assessment of the neuromuscular junction for synaptic phenotypes. Perhaps most impressive of the inherent *Drosophila* toolbox is the genetically tractable nature of fruit flies, which is unparalleled by any mammalian system. Many transgenic models of specific neurodegenerative diseases have been generated to study the progression of neurodegeneration, such as AD (Prüßing et al., 2013), PD (Whitworth, 2011), FTD (West, 2015), HD (Krench and Littleton, 2013), ALS (Casci and Pandey, 2015), and CTE (Aggarwal et al., 2022; Figure 1A). The ability to systematically probe protein function and control transgene expression in a cell-specific manner enables interrogation of various signaling mechanisms and opportunities for high-throughput screens. This approach has led to the study of cellular and molecular injury responses following axonal injury within the fly. Using a variety of genetic screens inherently accessible in *Drosophila* has led to the identification of several key genes and their respective proteins that are involved in axonal survival and degeneration (Collins et al., 2006; Xiong et al., 2010; Neukomm et al., 2014; Brace and DiAntonio, 2017); this includes Wallenda (Wnd), a fly homologue of dual leucine zipper kinase (a conserved mitogen-activated protein kinase (MAPK) important in cell-autonomous axonal degeneration following axonal transection). Importantly, these discoveries have translated to both murine (Osterloh et al., 2012; Shin et al., 2012; Watkins et al., 2013) and human cell-based (Tian et al., 2019) studies, which validate the utility of using *Drosophila* as a simple *in vivo* approach to investigate conserved neurobiological processes.

The use of fruit flies to model and investigate traumatic brain injury (TBI) was pioneered by Wasserman's group. To elicit traumatic injury, Katzenberger et al. (2013) developed a "high-impact trauma" (HIT) device (Katzenberger et al., 2013, 2015a), which features a metal spring-loaded fly vial that can swing to introduce hits to the flies in the vial (Figure 1B). This first report provided early characterization of fruit flies subjected to mechanical trauma, including mortality/lifespan effects, climbing deficits, neurodegeneration, and immune responses secondary to trauma exposure. It also took advantage of the high-throughput nature of *Drosophila* by performing a genetic screen to compare mortality outcomes in mutant lines for innate immunity. Loss of highwire, a protein involved in Wallerian degeneration, which was first studied within the fly, is protective against cell death and degeneration following head injury exposure (Hill et al., 2020). Additional *Drosophila* TBI models have been developed, including those that can deliver head-specific impacts (Barekat et al., 2016; Sun and Chen, 2017; Saikumar et al., 2020; Behnke et al., 2021; Saikumar et al., 2021; van Alphen et al., 2022; Figure 1B). A summary of different *Drosophila* TBI models and the key findings from research using these models is provided in Supplementary Table 1. These models have also been extensively covered by other reviews (Shah et al., 2019; Buhlman et al., 2021; Aggarwal et al., 2022). It should be noted that studies using *Drosophila* TBI models have recapitulated several key aspects of trauma found in humans and other preclinical models of trauma, including acute stress responses such as oxidative stress and lysosomal activity, progressive neurodegeneration, neuronal hyperexcitability, and glial-specific responses (Barekat et al., 2016; Sun and Chen, 2017; Saikumar et al., 2020; Behnke et al., 2021; Saikumar et al., 2021; van Alphen et al., 2022). The short lifespan of fruit flies also enables the lifelong monitoring of brain deficits elicited by early exposure to mild head traumatic impacts (Behnke et al., 2021). The findings that female adult flies exhibit higher elevations of neuronal activity and more severe brain deficits later in life than male flies indicate the existence of sex differences in age-dependent development of neurodegenerative

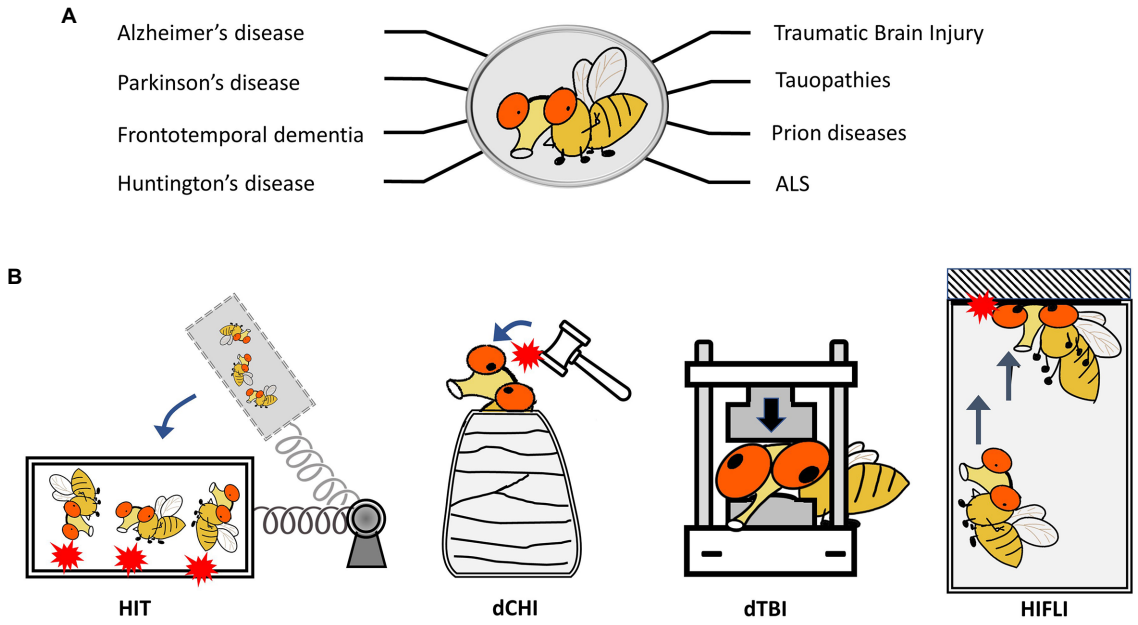

FIGURE 1
Drosophila melanogaster as a model to study neurodegenerative diseases and TBI. **(A)** Representative neurodegenerative diseases modeled in *Drosophila*. **(B)** Schematics showing four *Drosophila* models for TBI-related research. HIT: high impact trauma model by Katzenberger et al. (2013). dCHI: *Drosophila* controlled head-impact model by van Alphen et al. (2022). dTBI: *Drosophila* TBI model using a Piezoelectric actuator by Saikumar et al. (2020). HIFLI: Headfirst Impact FLy Injury model by Behnke et al. (2021).

conditions after mild TBI exposure (Behnke et al., 2021). Below we will discuss how *Drosophila* may be utilized to investigate aging effects and sex differences in late-life emergence of neurodegenerative conditions after mild head injuries.

Use of *Drosophila* melanogaster to understand aging effects

Aging usually refers to getting chronologically older, whereas normal aging or senescence is usually defined by the changes in physiology that occur naturally over time. *Drosophila* is a common model organism used in the study of aging (López-Otín et al., 2013). In 1916, Jacques Loeb and J. H. Northrop discovered that different ambient temperature can drastically alter *Drosophila* lifespan (Loeb and Northrop, 1916), and established that a 10°C reduction in temperature resulted in an approximate doubling of lifespan. This gave rise to the rate-of-living theory (Pearl, 1928), which suggests that an organism's lifespan may be determined by its rate of living, in other words the rate of energy expenditure or metabolism. In the next hundred years or so, research using *Drosophila* and other model organisms painted a more detailed picture of aging; aging results from a combination of interconnected mechanisms and interactions between different loss-of-function phenotypes. Genetics have a significant impact on the longevity of animals (Finch and Ruvkun, 2001), but non-genetic factors such as nutrition, environment, and lifestyle also play an important role. *Drosophila* is a great model organism for the study of both genetic and non-genetic factors that contribute to aging.

Many aspects of the genetic basis to aging are conserved across many species (Piper and Partridge, 2018). The expression patterns of over 20% of genes in the fly genome change with age (Pletcher et al., 2002; Davie et al., 2018). During aging, RNA content and cell size decrease drastically but cellular identity is unaffected in old brains (Lints et al., 1984; Le Bourg, 1987). There is an increase in glia, which is also seen in human aging (Soreq et al., 2017). Mild dietary restriction is found to extend lifespan in yeast, worms, flies, rats, and monkeys (Colman et al., 2009). It is believed that nutrient sensors and their downstream signaling pathways mediate these changes. Furthermore, the fly gut is also believed to affect fly lifespan as it is both important for nutrient absorption and defense against harmful microbes and toxins. As flies age, their gut becomes more prone to dysplasia and over-proliferation. Differentiation of stem cells is more likely to go wrong in old age, and more so in female flies (Wang and Jones, 2011). However, dietary restriction seems to have no substantive effects on senescence of behaviors such as olfactory avoidance (Bhandari et al., 2007). Additionally, there is a plethora of research highlighting other conserved metabolic processes and signaling pathways in aging, such as the insulin or insulin growth factor signaling pathway (Chen et al., 1996; Brogiolo et al., 2001; Tatar et al., 2001; Broughton et al., 2005; Toivonen and Partridge, 2009), genes involving oxidative stress (Liguori et al., 2018), and histone deacetylases (Yu et al., 2021), all of which can influence longevity and senescence. This suggests that aging is an evolutionarily conserved process and at the very least, some basic biological mechanisms that regulate aging are conserved between humans and flies.

Flies exhibit aging behavior comparable to human aging, such as the decline of locomotor activity, cognition, sensory responses, sleep, reproductive behavior, and the immune system (Iliadi and Boulianne, 2010) but over a much shorter time frame than human (Figure 2). Healthy fruit flies raised at 25°C have a median lifespan of 70–80 days and a maximum of around 100 days depending on the

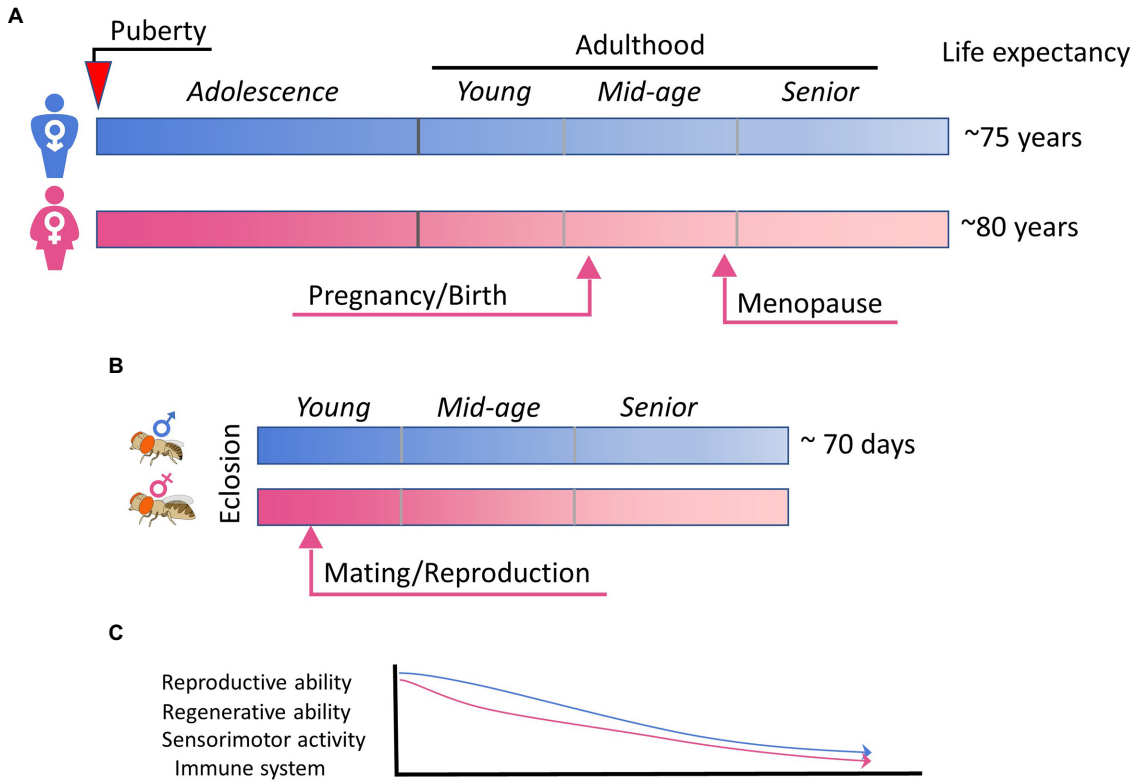

FIGURE 2
Aging and sex differences in human and fly. **(A)** A simplified summary of sex maturation, reproduction, and aging in human. Key events leading to physical, physiological, and hormonal changes in male and female are indicated arrows. **(B)** The schematic summary of the short lifespan of *Drosophila* flies, in which mating is known to trigger extensive changes in females. **(C)** A schematic plot highlighting the aging-associated functional declines that are common for human and fly, sexually dimorphic, and influenced by sex hormones and/or reproductive status.

genetic background. As flies age, their exploratory locomotor activity (Le Bourg, 1983) and negative geotaxis behavior (Grotewiel et al., 2005) gradually decrease. However, there seems to be no significant correlation between the amount a fly moves and their eventual lifespan (Lints et al., 1984; Le Bourg, 1987). There are significant sex differences in aging-related changes to locomotor activity; female motor activity declines earlier in life than male (Lints et al., 1984). Aging also affects sensory responses and learning behaviors such as those seen in olfactory avoidance in flies (Tamura et al., 2003). Additionally, circadian rhythm changes accompany aging in flies. The decrease in circadian rhythm intensity and fragmentation in sleep–wake patterns is conserved across different species (Koh et al., 2006). Altering sleep can decrease lifespan and spur senescence (Bushey et al., 2010). Sexual reproductive behavior is also closely tied with aging, where aging is generally associated with decrease in reproductive behavior and fecundity (Toivonen and Partridge, 2009). Increasing sexual activity decreases lifespan in both female (Partridge et al., 1987) and male flies (Partridge and Farquhar, 1981), whereas decreasing reproductive behavior can increase lifespan and resistance to environmental stress (Yamamoto et al., 2013). Finally, fly immune function shows age-related decline, like that seen in humans and other vertebrates. Unlike the vertebrate immune system, which consists of both innate and adaptive or acquired immunity (Vivier and Malissen, 2005), *Drosophila* only have innate immunity wherein pathogens are recognized using innate receptors that trigger activation of downstream signaling pathways and immune responses (Hoffmann, 2003). Aging eventually leads to a reduced ability to combat disease and infections. Interestingly, immune response genes are often found to be upregulated with age in *Drosophila* transcriptome studies (Pletcher et al., 2002; Zerofsky et al., 2005; Ramsden et al., 2008; Carlson et al., 2015; Kubiak and Tinsley, 2017), which results in a higher expression of antimicrobial peptides (AMPs). This age-related increase in AMP expression has been shown to be tightly linked to intestinal barrier dysfunction (Rera et al., 2012). The stronger net response to infections in older flies is assumed to stem from the fact that younger flies readily rid the body of the infection, whereas microbes in older flies live longer and thus continuously activate the immune system. In line with this, effects of chronic NF-κB signaling are associated with age-related neurodegeneration in the *Drosophila* brain and nervous system (Petersen and Wassarman, 2012), and interventions that reduce the age-associated dysregulation of NF-κB signaling extend lifespan (Guo et al., 2014). Interestingly, TBI in *Drosophila* has been shown to cause the acute upregulation of AMPs and activation of the NF-κB pathway (van Alphen et al., 2022), suggesting the involvement of the immune pathway in injury responses as well as intersection with the aging pathway. In humans, chronic inflammation is suggested to be one of the most important causes of post-traumatic neurodegeneration (Faden and Loane, 2015). Notably, there are also sex differences in immune senescence (Kubiak and Tinsley, 2017); in males this occurs principally due to age-related deterioration in barrier defenses, whereas in females

there are less changes related to barrier defense and more related to decreases in innate immunity.

The advantages of using flies in aging research are obvious; most prominently, the fly's short lifespan and genetic tractability. Disadvantages include difficulty in characterizing aging behaviors and the fact that flies do not have complex socioeconomical pressure. We also do not know what flies really die of (Piper and Partridge, 2018), though research in tissue and metabolic constraints are beginning to suggest some mechanisms. There are a handful of *Drosophila* studies of TBI that examine age as a risk factor for neurodegeneration that not only recapitulate injury-induced phenotypes but also reveal aging-related vulnerabilities. Studies using the HIT model (Katzenberger et al., 2013, 2015a) examined multiple age groups ranging from 0 to 28 days of age and found that age-associated processes lower the primary injury threshold of death within the first 24 h after injury. They also found that aging exacerbated injury-induced neurodegeneration in the flies that survived 14 days following injury. The piezo-electric actuator model (Saikumar et al., 2020, 2021) has been utilized to examine chronic time-points (up to 28 days after injury) in which chronic activation of the immune system promotes neurodegeneration after injury at a young age. In a similar vein, Behnke et al. (2021) examined the lifelong sensorimotor behaviors and brain structure after the flies receive mild head trauma at young age (3 day), and found that immediate elevation of neuronal activity contributes to chronic neurodegeneration. Future studies may examine intersections of mechanisms of aging and mTBI to better understand the molecular networks that connect aging and accelerated progression of long-term brain deficits after mild head trauma.

Sex dimorphism in *Drosophila* and neurodegeneration

Fruit flies are morphologically characterized as either male or female. In similar fashion to humans, sex determination in *Drosophila* is governed by the number of X chromosomes in the fertilized egg (Bridges, 1916). Two X chromosomes gives rise to a female fly, whereas XY gives rise to a male fly. The downstream pathways that determine sex dimorphism differ quite a bit between human and fly (Heller, 2010), though there are remarkable parallels when considering sex-specific variables such as the innate immune system and reproduction. Here, we will only discuss *Drosophila* pathways or mechanisms like those in humans which may contribute to sex differences in vulnerability to neurodegeneration.

In our previous discussion of possible sex-related mechanisms that affect after injury response, we highlighted the immune system as a mediator of later onset of neurodegeneration and a source of sex difference. In flies, sex differences exist in the immune system. Many of the genes in the innate immune pathways are found on the X chromosome and exhibit sex-specific induction following infection (Taylor and Kimbrell, 2007; Hill-Burns and Clark, 2009) as in the activation of Toll and immune deficiency signaling. Both of these signaling pathways are highly homologous to mammalian immune pathways (Kimbrell and Beutler, 2001). Though flies lack microglia, they do have ensheathing glia which are responsible for engulfing cellular debris following injury (Doherty et al., 2009). Unlike humans and other mammals, *Drosophila* do not have sex gonadal hormones such as estrogen, progesterone, and testosterone associated with sexual maturation and hormonal cycles associated with reproductive status. It is currently unclear whether sex-specific hormones in the fly, i.e., ecdysone, affect the immune system in the adult fly, though ecdysone is known to affect immunity during development (Meister and Richards, 1996; Flatt et al., 2008; Rus et al., 2013; Tan et al., 2014). Additionally, the fly immune system is known to be activated by TBI, though findings only pertain to male flies (Katzenberger et al., 2015b; Byrns et al., 2021). Sex differences in the immune response warrant the inclusion of female flies in the investigation of immune contributions to TBI-induced neurodegeneration.

Reproductive status is an important factor for sex differences in neurodegeneration due to remarkable changes in metabolism, fluctuations in female sex hormones, and alterations in the immune system. Here we discuss the fly post-mating response and its role in affecting the sexually dimorphic injury response. *Drosophila* are considered adults and are fertile within hours after eclosion. In humans, the reproductive system fully matures during puberty. In the female fly, mating elicits two types of major changes: increase in egg-laying and reduction in mating receptivity (Liu and Kubli, 2003). Other components of the post-mating response include changes in metabolism (increased intake of food (Carvalho et al., 2006), changes in food preferences for yeast and salt (Ribeiro and Dickson, 2010; Vargas et al., 2010; Walker et al., 2015), decreased intestinal transit (Cognigni et al., 2011; Apger-McGlaughon and Wolfner, 2013), changes in sleep–wake cycles (Isaac et al., 2010; Garbe et al., 2016; Dove et al., 2017) and altered immune system (Peng et al., 2005a; Short and Lazzaro, 2010; Short et al., 2012; Schwenke and Lazzaro, 2017). Some of these changes are mediated by ecdysone, a fly hormone structurally similar to estrogen in humans (Mangelsdorf et al., 1995). The seminal protein sex peptide (SP) from male flies plays a crucial role in eliciting the post-mating response in female flies. SP is an accessory fluid protein (Acps) produced by male flies in their accessory glands (Colucci et al., 2006; Jang et al., 2018) and received by female flies during copulation. A sustained post-mating response requires SP (Peng et al., 2005a,b; Apger-McGlaughon and Wolfner, 2013; Avila et al., 2015), and SP binding to its receptor is responsible for the switch in mating status in females. Overall, post-mating responses are considered evolutionarily beneficial as they maximize reproduction, though that may come with a cost; mating with males or just continuous exposure to male flies can significantly reduce lifespan in female fruit flies (Partridge et al., 1987; Fowler and Partridge, 1989). In humans, pregnancy elicits drastic changes to the body. These include changes in metabolism (Robinett et al., 2010), immune system (Aghaeepour et al., 2017), neurobiology (Hoekzema et al., 2017), and hormone fluctuations (Brunton and Russell, 2010), which are vital for the maintenance of pregnancy. Other sex-related changes in a healthy female's life such as puberty, menstruation, or menopause elicit changes on a much smaller scale when compared to pregnancy (Cognigni et al., 2011; Apger-McGlaughon and Wolfner, 2013). A higher number of pregnancies is believed to be linked to elevated risk of AD related dementia (Isaac et al., 2010; Garbe et al., 2016; Dove et al., 2017), suggesting that factors associated with reproductive status, such as higher levels of female sex hormones, could play a role in AD neurodegenerative pathology. Some other studies show contradictory findings, where pregnancy and reproduction can be neuroprotective (Fox et al., 2018). This highlights the need for animal studies with better controls of hormonal cycles and increased sampling points.

Metabolic changes are known to affect neurodegeneration (Muddapu et al., 2020). In all animals, the production of progeny requires a significant energy investment. Metabolic changes during pregnancy are important for ensuring a healthy development and delivery of offspring. Not only does the need for substrates such as glucose, lipids, and proteins increase, the requirement for water, iron and calcium also change (Soma-Pillay et al., 2016). Similarly, female flies alter aspects of nutrient intake and digestion to meet the energy demands of egg production and maintain energy homeostasis. Not only does food intake increase by almost double (Carvalho et al., 2006), but food preferences are also shifted towards salt (Walker et al., 2015) and yeast (Ribeiro and Dickson, 2010; Vargas et al., 2010; Walker et al., 2015), which increases reproductive output. It is likely that changes in metabolism are only one of several changes induced by reproduction that may affect the fly's vulnerability to developing neurodegeneration.

Along with metabolic changes, the female fly also experiences hormonal alterations after mating, which may affect neurodegeneration. Sex peptide is thought to induce release of steroid hormones, including Juvenile Hormone (JH) and ecdysone (20HE), due to the increase of 20HE titers in ovaries and a similar increase in the hemolymph of mated flies (Ameku and Niwa, 2016). Though the role of steroid hormones in developmental stages is better understood (Ameku and Niwa, 2016; Ahmed et al., 2020; White et al., 2021) than their role in reproduction, we now know that ecdysone produced by the ovaries is required for female fertility (Garen et al., 1977). Additionally, SP elicits steroid signaling from the ovaries to the gut to promote enlargement of the abdomen (Ameku and Niwa, 2016; Ahmed et al., 2020; White et al., 2021) and intestinal stem cell proliferation (Ahmed et al., 2020; Zipper et al., 2020). Although these transformations augment fecundity of the female fly by increasing energy uptake, they also increase female susceptibility to age-dependent tumors and thus potentially affecting overall health and reducing lifespan (Regan et al., 2016). In a similar vein, several studies in middle and older-age women suggest that high levels of overall hormone exposure accelerate brain aging and atrophy (Resnick et al., 2009; Kantarci et al., 2016; de Lange et al., 2020). However, there are other studies that suggest a protective role of female sex hormones (Ha et al., 2007; Song et al., 2020). The mechanisms underlying the effects of sex hormones on neurodegeneration are currently unclear, but future work using *Drosophila* and other animal models may help dissect this complex issue.

How does the sexually dimorphic immune system affect neurodegeneration? Changes in the immune system are known to alter trajectories of neurodegenerative disease such as CTE (Mason and McGavern, 2022). Sex differences in the immune system is evolutionarily conserved across many species and is strongly affected by age (Klein and Flanagan, 2016). Besides genetic factors, environmental factors and hormone cycles can also contribute to varying immune regulation pathways between the two sexes. In both flies and humans, immune responses are typically higher in females than in males (Klein and Flanagan, 2016). Within the female sex, reproductive status can drastically alter the immune system. In female flies, mating and exposure to SP reliably induces changes of the innate immune defense system (Short and Lazzaro, 2010; Short et al., 2012; Schwenke and Lazzaro, 2017). Female flies suffer a reduced ability to defend against certain bacterial pathogens after mating (Short and Lazzaro, 2010). Interestingly, when compared to virgins and females mated to sex peptide-less and sperm-less males, mated females exhibit lower survival rate and AMP expression. Females that fail to produce eggs demonstrate no effect of mating on immune defense (Short et al., 2012). This process has been shown to be mediated by steroid hormones. In virgin females, application of JH can phenocopy the immunosuppression observed in mated females while ablating JH and its downstream receptors induces virgin levels of resistance to bacterial infection (Schwenke and Lazzaro, 2017). This reproduction/ immune system tradeoff is seen also in humans (Westendorp et al., 2001; Abu-Raya et al., 2020). Notably, the risk and severity of certain infections such as urinary tract infections (Schnarr and Smaill, 2008) and pneumonia (Sheffield and Cunningham, 2009) are increased. This suggests that like in the fly, some energy from maintaining the mother's immune system may be diverted to maintaining pregnancy and health of the fetus.

Finally, reproduction also alters plasticity at the synaptic level. The reproductive status of the female fly affects their aversive long-term memory, suggesting long-lasting changes in the function of specific neurons with homologous modulatory functions to the hypothalamus (Scheunemann et al., 2019). Similarly, human pregnancy is associated with various changes to functional and structural plasticity such as increase in neurogenesis, remodeling of synaptic morphology, and alterations in connectivity (Hoekzema et al., 2017; Barba-Müller et al., 2019; Hoekzema et al., 2022). The impairment of synaptic plasticity has been implicated in the development of neurodegenerative diseases such as AD (Sheng et al., 2012). Therefore, it is likely that reproduction-related synaptic changes contribute to female vulnerability to developing neurodegenerative conditions.

Sex differences, particularly those related to reproduction, can affect response to neurodegenerative stimuli such as mTBI. In both fly and humans, we expect changes in metabolism, hormone levels, and immune responses to affect neurodegeneration after mTBI, though the specific mechanisms remain to be elucidated. To date, *Drosophila* studies overwhelmingly only use male flies or do not consider reproductive status. In a severe TBI model, the HIT model, no sex differences have been found in terms of survival (Katzenberger et al., 2015a). However, female flies show more gene transcript changes than males, particularly immune response genes and mitochondrial genes (Shah et al., 2020). The same research group also separately found sex differences in survival following injury in tau knock-out flies, though it appears that tau is not involved (Shah et al., 2021). In congruence, Behnke et al. found that behavior dysfunction and pathology after mild injury was more pronounced in female flies than male flies when injured at 3–5 days of age (Behnke et al., 2021). Therefore, there is a great potential in using the fly model to study sex as a contributing factor to neurodegenerative mechanisms. Advantages of using flies include short life cycles and easy manipulation of the reproductive process using available genetic tools. Disadvantages include some disparities between the species in terms of sex differences, including a limited number of *Drosophila* orthologs of human hormonal genes.

Summary and future studies

The complex nature of the inquiry into neurodegenerative diseases requires a variety of animal models and innovative

strategies. Studying mTBI-induced neurodegeneration is additionally challenging because even though animals or human patients can recover from acute concussive symptoms in the short term, other symptoms such as impaired cognition, depression, and dementia can appear much later in life. To gain mechanistic understanding of disease onset, progression, and contributing factors, one must venture beyond population observations and into cellular and molecular manipulations. Longitudinal human and animal studies that follow the entire disease progression may be several years to several decades in length and can be very costly and difficult to carry out, which contributes to the current dearth of mechanistic findings. When compared with other animal models, *Drosophila* possess unique advantages such as a short lifespan, simple but conserved nervous system, a variety of genetic tools that allows for target-specific manipulation and examination, and ease and affordability of care. In this review, we have highlighted several *Drosophila* mTBI models, which provide the groundwork from which mechanistic insights can be obtained. Several of these models inflict mild, non-invasive, and headfirst injuries that have been shown to elicit neurodegeneration later in life, phenocopying observations from human populations. Future work using these models or modifications of these models can fill in the gaps in our understanding of neurodegeneration caused by exposure to mTBI. Finally, we highlight the potential for *Drosophila* models to be utilized in investigating age and sex as contributing factors to mTBI-induced neurodegeneration. Vulnerable populations like the aging population and females may require different treatment and care after mTBI exposure. *Drosophila* mechanisms of aging are highly conserved with humans; therefore, it is highly likely that the mechanisms related to aging-induced vulnerability to neurodegeneration are also conserved. Furthermore, though *Drosophila* and human sexual dimorphism are very different, the two species share remarkable similarities in reproductive pathways, which are suggested to be implicated in neurodegeneration following mTBI. Further investigation of female vulnerability is warranted. Using available *Drosophila* models, we can identify possible therapeutic targets that are tailored for populations that are at higher risk of developing neurodegeneration. Once possible therapeutic targets are found, *Drosophila* also allows for high throughput genetic screening and drug screening before moving on to higher-order preclinical and clinical studies.

Author contributions

CY led the team effort in composing and completing this review article. CY and JB contributed a substantial amount of writing. KH and JZ contributed to the writing and editing. All authors contributed to the article and approved the submitted version.

Funding

This work is supported in part by research grants from National Institutes of Health to JZ (GM083889 and MH129019), a Pilot grant to JZ from the NIH-funded Emory Specialized Center of Research Excellence in Sex Differences (U54AG062334), a Diana Jacobs Kalman Scholarship from American Federation for Aging Research (AFAR) to CY, and a T32 training grant from National Institute of General Medicine to Emory BCDB program (1T32GM135060-01).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1150694/full#supplementary-material

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frontiers | Frontiers in Neuroscience

TYPE Review
PUBLISHED 11 April 2023
DOI 10.3389/fnins.2023.1166973

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 15 February 2023
ACCEPTED 17 March 2023
PUBLISHED 11 April 2023

CITATION
Ghalayini J and Boulianne GL (2023) Deciphering mechanisms of action of ACE inhibitors in neurodegeneration using *Drosophila* models of Alzheimer's disease. *Front. Neurosci.* 17:1166973. doi: 10.3389/fnins.2023.1166973

Deciphering mechanisms of action of ACE inhibitors in neurodegeneration using *Drosophila* models of Alzheimer's disease

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Alzheimer's disease (AD) is a devastating neurodegenerative disorder for which there is no cure. Recently, several studies have reported a significant reduction in the incidence and progression of dementia among some patients receiving antihypertensive medications such as angiotensin-converting enzyme inhibitors (ACE-Is) and angiotensin receptor blockers (ARBs). Why these drugs are beneficial in some AD patients and not others is unclear although it has been shown to be independent of their role in regulating blood pressure. Given the enormous and immediate potential of ACE-Is and ARBs for AD therapeutics it is imperative that we understand how they function. Recently, studies have shown that ACE-Is and ARBs, which target the renin angiotensin system in mammals, are also effective in suppressing neuronal cell death and memory defects in *Drosophila* models of AD despite the fact that this pathway is not conserved in flies. This suggests that the beneficial effects of these drugs may be mediated by distinct and as yet, identified mechanisms. Here, we discuss how the short lifespan and ease of genetic manipulations available in *Drosophila* provide us with a unique and unparalleled opportunity to rapidly identify the targets of ACE-Is and ARBs and evaluate their therapeutic effectiveness in robust models of AD.

KEYWORDS

renin angiotensin system, Alzheimer's disease, neurodegeneration, *Drosophila*, amyloid, angiotensin converting enzyme

Introduction

AD is a devastating neurodegenerative disorder that accounts for 70–80% of dementia cases worldwide (Barker et al., 2002). Dementia is a general term used to describe symptoms associated with a decline in cognitive functions including memory, thinking and social abilities that are distinguishable from normal ageing. Currently, around 55 million people have dementia worldwide and as the aging population continues to grow, this number is estimated to triple to 150 million by 2050 (Nichols et al., 2022). In its initial stages, AD is characterized by subtle changes in cognition. However, as the disease progresses, individuals present more severe symptoms including extreme memory loss, impaired spatial and temporal orientation, language disturbances, behavioral changes and motor deficits (Castellani et al., 2010). Ultimately AD renders patients unable to carry out simple day-to-day activities. These symptoms arise from extreme neuronal deterioration mainly in areas of the brain responsible for cognition such as

the hippocampus and cortex (Castellani et al., 2010; Holtzman et al., 2011).

AD was first characterized over a century ago, by psychiatrist and neuropathologist Alois Alzheimer who described a 51-year-old patient with memory and language deficits as well as severe disorientation and hallucinations (Graeber et al., 1997; Goedert and Ghetti, 2007). Although these symptoms matched the definition of what was then called dementia, it was atypical for them to be present in someone so young. A post-mortem autopsy revealed various abnormalities in the patient's brain including extensive atrophy of the cerebral cortex and abnormal protein deposits inside and between nerve cells (Graeber et al., 1997; Goedert and Ghetti, 2007). These protein deposits, known as amyloid plaques and neurofibrillary tangles (NFTs), soon became the pathological hallmarks of AD.

Amyloid plaques in the extracellular matrix of brain tissue are composed of amyloid-β (Aβ) peptides (Glenner and Wong, 1984). These peptides are derived from a precursor transmembrane protein known as APP, found mainly in neurons (Kang et al., 1987; van der Kant and Goldstein, 2015). APP undergoes sequential processing by enzyme complexes, β-secretase and γ-secretase, to produce a C-terminal fragment (C99) followed by an Aβ peptide, respectively (Vassar et al., 1999; Haapasalo and Kovacs, 2011; van der Kant and Goldstein, 2015). C99 may be cleaved at different sites by γ-secretase thus, amyloid-β peptides vary in size ranging from 38 to 43 residues, with Aβ40 and Aβ42 being the most prominent species (Takami et al., 2009). Aβ40 and Aβ42 only differ by two residues however, Aβ42 is more prone to aggregation and is found enriched in amyloid plaques (Miller et al., 1993; Iwatsubo et al., 1994; Mak et al., 1994). Furthermore, Aβ42 has a much higher level of neurotoxicity compared to Aβ40. The ratio of Aβ42/Aβ40 is often used as a reference to AD pathogenesis (Kuperstein et al., 2010). However, Aβ levels and deposition appear to plateau at the onset of clinical symptoms and correlate poorly with the degree of cognitive impairment in the dementia phases of AD (Masters et al., 2015). In contrast, measurements of biomarkers for neurofibrillary tangles (NFTs) strongly correlate with disease progression and severity at the onset of clinical manifestation (Masters et al., 2015).

NFTs are intraneuronal aggregates mainly composed of hyperphosphorylated Tau, a microtubule associated protein that interacts with tubulin to support its assembly into microtubules and to stabilize its structure (Barbier et al., 2019). Tau is also known to promote neurite outgrowth and axonal transport (Barbier et al., 2019). However, in AD, tau proteins are hyperphosphorylated, which causes them to assemble into filamentous bundles (Busche and Hyman, 2020). As a result, the structural integrity of microtubules is damaged, impairing axonal transport and neurite development, which leads to neuronal cell death (Busche and Hyman, 2020). Accordingly, NFTs and phosphorylated tau levels correlate strongly with the degree of neurodegeneration in AD patients and are predictive of disease severity (Masters et al., 2015).

The relationship between Aβ and NFTs is complex. Findings show high Aβ load - either as plaques or non-fibrillar, soluble, oligomeric forms, precede NFT formation and suggest that Aβ initiates AD in part by acting on pathophysiological mechanisms that lead to tau-hyperphosphorylation and aggregation into NFTs (Busche and Hyman, 2020). Consistent with this model, animal studies have demonstrated that Aβ peptides promote tau-hyperphosphorylation resulting in its aggregation into NFTs (Götz et al., 2001; Gomes et al., 2019). This concept is consistent with the amyloid-cascade hypothesis whereby Aβ is the primary initiator of AD (described below).

An understanding of how Aβ peptides lead to senile plaque formation was revealed through familial genetic studies of AD, which identified dominant mutations in APP and presenilin 1 and presenilin 2; the core catalytic components of γ-secretase (Holtzman et al., 2011; van der Kant and Goldstein, 2015). These mutations were shown to profoundly alter APP metabolism, increasing and favoring the production of aggregation prone Aβ species such as Aβ42 (Holtzman et al., 2011; van der Kant and Goldstein, 2015). Ultimately these studies led to the development of the amyloid cascade hypothesis of AD pathogenesis (Hardy and Selkoe, 2002). This long-standing hypothesis states that neurotoxic forms of β-amyloid peptides initiate AD pathology and precede all other disease hallmarks. However, familial AD (FAD), also known as early-onset AD (EOAD), accounts for only 1–5% of all cases (Holtzman et al., 2011). Most cases of AD fall under the "sporadic" late-onset form (LOAD), which lacks clearly defined genetic factors. However, genome-wide association studies (GWAS) have identified certain genetic risk factors including the apolipoprotein E gene (apoE) (Bertram and Tanzi, 2009). Individuals carrying the apoE e4 allele have up to a 12-fold increased risk of developing the disease (Bertram and Tanzi, 2009). As more genetic risk factors are discovered, it becomes increasingly clear that AD is a complex and multifactorial disorder.

Neuroinflammation has also emerged as a significant factor in the pathogenesis of AD, alongside amyloid plaques and NFT (Newcombe et al., 2018). Neuroinflammation refers to an inflammatory response that occurs within the CNS and involves the production of cytokines, chemokines, reactive oxygen species and secondary messengers. Microglial cells are the primary players in neuroinflammation and act as the resident macrophages of the CNS, serving several roles including acting as a first line of immune defence against brain injury or infection (Calsolaro and Edison, 2016). However, it has been suggested that the activation of microglia and the subsequent release of pro-inflammatory factors can cause significant neuronal damage. Although microglial activation can be protective within the CNS, if the stimulus for activation is not resolved, chronic inflammation can develop and contribute to neuronal dysfunction, injury and loss (Calsolaro and Edison, 2016; Fakhoury, 2018). Post-mortem studies of AD patient brains have revealed activated microglia co-localized with amyloid plaques. Amyloid peptides, fibrils and APP have also been shown to activate microglia, triggering an inflammatory response and the release of neurotoxic cytokines (Calsolaro and Edison, 2016; Fakhoury, 2018). PET studies have demonstrated a correlation between microglial activation and amyloid load in AD patients. Moreover, neuroinflammation has been detected before the onset of dementia, suggesting its early occurrence in AD pathology. Finally, GWAS studies have identified a relationship between components of the innate immune system and the incidence of sporadic AD, further supporting a link between the immune system and AD (Newcombe et al., 2018).

Despite distinct etiologies of sporadic and familial forms of AD, both exhibit comparable clinical manifestations, including rates of disease progression and similar biomarker profiles. As a result, genetic factors known in FAD are commonly employed to model the disease in research aimed at understanding its underlying causes. Moreover, these models are widely utilized in preclinical studies to evaluate the effectiveness of potential therapeutics.

Recently, there has been a significant advancement in the treatment of AD with the approval of two monoclonal antibodies, aducanumab and lecanemab, as potential therapies for AD (Tampi et al., 2021; Reardon, 2023). These drugs are designed to target and clear β-amyloid plaques. However, while clinical studies have demonstrated their efficacy in reducing Aβ levels, their impact on cognitive improvement has been limited (Tampi et al., 2021; Reardon, 2023). This suggests that targeting Aβ and tau alone may not be sufficient to effectively treat AD and that a more comprehensive approach that targets multiple pathways may be more effective. Therefore, ongoing research to develop therapies that target other aspects of AD is crucial. One promising avenue of research revolves around the potential benefits of drugs that target the renin angiotensin system (RAS).

RAS in AD

Systemic RAS (or peripheral RAS) is a hormonal system that plays a critical role in regulating blood pressure and fluid balance in the body (Yim and Yoo, 2008; Wu et al., 2018). It is made up of several hormones and enzymes, including renin, angiotensinogen, angiotensin (Ang) I, Ang II, and angiotensin converting enzyme (ACE). Renin is produced and released by the kidneys in response to low blood pressure or low blood volume and it acts on angiotensinogen (a pro-peptide produced by the liver) to produce Ang I (Yim and Yoo, 2008; Wu et al., 2018). Ang I is then converted to Ang II by ACE, a zinc- and chloride-dependent metallopeptidase, which is expressed in the lungs and other tissues (Yim and Yoo, 2008; Wu et al., 2018). Ang II is a potent vasoconstrictor, exerting its effects by binding to angiotensin II type 1 receptors (AT1Rs), while binding to angiotensin II type 2 receptors (AT2Rs) induces vasodilation (Yim and Yoo, 2008; Wu et al., 2018). Ongoing research of the RAS has led to the discovery of additional components, such as ACE2, a homolog of ACE. ACE2 cleaves Ang I or Ang II into the heptapeptide angiotensin 1–7 (Ang 1–7). This peptide was later found to bind to the Mas receptor (MasR), resulting in vasodilation (Yim and Yoo, 2008; Wu et al., 2018).

A pivotal discovery relating to the RAS, was the development of captopril, the first ACE inhibitor (ACE-I) drug in 1975 (Zheng et al., 2022). Captopril selectively targets ACE by binding to its active site and preventing the formation of Ang II thereby inhibiting the RAS. It marked a significant milestone in the treatment of hypertension paving the way for the development of additional ACE-Is with improved activity and bioavailability (Zheng et al., 2022). In addition, a new class of drugs known as angiotensin receptor blockers (ARBs), which selectively inhibit Ang II by competitive antagonism of the AT1Rs, were also developed (Barreras and Gurk-Turner, 2003). These drugs have proven to be clinically relevant in the treatment of various cardiovascular conditions, including hypertension, heart failure, diabetic nephropathy and continue to be an important therapeutic option for patients today (Barreras and Gurk-Turner, 2003; Zheng et al., 2022).

Well after its discovery, it became evident that the RAS system is also expressed in numerous organs including the brain (now referred to as local RAS) and possesses additional functionalities revealing its degree of complexity (Paul et al., 2006). Local synthesis of cerebral RAS is necessary due to the blood–brain barrier (BBB) preventing peripheral RAS components from accessing most regions of the brain (Jackson et al., 2018). Astrocytes are the primary source of angiotensinogen, which is constitutively secreted and cleaved into various neuroactive peptides (Jackson et al., 2018). As previously described and illustrated in w, renin converts angiotensinogen to Ang I, which is further processed by ACE to produce Ang II. Ang II is the main effector protein that binds to AT1R or AT2R. Ang II can also be further processed into Ang IV by aminopeptidases (AP-A and AP-N), which binds to AT4R (Jackson et al., 2018). Alternatively, ACE2 converts Ang II to Ang 1–7, which binds to MasRs. Ang 1–7 can also be produced through first processing of Ang I by ACE2 to produce Ang 1–9 and then by ACE (Jackson et al., 2018). AT1R and AT2R are present in neurons, astrocytes, oligodendrocytes and microglia of the cortex, hippocampus and basal ganglia both on the cell surface and intracellularly at mitochondrial and nuclear levels allowing for the regulation of oxidative stress, transcription and trafficking of receptor types (Jackson et al., 2018). Activation of AT1R has been associated with deleterious effects such as promoting neuroinflammation, oxidative stress and neuronal cell death (Jackson et al., 2018; Cosardereliogluet al., 2020). In contrast, AT2R appears to be neuroprotective, counteracting AT1R's effects by inhibiting neuroinflammation, reducing oxidative stress and influencing neuronal regeneration. Of note, while Ang II can bind both receptors, ACE upregulation specifically leads to increased AT1R activation (Jackson et al., 2018). MasR also located in these brain regions are expressed by neurons, astrocytes and microglia, and similar to AT2R, have both antioxidant and anti-inflammatory properties and promote cell survival (Jackson et al., 2018). The expression of AT4R is restricted to neurons localized in the cortex, hippocampus and basal ganglia, where it is believed to induce LTP and mediate learning and memory consolidation (Jackson et al., 2018; Cosardereliogluet al., 2020). The interplay between the receptors and enzymes of RAS in the brain has been suggested to work synergistically and is thought to be crucial in maintaining cognitive balance in a healthy brain. Accordingly, misregulation of RAS has been implicated in pathologies underlying neurodegenerative diseases including AD (Cosardereliogluet al., 2020; Gouveia et al., 2022). Changes in RAS components have been documented in brains of AD patients compared to control individuals (Savaskan et al., 2001). For example, studies have found increased levels of ACE in the hippocampus, frontal cortex and caudate nucleus of AD patients and its activity is increased and correlates positively with parenchymal Aβ load (Arregui et al., 1982; Miners et al., 2008; MacLachlan et al., 2022). While the expression of ACE appears to be upregulated in AD, the opposite has been reported for ACE2. Researchers observed a significant reduction in ACE2 activity in the mid-frontal cortex of AD patients and this reduction was inversely correlated with total β-amyloid and tau load as well as ACE activity (Kehoe et al., 2016). Similarly, a systematic analysis of ACE2 protein expression in different brain regions revealed a downregulation in the basal nucleus, hippocampus and entorhinal cortex, middle frontal gyrus, visual cortex and amygdala of AD patient brains (Cui et al., 2021). However, contrary to these findings, Ding et al. (2021) reported higher ACE2 protein expression levels in hippocampal tissues of AD patients compared to control subjects. Although, in a more recent study that evaluated ACE and ACE2 protein expression and enzyme activity in the frontal and temporal cortex in early AD stages, authors report that both ACE and ACE2 protein level are unchanged and that only ACE enzyme activity was elevated (MacLachlan et al., 2022). ACE and ACE2 act on different axes of the RAS having either

neurotoxic or neuroprotective properties, respectively and an imbalance between these axes may play a role in AD pathogenesis. Taken together, these discrepancies suggest the need for further analysis.

Genetic studies have also implicated ACE as a probable risk factor for AD (Alvarez et al., 1999; Elkins et al., 2004). Recent AD GWAS meta-analyses identified common genetic variants in the *Ace* locus outside of exonic regions, which are associated with an increased risk of AD (Marioni et al., 2018; Kunkle et al., 2019). In the past, genetic studies of *Ace* and AD have focused primarily on a common insertion/deletion (I/D) variant that influences ACE serum levels. Individuals with the D/D haplotype have higher serum ACE levels than those with I/I haplotype (Elkins et al., 2004). Most studies, Kehoe et al. (1999), Narain (2000), Kölsch et al. (2005), and Lehmann et al. (2005) although at times contradictory (Chou et al., 2016), have indicated that individuals carrying an insertion allele are at higher risk of AD than those with the D/D haplotype. It is important to note that ACE serum levels do not reflect ACE enzymatic activity levels. In a more recent genetic study, Cuddy et al. (2020) performed whole genome sequencing to identify rare coding variants in the *Ace* gene associated with AD. They selected one variant (R1297Q) for functional analysis to gain a better understanding of the role that ACE plays in AD. This was achieved by generating knock-in (KI) mice that harbored the cognate mutation, R1297Q, in the murine *Ace* gene. The authors report that while the mutation had no effect on blood pressure and cerebrovasculature, it did result in increased levels of neuronal ACE protein and activity, memory impairment, neuroinflammation and hippocampal neurodegeneration. Moreover, these reported phenotypes were exacerbated in an AD mouse model of amyloidosis. These findings strongly suggest that increased ACE activity is associated with AD pathogenesis.

Following this apparent genetic link between RAS and AD, epidemiological and clinical studies were performed to examine the effects of RAS targeting drugs including ACE-Is and ARBs on the incidence of AD. A retrospective study conducted by Barthold et al. (2018) assessed the risk of developing AD in patients being treated with either RAS targeting or non-RAS anti-hypertensive medication. The study findings indicated that RAS-acting drugs were more effective in reducing the risk of AD development compared to non-RAS acting drugs. The study also compared the effects between ARBs and ACE-Is and found that ARBs demonstrated superior preventative efficacy against AD than ACE-Is. These findings are in line with a previous prospective cohort study conducted by Li et al. (2010) that reported reduced incidence and progression of AD in participants taking ARBs compared to those taking other cardiovascular drugs and a nested case–control analysis following AD patients who were prescribed different anti-hypertensive drugs including ACE-Is and ARBs (Davies et al., 2011). Specifically, this study found a 53% decrease in AD incidence for ARB use and a 24% decrease in AD incidence from ACE-I use (Davies et al., 2011). Furthermore, ACE-Is and ARBs have also been evaluated for their ability to reduce the rate of cognitive decline and improve cognitive performance in AD patients. From a cross-sectional and retrospective cohort study of an elderly population, Hajjar et al. (2005) reported patients taking ARBs had improved cognitive performances while those taking ACE-Is presented a lower rate of cognitive decline. A follow up double-blind randomized clinical trial reported similar findings whereby the ARB, candesartan, was associated with improvement in cognition and outperformed the ACE-I, lisinopril (Hajjar et al., 2012). While studies continue to support the potential benefits of ARBs in AD (Ouk et al., 2021; Deng et al., 2022), a recent clinical study evaluating the effect of 12-month losartan (an ARB) treatment on brain atrophy in patients diagnosed with mild-to-moderate AD reported no significant reduction in brain volume loss (Kehoe et al., 2021). These findings suggest that further studies regarding ARB treatment duration and time of treatment are needed to determine when potential benefits of ARB use may arise. Regarding ACE-Is, their potential benefits for AD are not as clear. Even though a prospective cohort study by Soto et al. (2013) showed a slower rate of cognitive decline in older adults taking ACE-Is, it appears that ACE-Is as a pharmacological class do not reduce the risk of developing dementia or improve cognition in AD (Sink et al., 2009; Solfrizzi et al., 2013; O'Caoimh et al., 2014). However, a closer examination of subgroups of ACE-Is, such as those that can penetrate the BBB vs. those that cannot, imply potential beneficial effects may arise exclusively from drugs that can penetrate the BBB (Ellul et al., 2006; Gao et al., 2013; O'Caoimh et al., 2014). Indeed, in an observational study, O'Caoimh et al. (2014) found a decrease in the rate of cognitive decline in patients with mild to moderate AD receiving BBB-penetrating ACE-Is compared to those on no drug. Finally, it is important to note that the beneficial effects of ACE-Is and ARBs remain the same even after studies adjusted for blood pressure or hypertension suggesting that the beneficial effects are independent of blood pressure or hypertension regulation (Hajjar et al., 2008). While the precise mechanisms by which ACE-Is and ARBs exert their effects in AD remain unclear, their promise as potential therapeutics has inspired researchers to elucidate their mechanisms of action utilizing *in vivo* model systems.

Several studies have now examined the effects of ARBs on cognition animal models, as shown in Table 1. While the majority of studies have demonstrated that ARBs can improve learning and memory in AD mouse models (Wang et al., 2007; Takeda et al., 2009; Ongali et al., 2014; Royea et al., 2017; Torika et al., 2017) others have failed to demonstrate any or have shown limited beneficial effects (Papadopoulos et al., 2016; Trigiani et al., 2018). Nonetheless, improvements in memory performance and retrieval, spatial learning, and prevention of cognitive deficits have been documented through the use of different ARBs such as losartan, olmesartan, and telmisartan (Wang et al., 2007; Takeda et al., 2009; Ongali et al., 2014; Royea et al., 2017; Torika et al., 2017). These beneficial effects are suggested to result, in part, by the ability of ARBs to reduce β-amyloid load, neuroinflammation and oxidative stress in the brain (Wang et al., 2007; Takeda et al., 2009; Danielyan et al., 2010; Torika et al., 2017, 2018) all of which are neuropathological hallmarks of AD (Krstic and Knuesel, 2012; Cassidy et al., 2020). With the increasing number of studies in this field, it is evident that ARBs have strong therapeutic potential and efforts into revealing their mechanism of action are underway.

However, as summarized in Table 2, the effects of ACE-Is in AD are more ambiguous. Some studies suggest that ACE-Is may be detrimental as they led to an increase in Aβ accumulation in AD mice (Zou et al., 2007; Liu et al., 2019). For example, Liu et al. (2019) reported that treating Tg2576 AD mice with captopril, an ACE-I, for 11 months resulted in increased levels of Aβ42 and β-amyloid plaque deposition in the hippocampus and neocortex. In contrast, other studies have shown that captopril treatment reduced Aβ burden

(AbdAlla et al., 2013; Torika et al., 2016; Asraf et al., 2018). For example, AbdAlla et al. (2013) demonstrated that treating Tg2576 AD mice with captopril for 6 months led to reduced amyloidogenic processing of full-length APP resulting in slower accumulation of Aβ in the hippocampus. However, other studies reported no effect of ACE inhibition on Aβ (Hemming et al., 2007; Dong et al., 2011). These discrepancies make it difficult to conclude whether ACE inhibition may be beneficial in AD. However, an evaluation of the differences in a number of factors across these studies including, method of administration, drug dose and treatment duration may help explain the contrasting results. For instance, Hemming et al. (2007) evaluated the effect of different concentrations of captopril delivered by oral administration in AD mice over 28 days on its ability to inhibit ACE activity in the brain. They found that only high concentrations of captopril, approximately 10 times relative to the highest amount used in therapeutic doses in humans, led to a significant but modest reduction in ACE activity and there was no change in either cerebral Aβ levels or deposition. In a related study by Liu et al. (2019), captopril was administered orally at similar concentrations resulting in a significant reduction in systolic and diastolic blood pressure likely due to inhibition of peripheral ACE. However, no data was provided as to whether there was a significant effect on inhibiting brain ACE that could account for the increased levels of Aβ42 observed by the authors as a result of captopril treatment. In contrast, studies whereby captopril was administered intranasally reported reduced Aβ42 load in hippocampal and cortical areas (Torika et al., 2016; Asraf et al., 2018). It is worth noting that treatment duration also impacts the outcome of captopril treatment. Torika et al. (2016) found that when AD mice were treated with captopril for 3.5 weeks vs. 7 months, no changes in β-amyloid load were observed. However, this was not the case when they administered perindopril, another ACE-I, for a short duration suggesting that the type of ACE-I used could contribute to discrepancies in the literature. Finally, in addition to altering Aβ levels, ACE-Is appear to alter immune responses in brains of AD mice similar to that observed using ARBs (Dong et al., 2011; Torika et al., 2016; Asraf et al., 2018). For example, studies showed that ACE inhibition reduced the level of CD11b, a marker of activated microglial and reactive oxygen species (ROS) suggesting an overall reduction in inflammation (AbdAlla et al., 2013; Torika et al., 2016; Asraf et al., 2018). Altogether, these studies suggest that while ARBs and ACE-Is may have beneficial effects in AD animal models further studies are needed to decipher both the method of delivery and their mechanism of action.

Drosophila as a model for Alzheimer's disease

Drosophila has proven to be an excellent model system to study neurodegenerative diseases. The short life span of flies coupled with powerful genetic approaches has made it possible to generate models of disease that faithfully recapitulate many features observed in patients, including age-dependent neurodegeneration and progressive defects in synaptic plasticity and memory. Once a model has been generated it can also be used to perform genetic screens to identify modifiers of known disease-causing genes or drug screens to identify and evaluate novel therapies. Many of the genes associated with neurodegeneration are also conserved in *Drosophila* including those implicated in AD. For example, *Drosophila* possess a homolog of APP known as APP-like or APPL (Luo et al., 1992). Flies deficient in this gene exhibit a behavioral defect that can be partially rescued by expressing a human APP transgene suggesting functional homology between APP and APPL (Luo et al., 1992). However, APPL lacks the amyloidogenic Aβ peptide sequence at the C- terminus found in human APP and does not appear to be processed *in vivo* as is human APP (Prüßing et al., 2013). Similarly, while Presenilin and the γ-secretase complex are well conserved (Periz and Fortini, 2004) there is no clear homolog of β-secretase in flies (Prüßing et al., 2013).

Several transgenic fly lines based on the expression of human and/or *Drosophila* AD-related genes have been generated and are readily available to study disease processes. One such model is based on co-expression of human β-secretase along with human APP and fly presenilin, both of which possess FAD-linked mutations (Greeve et al., 2004). Co-expression of all three transgenes in the fly eye led to β-amyloid plaque formation and age-dependent neurodegeneration (Greeve et al., 2004) demonstrating that *Drosophila* could be used as a model for AD. Soon after, additional models were generated, whereby transgenic flies expressed different Aβ transgenes in specific tissues. The transgenes varied in several ways, including whether or not they possessed a signal sequence to allow for expression outside cells, the number of Aβ42 copies expressed in tandem, and whether they contained an FAD mutation (Prüßing et al., 2013). All exhibited similar phenotypic defects when expressed in neuronal tissue, including plaque formation, neurodegeneration, as well as motor and cognitive defects (Prüßing et al., 2013). The main difference between the models was the severity of the phenotypes, which often correlated with the levels of Aβ42 expression and the degree of protein aggregation. Transgenic flies that express Tau in neuronal tissue are also available and similarly show robust phenotypes reminiscent of those observed in AD (Prüßing et al., 2013). Functional genomic studies using various AD fly models have also facilitated our understanding of the role of cellular mechanisms including inflammation, oxidative stress, mitochondrial dysfunction and apoptosis in AD pathogenesis (reviewed by Jeon et al., 2020). Finally, the ability to perform large-scale genetic screens together with the availability of RNA interference for all genes annotated in the fly genome, make it possible to identify novel modifiers of Aβ42 and Tau (Jeon et al., 2020) that not only provide insight into the molecular and cellular pathways implicated in AD but also potential novel therapeutic targets for this devastating disease.

Ace and Drosophila AD models

The RAS has been well studied in humans and many mammalian model organisms due to its intricate role in regulating blood pressure. However, components of the RAS have also been found in non-mammalian organisms that lack a closed circulatory system (Fournier et al., 2012). ACE homologs have been identified in several invertebrate organisms, including *Drosophila*. There are six ACE-like factors in *Drosophila*, including Ance, Ance-2,-3,-4,-5, and Acer (Cornell et al., 1995; Taylor et al., 1996; Houard et al., 1998). Of these, only Acer and Ance are believed to be active zinc metallopeptidases as they possess an intact conserved active site motif (HExxH) (Coates et al., 2000). Their catalytic activity was demonstrated through

TABLE 1 Summary of effects of ARBs in mouse models of AD including Aβ levels and deposition, neuroinflammation and oxidative stress and, cognitive deficits.

Mouse model	Drug (*brain penetrant)	Treatment duration and age	Administration route	Dose	Results	References
APP/PSEN1 mice ($APP^{Swedish}/PSEN1^{1dE9}$)	Losartan*	2 months (every other day), starting at 7 months old	Intranasal administration	10 mg/kg	↓ Aβ plaques; ↓ Inflammation	Danielyan et al. (2010)
APP mice ($APP^{Swedish/Indiana}$)	Losartan*	3 months, starting at 4 months old	Drinking water	10 mg/kg/d	↑ Learning and memory; ↓ Inflammation	Royea et al. (2017)
A/T mice ($APP^{Swedish/Indiana}$, active TGF-β1 form)	Losartan*	3 months, starting at 3 months old	Drinking water	10 mg/kg/d	No improvement in learning or memory; no effect on Aβ levels and plaques; no effect on increased inflammatory response	Papadopoulos et al. (2016)
APP mice ($APP^{Swedish/Indiana}$)	Losartan*	3 months, starting at 15 months of age	Drinking water	10 mg/kg/d	↑ Memory; no effect on learning deficit; no effect on Aβ plaques; ↓ oxidative stress	Ongali et al. (2014)
5XFAD Tg mice ($APP^{Swedish/Florida/London}/PSEN1^{M146L, L286V}$)	Candesartan*	8 weeks, starting at 2 months of age	Intranasally	1 mg/kg/d	↓ Aβ plaques; ↓ Inflammation	Torika et al. (2018)
APP mice ($APP^{Swedish/Indiana}$)	Candesartan *	5 months, starting at 3–4 months of age	Drinking water	10 mg/kg/d	Limited cognitive improvement; no effect on Aβ plaques or oxidative stress; ↓ Inflammation	Trigiani et al. (2018)
APP23 mice	Olmesartan	4/5 weeks, starting at 12/13 weeks of age	Oral administration	1 mg/kg/d	↑ Memory and learning; ↓ oxidative stress; no effect on Aβ levels	Takeda et al. (2009)
5XFAD Tg mice ($APP^{Swedish/Florida/London}/PSEN1^{M146L, L286V}$)	Telmisartan*	5 months, starting at 8 weeks of age	Intranasal	1 mg/kg/d	↓ Aβ plaques;↓ Inflammation;↑ learning	Torika et al. (2017)
Tg2576 mice ($APP^{Swedish}$)	Valsartan*	5 months, starting at 6 months of age	Drinking water	10 and 40 mg/kg/d	↑ Memory andlearning; ↓ Aβ plaques	Wang et al. (2007)

Drugs marked with (*) indicate BBB penetrance.

biochemical assays that showed their ability to hydrolyze an ACE synthetic substrate, Hip-His-Leu, which mimics the C-terminal sequence of Ang I, to a similar degree as mammalian ACE (Houard et al., 1998; Coates et al., 2000).

ACE-Is have also been shown to be effective in inhibiting Acer and Ance catalytic activity although both enzymes structurally differ from their mammalian counterparts (Houard et al., 1998; Coates et al., 2000; Bingham et al., 2006; Akif et al., 2012). In mammals, the ACE gene is subjected to alternative splicing that gives rise to two distinct enzymes (Hubert et al., 1991). Somatic ACE (sACE), is widely distributed and contains two active sites (N and C-terminal) while germinal ACE (gACE), is expressed exclusively in the testes and only possesses the second active site (C-terminal) (Hubert et al., 1991; Shen et al., 2008). *Drosophila* Acer and Ance only possess a single catalytic domain (Houard et al., 1998; Coates et al., 2000). The active site of Acer is similar to the N-terminal active site of somatic ACE, whereas the active site of Ance is similar to the C-terminal active site of somatic ACE. Moreover, Acer and Ance lack a C-terminal transmembrane anchor that is found in mammalian ACE and may be proteolytically cleaved to yield soluble enzymes (Houard et al., 1998; Coates et al., 2000). However, both Acer and Ance possess a signal sequence that leads to their secretion outside the cell (Coates et al., 2000; Rylett et al., 2007; Carhan et al., 2011). A comparison between *Drosophila* and human ACE proteins is illustrated in Figure 2.

The first study to suggest a link between ACE and AD in *Drosophila* was a modifier screen aimed to identify genes that either enhanced or suppressed phenotypes resulting from over-expression of *Psn* followed by a secondary screen to determine if any of the *Psn* modifiers could also suppress phenotypes generated from expression of the truncated form of APP, called C99 (van de Hoef et al., 2009). Numerous candidates that suppressed or enhanced both the Psn and C99 phenotypes were identified, including some that had previously been shown to interact with Psn. More importantly, two ACE-like factors were identified: *Acer* and *Ance-5*. *Acer* and *Ance-5* modified Psn-dependent phenotypes while *Ance-5* also modified the C99-dependent phenotype (van de Hoef et al., 2009). This suggested that ACE-like factors might be involved in regulating Psn function and that further characterization of the interaction between psn, APP, and ACE might aid our understanding of AD pathogenesis and the potential of ACE-Is for AD therapeutic development. However, while there is increasing evidence that ACE-Is may be beneficial in AD patients, the mechanisms behind the beneficial effects of the drugs remain poorly understood. One reason is the inability to disentangle their effects on blood pressure from their direct effects on local RAS. As such, *Drosophila*, which does not have a conserved RAS pathway (Salzet et al., 2001; Fournier et al., 2012), provides a unique model to study the relationship between ACE-Is and AD. Toward this goal, Lee et al. (2020) evaluated the effects of ACE-Is and ARBs in fly lines expressing different AD-related transgenes. Using the GAL4/UAS system, fly lines were generated that expressed the following human transgenes: $C99^{WT}$ and $C99^{V717I}$ (a common mutation of APP found in FAD), and Aβ42 in CNS tissue, including the eye and brain.

TABLE 2 Summary of effects of ACE-Is in mouse models of AD including Aβ levels and deposition, neuroinflammation and oxidative stress and, cognitive deficits.

Mouse model	Drug (*brain penetrant)	Treatment duration and Age	Administration route	Dose	Results	Reference
Tg2576 mice ($APP^{Swedish}$)	Captopril*	11 months, starting at 6 months old	Oral administration	30 mg/kg/d	↑Aβ levels and plaques	Zou et al. (2007)
Tg2576 mice ($APP^{Swedish}$)	Captopril*	11 months, starting at 6 months old	Oral administration	30 mg/kg/d	↑Aβ levels and plaques	Liu et al. (2019)
3xTg-AD mice ($APP^{Swedish}$/$PSEN1^{M146V}$/Tau^{P301L}) J20 mice ($APP^{Swedish/Indiana}$)	Captopril*	1 months, starting at 16 months old	Drinking water	2 g/l	No effect on Aβ levels and plaques	Hemming et al. (2007)
PS2APP Tg mice ($APP^{Swedish}$/$PSEN2^{N141I}$)	Perindopril*	1 months, starting at 3 months old	Oral administration	1 mg/kg/d	↑ Memory; No effect on Aβ levels; ↓ Inflammation	Dong et al. (2011)
Tg2576 mice ($APP^{Swedish}$)	Captopril*	6 months, starting at 12 months old	Drinking water	20 mg/kg/d or 25 mg/kg/d	↓ Aβ plaques; ↓ oxidative stress	AbdAlla et al. (2013)
5XFAD Tg mice ($APP^{Swedish/Florida/London}$ /$PSEN1^{M146L, L286V}$)	Captopril*	2 months, starting at 8 weeks old	Intranasal administration	5 mg/kg/d	↓ Aβ plaques; ↓ Inflammation	Asraf et al. (2018)
5XFAD Tg mice ($APP^{Swedish/Florida/London}$ /$PSEN1^{M146L, L286V}$)	Captopril*	3.5 weeks, starting at 3 months old	Intranasal administration	5 mg/kg/d	No effect on Aβ plaques; ↓ Inflammation	Torika et al. (2016)
5XFAD Tg mice ($APP^{Swedish/Florida/London}$ /$PSEN1^{M146L, L286V}$)	Perindopril*	3.5 weeks, starting at 3 months old	Intranasal administration	1 mg/kg/d	↓ Aβ plaques; ↓ Inflammation	Torika et al. (2016)
5XFAD Tg mice ($APP^{Swedish/Florida/London}$ /$PSEN1^{M146L, L286V}$)	Captopril*	7 months, starting at 2 months old	Intranasal administration	5 mg/kg/d	↓ Aβ plaques	Torika et al. (2016)

Drugs marked with (*) indicate BBB penetrance.

TABLE 3 Summary of effects of ACE-Is and ARBs in *Drosophila* models of AD including Aβ levels and deposition, oxidative stress and, cognitive deficits.

Drosophila model	Drug (*brain penetrant)	Treatment duration and Age	Administration route	Dose	Results	Reference
hAPP, hBACE/+; elav-gal4/+	Lisinopril* (ACE-I)	5–7 days, 1 day old	Mixed in food	1 mM	↑ Learning and memory; ↑ climbing ability; ↓ oxidative stress (decrease H_2O_2 in thoracic)	Thomas et al. (2021)
elav-gal4/+; UAS-$APP^{C99V717I}$	Captopril* (ACE-I)	4 weeks, 1 day old	Mixed in food	5 mM	↑ Memory; ↓ cell death; no change in C99 levels	Lee et al. (2020)
elav-gal4/+; UAS-$APP^{C99V717I}$	Losartan* (ARB)	4 weeks, 1 day old	Mixed in food	1 mM	↓ cell death; no change in C99 levels	
elav-gal4/+; UAS-$APP^{Abeta42.B}$	Captopril* (ACE-I)	4 weeks, 1 day old	Mixed in food	5 mM	↑ Memory; ↓ cell death; No effect on Aβ levels and plaques	
elav-gal4/+; UAS-$APP^{Abeta42.B}$	Losartan* (ARB)	4 weeks, 1 day old	Mixed in food	1 mM	↑ Memory; ↓ cell death; No effect on Aβ levels and plaques	

They showed that all of the flies exhibit age-dependent defects, including neuronal cell death and impaired memory, with the mildest defects observed in $C99^{WT}$ flies and the most severe in Aβ42 flies. Importantly, both the cell death and memory defects observed in C99 and Aβ42 flies were suppressed when flies were fed either captopril (an ACE-I) or losartan (an ARB), as summarized in Table 3. Moreover, measurements of Aβ42 levels and plaques in drug treated flies were similar to untreated flies, suggesting that the observed beneficial effects are independent to changes in Aβ42 pathology. This is consistent with findings from some of the mouse studies mentioned previously. Finally, to confirm that ACE-Is work in *Drosophila* in a similar manner to mammals, Lee et al. (2020) evaluated the effects of an *Acer* null mutant on various transgenic AD-related lines mentioned above. They found that a complete loss of *Acer* recapitulated the effects of captopril demonstrating that captopril exerts its effects by specifically inhibiting Acer.

In a similar study, Thomas et al. (2021) aimed to determine whether the administration of lisinopril, an ACE-I, would have beneficial effects in a *Drosophila* model of AD that overexpresses human APP and human β-secretase in CNS tissue. Compared to control flies, these flies displayed deficits in learning and memory as well as in climbing ability, as determined through an aversive phototaxis suppression assay and a negative geotaxis assay, respectively. Upon lisinopril administration, AD flies displayed both

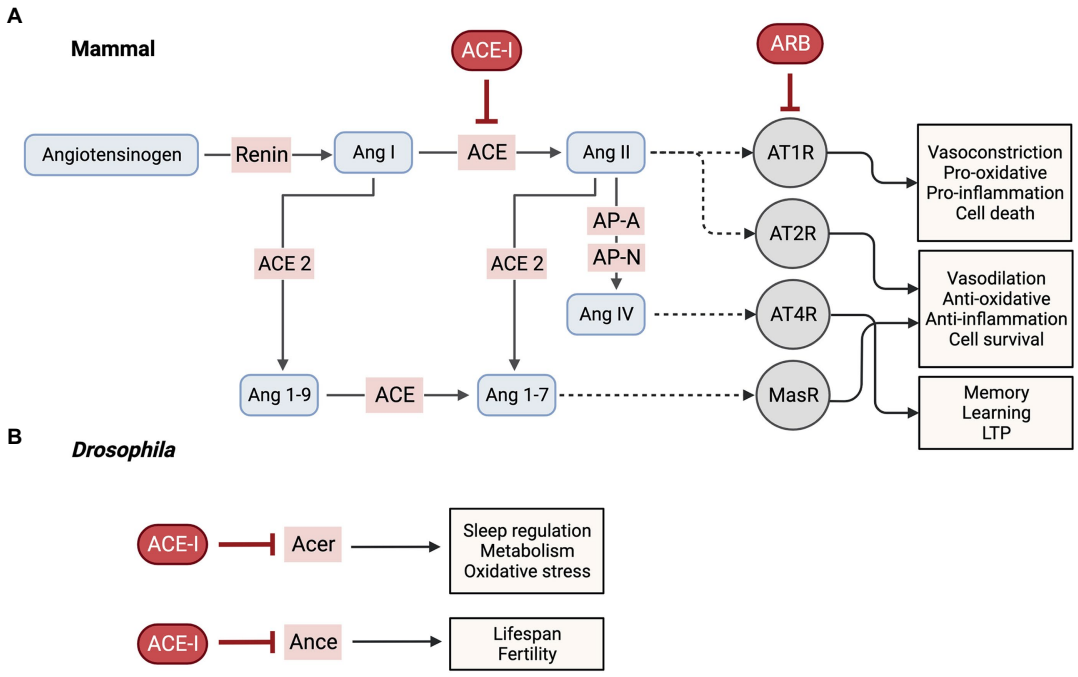

FIGURE 1
RAS in mammals vs. *Drosophila*. **(A)** Mammalian brain RAS pathways and its inhibitors. ACE, angiotensin converting enzyme; ACE-I, angiotensin converting enzyme inhibitor; Ang, angiotensin; AP-A, aminopeptidase A; AP-N, aminopeptidase N; ARB, angiotensin receptor blocker; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; AT4R, angiotensin 4 receptor; LTP, long-term potentiation; MasR, Mas receptor. **(B)** ACE-I inhibit Acer and Ance, the *Drosophila* ACE homologs. Acer, angiotensin-converting-enzyme related; Ance, angiotensin converting enzyme. (Created with BioRender.com).

improved cognition and climbing ability as well as a significant reduction in oxidative stress levels compared to untreated AD flies, as shown in Table 3.

To date, the mechanisms by which ARBs and ACE-Is function to suppress cell death and memory defects in *Drosophila* remain unknown. The effects of losartan are surprising given that their known target in mammals, AT1R, is not conserved in flies (Fournier et al., 2012). These findings suggest that losartan functions through an unknown and potentially novel target in *Drosophila*. This warrants further studies to help elucidate what its target(s) in flies might be. With regards to ACE-Is, Lee et al. (2020) demonstrated that the ability of captopril to suppress AD-related phenotypes in flies could be recapitulated by a null mutation in *Acer* demonstrating that Acer or a downstream effector of Acer, is the target of captopril. However, as previously mentioned, apart from ACE, RAS substrates do not appear to be conserved in *Drosophila* (Fournier et al., 2012) suggesting the existence of novel targets for ACE that extend beyond its conventional role in the canonical RAS. Such targets can be readily identified and validated in flies using a combination of biochemical and genetic approaches. Characterization of these targets will not only reveal the cellular pathways on which Acer acts in *Drosophila* but may also reveal novel roles for mammalian ACE beyond its canonical role in RAS and lead to the development of additional therapies for AD.

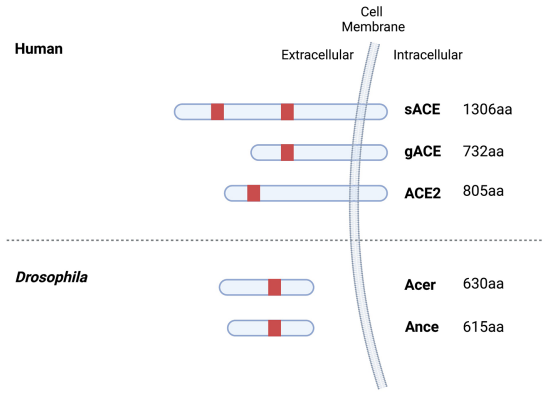

FIGURE 2
ACE family members in humans and *Drosophila*. Ance and Acer are homologous to ACE and share 61% (45% identity, 48% coverage) and 58% (41% identify, 45% coverage) amino acid similarity with ACE. Active site domains containing the conserved catalytic consensus zinc-binding motif (HEXXH) are indicated in red. sACE possess two active protein domains (N- and C- domain) whereas gACE, ACE2, Ance and Acer only have one. gACE is identical to that of the C domain of sACE except for its first 36 residues. Human ACE and ACE2 are integral-membrane proteins whereas *Drosophila* Ance and Acer lack a transmembrane domain. (Created with BioRender.com).

Although the molecular targets of Acer in *Drosophila* are unknown, several studies have begun to elucidate the physiological roles of *Acer* and a closely related gene, *Ance*, either through the use of ACE-Is or genetic nulls (Figure 1B). Such studies may provide insight into how inhibition of Acer using captopril or a null mutation, suppresses AD-related phenotypes. Both genes are broadly expressed in a variety of tissues throughout development and adult stages of flies (Chintapalli et al., 2007). However, only Ance is highly expressed in male accessory glands of the fly reproductive system suggesting a role in male fertility similar to that of mammalian gACE. Accordingly, males homozygous for hypomorphic alleles of *Ance* are infertile (Hurst et al., 2003; Rylett

et al., 2007). Not limited to this role, *Ance* has also been suggested to influence aging in flies. Specifically, Gabrawy et al. (2019) examined the effect of lisinopril (an ACE-I) on *Drosophila* lifespan and found that lisinopril extended life span due to inhibiting Ance. They demonstrated this by first showing that knockdown of *Ance* extended lifespan and that treatment of lisinopril failed to enhance this effect.

In contrast to *Ance*, *Acer* is highly expressed in adult heads, fat body (analogous to mammalian white adipose tissue and liver) and cardiac cells suggesting a role in different physiological processes, including cardiac function and metabolism (Crackower et al., 2002; Carhan et al., 2011). In fact, studies by Crackower et al. (2002) initially showed that an *Acer* mutation generated by a transposon (P-element) insertion gave rise to defects in heart morphogenesis that resulted in embryonic lethality. In line with these findings, Liao et al. (2013) presented a role for Acer in cardiac function by demonstrating that knock-down of *Acer*, specifically in adult heart tissue, results in heart defects such as impaired contractile properties. However, a contradictory study by Carhan et al. (2011) showed that flies homozygous for an *Acer* null mutation developed normally without significant heart defects, implying that previous heart development phenotypes observed by Crackower et al. (2002) may be attributed to a second-site mutation in the transposon-induced mutant line (Liao et al., 2013). Therefore, a role for *Acer* in *Drosophila* cardiac function remains to be established. Nevertheless, Carhan et al. (2011) identified a potential role for Acer in sleep regulation. Building upon a study that noted the cyclical expression of *Acer* in adult heads regulated by the circadian gene (*clock*) (McDonald and Rosbash, 2001; Carhan et al., 2011) hypothesized a possible role for Acer in circadian behavior. Indeed, they found *Acer* null flies exhibit a reduction in night-time sleep and greater sleep fragmentation. Moreover, this was also observed using an ACE-I, fosinopril.

Mechanisms underlying Acer's contribution to defective sleep patterns remain unclear. Though, there is speculation that it may be due to changes in metabolic processes. *Acer* expression is strong in the fat body, a tissue with various functions, including protein and carbohydrate metabolism, lipid storage, and hormone secretion (Arrese and Soulages, 2010). Recent studies have also indicated a role for the fat body in regulating complex behaviors, including sleep (Yurgel et al., 2018). Therefore, Acer potentially possesses a functional role in metabolic processes in the fat body that, when disrupted, result in sleep defects. Beyond its prominent role in metabolism, the fat body plays an integral role in innate immune response regulation. It is responsible for the humoral response, synthesizing and secreting antimicrobial peptides into the hemolymph. While there is evidence in AD mouse studies, that ACE-Is mitigate neuroinflammatory responses that are known to contribute to AD pathology, it remains to be determined whether *Drosophila* Acer plays a similar role in immune response regulation.

Recently, a study by Glover et al. (2019) identified a potential role for Acer in metabolism including glycogen storage. Stored levels of lipids and glycogen in *Drosophila* are known to respond to dietary intake of sugar and yeast. This study found that under certain dietary conditions, *Acer* null mutant flies exhibit reduced glycogen levels compared to controls. However, just as with its role in sleep modulation, the mechanisms underlying this role for Acer are unknown. Nevertheless, it does pose an interesting avenue for further research to investigate the role of Acer in AD.

A prominent feature of AD is a significant reduction in glucose metabolism that is believed to contribute to disease progression and underlie cognitive dysfunction (Kumar et al., 2022). A decrease in metabolism is suggested to result from poor cerebral uptake of glucose into the brain. Interestingly, a study by Niccoli et al. (2016), showed that increasing glucose uptake in neurons in a *Drosophila* AD model, alleviated neurodegeneration and extended lifespan. Therefore it is worth exploring whether Acer plays a role in maintaining proper glucose metabolism in the brain of flies that could explain how its inhibition results in increased levels that in turn rescues cell death and memory phenotypes found in AD models.

Conclusion

Looking for new strategies to treat AD is an unmet clinical need. Targeting the RAS system has great potential for AD therapeutics. While many studies in patients and animal models have shown promising beneficial effects from inhibiting this system, it remains unclear what mechanisms underlie these outcomes. The RAS is well studied for its peripheral role in regulating blood pressure, fluid and electrolytes. However, its role in organs such as the brain appears to be more complex with new components having been discovered. For this reason, *Drosophila* provides a unique opportunity to understand how ACE-Is may function in the context of AD. Given that only *ace* like factors have been identified in the fly, it is possible to study the role ACE has in AD in isolation without confounding effects from other RAS components. More so, it is evident from human studies that beneficial effects of ACE-Is arise from their ability to penetrate the BBB and act on central RAS. Therefore, their effects appear to be independent of their ability to regulate blood pressure. For that reason, using an invertebrate model such as Drosophila with an open circulatory system provides an advantage of disentangling the effects of ACE-Is from their vascular hemodynamic effects and focusing directly on their effects in the brain.

Author contributions

JG wrote the first draft of the manuscript. JG and GB wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by a grant from the Canadian Institutes of Health Research (PJT153063) to GB.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Neuroscience

TYPE Review
PUBLISHED 17 April 2023
DOI 10.3389/fnins.2023.1184080

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 10 March 2023
ACCEPTED 31 March 2023
PUBLISHED 17 April 2023

CITATION
Varte V, Munkelwitz JW and Rincon-Limas DE (2023) Insights from *Drosophila* on Aβ- and tau-induced mitochondrial dysfunction: mechanisms and tools.
Front. Neurosci. 17:1184080.
doi: 10.3389/fnins.2023.1184080

Insights from *Drosophila* on Aβ- and tau-induced mitochondrial dysfunction: mechanisms and tools

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Alzheimer's disease (AD) is the most prevalent neurodegenerative dementia in older adults worldwide. Sadly, there are no disease-modifying therapies available for treatment due to the multifactorial complexity of the disease. AD is pathologically characterized by extracellular deposition of amyloid beta (Aβ) and intracellular neurofibrillary tangles composed of hyperphosphorylated tau. Increasing evidence suggest that Aβ also accumulates intracellularly, which may contribute to the pathological mitochondrial dysfunction observed in AD. According with the mitochondrial cascade hypothesis, mitochondrial dysfunction precedes clinical decline and thus targeting mitochondria may result in new therapeutic strategies. Unfortunately, the precise mechanisms connecting mitochondrial dysfunction with AD are largely unknown. In this review, we will discuss how the fruit fly *Drosophila melanogaster* is contributing to answer mechanistic questions in the field, from mitochondrial oxidative stress and calcium dysregulation to mitophagy and mitochondrial fusion and fission. In particular, we will highlight specific mitochondrial insults caused by Aβ and tau in transgenic flies and will also discuss a variety of genetic tools and sensors available to study mitochondrial biology in this flexible organism. Areas of opportunity and future directions will be also considered.

KEYWORDS

Alzheimer's disease, amyloid beta, *Drosophila*, mitochondria, neurodegeneration, tau, mitophagy, Drp1

Introduction

Drosophila has been used in genetic research for over 100 years (Morgan, 1910). The conservation of genes between *Drosophila* and human (Rubin et al., 2000), the simplicity of genes with lesser isoform/redundancy in flies, and the smaller size of the *Drosophila* genome compared to the human counterpart, make *Drosophila* an excellent model organism. *Drosophila* also has the advantage of having a short life cycle, around 10 days from embryo to adulthood, is easy to maintain in a small space, and is very economical. Approximately 75% of human disease-causing genes are present in the fly (Rubin et al., 2000; Reiter et al., 2001;

Ugur et al., 2016) and its genome has been fully sequenced and extensively annotated (Adams et al., 2000). Fly genes can be over-expressed or knocked-down through RNAi, mutated by targeted knock-out or deletion of the gene, or replaced by knock-in of a gene. Another technical advantage in flies is the presence of the Gal4-UAS system, where the yeast transcription factor Gal4 can drive the expression of any gene of interest at any developmental stage in a tissue-specific manner (Brand and Perrimon, 1993). Thousands of Gal4 drivers and other valuable fly strains are available at stock repositories including the Bloomington *Drosophila* Stock Center (BDSC), the Harvard Transgenic RNAi Project (TRiP), the Vienna *Drosophila* Resource Center (VDRC), the Japan National Institute of Genetics (NIG) Stock Center, the Kyoto *Drosophila* Stock Center (DGGR), and the Zurich ORFeome Project (FlyORF) (Dietzl et al., 2007; Cook et al., 2010; Ni et al., 2011; Yeh et al., 2018). This is highly advantageous as researchers can easily procure essential stocks for experiments in a matter of days. Also, FlyBase is a regularly updated website that provides valuable information about any fly gene, which facilitates rapid progress in the field. All these *Drosophila* resources and tools make the fly system an ideal platform to approach the pathological complexity underlying neuronal dysfunction and degeneration.

Drosophila as a model to study neurodegenerative diseases

Drosophila melanogaster has been used to study mechanistic aspects of human neurodegenerative diseases for over two decades (Nayak and Mishra, 2022). This is facilitated by the functional conservation of many genes and pathways between humans and flies and their remarkable similarities in neuronal functions. The nervous system of *Drosophila* is quite complex and includes functionally distinct neurons in the eyes, olfactory, gustatory and auditory organs, ventral nerve cord, brain, as well as peripheral sensory neurons (Hirth, 2010). Multiple assays have been developed to assess neurodegeneration in many of these tissues. For instance, the fly eyes contain photoreceptor neurons and are made up of 800 ommatidia that are uniformly arranged with bristles. Human disease-causing genes can be overexpressed in the eye and the effects of these genes can be determined based on the degree of alterations in eye morphology, including the size of the eye, pigmentation, ommatidial organization, and cell death. All these phenotypic outcomes are very easy to score under a dissecting microscope, making the *Drosophila* eye the preferred screening platform for modifiers of neurodegenerative processes. Apart from the eye, neurodegeneration can be also assessed based on vacuolization of the brain, locomotor performance such as climbing and flight assays, analysis of neuromuscular junction morphology, life span analysis, as well as assessment of learning and memory decline (McGurk et al., 2015; Gevedon et al., 2019). Interestingly, the accumulation of amyloid aggregates and pathological tau, the main neuropathological hallmarks in AD, can be also assessed in the fly brain by staining with Thioflavin and with antibodies against phosphorylated and conformational tau (Greeve et al., 2004). Thus, neurodegeneration-related genes and their pathways can be easily studied at molecular and pathological level in *Drosophila* to understand their impact on disease progression. In the next sections, we will discuss the contributions of *Drosophila* as experimental platform to study mitochondrial dysfunction in Alzheimer's disease.

Alzheimer's disease, amyloid beta, and tau

Alzheimer's disease (AD) is an incurable neurodegenerative brain disorder that leads to cognitive impairment and memory deficits in affected individuals (Knopman et al., 2021) and displays a reduction in the hippocampal and temporal lobe of the brain (Ramos Bernardes da Silva Filho et al., 2017). It accounts for 50–60% of dementia (Blennow et al., 2006), affecting mostly people above 65 years of age. The disease is named after the German neurologist Alois Alzheimer who examined a 51 years old patient, Auguste Deter, suffering from memory loss, hallucinations, disorientation, and language problems. Her autopsy showed an accumulation of amyloid plaques and tangles in the cerebral cortex. The etiology of the disease is not well defined. However, the amyloid cascade theory is the predominant hypothesis where cognitive deficits are due to the deposition of amyloid beta peptides forming extracellular plaques (Morishima-Kawashima and Ihara, 2002; Cummings, 2004; de Vrij et al., 2004) and subsequent hyperphosphorylation of the microtubule associated protein tau, resulting in formation of neurofibrillary tangles (NFT) (Cummings, 2004; Blennow et al., 2006; Braak et al., 2011).

A variety of amyloid beta fragments (37–43 amino acid residues) are released into the extracellular space through proteolytic processing of APP by γ-secretase (Haass et al., 2012). Among these, Abeta 42 (hereafter referred to as Aβ) is the most toxic peptide as it is highly insoluble and more prone to aggregation (Moore et al., 2018). Monomeric Aβ aggregates into oligomers and protofibrils, while insoluble amyloid beta-fibril aggregates form amyloid plaques that interfere with signaling at the synapse (Chen and Yan, 2010; Crews and Masliah, 2010). Studies have shown that Aβ aggregation causes neuronal death by altering calcium homeostasis, elevating mitochondrial oxidative stress, and reducing energy metabolism. In addition, Aβ triggers microglial priming by interacting with the microglia, making it more prone to secondary inflammations. Aβ stimulates microglia to release pro-inflammatory cytokines, and these interfere with anti-inflammatory cytokines and transforming growth factor-beta1 (TGF-β1), which can induce neuroinflammation and neurodegeneration (Torrisi et al., 2019; Merlini et al., 2021). Aβ also causes a neuroinflammatory response by activating astrocytes to release various pro-inflammatory molecules (cytokines, interleukins, complement components, nitric oxide, and other cytotoxic compounds) (Brosseron et al., 2014; van Eldik et al., 2016; Arranz and DeStrooper, 2019). Polymerization of Aβ fibrils leads its aggregation into plaques and to the activation of Glycogen Synthase Kinase 3 (GSK3), causing hyperphosphorylation of microtubule-associated Tau and subsequent formation of neurofibrillary tangles (NFT) (Eftekharzadeh et al., 2018; Tiwari et al., 2019).

Tau is a microtubule-associated protein (MAP) that plays a role in the stabilization of neuronal microtubules and regulates axonal growth. Tau exists in a set of six isoform proteins (3R0N, 3R1N, 3R2N, 4R0N, 4R1N, and 4R2N) and is expressed in neurons

via alternate splicing of MAPT. Depending on the exclusion or inclusion of exon 10, the expression of tau isoforms will contain three (3R) or four (4R) microtubule-binding repeats, whereas isoforms with 0, 1, or 2 N-terminal inserts are determined by the inclusion of exons 2 and 3 (Goedert et al., 1989). The ratio of 3R to 4R is 1 in the healthy human brain (Kosik et al., 1989; Goedert and Jakes, 1990), but this ratio is altered in tauopathies (D'Souza and Schellenberg, 2005; Goedert and Jakes, 2005; Sergeant et al., 2005). Tau exists normally as soluble and unfolded protein (Mandelkow and Mandelkow, 2012) and interacts with tubulin, promoting its assembly into microtubules and helping stabilize the structure (Weingarten et al., 1975). It has the potential for multiple phosphorylation at Serine (S), Threonine (T), and Proline (P) residues within its Proline-rich region (PRG) or C-terminal region (CTR), and only 2–3 residues are phosphorylated in the healthy brain. However, there are around five to nine moles of phosphate per mole of tau in AD and other tauopathies (Grundke-Iqbal et al., 1986; Kopke et al., 1993; Holper et al., 2022). Hyperphosphorylation of Tau is associated with an aggregation of multimers and fibers (Despres et al., 2017) that seem to mediate cognitive defects. Microtubule-associated kinases such as Cyclin-dependent Kinase 5 (CDK5), Glycogen Synthase Kinase 3 (GSK3β), casein kinase II (CKII), Src-family tyrosine kinase Fyn, c-Abl tyrosine kinase (c-Abl), lemur tyrosine kinase 2 (LTK), dual specificity tyrosine-phosphorylation-regulated kinase 1A (Dyrk1A), and thousand-and-one amino acid kinases (TAOKs), casein kinase 1 (CK1), c-Jun amino-terminal kinase (JNK),extracellular signal-regulated kinases 1 and 2 (Erk1 and Erk2), adenosine-monophosphate activated protein kinase (AMPK), cyclic AMP (cAMP)-dependent protein kinase (PKA), protein kinase N1, tau-tubulin kinases 1 and 2 (TTBK1 and TTBK2), Ca2+/calmodulin-dependent protein kinase II (CaMKII) and microtubule-affinity regulating kinases (MARKs) are known enzymes to be involved in tau phosphorylation (Limorenko and Lashuel, 2022). In the AD brain, approximately 85 serine, threonine, and tyrosine residues have been found phosphorylated (Tavares et al., 2013; Limorenko and Lashuel, 2022). Therefore, deposition of extracellular Aβ plaques and intracellular accumulation of NFT constitute the main pathological hallmarks in AD (Knopman et al., 2021).

Mitochondrial dysfunction in Alzheimer's disease

The mitochondrion is a membrane-bound cytoplasmic organelle that carries out essential functions like ATP production through oxidative phosphorylation. It is critical for multiple cellular processes and interacts with several organelles to regulate energy metabolism (Rossmann et al., 2021; Collier et al., 2023). It is worth noting that the brain utilizes 25% of body glucose and 20% oxygen consumption, but it constitutes only 2% of body weight. As a result, the brain is very susceptible to changes in energy metabolism and, therefore, disturbances of mitochondrial functions are associated with neurodegenerative disorders (Lin and Beal, 2006; Wang et al., 2020; Zhang X. et al., 2021), including Alzheimer's disease (Wang et al., 2014; Swerdlow, 2018). Several studies have shown that mitochondrial abnormalities are early events in AD (Reddy et al., 2012; Swerdlow, 2018). Cell lines expressing mutant APP or treated with Aβ (Schmidt et al., 2007; Diana et al., 2008; Wang et al., 2008b, 2009; Calkins and Reddy, 2011; Manczak et al., 2011), AD mouse models (Smith et al., 1997; Li et al., 2004; Reddy et al., 2004; Caspersen et al., 2005; Manczak et al., 2006; Yao et al., 2009), and AD post-mortem brains (Parker et al., 1990; Gibson et al., 1998; Maurer et al., 2000; Butterfield et al., 2001, Dragicevic et al., 2010) are reported to have mitochondrial dysfunction. Brains from AD patients show decreased production of ATP, increased production of free radicals, lipid peroxidation, oxidative damage of DNA and protein, and cellular damage compared to control specimens (Parker et al., 1990; Gibson et al., 1998; Maurer et al., 2000; Wang et al., 2005; Devi et al., 2006; Dragicevic et al., 2010), suggesting that mitochondrial dysfunction is a main pathological feature of AD.

The mitochondrial cascade hypothesis was first reported in 2004 (Swerdlow and Khan, 2004). According to this hypothesis, mitochondrial dysfunction disrupts multiple pathways connected to AD, resulting in a variety of clinical phenotypes including cognitive decline. It also states that mitochondrial dysfunction alters Aβ homeostasis triggering its overproduction and accumulation, and that an overall bioenergetic dysfunction might be the main culprit in AD [reviewed in Swerdlow (2023)]. Accordingly, the mitochondrial ROS production, calcium homeostasis, mitochondrial morphology and number, transport along the neuronal axon, neurotransmitters levels, mitophagy, and mtDNA mutation and oxidation, are all clearly compromised in AD (Johnson and Blum, 1970; Hirai et al., 2001; Hauptmann et al., 2009; Wang et al., 2009; Calkins and Reddy, 2011; Butterfield and Halliwell, 2019; Wong et al., 2020; **Figure 1**). Next, we will examine specific mechanisms mediating mitochondrial dysfunction and abnormalities in the context of AD.

Mitochondrial ROS and oxidative stress

The mitochondrion contributes with approximately 90% of cellular reactive oxygen species (ROS), a by-product of electron transport of aerobic respiration in mitochondria (Balaban et al., 2005). When cellular homeostasis is disrupted, mitochondria produce less ATP and more ROS, resulting in oxidative stress (Gibson et al., 1998). AD patients are reported to have increased oxidative damage of proteins, nucleic acids, sugars, and lipids (Butterfield and Halliwell, 2019). For instance, markers of protein oxidation, such as protein carbonyl content, are significantly increased in the parietal lobe, superior, and middle temporal gyrus, and hippocampus of AD brains (Hensley et al., 1995; Lyras et al., 1997; Aksenov et al., 2001). Similarly, 3-nitrotyrosine, another protein oxidative modification, was significantly increased in various brain regions and cerebrospinal fluid from AD cases (Good et al., 1996; Smith et al., 1997; Tohgi et al., 1999; Castegna et al., 2003; Reed et al., 2009; Butterfield and Halliwell, 2019). Oxidative damage of DNA/RNA in AD brains causes double-strand breaks of DNA, crosslinking of DNA/DNA or DNA/protein, and extensive modification of DNA bases. Consequently, levels of DNA breaks in the AD hippocampus and cerebral cortex are found to be high (Mullaart et al., 1990; Anderson et al., 1996). Both

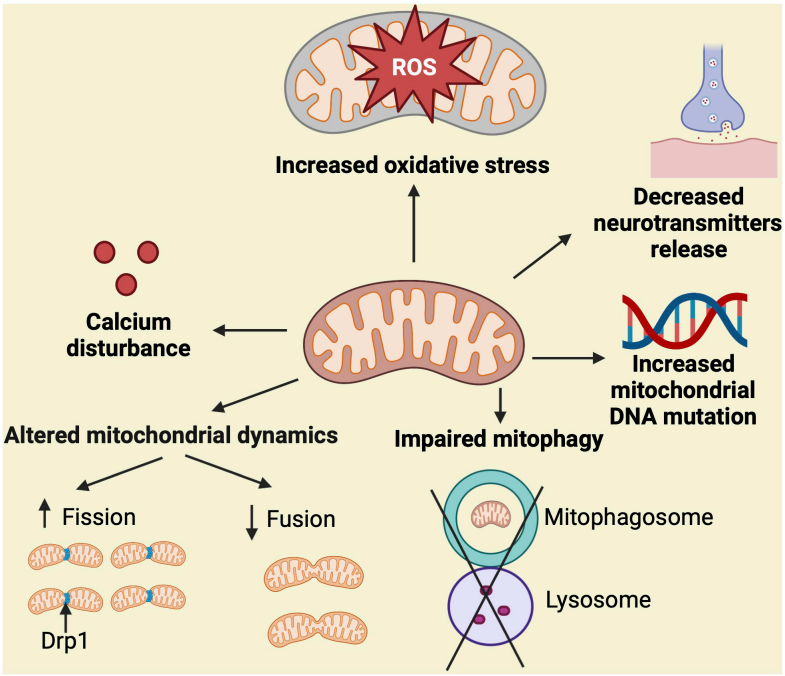

FIGURE 1
Simplified overview of mechanisms associated with mitochondrial dysfunction. Mitochondrial homeostasis is regulated by mitochondrial dynamics (fission and fusion), proper handling of calcium, control of ROS production, neurotransmitters function, maintenance of mtDNA replication, and elimination of damaged mitochondria through mitophagy. Stressful and persistent insults to the mitochondria, such as the presence of neurotoxic proteins, alter these processes resulting in the indicated abnormalities, which leads to mitochondrial dysfunction and potential cell death. Fission eliminates abnormal mitochondria from healthy ones and this process is mediated by Drp1.

mitochondrial DNA and nuclear DNA from AD brains have a significant increase of 8-hydroxydeoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG), markers of DNA oxidation (Mecocci et al., 1994; Good et al., 1996; Lyras et al., 1997). Similarly, the levels of oxidized rRNA or mRNAs are significantly elevated in AD cases (Shan et al., 2003; Ding et al., 2005; Honda et al., 2005). Moreover, lipid peroxidation products such as 4-hydroxynonal, malondialdehyde (MDA), and 2-propenal (acrolein) are increased in different regions of AD brains (Wang et al., 2014). In contrasts, the level of antioxidant factors and enzymes is decreased in AD brains compared to control specimens (Marcus et al., 1998; Doré, 2002; Kim et al., 2006; Venkateshappa et al., 2012). Altogether, these observations suggest that mitochondrial dysfunction and oxidative stress are closely linked and, thus, are considered primary triggers of neuronal death.

Calcium dyshomeostasis

The mitochondria play an essential role in the maintenance of calcium homeostasis in neurons which is essential for their survival (Jouaville et al., 1999). The level of mitochondrial calcium affects the activity of mitochondria and the supply of ATP. The mitochondrial calcium uniporter (MCU) protein complex conducts calcium transport into the matrix through MICU1 and MICU2/3 proteins (MICU gatekeepers) that sense calcium levels (Perocchi et al., 2010). AD patients have reduced expression of cytosolic calcium-binding proteins calmodulin, calbindin D28K, and parvalbumin which might cause activation of MCU after free calcium binds to MICU1 and MICU2/3 (McLachlan et al., 1987; Riascos et al., 2011; Ahmadian et al., 2015; Ali et al., 2019). Therefore, the level of calcium in mitochondria is elevated in AD. Consistently, exposure of cortical neurons to Aβ increases calcium concentration and promotes neurodegeneration, which is rescued by blocking MCU proteins (Hedskog et al., 2013). Aβ oligomers are also reported to form a calcium-permeable pore in the mitochondrial membrane that regulates calcium uptake and may disrupt the homeostasis of calcium in the mitochondria (Lashuel et al., 2002; Shirwany et al., 2007). In addition, Aβ oligomers promote the release of calcium stored in the ER to increase mitochondrial calcium levels, leading to mitochondrial dysfunction (Ferreira et al., 2015). Indeed, the expression of Ryanodine receptors (RyR), which regulate the release of intracellular Ca2 + in the ER, varies during AD progression and the RyR fly homologue has been found as modifier of Abeta and tau toxicity in transgenic flies (Casas-Tinto et al., 2011; Feuillette et al., 2020). Calcium homeostasis also depends on the sarcoplasmic/endoplasmic reticulum Ca2 + ATPase (SERCA), an essential ER protein that pumps Ca2 + into the ER, and modulation of SERCA expression modifies Aβ levels (Green et al., 2008). However, the direct role of RyR and SERCA in the AD-related mitochondrial malfunction remains to be elucidated. On the other side, it has been demonstrated that dyshomeostasis of mitochondrial calcium can be restored through calcium efflux pathways, mainly through the sodium-calcium exchanger (NCLX) (Boyman et al., 2013). Interestingly, AD pathology in mouse models is associated with the loss of mitochondrial NCLX expression

and function, and rescue of NCLX in these mice restores both cognitive decline and cellular pathology (Jadiya et al., 2019). Additional studies in AD mouse models demonstrated that an elevated mitochondrial calcium level is associated with induction of apoptosis, highlighting the importance of mitochondrial calcium concentration in this process (Sanz-Blasco et al., 2008; Calvo-Rodriguez et al., 2020). Of note, a recent study found an oscillation of cytosolic Ca2 + in primary neurons after incubation with tau K18, a protein fragment carrying the four repeat (4R) domain of the protein. As a result, both mitochondrial and cytosolic Ca2 + are increased, indicating that Tau also impairs Ca2 + homeostasis (Britti et al., 2020).

Mitochondrial fusion and fission

Mitochondria undergo fission and fusion in the cytoplasm to maintain proper distribution (Zhu et al., 2013), and disruption of either of these processes leads to neurological disorders (Mishra and Chan, 2014). The fission of mitochondria is regulated by the GTPase-related dynamin-related-protein1 (Drp1), also known as Dynamin-1-like protein (DLP1) in humans. It triggers the fragmentation of mitochondria and acts as a mitochondrial fission factor; however, its downregulation promotes mitochondrial fusion (Wenger et al., 2013). Of note, the structural damage in mitochondria was documented in AD brains over two decades ago (Hirai et al., 2001). Subsequent studies demonstrated that AD brains have a reduction of mitochondrial size and number (Wang et al., 2008b, 2009), which could cause a shortage of mitochondrial bioenergetics either by enhancing ROS generation (Yu et al., 2006) or by having a negative effect on electron transport chain (ETC) function (Liu et al., 2011; Zhou et al., 2017). AD patients are reported to have increased mitochondrial fission (Manczak et al., 2011; Kandimalla et al., 2016). Interestingly, the pathology of AD in the brain not only includes an interaction between oligomeric Aβ and DLP1, but also an interaction between hyperphosphorylated tau and DLP1 (Manczak et al., 2011). This suggests that these abnormal interactions induce mitochondrial fragmentation, which is supported by *in vivo* and *in vitro* models of AD. For instance, transgenic flies expressing Aβ display abnormal dynamics and distribution of mitochondria (Iijima-Ando et al., 2009; Zhao et al., 2010). In neuronal cell culture, where Aβ or APP is overexpressed, mitochondria undergo fragmentation with altered distribution (Wang et al., 2008b, 2009; Manczak et al., 2011). In M17 neuroblastoma cells and primary neurons, wild-type or mutant APP overexpression also induces fragmentation of mitochondria (Wang et al., 2008b, 2009). Interestingly, APP- or Aβ-induced mitochondrial deficit in neurons is rescued by blocking mitochondrial fission, confirming the role of this process in AD pathogenesis (Wang et al., 2008b, 2009). On the other hand, mitochondria fusion is regulated by dynamin-related GTPase proteins mitofusin-1 (Mfn1), mitofusin-2 (Mfn2), and the optic atrophy type 1 (OPA1) protein where Mfn1 and Mfn2 bind to the outer membrane of mitochondria and OPA1 binds to the inner mitochondrial membrane and mediates the fusion of the inner mitochondrial membrane (Koshiba et al., 2004; Song et al., 2009). In mice cortexes and the hippocampus, knockout of Mfn2 caused structural and functional damage to mitochondria as well as neuroinflammation, oxidative stress and neuronal death, illuminating the role of mitochondrial fragmentation in AD pathology (Jiang et al., 2018; Han et al., 2020). Confocal and electron microscopy studies from another group confirmed the structural damage and fragmentation of mitochondria in the brain of CRND8 APP transgenic mice at 3 months of age, much before visible amyloid deposition, suggesting that abnormal mitochondrial dynamics is an early event in AD pathogenesis (Wang et al., 2017). It is worth noting that overexpression of Tau also causes abnormal mitochondrial fusion (Li et al., 2016; Kandimalla et al., 2018). A study showed that overexpression of human wild-type tau alters mitochondrial dynamics and results in mitochondrial elongation by increasing the fusion proteins OPA1, Mfn1, and Mfn2, which reduces neuronal viability (Li et al., 2016; Szabo et al., 2020). In addition, the knockdown of mfn2 reduced the human tau-enhanced mitochondrial fusion and restored mitochondrial function, suggesting that Mitofusin-associated mitochondrial fusion might contribute to tau toxicity (Szabo et al., 2020). Another group has shown that expression of caspase-cleaved tau in cortical neurons from tau-/- knockout mice, as well as in immortalized cortical neurons, led to mitochondrial fragmentation with a decline in OPA1 levels (Pérez et al., 2017; Szabo et al., 2020), indicating the impact of tau on mitochondrial fusion.

Axonal trafficking deficits and abnormal mitochondrial distribution

In addition to defects in mitochondrial structure, reduced expression of mitochondrial fission and fusion proteins in AD can lead to the absence of mitochondria in axons or dendritic segments (Wang et al., 2009; Pickett et al., 2018). Mitochondria undergo anterograde and retrograde transport that helps maintain healthy mitochondria by inhabiting axons with fresh mitochondria and recycling the damaged ones (Sheng and Cai, 2012; Lin et al., 2017). Alteration of either of these processes causes a reduction of healthy mitochondria, which leads to impaired mitochondrial function. Dysregulation of axonal mitochondrial transport contributes to AD as this transport is crucial for the maintenance of neurons and their synaptic function (Hollenbeck and Saxton, 2005). In mammals, Milton (OIP106 and GRIF1) and Miro (Miro1 and Miro2) regulate the attachment of mitochondria to microtubules via kinesin heavy chains (Fransson et al., 2006). In *Drosophila*, Milton and Miro proteins perform similar functions (Guo et al., 2005) and, thus, mitochondria are absent in synaptic terminals and axons when there is no Milton or Miro (Stowers et al., 2002). Interestingly, it has been reported that the number of mitochondria is reduced in the axons of hippocampal neurons upon Aβ treatment (Du et al., 2010), which is consistent with a lower number of mitochondria in axons from AD brains (Stokin et al., 2005). Mislocalization of mitochondria and its decrease in dendrites, axons, and soma (Iijima-Ando et al., 2009) was observed as consequence of Aβ expression in flies. Similar results were reported in neuronal cell cultures exposed to Aβ oligomers, which led to reduced mitochondrial trafficking/motility in axons (Du et al., 2010; Wang et al., 2010; Calkins and Reddy, 2011; Rui and Zheng, 2016). On the other hand, tau has been also associated

with abnormal mitochondrial trafficking. For instance, earlier investigations showed that over-expression of tau in neuroblastoma cells alters the kinase-dependent anterograde axonal transport of mitochondria by enhancing the binding of microtubules, resulting in neurites almost devoid of these organelle (Ebneth et al., 1998). *In vivo* studies in transgenic mice confirmed the reduced mitochondrial movement in axons upon overexpression of human tau (Stoothoff et al., 2009; Vossel et al., 2015). Intriguingly, not only the expression of tau but also its phosphorylation impairs mitochondrial transport. For instance, in neuronal PC12 cells and mouse cortical neurons, phosphorylation of tau at AT8 sites (Ser 199, Ser202, and Thr 205) inhibits mitochondrial movement (Shahpasand et al., 2012). This is consistent with abnormal mitochondrial distribution in neurons positively stained with the anti-phospho tau Alz50 antibody in AD brains (Kopeikina et al., 2011), suggesting that tau plays a key role in the mitochondrial loss observed in AD (Wee et al., 2018).

Neurotransmitters and mitochondrial dysfunction

Mitochondrial dysfunction affects a number of neurotransmitters in AD. For instance, memory and learning deficits result from insufficient cholinergic transmission. In this case, the hyperpermeability of the mitochondrial membrane leads to degeneration of cholinergic neurons and deficiency of acetylcholine (ACh) (Wong et al., 2020). Dysfunctional mitochondria also alter the activity of acetylcholine esterase (AChE) and recycling of choline from the synapse is hampered by mitochondrial-induced oxidative stress via nitrosative stress, which results in ACh deficiency (Wong et al., 2020). Serotoninergic, dopaminergic, norepinephrinergic, and histaminergic systems comprise the diverse monoaminergic neurotransmission network. Through membrane permeabilization and altered serotoninergic metabolism, mitochondrial dysfunction in AD causes serotoninergic inefficiency (Yamamoto and Hirano, 1985; Lai et al., 2011). Mitochondrial dysfunction also causes the loss of serotoninergic neurons via caspase-dependent apoptosis resulting in the reduction of 5-hydroxytryptamine (5-HT) or Serotonin neurotransmission (Wong et al., 2020). In AD, an excessive 5-HT breakdown is the result of mitochondrial dysfunction, which leads to a 5-HT deficit. The inadequate serotoninergic transmission also contributes to AD progression by causing ROS accumulation and further mitochondrial dysfunction (Wong et al., 2020).

Impaired mitophagy

Mitophagy is a mechanism that eliminates damaged mitochondria by activating PINK1 at the outer mitochondrial membrane (Nguyen et al., 2016). Studies have shown that Parkin, an E3-ubiquitin ligase, is drawn to the mitochondria by PINK1 and phosphorylated there to initiate the mitophagy pathway for protein ubiquitination and degradation (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). Accumulation of damaged mitochondria is due to insufficient mitophagy to remove them (Ye et al., 2015) or to defects in lysosomal degradation (Martín-Maestro et al., 2016, 2017; Kerr et al., 2017; Sorrentino et al., 2017). Nonetheless, it has been suggested that AD is mainly associated with mitophagy impairment (Nixon and Yang, 2011). For instance, post-mortem hippocampal brain samples from AD patients showed a reduction of mitophagy by 30–50% compared to control patients (Fang et al., 2019). Proteins believed to be involved in autophagy and mitophagy processes, such as Optineurin (OPTN), ATG5, ATG12, Beclin-1 (Bcl-1), PI3K class III, ULK1, AMBRA1, BNIP3, BNIP3L, FUNDC1, VDAC1, and VCP/P97 were decreased in AD brains (Martín-Maestro et al., 2017). The mitochondrial structure and function in the brains of AD patients and mouse models change into a swollen round shape with deformed cristae, low ATP production, reduced LC3 recruitment to the mitochondria, dysfunctional AMP-activated protein kinase (AMPK), and inhibition of its targets ULK1 and TBK1, which collectively impairs the mitophagy process (Hirai et al., 2001; Martín-Maestro et al., 2016; Wang et al., 2017; Fang et al., 2019). Parkin's ability to translocate to damaged mitochondria is also affected by abnormal contacts between the projection domain of tau protein and Parkin, which prevents mitophagy (Cummins et al., 2019). Moreover, addressing mitophagy with urolithin A or actinonin improves memory in APP/PS1 mice and *C. elegans* expressing Aβ or tau, emphasizing the role of mitophagy in AD pathogenesis (Fang et al., 2019).

Mitochondrial genome abnormalities

Mitochondria have their own DNA called mtDNA that codes for the 13 mitochondrial core proteins of the electron transport chain complexes, two rRNAs, and 22 tRNAs (Taanman, 1999; D'Souza and Minczuk, 2018). Although mitochondrial DNA plays a crucial role in mitochondrial function, it is prone to mutations due to the lack of histone proteins necessary to protect DNA and to mediate DNA repair mechanisms (Yana et al., 2013; Boczonadi et al., 2018). MtDNA mutations, via inheritance or gradual somatic mutation, affect mitochondrial function, which leads to cell death and disease (Swerdlow, 2018). Like AD patients, individuals with mtDNA mutations are reported to have similar cognitive deficits.

(Inczedy-Farkas et al., 2014), suggesting the potential role of mtDNA in cognition. In the case of AD, affected individuals have an increase in mtDNA mutations, possibly due to higher oxidative damage (Swerdlow, 2018). Indeed, AD patients have 10-fold higher levels of oxidized bases in mtDNA than nuclear DNA compared to healthy controls (Mecocci et al., 1994; Wang et al., 2005). This is consistent with higher oxidized nucleic acid in mtDNA in MCI patients and preclinical AD (Lovell et al., 2011). All these observations illustrate the role of mitochondrial genome abnormalities in AD pathogenesis.

Assays for studying mitochondrial biology

Many assays have been developed and improved over the years to analyze the structure and function of the mitochondria.

Flow cytometry is a standard assay to record mitochondrial mass (Doherty and Perl, 2017). However, it was only in recent decades that this procedure could be performed independently of mitochondrial potential due to the production of the MitoTracker family of dyes in the mid-1990s. Specifically, MitoTracker probes-green (MTG) is used for its ability to accumulate inside the mitochondria despite the measured membrane potential, unlike the positively charged red dye (Doherty and Perl, 2017; Rana et al., 2017). MTG collects inside the mitochondrial matrix and binds to free thiol groups in cysteine residues located on the mitochondrial membrane proteins; the amount of accumulated dye can then be quantified via fluorescence relative to the mitochondrial size (Presley et al., 2003; Doherty and Perl, 2017).

Along with the size, it is also important to measure the mitochondrial membrane potential (MMP) to assess the global mitochondrial function, as this parameter directly correlates with the ability of the mitochondria to generate ATP for the cells. The MMP is created from the electrochemical gradient formed through a series of coupled redox reactions in the electron transport chain steps; decreases in the MMP can be detected using lipophilic cationic fluorescent dyes (Sakamuru et al., 2012). Other membrane-potential dependent fluorescent dyes that can be used to measure MMP include tetramethylrhodamine, methyl ester (TMRM), and tetramethylrhodamine, ethyl ester (TMRE). The more polarized the mitochondria, the brighter the signal when measured due to higher accumulation of dye (Perry et al., 2011). Other dyes have been developed to analyze and measure oxygen consumption, which is directly related to oxidative phosphorylation (OXPHOS) activity (Yarosh et al., 2008). Spectrophotometric assays have also been developed to measure the activity of mitochondrial enzymes or the concentrations of cellular metabolites. For instance, by measuring enzyme activity through spectrophotometry, it was found that the activity of the mitochondrial complex I is slightly reduced in the brain during aging, but highly reduced in the context of neurodegeneration (Pollard et al., 2016).

In many cell types, the mitochondria create a complex, reticular network to support its critical functions, such as energy production. These networks undergo constant dynamic changes, which require the complementary processes of fission and fusion to occur (Hoppins et al., 2020). Several *in vitro* and *in vivo* studies were performed to assess mitochondrial fission and fusion and earlier assays provided evidence that mitochondrial fission involves multiple constriction steps that are marked by the endoplasmic reticulum (ER) and dynamin-related proteins (DRPs) (Friedman et al., 2011; Anand et al., 2014). Recent advances in light and electron microscopy demonstrated that the inner and outer membrane dynamics may be uncoupled during fission and fusion, which suggests that both processes are uniquely required to uphold the morphology of the mitochondria during its dynamic equilibrium; problems in this relationship can cause a diverse array of diseases as mitochondrial fission and fission are required for mitochondrial replication, a process critical to maintain energy levels and overall cell health (Cho and Sun, 2020; Hoppins et al., 2020). To correctly visualize mitochondrial fusion, stable cell lines that express fluorescent mitochondrial matrices via targeted proteins must be generated; once this is done, the mitochondria can be isolated, and the fusion assay can be performed (Hoppins et al., 2020). The fusion efficiency of the mitochondria can be quantified by dividing the number of fused mitochondria by the total number of mitochondria seen in the microscope field. It is anticipated that about 15–20% of total mitochondria are fused in wild-type control specimens (Hoppins et al., 2020).

Mitochondrial fission is required for growing and dividing cells and is mediated by the cytosolic dynamin family member, Drp1, in *Drosophila* and mammals (Youle and van der Bliek, 2012). Assays to measure fission *in vitro* involve quantification of the GTPase cycle kinetics and biochemical activity of Drp1 (Ingerman et al., 2005). Electron microscopy has also been included to study mitochondrial fission through Drp1-mediated liposome tubulation and constriction assays. Once images are obtained, the ultrastructural changes can be analyzed and measured using the ImageJ software (Schneider et al., 2012).

Mitochondrial biology in *Drosophila*

Drosophila is a prominent model for studying mitochondrial diseases because researchers can manipulate the mitochondrial genome to express the characteristics of many human mitochondrial disorders (Chen et al., 2019). By utilizing certain restriction enzymes on *Drosophila* mitochondrial DNA, it has been observed that many mitochondrial mutations are heritable by isolation of specific disease-causing genes; when these methods are used with genetic drivers, such as GMR-GAL4, heteroplasmic flies with different mitochondrial genomes can be produced in order to study the molecular and phenotypic effects of a specific disease gene (Xu et al., 2008; Chen et al., 2015).

From a genomics perspective, human and *Drosophila* genomic mtDNA are very similar despite the human genome being about 3 kb shorter (Lewis et al., 1995). The 16,559 kb human mtDNA genome encodes for 13 proteins, 22 tRNAs, and two rRNA; all thirteen of these mitochondrial proteins make up components of the four complexes found in the electron transport chain, and almost all the DNA sequences in each specific protein have small introns that must be spliced out before translation can occur. *Drosophila* mtDNA encodes the same transcripts as its human counterpart but has a slightly different genomic order, predominantly due to its expanded "A + T-rich" regions (Sen and Cox, 2017). Crucial molecular functions of the mitochondria, including transport, oxidative phosphorylation (OXPHOS), and nucleotide biosynthesis, have highly conserved nuclear-encoded genes across both species, making *Drosophila* an excellent model for studying metabolic issues (Chen et al., 2019). Each of the 13 mitochondrial proteins is translated in the mitochondrial matrix using mtDNA-encoded tRNAs and most of the mRNA sequences for the mitochondrial proteins are separated by at least one mtDNA-encoded tRNA. It is critical that each mtDNA-encoded tRNA is properly and systematically excised since both human and *Drosophila* mtDNA are transcribed in a polycistronic manner. Without proper excision of each previous transcript, normal translation and processing of the proteins cannot occur which can lead to illnesses or even the death of the organism (Sen and Cox, 2017).

Drosophila research can also be used to define phenotypes associated with defective mitochondria. It has become clear that the number of mitochondria present in different organisms can fluctuate and the structure of the organelle dynamically changes

depending on which type of cell they reside in Zhang et al. (2016). Neuronal cells are in a constant state of high energy demand so they are considered to be especially vulnerable to dysfunction in the mitochondrial equilibrium as this can lead to abnormally low levels of ATP; specifically, there is an abundance of mutations in the MFN2 gene that have been commonly linked to a peripheral neuropathy caused by Charcot-Marie-Tooth disease Type 2A (CMT2A) (Kyriakoudi et al., 2021). Severe symptoms of this disease, most likely caused by mutations in the functional domains of the gene, include distal limb atrophy leading to loss of leg function as well as distal sensory loss in the limbs (Züchner et al., 2004). Other mutations in the *MFN2* gene can be linked to other CMT diseases, which can damage the optic nerve and other crucial neurons in different sensory pathways (Zhou et al., 2019). In addition to genetic research, behavioral assays such as flight and climbing tests can be performed in flies as behavioral defects correlate with key features observed in patients with certain mitochondrial disorders (Jacobs et al., 2004).

Tools for studying mitochondrial biology in flies

Genetically encoded sensors have been generated in *Drosophila* to monitor mitochondrial structure, function, and metabolites. These sensors are placed under the control of the UAS promoter and have been used to perform mitochondrial assessments in different fly tissues using tissue-specific Gal4 drivers such as C155-Gal4 (pan-neuronal), MB-GS Gal4 (mushroom body), MB296B-Gal4 (dopaminergic neurons), TH-Gal4 (dopaminergic neurons), vGlut^{OK371} Gal4 (glutamatergic neurons), esg^{ts}-Gal4 (intestinal stem cell), apt-Gal4 (perineurial glial cells), and vGlut^{VGN6341} Gal4 (glutamatergic interneurons) (Hwang et al., 2014; Arce-Molina et al., 2020; Morris et al., 2020; Sharma and Hasan, 2020; Cho et al., 2021; Hartwig et al., 2021; Wong et al., 2021; Houlihan et al., 2022). Some sensors are substrate-dependent, while some are light-dependent. On the other hand, UAS-mitoGFP is used as a mitochondrial marker to visualize mitochondria in any tissue because, in this case, the mitochondrial import sequence is fused to GFP (Lutas et al., 2012; DeVorkin et al., 2014; Morris et al., 2020; Hartwig et al., 2021; **Figure 2A**).

Substrate-dependent sensors

Various substrate-dependent sensors were created to detect substrates like hydrogen peroxide, glutathione, pyruvate, ATP, NAD/NADH, and calcium. To see redox changes in live mitochondria, the UAS-mito-roGFP2-Grx1 is available, which encodes a redox-sensitive GFP cassette fused to glutaredoxin-1 (Grx1) (Albrecht et al., 2014; Krzystek et al., 2021; Houlihan et al., 2022; **Figure 2B**). The sensor for pyruvate is known as UAS-mito-PyronicSF and consist of a circularly permuted GFP fused to the bacterial pyruvate-sensitive transcription factor PdhR. It binds to pyruvate and induces conformational changes that increase the readout of FRET signal (Arce-Molina et al., 2020). Therefore, this sensor can be used to study mitochondrial metabolism (**Figure 2C**).

To monitor calcium, the UAS-mito-GCaMP3 was designed to express a circularly permutated EGFP M13/Calmodulin fusion protein under control of the UAS promoter. Upon binding to calcium, this fusion protein undergoes conformational changes that elicit GFP signal (**Figure 3A**; Lutas et al., 2012; Morris et al., 2020; Sharma and Hasan, 2020). UAS-AT1.03NL is an ATP sensor that consists of two fluorescent proteins (mVenus and mseCFP) linked to the ε subunit from bacterial F0F1-type ATP synthase. The ε subunit has two C-terminal helices and an N-terminal barrel domain. Low FRET efficiency results from the loose and flexible subunit separating two fluorescent proteins in ATP-free mode. When ATP is bound, the ε subunit configuration switches from open to close, bringing the two fluorescent proteins closer together and increasing FRET efficiency (Imamura et al., 2009; Tsuyama et al., 2013; Dong and Zhao, 2016; Cho et al., 2021; **Figure 3B**). UAS-PercevalHR is also used as ATP/ADP sensor and contains the ATP-binding protein GlnK1 from *Methanocaldococcus jannaschii* and circularly permutated monomeric Venus (CpmVenus) connected by a peptide linker. Upon binding to ATP, the T-loop of GlnK1 undergoes a dramatic conformational change from a loose, disordered structure to a tight, ordered loop, which integrates CpmVenus into the T-loop for sensing ATP (Berg et al., 2009). It has an excitation peak at 405 nm for ATP binding and 488 nm for ADP binding (**Figure 3C**; Broyles et al., 2018; Morris et al., 2020; Wong et al., 2021). Lastly, the UAS-SoNaR transgene is used as NADH/NAD + sensor. It has the NADH-binding domain of Rex protein from *Thermus aquaticus* (T-Rex) connected to a circularly permuted yellow fluorescent protein (cpYFP) (**Figure 3D**). It has excitation at 420 or 485 nm with emission at 528 nm to determine NADH/NAD + ratio (Zhao et al., 2015; Bonnay et al., 2020; Morris et al., 2020).

Light-dependent sensors

UAS-Dendra2.mito is a light-dependent sensor where the photoconvertible protein Dendra2 (green) from the filamentous fungus *A. nidulans* is tagged to the mitochondrial matrix. The green fluorescence of this tagged protein can be localized and examined prior to irradiation. The region of interest within a cell or the entire cell is then exposed to UV (405 nm) or blue light (488 nm) lasers. Dendra2 is immediately photo-converted from green to red fluorescence by UV or blue light (**Figure 4**). It is used to monitor mitochondria over time (Hwang et al., 2014; Perez-de-Nanclares-Arregi and Etxebeste, 2014; Bertolin et al., 2018). This sensor allows easy tracking of green signal before photoconversion and the corresponding shift to red fluorescence upon UV or blue light irradiation. Importantly, with excitation and emission occurring at 553 and 573 nm, respectively, the activated red Dendra2 signal exhibits high photo-stability (Perez-de-Nanclares-Arregi and Etxebeste, 2014).

Mitochondrial dysfunction in Aβ-expressing flies

Wang and Davis (2021) recently assessed mitochondrial function and dynamics in flies expressing Aβ in the mushroom

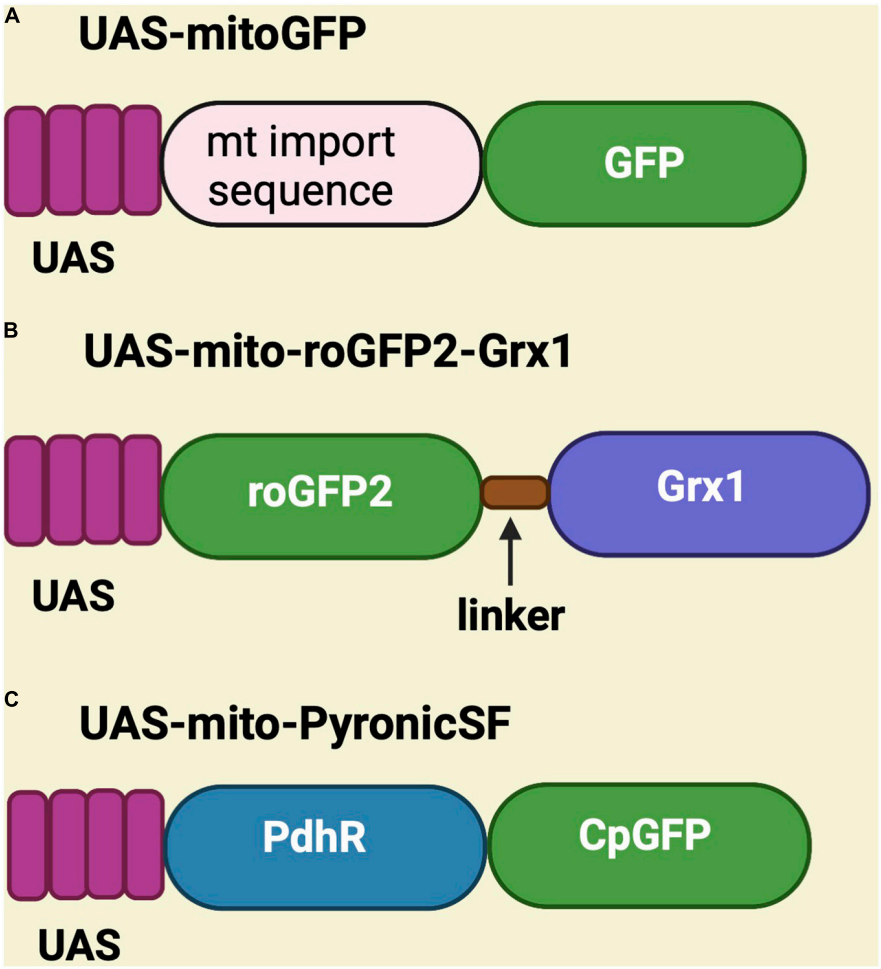

FIGURE 2
Schematic representation of UAS-mitoGFP, UAS-mito-roGFP2-Grx1 and UAS-mito-PyronicSF constructs. **(A)** UAS-mitoGFP contains a mitochondrial import sequence fused to GFP and serves as mitochondrial marker. **(B)** UAS-mito-roGFP2-Grx1 is a redox sensor transgene in which glutaredoxin-1 (Grx1) is fused to redox-sensitive GFP. **(C)** UAS-mito-PyronicSF is a pyruvate sensor containing the bacterial pyruvate-sensitive transcription factor PdhR linked to circulated permuted GFP (CpGFP), which allows real-time assessment of mitochondrial pyruvate transport.

body neurons through super-resolution microscopy, calcium imaging and behavioral assays. They found that Aβ induces mitochondrial fragmentation and dysfunction at a very early age, consistent with detectable apoptosis. Interestingly, learning was impaired much later than the initial mitochondrial abnormalities, confirming the proximal role of mitochondria in AD pathogenesis. In terms of structural changes, Aβ was found to induce formation of aberrant mitochondria with a build-up of vacuoles or damaged cristae in the pre-synapse of the fly dorsal longitudinal flight muscle (DLM). In this case, Aβ decreased the age-dependent anterograde and retrograde axonal trafficking of mitochondria (Zhao et al., 2010). To determine if manipulation of mitochondrial fission could modify Aβ-induced phenotypes, the fission regulator Drp1 was pan-neuronally co-expressed with Aβ. This work demonstrated that overexpression of Drp1 improves survival, climbing capacity, neuronal degeneration and ATP levels in Aβ flies (Lv et al., 2017). On the other hand, over-expression of Aβ in all neurons decreases *drp1* and m*arf* mRNA levels in older flies (Abtahi et al., 2020).

Another protein essential for mitochondrial function and transport is Milton. It connects Miro to kinesin, (Stowers et al., 2002) and enables them to move in axons and dendrites (Glater et al., 2006). Knock-down of Milton enhanced Aβ-induced locomotion defects. Consistently, heterozygous *miro* mutants led to an enhancement of Aβ-induced locomotor impairment associated with mitochondrial mislocalization (Iijima-Ando et al., 2009), whereas over-expression of Miro improved the eye phenotype, climbing performance and ATP levels in Aβ flies (Panchal and Tiwari, 2020).

To understand the effects of Aβ on mitochondrial distribution, Aβ was expressed in glutamatergic motor neurons of the fly leg. Aβ significantly reduces the number of mitochondria in the motor neurons and shortens the fly lifespan when overexpressed in glutamatergic neurons (Fernius et al., 2017). This suggests that Aβ affects mitochondrial distribution in the neuron, possibly contributing to the reduction in life span.

Mitochondria and endoplasmic reticulum connect to create mitochondria-ER contact sites (MERCs), which facilitates the exchange of lipids and calcium ions. However, MERCs dysfunction or miscommunication affects ATP generation and mitochondrial division by disturbing calcium shuttling and possibly through

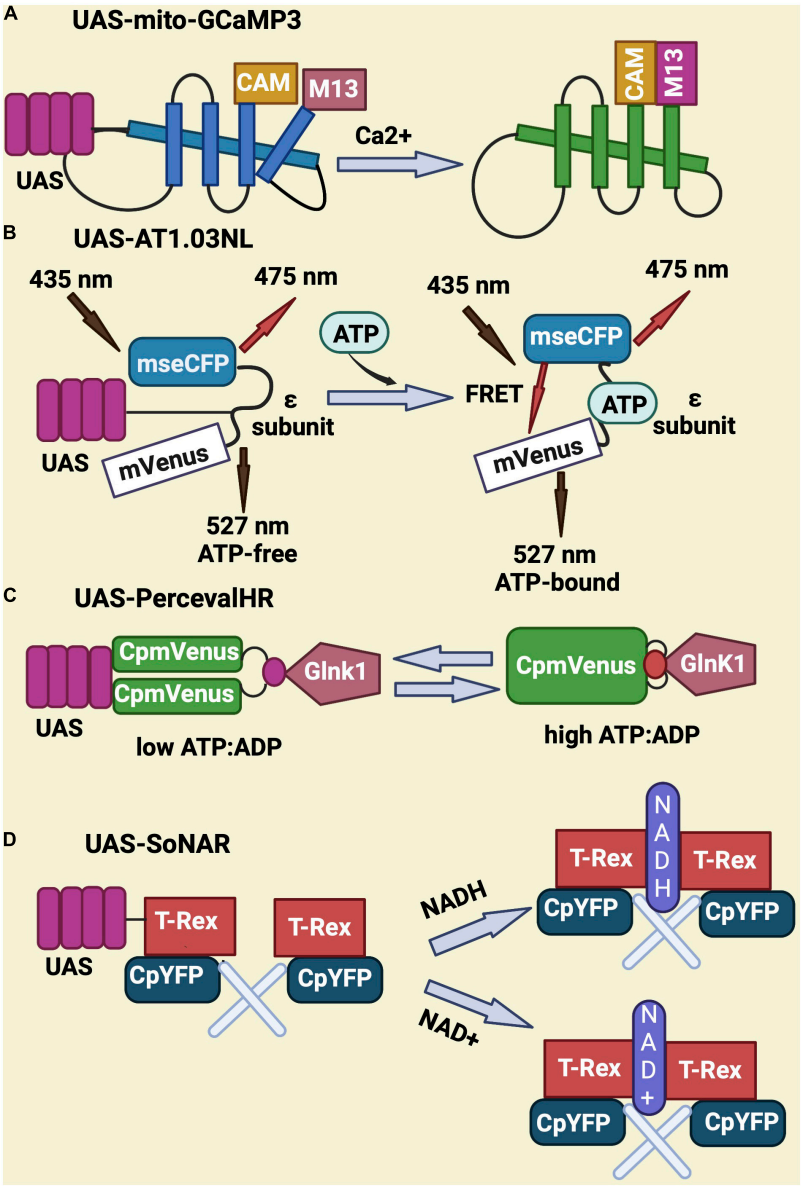

FIGURE 3
Schematic representation of genetically-encoded sensors for Calcium, ATP, ATP/ADP, NADH, and NAD. **(A)** UAS-mito-GCaMP3 encodes for a circularly permutated EGFP M13 and calmodulin fusion protein that gives GFP signal upon binding to calcium. **(B)** UAS-AT1.03NL consist of two fluorescent proteins (mVenus and mseCFP) and an ATP binding sequence (ε subunit) that elicits a FRET signal upon binding to ATP. **(C)** UAS-PercevalHR contains the ATP-binding protein GlnK1 from *Methanocaldococcus jannaschii* linked to circularly permutated mVenus and allows ATP sensing through conformational changes in GlnK1. **(D)** UAS-SoNar serves as NADH/NAD + sensor and encodes for a fusion of the NADH-binding domain of T-Rex (Rex protein from *Thermus aquaticus*) with a circularly permuted yellow fluorescent protein (cpYFP).

interaction with Drp1 and mitochondrial fission factor (MFF), although the molecular mechanism is not well understood (Rowland and Voeltz, 2012; Wilson and Metzakopian, 2021). This is relevant because alterations of the mitochondria–endoplasmic reticulum contacts have been reported in AD (Schon and Area-Gomez, 2013). In an effort to improve contacts between mitochondria and ER, synthetic linkers have been designed to enhance the proximity between both organelles. For instance, over-expression of a synthetic linker carrying mitochondrial and ER targeting sequences extended lifespan and suppressed climbing deficits in Aβ flies, which suggests that improving the interaction between mitochondria and ER could alleviate AD pathologies associated with mitochondrial dysfunction (Garrido-Maraver et al., 2020). However, a different team recently demonstrated that the knockdown of *pdzd8*, a putative *Drosophila* homolog of the mammalian MERC tethering protein, decreases contacts between the ER and mitochondria and restores locomotor deficits in Aβ flies (Hewitt et al., 2022). Thus, further research is needed to clarify these contradictory findings.

Calcium homeostasis is very important for proper mitochondrial function and must be preserved. Flies expressing human Aβ in the mushroom body have significantly reduced calcium import compared to control flies, suggesting an Aβ-mediated impairment of mitochondrial function in mushroom

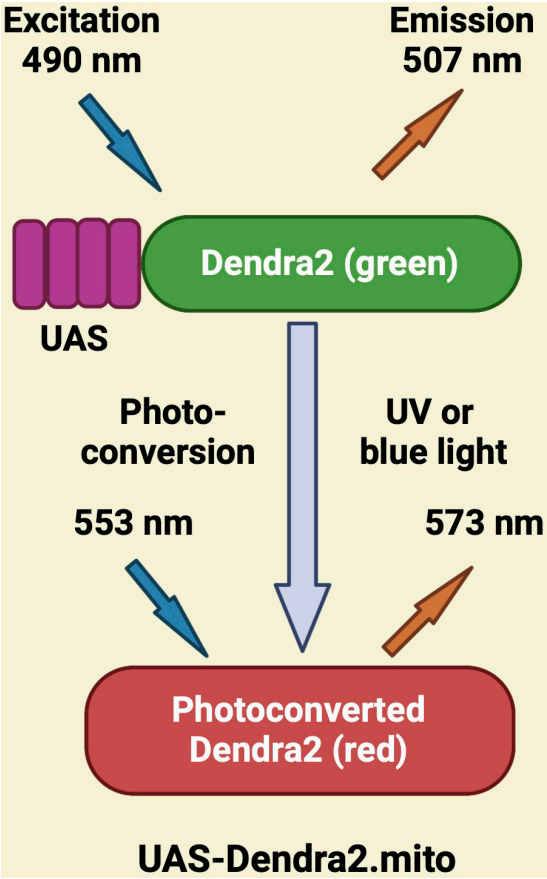

FIGURE 4
Schematic representation of the photo-convertible process associated with UAS-Dendra2.mito. Green Dendra2 is photo-converted to the red form upon UV or blue light exposure.

body neurons (Wang and Davis, 2021). In addition, pan-neuronal expression of Aβ reduced the levels of phosphoproteins predicted to be substrates of PKA, indicating that Aβ42 might modulate cAMP/PKA signaling (Iijima-Ando et al., 2009). This cAMP/PKA signaling is known to be affected by mitochondrial dysfunction which, in turn, reduces synaptic strength and prevents synaptic vesicle movement in the presynaptic terminal (Verstreken et al., 2005).

NDUFS3, a core component of the mitochondrial complex, is involved in the electron transport chain. Its expression is down-regulated in Aβ-expressing flies, which causes a decrease in the generation of ATP (Lin et al., 2021). Given that vitamin K serves as a mitochondrial electron transporter during oxidative respiration (Vos et al., 2012), there is an interest in investigating its therapeutic potential in fly models of AD. For instance, Lin et al. (2021) treated Aβ flies with vitamin K, which led to an improvement of mitochondrial function, reduction of Aβ neurotoxicity, and autophagy activation with concomitant increase in NDUFS3 expression and ATP levels.

Taken together, all these findings demonstrate that Aβ over-expression in flies triggers abnormal structural and functional changes in mitochondria, disrupts their dynamics and transport, and impairs learning and memory in late stages of the disease, all of which is relevant to understand AD pathogenesis (**Figure 5**).

Mitochondrial dysfunction in tau-expressing flies

Tau is a protein involved in the polymerization and stabilization of microtubules and is also associated with the axonal transport of sub-cellular organelles (Gendron and Petrucelli, 2009; Dolan and Johnson, 2010). Phosphorylation of tau reduces its binding affinity for the tubulin subunits of microtubules, which enhances the self-aggregation and fibrillization of phosphorylated tau (Cohen et al., 2011; Cisek et al., 2014; Singh et al., 2015) and leads to defects in axonal transport of mitochondria (Ittner and Götz, 2011; Mondragón-Rodríguez et al., 2013; Mietelska-Porowska et al., 2014). Over-expression of human wild-type and mutant tau R406W in flies induces elongation of mitochondria, resulting in mitochondrial dysfunction and apoptotic neurodegeneration with cell cycle activation (Wittmann et al., 2001; Khurana et al., 2006). Tau-dependent activation of the cell cycle requires tau phosphorylation and it has been shown that TOR signaling is also involved in the cell-cycle activation that mediates tau-induced neurodegeneration (Khurana et al., 2006). Interestingly, stimulation of mitochondrial fission by concomitantly increasing the expression of *Drp1* and reducing *Marf* levels reversed mitochondrial elongation and alleviated tau neurotoxicity in flies, suggesting that restoring the proper balance of mitochondrial fission and fusion is necessary to alleviate mitochondrial dysfunction and cell cycle-mediated cell death (DuBoff et al., 2012). In contrast, increasing fusion by upregulation of *Marf* and downregulation of *Drp1* further increased the mitochondrial length in tau flies, resulting in more aggressive neurodegeneration (DuBoff et al., 2012).

Dynamin-related-protein1 is a cytoplasmic protein that translocates to the mitochondrial outer membrane to drive mitochondrial fission; however, in the context of mutant tau overexpression, Drp1 staining does not colocalize with mitoGFP and stays primarily in the cytosol, as evidenced by its distribution in cytoplasmic and mitochondrial fractions from fly heads (Frank et al., 2001). This tau-related blockage of Drp1 translocation is thought to be mediated by stabilization of actin because reversing actin stabilization rescues tau-induced mitochondrial defects (DuBoff et al., 2012). Another study demonstrated that over-expression of leucine-rich repeat kinase 2 (LRRK2) also increases tau neurotoxicity through excessive actin stabilization and subsequent mislocalization of Drp1 (Bardai et al., 2018). This seems to be a relevant pathway as pharmacological suppression of actin polymerization was found to reverse neurodegeneration and mitochondrial impairments in tau transgenic flies (Bardai et al., 2018). On the other hand, given that the balance between fusion and fission is disturbed in AD (Wang et al., 2008a), Abtahi and coworkers looked at how tau affects the expression of *Marf* and *Drp1*, which are essential for mitochondrial fusion and fission, respectively, (Abtahi et al., 2020). The authors found that pan-neural expression of both wild-type and mutant tau R406W in flies decreased the expression of *Marf* mRNA in older flies, suggesting that the decline in the mitochondrial fusion process takes place at a later stages in AD. The expression of *Drp1* is modulated differently by wild-type and mutant tau; wild-type tau up-regulates *Drp1* mRNA, whereas mutant tau down-regulates it. Despite the fact that mutant and wild type tau express *Marf* and *Drp1* differently,

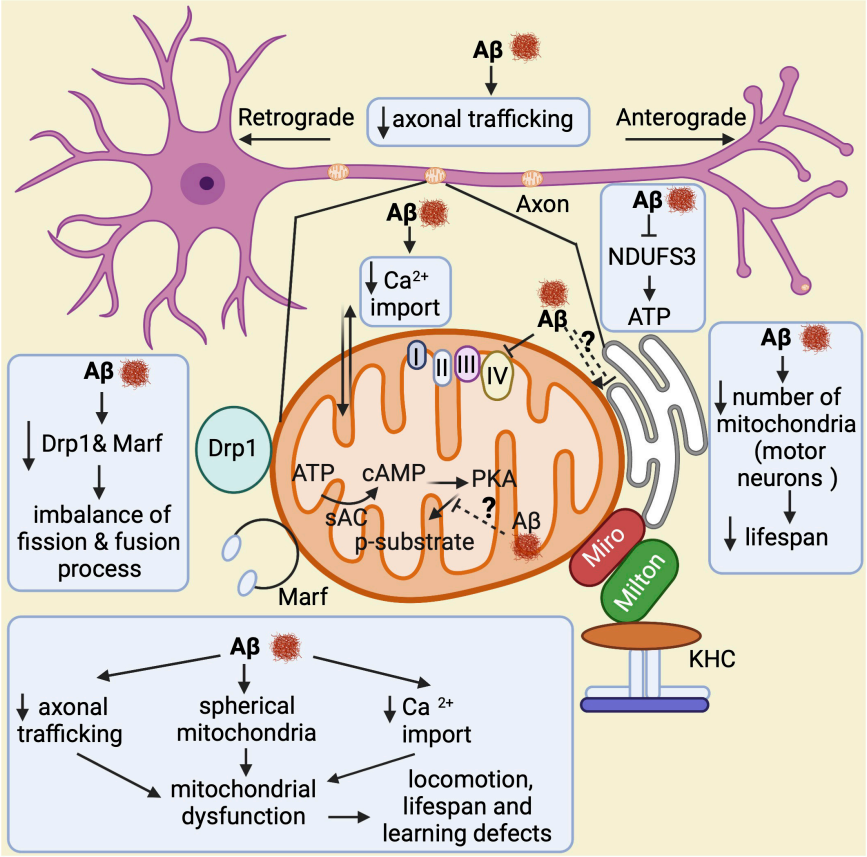

FIGURE 5
Overview of mitochondrial abnormalities found in Aβ-expressing flies. Aβ induces formation of spherical mitochondria, which disrupts their function. It also decreases calcium import into the mitochondria and affects their axonal trafficking, which impacts learning, lifespan, and locomotor behaviors. Aβ down-regulates NDUFS3, a crucial factor for the electron transport chain, resulting in low ATP production. In motor neurons, it reduces the number of mitochondria leading to shortened lifespan. Furthermore, Aβ decreases Drp1 and Marf expression, which causes an imbalance in the fission and fusion processes. Lastly, it seems to alter the mitochondria–endoplasmic reticulum contacts, although contradictory results have been found in this regard. Thus, this is highlighted with a question mark.

the increased ratio of *Drp1*/*Marf* suggests that both wild type and mutant tau display more mitochondrial fission (Abtahi et al., 2020), uncovering an imbalance between fusion and fission in tau flies.

In *Drosophila* larval motor neurons, overexpression of human tau (0N3R) disrupts axonal transport as well as the morphology and function of neuromuscular junctions (Chee et al., 2005). This is caused by a marked decrease in the amount of detectable mitochondria in the pre-synaptic terminal, which causes synaptic dysfunction accompanied by a lower number of functional mitochondria (Chee et al., 2005). To understand the effects of tau on mitochondrial distribution, both UAS-tau^{0N4R} and UAS-tau$^{0N4R-E14}$ were expressed in the fly leg neurons. The results showed that "clump-like" aggregation of mitochondria is seen in the motor neurons projecting into the muscles while the distribution of mitochondria is even in control flies, indicating that tau affects mitochondrial distribution (Fernius et al., 2017). Accordingly, wild-type UAS-tau^{0N4R} and UAS-tau$^{0N4R-E14}$ expression in glutamatergic neurons significantly shorten the fly lifespan (Fernius et al., 2017).

A recent genome-wide RNAi screen in flies expressing human mutant tau pan-neuronally led to the identification of several modifiers involved in the mitochondrial pathway, such as biotinidase, *NDUFS4*, *ALDH6A1*, and *TFB1M* (Lohr et al., 2020). The authors found that the knock-down of biotinidase in tau flies disrupts the structure and function of mitochondria, the function of carboxylase enzymes, and leads to a more aggressive neurodegeneration. Interestingly, administration of biotin through feeding rescues toxicity of both wild-type and mutant tau in transgenic flies (Lohr et al., 2020). This is relevant because the authors also found reduced carboxylase biotinylation in the brain of some AD patients. However, the extent to which biotin levels contribute to AD pathogenesis is largely unknown. On the other hand, to understand the possible interaction between toxicity of tau and axonal mitochondria, the adaptor proteins essential for axonal mitochondrial transport, Milton and Miro, were knocked-down in flies expressing wild type tau. Knock-down of either Milton or Miro enhances tau-induced neurodegeneration. Moreover, Milton knock-down accelerates the accumulation of autophagic bodies and vacuole formation in presynaptic vesicles and axons (Iijima-Ando et al., 2012). Additionally, it increases tau phosphorylation at Ser262 via the partitioning defective-1 (PAR-1) protein, which decreases tau ability to bind microtubules. This suggests that both tau phosphorylation at Ser262 and PAR-1 are essential for

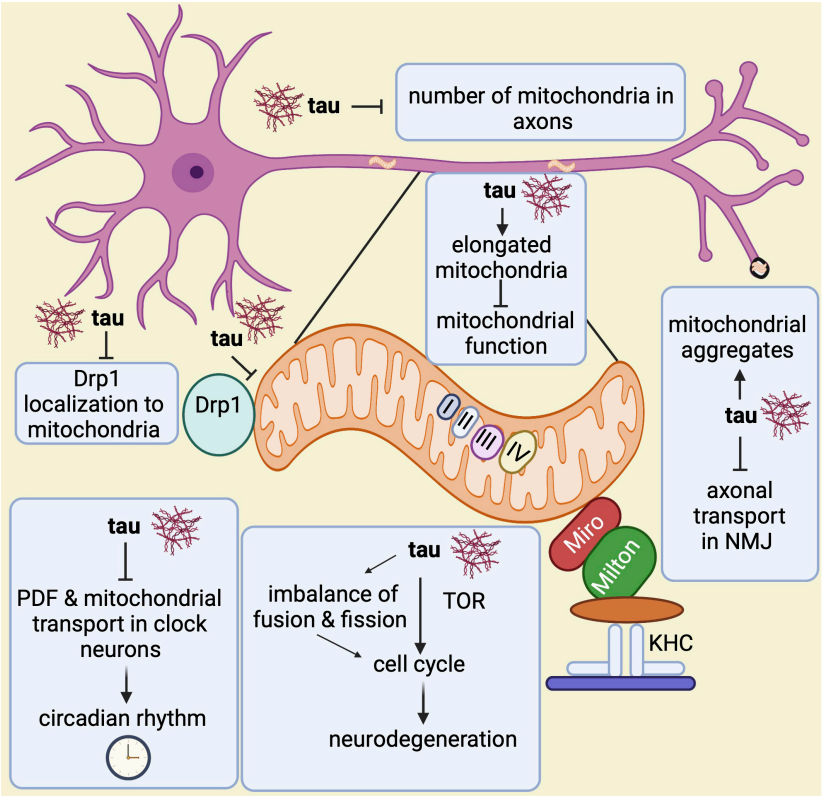

FIGURE 6
Overview of mitochondrial abnormalities found in tau-expressing flies. Tau induces elongated mitochondria, which causes mitochondrial dysfunction and apoptotic neurodegeneration with cell cycle activation. The cell cycle activation brings neurodegeneration via TOR and through an imbalance in the fusion and fission processes. Tau disrupts the circadian rhythm by inhibiting the neuropeptide pigment dispersing factor (PDF) and reducing mitochondrial transport in axons of clock neurons. Additionally, it prevents Drp1 from localizing to the outer mitochondrial membrane, which affects the fission process. Tau also disrupts axonal transport in the neuromuscular junction (NMJ) leading to mitochondrial aggregation. Lastly, it has been found that tau also reduces mitochondrial numbers in axons.

enhancing tau-induced axon degeneration in Milton knockdown. Whereas pan-neuronal knockdown of Milton or Miro results in age-dependent neurodegeneration in the fly brain, knock-down of PAR-1 or endogenous fly tau suppresses Milton knockdown-induced neurodegeneration, demonstrating that both PAR-1 and tau participate in Milton knockdown-mediated neuropathology (Iijima-Ando et al., 2012).

Lastly, another study showed that pan-neuronal expression of wild-type and phosphomimetic mutant tau (tauE14) disrupts the circadian rhythm (Zhang et al., 2022). While wild type tau expression in clock neurons reduces the levels of neuropeptide pigment dispersing factor (PDF), a neurotransmitter essential for circadian function in *Drosophila*, expression of tauE14 in clock neurons disrupts the circadian rhythm and reduces PDF distribution in the dorsal axonal projections. Interestingly, tauE14 also induces a complete loss of mitochondria in dorsal projections indicating that tauE14 impairs axonal transport of neuropeptides and mitochondria in circadian pacemaker neurons, affecting circadian rhythm (Zhang et al., 2022). Further studies will be required to better understand the association between circadian rhythm, mitochondrial biology and Alzheimer's disease. In summary, all these observations highlight multiple ways in which abnormal tau contributes to mitochondrial malfunction (**Figure 6**).

Concluding remarks and future directions

Alzheimer's disease is a devastating neurodegenerative brain disorder characterized by extracellular Aβ plaques and intracellular aggregates of hyperphosphorylated tau, along with progressive cognitive decline. Despite decades of research and impressive efforts from multiple groups, there is still no treatment available for this dreadful disorder. Recent evidence suggested that AD and other neurodegenerative diseases are impacted by mitochondrial dysfunction, an area that may provide new targets for future therapeutic strategies.

In this review, we emphasized how *Drosophila* closely resembles molecular and pathological features of Aβ- and tau-related mitochondrial disfunction observed in AD. We compiled relevant studies in flies showing how Aβ42 or tau affect the structure of mitochondria, mitochondrial dynamics, calcium homeostasis, axonal transport of mitochondria, cAMP/PKA signaling, mitochondria-ER contact sites as well as the expression of several mitochondrial factors. We also discussed available assays and tools for examining mitochondrial function and dynamics in *Drosophila*. Considering the flexibility and power of *Drosophila* genetics, it is clear that this model organism will continue

improving our understanding of the critical role of mitochondria in AD pathogenesis and its crosstalk with other pathomechanisms.

It is worth noting that there are still many opportunities for *Drosophila*-based research in this field. For instance a recent study found that the glutathione S-transferase (GST) Gfzf prevents mitochondrial hyperfusion in axons and regulates mitochondrial dynamics (Smith et al., 2019). This is important because several GST polymorphisms in humans have been associated with the development of AD (Allen et al., 2012). Thus, future manipulation of Gfzf in fly models of AD will help understand the potential contribution of GST activity to this devastating disorder.

One limitation of the studies discussed here is that they were performed in either Aβ42- or tau-expressing flies. Since Aβ42 and tau display synergistic interactions (Zhang H. et al., 2021), it will be imperative to study mitochondrial dynamics and function in flies co-expressing Aβ42 and tau to provide a more physiological context. This is because a recent longitudinal positron emission tomography (PET) study found that Aβ accelerated tau deposition in the inferior temporal cortex of older people with cognitively normal function over a 7-year follow-up period and that the rate of such accumulation was linked to the degree of cognitive decline (Hanseeuw et al., 2019). This finding is consistent with another PET study in cognitively healthy individuals showing that Aβ-tau interactions (rather than Aβ or tau alone) accelerated cognitive decline (Sperling et al., 2019). Moreover, individuals with primary age-related tauopathy, who exhibited equivalent tau burdens but negligible amounts of Aβ, had lesser high-molecular-weight (HMW) tau levels than patients with AD, who showed typical Aβ-plaque and tau-tangle burden (Bennett et al., 2017). Taken together, these studies confirm the synergistic interaction between Aβ and tau in AD pathology and emphasize the need of using fly models with concurrent Aβ and tau pathologies. On the other hand, TDP-43, an RNA/DNA binding protein linked to frontotemporal lobar degeneration and amyotrophic lateral sclerosis, was recently found to mediate prominent structural and functional damage to mitochondria along with activation of the mitochondrial unfolded protein response (UPR^{mt}) (Wang et al., 2019). This is also relevant because more than 50% of AD cases display TDP-43 pathology in the brain (Meneses et al., 2021). It is, therefore, critical to concurrently manipulate Aβ42, tau and TDP-43 in transgenic flies to decipher their overall mitochondrial insults in AD cases with TDP-43 pathology. Approaching these and other unknown aspects of mitochondrial dysfunction with *Drosophila* will provide a more comprehensive portrait of molecular abnormalities and, thus, may lead to the identification of multiple and promising therapeutic targets in the years to come.

Author contributions

VV and DER-L conceived the original idea and revised the manuscript. VV and JM designed the outlines of the study, performed the literature review, wrote the first draft, and prepared the figures. All authors read and approved the final manuscript.

Funding

This work was supported by the National Institutes of Health grants R01AG059871, R01AG077534, and R21AG069050 to DER-L.

Acknowledgments

We thank all members of the DER-L's laboratory for critical comments and Deepak Chhangani for suggestions in the preparation of figures. All figures in this review article were created with BioRender.com.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Neuroscience

TYPE Review
PUBLISHED 18 May 2023
DOI 10.3389/fnins.2023.1082047

OPEN ACCESS

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RECEIVED 27 October 2022
ACCEPTED 14 April 2023
PUBLISHED 18 May 2023

CITATION
Santarelli S, Londero C, Soldano A, Candelaresi C, Todeschini L, Vernizzi L and Bellosta P (2023) *Drosophila melanogaster* as a model to study autophagy in neurodegenerative diseases induced by proteinopathies.
Front. Neurosci. 17:1082047.
doi: 10.3389/fnins.2023.1082047

Drosophila melanogaster as a model to study autophagy in neurodegenerative diseases induced by proteinopathies

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Proteinopathies are a large group of neurodegenerative diseases caused by both genetic and sporadic mutations in particular genes which can lead to alterations of the protein structure and to the formation of aggregates, especially toxic for neurons. Autophagy is a key mechanism for clearing those aggregates and its function has been strongly associated with the ubiquitin-proteasome system (UPS), hence mutations in both pathways have been associated with the onset of neurodegenerative diseases, particularly those induced by protein misfolding and accumulation of aggregates. Many crucial discoveries regarding the molecular and cellular events underlying the role of autophagy in these diseases have come from studies using Drosophila models. Indeed, despite the physiological and morphological differences between the fly and the human brain, most of the biochemical and molecular aspects regulating protein homeostasis, including autophagy, are conserved between the two species.In this review, we will provide an overview of the most common neurodegenerative proteinopathies, which include PolyQ diseases (Huntington's disease, Spinocerebellar ataxia 1, 2, and 3), Amyotrophic Lateral Sclerosis (C9orf72, SOD1, TDP-43, FUS), Alzheimer's disease (APP, Tau) Parkinson's disease (a-syn, parkin and PINK1, LRRK2) and prion diseases, highlighting the studies using Drosophila that have contributed to understanding the conserved mechanisms and elucidating the role of autophagy in these diseases.

KEYWORDS

protein-aggregate, protein-misfolding, autophagy, neurodegeneration, proteinopathies, non-autonomous signaling, animal model, *Drosophila melanogaster*

1. Introduction

Drosophila melanogaster is an established model organism for developmental studies and due to the remarkable conservation of the signaling regulating autophagy, it has been used to better understand the relationship of this catabolic pathway with the genetic conditions that in humans are responsible of a class of neuronal diseases called proteinopathies (PPs). Autophagy is a key cellular pathway that, together with the ubiquitin-proteasome system (UPS), controls protein homeostasis by degrading misfolded proteins or exhausted organelles otherwise detrimental to the cells (Kocaturk and Gozuacik, 2018). Autophagy and UPS are closely linked,

in fact protein ubiquitination is a key step for the cargo recognition by the autophagic receptors and alterations in one pathway may affect the activity of the other (Waite et al., 2022). Both pathways are crucial for cell survival particularly in neurons where their perturbation causes age-associated disorders including neurodegenerative diseases (Rai et al., 2022). In this review, we will illustrate the contribution of *Drosophila* studies to the understanding of the role of autophagy in PPs induced by mutations in genes responsible for the most common neurodegenerative diseases (summarized in Table 1). Furthermore, we will discuss how flies could be used to further improve our understanding of the mechanisms that control these diseases, particularly those that are linked to mutations in genes that are physiologically involved in the control of the autophagic-proteostatic pathway.

2. *Drosophila* versus human brain

Drosophila 's studies played a crucial role in understanding brain function and development, leading to significant advances in neuroscience. *Drosophila*'s brain contains about 200,000 neurons, which are quite similar to mammalian neurons in terms of electrophysiological properties, and form a complex network of interactions recently mapped at high resolution (Zheng et al., 2018), and glial cells that represents 5–10% of the total cell population within the central nervous system (CNS). The CNS is composed of multiple specific and distinct brain compartments (Ito et al., 2014) that are interconnected and synergistically cooperating to control complex behaviors, such as learning, flight control, courtship, grooming, and memory-driven behaviors. Despite the small size, the overall brain organization and regional division shows fundamental similarities to the network structure of the mammalian brain. Indeed, the neural organization underlying primitive functions, such as the perception of odors, taste, vision, sound, gravity, and the circuits regulating feeding and satiety are very similar to those in humans (Tsubouchi et al., 2017; Jayakumar and Hasan, 2018). *Drosophila* CNS can be divided into two histological regions: the neuronal cell cortex, where all the neurons cell bodies are located, and the neuropil, where axons and dendrites project. This represents a major difference with vertebrates, where the cell bodies are in different areas of the brain depending on the circuit that they are regulating. In addition, in the fruit fly, the lateral connections between neighboring projection-neurons of the same compartment are made not only through interneurons (Liou et al., 2018) but also through dendro-dendritic synapses, which are rare or absent in most vertebrate neuropiles. In terms of morphology, while in vertebrates most neurons are multipolar, in invertebrates there are mostly unipolar (Rolls, 2011; Shah et al., 2016; Smarandache-Wellmann, 2016). Despite these differences, both vertebrate and invertebrate neuronal circuits are plastic which means that their structure and physiology are modified in response to stimuli, both extrinsic and intrinsic, during development and in adult life (Holtmaat and Svoboda, 2009; Lin et al., 2013; Peretti et al., 2015). The electrophysiological properties of the fruit fly neurons are also very similar to those in mammals. Their firing activity depends on Na^+ and K^+ fluxes that affect the membrane potential, and they communicate using vesicle release of conserved neurotransmitters (i.e., acetylcholine, GABA, glutamate) and neuromodulators (i.e., biogenic amines and neuropeptides) at the synapses. In *Drosophila* there are several classes of glia, mostly classified based on their morphology function and association with neurons. The perineural glia (PNG), which surrounds the central nervous system, is required to filter nutrients, while the sub-perineural glia (SPG) forms the septate junctions and together form the blood–brain barrier (BBB). This is different than in humans, where the BBB is composed of astrocytes and microglia, however also in *Drosophila* the BBB prevents paracellular diffusion and controls the influx and efflux of soluble molecules (Hindle and Bainton, 2014; Babatz et al., 2018; Kim et al., 2020). Within the CNS there are other classes of glia cells such as the cortex glia, associated with the neuronal cell bodies, unsheathing glia, that surrounds neuropils and astrocytes that associates closely with the synaptic neuropils and support neurons, similarly to those of the mammalian glia. These classes of glia are abundant and are involved in nervous system development, circuit assembly, synaptic plasticity and neurons support (Freeman, 2015).

3. *Drosophila's* tools to study proteinopathies (PPs)

The availability of a wide variety of transgenic strains, advanced genetic tools and databases have enabled the rapid development of *Drosophila* models for nearly 75% of human neuronal diseases including proteinopathies PPs (Ugur et al., 2016). The simplicity of fly's brain architecture and the highly conserved function of genes involved in neuronal development, highlights another advantage for its use. Furthermore, *Drosophila*'s genome generally harbors only one orthologue of the human counterpart therefore, mutation of a single gene generally leads to loss-of-function phenotypes, without redundant effects due to the presence of compensatory paralogues. The availability of unique and advanced genetic techniques allows for the rapid generation of transgenes in which the manipulation of the gene of interest (GOI) is performed in a short time and with unique precision in specific tissues and organs. The most common approach used is based on the yeast derived UAS/Gal4 system, in which a line carrying a tissue specific promoter fused with the transactivating domain of Gal4, is crossed with another line carrying the GOI (overexpression or its RNAi) cloned under the control of the UAS (Upstream regulating sequence). In the progeny, the binding of Gal4 to the UAS sequence will express or reduce the GOI in the tissue of interest (Brand and Perrimon, 1993). If manipulation of the GOI induces lethality, tissue expression control can be achieved by co-expression of the temperature sensitive inhibitor $GAL80^{ts}$, which acts as a transcriptional repressor of Gal4 at its permissive temperature, or by the Gene-Switch system which relies on a modified version of Gal4 which can be temporally induced by the administration of the drug RU486 (Walters et al., 2019). Variants of the UAS/Gal4 system, such as LexA/Op and QUAS, can be used to drive the expression of different transgenes concurrently with the UAS/Gal4. This is particularly useful for example to study the presence of non-autonomous signals between different organs, tissues or cells *in vivo* (Potter et al., 2010). Additionally, loss-of-function mutations or overexpression of genes can be easily obtained using CRISPR/Cas9 technology and commercially available sgRNA lines deposited at the Bloomington Stock Center (Ewen-Campen et al., 2017; Zirin et al., 2022). More recently, novel optogenetic techniques have been successfully developed to study protein misfolding *in vivo* in the brain

using *Drosophila* models of Alzheimer's disease (AD), Parkinson's disease (PD), and TDP-43/ALS (Lim et al., 2021). The morphological defects induced by the expression of genes responsible for human PPs (Figures 1A,B) can be easily analyzed after their expression in *Drosophila* (Figure 1C) using *ex vivo* dissection of the entire larval or in adult-fly brains, using different techniques ranging from immunofluorescence to super resolution microscopy (SEM or TEM; Figure 1D). Furthermore, motor defects caused by neuronal degeneration can be characterized by analyzing larval motility defects or by analyzing the decline of negative geotaxis in adults, which is the natural ability of flies to climb against gravity (Figure 1E). PPs are characterized by the formation of protein aggregates, which can be visualized by immunofluorescence using specific antibodies, or with the expression of fusion proteins where the gene of interest is fused with fluorochromes such as GFP or RFP (Figure 1F). Biochemical assays, using techniques such as western blot or filter-trap analysis, can show variations in molecular weight and the presence of large insoluble aggregates (Figure 1K). Cell death or induced inflammation at sites of aggregate formation can be studied directly by immunofluorescence assays using appropriate markers (e.g., cleaved caspase, TUNEL assay, or the presence of secreted immune-modulators, like eiger/TNFα or SPARK). Finally, one of the great advantages of using fruit flies as a model for PPs is that the onset of these diseases is relatively rapid, for example the expression of human mutant forms of Huntingtin *HTTQ97GFP*, in neurons leads to the formation of aggregates which are already visible within 48–72 h of larval development (Figure 1F). This is extremely advantageous since it allows to perform genetic or chemical screening to identify in a short time genes or drugs that reduce the formation of the aggregates (Figures 1G,J). These inhibitors can be chemically optimized (Figure 1H) to improve their qualities and finally be tested on human cells derived from patients (Figure 1I). However, questions remain as to why flies develop aggregates so early in their development, compared to vertebrates. One hypothesis is because flies lack a more complex, adaptive immune system that could control the onset of these diseases as it does in other model systems. In summary, the possibility to target specific mutations into subpopulation of neurons or glia and the ability to rapidly observe their effect cell autonomously or across the neural networks, combined with the short life cycle and the propensity to produce many offspring confirms *Drosophila* as an excellent model for studying human diseases including proteinopathies.

4. Mechanism of autophagy

Autophagy is a conserved mechanism with different specific catabolic functions in different tissues and cells. For example, in conditions of nutrient deficiency it represents a survival mechanism that generates amino acids and bioenergetic substrates to allow cell survival. However, autophagy is also used to eliminate toxic debris and exhausted organelles to maintain cellular clearance, particularly relevant in aging neurons, where physiological reduction in autophagy can cause their premature loss. Autophagy can occur in three different forms: microautophagy that is mediated by small cargo-containing vesicles on the lysosomal membrane; chaperone-mediated autophagy (CMA), in which the chaperone Hsc70, a member of the Hsp70 heat shock protein family, recognizes cargo proteins containing KFERQ-like motifs and delivers them to the lysosomes via the receptor lysosome-associated membrane protein 2A (LAMP2A); and the most studied macroautophagy herein referred as autophagy, mediated by a subset of ATG proteins encoded by the *ATG* gene-family, originally identified in yeast in response to nutrient starvation (Fleming et al., 2022). The product of the *ATGs* genes is responsible for the initial formation of the phagophore that envelops cytoplasmic cargoes in a double membrane (autophagosomes) which subsequently fuses with the lysosomes for the degradation of the cargo by hydrolases. Due to the specificity of different cargoes, autophagy is now studied and classified into more specific pathways (selective autophagy) such as: lipophagy (lipid droplets), ERphagy (endoplasmic reticulum), mitophagy (mitochondria), pexophagy (peroxisomes), aggrephagy (protein aggregates) and xenophagy (bacteria and viruses; Galluzzi et al., 2017). An important step in selective autophagy is the interaction of the ubiquitinated cargo-proteins with autophagic receptors, such as p62/SQSTM1 (Ref2(P) in flies) that by binding LC3 (ATG8a in flies) via the LIRs (LC3-interacting regions) sequences, recruits cargoes into the autophagosome for degradation. The amino acids produced in the lysosome by hydrolysis of the cargo can directly re-activate the TORC1 complex, located on the lysosomal membrane, thus blocking the autophagic flux (Abu-Remaileh et al., 2017; Wyant et al., 2017). Indeed, TOR kinase, which is part of the TORC1 complex, acts as negative regulator of autophagy by phosphorylating specific sites of the Ser/Tre kinase ULK1/2 (ATG1 in flies) thus destabilizing the initiator complex of the autophagy process that is composed by ATG13, ATG101, FIP200 (Figure 2). This is an essential step for the phagophore assembly since this complex activates local phosphatidylinositol-3-phosphate (PI3P) production at membrane structures called omegasome, that recruits the WIPI2 (WD repeat domain phosphoinositide-interacting proteins) and DFCP1 (zinc-finger FYVE domain-containing protein 1). These proteins are important for the recruitment of the ATG6L1-ATG5-ATG12 complex, responsible for the ATG3-mediated conjugation of LC3 proteins to membrane phosphatidylethanolamines (PEs) to form the membrane double bond. In this process, LC3-I is cleaved at the membrane and converted into LC3-II, process that is also used as a signature of autophagic flux efficiency and typically visualized in western blot assays (Figure 2A). Ectopic expression of LC3 fused to a Fluorescent Proteins is used as a marker of the autophagosome and to quantify the autophagic vesicles (in Figure 2B is shown *Atg8a-mCherry* expressed in neurons of the calyx together with *GFP* using the *Elav-Gal4* promoter). Autophagy is also regulated by AMPK, a kinase that is activated by cellular stress and low levels of ATP. AMPK phosphorylates ULK1/2 (ATG1 in flies) at sites different than those targets of TORC1. This promotes the formation of the initiator complex of autophagy (Figure 2; Kim et al., 2011). Therefore, molecules capable of inducing basal autophagy, such as rapamycin (a potent TORC1 inhibitor), or metformin (which activates AMPK) represent important therapeutic targets in proteinopathies. The use of simple animal models such as *Drosophila* was particularly relevant to the discovery and initial characterization of such molecules.

4.1. *Drosophila*'s autophagy a link to neuronal degeneration

Most of the steps in the autophagy pathway were first identified and characterized in the *Drosophila* fat body, where

there is a physiological induction of autophagy during metamorphosis to allow the animal to survive starvation (Rusten et al., 2004; Scott et al., 2004). Furthermore, because of the big size of the fat cells (50 μm), the fat body was used to better visualize the autophagosome maturation using ATG8a conjugated with mCherry or GFP (Figure 1B). Subsequently, genetic studies have delineated the important control of autophagy in longevity (Maruzs et al., 2019). First, *Atg7*^{d77} mutants showed increased oxidative stress and levels of ubiquitinated proteins, and presented defects in climbing and shortening of lifespan (Juhasz et al., 2007). Similar data were obtained with *ATG8a* mutants, while its overexpression was shown to promote neuronal survival by controlling oxidative stress and to induce longevity (Simonsen et al., 2008). Work in mice confirmed the role of ATG5 in promoting the survival of Purkinje cells and of ATG7 in preventing axonal degeneration (Komatsu et al., 2007; Nishiyama et al., 2007). Recently, GWAS studies in mice and human cells confirmed ATG5, ATG7 and identified ATG101 and ATG16L1 as crucial for maintaining neuronal survival in particular, ATG7 was associated with complex neurodevelopmental disorders in patients, confirming the role of these autophagic genes in the control of neuronal homeostasis (Wertz et al., 2020; Collier et al., 2021).

5. Neurodegenerative proteinopathies or (PPs)

Neurodegenerative Proteinopathies (PPs) are a large family of diseases that share common pathogenic features such as misfolded protein aggregations, neuroinflammation, oxidative stress and mitochondrial dysfunction that contribute to cellular degeneration of the neural tissue (Fleming et al., 2022). Either induced by genetic alteration or by an age-related or stochastic event, alterations of the natural conformation of proteins leads to the formation of oligomers or aggregates with consequent loss of the physiological function of the protein (Rai et al., 2022). Accumulation of aggregates in PPs is commonly due to different mechanisms that include: (i) irreversible formation of aggregates with new strong intermolecular interactions; (ii) inefficacious cell clearance; (iii) seeding and spread of pathological aggregates between cells, evident for prion-like diseases, but identified also in other proteinopathies where the presence of old aggregates function as a seed for the new one (seeds effect; Soto and Pritzkow, 2018). Thus, the central pathogenic role of protein aggregation in these diseases underlines the importance of endogenous mechanisms that control the proteostasis networks, such as the unfolded protein response (UPR), autophagy-lysosome pathway and chaperone activity (Klaips et al., 2018). In fact, age-related disruption of these proteostasis networks or their genetic alterations, contribute to the early onset of these diseases (Fleming et al., 2022; Rai et al., 2022). Deregulation of autophagy has been linked to the pathogenesis on many neuronal diseases thanks to the use of several animal models. In the current review, we will discuss only the most conserved and frequently mutated genes that are the cause of PPs (summarized in Table 1) whose characterization in flies has shown their role in protein homeostasis and autophagy, and those include HD, SCAs, ALS, AD, PD, and prion-like diseases (Pr-D).

5.1. Huntington's disease

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by a dominant mutation in the first exon of the *huntingtin* (*htt*) gene that leads to an expansion of the CAG trinucleotide sequence (longer than 35 repeats), resulting in a protein with a long polyglutamine-Q stretch. The clinical onset of the disease is generally at middle age, however the length of the mutation is inversely proportional to the onset of the disease in fact, patients with long CAG show signs already at a young age (Gusella and MacDonald, 1995). The human *htt* gene encodes for a protein of 350 KDa that contains four HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A and TOR kinase) domains, structurally related to the ARM (armadillo) repeats, and few PEST (peptide sequence rich in proline, glutamic acid, serine, and threonine) domains, that act as substrates for proteolytic enzymes, including caspases or calpain that cleave at amino acids 552 and 586 to produce an N-terminal fragment containing the polyQ domain (Ehrnhoefer et al., 2011). Post translational modifications (PTMs) regulates these proteolytic events and modification of the N-terminus region, such as arginine methylation (Migazzi et al., 2021; Ratovitski et al., 2022), neddylation (Ghosh and Ranjan, 2022), acetylation (Gottlieb et al., 2021), palmitoylation (Lemarie et al., 2021) and phosphorylation at Ser13 and Ser15, are relevant to modulate HTT localization and aggregate formation (Arbez et al., 2017; Chatterjee et al., 2021). These proteolytic cleavages occur also in the mutant HTT (mHTT) resulting in the formation of N-terminus truncated fragments that compete with endogenous HTT and favor the formation of toxic aggregates (Graham et al., 2006; Barbaro et al., 2015; Koyuncu et al., 2017; Ast et al., 2018). The presence of mutant HTT protein induces cellular defects leading to cell death, particularly of neurons in the striatum and cerebral cortex, leading to motor defects and rapid cognitive decline (Bates et al., 2015).

5.1.1. Huntingtin and autophagy

The physical interaction between endogenous HTT and p62/SQSTM1 was shown to facilitates the binding of HTT to ULK1/Atg1 releasing its negative regulation by mTOR, thus promoting autophagy (Rui et al., 2015). The fact that HTT protein contains sequences with structural homology to Atg23, vacuole protein 8 (Vac8ar) and Atg11, containing LC3-interacting repeats (LIRs) sequences (WxxL) present only in cargo receptors suggested a role for endogenous HTT to control autophagy (Ochaba et al., 2014; Martin et al., 2015). Further data showed that myristylation at its N-terminus directed the truncated HTT to the endoplasmic reticulum to initiate autophagosome-formation (Martin et al., 2014). Conversely, the inefficient myristoylation of the mutant HTT promoted the formation of large aggregates that bind p62/SQSTM1 but were not transported to the cargo resulting in an empty autophagosome (Martinez-Vicente et al., 2010; Martin et al., 2015). The human *huntingtin* gene (*htt*) is conserved across evolution and its homologue in flies, *dhtt,* encodes for a protein that shares an overall 24% identity with the human counterpart but contains only one CAG triplet positioned at its N-terminus (Li et al., 1999). While in vertebrates the function of *htt* is essential for development and is required for neuronal survival (Nguyen et al., 2013), flies *dhtt* is not necessary for development. However, *dhtt* knockout animals exhibit a reduced Mushroom body, learning impairment, age-related impaired of motility and shortened

lifespan (Zhang et al., 2009). The role of endogenous HTT in autophagy was first outlined in *dhtt* mutant animals that showed defective developmental autophagy and increased ubiquitination of p62/Ref(2)P (*Drosophila*'s p62/SQSTM1; Ochaba et al., 2014) and like its orthologue in vertebrates, Ref(2)P accumulated in brains of aged animals (Nezis et al., 2008; Bartlett et al., 2011). Furthermore, endogenous *dhtt* was shown to counteract the toxic effect of ectopic expression of the mutant human *htt-exon-1*-Q93 (Zhang et al., 2009), corroborating the protective role of endogenous HTT evidenced also in mice (Van Raamsdonk et al., 2005). More recently, loss of function mutation in the *dhtt* gene was shown to enhance defects in axon outgrowth in the Mushroom body due to decreased function of the *App* gene, the *Drosophila* orthologue of the human *amyloid beta-precursor protein* (*APP*) gene, responsible of Alzheimer's disease, suggesting an interaction between those genes that may control the onset of AD (Marquilly et al., 2021).

5.1.2. Drosophila models of HD

A few models of *Drosophila* HD have been generated over the years (Lewis and Smith, 2016). They are mostly based on the expression of the N-terminal fragments of the human *htt* gene or on the use of *htt-exon-1* mutated to carry different lengths of the CAG tract; while few models have been generated using the full-length mutant human gene due to the high toxicity of its expression when carrying the polyQ expansion tract (Romero et al., 2008). Because of their viability, flies carrying mutations in the *htt-exon-1* (mHTT) were widely used to study the function of the polyQ, and to demonstrated that the length of the CAG tract is proportional to a progressive neuronal degeneration and decline in animal motility (Jackson et al., 1998; Marsh et al., 2000; Krench and Littleton, 2017). Furthermore, expression of mHTT in neurons showed a proportional increase in the number and size of aggregates that were already visible at 48–72 h of development (Figure 1F), indicating that *Drosophila* could be an efficient model to study the kinetic and inhibitors of aggregates formation *in vivo*. Indeed, genetic screens for aggregate-phenotype modifiers, identified several pathways including TOR signaling (Ravikumar et al., 2004; Sarkar et al., 2007), the chaperons CCT (Sajjad et al., 2014; Pavel et al., 2016), histone deacetylase (Pallos et al., 2008; Jia et al., 2012), antioxidant pathways (Mason et al., 2013), deubiquitinating enzymes (Aron et al., 2018), enzymes involved in glutamine metabolism (Vernizzi et al., 2020), early endosomal protein Rab5 (Ravikumar et al., 2008), Puromycin-sensitive aminopeptidase (PSA; Menzies et al., 2010) and very recently the novel chemical compounds that function as linkers between mHTT and LC3 able to target only the mutant form of HTT to the autophagosome leaving intact the wild-type allele in a selective manner (Li et al., 2019).

5.2. Spinocerebellar ataxias

Spinocerebellar ataxias (SCAs) are a heterogeneous group of inherited disorders that affects the spinal cord and the cerebellum and are characterized by loss of Purkinje cells and cerebellar atrophy. SCAs present mutations in 40 different genes many of which carry an expansion of the polyglutamine tract such as SCA1, SCA2, SCA3/MJD (Machado-Joseph disease), SCA6, SCA7, SCA17 and DRPLA (Dentatorubral-pallidoluysian atrophy; Klockgether et al., 2019). Here we will focus only on the functional mechanisms characterized in *Drosophila* models for SCA1-3 (Warrick et al., 2005), since models for *CACNA1A* responsible for SCA6 (Tsou et al., 2016; Sujkowski et al., 2022), SCA7 (Jackson et al., 2005), SCA17 (Ren et al., 2011) and *DRPLA* (Charroux and Fanto, 2010) only describe motility defect in the adult flies.

5.2.1. SCA1

SCA1 is an autosomal dominant disease caused by an expansion of the CAG triplet in the coding region of the *Ataxin1 (ATXN1)* gene. This gene encodes for a protein with RNA binding capacity that associate to transcriptional regulators at promoter regions (Yue et al., 2001). Patients affected by SCA1 represent 6% of the individuals affected by cerebellar ataxias, and their mutant *ATXN1* carries a tract of more than 39 *CAG* as compared to approximately 20 in the wild-type gene. The mutation leads to the production of a protein with a long polyQ that forms insoluble aggregates, visible in the nuclei of the Purkinje cells (Stoyas and La Spada, 2018). Furthermore, *ATXN1* has been shown to favor the toxicity of human pathogenic *ATXN2* in mice-models; on the contrary loss of function of *ATXN1* increased the stability of *BACE1-mRNA*, enhancing amyloidogenic cleavage of APP in a mouse model of AD (see Section 5.4.1) and outlining a function of ATXN1 also in AD (Suh et al., 2019).

5.2.1.1. Ataxin1 and autophagy

A specific function for Ataxin1 in controlling the autophagic pathway in flies has not been described, however TOR inhibitors in flies were shown to ameliorate the toxic effect of mutant Ataxin1 through the release of the inhibitory role or TORC1 on the lysosomal transcription factor Mitf (*Drosophila* homologue of human TFEB; Bouche et al., 2016). *Drosophila* harbors a gene encoding for *Ataxin1* (*dAtx*) that lacks the polyQ domain, nevertheless its overexpression led to phenotypes like those obtained by human *ATXN1* overexpression, but different from those observed upon overexpression of the polyQ alone. Genetic experiments showed that Atx1 interacts through its conserved AXH domain with the zinc-finger transcription factor Senseless (Sens). This mechanism is conserved for its mammal's homolog, the growth factor independence-1 (Gfi-1) and proposed as a potential pathogenic mechanism for SCA1 (Tsuda et al., 2005).

5.2.1.2. *Drosophila* models of SCA1

Expression of the human mutant *ATXN1* gene in *Drosophila*'s neurons leads to retinal degeneration and loss of axonal projections of the interneurons in the ventral nerve cord of the brains (Fernandez-Funez et al., 2000), expression of the pathogenic form of SCA1 or SCA3 in *Drosophila* larval dendritic neurons reduced their arborization with disruption of F-actin dendritic structures, defect that is partially rescued by Rac-PAK signaling activation (Lee et al., 2011). *In vivo* screens using the adult eye helped define the role of chaperones to proteins with an expanded polyQ. In particular, the chaperone-dependent E3 ubiquitin (Ub) ligase CHIP (carboxyl terminus of Hsp70-interacting protein) and the NAD synthase nicotinamide mononucleotide adenylyl transferase (NMNAT) together with Hsp70 mediate activation of the proteosome pathway (Zhai et al., 2008). Genetic experiments have delineated the role of transglutaminases (TGs), a class of enzymes that catalyzes the formation of cross-links between glutamine

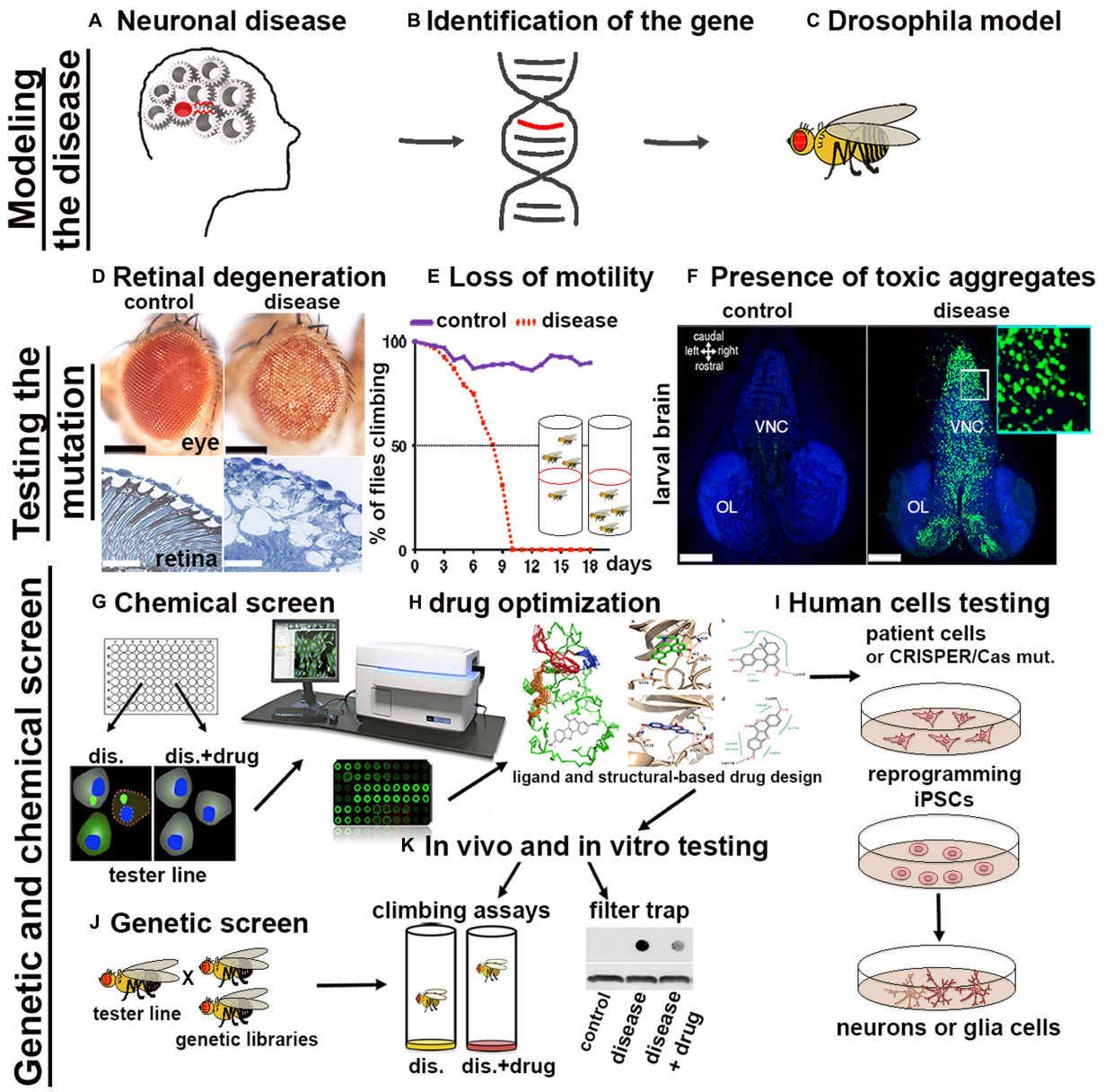

FIGURE 1
Scheme of a pipeline to characterize genes associated with proteinopathies and to perform High-throughput Screens with small chemical compounds, to develop new therapeutical strategies in humans. **(A–C)** From the identification of a gene related to a human disease to the generation of the transformants carrying the GOI. **(D–F)** The effect of the mutant-disease genes can be tested at cellular and behavioral levels. **(D)** For example, the expression of *exon1* of the human mutant *Huntingtin* containing 93-CAGs (*HTTQ93*) in the retina using the *GMR-Gal4* promoter leads to loss of the pigmentation in the ommatidia of the compound eye (as seen in the upper image obtained using a stereo microscope) and to retinal degeneration accompanied by defects in tissue morphology and neuronal death [outlined by the white spot of missing tissues visible by transmission electron microscopy (TEM) showed in the images below; (Vernizzi et al, 2020)]. **(E)** Expression of mutant HTT in neurons using *ELAV-Gal4* induces neuronal defects that can be indirectly quantified by measuring the decline over time of the animal motility (negative geotaxis assay). **(F)** Using the *Elav promoter* we can express the human *HTT exon-1 with 97 CAGs* as a fusion protein with GFP *(HTT-GFP)*, and show the formation of HTT-GFP aggregates in larval neurons already at 48-72 hours of age. Photo in panel **F**, to the right is shown a larval brain of *Elav-LexOP-HTTGFP-LexA larvae at 72 hrs AEL*, control is to the left. BLUE stains the nuclei (inset 63x). **(G–K)** Potential pipeline for a High throughput screen (HTS) to identify drugs that reduce the formation of toxic aggregates. **(G)** *Drosophila* cells, induced to express the *HTTGFP* construct are cultured in medium containing small chemical compounds (libraries); analysis of the changes in GFP expression can be quantified using a microplate reader (TECAN). Compounds capable of reducing GFP expression will highlight potential pathways that could be involved in the reduction of mHTT aggregates. **(J)** *Drosophila* HD models can be used to perform genetic screens to analyze *in vivo* the expression of components of these pathways; **(K)** *Drosophila* HD models will be fed with the small compounds identified to analyze their effect in ameliorating animal motility or in reducing the size of mHTT aggregates; analyzed directly by immunofluorescence in the brain or by filter-trap assays using either organs or from whole animals. **(H)** For better performance, chemical drugs can be modified using ligand and structure-based drug design to improve their characteristics and then they can be tested again *in vivo* in *Drosophila* HD models such as in K. **(I)** Finally, the candidate drugs will be tested using human cells differentiated to iPSCs and then to neurons of glia, or directing to neurons iNs (Hong and Do, 2019) starting from cells of patients or from cells from healthy donors in which the specific mutation is introduced using the CRISPR/CAS9 technique.

residues within proteins thus increasing their stiffness and insolubility (Muma, 2007). The relevance of these enzymes in proteotoxicity and autophagy was first demonstrated using a *Drosophila* model for PD and later also in HD, where TG2 phosphorylation by PINK blocked its degradation favoring proteotoxicity and mitochondria degradation. Further experiments, both in *Drosophila* and in human cells, showed that TG2 reduction ameliorates the defects in both PD and HD (Karpuj et al., 2002; McConoughey et al., 2010; Min et al., 2015). Similarly, a role for TG5 in enhancing the toxicity of mutant *ATXN1* was shown in *Drosophila* models and in cells from SCA1-patients where TG5 was shown to colocalize with Ataxin1 (Lee et al., 2022b) further supporting a functional role for this enzymes in the disease. Genetic screen and chemical inhibitors revealed the RAS-MAPK–MSK1 as an important axis in the control of Ataxin1 aggregate formation (Park et al., 2013). Using small inhibitors of the MSK1/MAPK complex it has been demonstrated in mice and flies that the phosphorylation of Ataxin1 on Ser776 is relevant for its stability, while mutation of this residue in the wt protein reduced the level of Ataxin1-82Q in mice suggesting that Ser776 could represent a novel substrate for both drug and allele-specific therapies (Nitschke et al., 2021).

5.2.2. SCA2

Mutations in the human *Ataxin2* (*ATXN2*) gene lead to spinocerebellar ataxia type 2 (SCA2) which accounts for 13% of spinocerebellar ataxias (Klockgether et al., 2019). Mutations in *ATXN2* with an expansion greater than 32 CAG repeats forms insoluble cytoplasmatic aggregates visible in Purkinje and granule cells, leading to gliosis and neuronal death (Huynh et al., 2000). Additionally, mutations in *ATXN2* were found to be a risk also for ALS due to their effect on increasing TP-43 toxicity (van den Heuvel et al., 2014).

5.2.2.1. Ataxin2 and autophagy

Not many studies ruled out the function of autophagy in SCA2 until recently, when it was shown that autophagy ameliorates mitochondrial dysfunction and oxidative stress in mice models for SCA2 and in cells from patients (Wardman et al., 2020). In addition, it was shown that cordycepin, a drug that activates AMPK and autophagy, reduces the abnormal accumulation of p62/SQSTM1 and

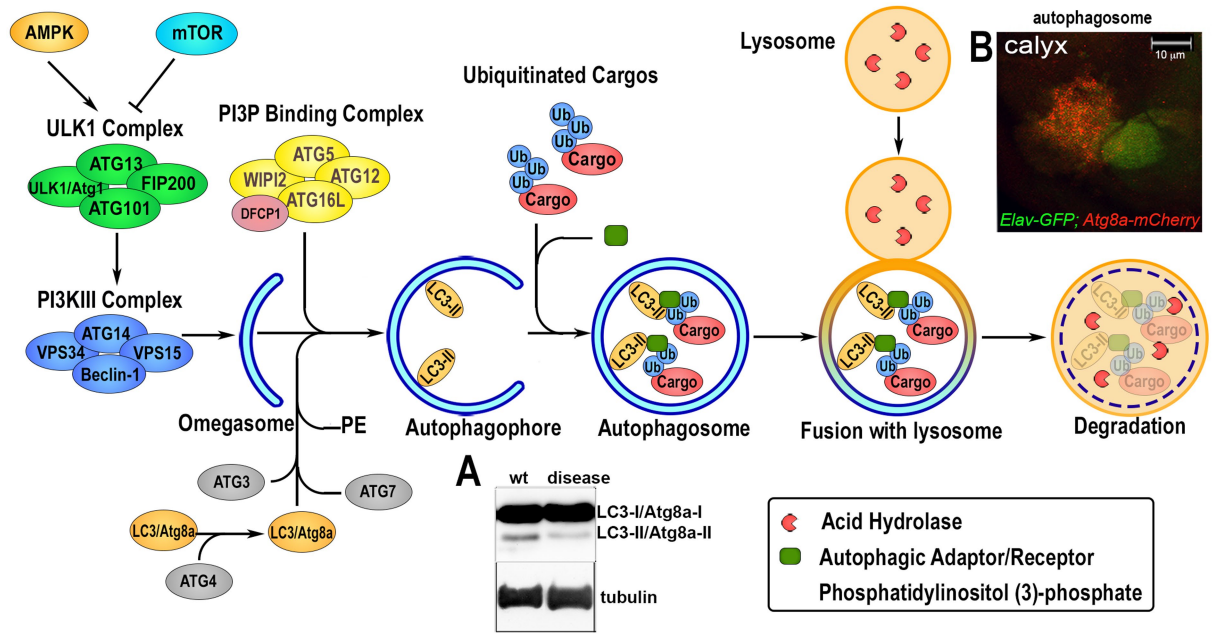

FIGURE 2
Overview of the autophagic flux. The induction of the autophagic process is regulated upstream by 5t'-AMP-activated Protein Kinase (AMPK) and Target of Rapamycin (TOR), that modulate the activation of the Unc51-like Kinase 1 (ULK1/Atg1) via phosphorylation and the formation of the initiator the ULK1-Complex composed by ULK1, Autophagy-related protein 13 (ATG13), ATG101 and FIP200. Upon activation, ULK1/Atg1 Complex in turn activates via phosphorylation the phosphatidylinositol-3-kinase III (PI3KIII) Complex, which comprises vacuolar protein sorting 34 (VPS34), VPS15, ATG14 and Beclin-1. This allows its translocation on the ER membrane and the production of an isolation membrane enriched in phosphatidylinositol (3)-phosphate (PI3P). PI3P induces the recruitment of the PI3P Binding Complex, consisting of ATG5, ATG12, ATG16L and WD Repeat Domain, Phosphoinositide Interacting 2 protein (WIPI2), on the growing autophagophore (omegasome) and the Double FYVE-containing protein 1 (*DFCP1*). This process enhances the ATG3-mediated binding of Microtubule-associated protein 1A/1B-light chain 3 (LC3) to phosphatidylethanolamine (PE) on the autophagosomal membrane where is lipidated. This leads to its cleavage from LC3-I/ATG8a-I to LC3-II/ATG8-II, which is considered a feature of an active autophagic flux and can be visualized and quantified by western blot analysis (panel **A**). Ubiquitin-tagged proteins are recognized by specific autophagic adaptors/receptors, such as p62/SQSTM1/Ref2(P), with a mechanism that is selective for each different organelles or cellular structure called selective autophagy. The cargo receptors bind LC3/ATG8a and transport the cargo into the autophagophore where its content is hydrolyzed upon fusion with lysosome. Autophagosome formation can be visualized *in vivo* by ectopic expression of LC3/Atg8a fused to GFP or mCherry fluorescent proteins that form small "puncta" on the autophagosome membrane, and fluorescence can be quantified using imaging processing programs. Panel **B** shows the *UAS-Atg8a-mCherry* staining pattern under physiological conditions analyzed in the calyx region of *Drosophila* larval brain, where neuronal cells are visualized by expressing *UAS-GFP* using the *ELAV-Gal4* promoter. Co-localization of mCherry puntae within GFP is used to quantify the presence of the autophagosome in neurons.

of LC3 observed in cells form SCA2 patients, indicating a dysfunction in the autophagic flux in the disease (Marcelo et al., 2021). *Drosophila* possess one orthologue of the *ATXN2* gene *(Atx2)* which encodes for a protein Atx2 that shares 23% of identity and 36% of similarity with the human Ataxin2 protein, but differently *Drosophila* Atx2 does not contain a long polyQ stretch but three short separate segments of glutamines. Structurally human Ataxin2 contains the Lilke-Sm (Lsm) and Lsm-AD domains found in RNA binding proteins (Satterfield and Pallanck, 2006), and further experiments in human and flies confirm that both Ataxin2 and Atx2 physically bind with the polyribosome suggesting that Ataxin2 may be involved in the control of translation (Satterfield et al., 2002).

5.2.2.2. *Drosophila* models of SCA2

Expression of human mutant *ATXN2* in neurons resulted in the formation of aggregates leading to neuronal degeneration that (Lessing and Bonini, 2008). Further work demonstrated that overexpression of *Drosophila* Atx1 promotes the accumulation of human mutant Ataxin2 aggregates, indicating that the interaction between the two proteins could contribute to the pathogenesis of SCA1 and SCA2. This hypothesis is in line with the idea of a cooperative mechanism between Ataxin1 and Ataxin2, also suggested by the presence of Ataxin2-enriched aggregates in postmortem neurons of patients with SCA1 (Al-Ramahi et al., 2007). Further studies should be conducted using *Drosophila's* model of SCA2 to better understand the physiological role of Ataxin2/Atx2 in the control of autophagy and how it could be perturbed in SCA2, to reveal novel mechanisms of intervention.

5.2.3. SCA3

Also known as Machado-Joseph disease, SCA3 is the most common type of spinocerebellar ataxia. It is characterized by a progressive neurodegeneration caused by an expansion of CAG at the 3′ end of the coding region of the *ATXN3* gene, which from 12 to 40 repeat in the wild-type reaches more than 55 CAGs in the mutant form (Klockgether et al., 2019). The gene encodes for a deubiquitinase (DUB), a class of enzymes that counteracts the action of ubiquitin and important for the control protein stability of TP-43 and HTT (Doss-Pepe et al., 2003; van Well et al., 2019; Tran and Lee, 2022), responsible when mutated of ALS and HD, making DUBs potential targets for proteinopathies.

5.2.3.1. Ataxin3 and autophagy

Autophagy is compromised by mutation of *ATXN3* since the expression of the autophagic markers p62/SQSTM1 and LC3 was found abnormal in the brain of patients with SCA3. Furthermore, transgenic mice expressing *ATXN3-71Q* exhibit large aggregates that are reduced in the presence of the autophagy-promoting gene Beclin1 (Nascimento-Ferreira et al., 2011). Puromycin-sensitive aminopeptidase (PSA), a cytosolic enzyme able to digest polyQ sequences (Menzies et al., 2010) was shown to ameliorate also Ataxin3 phenotypes both in flies and mice and to induce autophagy (Menzies et al., 2010). *Drosophila*, like mammals has two isoforms of Atx3 proteins that contain either two or three ubiquitin interacting motifs (2UIM) with an additional at its C-terminus (3UIM; Johnson et al., 2019). Studies in mammals and flies highlighted the different ability of these isoforms to form aggregates when carrying the polyQ expansion. In particular, 2UIM is more prone to form aggregates than 3UIM, that is rapidly degraded mainly through the proteasome pathway (Harris et al., 2010). Further studies in flies showed that the UIMs motifs of Atx3 interact with the heat shock protein cognate-4 (Hsc70-4) to enhance Ataxin3 mutant aggregation and toxicity (Johnson et al., 2021). This data complement those showing that the auto-protective function of *Drosophila* endogenous Atx3 depends on its deubiquitinating catalytic activity that indirectly reduce the folding of the toxic polyQ present in mutant Ataxin3 proteins as in others polyQ proteins (Warrick et al., 2005), rather than on the activation of the proteasomal degradation pathway (Tsou et al., 2015).

5.2.3.2. *Drosophila* models of SCA3

The most widely used models for SCA3 express the C-terminal fragment of the human *ATXN3* gene with 78CAGs or 82CAGs repeats, that in the retinal neurons cause abnormal eye morphology due to neuronal and tissues degeneration and motility defects (Warrick et al., 1998, 2005). Genetic screens for modifiers of the Ataxin3 mutant phenotypes led to the identification of several genes of the ubiquitin pathways, members of Hsp70/chaperone proteins, and potential regulators of RAN translation, a Repeat-associated non-AUG (RAN) translation that generates toxic dipeptide repeat proteins (DPRs) from pathological repeat RNA expansions hat do not contain the classical methionine start codon (Cleary et al., 2018), see also for C9orf72-ALS (Section 5.3.1). Genetic screen in flies, using the eye, identified members of the ubiquitin ligase family, such as Cullins and of Praja1, that protect against the effect of mutant Ataxin3 strongly supporting the relevance of the ubiquitin and of the DUBs in the disease (Chen et al., 2019; Ghosh et al., 2021). This screens also identified selective modifiers of Ataxin3 able to rescue also Tau-R406W-AD mutation (Section 5.4.2) supporting the existence of common pathways that converge and contribute to neuronal degeneration, controlled by the Ataxin3 DUB-activity (Bilen and Bonini, 2007). In addition, Ataxin3 DUB-activity controls parkin's ubiquitination in the modulation of "physiological" mitophagy (Durcan and Fon, 2011), and ubiquitination of K63 in the formation of SOD1 aggregates (Wang et al., 2012). Treatment with antioxidant drugs such as the Nrf2-inducers caffeic acid and resveratrol have also been shown to indirectly reduce apoptosis and to induce autophagy, both in human cells and in *Drosophila* models of SCA3 (Wu et al., 2017).

5.3. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a rare disease, with an incidence of 2–3 per 100,000 and a variable progression rate. ALS patients are diagnosed based on the degeneration of motor neurons, however, the pathophysiological heterogeneity of the disease is accompanied by a variety of other factors that are not completely understood. ALS is often associated with frontotemporal dementia (FTD), a disease that presents common mutant genes with ALS resulting in mental disability without motoneurons degeneration or movement impairment. Genetics ALS accounts for only 10–15% of all ALS cases, while the remaining are of sporadic, however up to now, more than 50 genes have been implicated in ALS and the pool

of loci associated with this disease is expanding due to GWAS and whole-exome/genome sequencing. Among new pathways identified there are members of sphingolipid signaling and actin polymerization, identified in the vesicles transport network (Brenner and Freischmidt, 2022) suggesting how complex is the physiopathology of this disease. Autophagy in ALS. Deregulation of autophagy has been linked to pathogenesis of ALS thanks to the use of several animal models. ALS patients present accumulation of autophagosomes in the cytoplasm of spinal cord neurons and harbor mutations in genes involved in the autophagy machinery. Many genes have been associated to ALS and here, we will focus on C9orf72, SOD1, TD-P43, FUS since their characterization in flies has been of crucial importance to show their role in protein homeostasis and autophagy.

5.3.1. C9orf72

Mutations in the *C9orf72* gene are characterized by an expansion of the hexanucleotide sequence GGGGCC (G4C2) in the first intron of the gene, which can reach up to thousands of repeats in the most affected patients (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The gene encodes for a guanine nucleotide exchange involved in cellular processes such as vesicular trafficking, autophagy, lysosomal function and in the control of the immune system and recently described as part of the lysosomal membrane-complex that binds RABs for a correct lysosomal function (Taylor et al., 2016; Root et al., 2021). *C9orf72* mutation causes toxicity and alteration of the autophagy-lysosomal pathway contributing to disease pathology (Beckers et al., 2021). Hypothesis regarding the consequences of *C9orf72* mutation range from loss of gene function due to epigenetic transcriptional silencing of the locus, to RNA-mediated toxicity caused by the formation of RNA foci that trap both RNA transcripts and RNA binding proteins, and to the formation of toxic dipeptides (DPR) produced by non-AUG translation (RAN) associated with intronic hexanucleotide repeat expansion (Kwon et al., 2014; Wen et al., 2014; Freibaum and Taylor, 2017). The presence of DPRs also compromises the nucleocytoplasmic transport, resulting in the accumulation of toxic debris in the cytoplasm leading to neuronal death (Freibaum et al., 2015; Zhang et al., 2015). Here we have outlined few of these events and discussed their relationship with autophagy.

5.3.1.1. *C9orf72* and autophagy

The relationship between *C9orf72* and autophagy is somewhat controversial, since some data showed that mutation in *C9orf72* cause toxicity and alteration of the autophagy-lysosomal pathway (Beckers et al., 2021), others showed that *C9orf72* acts as a negative regulator of autophagy as mice mutant for the *C9orf72* gene showed reduction TOR activity with nuclear translocation of TFEB and activation of the autophagy flux (Ugolino et al., 2016). Moreover, DPRs induce dysfunctions of the autophagic-lysosomal pathway by synergizing with other mechanisms of toxicity increasing the pathogenesis of the disease (Beckers et al., 2021). Growth factors and insulin/IGF influences neurodegenerative diseases including AD, PD and ALS. indeed *C9orf72-*G_4C_2 mutation has been shown to downregulate insulin/IGF signaling in both fly and human cells while activating insulin/IGF signaling enhances the toxicity of poly-GR repeats (Atilano et al., 2021). IGFs also act as neurotrophic factors for the survival of motor neurons and have therapeutic effects in a mouse model for SOD1-ALS, and in the murine motoneurons NSC34 cells expressing mutant *C9orf72-*G_4C_2 (Kaspar et al., 2003; Stopford et al., 2017). Using high-throughput screen to identify chemical modulators of DPRs, we also found that cells expressing mutant *C9orf72-*G_4C_2 have increased cAMP levels resulting in protein kinase A (PKA) activation, event that was partially rescued by pharmacological or genetic inhibition of PKA (Licata et al., 2022). Since PKA is activated by growth factors, this observation raises the question of a connection between growth pathways and C9orf72 in ALS and, although the mechanisms are not yet fully understood, we presume that they may converge to the control of cellular proteostasis or autophagy.

5.3.1.2. *Drosophila* models of C9orf72

Although *Drosophila* does not have a *C9orf72* orthologue, transgenic models have been established and mimic the toxicity in humans due to its gain of function effect in flies (Mori et al., 2013; Xu et al., 2013; Mizielinska et al., 2014). The insertion of repeating sequences of GGGGCCs at different lengths was used to determine their different toxicity and ability to form structures called foci that include RNA-binding proteins and RNA (Mori et al., 2013; Mizielinska et al., 2014). RNA of the DPRs obtained by RAN translation was optimized *in vitro* to express as specific peptides (poly GA, GP, GR, PA, alone or fused with GFP or FLAG tags; Cleary et al., 2018). The difference in toxicity for each DPR is related to their different chemical composition and atomic charge. Indeed, highly arginine-rich GR and PR have been shown to alter the phase separation of LCD-containing proteins by changing the charge and structure of membrane organelles and their dynamics and functions (Wen et al., 2014; Lee et al., 2016). Works in flies evidenced a direct role of *C9orf72-*G_4C_2 in autophagy that led to the identification that its expression induced the accumulation of Ref(2)P and of poly-ubiquitinated proteins both in motor neurons and in whole larvae, due to defects in cargo protein degradation. These flies showed defects in lysosome formation, accompanied by reduced Mitf/TFEB nuclear localization (Cunningham et al., 2020). Recent work has demonstrated that nuclear transport of TFEB is mediated by nucleoporins (nuclear import proteins) and their expression is based on the molecular chaperone SIGMAR1/Sigma-1 (sigma-1 intracellular non-opioid receptor) mutated in ALS (Wang et al., 2022). Overexpression of SIGMAR1 in flies restores proper cellular transport, outlining the relevance of this chaperonin in nuclear import in this disease (Lee et al., 2020a).

5.3.2. Superoxide dismutase

Superoxide dismutase (SOD1) is the second most frequently mutated gene in ALS, representing approximately 20% of familial ALS. This gene encodes for SOD1 a ubiquitously cytosolic Cu/Zn-binding enzyme that homodimerizes and catalyzes the dismutation of superoxide radicals to hydrogen peroxide counteracting the toxic effect of free reactive oxygen species (ROS). SOD1 modulates protein quality control, autophagy, mitochondrial function, and axonal transport. SOD1 mutations may lead to both loss or gain of function phenotypes, making difficult to interpret the mechanisms through which its mutation leads to ALS (Brenner and Freischmidt, 2022).

5.3.2.1. Superoxide dismutase-ALS and autophagy

wt SOD1 was found in a complex with Atg9/Beclin1 to control P62/SQSTM1 expression, indeed mice mutant for SOD1-ALS show reduced Atg9/Beclin1 and increased level of P62/SQSTM1 and of ubiquitinated proteins (Nassif et al., 2014). Work in *Drosophila* as well as in *C. elegans*, identified a role for *lethal(2)* malignant brain tumor (L3MBTL1) a histone methyl-lysine protein, and SET domain-containing protein-8 (SETD8) in the control protein misfolding and degradation as their reduction alleviate the phenotypes induced by SOD1. This mechanism is conserved also in mammals underlining the role of chromatin modification in the control of protein quality control (Lu et al., 2019). As we know the interplay of ubiquitin-mediated autophagy is controlled also by deubiquitinases (Clague et al., 2019) and studies in *Drosophila,* as in other invertebrate models, outlined the negative role of the DUB-USP7 that by reverting the ubiquitination of the E3-ligase NEDD4L, reduced autophagy and the clearance of misfolded SOD1 and TDP-43 proteins by reducing the SMAD2/TGF-beta pathway (Zhang et al., 2020).

5.3.2.2. *Drosophila* models of SOD1

Drosophila endogenous *dSOD1* is necessary for neuronal health as its null mutation results in neuronal loss. Co-expression of a mutant *dSOD1* allele, carrying specific structural LOF mutations, results in formation of unfunctional wt-SOD1/dSOD1 heterodimers indicating that functional dimers are necessary for the activity of the enzyme (Phillips et al., 1995). Studies in *Drosophila* and in mice demonstrated that expression of human SOD1 in motor neurons reduced ROS activity, leading to longevity (Parkes et al., 1998; Missirlis et al., 2001). Further studies in flies demonstrated that the enzymatic activity of human SOD1 is not required for its toxicity: In fact, homozygous inactive SOD1 mutants (G85R, H48R, and H71Y) expression in *dSOD1* null flies results in neurodegeneration, motor defects, and shortened life span, suggesting that these phenotypes are associated to SOD1-mutations rather than SOD1 activity (Şahin et al., 2017; Agudelo et al., 2020). Expression of mutants SOD-1 results in deposition of protein aggregates visible in neurons, glia and in skeletal muscles in both mice-of ALS and cells from ALS patients (Dirren et al., 2015). Mutations in SOD1 lead to mitochondrial impairment, a defect that also occurs in *Drosophila,* in fact mutant SOD1 accumulates in the intermembrane space of the mitochondrial membranes modifying their structure, reducing the production of ATP and causing metabolic dysfunctions particularly relevant for the activity of motor neurons (Tafuri et al., 2015; Gallart-Palau et al., 2016). A distinctive characteristic of SOD1 mutations is the presence of *non-autonomous signals from glia* that induce lethality in neighboring cells. This effect was demonstrated *in vivo* in *Drosophila* and mice models for SOD1-ALS (Boillee et al., 2006; Watson et al., 2008) and supported by data showing that ablation of the glia ameliorate the disease in mice mutants for SOD1-ALS (Guttenplan et al., 2020). The importance of non-autonomous signaling has been strengthened by recent work demonstrating that motor neurons from SOD1-G93A mutation-bearing mice exhibit metabolic changes that affect surrounding myocytes (Peggion et al., 2022), and likewise between glia and neurons to induce survival of neurons (Chua et al., 2022). Understanding the nature of the non-autonomous signals is relevant to better define the mechanisms in the pathogenesis of ALS and to identify new components and modalities of therapeutic intervention. Thus, the design of Drosophila ALS models using the combination of the two binaries, LexA-LexOp with UAS-Gal4, may allow for the expression of SOD1 mutations in a specific tissue, for example motor neurons, and the concomitant manipulation of glia, using the UAS-Gal4 where many lines are available at stock centers. This would allow to rapidly identify genes that can interfere/suppress SOD1 neuron lethality similar to the approach used for identify mHTT non-autonomous interactors (Bason et al., 2019).

5.3.3. TAR DNA-binding protein 43

TAR DNA-binding protein 43 (TDP-43) is a DNA/RNA-binding protein that belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP) family with both nuclear and cytoplasmic functions. Functionally, TDP-43 is involved in modulation of several aspects of RNA life such as transcription, splicing, stability and turnover, degradation, alternative polyadenylation, transport, translation, and microRNA biogenesis [165]. TDP-43 is considered a key protein in ALS for two main reasons: firstly, TDP-43 is the major component of the ubiquitin-positive cytoplasmic inclusions found in spinal motor neurons of ALS patients, secondly, mutations in its gene (*TARDBP*) occur in around 0.9% of ALS patients.

5.3.3.1. TAR DNA-binding protein 43 and autophagy

Autophagy plays an important role in the clearance of the cytoplasmic inclusions in fact, it has been shown that the TDP43 aggregates colocalize with markers of autophagy and inhibition of autophagy increases their aggregates formation (Brady et al., 2011). Moreover, TDP-43 itself modulates autophagy, therefore creating a complex scenario whose perturbation contributes to ALS (Huang et al., 2020). Despite the recognized importance of TDP-43 in ALS, it is still unclear whether the pathogenesis of ALS is related to its reduced physiological function, since wild-type TDP-43 is sequestered in inclusion bodies and unable to interact with its physiological targets, or if it is due to the formation of toxic aggregates containing TDP-43 (Scotter et al., 2015). Loss of *TARDBP* in mammals downregulates histone deacetylase 6 (HDAC6), an enzyme that controls ubiquitinated protein and autophagy (Fiesel et al., 2010). Controversial results have been obtained in *TARDBP* knockout cells, where although the lysosomal transcription factor TFEB is upregulated, accumulation of immature autophagic vesicles and reduced expression of Atg7 are observed, suggesting that reduction of *TARDBP* affects other signals/genes necessary for a functional autophagy (Xia et al., 2016).

5.3.3.2. *Drosophila* models of TDP-43

Drosophila harbors a *TARDBP* orthologue, namely *TBPH,* and several groups have investigated its function to gain insights into ALS pathogenesis by performing loss of function studies. Interestingly, *Atg7* overexpression suppresses the semi-lethality of *TBPH* null flies, ameliorating motility, lifespan and reducing the accumulation of Ref2(P)/P62 inclusions at the NMJ (Donde et al., 2020). Other studies in flies addressed the consequences of overexpressing wild-type or disease forms of TDP-43. Overexpression of a cytoplasmic wt-TDP-43 or of TDP-43-M337V mutant in the fly's retina results in neurodegeneration that can be rescued by co-expression the heat shock protein HSP67Bc that promotes autophagy (Crippa et al., 2016). More recently, HEXA-018, a novel chemical compound that induces

autophagy in a TOR-independent manner and ameliorates climbing activity and lifespan was identified as a suppressor of TDP-43 overexpression in flies (Lee et al., 2021).

5.3.4. Fused in sarcoma

Fused in sarcoma (FUS) is a DNA/RNA-binding protein involved in DNA repair and RNA processing, often found in stress granules (SGs) dense structures present in the cytosol composed of RNA and RNA binding proteins (Wolozin and Ivanov, 2019). Over 70 mutations in the FUS gene have been identified in patients with familial and sporadic ALS, the vast majority of which are heterozygous mutations with autosomal dominant inheritance. Considering that most mutations influence the nuclear localization signal (NLS) of the protein, recent studies have delineated the presence of intrinsically disordered regions often enriched with arginine and glycine repeats that may be prone to promote protein aggregation, when mutated in ALS perturbating SGs dynamics that may be at the heart of ALS (Vance et al., 2009; Rhine et al., 2020). RNA binding proteins have multivalent domains important for their behavior; indeed proteins like TDP-43 and FUS, that exhibit spontaneous liquid–liquid demixing upon interaction with specific targets during phase separation, have been found to interact genetically when mutated, further accelerating neurodegeneration (Lanson et al., 2011). WT and mutant FUS are incorporated into a variety of RNA granules and they accumulate in *de novo* paraspeckles described in spinal motor neurons of ALS-patients (An et al., 2019).

5.3.4.1. Autophagy and FUS

Similarly, to TP-43, autophagy seems to play a crucial positive role in the elimination of toxic aggregates also in FUS-related ALS. Expression of wild-type FUS and ALS-related FUS mutations triggers mechanisms that lead the accumulation of toxic cytoplasmic aggregates and to inhibition of autophagy (Brunet et al., 2021). Furthermore, work in flies and human iPSCs demonstrated that the P525L-FUS mutation alters stress granule dynamics resulting in inhibition of PI3K/AKT/TOR signaling which indirectly increases autophagy by a yet unknown mechanism (Marrone et al., 2018).

5.3.4.2 Drosophila models of FUS

Genetic screens using flies identified Mask, an Ankyrin-repeat containing protein, that promotes the expression of the proton-pumping vacuolar (V)-type ATPases, favoring the elimination of FUS aggregates via lysosomal autophagy (Zhu et al., 2017). Recently, attention has been placed on post translational modifications that interfere with the protein phase separation of FUS, indeed the enzyme glutathione transferase omega 2 (GstO2) was shown to reduce cytoplasmic FUS level and the formation of FUS aggregates both in *Drosophila* and in mouse neuronal cells overexpressing FUS. Glutathionylation of FUS promotes its separation into the liquid phase suggesting how an accurate analysis of the mechanism driving protein phase separation might be a promising target for novel therapeutic strategies (Cha et al., 2022).

5.4. Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia in elders (Gonzales et al., 2022), characterized by progressive neurodegeneration and cognitive impairment. This pathology is considered a proteinopathy because it is characterized by the accumulation of extracellular amyloid plaques, enriched in amyloid-β peptide (Aβ42), intracellular neurofibrillary tangles, composed of hyperphosphorylated Tau, and reactive gliosis. Most cases of AD are sporadic and only a small percentage shows clear familial autosomal dominant inheritance: the familial cases are predominantly early-onset forms associated with fully penetrant mutations in the Amyloid precursor protein (APP), and the γ-secretases Presenilin 1 and 2 (PSEN1 and 2; Karch et al., 2014). *APP* encodes for a type I transmembrane protein involved in several neuronal functions, many of which have been discovered and studied in *Drosophila* (Gunawardena and Goldstein, 2001). APP can be processed following two different pathways: the so-called amyloidogenic pathway, consisting in a subsequent action of β- and γ-secretases (BACE and PSENs) that leads to the formation of several fragments including the Aβ, and the non-amyloidogenic pathway, where the α- and γ-secretases lead to the production of different APP fragments (Vassar et al., 2009; Zhang et al., 2014). The fruit fly represents a powerful model to study AD in fact, the genes associated with AD are evolutionary conserved and models obtained by overexpression of human Aβ peptide, Tau or of APP carrying pathological mutations mimic neurodegeneration and deficit in memory/cognitive abilities.

5.4.1. Amyloid precursor protein and amyloidopathies

Mutations in *APP*, *PS1*, and *PS2* genes, favor the production of the Aβ42 peptides and this, together with the presence of the amyloid plaques, shaped the "Amyloid Hypothesis" that proposes the imbalance between production and clearance of Aβ42 as the main cause of AD development (Selkoe and Hardy, 2016). To date, therapies for AD are still missing, underlying the need to better understand APP function/processing and the consequences of Aβ42 and tau aggregation.

5.4.1.1 Autophagy in APP and amyloidopathies

Autophagy plays a complex and dual role in AD since it regulates both Aβ eneration and clearance (Mputhia et al., 2019). Several studies support a protective role: Atg5, Beclin1, and ULK1 were shown to be involved in Aβ degradation (Tian et al., 2011) and the adaptor protein AP2, a member of a complex responsible for the internalization of cargos in clathrin-mediated endocytosis, was shown to induce the degradation of the APP-βCTF (C-terminal fragment) via autophagy, affecting Aβ production (Tian et al., 2013). Moreover, autophagic degradation of APP-CTF and Aβ is promoted by NRBF2, a member of the class of III phosphatidylinositol 3-kinase (PtdIns3K) complex that regulates autophagosome maturation (Cai et al., 2021). On the other hand, activation of autophagy might play a detrimental role in AD depending on the context. For example, activation of TFEB, plays opposite effects on Aβ production in neurons depending on the expression level of APP or of the βCTF (Yamamoto et al., 2019; Song et al., 2020).

5.4.1.2 Drosophila models of APP and amyloidopathies

Drosophila harbors an orthologue of *APP*, namely *APPL* and since the Aβ sequence is not conserved *Drosophila*' models of AD that contributed to gain insight into the role of autophagy were mostly obtained by overexpression of either human Aβ peptide or of human

AD-associated genes. The protective action of autophagy has been proven by modulating the levels of autophagic activity in flies expressing human Aβ peptide. For example, lowering the expression of autophagy-related genes, such as *Atg1*, *Atg8a* and *Atg18*, strongly enhances the neuronal toxicity caused by Aβ expression (Omata et al., 2014). In line with this, *Atg8a* overexpression in an Alzheimer *Drosophila* model obtained by overexpression of human amyloid precursor (hAPP) and beta-secretase 1 (hBACE1), enhances stress-resistance, slows down neurodegeneration and improves lifespan (Tsakiri et al., 2021). In a similar AD model, promotion of autophagy by the Nicotinamide mononucleotide adenylyltransferase (NMNAT) reduced amyloid plaques formation (Zhu et al., 2022). In flies overexpressing Aβ42, the protective role of autophagy was demonstrated by the expression of Thioredoxin-80 (Trx80), which prevents the accumulation of the toxic peptides in the brain and rescues lifespan by inducing autophagosome formation (Gerenu et al., 2021). On the other hand, Ling and colleagues showed that dysfunctional AEL (autophagy-endosomal-lysosomal) plays a crucial role in Aβ42 accumulation. In fact, the abnormal accumulation of Aβ42 within AEL vesicles was reduced when the functional autophagy was decreased (modulation of *Atg5* or *Atg12* levels), suggesting that dysfunctional autophagy–endosomal–lysosomal may cause Amyloid-like plaque formation (Ling et al., 2014). Moreover, it was recently shown that ectopic APP expression is sufficient to trigger aberrant autophagy through its interaction with the carboxyl-terminus of Hsc70-interacting protein (CHIP), a U-box type chaperone associated E3 ubiquitin ligase via transcriptional upregulation of *BACE1* and *PSEN* inducing an AD-like neurodegeneration (Zhuang et al., 2020).

5.4.2. Tau and tauopathies

Tau is a group of six microtubule-associated proteins all derived from alternative splicing of the *MAP/Tau* gene, they are abundant in the CNS, where they are necessary for the stabilization of the microtubules important for axonal transport. In the presence of specific mutations or during aging, Tau becomes hyperphosphorylated and changes its structure, decreases its ability to bind microtubules, leading to the formation of insoluble filaments that accumulate as neurofibrillary tangles resulting in tauopathies, a hallmark of AD (Goedert et al., 2017; Hernandez et al., 2022). Recent work pointed to a role of Tau within the nucleus where it associates with nucleolar proteins, suggesting the presence of other mechanisms activated by mutation of Tau (Anton-Fernandez et al., 2022).

5.4.2.1. Autophagy in tauopathies

Autophagy plays a protective role in tauopathies, as it favors the clearance of the soluble and the insoluble Tau form the last present in the aggregates. Furthermore, autophagy helps the movement of Tau through vesicles involved in its transport into neurons.

5.4.2.2. *Drosophila* models of tauopathies

Drosophila possesses a *tau* gene with 46% identity and 66% similarity to the corresponding human *Tau*. Flies have been successfully used to pin down numerous pathways that control Tau toxicity via autophagy. For example, insulin signaling can induce abnormal Tau phosphorylation and accumulation, inhibiting its autophagic clearance and worsening the disease (Chatterjee et al., 2019). Silencing calpain, a family of proteases that inhibit autophagy, is protective against Tau toxicity in the *Drosophila*-eye, an effect that requires a functional autophagy since it was lost in *Atg8a* mutant flies (Menzies et al., 2015). A microRNAs screen aimed at identifying suppressors of Tau-mediated phenotypes, clarified that *CG11070*, a target of miR-9a mediates Tau ubiquitination and degradation primarily via the autophagy-lysosome system. Interestingly, this function is conserved for the human orthologue *UBE4B* in cells of neuroblastoma (Subramanian et al., 2021). In AD patients Tau protein is often hyper-phosphorylated and one of the kinases responsible is PTK2/FAK (focal adhesion kinase; Lee et al., 2022a). PTK2 plays a critical role in proteinopathies as it favors abnormal phosphorylation of proteins, including TDP-43 and p62/SQSTM1 leading to the formation of ubiquitinated aggregates and neurotoxicity induced by the dysregulation of the ubiquitin–proteasome system (UPS; Lee et al., 2020b). This mechanism is conserved for Tau (wt or mutant P301L) and also present in *Drosophila* where it was shown that attenuation of PTK2 expression reduces the phosphorylation of Ref2(P)/p62 ameliorating the motility of Tau expressing flies (Lee et al., 2022a). This also suggests the presence of a feed-back loop between PTK2 and p62 dysregulated in tauopathies.

5.5. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease and another example of tauopathy (Poewe et al., 2017). PD symptoms include slow movement, postural imbalances, resting tremor, and muscle rigidity. Non-motor symptoms are also common and include memory loss, psychiatric symptoms, sleep problems, and pain. These symptoms stem from dopaminergic (DA) neuron loss in a part of the brain known as the substantia nigra pars compacta (SN), which leads to a lack of dopamine in other regions of the brain including the striatum. Both the affected regions are involved in movement and muscle control (Maiti et al., 2017). Treatment with the dopamine precursor levodopa or drugs such as dopamine agonists can temporarily improve many of the motor symptoms of PD however, no treatment currently exist able to prevent the neurodegenerative processes, underlying the importance to study the mechanisms responsible for DA neuron loss (Poewe et al., 2017). PD is characterized by the presence of Lewy Bodies, cytoplasmic inclusions consisting of a variety of misfolded proteins including α-synuclein and phosphorylated tau, in the SN and throughout the brain. As for Alzheimer's Disease, most cases of PD are sporadic, thought to be triggered by environmental factors. However inherited forms of PD, linked to mutations in several different genes including *α-synuclein, LRRK2, GBA, Parkin, PINK1*, and *Vps35* do exist, and their pathology and progression is very similar or indistinguishable from the sporadic one.

5.5.1 Autophagy in AD

The misfolded proteins accumulated in the Lewy Bodies are thought to be toxic, triggering neuroinflammation and cell death (Lashuel et al., 2013), and even though the exact mechanisms leading to their accumulation and toxicity are not completely understood, it seems that they include mitochondrial dysfunction, oxidative stress, and disruption of autophagy-mediated protein clearance (Karabiyik et al., 2017; Maiti et al., 2017). Interestingly, most of the genes

associated with PD, such as *Parkin, PINK1,* and *LRRK2*, control mitochondria activity and are involved in autophagy-mediated degradation/ubiquitination of proteins to control the turnover of damaged mitochondria (mitophagy; Winklhofer, 2014; Rahman and Morrison, 2019). Most of the PD-associated genes are expressed and functionally conserved in *Drosophila*, therefore both loss of function of endogenous genes or models harboring different human mutations were generated (Hewitt and Whitworth, 2017). Work performed in the fruit fly has largely contributed to clarifying the role of these genes in autophagy/mitophagy. In the following paragraphs, we will discuss the most common *Drosophila* models of PD used to study autophagy modulation in this disease.

5.5.2. Alpha-synuclein (α-syn)

The *SNCA* gene encodes for α-synuclein (α-syn), a protein that is abundantly expressed in the nervous system where it is found in membranes and is a major components of the Lewy Bodies. Mutations in α-syn have been identified in both familial and sporadic cases of PD (Lesage and Brice, 2009). While some carry single-amino acid substitutions that increased α-synuclein secretion (Guan et al., 2020), most mutations promote α-syn aggregation and fibrils formation (i.e., E46K, H50Q, A53T), while others (i.e., G51D and A53E) decelerate α-syn aggregation (Flagmeier et al., 2016; Guan et al., 2020). This suggests that the effects of PD-associated pathogenic mutations on α-syn behavior are quite complex and probably modulated by different signaling.

5.5.2.1. *Drosophila* models of α-synuclein

Flies do not possess an orthologue of *α-syn* therefore models have been produced by ectopically expressing human mutants of *α-syn*. A large part of studies conducted in flies point to a dysfunction of autophagy in presence of *α-syn* mutation and to a positive role of autophagy in α-syn induced degeneration: ectopic expression of human α-syn leads to F-actin-mediated impairment in autophagic flux, accumulation of abnormal autophagosomes, impairment in mitophagy, leading to defects in cellular bioenergetics (Sarkar et al., 2021). Treatment of flies overexpressing α-syn with spermidine, a naturally occurring polyamine known to prolong lifespan by inducing autophagy, protects against α-syn induced neurotoxicity (Buttner et al., 2014). Overexpression of the human lysosomal membrane protein LAMP2A fully prevents the behavioral defects induced by neuronal expression of a PD-associated mutant form of α-syn (SNCA/A30P) as well as reduces the α-syn accumulation in older flies. Moreover, LAMP2A expression upregulates Atg5 that stimulates macroautophagy (Issa et al., 2018). Similarly, the progressive locomotor decline and the loss of dopaminergic (DA) neurons caused by the human α-syn-A30P variant is reduced by pan-neuronal overexpression of dDOR (orthologue of the human tumor protein 53-induced nuclear protein 1 (TP53INP1), able to activate autophagy and basal mitophagy (Dinh et al., 2021). Another group demonstrated that knockdown of inositol-requiring enzyme 1 (IRE1) or Atg7 reverses α-syn-A30P-induced neurodegeneration in terms of lifespan, locomotor activity, and DA neuron loss (Yan et al., 2019). This suggests that IRE1 and Atg7 may play different roles in the classical autophagy mechanism of survival and their loss activates protective mechanisms thereby explaining the reduction of toxic proteins and neuron survival in PD.

5.5.3. Parkin/Pink1

One of the molecular characteristics of PD are defects in mitophagy, the process that allows the elimination of damaged mitochondria, and this leads to accelerated neurodegeneration.

5.5.3.1. *Drosophila* models of Parkin/Pink1

Parkin and Pink1 are involved in mitochondria quality control and turnover (Shimura et al., 2000), the recruitment of the E3 ubiquitin ligase Parkin on the damaged mitochondrial membrane by the kinase Pink1, controls mitochondrial turnover by inducing a physiological level of mitophagy (Truban et al., 2017). *Drosophila* harbors only one *parkin* and *PINK1* orthologue (Clark et al., 2006) and their function is highly conserved. Several models have been created both using endogenous *parkin* and *PINK1* null mutants and by overexpressing human genes harboring pathogenic mutations, that closely recapitulate many PD features including DA loss, decreased lifespan, and motor defects (Ishihara-Paul et al., 2008; Hewitt and Whitworth, 2017). In particular, these models have been fundamental to understand the molecular mechanisms linking parkin and Pink1 to mitophagy. Studies using flies' flight muscles identified their relevance in the control physiological mitophagy (Greene et al., 2003), furthermore, new studies outline how the increase in mitophagy observed in aging depends on the parkin/Pink1 interaction and on the activity of two deubiquitinases USP15 and 30 (Cornelissen et al., 2018). The mitochondria phenotype observed in *parkin* null mutants recapitulates general autophagy inhibition obtained by loss of *Atg7*, supporting the physiological role of parkin in the activation of mitophagy as clearance pathway (Vincow et al., 2013). Many proteins have been identified that may affect parkin/Pink1 modulation of mitophagy: Fbxo7, whose gene *PARK15* is mutated in early-onset autosomal recessive forms of Parkinson, directly interacts with parkin to control its translocation to the mitochondrial membrane and functionally interacts with Pink1 to regulate parkin-dependent mitophagy (Burchell et al., 2013). The mitochondrial protein BNIP3L, a BH3 protein member of the Bcl2 family, interacts with Pink1 to induce mitophagy, and work in flies' muscles helped to understand the role of human BNIP3 as suppressor of Pink1-induced-mitophagy (Zhang et al., 2016). BNIP3L was identified as a substrate for PARKIN2, and this ubiquitination recruits the autophagic cargo receptor NBR1 to the mitochondria to induce PARKIN2-mediated mitophagy. Even if PARKIN2 is present only in mammals, it was demonstrated using *Drosophila* that ectopic expression of BNIP3L rescues the mitochondrial defects of *PINK1* mutant but not the effect of *parkin* mutant, outlining that PARKIN2 is the principal substrate for BNIP3L and it is necessary for BNIP3L clearance of mitochondria (Gao et al., 2015). Using flies, it was also demonstrated how the reduction of the human orthologue of Mask (ANKHD1), a scaffolding protein that inhibits mitophagy, rescues *parkin/PINK1* mutant defects, suggesting that the human ANKHD1 might represent an interesting novel therapeutic target for treating PD associated to parkin/Pink1 mutations (Zhu et al., 2015). A novel mechanistic link between mitophagy and translation lies in the evolutionarily conserved function of EFTU, a mitochondrial translation elongation factor, that acts as substrate and interacts with Pink1 independently of parkin. Pink1 regulates mitophagy by restraining EFTU in the cytosol after phosphorylation on Ser222, inhibiting mitophagy by interacting with the Atg5-Atg12 complex formation (Lin et al., 2020). Despite several studies strongly linking parkin/Pink1 to mitophagy, Lee et al. (2018)

demonstrated that basal mitophagy is not strongly reduced in the muscles of *parkin/PINK1* mutant animals, and similar results were obtained using mouse models for PD under conditions of high metabolic demand. A possible explanation could be that the metabolic state of cells could influence mitophagy, particularly in animals carrying *parkin/PINK1* mutations (McWilliams et al., 2018).

5.5.4. Leucine rich repeat kinase-2

Leucine rich repeat kinase-2 (LRRK2) is a leucine-rich repeat cytoplasmic kinase found also on the mitochondrial outer membrane, which contains a central core with a ROC-GTPase and other protein–protein interaction domains, suggesting multi-functional activity. LRRK2 is involved in several signaling pathways including vesicle trafficking, mitochondrial function, autophagic and lysosomal pathways, protein translation, neurite outgrowth, and cytoskeletal arrangement (Cookson, 2010). Mutations in LRRK2 are the most common genetic cause of both familial (41%) and sporadic (1–3%) PD (Jia et al., 2022; Oun et al., 2022) and among the function of LRRK2 that have been already clarified, it seems that its role in endomembrane trafficking may play a role in the pathogenesis of PD (Roosen and Cookson, 2016). Notably, recent experiments in human cells, also demonstrated that LRRK2 reduction is protective against the formation of α-syn aggregates suggesting a common signaling between these proteins. These studies pointed to glial cell function, in which the LRRK2 mutation interferes with the ability of glia to clear neuronal α-syn aggregates with a mechanism that is still unclear. The relevance of astrocytes in PD and proteinopathies is an important topic that needs attention (see Section 6) as more and more data delineate the presence/relevance of non-autonomous signals between astrocytes and neurons critical for neuronal clearance and survival (Streubel-Gallasch et al., 2021).

5.5.4.1. *Drosophila* models of LRRK2

Drosophila harbors a single *LRRK2* orthologue gene (*dLRKK*) therefore it has been used to better understand the physiological functions of LRRK2 using loss of function models or overexpression of human *LRRK2* variants responsible for PD (Hewitt and Whitworth, 2017; Seegobin et al., 2020). Overexpression of pathogenic mutant forms of *LRRK2* causes defects in endolysosomal and autophagic assembly, and LRRK2-targeted therapies in PD are based on this molecular event. However, several studies in *Drosophila* have shown that *dLRKK* reduction affects lysosomal compartments and leads to the accumulation of dysfunctional autophagosomes, suggesting that proper expression of LRRK2 is required for functional signaling of autophagy and vesicle trafficking (Dodson et al., 2014; Seegobin et al., 2020). LRRK2 controls the phosphorylation of several small GTPases of the Ras-associated binding protein (Rab) family responsible for vesicle trafficking at the synapse level (Steger et al., 2016; Bellucci et al., 2022). Another relevant observation is the interaction of LRRK2 with other proteins leading to PD. Indeed, Ng and colleagues demonstrated that overexpression of the disease-associated LRRK2/G2019S mutant in flies flight muscles induces mitochondrial pathology and impairs locomotion by phenocopying the behavior of *parkin*-null flies, whereas in DA neurons it leads to significantly enlarged mitochondria. In both cases, the toxic effects of LRKK2 mutants were rescued by co-expression of parkin or activation of AMPK, known to reduce TOR signaling and to activate autophagy (Ng et al., 2012), suggesting a cooperative function in mitochondria between LRRK2 and parkin.

5.6. Prion diseases

Prion diseases (PrD) are a group of disorders characterized by the presence of aberrantly shaped proteins, called prions (*proteinaceous infectious particles*) encoded by the *PRNP* gene, that cause the accumulation of misfolded aggregated proteins in the central nervous system (Geschwind, 2015). Some forms occur sporadically, others are inherited or acquired through and include Creutzfeldt-Jacob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS), Fatal Familial Insomnia (FFI), and Kuru disease. Their characteristic is to induce transmissible spongiform encephalopathies (TSE) which lead to inflammation and neuronal death, with symptoms and severity depending on the type of prion and the animal species (Geschwind, 2015).

5.6.1 Autophagy in PrD

PrP^{C}, responsible for this class of diseases, is an abundant protein inserted into the cell membrane via a GPI domain located at its C-terminus while the N-terminal tail contains functional glycosylation sites (Scheckel and Aguzzi, 2018). PrP^{C} undergoes conformational changes transforming into an infectious misfolded isoform, named scrapie-associated prion protein (PrP^{Sc}) responsible for the toxic effect of the infection (Sandberg et al., 2011). *PrD and autophagy*. Proteostasis and autophagy play a central role in protecting neurons from the toxic effect of PrP^{Sc} (Thellung et al., 2022). Several studies have outlined a direct role of PrP^{C} in the control of autophagy: in fact, the reduction of PrP^{C} impairs the cellular response to oxidative stress with consequent dysregulation of the autophagic flux. Conversely, ectopic expression of PrP^{C} appears to have a protective role by inducing autophagy under stressful conditions (Jeong and Park, 2015). Inducing autophagy is beneficial for the disease indeed, treating mice expressing the infective prions with rapamycin prolong their survival, while in yeast the induction of autophagy reduces the formation of prion protein PrP^{Sc} (Speldewinde et al., 2015). Similarly, transcriptomic analysis using the *Drosophila* model of PrP^{Sc} revealed a perturbation in genes component of TOR signaling suggesting a role in the control of translation by mutant PrP^{Sc} (Thackray et al., 2020). Furthermore, in the brains of prion-infected rodents the level of p62/SQSTM1 increases and co-localizes with PrP^{Sc} in large aggregates surrounding the perinuclear region of the cells (Homma et al., 2014). This mechanism resembles that observed in HD, where p62/SQSTM1 accumulates in the perinuclear area due to the formation of large ubiquitinated aggregates thus blocking its ability to transport cargo proteins into the autophagosome. However, the ubiquitin-binding protein p62/SQSTM1 not only mediates autophagy but also controls the activation of the Nuclear factor erythroid 2 (Nrf2), a key regulator of the cellular antioxidant response (Bellezza et al., 2018; Gureev et al., 2020). Thus inactivation of p62/SQSTM1 in prion diseases may contribute to the higher oxidative stress present in the cells (Shah et al., 2018). Recently, p62/SQSTM1 was found in cytoplasmic inclusions together with PrP^{C} and the E3-ligase/TRAF6. Moreover, its activity was required for proper redistribution of PrP^{C} into the insoluble cell fraction suggesting a novel physiological interaction between p62/SQSTM1 and PrP^{C} (Masperone et al., 2022). Recent evidence suggested that neurodegeneration in prion diseases may be the

TABLE 1 *Drosophila* models of human proteinopathies discussed in this article, their principal mechanism, and components of pathways (modifiers) that can either suppress on enhance the toxic phenotype.

Disease	*Drosophila* model	Principal mechanism	Modifiers
Huntington's disease (HD)	**HTT** Ectopic expression of *human HTT-Exon1-polyQ* (e.g., *HTT-Q93Q-ex1; HTT-Q72-GFP-ex1* Ectopic expression of human HTT-*full-length,* e.g., *HTT-Q128-fl* (Romero et al., 2008)	• The length of the CAG correlates with progressive motor decline and neuronal death (Marsh et al., 2000) • Kinetic of aggregate formation (Weiss et al., 2012) • Huntingtin role in autophagy (Ochaba et al., 2014) • PolyQ oligomers forms "*de* novo" aggregates and increase their original size by directly using the endogenous prion-forming protein Rnq1 in its amyloid-like prion conformation (Gropp et al., 2022)	**Suppressor:** • Endogenous *dhtt* (Zhang et al., 2010) • TOR inhibition (Ravikumar et al., 2002; Sarkar et al., 2009) • Chaperones (Sajjad et al., 2014; Pavel et al., 2016) • Histone deacetylase (Pallos et al., 2008; Jia et al., 2012) • Antioxidant pathway (Mason et al., 2013) • deubiquitinating enzymes (Aron et al., 2018) • Glutamine metabolism (Vernizzi et al., 2020) • Rab5 (Ravikumar et al., 2008); PSA (Menzies et al., 2010) • Compounds that target mHTT to autophagosomes (Li et al., 2019)
Spinocerebellar ataxias (SCAs)	**SCA1** Ectopic expression of human mutant *ATXN1*	• Retinal degeneration and loss of interneurons projections (Fernandez-Funez et al., 2000) • Reduced dendritic neurons arborization (Lee et al., 2011) • Role of chaperones respect to polyQ containing proteins (Zhai et al., 2008)	**Suppressor:** • TOR inhibition (Bouche et al., 2016) • Rac-PAK pathway (Lee et al., 2011) • CHIP and NMNAT (Zhai et al., 2008) • Transglutaminases 5 (Lee et al., 2022b) • Ataxin1 phosphorylation on Ser776 (Nitschke et al., 2021)
	SCA2 Ectopic expression of human mutant *ATXN2*	• Atx2 is involved in translational control (Satterfield and Pallanck, 2006) • Aggregate formation and neuronal degeneration, critical for SCA3 (Lessing and Bonini, 2008)	**Enhancer:** • Atx1 promotes Ataxin2 aggregation (Al-Ramahi et al., 2007)
	SCA3 Ectopic expression of human mutant C-terminal *ATXN3*	• Atx3 enhances the aggregation of the mutant *ATXN3* (Johnson et al., 2019) • Catalytical activity of Atx3 is necessary for its autoprotective role (Warrick et al., 2005) • Mutant *ATXN3* expression leads to abnormal eye morphology and motility defects (Warrick et al., 1998)	**Suppressor:** • Functional *ATXN3* deubiquitination activity (Warrick et al., 2005) • Ubiquitin ligases as Cullins and Praja1 (Chen et al., 2019; Ghosh et al., 2021) • Antioxidant drugs (Wu et al., 2017) **Enhancer:** • Hsc70-4 (Johnson et al., 2020)
Amyotrophic lateral sclerosis (ALS)	***C9orf72*** *C9orf72* transgenic flies carrying different length of 'pure' GGGGCC sequence contained in the human gene (e.g., *GGGGCCx36* or *GGGGCCx103*) Expression of polypeptides containing GA, GP, GR or PA repeats (that may carry GFP or FLAG tags)	• Toxicity of different numbers of GGGGCC repeats and formation of RNA foci (Mori et al., 2013; Xu et al., 2013; Mizielinska et al., 2014) • Polypeptides containing GR and PR repeats are the most toxic role of *C9orf72* in autophagy (Wen et al., 2014; Lee et al., 2016; Cleary et al., 2018; Cunningham et al., 2020)	**Suppressor:** • overexpression of SIGMAR1 (Lee et al., 2020a)
	SOD1 *SOD1* LOF mutations in *Drosophila*'s *SOD1* gene or *null dsod* mutants Expression of human mutant *SOD1*	• SOD1 is involved in protein misfolding, and it is necessary for neuronal health (Şahin et al., 2017; Agudelo et al., 2020) • SOD1 was found in a complex with Atg9/Beclin1 to control P62/SQSTM1 accumulation (Nassif et al., 2014) • Mutations in *SOD1* lead to mitochondrial impairments onomous signaling from glial to neurons and in MNs (Tafuri et al., 2015)	**Enhancer:** • DUB-USP7 (Zhang et al., 2020) • L3MBTL1 and SETD8 (Lu et al., 2019) • USP7 which reduces SMAD2/TGF-β pathway (Zhang et al., 2020)

(Continued)

10.3389/fnins.2023.1082047

TABLE 1 (Continued)

Disease	*Drosophila* model	Principal mechanism	Modifiers
	TDP-43 TDP-43 expression of human TDP-43 or its mutant form (TDP-43-M337V, with unfunctional NLS) LOF variants of *Drosophila*'s *TBPH*	• TBPH-null mutant is semi-lethal in flies (Donde et al., 2020).	**Suppressor:** • Atg7 (Donde et al., 2020) • HSP67Bc (Crippa et al., 2016). • HEXA-018 treatment (Lee et al., 2021)
	FUS *FUS* expression of mutant forms of human *FUS* (e.g., FUS-518 K, -R521C or -R521H)	• Expression of wild-type FUS and of ALS-related FUS mutations triggers the accumulation of toxic aggregates that inhibits autophagy (Brunet et al., 2021) • FUS and TDP-43 interact to induce neurodegeneration (Lanson et al., 2011)	**Suppressor:** • Mask promotes autophagy by expression of the proton-pumping vacuolar (V)-type ATPases (Zhu et al., 2017) • Inhibition of PI3K/AKT/TOR ameliorate P525L-FUS mutation due to induction of autophagy (Marrone et al., 2018). • Glutathionylation by Glutathione transferase omega 2 promotes FUS degradation (Cha et al., 2022)
Alzheimer's disease (AD)	**APP** Expression of human Aβ peptides or human AD-associated genes (e.g., human *APP* and *BACE1*)	• Lowering the expression of *Atg1*, *Atg8a* and *Atg18*, enhances the neuronal toxicity caused by Aβ expression • Dysfunctional AEL (autophagy-lysosomal-endosome vesicles) induces amyloid-plaque formation • Ectopic APP expression leads to aberrant autophagy (Ling et al., 2014; Omata et al., 2014; Zhuang et al., 2020; Tsakiri et al., 2021; Zhu et al., 2022)	**Suppressor:** • NMNAT (Zhu et al., 2022). Trx80 (Gerenu et al., 2021) **Enhancer:** • Lowering of autophagy-related genes (Omata et al., 2014)
	Tau modulation of the expression of full-length human *Tau* gene	• Insights in Tau toxicity and autophagy (Chatterjee et al., 2019; Subramanian et al., 2021)	**Suppressor:** • Ataxin3 deubiquitinase activity (Bilen and Bonini, 2007) • Calpain silencing (Menzies et al., 2015) • CG11070 (Subramanian et al., 2021) • Decrease in PTK2 expression (Lee et al., 2022a) **Enhancer:** • Insulin signaling (Chatterjee et al., 2019)
Parkinson's disease (PD)	**α-synuclein** expression of mutant human *SCNA* gene (e.g., α-syn-A30P; α-syn-A53T)	• Impairments in the autophagic flux and in mitophagy (Sarkar et al., 2021).	**Suppressor:** • Spermidine (Buttner et al., 2014) • LAMP2A (Issa et al., 2018) • dDOR (Dinh et al., 2021) • knockdown of inositol-requiring enzyme 1 (IRE1) and Atg7 (Yan et al., 2019)
	Parkin/PINK1 *Parkin/PINK1* null mutants for *Drosophila*'s *parkin* and *pink* Ectopic expression of mutant human *parkin* and *PINK*	• Relevance of Parkin and Pink in mitophagy molecular process (Greene et al., 2003; Ishihara-Paul et al., 2008; Hewitt and Whitworth, 2017) • The age-related increase in mitophagy depends on the interaction of parkin and Pink with UPS-15 and −30 (Cornelissen et al., 2018) • Mitochondria phenotype in *parkin* null mutants recapitulates autophagy inhibition in loss of *Atg7* (Vincow et al., 2013)	**Suppressor:** • BNIP3L (Gao et al., 2015) • Downregulation of ANKHD1 (Zhu et al., 2015)

(Continued)

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TABLE 1 (Continued)

Disease	*Drosophila* model	Principal mechanism	Modifiers
	LRRK2 LOF *dLRRK* models Expression of human *LRRK2* mutants (e.g., LRRK2-G2019S)	• Overexpression of human mutant LRRK2 induces defects in autophagy (Hewitt and Whitworth, 2017; Seegobin et al., 2020; Dodson et al., 2014; Steger et al., 2016; Bellucci et al., 2022) • *dLRRK* is essential for functional autophagic flux and vesicle trafficking, also at the synaptic level (Ng et al., 2012; Steger et al., 2016; Bellucci et al., 2022)	**Suppressor:** • Expression of parkin and AMPK activation (Ng et al., 2012)
Prion Diseases (PrD)	**PrP** Expression of mutant PrP forms of human or murine genes (e.g., Prp-PG14, Prp-P101L, PrP-3F4, PrP-M129, PrP-V129 and from human diseases PrP-GSS, FFI and CJD)	• Insights in PrP misfolding process (Thackray et al., 2020) • Reversibility of the human PrP-GSS phenotype using inducible system (Murali et al., 2014) • Transferability of the pathology between flies or from mice to flies (Thackray et al., 2017) • Toxicity of different aminoacids substitutions in PrP protein (Myers et al., 2022) • Perturbation of TOR signaling-related and cell-cycle related genes expression (Thackray et al., 2020) • Co-localization of PrP^{Sc} in large aggregates with p62/SQSTM1 (Homma et al., 2014)	**Suppressor:** • 4E-BP activity suppresses human PrP-M129 and PrP-V129 mutations (Myers et al., 2022)

Their link to autophagy is also outlined.

consequence of defective glia as astrocytes have been shown to deposit mutant PrP aggregates and propagate prions to neurons and other cells (seed effect; Tahir et al., 2022). To this end we would like to suggest that *Drosophila* could be an optimal model to start exploring the contribution of glia to neuronal survival/death during prion pathogenesis using the combination of binary systems (see Sections 2 and 6).

5.6.2 Drosophila models of PrD

Drosophila models of PrD. *Drosophila* genome does not contain a *PrP* orthologue, making flies ideal for identifying the gain-of-function mechanisms associated with PrP misfolding (Fernandez-Funez et al., 2017). Common PrP mutations were first created in *Drosophila* using a mutation found in the *CJD-Prp-PG14* family with nine additional repeats (Deleault et al., 2003). *Prp-PG14* was expressed in neurons of the brain, in photoreceptors and pigmented cells of the eye but surprisingly, Prp-PG14 did not accumulate in the neurons and flies showed no behavioral or neuropathological abnormalities. In contrast, the expression of *Prp-P101L* (the murine orthologue of human *PrP-P102L*) resulted in locomotor defects and accumulation of PrP-P101L aggregates and vacuolation similar to what observed in neurons of GSS patients (Gavin et al., 2006). Surprisingly, using an inducible system it was shown that PrP-GSS phenotypes could be reverted and its level decreased when its expression was blocked, representing the first demonstration of reversibility of a phenotype reported in a genetic model for prion disease (Murali et al., 2014). *Drosophila* is also susceptible to exogenous sources of prions, indeed, it has been shown that PrP toxic phenotype can be transferred from fly to fly and from mice to fly. In these experiments, *Drosophila* larvae expressing variants of the PrP^{WT} or carrying the toxic PrP^{3F4} mutant epitope were exposed to a homogenate of mouse or *Drosophila* brains expressing amino acid substitutions associated with the human prion diseases *FFI* and *CJD* (Thackray et al., 2017). These experiments demonstrated that both *Drosophila* adult-animals PrP^{WT} or PrP^{3F4} exposed to prions show a significant decline in locomotor ability and of survival, indicating that the cellular and molecular components for prion replication and toxicity, were present and functional also in flies (Thackray et al., 2017). Furthermore, transcriptomic analysis using a *Drosophila* model of mutant PrP revealed a perturbation in cell cycle genes, regulator of protein synthesis and mitochondrial function, revealing data very similar to those in mammalian hosts undergoing to prion disease, further supporting the idea that flies are a well-established animal model to study mammalian prion biology (Thackray et al., 2020). More recently, new transgenic flies expressing different mutations of PrP in neurons highlight the function of PERK (EIF2AK3 in humans) and of the activating transcription factor 4 (ATF4), members of the UPR response in mediating PrP toxicity. A partially protective activity was shown for 4E-BP (EIF4EBP2 in humans), in the disease induced by human PrP-M129 and PrP-V129 mutations, on the contrary mutations in human PrP-(N159D, D167S, N174S) revealed a partial reduction of the toxic activity induced by co-expression of N159 mutation (Myers et al., 2022), highlighting how *Drosophila* can be used to study important amino acid substitutions to link PrP structural propensities to its toxicity.

6. Why using *Drosophila* to study PPs

6.1. To study non autonomous glial-neuron signaling *in vivo*

Cell-autonomous degeneration of neurons was considered the main outcome of neurodegenerative proteinopathies until several studies highlighted the causal role of glia and the relevance of non-cell autonomous signals that may influence neuronal health (Hickman et al., 2018; Yamanaka and Komine, 2018; Acioglu et al., 2021). Glial cells play important physiological functions in the CNS trough non-autonomous signals such as release of small molecules necessary for neuronal survival or inducing toxicity causing their death (Acioglu et al., 2021; Goodman and Bellen, 2022). The exploitation of cell type-specific expression systems, such as UAS-Gal4 and LexA-LexOp, would make fruit flies a suitable model to study the non-autonomous interactions between cells (see section 2). Indeed, it has been demonstrated that non-cell autonomous mechanisms play a key role in mutation for SOD1 (ALS), LKRR2 (PD) and in HD (Van Harten et al., 2021), with mechanisms not totally clear. Phagocytic glia may be activated by the dying neurons promoting their clearance but this may also worsen the disease as glia releases inflammatory neurotoxic cytokines toxic for the cells as shown for HD (Crotti et al., 2014; Creus-Muncunill and Ehrlich, 2019). A non-autonomous mechanisms is activated by the expression in glia of the chaperon DNAJB6, ortholog of the human HSP-DNAJ, that protects neuronal degeneration and extend lifespan (Bason et al., 2019), A new component is the Triggering *Receptor* Expressed on Myeloid cells (TREM2), a microglial phagocytic transmembrane receptor, conserved also in flies, for which genetic variations have been associated with senile dementia and increased risk of AD (Filipello et al., 2022). Data in mammals showed that its N-terminal cleavage blocks amyloid-β oligomerization in neurons exerting a protective role in AD. TREM2 also enhances glia metabolism and its function in clearance (Filipello et al., 2022); we may speculate that in AD TREM soluble-extracellular domain may act non-autonomously to activate its protecting signals on Aβ oligomerization.

6.2. To study protein-aggregate formation *in vivo*

Aggregates can be visualized *ex vivo* in organs (brain, muscles) either by immunofluorescence or by using the gene expressed as fusion protein with fluorescent proteins. The size and number of aggregates can be easily quantified with imaging techniques and applied to visualize changes upon performing: edible screens using chemical libraries; genetic screens for interactors or to identify signals, i.e., between glia and neurons that modify aggregates formation and dimensions. Glia-mediated phagocytosis might also favor prion-like seeding mechanisms characteristic of some PPs that drive aggregate formation (Pearce et al., 2015; Soto and Pritzkow, 2018; Donnelly et al., 2022). The seed-mechanism occurs also in flies and it was demonstrated in a model for HD using FRET/FRASE where the migration of mHTT aggregates was monitored *in vivo* in fly brains, and it has been proposed that glia phagocytosis might be involved in the rapid grow of mHTT aggregates *in vivo* (Ast et al., 2018). This observation is corroborated by a new mechanism in yeast showing that polyQ oligomers forms "*de novo*" aggregates and increase their original size by directly using the endogenous prion-forming protein Rnq1 in its amyloid-like prion conformation (Gropp et al., 2022).

6.3. To study the cross-talks of genes acting in the same proteinopathies

As we have seen, mutations in some PP can affect other genes suggesting common pathways to be analyzed. For example, Dewan and colleagues identified pathogenic HTT repeat expansions in patients diagnosed with FTD/ALS neurodegenerative disorders with mutations in *TDRPH* (Dewan et al., 2021), while polyQ expansions in the *ATXN2* gene, that are normally associated with the onset of SCA2, have been observed in some forms of ALS (van den Heuvel et al., 2014). In addition, increasing evidence points to prion-like transmission as a mechanism underlying the development of many proteinopathies, driven by α-syn, HTT, SOD1, Tau, TDP-43, which can be uptake by the cytoplasm of acceptor cells from the brain (i.e., phagocytic microglia; Bayer, 2015; Pearce et al., 2015; Jo et al., 2020; Holec et al., 2022; Trist et al., 2022). Again *Drosophila* models can be easily engineered to co-express different mutations and their cross-talks with specific pathways such as autophagy or cell death, or their response to specific chemical drugs, can be easily analyzed *in vivo*.

6.4. To identify biomarkers

Nowadays much attention is paid to the identification of biomarkers for an early diagnosis/detection of proteinopathies (Doroszkiewicz et al., 2022). To analyze the direct secretion of non-autonomous signals (see section 6.1), or conveyed by Extracellular Vesicles (EV; Quiroz-Baez et al., 2020; Picca et al., 2022) or by exosomes (Beatriz et al., 2021; Zhang et al., 2021; Kumari and Anji, 2022) could identify molecules that may have a pathological role in the diseases (Jackson et al., 2022). In *Drosophila,* markers are available to identify EVs or exosomes, this, together with the advantage of generating a large progeny and the use of advanced biochemical tools, could accelerate this area of intervention.

In conclusion, the field of proteinopathies is in continuous evolution and there is still much to uncover about the misfolding process that is at the basis of the protein accumulation and disease propagation. *Drosophila* represents a powerful tool to dissect the many aspects that we still need to understand and represent an important alternative to the use of other model organisms. For example, the short time frame needed for the formation of the toxic aggregates in flies is an incredible advantage when studying the dynamic of aggregates formation upon modulation of signaling pathways or with chemical compounds. These data can be complemented with those obtained directly from drug screen using 3Dculture of patients-derived organoids rather than using 1D tissue culture lines. *Drosophila* can also by-pass mouse models for proteinopathies in the initial phase of characterization of novel human genes whose

function is still unknown, using genetic screens. However, there are also some pitfalls to consider since the lack of an immune system and of proper microglia limits a deeper understanding of the physiology of the PPs. Nevertheless, we would like to underline the relevance of implementing *Drosophila* studies of the function of glia in PPs since it is more targetable for therapies than neurons, and because many data suggest the presence of non-autonomous cellular mechanisms, that are difficult to address in the available mammalian models.

Author contributions

SS, CL, CC, LT, and LV wrote and revised the review. PB and AS organized the topics and wrote and revised the review. All authors contributed to the article and approved the submitted version.

Funding

5 × 1000 tax campaigned for the research on neurodegenerative diseases (University of Trento).

Acknowledgments

We thank all members of Bellosta's and Soldano's laboratories for their comments on the review. We apologize to colleagues whose work was not cited in this manuscript due to space limitations.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Neuroscience

TYPE Brief Research Report
PUBLISHED 09 June 2023
DOI 10.3389/fnins.2023.1164251

OPEN ACCESS

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RECEIVED 12 February 2023
ACCEPTED 16 May 2023
PUBLISHED 09 June 2023

CITATION
Borg R, Herrera P, Purkiss A, Cacciottolo R and Cauchi RJ (2023) Reduced levels of ALS gene *DCTN1* induce motor defects in *Drosophila*. *Front. Neurosci.* 17:1164251. doi: 10.3389/fnins.2023.1164251

Reduced levels of ALS gene *DCTN1* induce motor defects in *Drosophila*

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Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neuromuscular disease that has a strong genetic component. Deleterious variants in the *DCTN1* gene are known to be a cause of ALS in diverse populations. *DCTN1* encodes the p150 subunit of the molecular motor dynactin which is a key player in the bidirectional transport of cargos within cells. Whether *DCTN1* mutations lead to the disease through either a gain or loss of function mechanism remains unresolved. Moreover, the contribution of non-neuronal cell types, especially muscle tissue, to ALS phenotypes in *DCTN1* carriers is unknown. Here we show that gene silencing of *Dctn1*, the *Drosophila* main orthologue of *DCTN1*, either in neurons or muscles is sufficient to cause climbing and flight defects in adult flies. We also identify Dred, a protein with high homology to *Drosophila* Dctn1 and human DCTN1, that on loss of function also leads to motoric impairments. A global reduction of Dctn1 induced a significant reduction in the mobility of larvae and neuromuscular junction (NMJ) deficits prior to death at the pupal stage. RNA-seq and transcriptome profiling revealed splicing alterations in genes required for synapse organisation and function, which may explain the observed motor dysfunction and synaptic defects downstream of *Dctn1* ablation. Our findings support the possibility that loss of *DCTN1* function can lead to ALS and underscore an important requirement for DCTN1 in muscle in addition to neurons.

KEYWORDS

***Drosophila*, amyotrophic lateral sclerosis, DCTN1, DCTN1-p150, DRED, CG9026, dynactin, CG9279**

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing, fatal neurodegenerative disease. Patients mostly present with weakness in either the limb or bulbar muscles resulting from degeneration of upper and/or lower motor neurons. This leads to a gradual decline in their mobility with death typically occurring around 3 years following clinical onset, mostly due to respiratory failure (Brown and Al-Chalabi, 2017; Van Es et al., 2017). Genetics plays a strong role in ALS pathoaethiology, hence, to date, more than 40 genes have been associated with the disease (Goutman et al., 2022). In admixed populations, a sizable number of ALS cases can be explained by dominant causal variants residing in *C9orf72*, *SOD1*, *TARDBP* and *FUS* genes (Zou et al., 2017). Deleterious variants in several other genes including the *DCTN1* gene are a rare cause of ALS globally although a founder effect can inflate their contribution to ALS in homogeneous populations (Borg et al., 2021; Farrugia Wismayer et al., 2023).

The *DCTN1* gene encoding the p150 subunit of the molecular motor dynactin has been identified as an ALS gene nearly two decades ago (Puls et al., 2003; Munch et al., 2004). The discovery underscored impaired axonal transport as a mechanism for motor neuron degeneration in ALS. Dynactin is a key player in the bidirectional transport of cargos including vesicles, organelles, RNAs and proteins along microtubules mediated through either dynein or kinesin (Deacon et al., 2003; Schroer, 2004; Ross et al., 2006; Haghnia et al., 2007). In mice, dominant missense mutations in the *DCTN1* homologue lead to a late-onset, slowly progressive motor neuron disease characterised by gait abnormalities, motor neuron loss, neuromuscular junction (NMJ) defects and, eventually, paralysis (Laird et al., 2008). Heterozygous mutations in *Dctn1* (also referred as *DCTN1-p150*), the homologous gene in *Drosophila* also leads to age-dependent motor deficits, reduced survival and synaptic abnormalities at the NMJ (Eaton et al., 2002; Lloyd et al., 2012). It is thought that missense mutations in the *DCTN1* gene lead to ALS phenotypes through a gain of function mechanism. However, it remains as yet unclear whether haploinsufficiency of DCTN1 plays a role in the disease process. Furthermore, the contribution of non-neuronal cell types, specifically muscle tissue, to ALS phenotypes in patients harbouring *DCTN1* mutations is not known.

Here, we present data demonstrating that reduced levels of Dctn1 specifically in neurons leads to an age-progressive decline in motoric ability and survival in *Drosophila*. Muscle-selective knockdown leads to similar phenotypes that nonetheless manifest in younger adult flies. We further identify a gene, named here as *Dctn1-related* or *Dred*, as having homology to *Drosophila Dctn1* and human *DCTN1*. Loss of *Dred* function in either muscle or neurons also leads to motor impairments. Interestingly, a global reduction of Dctn1 leads to paralysis of larvae and NMJ defects. Finally, RNA sequencing (RNA-seq) of these organisms followed by transcriptome analysis led to the identification of alterations in genes required for synapse organisation and function, hence allowing us to speculate that these may explain the motor dysfunction and synaptic defects downstream of *Dctn1* deficiency.

2. Methods

2.1. Fly culture and stocks

Flies were cultured on food consisting of sugar, corn meal, yeast and agar in plastic vials at an incubation temperature of 25°C under 12 h day/night cycles. The RNAi transgenic constructs *Dctn1-IR*^{1} (ID: 3785), *Dred-IR*^{1} (ID: 105109) and *Dred-IR*^{2} (ID: 45052) were obtained from the Vienna *Drosophila* Resource Centre, Austria (Dietzl et al., 2007). The *Dcr-2* transgene and the GAL4 drivers were obtained from the Bloomington *Drosophila* Stock Centre (NIH P40OD018537) at Indiana University, United States. Constitutive expression of transgenes was driven by the *Act5C*-GAL4 whereas the *Mef2*-GAL4 and *elav*-GAL4 drivers were employed to induce expression specific to muscle and neurons, respectively. Combination of the various genetic tools was performed according to standard genetic crossing schemes.

2.2. Protein alignment

To determine % amino acid similarity and identity between human DCTN1 (NP_004073.2) and its *Drosophila* orthologues Dctn1 (NP_524061.1) and Dred (NP_649124.1), we utilised the DRSC Integrative Ortholog Prediction Tool (DIOPT, https://www.flyrnai.org/diopt). Alignment of the *Drosophila* proteins with their human counterpart was performed by Clustal Omega (EMBL-EBI).

2.3. Neuromuscular function assays

Larval mobility was assessed at 72 h (L3a) and 96 h (L3b) after egg laying. Briefly, third instar (L3) larvae (sex ratio, 1:1) of the appropriate genotype were first place on a 0.7% agar plate and allowed to acclimatise for 5 min. Subsequently, the number of forward body wall contractions exhibited by the organism in 30 s were counted. Each larva was assessed three times before an average was taken. A minimum of 15 larvae per genotype were assayed.

Climbing performance of male adult flies was assessed at different timepoints following eclosion. In brief, two empty polystyrene tubes were vertically joined by tape facing each other. Flies (15–20) were then transferred to the lower tube and allowed to acclimatise. Flies were then gently tapped down to the bottom of the tube. To determine the percentage climbing success rate, the number of flies per group, that climb above the 8 cm mark by 10 s were counted. For determination of the time for first fly, the time taken for the first fly within a group to cross the 8 cm mark was observed. Four trials were performed for each group of flies and a minimum of four groups were assayed per genotype.

Assessment of flight performance was determined on male adult flies through the use of the Droso-Drome apparatus as described previously (Lanfranco et al., 2017). This consisted of a 1 L glass bottle coated with an alcohol-based sticky fluid, and divided into 4 sectors, of 5 cm each, spanning a total height of 20 cm. In short, flies first underwent a 'warm-up' by inducing negative geotaxis in an empty tube for 3 times. Organisms were then dropped into the Droso-Drome to induce flight. The number of flies distributed in each sector was next counted, divided by the total number of flies dropped and multiplied by 100 to generate the percentage number of flies per sector. Fight ability correlates with the sector in which flies are distributed on landing, hence, fly percentages that are skewed towards the lower sectors of the Droso-Drome are indicative of reduced flight capacity. Four trials were performed for each group of flies and a minimum of four groups were assayed per genotype.

2.4. Assessment of adult fly survival

Male adult flies were maintained in vials at a density of 15 to 20 flies per vial. The percentage number of flies alive at each time point measured was determined by dividing the number of flies still alive by the initial number of flies in the vial and multiplying the value by 100. During their adult lifespan, flies were transferred to new vials routinely.

2.5. Immunohistochemistry of NMJs

Wandering L3 larvae were dissected in phosphate buffered saline (PBS) to expose the body wall muscles, then fixed in 4% paraformaldehyde in PBS and washed in PBS + 0.1% Triton X-100 (PBT). Tissues were then stained overnight at room temperature by mouse anti-Discs large antibody (1:1,000; Developmental Studies Hybridoma Bank, University of Iowa, United States). On the following day, tissues were washed in PBT and stained overnight at room temperature with anti-mouse Alexa Fluor 488-conjugated secondary goat antibody (1:50). After a final wash in PBT, the samples were mounted in 90% glycerol with anti-fade. Imaging was performed with the Optika B-600TiFL microscope (20x or 40x objectives) using brightfield and fluorescent light channels.

2.6. Analysis of NMJ morphology parameters

Analysis of NMJ morphology was done as described previously (Cacciottolo et al., 2019). The area of NMJs innervating ventral longitudinal muscles 6 and 7 derived from abdominal segments 2–3 was quantified by the ImageJ software (NIH). Branch number was determined by counting the number of arborisations containing at least two boutons within a single NMJ. To determine bouton numbers, all boutons were counted within a single NMJ.

2.7. RNA extraction

RNA was extracted from 12–15 L3b larvae of the desired genotype using the Qiagen RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. For quantitative RT-PCR, sex ratio of larvae was 1:1 whereas for RNA-seq, RNA was extracted from females larvae only. In brief, whole larvae were homogenized and lysed. Tissue lysates were then spun through genomic DNA eliminator spin columns to remove genomic DNA and RNeasy Mini spin columns were subsequently used to purify total RNA.

2.8. Quantitative RT-PCR

Quantification of *Dctn1* and *Dred* expression levels was achieved by amplifying the corresponding cDNA using the SOLIScript 1-step SolisGreen kit (Solis Biodyne, Tartu, Estonia) following manufacturer's instructions. The primers were from Integrated DNA Technologies (Leuven, Belgium) and were specific for *Dctn1* (forward: 5′ – CGCACCAAGGAGAAGCTTAG – 3′; reverse: 5′ – GGTCGCGATCATAGATGGTT – 3′), *Dred* (forward: 5′ – CACGGCAGCATTTACTTCAA – 3′; reverse: 5′ – GAGTCGCCAAAAATTTTCCA – 3′) and housekeeping gene *RpL32* (forward: 5′ – TACAGGCCCAAGATCGTGAA – 3′; reverse: 5′ – GACAATCTCCTTGCGCTTCT – 3′). The transcriptional levels were calculated by the 2–ΔΔCt (Ct, cycle of threshold) method. ΔΔCt = ΔCt of experimental group – mean ΔCt of control groups. ΔCt = Ct (gene of interest) – Ct (housekeeping).

2.9. RNA-seq and data analysis

RNA-seq libraries from RNA samples (derived from female L3 larvae) were prepared and sequenced at the Beijing Genomics Institute, Denmark as described previously (Borg et al., 2023). Briefly, poly(A) mRNA was enriched using poly(T) oligo-attached magnetic beads. This was followed by fragmentation and subsequent first strand cDNA synthesis using random hexamer N6 primers and reverse transcriptase. Following end repair and adaptor ligation, cDNA fragments were PCR amplified and purified to generate single-stranded DNA circles in a final library. DNA nanoballs were finally generated by rolling circle replication, which underwent paired end sequencing (100 bp) on the BGI DNBseq platform.

Raw reads were filtered using SOAPnuke (Li et al., 2009) and clean reads were mapped to the reference *Drosophila* genome using HISAT2 (Kim et al., 2019). Transcript quantification was obtained using RSEM and normalized as fragments per kilobase of transcript per million mapped reads (FPKM) (Li and Dewey, 2011). Differentially expressed genes (DEGs) were identified by the DESeq2 algorithm with *p*-values adjusted for multiple comparisons by the Benjamini and Hochberg procedure, and differential expression of the genes determined using a false discovery rate (FDR) cut off of <0.05 (Love et al., 2014). DEGs with a > 2 fold change (log2FC > 1) were selected. Differentially spliced genes (DSGs) were detected using rMATS (Shen et al., 2014) and five types of alternative splicing events including skipped exon (SE), alternative 5′ splicing site (A5SS), alternative 3′ splicing site (A3SS), mutually exclusive exons (MXE) and retained Intron (RI) were defined. GO biological pathway analysis on DSGs and upregulated or downregulated DEGs was carried out using ShinyGO (Ge et al., 2020).

2.10. Statistical analysis

Values are presented as means ± SEM unless otherwise indicated. The unpaired *t*-test was used to compare measures between 2 groups whereas two-way ANOVA, followed by Dunnett's *post hoc* test, was used for multiple comparisons with control (GraphPad Prism v9.4.1). Differences were deemed statistically significant if $p < 0.05$.

3. Results

3.1. Knockdown of the Drosophila DCTN1 orthologues *Dctn1* and *Dred*

The top-most predicted orthologue of DCTN1 in *Drosophila* is Dctn1 (CG9206). Compared to its human counterpart, Dctn1 has an amino acid similarity and identity of 54 and 33%, respectively (93% coverage) (Supplementary Figure S1A). Nonetheless, within the *Drosophila* genome we were able to identify a second gene (*CG9279*), named here as *Dctn1-related* or *Dred,* that encodes a protein that also has a high homology to DCTN1. Hence, Dred is 41% similar and 25% identical (74% coverage) to human DCTN1 (Supplementary Figure S1B). Dred and Dctn1, which compared to each other have 48% amino acid similarity and 31% amino acid identity (77% coverage), can be considered as paralogues. It must also be noted that the locus of *Dred* is shared with another gene (*CG46434*).

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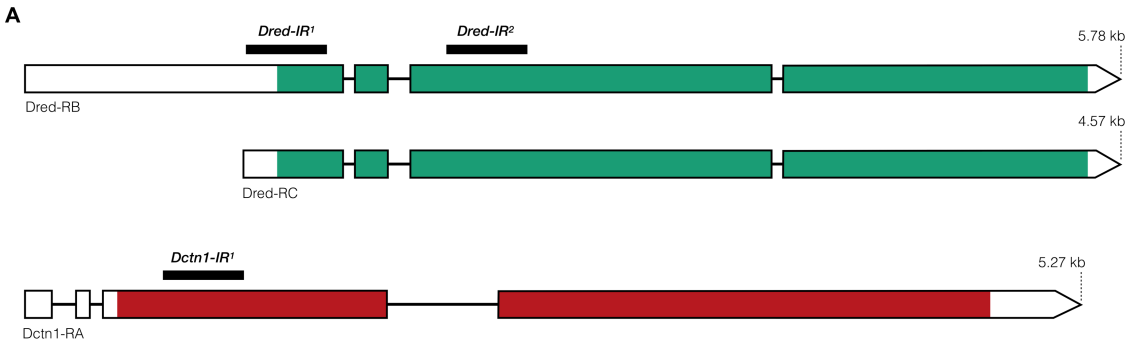
We employed the *UAS*/GAL4 system to activate RNAi transgenes targeting either *Dctn1* or *Dred* predicted mRNA transcripts (Figure 1A). On RNAi activation in the whole organism (*Act5c*-GAL4), constitutive knockdown of *Dctn1* was found to halt developmental progression, leading to flies perishing at the pupal stage. The stage of death is therefore later than that observed for null mutations in *Dctn1*, which are embryonic or early larval recessive lethals (Harte and Kankel, 1982). Global silencing of *Dred* had no effect on adult fly viability (Figure 1B). Selective expression of the *Dctn1*-specific RNAi transgene in either neurons (*elav*-Gal4) or muscle (*Mef2*-Gal4), even when knockdown was enhanced by co-expression of the *Dcr-2* transgene, was found to bypass lethality, hence, leading to viable adult flies (Figure 1B).

We next assessed gene knockdown efficiency and specificity by performing quantitative RT-PCR (qRT-PCR) on RNA extracted from third instar (L3) larvae with constitutive expression of each transgenic construct. We show that activation of the *Dctn1-IR*^{1} transgene, which targets the 5′ coding sequence of the *Dctn1* transcript, leads to a strong reduction in *Dctn1* transcript expression (20%) (Figure 1C). A robust knockdown was also observed on constitutive activation of the *Dred-IR*^{1} transgene (23%) which targets the 5′ untranslated region and part of the exon 1 of the *Dred* transcript (Figure 1C). Moderate reduction in transcript levels (71%) were however noted for the *Dred-IR*^{2} transgene which targets a downstream coding region of the *Dred* transcript (Figure 1C).

3.2. Motor impairment in adult flies with loss of *Dctn1* or *Dred* function

Given that the *Dctn1-IR*^{1} and *Dred-IR*^{1} transgenic constructs we identified were sufficient to decrease the respective transcript levels to a high degree, we asked whether loss of function of either *Dctn1* or *Dred* in disease-relevant tissues leads to an impairment in motoric ability, which is considered as the most obvious outward feature of ALS. First, we induced a knockdown of either *Dctn1* or *Dred* in muscle tissue, enhanced by co-expression of Dcr-2. Interestingly, the resulting adult flies had severe flight defects as early as day 5 post-eclosion as observed by a significant percentage that was distributed to the lower most sector (sector 1) of the Droso-Drome apparatus (Figure 2A). Climbing ability was also profoundly reduced in flies with muscle-specific *Dctn1* knockdown and totally abolished in young flies with muscle-exclusive loss of *Dred* function (Figure 2B). To better document the climbing defects we then assessed the time taken for the first fly out of a sample population to reach a predetermined threshold. Compared to control organisms, we observed a 2-fold or 6-fold increase in the time taken for flies with *Dctn1* or *Dred* muscle-selective gene silencing, respectively (Figure 2C). Although not effected in day 5 old flies, survival underwent a drastic reduction at day 15 post-eclosion only in flies with muscle-selective *Dred* knockdown (Supplementary Figure S2). We also note that motoric defects were specific to the adult stage



B

RNAi line	Viable until				
	Constitutive *Act5C*-GAL4	Muscle *Mef2*-GAL4	Muscle^{E} + *Dcr-2*	Neurons *elav*-GAL4	Neurons^{E} + *Dcr-2*
Dctn1-IR^{1}	Pupa	Adult	Adult	Adult	Adult
Dred-IR^{1}	Adult	Adult	Adult	Adult	Adult
Dred-IR^{2}	Adult	Adult	Adult	Adult	Adult

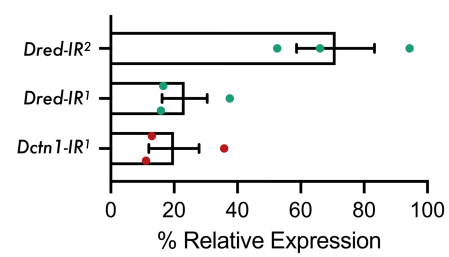


FIGURE 1
Viability outcomes and transcript expression on activation of RNAi transgenes targeting *Dctn1* or *Dred*. **(A)** Predicted *Dred* and *Dctn1* mRNA transcripts in *Drosophila* and regions targeted by RNAi constructs. **(B)** Viability outcomes of flies in which each RNAi transgene was activated either constitutively or in specific tissues by the indicated GAL4 drivers. Co-expression of Dcr-2, as indicated by E in superscript, was employed to enhance tissue-specific knockdown. **(C)** Expression of *Dctn1* or *Dred* relative to the housekeeping *RpL32* gene in L3b larvae in which the indicated RNAi transgene was constitutively expressed as determined by qRT-PCR. Each bar represents the mean±SEM of three biological replicates with the respective data points superimposed on the bars.

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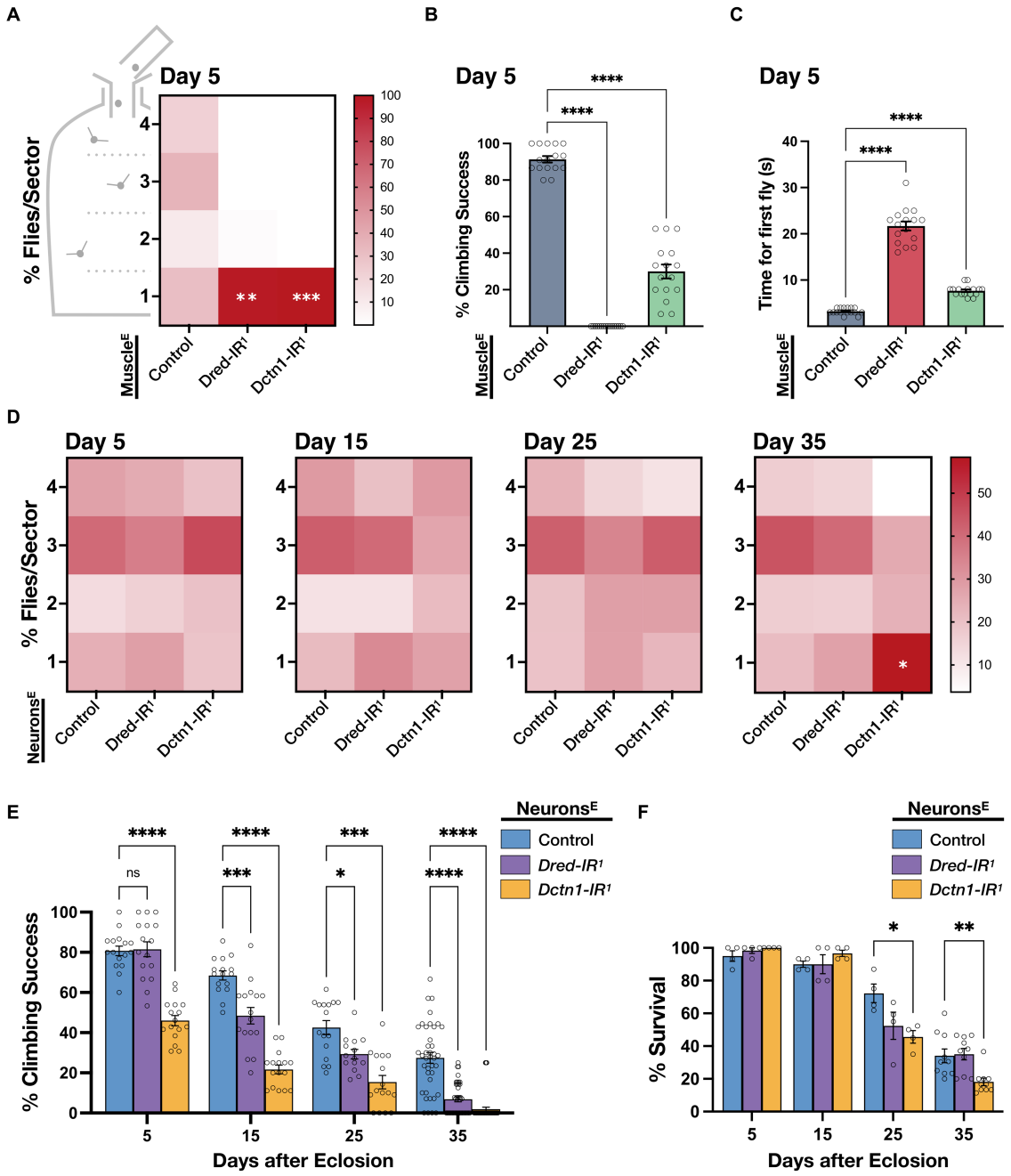

FIGURE 2
Knockdown of *Dctn1* or *Dred* induces motor deficits. **(A)** Heat map showing percentage distribution of flies landing in either of four sectors (4, top, 1, bottom) of the Droso-Drome apparatus after drop-off (4 replicates/genotype, *n* =15 flies/replicate). Young adult flies with muscle-selective (*Mef2*-GAL4) expression of the indicated RNAi transgenes, enhanced by *Dcr-2*, had significant flight defects compared to the control. **(B)** Climbing ability of young adult flies with enhanced reduction of Dctn1 or Dred in muscle was significantly impaired compared to the control. **(C)** Time taken for the first fly out of a sample population to reach a predetermined threshold was significantly longer in flies in which *Dred-IR*^{1} or *Dctn1-IR*^{1} activation was restricted to muscle tissue. **(D)** Heat maps showing percentage number of organisms per sector for flies with neuron-selective (*elav*-GAL4) expression of the indicated RNAi transgenes, enhanced by *Dcr-2*, that were assessed at different timepoints throughout adulthood and compared to age-matched controls (≥4 replicates/genotype, *n* ≥15 flies/replicate). Flight defects were apparent on brain-specific knockdown of *Dctn1* in old flies. **(E)** Climbing ability of adult flies with enhanced brain-exclusive *Dctn1* or *Dred* loss of function compared to control and assessed at different time points. **(F)** Survival of adult flies in which Dctn1 or Dred RNAi was induced specifically in neurons. Flies with neuron-specific *Dctn1* knockdown have reduced survival during late stages of adulthood. For graphs, each bar represents the mean±SEM of at least 4 independent experiments superimposed on the bars (for each genotype, *n* ≥15 flies/replicate). Significance was tested by two-way ANOVA with Dunnett's *post hoc* test and for all data, **p* =0.01, ***p* <0.01, ****p* <0.001, and *****p* <0.0001. Enhanced knockdown was achieved by co-expression of *Dcr-2* (indicated by E in superscript). Control expressed both driver and *Dcr-2*.

given that we did not detect any locomotor abnormalities in larvae with the respective genotype compared to control (Supplementary Figure S3).

Next we generated adult flies with neuron-specific knockdown of *Dctn1* or *Dred* enhanced by co-expression of Dcr-2. We observed that flies had no significant flight behaviour differences compared to

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control until late adulthood, where a significant number of flies with brain-selective knockdown of *Dctn1* but not *Dred* were flight impaired which is indicative of an age-dependent decline in flight capacity (Figure 2D). On assessment of climbing ability, we noticed that reduced levels of either Dctn1 or Dred, specifically in neurons, induced an age-progressive decline in the climbing performance success rate, which started earlier and was relatively worse in flies with *Dctn1* knockdown (Figure 2E). Survival was also negatively affected in day 25- and day 35-old adult flies with neuron-selective activation of the *Dctn1-IR*^{1} but not *Dred-IR*^{1} transgene (Figure 2F). We did not observe any motoric defects in larvae with neuron-selective loss of either *Dred* or *Dctn1* (Supplementary Figure S3). In summary, these findings support an important role for Dctn1 or Dred in normal neuromuscular behaviour of adult flies, required in both compartments of the motor unit.

3.3. Constitutive *Dctn1* deficiency induces reduced muscle contraction and NMJ defects in larvae

Considering that constitutive knockdown of *Dctn1* induced lethality prior to the adult stage, we wondered whether we could also uncover neuromuscular deficits in larvae during an earlier developmental stage. To this end, we focused on third instar (L3) larvae and assessed their mobility. We show that flies with global *Dctn1* knockdown experienced a significant drop in the body wall contraction rate at the early L3a stage (72 h after egg laying) that declined further after the next 24 h (L3b wandering stage) (Figure 3A). We next questioned whether we can link the motor deficits observed on loss of *Dctn1* function with defects in motor synapses, which are a well-recognised sign of ALS pathophysiology (Dadon-Nachum et al., 2011; Verma et al., 2022). To this end, we dissected wandering L3b larvae and examined the NMJs of motor neurons innervating their abdominal muscles. On visual inspection, we observed that loss of *Dctn1* function induced an obvious decrease in NMJ span and complexity (Figure 3B). To quantify these defects, we measured several NMJ morphology parameters including area, number of branches and bouton numbers which were all significantly depressed upon reduction in Dctn1 levels (Figure 3C). In sum, we present data that underscore a role for Dctn1 in synapse organisation and function within the NMJ.

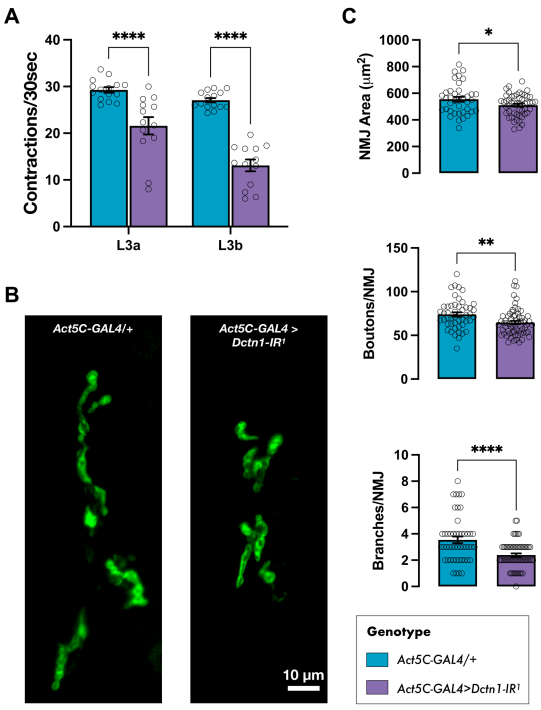

FIGURE 3
Loss of *Dctn1* disrupts larval mobility and induces NMJ defects. **(A)** Body wall contraction rate of L3 larvae with global downregulation of *Dctn1* assessed at 72h (L3a) and, subsequently, 96h (L3b) after egg laying ($n \geq 13$/genotype). **(B)** Representative DLG-stained NMJs innervating ventral longitudinal muscles 6 and 7 in L3b larvae with constitutive knockdown of *Dctn1*. **(C)** Quantification of various NMJ parameters including synaptic area, bouton numbers and number of branches per NMJ ($n \geq 38$ NMJs derived from ≥ 15 larvae/genotype). For graphs, each bar represents the mean±SEM of several independent experiments superimposed on the bars. Significance was tested by two-way ANOVA with Dunnett's *post hoc* test or the unpaired *t*-test and for all data, **p* =0.01, ***p* <0.01, and *****p* <0.0001.

3.4. Transcriptional response to loss of *Dctn1*

Finally, to identify the molecular changes responsible for the neuromuscular deficits downstream of *Dctn1* loss of function, we carried out RNA-seq in larvae with constitutive downregulation of *Dctn1*. We found 317 differentially expressed genes (DEGs) of which 176 were downregulated and 141 were upregulated (Figure 4A, Supplementary Material Dataset S1). Only 2 downregulated transcripts were annotated as novel. Gene Ontology (GO) biological pathway enrichment analysis on DEGs revealed a downregulation of processes associated with cuticle development, cytolysis and metabolism, and an upregulation in the innate immune response (Figure 4B). Interestingly, RNA-seq also revealed 59 differentially spliced genes (DSGs) upon *Dctn1* gene silencing, of which 10 had an alternative 3′ splice site (A3SS), 17 had an alternative 5′ splice site (A5SS), 10 had a mutually exclusive exon (MXE), 23 had a retained intron (RI) and 14 had a skipped exon (SE) (Supplementary Material Dataset S2). Several transcripts were subjected to more than one mode of alternative splicing, and the transcript encoded by the polyubiquitin gene *Ubi-p63E* (*CG11624*) was affected by all modes (Supplementary Figure S4). GO biological pathway analysis on DSGs revealed an enrichment of terms associated with synapse signalling and organisation including protein localisation (Figure 4C, Supplementary Material Dataset S3). The synapse was also one of the most enriched GO cellular component terms (Figure 4D). Overall, RNA-seq data revealed several transcriptome alterations that may explain the synaptic deficits and the consequential motor dysfunction resulting from loss of *Dctn1* function.

4. Discussion

Mutations in *DCTN1* have been detected in ALS patients of diverse ancestries (Puls et al., 2003; Munch et al., 2004, 2005; Liu et al.,

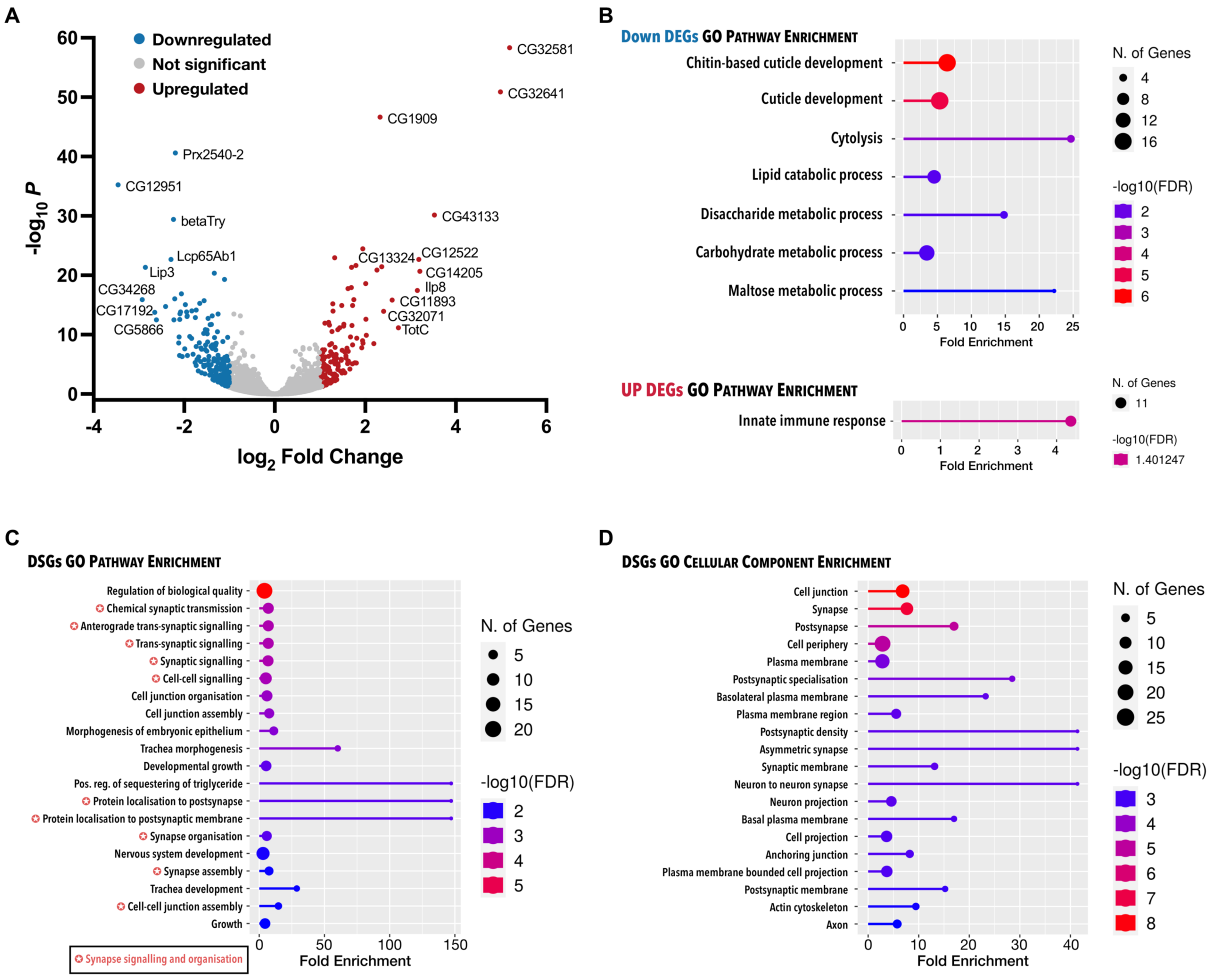

FIGURE 4
Analysis of all genes with expression and splicing changes in response to *Dctn1* gene silencing. **(A)** Volcano plot showing DEGs in L3b larvae with ubiquitous *Dctn1* knockdown compared to the driver only control (n =3 biological replicates, sex=females). Topmost significant DEGs have been annotated. **(B)** Lollipop plot presenting significant molecular pathway terms enriched in downregulated or upregulated DEGs upon gene ontology (GO) analysis. **(C)** Lollipop plot showing the most significant GO molecular pathways terms enriched in DSGs. **(D)** Lollipop plot exhibiting the most significant GO cellular component terms enriched in DSGs. In **B–D**, GO terms are sorted by FDR (<0.05) with the colour of the lollipops representing the values of the enrichment analysis relative to the other displayed terms (brighter red is more significant) and the size of the dots represent the number of genes that consist the term. GO terms tagged with a colour-coded star indicate pathway overlap.

2017; Ryan et al., 2019; Wei et al., 2019; Borg et al., 2021; Farrugia Wismayer et al., 2023). It is well known that the protein encoded by *DCTN1* plays a crucial role in the bidirectional transport of cargos along microtubules in axons of motor neurons (Schroer, 2004). Studies in mice have shown that disruption of *DCTN1* induces ALS-like phenotypes which are accompanied by defects in vesicular transport including excessive synaptic vesicle protein accumulation at NMJs (Lai et al., 2007; Laird et al., 2008). However, it remains unresolved whether loss as opposed to gain of DCTN1 function is a contributing factor to the disease process. Moreover, the contribution of cell types other than neurons to the phenotype in ALS patients carrying *DCTN1* mutations is still unclear. Here, we attempted to address these questions by inducing RNAi-mediated gene silencing of two putative orthologues of DCTN1 in flies. Our findings demonstrate that, indeed, loss of *Dctn1* or *Dred* function is sufficient to induce ALS-like phenotypes in *Drosophila*. Interestingly, we find that in addition to neurons, disruption of DCTN1 orthologues in muscle tissue also impaired motoric ability. *Dctn1* deficiency was also found to induce NMJ defects that overlapped with those observed in animal models carrying *DCTN1* mutations (Eaton et al., 2002; Chevalier-Larsen et al., 2008; Laird et al., 2008; Lloyd et al., 2012). Splicing alterations in genes with a function in synapse organisation and function identified through transcriptome profiling may explain the motor dysfunction and synaptic defects observed in flies with loss of *Dctn1* function.

Considering that missense mutations in *DCTN1* have been associated with ALS under a dominant disease model, a gain of function mechanism has predominated the view of how disease arises in patient carriers. This is supported by reports showing that ALS-linked mutations in *DCTN1* disrupt the folding of its encoded protein to induce aggregates that are toxic to motor neurons (Levy et al., 2006; Laird et al., 2008). Nonetheless, there is evidence that *DCTN1* mutations can disrupt the binding between dynactin and microtubules, which can lead to impaired dynein/dynactin-based transport along microtubules (Levy et al., 2006). *DCTN1* was also

found to be downregulated in spinal motor neurons isolated from autopsied patients with sporadic ALS (Jiang et al., 2005). These findings raise the possibility that a loss of function mechanism can also lead to the disease, at least in combination with a toxic gain of function. Here, we show that loss of *Dctn1* function in flies is alone sufficient to induce ALS-like phenotypes and pathology including impaired motoric ability and NMJ defects. Our study is therefore supportive of the possibility that haploinsufficiency arising from *DCTN1* mutations can lead to ALS. Our findings are corroborated by reports demonstrating severe motoric behavioural defects and/or NMJ instability, similar to those described here, upon depletion of the DCTN1 homologues in *C. elegans* (Ikenaka et al., 2013), zebrafish (Bercier et al., 2019) or mouse (Yu et al., 2018). We must however note that the phenotypes we have observed here are resulting from reductions in *Dctn1* levels well below the 50% reduction that is expected in ALS patients harbouring damaging variants in one copy of the *DCTN1* gene. Nonetheless, expression levels of ubiquitous or housekeeping proteins vary between cell types (Groen et al., 2018) so it is plausible that motor neurons or muscle might have lower DCTN1 expression and haploinsufficiency can therefore lead to negative consequences that are greater in these tissues.

Missplicing of several genes involved in synapse organisation and function may explain the motor behavioural phenotypes and NMJ defects we observed in flies with loss of *Dctn1* function. However, it is still unclear how reduced levels of a protein involved in intracellular transport leads to such consequential changes. There is emerging evidence that pre-mRNA splicing can occur outside the nucleus including in axons where spliceosome components and splicing factors, that retain their potential to promote pre-mRNA splicing, have been localised (Glanzer et al., 2005; Giorgi et al., 2007; Konig et al., 2007; Racca et al., 2010; Cosker et al., 2016; Thomas-Jinu et al., 2017; Poulopoulos et al., 2019). To this end, SNRNP70, a component of the major spliceosome, was recently found localised in RNA-associated granules in axons of zebrafish motor neurons and its extra-nuclear requirement for alternative pre-mRNA splicing was found to be important for neuromuscular synaptogenesis (Nikolaou et al., 2022). This also ties well with the identification of intron-retaining transcripts in the dendrites of mature neurons (Giorgi et al., 2007; Ortiz et al., 2017; Sharangdhar et al., 2017). It is therefore plausible that *Dctn1* deficiency can impair anterograde transport of splicing regulators reducing their availability within the cytoplasmic pool with this having a negative impact on the correct RNA processing of proteins required for assembly and operation of the NMJ.

Our work also highlights an important requirement for DCTN1 in muscle tissue in addition to neurons. Data showing rapid motor behavioural abnormalities in young flies with muscle-selective knockdown indicates that muscle appears to be more vulnerable to reduced levels of Dctn1 or Dred. The contribution of muscle tissue to ALS pathophysiology is supported by various studies in animal models (Loeffler et al., 2016; Anakor et al., 2022) including *Drosophila*, where for instance, motor abnormalities that overlap with those described here were reported on muscle-selective disruption of TDP-43, which is itself found aggregated in the majority of ALS patients (Diaper et al., 2013). Whether ALS originates in skeletal muscle leading to motor neuron death through a retrograde signalling cascade, the so-called 'dying-back' hypothesis, remains controversial and highly debatable. However, it is highly likely that muscle provides trophic support to motor neurons (Kablar and Belliveau, 2005) and, these signals may be absent either because they fail to be retrogradely transported from the periphery to the cell bodies in motor neurons or because they are not being produced by the muscle itself. The latter hypothesis might be especially true for patients with *DCTN1* mutations. In support, muscle-targeted knockout of BICD2, the causative factor of Spinal Muscular Atrophy Lower Extremity Predominant (SMALED), a lower motor neuron disease, was found to be a major driver of the motor neuron loss in a mouse model (Rossor et al., 2020). BICD2 is a cargo adaptor protein, which binds to the dynein/dynactin transport complex.

In conclusion, our evidence is supportive of the possibility that loss of *DCTN1* function is a likely contributor to ALS with changes in muscle in addition to neurons driving motor system dysfunction. Future work needs to address the tissue-specific contributions to the NMJ deficits observed on ubiquitous *Dctn1* knockdown, focusing on pre-synaptic terminals in addition to post-synaptic NMJ structures. We find it plausible to speculate that impaired splicing of genes required for motor synapse assembly, structure and function lead to the motor dysfunction phenotypes as well as the evident NMJ defects downstream of *Dctn1* loss of function. In addition to future work directed at confirming this link, we therefore anticipate that our study will be a stimulus for further investigations into the mechanisms through which loss of *DCTN1* triggers neuromuscular-specific splicing changes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/geo/, GSE225648.

Author contributions

RuC conceptualized and designed the experiments. RB, AP, RC, PH, and RuC performed experiments. RuC, RB, and AP analysed and interpreted the data. RuC wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Malta Council for Science & Technology Fusion R&I Research Excellence Programme, the University of Malta Research Seed Fund, the Malta Council for Science & Technology Internationalisation Partnership Award, a Tertiary Education Scholarship, and the Anthony Rizzo Memorial ALS Research Fund facilitated by the Research Trust (RIDT) of the University of Malta.

Acknowledgments

The authors are indebted to Matthew Camilleri for unwavering technical support. For fly stocks, we are grateful to the Vienna *Drosophila* Resource Center (Austria) and the Bloomington *Drosophila* Stock Centre (USA).

Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1164251/full#supplementary-material

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frontiers | Frontiers in Neuroscience

TYPE Methods
PUBLISHED 26 June 2023
DOI 10.3389/fnins.2023.1158858

OPEN ACCESS

EDITED BY
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RECEIVED 04 February 2023
ACCEPTED 03 May 2023
PUBLISHED 26 June 2023

CITATION
Ayajuddin M, Chaurasia R, Das A, Modi P, Phom L, Koza Z and Yenisetti SC (2023) Fluorescence microscopy-based sensitive method to quantify dopaminergic neurodegeneration in a *Drosophila* model of Parkinson's disease.
Front. Neurosci. 17:1158858.
doi: 10.3389/fnins.2023.1158858

Fluorescence microscopy-based sensitive method to quantify dopaminergic neurodegeneration in a *Drosophila* model of Parkinson's disease

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Death of dopaminergic (DAergic) neurons in the *substantia nigra pars compacta* of the human brain is the characteristic pathological feature of Parkinson's disease (PD). On exposure to neurotoxicants, *Drosophila* too exhibits mobility defects and diminished levels of brain dopamine. In the fly model of sporadic PD, our laboratory has demonstrated that there is no loss of DAergic neuronal number, however, a significant reduction in fluorescence intensity (FI) of secondary antibodies that target the primary antibody-anti-tyrosine hydroxylase (TH). Here, we present a sensitive, economical, and repeatable assay to characterize neurodegeneration based on the quantification of FI of the secondary antibody. As the intensity of fluorescence correlates with the amount of TH synthesis, its reduction under PD conditions denotes the depletion in the TH synthesis, suggesting DAergic neuronal dysfunction. Reduction in TH protein synthesis is further confirmed through Bio-Rad Stain-Free Western Blotting. Quantification of brain DA and its metabolites (DOPAC and HVA) using HPLC-ECD further demonstrated the depleted DA level and altered DA metabolism as evident from enhanced DA turnover rate. Together all these PD marker studies suggest that FI quantification is a refined and sensitive method to understand the early stages of DAergic neurodegeneration. FI quantification is performed using ZEN 2012 SP2, a licensed software from Carl Zeiss, Germany. This method will be of good use to biologists, as it with few modifications, can also be implemented to characterize the extent of degeneration of different cell types. Unlike the expensive and cumbersome confocal microscopy, the present method using fluorescence microscopy will be a feasible option for fund-constrained neurobiology laboratories in developing countries.

KEYWORDS

dopamine, neurodegeneration, fluorescence intensity, tyrosine hydroxylase, *Drosophila*

Introduction

The first study on the α-synuclein-mediated *Drosophila* model of Parkinson's disease (PD) demonstrated that misexpression of human α-synuclein causes a progressive age-dependent locomotor dysfunction and concurrent loss of dopaminergic (DAergic) neurons, similar to pathological and clinical manifestations of PD in humans (Feany and Bender, 2000). Since then, numerous laboratories have been using the fly model to examine the effects of gene mutations or over-expression in PD (Auluck et al., 2002; Pesah et al., 2005; Park et al., 2006; Botella et al., 2009; Navarro et al., 2014; Sur et al., 2018; Bordet et al., 2021; Maitra et al., 2021; Rai and Roy, 2022).

Drosophila models of PD recapitulate critical PD phenotype, i.e., loss of DAergic neurons (Feany and Bender, 2000; Auluck et al., 2002; Chen and Feany, 2005; Cooper et al., 2006; Trinh et al., 2008, 2010; Barone et al., 2011; Hernandez-Vargas et al., 2011; Sur et al., 2018; Maitra et al., 2021). However, some groups reported no loss in neuronal numbers (Pesah et al., 2004, 2005; Menzies et al., 2005; Meulener et al., 2005; Whitworth et al., 2005; Navarro et al., 2014; Ayajuddin et al., 2022). Neurotoxins such as rotenone (ROT) and paraquat (PQ) have been used to develop the *Drosophila* model of sporadic PD. In them, specific loss of DAergic neurons was found with different concentrations of toxins (Coulom and Birman, 2004; Chaudhuri et al., 2007; Wang et al., 2007; Maitra et al., 2021; Chaouhan et al., 2022) or no alteration in the number of neurons was also reported (Menzies et al., 2005; Meulener et al., 2005; Navarro et al., 2014; Ayajuddin et al., 2022). The tyrosine hydroxylase (TH) immunostaining and green-fluorescent protein (GFP) reporter-based techniques have typically been approached to measure DAergic neurons in the whole *Drosophila* brain. The reduction in fluorescence intensity (FI) of TH immunostaining or GFP signal has been referred to as "neuronal dysfunction" (Navarro et al., 2014).

In the present study, we employed the PQ-induced *Drosophila* model of PD that was developed in our laboratory (Phom et al., 2014) and demonstrated that in PD brain DAergic neuronal number remains unaffected but the FI of the secondary antibody that targets the primary anti-TH antibody (TH is a rate-limiting enzyme in the dopamine synthesis and marker protein for DAergic neurons) decreased as compared with control suggesting the neurodegeneration. This is further confirmed by quantifying the brain TH protein through western blotting and altered brain DA metabolism using HPLC-ECD.

Here, we describe a sensitive fluorescence microscopy-based assay, which is less expensive and user-friendly as compared to cumbersome confocal microscopy, to characterize DAergic neuronal dysfunction even in the absence of loss of neuronal cell body, which enables the researcher to follow the progress of neurodegeneration.

Materials and methods

Fly husbandry

The male Oregon K (OK) flies of the *D. melanogaster* were used in the present study (OK strain procured from National *Drosophila* Stock Center, Mysuru University, Mysuru, Karnataka, India). The flies were reared in a fly incubator at 22°C ± 2°C with a 12-h (Hr) light/dark cycle (Percival, United States). A culture media constituting sucrose, yeast, agar-agar, and propionic acid was used to feed the flies (Phom et al., 2014). The flies were collected by mildly anesthetizing them with a few drops of diethyl ether. Each vial with fresh culture media contained not more than 25 flies. Every 3rd day, the collected flies were moved to a fresh media vial. The early health phase (4–5 days old) flies were used in this experiment.

Chemicals

The required chemicals *viz.*, Sucrose (SRL, Maharashtra, India, Cat: 84973), Methyl viologen dichloride hydrate /Paraquat (PQ; Sigma-Aldrich, St. Louis, MO, United States, Cat: 856177) were used for feeding procedures. Phosphate buffered Saline (PBS; HiMedia, Maharashtra, India, Cat: ML023), Paraformaldehyde (Sigma-Aldrich, St. Louis, MO, United States, Cat: I58127), Triton X-100 (TX-100, Sigma-Aldrich, St. Louis, MO, United States, Cat: T8787), Normal Goat Serum (NGS; Vector Labs, CA, United States, Cat: S1000), VECTASHIELD® mounting medium (Vector Labs, CA, United States, Cat: H1000), Rabbit anti-Tyrosine hydroxylase (anti-TH) polyclonal primary antibody (Millipore, MA, United States, Cat: Ab152), and Goat anti-rabbit IgG H&L (TRITC labeled) polyclonal secondary antibody (Abcam, MA, United States, Cat: Ab6718) were used for immunostaining. NGS (Sigma-Aldrich, St. Louis, MO, United States, Cat: 2153), Protease inhibitor cocktail tablets (Sigma-Aldrich, St. Louis, MO, United States, Cat:S8830), RCDC assay reagent (Bio-Rad, CA, United States, Cat: 500-0120), TGX stain-free fast cast acrylamide (Bio-Rad, CA, United States, Cat:161-0183TA), Pre-stained plus dual color protein standard (Bio-Rad, CA, United States, Cat:161-0374), PVDF membrane (Bio-Rad, CA, United States, Cat: 162-0174), Goat anti-rabbit HRP antibody (Abcam, MA, United States, Cat: 205718), Clarity western ECL substrate (Bio-Rad, CA, United States, Cat: 170-5060) were used in western blotting. Trichloro Acetic Acid (TCA; SRL, Maharashtra, India, Cat: 204842), Dopamine (DA; Sigma-Aldrich, St. Louis, MO, United States, Cat: H8502); 3,4-Dihydroxyphenylacetic acid (DOPAC; Sigma-Aldrich, St. Louis, MO, United States, Cat: 11569); Homo vanillic acid (HVA; Sigma-Aldrich, St. Louis, MO, United States, Cat: 69673), MD-TM mobile phase (Thermo-Scientific, Waltham, United States, Cat: 701332) were used for quantifying DA and its metabolites.

Treatment of flies

Treatment of the flies with PQ was done as described by Phom et al. (2014). Whatman filter paper No.1 was used for disc-feeding experiments. Briefly, 10 mM PQ was prepared in 5% sucrose solution, and 275 μL of the solution was poured on filter paper. Twenty-five flies of the same age groups were placed in each vial. The climbing ability was noted every 24 h.

Negative geotaxis assay

A negative geotaxis assay (climbing assay) was performed as described by Botella et al. (2008) and Phom et al. (2021). Briefly, an individual fly was placed into the plastic tube and given 2 min to acclimatize. The fly was then taped to the bottom of the tube, and the

height it climbed in 12 s was recorded. A minimum of 12 flies were scored for each group, and the experiment was performed three times with each fly. The time point at which the fly showed significant mobility defect but not mortality was chosen to analyze the DAergic neuronal system.

Immunostaining of the whole *Drosophila* brain

Quantification of DAergic neurodegeneration of the fly brain was done as described in Bayersdorfer et al. (2010) and Ayajuddin et al. (2022). Elaborately, the brains of male Oregon K flies were fixed in 4% paraformaldehyde (PFA) containing 0.5% TritonX (TX)-100, at room temperature for 2 h, and then washed five times after every 15 min (5 × 15 min) in phosphate-buffered saline (PBS) with 0.1% TX-100 (PBST), at room temperature (RT). Blocking was performed using PBS containing 0.5% TX-100 and 5% normal goat serum (NGS) for 120 min at room temperature (RT). Then, primary antibody (anti-TH) incubation was done for the brains with a dilution of 1:250 for 72 h at 4°C. The excess primary antibody was washed off from the brains for 5 × 15 min with PBST. Brains were then incubated with 1:250 dilution of secondary antibody (TRITC labeled) for 24 h at RT under dark conditions. After thorough washing for 5 × 15 min in PBST to remove the excess secondary antibodies, brains were mounted in VECTASHIELD® mounting medium and then topped with cover glass (Electron Microscopy Sciences, PA, United States), and image acquisition was done on the same day.

Image acquisition

Prepared/stained brains were viewed under a fluorescence microscope (Axio Imager M2 with 100 W Mercury lamp, Carl Zeiss, Germany) at a 40 X lens (Supplementary Figure S1). The image was scanned using a monochromatic camera with a Rhodamine filter (Supplementary Figure S2). The image acquisition at 40X was done by using a red dot test for visibility of neuron(s) and assessing saturation using the control brain and reusing the same exposure time for all other brain samples (Supplementary Figure S2). Then, Z-stack programming with constant intervals was performed (Supplementary Figure S3). For image processing in 2D, on the method column, image subset and maximum intensity projection (MIP) with X–Y Plane was created (Supplementary Figure S4). Supplementary Figure S5 illustrates the merged fly brain image in 2D, which was used for presentation.

Fluorescence intensity quantification

FI quantification is performed using ZEN 2012 SP2 software from Carl Zeiss, Germany. ZEN 2012 SP2, Carl Zeiss software is a single user and a license must be acquired to utilize the imaging system to interactively control image acquisition, image processing, and analysis. The FI quantification was done using 3D scan images. Briefly, PAL, PPL1, PPL2, PPM1/2, PPM3 (PAL, Protocerebral anterior lateral; PPL, Protocerebral posterior lateral; PPM, Protocerebral posterior medial) brain regions were selected (Supplementary Figure S6). The images were enlarged to see clear neurites, then from graphics appropriate tools draw spline contour were selected and a line was drawn around the neuron giving intensity mean and area (Supplementary Figure S7) and intensity sum was created by selecting more options (Supplementary Figure S8). From the measure tab on the left side of the panel; the list, view all, and create document options were selected (Supplementary Figure S9). The area and FI sum were recorded for each scan of a neuron in .xml format (Supplementary Figure S10). For quantification of FI of a single neuron, a total of 11 scans with an interval of 1.08 µm for each scan, meaning the cumulative of 11.08 µm width was considered (Supplementary Figure S11). The same process was followed for all the neurons located in different clusters. The intensity sum of all the neurons in a specific cluster gives the total FI of that particular region (cluster-wise). The total FI is the sum of the FI of all the neurons belonging to all the **DAergic neuronal** clusters. The fly brain with the same orientation was carefully chosen for FI quantification.

Protein extraction from *Drosophila* brains

Fifty fly heads per group were homogenized in 160ul of RIPA buffer (50 mM Tris HCL, 1% Triton, 0.5% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 2 mM EDTA) with protease inhibitor cocktail (10% working concentration). Homogenates were then sonicated for 20s (with a pulse of 10s and amplitude at 30%) using a sonicator (Qsonica, OHIO industries, OH, United States). The samples were centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was re-centrifuged at 13,000 rpm for 5 min at 4°C.

Protein quantification

Protein quantification was performed using the Bio-Rad RCDC assay reagent. BSA at a concentration of 1 mg/mL was used as the standard and 5 uL of the extracted protein lysates were used for quantification of the samples. Absorbance was read at 750 nm using the NanoDrop 2000C (Thermo Scientific, MA, United States). Lysates were stored at −80°C until further use.

Western blotting

Bio-Rad proprietary stain-free western blotting

The casting of Bio-Rad stain-free gels: SDS-polyacrylamide gels were cast using the Bio-Rad TGX stain-free fast-cast acrylamide kit (10% and 1.5 mm thickness). In brief, the procedure is as follows: Resolving gel solution is prepared by mixing equal volumes of resolver A and resolver B solution (as described by the manufacturer). Added TEMED and freshly prepared 10% APS to the combined resolver, mixed well, and dispensed the solution into the glass plates. Filled the cassette to 1 cm below the bottom of the teeth of the comb. Then prepared stacking gel acrylamide solution by combining equal volumes of stacker A and stacker B solution (as described by the manufacturer). Added the TEMED and 10% APS to the stacker solution, mixed well, and pipetted the solution in the middle of the cassette, filling to the top of the plate (applied slowly and steadily to prevent mixing with resolving solution). Allowed the gel to polymerize for 30–45 min before starting the run.

Preparing the fly brain protein lysate for SDS-PAGE

40 ug of each sample lysate was mixed with 20 uL of sample buffer (0.5 M Tris HCl pH6.8, 10%SDS, glycerol, 0.1% bromophenol blue, β-mercapto ethanol) in a total volume of 40 uL and denatured for 5 min at 95°C. A 10X stock of running buffer (Tris, Glycine, and SDS) was used for preparing a 1X running buffer. The gel was run at a current of 20mAmp at room temperature using a Bio-Rad powerpack basic power system. Before electro-blotting, the stain-free gels were scanned and activated (2.5 min) using the Bio-Rad fluorescent documentation system. PVDF membrane was used after activation by methanol before setting up the transfer sandwich. The transfer was carried out at a voltage of 90 V for the duration of 90 min using chilled 1X transfer buffer (Glycine, Tris, and methanol) with continuous stirring using a magnetic bead. To create a cold temperature condition the transfer tank was placed inside a bucket filled with ice.

Membrane blocking and antibody treatment

Post-transfer the PVDF membrane was scanned using the Bio-Rad fluorescent documentation system and incubated in the blocking buffer of 5% BSA in 1X TBS-T (0.05%) for a duration of 90 min at room temperature with gentle rocking. Rabbit polyclonal anti-TH antibody was used in the dilution of 1:1,000 and the membrane was incubated at 4°C for 48 h. Post-primary antibody incubation, the membrane was washed 3X in 1X TBS-T (0.05%) for 15 min at room temperature. The secondary antibody Goat anti-rabbit HRP was used in a dilution of 1:5,000 and the membrane was incubated for 24 h at 4°C. The membrane was washed 5X in 1XTBS-T (0.05%) for 15 min and developed using ECL substrate. Scanning was performed with Bio-Rad fluorescent documentation system. TH protein amount was quantified using the whole protein normalization method (using Bio-Rad proprietary stain-free gels) that requires no loading control. Data analysis was performed using Bio-Rad ImageLab 5.2.1 version software.

(Details of WPN (whole protein normalization method) and calculation of TH protein amount in a brain sample are presented in Supplementary material 2).

Quantification of DA and metabolites (DOPAC and HVA) using high-performance liquid chromatography with an electrochemical detector

Brain-specific DA and its metabolites were quantified using high-performance liquid chromatography with an electrochemical detector (HPLC-ECD; HPLC-Thermo Scientific, Dionex Ultimate 3000) following the protocol described by Ayajuddin et al. (2021). The control group and PQ-exposed group of flies were immediately frozen following 24 h of exposure. To avoid thawing of tissue and degradation of biomolecules, frozen flies were placed on an ice tray containing a chilled metal surface and 15 fly heads were decapitated quickly with a sharp scalpel. Head tissue homogenate was prepared in 300 µL of chilled PBS. Sonication of the homogenate was performed at 30% amplitude for 20 s with 5-s intervals. Then homogenate was centrifuged at 6,000 rpm, 4°C for 10 min. After centrifugation, 50 µL of the supernatant was set aside for protein quantification. The remaining was combined with 5% TCA (prepared in HPLC grade or enzyme-free water) in a 1:1 ratio and kept in ice. Standard DA, DOPAC, HIAA, and HVA were prepared in PBS, each having a concentration of 200 ng/mL. The standard solution was mixed with 5% TCA in a 1:1 ratio and kept on ice to prevent catecholamine degradation. For quantification, 50 µL of the tissue sample and 20 µL of the composite standard were loaded into the HPLC. MCM 15 cm × 4.6 mm, 5 µ C-18 packed columns (Thermo-Scientific, Waltham, United States, Cat: 70-0340) was used as the stationary phase for elution of the catecholamines, and MD-TM served as the mobile phase. To detect the catecholamines, the reduction and oxidation potentials within the two cells of primary ECD were kept at −175 and +225 mV, respectively. The secondary ECD module acting as a third cell also known as Omnicell, was set to +500 mV to reduce background noise. Data was gathered at a rate of 5 Hz. Chromatogram analysis was done using Chromeleon® 7 from Thermo-Scientific (Waltham, United States). Comparisons were made between sample and standard chromatograms for a catecholamine's retention time. To precisely pinpoint the peaks corresponding to DA, DOPAC, and HVA in the sample, 10 µL of the composite standard was added to a sample and run through the HPLC once again. The spiked peaks according to the detection sequence in the standard solution were recognized as the catecholamines of interest in the sample.

Quantification and normalization of catecholamines were described by Ayajuddin et al. (2021).

In brief, (1) The concentration of a catecholamine is: C_{Std} (ng/mL), (2) the area of a catecholamine in the composite standard chromatogram is: $A_{Std,}$ and the injection volume of the composite standard solution is: I_{Std} (µL), (3) the area of the catecholamine in the tissue extract chromatogram is: A_{Samp} and the injection volume of the tissue extract is I_{Samp} (µL), (4) total number of fly heads for protein extraction: N, (5) the total protein concentration of the tissue extract is: P_{Samp} (µg/µL).

Calculation steps (Supplementary material 3):

1. The standard catecholamine concentration in I_{Std} (µL) injection volume: (C_{Std} X I_{Std})/1,000 = V1 (ng).
2. The catecholamine concentration in tissue extract: (A_{Samp} X V1)/A_{Std} = V2 (ng).
3. Total protein in I_{Samp} (µL) injection volume of tissue extract: (P_{Samp} X I_{Samp}) = V3 (µg).
4. The catecholamine concentration per 1 µg in the injected tissue extract: V2/V3 = V4 (ng/1 µg).
5. The catecholamine concentration per fly head = V5/N = V6 (ng).
6. Injected tissue extract and the standard solution was mixed with 5% TCA in a 1:1 ratio. Therefore, the actual catecholamine concentration per fly head (V6/2) = V7 (ng) or (V7 X1000) = V8 (pg).

(Detailed calculation of the concentration of catecholamines in a brain sample with an example is presented in Supplementary material 3).

Data analysis

GraphPad Prism 5.0 software was used to perform statistical analysis and preparation of the graphs, which were expressed as

mean ± standard error of the mean (SEM). For the two-group data, statistical significance was determined by a two-tailed unpaired t-test. A one-way analysis of variance (ANOVA) followed by the Newman–Keuls Multiple Comparison Test was carried out for the data with more than two groups. p-value < 0.05 was considered significant.

Results

PQ induces Parkinsonian symptoms as indicated by negative geo-taxis assay

A PQ-induced fly model of sporadic PD was developed in our laboratory (Phom et al., 2014) and the same is employed in the present study. Flies were fed with 10 mM PQ prepared in 5% sucrose to induce PD, whereas the control flies remained in 5% sucrose. The negative geotaxis assay was used to measure mobility impairments. Results revealed that 90% of the flies could reach the top of the column in 12 s under normal conditions, but PQ-treated flies were unable to do so. PQ-intoxicated *Drosophila* exhibited resting tremors and bradykinesia, which are the distinctive clinical symptoms of PD in human patients. The flies failed to hold their grip and slipped to the bottom as they tried to climb on the wall. Further, the climbing speed of the flies declined by 33% after 24 h and by 60% after 48 h of exposure to the neurotoxicant (Figures 1A,B). No mortality was observed after either 24 or 48 h of exposure window, yet flies showed significant mobility defects. Therefore, an exposure window of 24 h was chosen to characterize the DAergic neurodegeneration.

Fly model of PD shows no loss of DA neurons but depletion in TH synthesis: insights from whole brain immunostaining and western blotting

The adult *Drosophila* brain comprises six countable DAergic neuronal clusters in each hemisphere of the brain (Figures 2A,B). To probe into DAergic neuronal dysfunction in PQ-administered flies, the brains were immuno-stained for TH (Figure 3A). Quantification of DAergic neuronal number reveals no significant difference in all the five clusters analyzed, and *in toto* in PD-induced brains as compared to the control (Figures 3B,C). However, there are slight changes in the neuronal number even among the control groups, which can be accredited to natural variation. This result is consistent with the earlier comprehensive study performed by Navarro et al. (2014). To comprehend whether there is a change in the level of TH synthesis, the FI of the DAergic neurons (fluorescently labeled secondary antibody (ab) targets the primary anti-TH ab, and hence FI is correlated to the level of TH protein synthesis) was quantified. In PQ-administered flies, the FI of DAergic neurons in the PAL, PPL1, PPL2, and PPM3 clusters acutely decreased except for PPM1/2 (Figure 3D). The FI of DAergic neurons (five quantifiable DA neuronal clusters) of the whole brain mount exhibited a significant reduction (30%–35%) as compared to the control group (Figure 3E). Further, by employing the Bio-Rad Stain-Free Western Blotting (BR-SFWB), fly brain protein lysate was probed to quantify the level of TH protein (BR-SFWB enables total protein normalization method and requires no loading control). Results illustrate a reduction in brain TH protein upon PQ treatment (15% depletion; Figure 3F). Immunostaining together with western blotting, results elucidate that in the fly PD model, though there is no loss in the number of DA neurons, the TH protein synthesis is diminished suggesting the "neuronal dysfunction."

PQ exposure diminishes brain DA and enhances its oxidative turnover rate

TH is the rate-limiting enzyme in the synthesis of DA. Therefore, to further understand the implication of diminished TH synthesis in the brain, the DA level was also quantified (Figures 4A,B). The result demonstrated that PQ exposure diminishes brain DA levels (Figure 4C), which can be attributed to diminished TH synthesis under PD conditions. Further, oxidative turnover of DA is also linked with the onset of Parkinsonian symptoms. Therefore, downstream

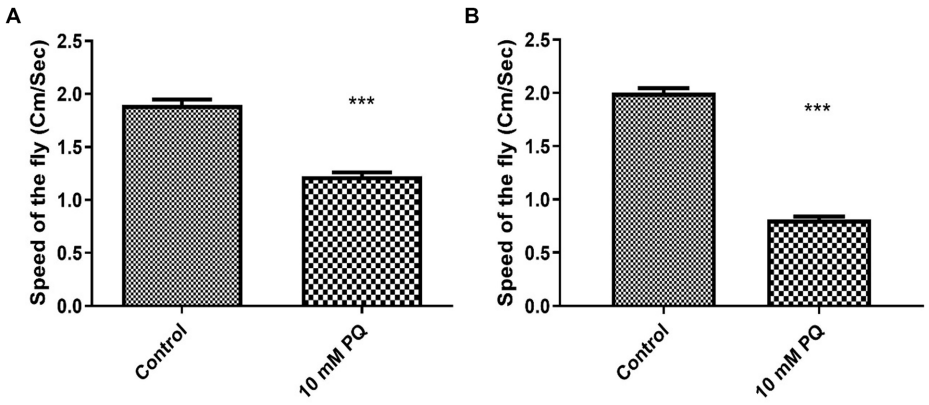

FIGURE 1
Assessing the climbing ability of *Drosophila* by negative-geotaxis assay after exposure to PQ: the climbing speed of flies within 12s was assayed. Ingestion of 10mM PQ caused severe mobility defects after 24 **(A)** and 48 h **(B)** of exposure. Mobility defects enhanced with exposure duration, but no mortality was observed for the exposure duration of 24 and 48 h. Hence, an exposure window of 24h was selected for further experiments. Unpaired *t*-test reveals a significant reduction in mobility of PQ exposed fly compared to control. ***p<0.001.

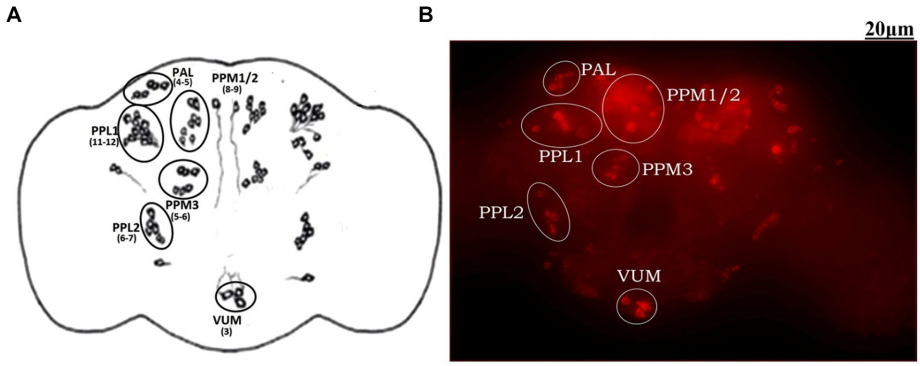

FIGURE 2
Demonstration of DAergic neurons in the whole fly brain: cartoon of *Drosophila melanogaster* brain illustrating the position of quantifiable DAergic neurons **(A)** and image of the whole-brain mount of 5days old male *Drosophila* captured using *ZEN software* of Carl Zeiss Fluorescence Microscope using fluorescently labeled secondary antibody targeted against the primary anti-TH antibody **(B)**. In the fly brain, ~140 DAergic neurons (including ~100 neurons of the PAM cluster which cannot be quantified due to the high neuronal density) are arranged in different clusters in each hemisphere. The Scale bar of the brain image in the panel is 20µm (PAL, Proto-cerebral Anterior Lateral; PAM, Proto-cerebral Anterior Medial; PPL, Proto-cerebral Posterior Lateral; PPM, Proto-cerebral Posterior Medial; VUM, Ventral Unpaired Medial).

metabolites of DA, i.e., DOPAC and HVA levels were also assayed. The result revealed that the neurotoxicant exposure promoted DOPAC depletion which was not as much as DA depletion (Figure 4C), whereas the HVA level was enhanced in the induced PD condition (Figure 4C). This observation signified enhanced oxidative turnover of DA under the induced PD condition (Figure 4D). Insight suggests that the onset of Parkinsonian symptoms underlies the depletion of TH synthesis in DAergic neurons contributing to diminished DA levels in the fly brain. Further, such induced PD conditions also promote oxidative turnover of DA.

Discussion

Two methods have been usually used in *Drosophila* models to envisage the DAergic neuronal system: (1) anti-TH immunostaining or (2) the targeted over-expression of a GFP reporter gene under the control of either TH-GAL4 or Ddc-GAL4 transgenes. The pattern of TH-GAL4 expression and anti-TH immunoreactivity in the adult protocerebrum was originally described in Nässel and Elekes (1992) and Friggi-Grelin et al. (2003) and further characterized by Mao and Davis (2009) and White et al. (2010).

The negative geotaxis assay clearly shows a significant decrease in climbing ability showing Parkinsonian symptoms which might be induced by DAergic neurodegeneration (Figure 1). But, the numbers of DAergic neurons remain unchanged as we count all the clusters which is consistent with other studies (Meulener et al., 2005; Navarro et al., 2014).

Loss of DAergic neurons *per se* in fly PD models has been an issue of debate and it was re-evaluated in both the sporadic and genetic fly models of PD and resolved that there is no loss of DAergic neurons; however, there is a diminished level of TH synthesis (Navarro et al., 2014). In the present method, we have used anti-TH immunostaining. We found that the number of DAergic neurons in the whole fly brain under PQ-induced PD condition remains unchanged. Nevertheless, the FI of the secondary antibody which is targeted against the primary anti-TH antibody is diminished suggesting decreased TH synthesis. Decreased TH protein synthesis is further confirmed through western blotting.

Quantification of TH protein using whole brain immunostaining reveals that it is about 30–35% depletion in the PQ-treated brain and the western blotting result illustrates that it is a 15% reduction. The observed variations in the level of TH depletion can be attributed to the following reasons:

1. It can be due to the variation/limitation in the sensitivity levels of the two methods.
2. It is important to note that the quantification of FI was limited to only five quantifiable clusters in the whole fly brain (quantifiable five clusters together constitute ~40 neurons only out of a total of ~140 DAergic neurons in one hemisphere of the fly brain! ~ 100 neurons of the PAM cluster cannot be quantified due to the high neuronal density). Whereas western blotting is done in whole brain protein lysate (that includes all the DA neuronal clusters).
3. It is worth mentioning that it has been demonstrated in fly models of PD that the extent DAergic neurodegeneration varies among different DA neuronal clusters and degeneration is also can be cluster-specific (Coulom and Birman, 2004; Chaudhuri et al., 2007; Wang et al., 2007; Maitra et al., 2021; Chaouhan et al., 2022).

This approach allowed us to accurately assess the neurodegeneration of DAergic neurons in the fly PD model. While using this methodology, it was comprehended that the orientation of the brains mounted for fluorescence microscopy could impact the quantification of FI of DAergic neurons. Therefore, for an optimal imagining of the DAergic neuronal clusters, neurons that confine to the posterior protocerebrum were taken into consideration, and slightly damaged or torn brains were not examined. In this study, all brains were analyzed in the same orientation.

Auluck et al. (2002) studied both the paraffin sections and the whole brain mount. They observed a change in the number of DAergic neurons in the PPM1/2 cluster in the paraffin section which

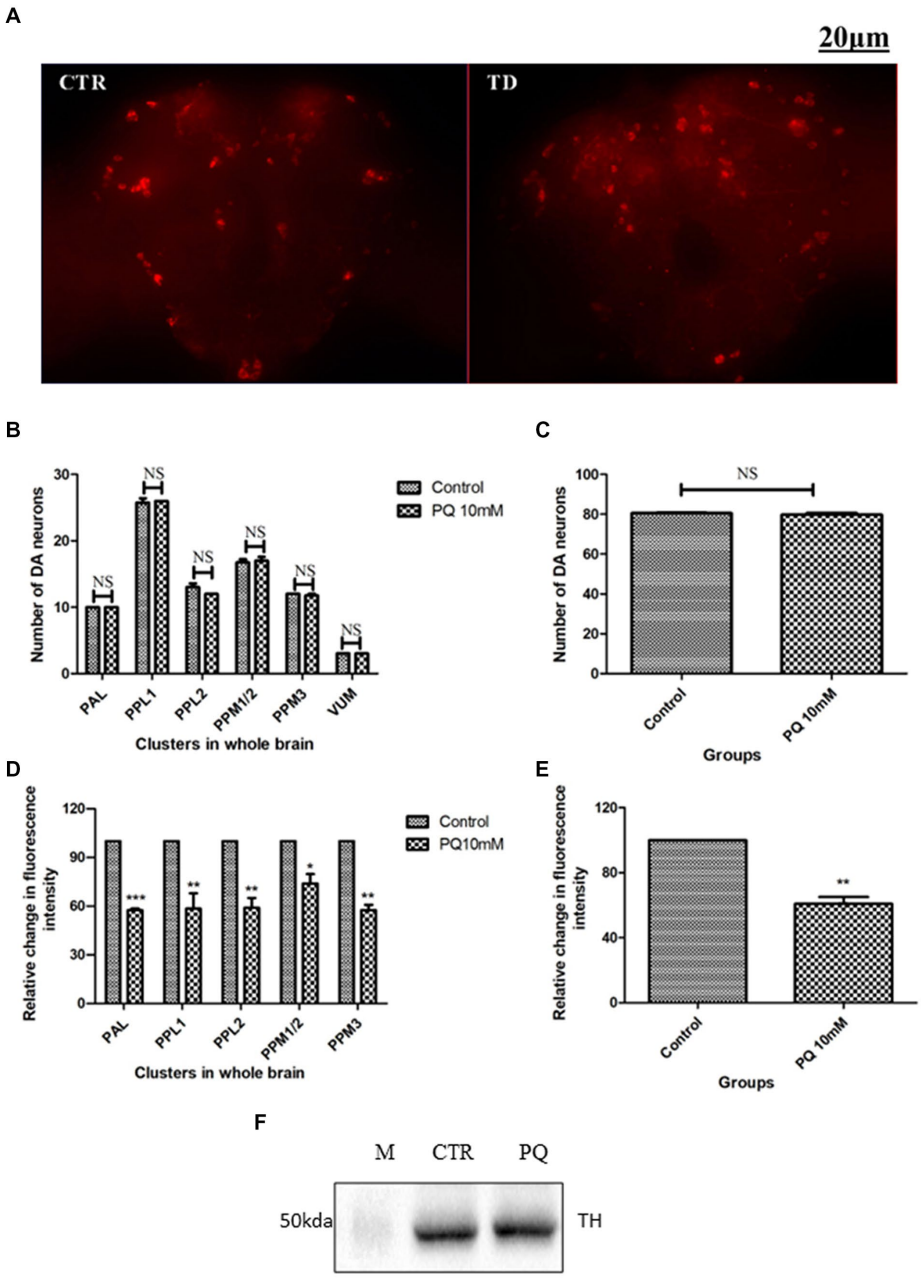

FIGURE 3
Characterization of DAergic neurodegeneration in the whole fly brain through anti-TH antibody immunostaining and quantification of brain TH protein using western blotting: the image depicts the whole brain mount of the adult *Drosophila* under control and PQ-treated conditions **(A)** (CTR, Control; TD, PQ-treated). Quantification of DAergic neurons reveals that the neuronal number remains unaffected **(B,C)**, whereas the fluorescence intensity (of fluorescently labeled secondary antibodies that target primary antibody anti-TH) is significantly decreased in all the clusters **(D)**, and *in toto* **(E)** between the control and treated group. The scale bar of the brain images in the panel is 20µm. (CTR, Control; TD, Treated with 10mM PQ; Represented images are "merged" Z-stacking images; however, the quantification of DA neuronal number and fluorescence intensity is performed in 3D Z-stack images; PAL, Protocerebral anterior lateral; PPL, Protocerebral posterior lateral; PPM, Protocerebral posterior medial). **(F)** Stain Free Western Blot analysis shows a reduction of brain TH protein (15%) upon PQ treatment in the fly model of PD [Bio-Rad Stain-Free Western Blotting using total protein normalization method (TPN) (M-protein ladder; CTR-control; PQ- paraquat treated)]. Statistical analysis was performed using a *t*-test (compared to control). *p<0.05, **p<0.01, ***p<0.001; NS, not-significant.

is not observed in the whole brain mount showing the unreliability of the previous method. Using fluorescence microscopy, we quantified both the DAergic neuronal number and FI. Results reveal that FI was significantly down-regulated under PQ-induced conditions as compared with controls (Figure 3E). The result was consistent with the observed FI of GFP reporter genes (Navarro et al., 2014). Grasping the idea from their study, we have assessed the neuroprotective efficacy of curcumin in the *Drosophila* model of PD

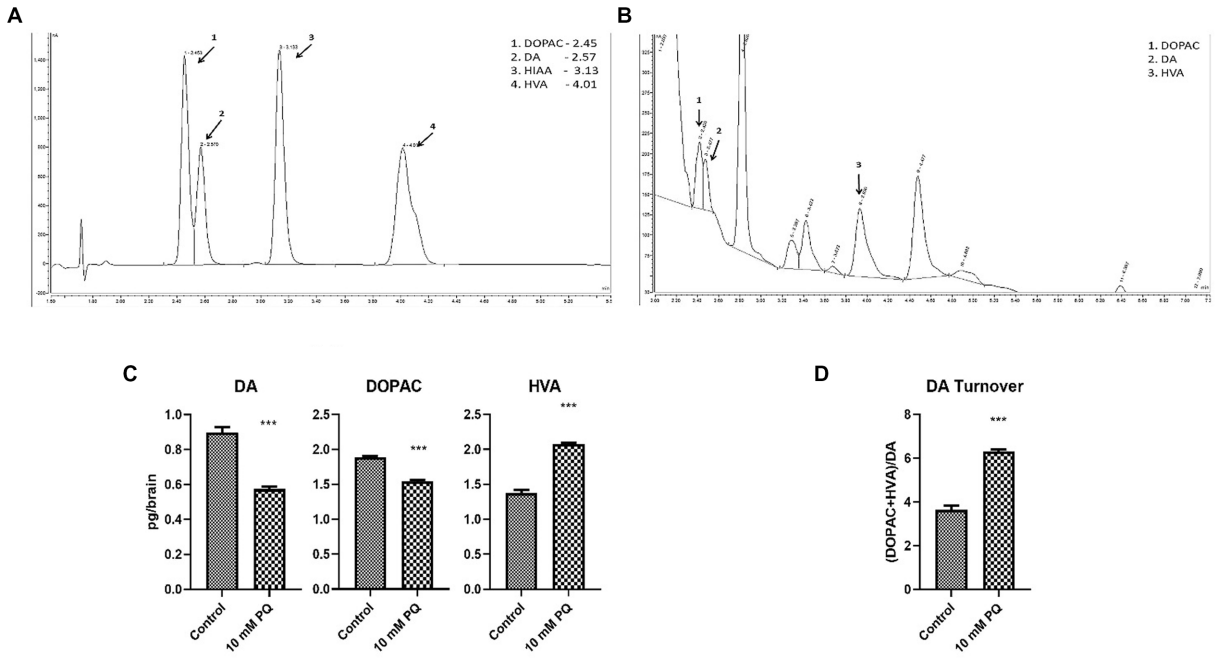

FIGURE 4
Quantification of DA and its metabolites (DOPAC, HVA) in fly brain tissue extract using HPLC-ECD: the retention time of standard DA, DOPAC, and HVA is shown in the chromatogram **(A)** and chromatogram for the fly head tissue extract shows the detected catecholamines **(B)**. Quantification of the brain-specific catecholamines revealed that PQ exposure for 24 h depleted brain DA and DOPAC levels, whereas the HVA level is significantly enhanced **(C)**. The result also revealed that in the induced PD condition there is a higher oxidative turnover of DA to its metabolites (DOPAC and HVA) **(D)**. Statistical analysis was performed using an unpaired *t*-test (compared to the control), *** $p<0.001$.

(Ayajuddin et al., 2022), which indicates the reliability of the current method of quantification.

TH is the rate-limiting enzyme for DA synthesis. Therefore, to further validate the current methodology we also quantified the levels of brain DA and its metabolites (DOPAC and HVA). The result demonstrated that the induced PD condition depleted brain DA levels (Figure 4C). Diminished brain DA level is a prime characteristic of PD in human and PQ-induced animal models of PD (Goldstein et al., 2011; Phom et al., 2014). It has been demonstrated that oxidative turnover of DA to DOPAC and HVA contributes to the onset of PD, owing to the neurotoxic nature of the process (Zhang et al., 2019; Cao et al., 2021). Observation in the current study demonstrated that DA turnover is increased in the induced PD condition (Figure 4D) which further explains the enhanced oxidative stress and neurotoxicity under the induced PD condition. Insights from the catecholamine quantification with the HPLC-ECD method corroborate neurophysiological changes associated with PD and further validate the reliability of the FI quantification method.

Demonstration of mobility defects, reduced FI of DA neuronal clusters, reduction in TH protein synthesis, depleted brain DA, and enhanced DA turnover phenotypes illustrates the robustness of the present method of quantification of DAergic neurodegeneration through quantification of FI using fluorescence microscopy.

Fluorescence microscopy, on the other hand, is much simpler to handle, easier to operate, and also gives precise results whereas confocal microscopy is cumbersome and costlier. As we do not have access to expensive confocal microscopy, we worked on and developed this method which can be of use to neurobiologists working in fund-constrained academic institutions of developing countries.

Through this assay, it is possible to characterize the incipient DAergic neurodegeneration that will be of great support in following the progression of the disease which is a critical necessity to screen novel neuroprotective molecules, in order to develop smart and successful therapeutic strategies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MA, RC, and ZK performed experiments relating to whole-brain immunostaining. MA, AD, and LP performed experiments relating to the quantification of DA and its metabolites using HPLC-ECD. PM performed experiments relating to the quantification of brain TH protein through western blotting. AD conducted PQ treatments and performed fly mobility experiments. MA, AD, PM, and RC involved in acquisition, analysis of the data, and manuscript drafting. SY contributed to conception and design of the study, obtained funding, involved in interpretation of the data, manuscript revision, and supervision. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the Department of Biotechnology (DBT), India (R&D grant nos. BT/405/NE/U-Excel/2013 and BT/PR16868/NER/95/328/2015) and the Science and Engineering Research Board (SERB) of the Department of Science and Technology (DST) India (R&D grant no. EMR/2016/002375, 27-3-2017). LP, MA, PM, AD, and RC received the DBT-JRF (Junior Research Fellowship); LP, PM, AD, and RC received DBT-SRF (senior research fellowship); ZK received MANF (Maulana Azad National Fellowship) of the Ministry of Minority Affairs, India; and MA and RC received ICMR (Indian Council of Medical Research)-SRF.

Acknowledgments

SY thanks Stephan Schneuwly for the given opportunity as a visiting scientist in his lab at the University of Regensburg, Germany and acknowledges the support of Jose A. Botella (Univ. Regensburg, Germany) and Juan A. Navarro (INCLIVA Biomedical Research Institute—Universitat de València, Spain) in understanding the critical methods of *Drosophila* neurobiology.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1158858/full#supplementary-material

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frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 29 June 2023
DOI 10.3389/fnins.2023.1204068

OPEN ACCESS

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RECEIVED 11 April 2023
ACCEPTED 09 June 2023
PUBLISHED 29 June 2023

CITATION
Rozich E, Randolph LK and Insolera R (2023) An optimized temporally controlled Gal4 system in *Drosophila* reveals degeneration caused by adult-onset neuronal Vps13D knockdown. *Front. Neurosci.* 17:1204068. doi: 10.3389/fnins.2023.1204068

An optimized temporally controlled Gal4 system in *Drosophila* reveals degeneration caused by adult-onset neuronal Vps13D knockdown

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Mutations in the human gene VPS13D cause the adult-onset neurodegenerative disease ataxia. Our previous work showed that disruptions in the Vps13D gene in *Drosophila* neurons causes mitochondrial defects. However, developmental lethality caused by Vps13D loss limited our understanding of the long-term physiological effects of Vps13D perturbation in neurons. Here, we optimized a previously generated system to temporally knock down *Vps13D* expression precisely in adult *Drosophila* neurons using a modification to the Gal4/UAS system. Adult-onset activation of Gal4 was enacted using the chemically-inducible tool which fuses a destabilization-domain to the Gal4 repressor Gal80 (Gal80-DD). Optimization of the Gal80-DD tool shows that feeding animals the DD-stabilizing drug trimethoprim (TMP) during development and rearing at a reduced temperature maximally represses Gal4 activity. Temperature shift and removal of TMP from the food after eclosion robustly activates Gal4 expression in adult neurons. Using the optimized Gal80-DD system, we find that adult-onset *Vps13D* RNAi expression in neurons causes the accumulation of mitophagy intermediates, progressive deficits in locomotor activity, early lethality, and brain vacuolization characteristic of neurodegeneration. The development of this optimized system allows us to more precisely examine the degenerative phenotypes caused by *Vps13D* disruption, and can likely be utilized in the future for other genes associated with neurological diseases whose manipulation causes developmental lethality in *Drosophila*.

KEYWORDS

VPS13D-related disorders, mitophagy, ataxia, neurodegenerative disease, VPS13D, mitochondria

Introduction

Neurodegenerative diseases are characterized by the progressive loss of specific neuronal populations, ultimately resulting in substantial age-dependent neurophysiological and cognitive deficits. The underlying cause of most neurodegenerative disease is thought to derive from a complex combination of genetic and environmental factors (Wilson et al., 2023). However, a growing number of neurodegenerative diseases have been associated with the inheritance of mutated genes causal to the progressive loss of neurons. These disease-causing genes provide a

unique opportunity for researchers to genetically model neurodegenerative diseases, and investigate the perturbed biology associated with alterations in disease-causing genes.

A model organism well-suited for interrogating the function of disease-causing genes is the common fruit fly, or *Drosophila melanogaster*. It is estimated that ~60% of *Drosophila* genes have human homologs (Yamamoto et al., 2014; Bellen and Yamamoto, 2015), and approximately 75% of human disease-causing genes have *Drosophila* homologs (Reiter et al., 2001; Pandey and Nichols, 2011). A wide-array of powerful genetic tools in *Drosophila* have been continuously innovated and refined over the past few decades to support research at the forefront of understanding the function of human disease-causing genes (Brand and Perrimon, 1993; Duffy, 2002; Dietzl et al., 2007; Ni et al., 2009; Zirin et al., 2020). These advantages have allowed researchers to use *Drosophila* to model a variety of neurodegenerative diseases (Bolus et al., 2020; Ma et al., 2022).

The most significant risk factor associated with the onset of neurodegenerative diseases is aging, which is proposed to be due to the susceptibility of post-mitotic, non-regenerative neurons to cumulative damage (Hou et al., 2019). This causes the progressive deterioration of neuronal function. One of the challenges of modeling human neurodegenerative diseases in *Drosophila* is their developmental stages that complicate the study of long-term consequences of manipulating disease-causing genes.The fly's life-cycle involves a transient embryonic and larval stage (5–6 days) before developmental rearrangement during metamorphosis in the pupal stage (4–5 days). This culminates in the development of the adult fly, which is the longest stage of the life cycle (~40–60 days). Therefore, exploring the long-term consequences of manipulating disease-associated genes would be best achieved in the adult fly. However, disease-causing genes are often essential. Thus, genetic manipulation is likely to cause developmental defects during the transient developmental stages and/or metamorphosis, and can often result in lethality prior to adult stages. This feature of *Drosophila* development can limit the ability to model the chronic features of neurodegenerative disease.

Fortunately, genetic tools have been developed in *Drosophila* research to circumvent this challenge. Cell-type specific manipulation through the bipartite Gal4/UAS system (Brand and Perrimon, 1993; Duffy, 2002) allows for the spatial control of transgenes in living flies. Often utilized for the study of essential disease-genes is Gal4/UAS-dependent manipulation of neurons in an adult-specific, non-essential tissue, such as the *Drosophila* eye (McGurk et al., 2015; Ma et al., 2022). Degeneration of this tissue can be observed through the progressive atrophy of the tissue at a macroscopic level, and visualization of the loss of ommatidia at a microscopic level (Burr et al., 2014). However, this system assays the degeneration of photoreceptor neurons, which are a neuronal subtype executing the unique function of phototransduction (Hardie and Juusola, 2015). This specialized function is not shared by neurons of the central nervous system (CNS), which are primarily lost in neurodegenerative diseases.

Manipulation of *Drosophila* CNS neurons can be achieved through the Gal4/UAS system (Jenett et al., 2012; Simpson, 2016), but the proper function of the CNS is required for embryonic and larval survival. Therefore, the manipulation of essential genes can lead to lethality during development, limiting the examination of long-term phenotypic consequences. Developmental lethality can be bypassed with tools that temporally control Gal4. One widely used tool, known as GeneSwitch (GS), utilizes a steroid hormone-inducible Gal4 (Osterwalder et al., 2001; Roman et al., 2001). In practice, Gal4-GS is activated when flies are fed the antiprogestin drug RU486, which can be fed to flies only in adult stages to skirt developmental lethality. However, there are cautionary reports of "leaky" Gal4-GS expression in the absence of RU486 (Poirier et al., 2008; Scialo et al., 2016). Furthermore, administration of the drug RU486 alone, independent of the actions on Gal4-GS, was capable of eliciting negative neurophysiological consequences (Li and Stavropoulos, 2016), and perturbing mitochondrial function (Robles-Murguia et al., 2019). To avoid these issues with RU486, other tools have been developed that utilize distinct chemical-inducible systems for temporal control of Gal4. These involve pharmacologically controlling the stability of the Gal4 repressor protein Gal80 (Sethi and Wang, 2017; McClure et al., 2022). These newly developed tools have not yet been adopted in the neurodegenerative disease field to bypass developmental lethality and examine long-term consequences of adult-onset gene manipulation.

We have previously examined the cell-biological ramifications of knocking down expression of the ataxia-associated gene *Vps13D* in *Drosophila* neurons (Seong et al., 2018; Insolera et al., 2021). We found that knockdown of *Vps13D* caused a "two-hit" defect in neuronal mitochondrial quality control: (1) mitophagy is induced, and (2) completion of mitophagy is inhibited. The resultant phenotype is the accumulation of stalled mitophagy intermediates in *Drosophila* neurons (Insolera et al., 2021). Damage to mitochondria is thought to be a natural byproduct of aging, but mitochondrial quality control mechanisms, such as mitophagy, prevent the build-up of damaged mitochondria (Evans and Holzbaur, 2020). However, accumulating damaged mitochondria by combining age-dependent damage with a diminished capacity to degrade mitochondria is believed to be a common condition associated with neurodegenerative disease (Pickrell and Youle, 2015). Therefore, knockdown of *Vps13D* in *Drosophila* neurons can be used as a model to scrutinize the cellular consequences of accumulating damaged mitochondria in an *in vivo* system.

Constitutive, pan-neuronal knockdown of *Vps13D* caused strong developmental lethality during pupal stages (Seong et al., 2018; Insolera et al., 2021), limiting the ability to investigate the chronic effects of accumulating damaged mitochondria in neurons. We hypothesized that bypassing this developmental lethality with genetic tools that permit adult-onset *Vps13D* RNAi expression would provide a more accurate model of the progressive neurodegenerative phenotypes of patients with mutations in VPS13D (Gauthier et al., 2018; Seong et al., 2018; Koh et al., 2020; Durand et al., 2022; Huang and Fan, 2022; Pauly et al., 2023). To this end, we examined two temporally-controlled Gal4 tools for adult-onset knockdown of *Vps13D*. We found that the elav-GS (Osterwalder et al., 2001) tool was leaky in the absence of an inducer during development, but the Gal80-DD (Sethi and Wang, 2017) tool was a viable alternative. With an optimized protocol we describe here, the Gal80-DD tool can be utilized for precise adult-onset Gal4 activation. Using this method, we found that adult-onset neuronal *Vps13D* knockdown causes progressive deficits in locomotor activity and early lethality. These defects correlate with cumulative mitophagy defects and neurodegeneration. We believe that this method can be widely used for accurate adult-onset knockdown of essential disease-genes to enable exploration of phenotypic consequences in the post-developmental adult fly brain.

Materials and methods

Fly husbandry and stocks

Fly stocks were maintained on standard Semi-defined yeast-glucose media. All experiments were performed in a standard 12:12 h light: dark cycle incubator.

The following stocks, (Bloomington (BL) stock numbers provided) were used in this study: *Vps13D* RNAi (BL #38320), luciferase RNAi (Control RNAi) (BL #31603), nSybGal4 [on II chromosome (Pauli et al., 2008)], nSyb-Gal80-DD (BL #79028), UAS-nucLacZ (BL #3956), elav-GS (BL# 43642). The most useful stock for crossing to any UAS line for adult-onset Gal4 expression using the methods described here (;nSybGal4;nSyb-Gal80DD) can be provided by the corresponding author, and will be deposited at the Bloomington Drosophila Stock Center.

Preparation of food containing TMP

Standard fly food was melted in a microwave until fully molten, and cooled to 50°C in a water bath. Trimethoprim (TMP) (Sigma-Aldrich T7883) was added as a solid to a measured volume of molten fly food at 50°C to generate a final concentration of 1 mM and vigorously mixed with a vortex (for approximately 10 s at the highest level). Following vortexing, 1 mM TMP food was distributed to empty vials (typically 5 mL/vial) and cooled. Vials containing 1 mM TMP were stored covered in 4°C, and used within 1 week of preparation.

Negative geotaxis assays

Negative geotaxis assays were performed as previously described (Barone and Bohmann, 2013). Briefly, cohorts of flies (10–15) were anesthetized, transferred to vials without food, and allowed to acclimate for 1 h following anesthesia. Vials were placed in front of a ruler and tapped on the counter three times to knock down the flies, while a video was recorded. The % of flies able to cross the 2 cm threshold within 10 s was counted, with each trial repeated five times on the same cohort. Flies were allowed to rest for 1 min between trials. The average % of 5 trials of one cohort was used as a single n value, with each experiment being tested containing at least 4 independent cohorts. Only male flies were used for negative geotaxis assays.

Eclosion and lifespan assays

For eclosion assays, pupae of the proper genotype (based on morphology and fluorescence to select against balancer chromosomes) were selected 8 days following egg laying, and these pupae were followed for a total of 7 days. Successful eclosion was scored as complete exit from the pupal case, leaving an empty case. Flies that died while partially eclosed were counted as failed. For experiments in which animals were reared at 18°C, timing of pupal selection and eclosion counts was extended to account for slower development. Typically, pupa were scored for their proper genotype 14 days following egg laying, and followed for successful eclosion for 12 days.

For lifespan assays, adult flies were collected on the day of eclosion, kept at low density (15 flies per vial) and kept on standard food at 25°C in an incubator with a 12:12 h light: dark cycle. Flies were transferred to new vials every 2–3 days and the number of deaths were recorded until all flies of one condition were dead. Only male flies were used for lifespan assays.

Immunostaining of larval and adult fly brains

For larval VNC staining, dissections and stainings were performed as previously described (Insolera et al., 2021). Briefly, third instar larvae were dissected in cold 1x PBS and fixed in either 4% paraformaldehyde for 20 min or Bouin's fixative (Ricca Chemical, 1120-16) for 7 min at room temperature (RT). Larvae were washed in 1x PBS three times for either 5 or 10 min, respectively, followed by three five minute washes with 1x PBS with 0.1% Triton X-100 (PBST). The larvae were incubated in blocking buffer (PBST with 5% normal goat serum and 0.02% sodium azide) for 30 min at RT, and then incubated in primary antibodies diluted in blocking buffer overnight at 4°C. After washing in PBST, larvae were incubated in secondary antibodies diluted in blocking buffer for 2 h at RT, followed by PBST washes. Dissected larvae were mounted in Vectashield (Vector Laboratories).

Adult brains were dissected in 1x PBS and fixed in 4% paraformaldehyde in 1x PBS for 30 min at room temperature. Brains were washed in PBST two times for 10 min each and permeabilized for 30 min in 1x PBS, 0.3% Triton X-100 at RT. Blocking was done in PBST with 1% BSA and 0.01% sodium azide with three washes for 20 min each at RT, and brains were incubated in primary antibodies diluted in blocking solution overnight at 4°C. After three 20 min washes in PBST at RT, brains were incubated with secondary antibodies diluted in 1x PBS, 0.3% Triton X-100, 0.1% BSA, 2% normal goat serum, and 0.01% sodium azide overnight at 4°C. Brains were washed in PBST three times for 10 min each at RT and mounted in Vectashield (Vector Laboratories). Brains stained with DAPI (Life Technologies) were incubated with 1 μg/mL DAPI in PBST for 10 min at RT, followed by three 10 min PBST washes.

Primary antibodies (with included manufacturers and catalog numbers) were diluted as follows: mouse anti-LacZ at 1:100 [Developmental Studies Hybridoma Bank (DSHB), cat no. 40-1a], mouse anti-ATP5A at 1:1000 (Abcam, cat no. ab14748), rat anti-Elav at 1:100 DSHB, cat no. 7E8A10), and rabbit anti-Ref(2)p at 1:500 (Abcam, cat no. ab178440). All secondary antibodies were diluted 1:1000 and used as follows: goat anti-mouse IgG1 Alexa Fluor 555, goat anti-rat Alexa Fluor 555, and goat anti-rabbit Alexa Fluor 488 or Alexa Fluor 647 (all Life Technologies).

Brain vacuolization assay

Adult flies were aged at 25°C and brains were dissected as described above. Brains were stained as previously described (Behnke et al., 2021a,b), except that phalloidin Alexa Fluor 594 (Invitrogen, A12381) was used at the manufacturer's suggested concentration of 16.5 μM.

Imaging, quantification, and statistics

Images were acquired with a Leica SP8 confocal microscope with a 20x (0.75NA) objective lens for adult fly brains or 63x (1.3NA glycerol immersion) objective lens for larval VNC images. Control conditions were imaged first to determine the appropriate imaging settings for each experiment, and kept consistent between conditions that were directly compared for analysis. Images of brain vacuoles were obtained with a Leica SP5 multiphoton microscope with a 20x objective lens as previously described (Behnke et al., 2021a,b).

Image quantification was performed with FIJI (NIH). Ref(2)p intensity was determined by thresholding Z-projections of images with the maximum entropy algorithm. The mean intensity was measured in four defined regions of interest (ROI) in the central brain per brain, and averaged. LacZ expression in larval motoneurons was quantified using a circular region of interest to sample LacZ intensity within 20 individual nuclei in the midline motoneuron population per VNC, and averaged per animal. In adult brains, LacZ expression was quantified by measuring the mean intensity for the whole central brain. The percentage of neurons that contained mitophagy intermediates was quantified as previously described (Insolera et al., 2021). Vacuolization of adult brains was quantified as previously described (Behnke et al., 2021a,b).

Statistical analysis and graphs were generated using GraphPad Prism. All statistical methods utilized are listed in the Figure legends. Two-tailed, unpaired t-tests assuming parametric distributions were used to compare statistical significance when two conditions were compared. When >2 conditions were being compared, one-way ANOVA was used with Tukey's multiple comparisons to test the means of each condition against all others. All error bars represent standard error of the mean (SEM), and individual data points are included to indicate the distribution of the data. The statistical significance of successful eclosion were determined using a Fisher's Exact Test to compare two individual genotypes at a time. Statistical significance of lifespan difference was determined using a Log-Rank (Mantel-Cox) Test. Sample sizes were determined based on previous literature.

Results

The elav-GS tool partially repressed Vps13D RNAi expression during development, but is leaky in the absence RU486

Our previous study demonstrated that constitutively driving expression of UAS-*Vps13D* RNAi (hereafter referred to as *Vps13D* RNAi) pan-neuronally via the nSyb-Gal4 caused significant pupal lethality, in which only ~5% of flies eclosed as adults (Insolera et al., 2021). To bypass this developmentally lethality, we utilized the inducible Gal4-GS tool, under the control of a different pan-neuronal promoter, elav-GS (Osterwalder et al., 2001). We found that rearing flies on food lacking the inducer molecule RU486 during development rescued the pupal lethality caused by pan-neuronal *Vps13D* knockdown, such that 96% of flies eclosed as adults (Figure 1A). However, we observed that eclosing flies from the *Vps13D* RNAi condition were lethargic and uncoordinated compared to control flies (data not shown), without ever introducing food containing RU486. We suspected that leaky expression of GS-Gal4 in the absence of induction (Poirier et al., 2008; Scialo et al., 2016) could be an explanation for this effect. Hence, we assessed the lifespan of the eclosing adult flies expressing *Vps13D* RNAi in the absence of RU486 throughout their life, and found that these flies are extremely short-lived (median survival of 10 days) (Figure 1B). These data indicate that while RNAi expression is repressed enough to rescue the eclosion defects, leaky expression of elav-GS in the absence of RU486 is partially knocking down *Vps13D* throughout development and eliciting a significant phenotype.

Knockdown of *Vps13D* results in enlarged mitochondrial morphology (Anding et al., 2018; Seong et al., 2018) and the accumulation of stalled mitophagy intermediates in larval motoneurons (Insolera et al., 2021). Therefore, we examined the mitochondrial morphology and stalled mitophagy intermediates in motoneurons of larvae with elav-GS reared in the absence of RU486. We found that leaky elav-GS expression of *Vps13D* RNAi produces significant enlargement of mitochondrial morphology, as visualized by immunostaining with an antibody against the mitochondrial protein ATP5A, compared to neurons expressing a control RNAi targeted against a non-endogenous gene in *Drosophila (Luciferase)* (Figures 1C,D). Leaky expression also causes the accumulation of stalled mitophagy intermediates in larval motoneurons, which were labeled by ATP5A+ mitochondria co-localizing with the *Drosophila* homolog of p62 known as Ref(2)p (Nezis et al., 2008; Insolera et al., 2021), in 22.8% of larval motoneurons. This represented a partial reduction in the percentage of neurons we have previously identified as containing stalled mitophagy intermediates (59%) (Insolera et al., 2021), albeit with a different, non-inducible pan-neuronal Gal4 driver under control of the same promoter (elav-Gal4). Regardless of the direct comparison to the constitutive Gal4, these results infer leaky elav-GS expression of *Vps13D* RNAi during larval stages.

The Gal4/UAS system has decreased activity at lower temperatures (Duffy, 2002; Nagarkar-Jaiswal et al., 2015), although this temperature-dependence has not been thoroughly examined with GS. We therefore tested whether rearing larvae at a lower temperature (18°C) would decrease the leaky expression of *Vps13D* RNAi during development caused by elav-GS in the absence of RU486. Lower temperature rearing of larvae containing elav-GS and *Vps13D* RNAi in the absence of RU486 resulted a slight, but non-significant decrease in the % of neurons containing mitophagy intermediates (Figures 1C,D), and did not decrease enlarged mitochondrial morphology. Therefore, this suggests that lower temperature rearing had no effect on lowering elav-GS leaky expression of *Vps13D* RNAi during development.

Finally, we stained adult brains for Ref(2)p to visualize the accumulation of stalled mitophagy intermediates to confirm the lethality was caused by a neuronal phenotype, as previous reports have noted non-neuronal expression of elav-GS in the absence of RU486 (Poirier et al., 2008). We found that three-day-old elav-GS adult flies expressing *Vps13D* RNAi in the absence of RU486 throughout their life had significant accumulation of Ref(2)p in the brain compared to flies expressing control RNAi (Figures 1E,F). These results suggest that, consistent with others (Poirier et al., 2008; Scialo et al., 2016),

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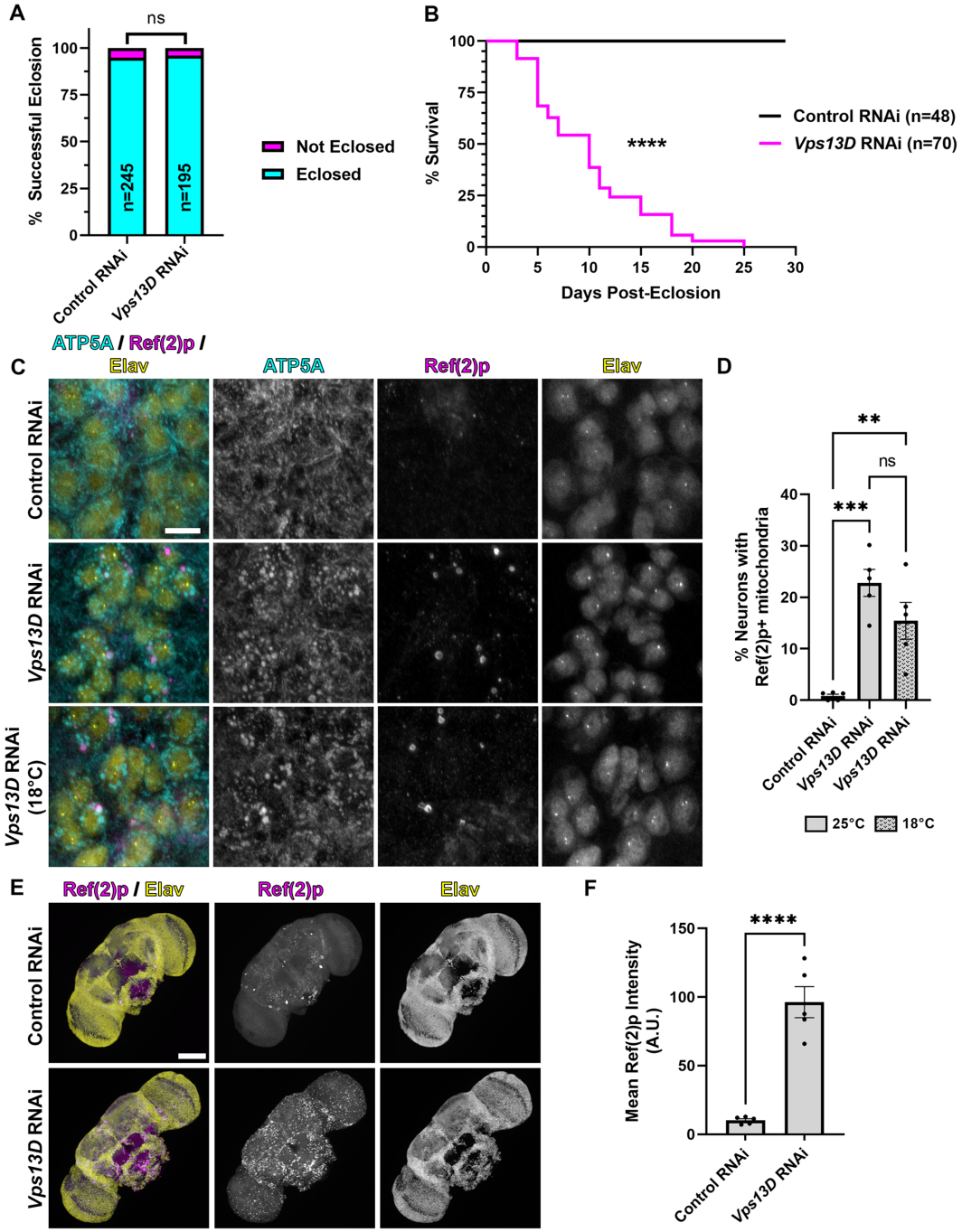

FIGURE 1
elav-GS is leaky during development in the absence of RU486. **(A)** Quantification of the % of successful eclosion events among total pupa indicated in bars of the graph when elav-GS is driving expression of the indicated RNAi constructs in the absence of RU486 during development (embryonic, larval, and pupal stages). "ns" p >0.05 (Fisher's exact test). **(B)** Lifespan curve of adult flies in days post-eclosion with elav-GS driving expression of the indicated RNAi constructs in the absence of RU486 throughout their life. Total number of flies assessed is indicated in the key. ****p <0.0001 (Log-Rank (Mantel-Cox) test). **(C)** Representative images of motoneurons in the larval VNC, which express the indicated RNAi driven by expression of elav-GS in the absence of RU486. Bottom panel images are from larvae reared at a lower temperature (18°C), compared to standard conditions (25°C) in the top two panels. Tissue was stained for the mitochondrial protein ATP5A (cyan), p62 homolog Ref(2)p (magenta), and neuronal transcription factor Elav (yellow). Scale bar: 10 µm. **(D)** Quantification of the percentage of dorsal midline motoneurons containing a stalled mitophagy intermediate (Ref(2)p+/ATP5A+ mitochondria) in the indicated conditions of RNAi, with fill pattern representing the rearing temperature (25°C or 18°C). Black points represent the % of neurons from one animal (n =5 animals for each condition). Bars represent the mean±standard error of the mean (SEM). **p <0.01, ***p <0.001, "ns" p >0.05 (One-way ANOVA). **(E)** Representative images of 3day old whole-mount adult fly brains, which express the indicated RNAi driven by expression of elav-GS in the absence of RU486 throughout life. Tissue was stained for the p62 homolog Ref(2)p (magenta), and neuronal transcription factor Elav (yellow). Scale bar: 100 µm. **(F)** Quantification of the mean Ref(2)p intensity in the brains of the indicated conditions of RNAi. Black points represent the average of 4 defined regions of interest (ROI) of one animal (n =5 animals for each condition). Bars represent the mean±SEM. ****p <0.0001 (T-test).

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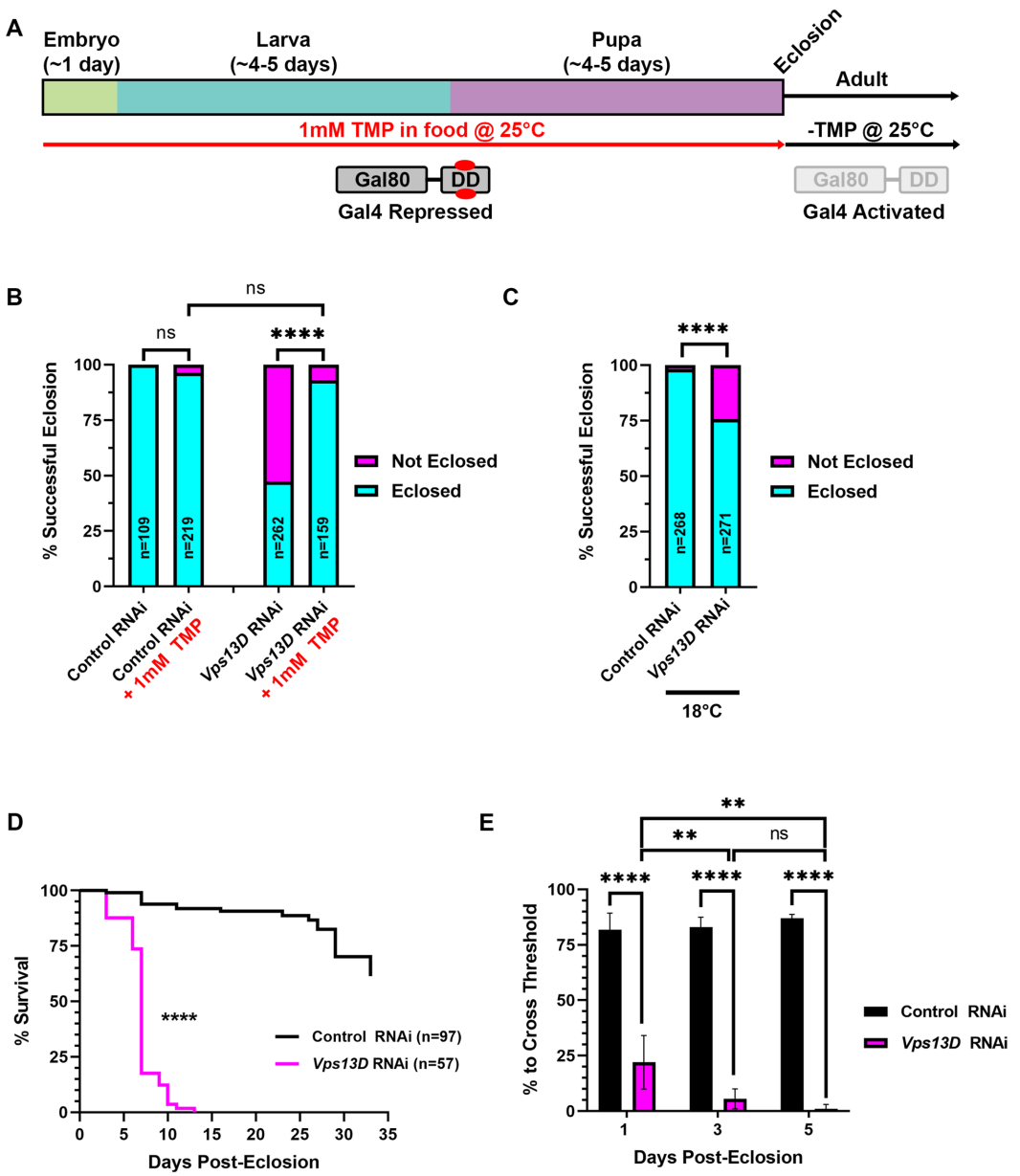

FIGURE 2
Gal80-DD partially represses Gal4 activity when TMP is administered during development. **(A)** Cartoon indicating the administration of 1 mM TMP in the food of developing flies for temporal control of Gal4. Flies express both Gal4 and Gal80-DD under the control of the nSyb promoter (from separate transgenes). During development, larvae consuming food laced with TMP (red) causes the stabilization of Gal80-DD (referred to in text as "stabilized Gal80-DD"), and repression of Gal4. After eclosion, TMP is no longer present in the food, Gal80-DD is degraded, and Gal4 is activated. **(B)** Quantification of the % of successful eclosion events among total pupa indicated in bars of the graph with nSyb driven Gal4 and Gal80-DD expressing the indicated RNAi constructs, with (indicated in red) or without 1mM TMP in the food during development. "ns" $p>0.05$, ****$p<0.0001$ (Fisher's exact test). **(C)** Quantification of the % of successful eclosion events among total pupa indicated in bars of the graph when nSyb-Gal4 is driving expression of the indicated RNAi constructs in the presence of Gal80-DD when flies are reared at 18°C (in the absence of TMP). ****$p<0.0001$ using the Fisher's exact test. **(D)** Lifespan curve of adult flies in days post-eclosion with nSyb driven Gal4 and Gal80-DD expressing the indicated RNAi constructs with 1mM TMP in the food during development. Total number of flies assessed is indicated in the key. ****$p<0.0001$ (Log-Rank (Mantel-Cox) test). **(E)** Quantification of the negative geotaxis assay (see Methods), indicating the % of flies able to cross a threshold as an indication of locomotor ability. Bar colors indicate the RNAi construct expressed. Adult flies were tested at three different ages post-eclosion (indicated on X-axis). All bars represent the mean score of multiple cohorts of flies, with each bar representing an n≥4 cohorts ± SEM. **$p<0.01$, and ****$p<0.0001$ (*T*-test, for pairwise comparison of *Vps13D* to Control RNAi in each time-point; One-way ANOVA for statistical significance of comparing within genotypes across the three time points. Not specified in the graph is that the Control RNAi changes across the three time periods was "ns" $p>0.05$ by one-way ANOVA).

leaky expression of elav-GS in the absence of RU486 is capable of driving some level of *Vps13D* RNAi expression during development and adult stages. These results motivated us to test another temporally-controlled, pan-neuronal Gal4 tool for more precise control of adult-onset *Vps13D* knockdown.

The Gal80-DD tool provides an alternative method for temporal control of Gal4

We next tested the efficiency of a chemically-inducible repressor of Gal4, Gal80, to suppress pupal lethality caused by pan-neuronal

expression of *Vps13D* RNAi. In this previously engineered tool, Gal80 was fused to an engineered destabilization domain (DD) from *Escherichia coli* dihydrofolate reductase (ecDHFR) to induce its continuous degradation (Sethi and Wang, 2017). The so-called "Gal80-DD" protein is expressed in neurons, as it is driven by the promoter region of *Drosophila* neuronal synaptobrevin (nSyb) gene (Sethi and Wang, 2017). Gal80-DD is "stabilized" to repress Gal4 activity in the presence of the antibiotic molecule TMP. We reasoned that rearing larvae on food containing 1 mM TMP will suppress Gal4 activity during development, and a shift to normal (-TMP) food after eclosion will turn on Gal4 in adult neurons (Figure 2A). For all subsequent experiments using this method, we utilized the pan-neuronal Gal4 driven by the same promoter (nSyb-Gal4 on second chromosome, see Methods), thus yielding a relatively similar ratio of Gal80 to Gal4 protein. We found that expression of Gal80-DD, while feeding TMP in larval stages, rescued pupal lethality caused by *Vps13D* RNAi expression from a 47.3% success rate in the absence of TMP to a 93.1% success rate in the presence of TMP (Figure 2B). Flies expressing control RNAi had negligible pupal lethality, which was not significantly altered by the ingestion of 1 mM TMP (Figure 2B).

Because lower rearing temperatures (18°C) decreases Gal4 activity (Duffy, 2002; Nagarkar-Jaiswal et al., 2015), we tested whether lower temperature alone can restrict the activity of nSyb-Gal4, as compared to the repression by stabilized Gal80-DD. Temperature-dependent repression of Gal4 yielded a successful eclosion rate of 75.7% (Figure 2C), indicating that lower temperature rearing indeed represses Gal4 in this scenario. However, temperature-dependent repression alone was weaker than that of stabilized Gal80-DD, based on eclosion success. These results suggest that stabilized Gal80-DD represses Gal4 activity during development stronger than temperature-dependent repression of Gal4.

We next examined the resulting adult flies produced from conditions of *Vps13D* RNAi expression in the presence stabilized Gal80-DD during development. After eclosion, adult flies were shifted to normal fly food lacking TMP (-TMP) to induce Gal80-DD degradation and activate Gal4 (Figure 2A). Similar to the elav-GS conditions, we observed that eclosing flies from these conditions were lethargic and uncoordinated. These flies were short-lived, with a median lifespan of only 7 days (Figure 2D). This suggested that Gal80-DD was only partially repressing Gal4 activity during development. Negative geotaxis assays (Barone and Bohmann, 2013) confirmed that flies only 1 day post-eclosion had significant locomotor impairment that dramatically declined over the course of 5 days (Figure 2E). This immediate impairment is not consistent with adult-onset knockdown, as we reasoned that Gal4 induction and RNAi mediated knockdown would require >24 h to elicit such a strong phenotype. We therefore sought to optimize the Gal80-DD system further for less leaky expression, and more precise adult-onset knockdown.

Combining Gal80-DD with low temperature rearing during development strongly represses Gal4

We posited that one contributing factor to the incomplete repression of Gal4 by stabilized Gal80-DD during development could be the embryonic and pupal stages when the flies are not ingesting the TMP-containing food. To combat this, we took advantage of the partial temperature dependence of the Gal4/UAS system (Duffy, 2002; Nagarkar-Jaiswal et al., 2015), which we presumed could be utilized in combination with stabilized Gal80-DD for "dual repression" of Gal4. To test this hypothesis, we chose to directly read out Gal4 activity in conditions of singular and dual repression with a UAS-driven nuclear localized LacZ (nucLacZ) reporter, staining wandering third instar larval ventral nerve cords (VNCs) with antibodies against beta-galactosidase. In larvae expressing nSyb-Gal4 and Gal80-DD, the addition of 1 mM TMP to the food yielded an average 34% reduction in nucLacZ reporter intensity compared to-TMP conditions, but this difference did not reach statistical significance ($p > 0.05$) (Figures 3A,B). When we reared larvae at a lower temperature (18°C), the average nucLacZ levels were significantly lower than larvae reared in standard conditions (25°C) due to the apparent temperature-dependence of Gal4. Stabilized Gal80-DD repression at 18°C was significantly more effective, reducing expression of Gal4 by an average of 85% compared to -TMP conditions alone at this temperature (Figures 3C,D). No single acquisition setting could accommodate the dynamic range of nucLacZ intensity within the extreme ends of this experiment, preventing a direct comparison of the highest reporter expression levels to the lowest reporter expression levels (-TMP at 25°C vs. 1 mM TMP at 18°C). To best estimate the total level of repression, we performed a direct comparison of nucLacZ expression in conditions of TMP food in which Gal4 was repressed by stabilized Gal80-DD. Larvae raised in 18°C compared to 25°C resulted in on average a 93% reduction in nucLacZ expression (Figures 3E,F). These results indicate that dual Gal4 repression with stabilized Gal80-DD and low temperature rearing (18°C) provided significantly higher levels of repression in larval stages compared to stabilized Gal80-DD at standard rearing temperatures (25°C).

We next assessed the mitochondrial morphology and stalled mitophagy intermediates in larval motoneurons in conditions of stabilized Gal80-DD repression of *Vps13D* RNAi expression during development. In the absence of TMP, when larvae were reared at 25°C, mitochondrial morphology is enlarged, and stalled mitophagy intermediates were present in 30% of larval motoneurons containing *Vps13D* RNAi (compared to 1.4% of neurons expressing control RNAi) (Figures 4A,C). The addition of TMP had no observable effect on decreasing mitochondrial enlargement in *Vps13D* RNAi conditions, but significantly reduced the percentage of neurons containing stalled mitophagy intermediates to 22.3% (Figures 4A,C). Rearing larvae in 18°C in the absence of TMP significantly reduced the percentage of neurons containing stalled mitophagy intermediates more significantly than TMP addition at 25°C, to 13.2% of neurons; however, enlargement of the mitochondria was still prominent. Lastly, conditions of dual Gal4 repression, employing both stabilized Gal80-DD and low temperature (18°C) rearing, yielded the most significant reduction in morphological enlargement of mitochondria, and reduced the percentage of neurons containing stalled mitophagy intermediates to 4.1%, which is not statistically different from the control condition (Figures 4B, bottom, C). However, the modest mitochondrial enlargement and low-frequency presence of stalled mitophagy intermediates suggested some minor leaky RNAi expression in these conditions. Overall, these results demonstrate that lower temperature rearing, combined with stabilized Gal80-DD during larval stages maximally represses leaky *Vps13D* RNAi expression during development.

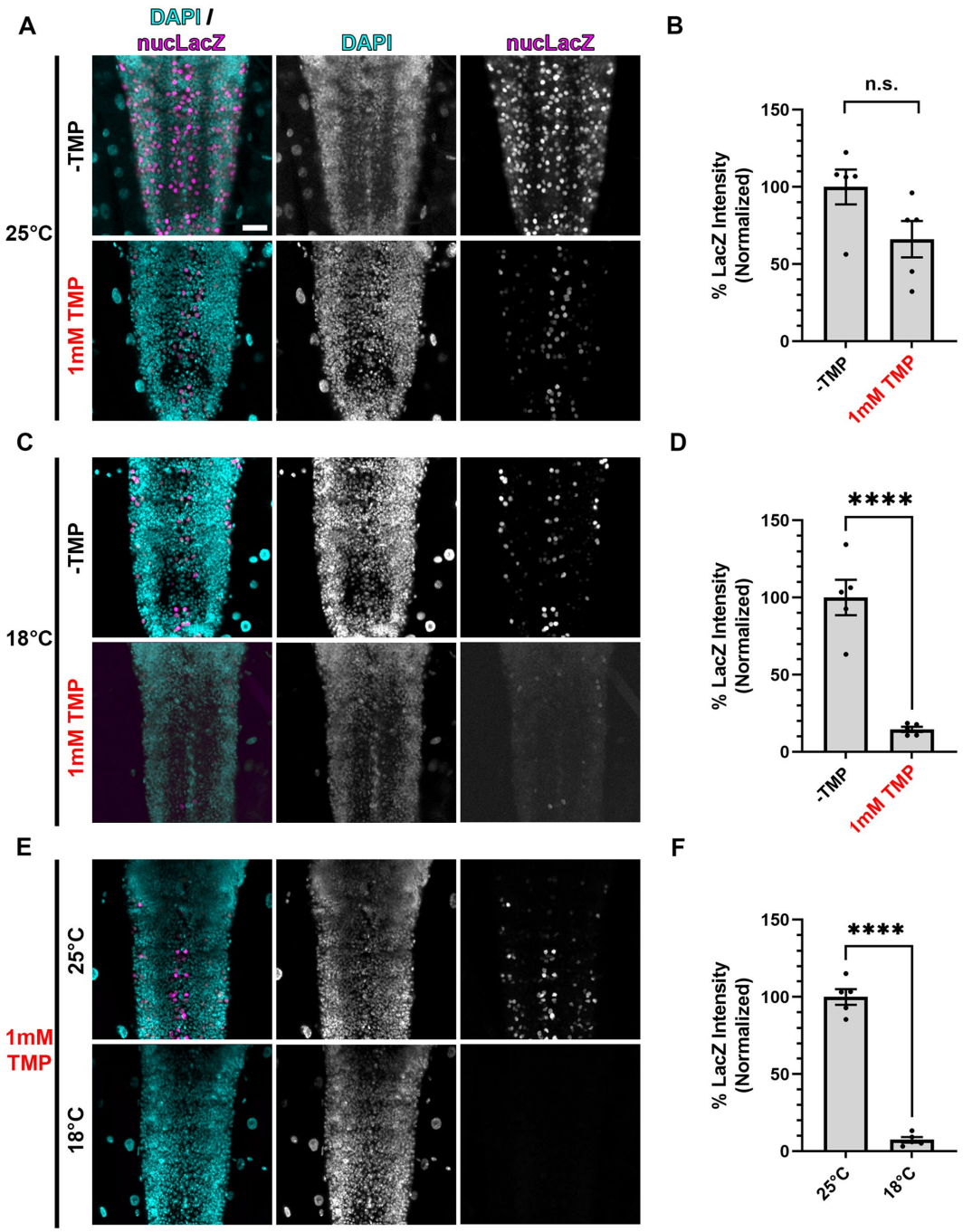

FIGURE 3
Gal80-DD repression of Gal4 in the presence of TMP is more complete at lower temperatures. **(A,C,E)** Representative images of motoneurons in the larval VNC, with nSyb driven Gal4 and Gal80-DD expressing the UAS-nucLacZ reporter. Tissue was stained for DAPI (cyan) and beta-galactosidase (magenta). Conditions (temperature or presence of 1mM TMP, indicated in red) in each panel are indicated on the left. Scale bar: 10 μm. **(B,D,F)** Quantifications of the mean nucLacZ intensity in the indicated conditions corresponding to the image panel to the left of the graph **(A** with **B**, **C** with **D**, and **E** with **F)**. Black points represent the average from one animal (n =5 animals for each condition). Normalized to the condition in the left bar. Bars represent the mean±SEM. "ns" p >0.05, ****p <0.0001 (T-test).

Dual repression conditions followed by post-eclosion shift produces precise adult-onset Gal4 expression

To assay the induction of Gal4 in adult brains, we measured the expression of the nucLacZ reporter in adult brains expressing nSyb-Gal4 and Gal80-DD reared at 18°C on TMP food during development, and shifted to 25°C and normal food (-TMP) post-eclosion (Figure 5A). In one-day-old adult flies, we observed expression of nucLacZ in adult neurons (Figure 5B). This expression was concentrated in the central brain region, with lower expression in the optic lobes. Expression levels of nucLacZ significantly increased by 50% in 7 day old flies, demonstrating activation and sustainment of Gal4 activity in adult stages (Figures 5B,C).

We next examined the phenotype of resulting adult flies reared with the optimized adult-onset induction system (Figure 5A) containing

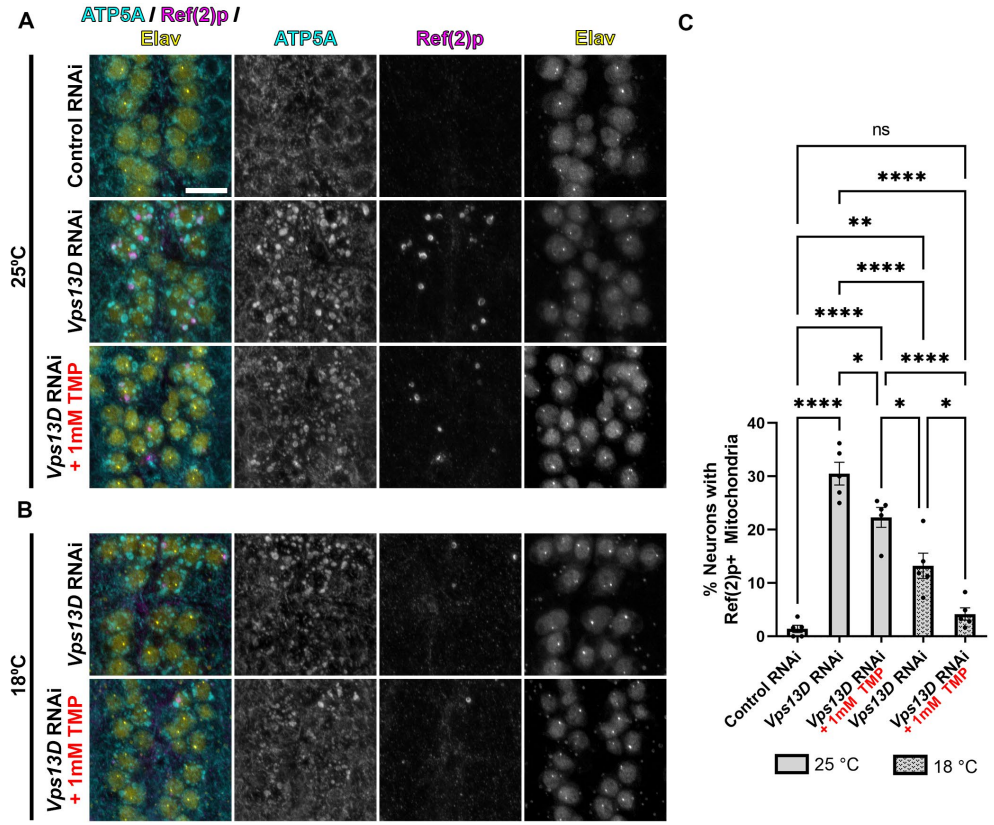

FIGURE 4
Minimal leaky expression of RNAi in conditions of low temperature rearing and stabilized Gal80-DD. **(A,B)** Representative images of motoneurons in the larval VNC with nSyb driven Gal4 and Gal80-DD expressing the indicated RNAi, with (indicated in red) or without TMP. Temperature of rearing conditions during developmental stages is indicated to the left of the image panels in **A** (25°C) and **B** (18°C). Tissue was stained for the mitochondrial protein ATP5A (cyan), p62 homolog Ref(2)p (magenta), and neuronal transcription factor Elav (yellow). Scale bar: 10 μm. **(C)** Quantification of the percentage of dorsal midline motoneurons containing a stalled mitophagy intermediate (Ref(2)p+/ATP5A+ mitochondria) in the indicated conditions, with fill pattern representing the rearing temperature (25°C or 18°C). Black points represent the % of neurons from one animal (n =5 animals for each condition). Bars represent mean±SEM. *p <0.05, **p <0.01, ****p <0.0001, "ns" p >0.05 (One-way ANOVA).

Vps13D RNAi. Negative geotaxis assays revealed that locomotor activity of newly eclosed flies (one-day-old) is not significantly different between flies expressing control versus *Vps13D* RNAi (Figure 6A, first two bars). However, flies with adult-onset neuronal *Vps13D* RNAi expression presented with progressive locomotor deficiencies that gradually declined at 1 week old, and further at 2 weeks of age (Figure 6A). By 3 weeks old, adult-onset *Vps13D* knockdown flies were either dead or incapable of responding to the negative geotaxis stimulus due to severe locomotor impairment. Flies with adult-onset *Vps13D* knockdown had significantly shorter lifespan compared to flies expressing control RNAi (Figure 6B) (median lifespan of 20 days). We interpret the more gradual decline in locomotor activity and lethality observed in flies reared in these optimized repressive conditions to be indicative of precise adult-onset knockdown, not confounded by developmental defects caused by leaky expression of Gal4.

Adult-onset Vps13D knockdown causes progressive accumulation of mitophagy intermediates and neurodegeneration

We predicted that cellular defects in the adult fly neurons expressing *Vps13D* RNAi would correlate with the early lethality and neurophysiological defects. We first assessed accumulation of stalled mitophagy intermediates by immunostaining for Ref(2)p (Nezis et al., 2008; Insolera et al., 2021). In one-day-old flies expressing *Vps13D* RNAi, there was a significant accumulation of Ref(2)p compared to flies expressing control RNAi (Figures 7A,B). We infer that this early phenotype in flies 24 h following Gal4 induction is likely caused by some leaky RNAi expression initiating during pupal stages in the absence of active TMP ingestion. Importantly, levels of Ref(2)p significantly increase in 7-day-old flies (Figures 7A,B). These results suggest that adult-onset *Vps13D* knockdown causes the progressive accumulation of stalled mitophagy intermediates.

Finally, the loss of locomotor activity and early lethality suggested that adult-onset *Vps13D* knockdown was inducing neurodegeneration. A common histological feature of neurodegeneration in *Drosophila* brains is vacuolization (Sunderhaus and Kretzschmar, 2016). Therefore, we performed a newly described protocol for visualization of vacuoles in whole-mount adult fly brains (Behnke et al., 2021a,b). We chose to compare vacuolization of brains at 14 days old, when death from adult-onset *Vps13D* knockdown began to accelerate to correlate lethality with neurodegeneration. Brains were stained for phalloidin to detect actin in regions of neuropil and DAPI to detect nuclei. Voids in staining of both markers (apart from defined physiological holes) were considered vacuoles (Behnke et al., 2021a).

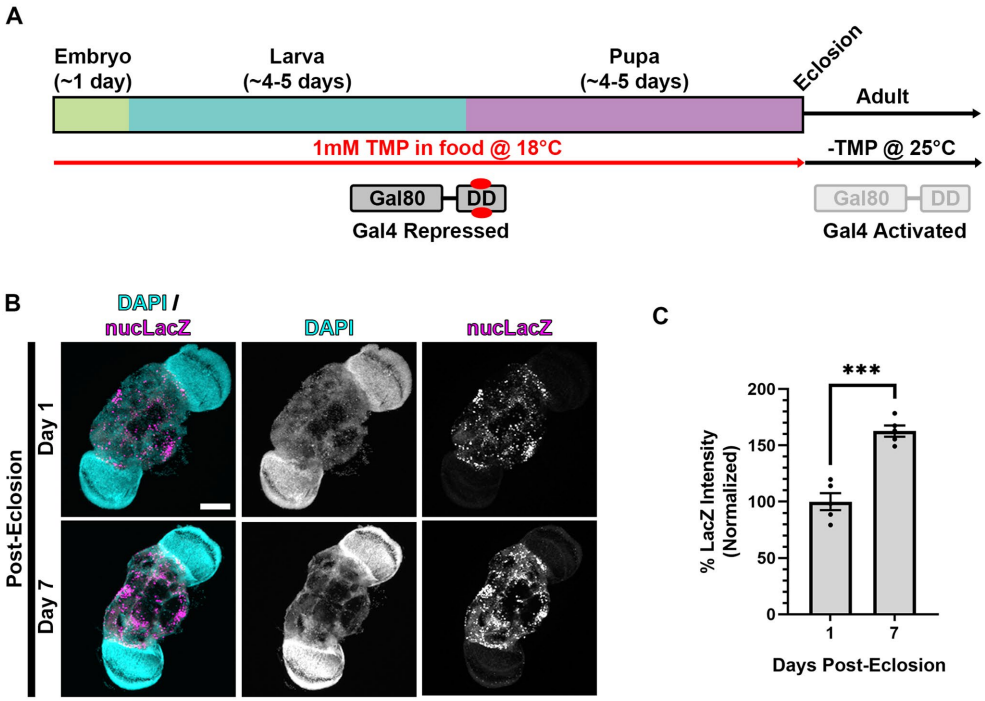

FIGURE 5
Gal4 expression is activated in adult animals following dual repression of Gal4 during development. **(A)** Cartoon indicating "dual repression" scheme. Similar to Figure 3A, but with the addition of lower temperature rearing (18°C) during development to additionally repress Gal4 (in addition to stabilized Gal80-DD), and shift to normal temperature (25°C) and-TMP food after eclosion. **(B)** Representative images of adult brains, with nSyb driven Gal4 and Gal80-DD from the protocol represented in panel **(A)**, expressing the UAS-nucLacZ reporter. Tissue was stained for DAPI (cyan) and beta-galactosidase (magenta) at the indicated ages post-eclosion. Scale bar: 100 μm. **(C)** Quantification of the mean nucLacZ intensity from conditions indicated in panel **(B)**. Black points represent the mean nucLacZ intensity from the central brain region of one animal (n =5 animals for each condition). Normalized to the day 1 condition. Bars represent mean±SEM. ***p <0.001 (T-test).

Brains expressing *Vps13D* RNAi showed significantly higher total areas of vacuolization at 14 days old compared to control RNAi (Figures 7C,D). These results indicate that adult-onset neuronal knockdown of *Vps13D* causes progressive mitophagy defects that correlates with neurodegeneration.

Discussion

Here, we optimized a protocol for the use of Gal80-DD (Sethi and Wang, 2017) for precise adult-onset knockdown of *Vps13D* in the *Drosophila* brain. We were previously able to investigate subcellular perturbations associated with *Vps13D* loss only in larval neurons (Insolera et al., 2021) because pan-neuronal genetic manipulation of *Vps13D* caused lethality in pupal stages. This optimized method now allows us to bypass this developmental lethality, and explore the progressive defects in neurons lacking *Vps13D* over the course of a few weeks, instead of in the 3–4 days of the transient larval stage. We believe this better models the human disorder, and enables the leveraging of the powerful genetic tools available in *Drosophila* to screen for suppressors of the neurodegenerative phenotype as a potential avenue for therapeutics.

We initially sought to utilize the elav-GS tool for adult-onset pan-neuronal *Vps13D* knockdown (Osterwalder et al., 2001), but we directly observed leaky expression of *Vps13D* RNAi in the absence of RU486 during development based on the robust mitochondrial phenotype observed in larval motoneurons upon *Vps13D* loss (Figure 1). We tested this because previous reports have documented that leaky expression of UAS-driven transgenes is significantly higher when GS is driving RNAi constructs in comparison to when GS is driving protein-coding sequences (Scialo et al., 2016).This intriguing divergence in the level of leaky expression in protein-coding versus RNAi encoding UAS-driven transgenes has major implications on interpretation of results when using this tool. This motivated our characterization of Gal80-DD beyond what was described in the original manuscript (Sethi and Wang, 2017), which examined the utility of Gal80-DD to repress Gal4 driving a UAS-transgene that was protein-coding (UAS-GFP). We expanded on these prior results with quantitative measurements of an independent protein-coding reporter (UAS-nucLacZ, Figure 3), in which tissue was immunostained for LacZ to enhance sensitivity of detecting low-levels of protein. In the original report (Sethi and Wang, 2017), UAS-GFP expression was measured in fixed brain tissue without immunostaining, which has less sensitivity to detect low levels of GFP protein. We found that repression of the protein coding UAS-nucLacZ transgene was significantly more effective during development when the TMP is combined with low temperature rearing (Figure 3), and robustly activated when TMP is withdrawn and temperature is shifted up post-eclosion (Figure 5). Finally, we validated that our RNAi driven transgene of interest (*Vps13D* RNAi) followed a similar pattern of enhanced repression of leaky RNAi expression when TMP is fed in combination with low temperature rearing (Figure 4). We reasoned that the easily observable phenotype in larval motoneurons

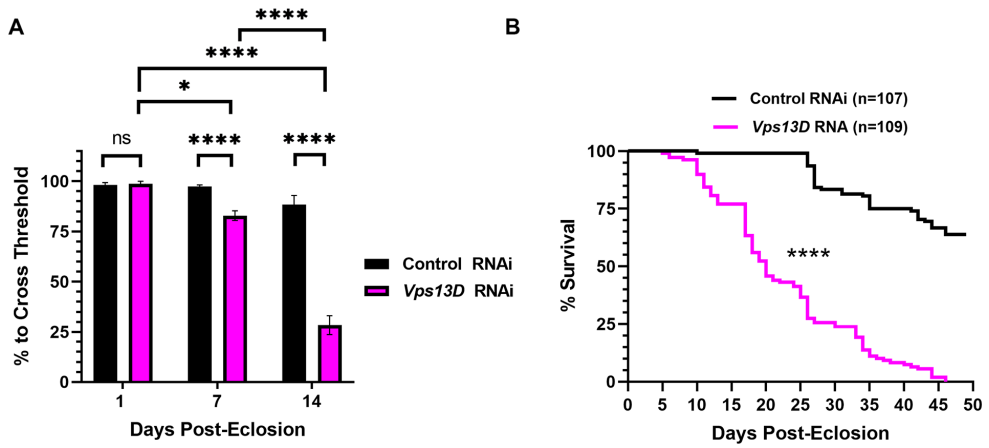

FIGURE 6
Dual repression of Gal4 results in adult-onset *Vps13D* knockdown that causes progressive locomotor deficits and early lethality. **(A)** Quantification of the negative geotaxis assay of adult flies resulting from conditions described in Figure 5A. Graph indicates the % of flies able to cross a threshold as an indication of locomotor ability. Bar colors indicate the RNAi construct expressed. Adult flies were tested at three different ages post-eclosion (indicated on X-axis). All bars represent the mean score of multiple cohorts of flies, with each bar representing an $n \geq 5$ cohorts ± SEM. "ns" $p > 0.05$, *$p < 0.05$, and ****$p < 0.0001$ (*T*-test, for pairwise comparison of Vps13D to Control RNAi in each time-point; One-way ANOVA for statistical significance of comparing within genotypes across the three time points.Not specified in the graph is that the Control RNAi changes across the three time periods was "ns" $p > 0.05$ by one-way ANOVA). **(B)** Lifespan curve of adult flies resulting from conditions described in Figure 5A expressing the indicated RNAi constructs. Total number of flies assessed is indicated in the key. ****$p < 0.0001$ based on a Log-Rank (Mantel-Cox) test.

caused by *Vps13D* knockdown provided us with a sensitive output measure, albeit indirect, to observe potential leakiness of the tool. Ultimately, our results show that the combination of Gal80-DD with TMP feeding and low temperature rearing during development strongly represses leaky *Vps13D* RNAi expression to yield healthy adult flies for adult-onset knockdown.

A limitation of this study is that we were unable to quantitatively measure the degree of knockdown of *Vps13D* expression as a consequence of leaky or induced Gal4 expression due to technical constraints. We do not have a reliable antibody against *Drosophila* Vps13D to test for the degree of protein knockdown via immunostaining in neurons. Measuring levels of *Vps13D* transcript expression requires the harvesting of RNA from fly heads that contains contaminating cell/tissue types (such as glial cells, fat body, digestive tissue, and muscles in the proboscis) not expressing *Vps13D* RNAi, complicating the interpretation of knockdown efficiency. Historically, others have tested for the efficiency of RNAi knockdown in flies through Gal4-driven expression of an RNAi from a ubiquitous promoter, and harvesting RNA samples from the whole animal (Scialo et al., 2016). Unfortunately, this cannot be performed in this circumstance because ubiquitous expression of *Vps13D* RNAi causes significant lethality in early larval stages (Seong et al., 2018). Techniques like Translating Ribosome Affinity Purification (TRAP) are available that would permit cell-type specific RNA isolation (Thomas et al., 2012; Chen and Dickman, 2017). However, these methods require active Gal4 to express UAS-driven tagged ribosomal subunits, which would be repressed in uninduced conditions, negating the use of the tool to examine *Vps13D* transcript levels that result from leaky RNAi expression. Nevertheless, the RNAi stock line targeting *Vps13D* used in this study has been previously characterized, and shown to efficiently knockdown Vps13D protein expression in *Drosophila* midgut cells (Anding et al., 2018). Further, the resultant phenotype of knockdown via RNAi expression from this fly line is highly similar to the phenotype of *Vps13D* mutant larvae (Seong et al., 2018) and mutant cells in mosaic tissue (Anding et al., 2018). Additionally, the 21 base pair short hairpin loop expressed in this fly stock targets a sequence unique to *Drosophila* Vps13D that is not shared by other vps13-protein family genes encoded in the *Drosophila* genome (*vps13* and *Vps13B*).

Without a quantitative measurement of RNA transcript levels or protein levels of *Vps13D* neurons, we cannot directly compare the level of leakiness of elav-GS to the optimized system described in this manuscript. Additionally, the drivers are under the control of different promoters, albeit both widely considered as pan-neuronal, preventing a fair comparison of phenotypes. Our overall motivation was not to directly compare these systems, but instead to develop a strategy to induce precise adult-onset neuronal *Vps13D* knockdown. When we combined the observed leakiness of the elav-GS with previous reports of the detrimental effects caused by RU486, especially on mitochondrial function (Robles-Murguia et al., 2019), we determined that the elav-GS tool would not be ideal for our future studies. As noted in the original work describing the tool (Sethi and Wang, 2017), other advantages to Gal80-DD are that we can utilize Gal80-DD with existing Gal4 lines, if we choose to focus on a specific neuronal subset in the future. TMP is also significantly more cost effective than RU486. Further, TMP can be directly dissolved in molten fly food at 1 mM concentrations used in the experiments described here, avoiding the detrimental effects of solvent consumption (RU486 is typically dissolved in ethanol).

Other temporally activated Gal4 systems are available for use in *Drosophila* research, including the Gal80^{ts} tool (McGuire et al., 2003). We tried this tool in the context of adult-onset, pan-neuronal *Vps13D* knockdown. We found that adult flies reared at the higher temperature needed to activate Gal4 (29°C) were short-lived in control conditions, which was indeed more prominent in conditions of *Vps13D* knockdown (data not shown). High-temperature rearing of flies results in the acceleration of aging and inflammation (Miquel et al., 1976; Kounatidis et al., 2017). We ultimately chose not to pursue the

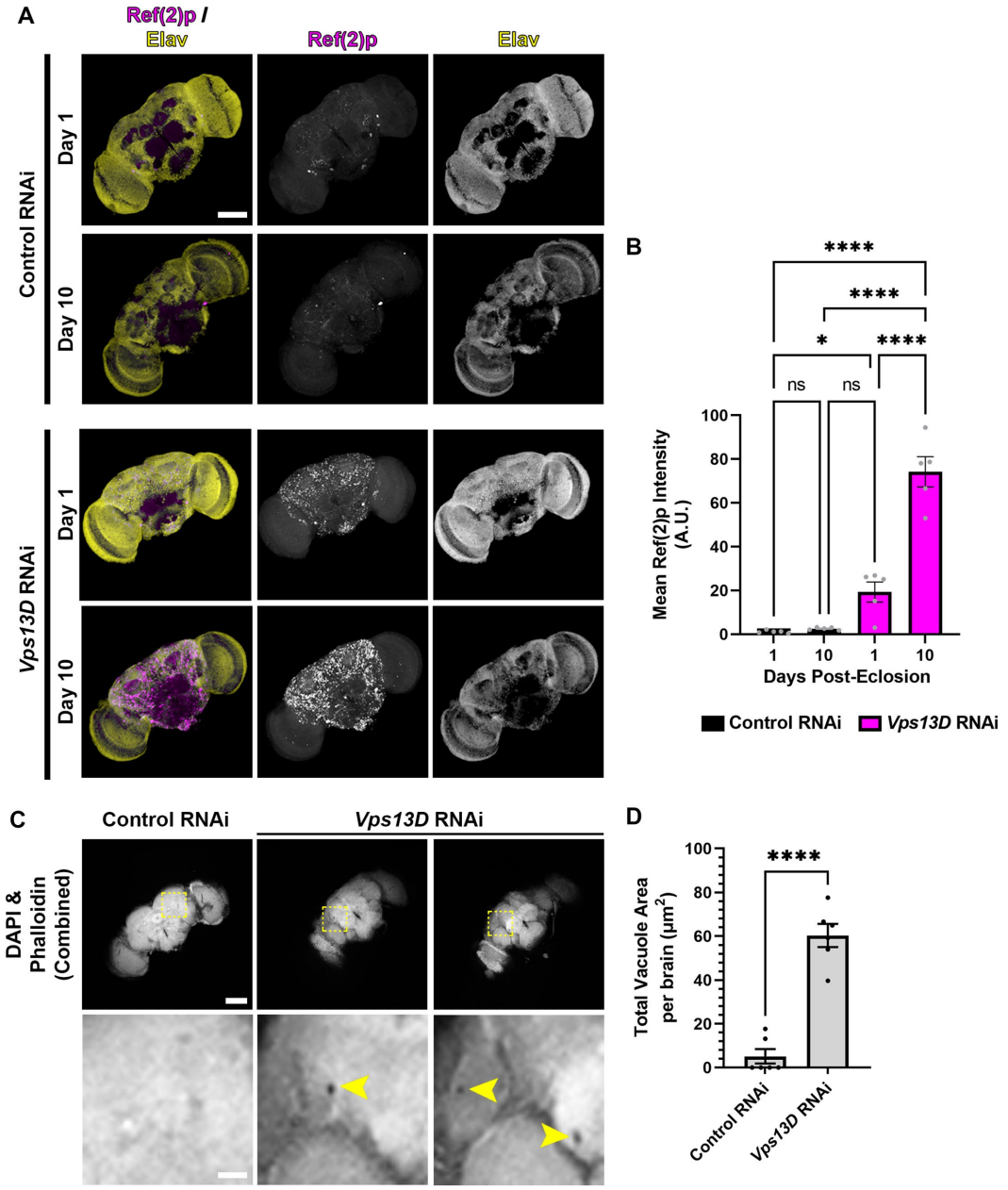

FIGURE 7
Adult-onset *Vps13D* knockdown causes progressive accumulation of stalled mitophagy intermediates and neurodegeneration. **(A)** Representative images of one and 10day old whole-mount adult fly brains, which express the indicated RNAi using optimized adult-onset Gal4 activation conditions described in Figure 5A. Tissue was stained for the p62 homolog Ref(2)p (magenta), and neuronal transcription factor Elav (yellow). Scale bar: 100 μm. **(B)** Quantification of the mean Ref(2)p intensity in the brains of the indicated ages and conditions of RNAi. Bar colors indicate the RNAi construct expressed. Grey points represent the average of 4 defined regions of interest (ROI) of one animal (n =5 animals for each condition). Bars represent the mean±SEM. *p <0.05, ****p <0.0001, "ns" p >0.05 (One-way ANOVA). **(C)** Representative images of 14day old whole-mount adult fly brains which express the indicated RNAi using optimized adult-onset Gal4 activation conditions, stained for DAPI and phalloidin (combined into a single greyscale picture). Bottom panel represents a magnified view of the region indicated by dashed lines in a single Z-plane. Two different brains of *Vps13D* RNAi condition are included to demonstrate examples of vacuoles. Yellow arrowheads indicate vacuoles. Scale bars: 100μm (top), 20 μm (bottom). **(D)** Quantification of the total vacuole area per brain in conditions of the indicated RNAi. Black points represent the total vacuole area from the brain of a single animal (n =5 animals for each condition). Bars represent the mean±SEM. ****p <0.0001 (T-test).

use of this tool due to these confounding variables, and focused instead on the optimization of Gal80-DD described here. A major advantage for optimizing the Gal80-DD tool was that the flies of interest (aged flies following adult-onset Gal4 activation) were reared at the time of our experiments in standard culture conditions (25°C) on normal food lacking potentially confounding chemical and environmental variables.

The newly published AGES system (McClure et al., 2022) shares many of the benefits of Gal80-DD, and shows inducible expression of GFP and other proteins of interest in adult neurons using a number of different Gal4s. We would be interested to examine the level of leaky expression of RNAi constructs in the absence of induction with AGES during development, similar to the experiments we performed here with Gal80-DD. We found that detection of reporter protein

expression (nucLacZ) was not as sensitive for detecting leaky expression as the phenotype caused by low levels of expression of *Vps13D* RNAi. While we found that we could not detect reporter expression in larval neurons containing stabilized Gal80-DD reared at 18°C (Figure 3E), we were able to detect a subtle mitochondrial phenotype caused by low level expression of *Vps13D* RNAi in these conditions (Figure 4B). Additionally, one difference of AGES compared to Gal80-DD is that flies are fed the chemical inducer (auxin) in order to activate (not inactivate) Gal4; therefore, flies are fed inducer during the time of Gal4 activation. The initial report described the relatively negligible effects of auxin consumption on *Drosophila* physiology at the concentrations needed for induction (McClure et al., 2022), and the effects of auxin consumption can be accounted for with proper controls. Overall, it's unlikely that any temporally controlled Gal4/UAS system is going to provide perfect on/off control, and having a number of tools to choose from for specific experimental goals is beneficial.

The usefulness of Gal80-DD in the field of neuroscience was previously described by the original report (Sethi and Wang, 2017). Here, we optimized this tool to allow for adult-onset neuronal knockdown of *Vps13D*. While this newly optimized tool can be a benefit to researchers in the field of neuroscience and neurodegenerative diseases, the Gal80-DD expression is restricted to neurons due to its neuronal promoter. Future studies utilizing this same Gal80-DD construct driven by a strong, ubiquitous promoter would presumably allow for the temporal control of Gal4 in a number of tissues. There are also disadvantages to the method we describe here. First, the temperature shift of the flies during development will not be ideal for certain fields of research including researchers interested in adaptive changes caused by temperature, or circadian research. Additionally, the practice of feeding flies TMP, which is an antibiotic, will likely create confounds for researchers interested in the endogenous immune system and commensal microbiota. In a different system utilizing ecDHFR, a developmental delay was noted in *Drosophila* ingesting TMP (López Del Amo et al., 2020) potentially due to alterations in commensal microbiota. This confound may be circumvented with non-antibiotic molecules capable of stabilizing ecDHFR DD that have been recently developed (Peng et al., 2019).

The temperature dependence of Gal4 has been reported by some (Duffy, 2002; Nagarkar-Jaiswal et al., 2015; Lee et al., 2018), but other reports have shown no temperature-dependent effects of Gal4 activity (Mondal et al., 2007; Riabinina et al., 2015). Our results demonstrated that the Gal4 we used (nSyb-Gal4, see Methods) was significantly lower in activity at reduced temperatures (Figures 3C, 4B), which is consistent with a previous observation of lower activity at 18°C with same nSyb-Gal4 line (Nagarkar-Jaiswal et al., 2015). The prevailing view of the temperature-dependence of Gal4 is not necessarily based on the activity of the Gal4 transcription factor at varying temperatures (Mondal et al., 2007), but instead on temperature-responsive regulatory elements present in transgenic constructs used to generate the Gal4 and UAS lines. Most importantly for our results was that lower temperature rearing allowed for a more complete repression of nSyb-Gal4 in the presence of stabilized Gal80-DD. This is potentially due to some Gal4 repression during stages in the life-cycle in which the fly is not consuming TMP containing food (embryonic and pupal stages), and/or lowering the expression level of Gal4 protein to a threshold level that is more completely repressed by the level of stabilized Gal80-DD protein expressed. There is also the possibility that metabolism of TMP is slower at lower temperatures, thus yielding a more stabilized Gal80-DD for repression, but we were unable to empirically test this.

In summary, we described an optimized protocol to utilize the Gal80-DD tool to induce adult-onset expression of *Vps13D* RNAi. Based on this result, we predict that this system will be compatible for use with knockdown of other essential genes that typically cause developmental lethality, and will permit the investigation of manipulating disease-genes precisely in adult stages of the fly CNS in the future. We suspect that this system will be of value to other researchers due to the availability of reagents, simplicity, and low cost. With this optimized tool, we have created a model to investigate the progressive neurological and locomotor defects associated with *Vps13D* perturbation in fly neurons, better modeling the clinical ataxia phenotype described in patients with mutations in VPS13D (Gauthier et al., 2018; Seong et al., 2018; Koh et al., 2020; Pauly et al., 2023).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

RI conceived the project and designed the experiments with input from LR and ER. ER, LR, and RI performed the experiments. RI wrote the manuscript. ER and LR edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by grants from the National Institutes of Health (NINDS) [R00NS111000 (to RI)]; (NEI) [P30EY004068 (NEI Core Grant)] and an unrestricted grant from Research to Prevent Blindness to the Department of Ophthalmology, Visual and Anatomical Sciences at WSU SOM.

Acknowledgments

Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. We thank Catherine Collins (Case Western Reserve University) for her assistance in supporting the research in this manuscript. We also thank Catherine Collins, and Sokol Todi (Wayne State University) for their useful feedback on the manuscript.

Conflict of interest

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

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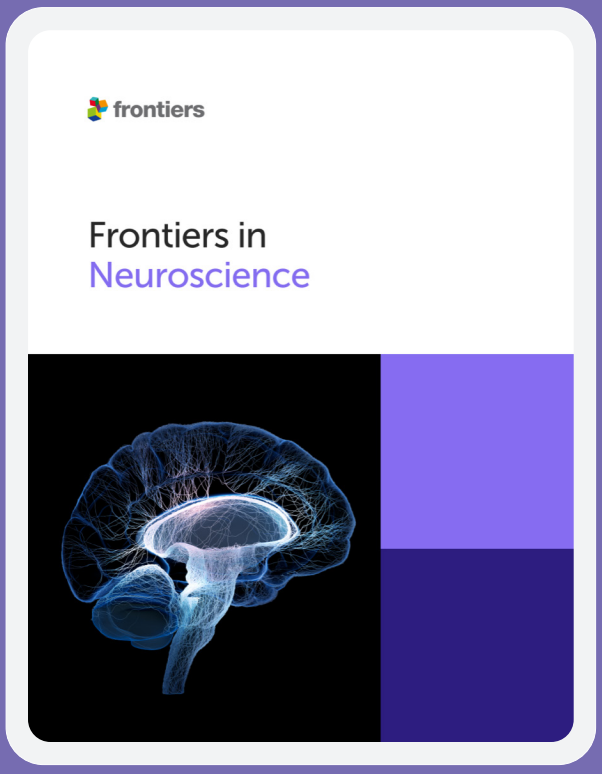

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