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Glial α-synuclein promotes neurodegeneration characterized by a distinct transcriptional program in vivo

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Abstract

α-Synucleinopathies are neurodegenerative diseases that are characterized pathologically by α-synuclein inclusions in neurons and glia. The pathologic contribution of glial α-synuclein in these diseases is not well understood. Glial α-synuclein may be of particular importance in multiple system atrophy (MSA), which is defined pathologically by glial cytoplasmic α-synuclein inclusions. We have previously described *Drosophila* models of neuronal α-synucleinopathy, which recapitulate key features of the human disorders. We have now expanded our model to express human α-synuclein in glia. We demonstrate that expression of α-synuclein in glia alone results in α-synuclein aggregation, death of dopaminergic neurons, impaired locomotor function, and autonomic dysfunction. Furthermore, co-expression of α-synuclein in both neurons and glia worsens these phenotypes as compared to expression of α-synuclein in neurons alone. We identify unique transcriptomic signatures induced by glial as opposed to neuronal α-synuclein. These results suggest that glial α-synuclein may contribute to the burden of pathology in the α-synucleinopathies through a cell type-specific transcriptional program. This new *Drosophila* model system enables further mechanistic studies dissecting the contribution of glial and neuronal α-synuclein in vivo, potentially shedding light on mechanisms of disease that are especially relevant in MSA but also the α-synucleinopathies more broadly.

Keywords

Drosophila; glia; multiple system atrophy; Parkinson's disease; α-Synuclein

1 | INTRODUCTION

The α-synucleinopathies are a family of neurodegenerative diseases characterized by pathologic accumulation of misfolded α-synuclein (Fujiwara et al., 2002; Uversky, 2008; Vilar et al., 2008). Postmortem studies demonstrate that α-synuclein inclusions can be found

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

in neurons and glia, to varying extents, in all of the α-synucleinopathies (Brück, Wenning, Stefanova, & Fellner, 2015). Specifically, in Parkinson's disease (PD) and dementia with Lewy bodies (DLB), inclusions are predominantly identified in neurons in the form of Lewy bodies and Lewy neurites (Baba et al., 1998; Beyer & Ariza, 2007) but also to a lesser extent in astrocytes (Braak, Sastre, & Del Tredici, 2007; Song et al., 2009; Wakabayashi, Hayashi, Yoshimoto, Kudo, & Takahashi, 2000), whereas in multiple system atrophy (MSA) inclusions are found in oligodendrocytes in the form of glial cytoplasmic inclusions, but also in neurons and astrocytes (Cykowski et al., 2015; Gai, Power, Blumbergs, & Blessing, 1998; Papp & Lantos, 1994). Despite these consistent pathologic observations, whether glial α-synuclein serves as a pathologic driving force or is merely a bystander in the development or progression of these diseases remains unclear.

Drosophila offers many advantages as a model system and has been used successfully to model multiple diseases with prominent or exclusive glial pathology, including gliomas (Kim et al., 2014; Read et al., 2013; Witte, Jeibmann, Klämbt, & Paulus, 2009), glial tauopathies (Colodner & Feany, 2010), Alexander disease (Wang, Colodner, & Feany, 2011), and complex I deficiency (Hegde, Vogel, & Feany, 2014). Similar to mammalian glia, *Drosophila* glia include multiple specialized cell types (Kremer, Jung, Batelli, Rubin, & Gaul, 2017) and are essential for neuronal development (Booth, Kinrade, & Hidalgo, 2000; Sepp, Schulte, & Auld, 2001) and maintenance (Xiong & Montell, 1995). In the adult nervous system, they serve many of the same specialized functions as mammalian glia, including phagocytic clearance of cellular debris (Doherty, Logan, Taşdemir, & Freeman, 2009; MacDonald et al., 2006), participation in innate immunity (Kounatidis & Chtarbanova, 2018), blood–brain barrier formation (DeSalvo et al., 2014), glutamate recycling (Farca Luna, Perier, & Seugnet, 2017; Rival et al., 2004), protection of axons in white matter (Logan et al., 2012), and protection of neurons from reactive oxygen species through lipid droplet formation (L. Liu, MacKenzie, Putluri, Maletić -Savatić, & Bellen, 2017; L. Liu et al., 2015).

The Feany laboratory has previously published *Drosophila* models of neuronal α-synucleinopathy (Feany & Bender, 2000; Ordonez, Lee, & Feany, 2018). These flies recapitulate many features of human α-synucleinopathies, including progressive locomotor impairment, neurodegeneration (including death of dopaminergic neurons), and accumulation of α-synuclein inclusions. Here we expand on this model to investigate the pathologic contribution of glial α-synuclein. We make use of two bipartite expression systems, the UAS–GAL4 system (Brand & Perrimon, 1993) and the Q system (C. J. Potter, Tasic, Russler, Liang, & Luo, 2010) to independently express human α-synuclein in glia or neurons using the pan-glial driver *repo-GAL4* or the pan-neuronal driver *Syb-QF2*, respectively. We determine that glial α-synuclein forms inclusions, impairs locomotion, causes autonomic dysfunction, and induces death of dopaminergic neurons. Additionally, flies expressing α-synuclein in both neurons and glia develop more α-synuclein inclusions in neurons than those expressing α-synuclein in neurons alone. Finally, we identify unique transcriptional programs induced by glial and neuronal α-synuclein, suggesting that the cellular context of α-synuclein matters for gene expression. Importantly, many of the differentially expressed genes we identify in *Drosophila* have orthologs that have been recognized as causally important in mammalian models of MSA or in patients, supporting

the applicability of this model for uncovering human disease mechanisms. Our work represents a novel model system for studying glial α-synucleinopathy and uncovering glial-based therapeutic targets.

2 | METHODS

2.1 | Drosophila

All fly crosses and aging were performed at 25°C. All experiments include flies in which wild type human α-synuclein is expressed in either neurons or glia using the pan-neuronal driver *synaptobrevin* (*Syb*) or the pan-glial driver *reversed polarity* (*repo*), respectively. Control flies include the drivers but lack transgenic human α-synuclein. The exact genotypes for all experiments are as follows: (a) Control = *Syb-QF2*, *repo-GAL4/+*, (b) Glia = *Syb-QF2*, *repo-GAL4/UAS-α-synuclein*, (c) Neurons = *Syb-QF2*, *repo-GAL4*, *QUAS-α-synuclein/+*, and (d) Both = *Syb-QF2*, *repo-GAL4*, *QUAS-α-synuclein/UAS-α-synuclein*. All experiments were performed at 10 days post-eclosion unless otherwise noted in the figure legends.

2.2 | Immunohistochemistry and immunofluorescence

Flies were fixed in formalin and embedded in paraffin. Either 2 or 4 μm serial frontal sections were prepared through the entire fly brain. Slides were processed through xylene, ethanols, and into water. For neuron counts, slides were stained with hematoxylin. For immunohistochemistry, microwave antigen retrieval with 10 mM sodium citrate, pH 6.0, was performed. Slides were blocked in 2% milk in PBS with 0.3% Triton X-100 for 1 hr then incubated with appropriate primary antibody in 2% milk in PBS with 0.3% Triton X-100 at room temperature overnight. Primary antibodies used include repo (1:5, mouse, Developmental Studies Hybridoma Bank, Iowa City, IA), elav (1:5, mouse, Developmental Studies Hybridoma Bank), tyrosine hydroxylase (1:200 to 1:500, mouse, Immunostar, Hudson, WI), α-synuclein hSA-2 (1:1000, rabbit, provided as a kind gift from Dr. Michael Schlossmacher, Boston, MA), and α-synuclein (1:1,000 to 1:10,000, rat, provided as a kind gift from Biolegend, San Diego, CA). α-Synuclein hSA-2 recognizes both monomeric and oligomeric α-synuclein by immunoblotting as well as α-synuclein aggregates by immunostaining. The α-Synuclein antibody from Biolegend was raised against aggregated α-synuclein and recognizes aggregates and total α-synuclein by immunostaining. For immunohistochemistry, slides were incubated in biotin-conjugated secondary antibodies in 2% milk in PBS with 0.3% Triton X-100 for 1 hr (1:200, Southern Biotech, Birmingham, AL) followed by avidin–biotin–peroxidase complex (Vectastain Elite) in PBS for 1 hr. Histochemical detection was performed with diaminobenzidine (ImmPACT DAB, Vector, Torrance, CA). For immunofluorescence, slides were incubated with fluorophore-conjugated secondary antibodies in 2% milk in PBS with 0.3% Triton X-100 for 1 hr (1:200, Alexa 488 or Alexa 555, Invitrogen, Waltham, MA) then mounted with DAPI-containing Fluoromount medium (Southern Biotech). Immunofluorescence microscopy was performed on an Olympus FV1000 confocal microscope through the Harvard Neurobiology Imaging Facility or on a Zeiss LSM 800 confocal microscope. Images were processed using Fiji.

2.3 | Western blotting

Fly heads were homogenized in 2× Laemmli buffer, boiled for 10 min, and centrifuged. SDS-PAGE was performed (Lonza, Basel, Switzerland) followed by transfer to nitrocellulose membrane (Bio-Rad, Hercules, CA) and microwave antigen retrieval in PBS. Membranes were blocked in 2% milk in PBS with 0.05% Tween-20 for 1 hr, then immunoblotted with appropriate primary antibody in 2% milk in PBS with 0.05% Tween-20 overnight at 4°C. Primary antibodies used include α-synuclein H3C (1:10,000 to 1:100,000, mouse, Developmental Studies Hybridoma Bank), GAPDH (mouse, 1:25,000 to 1:100,000, Invitrogen), GFP N86/8 (mouse, 1:100, Neuromab, Davis, CA). Membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:50,000) in 2% milk in PBS with 0.05% Tween-20 for 3 hr. Signal was developed with enhanced chemiluminescence (Thermo Scientific, Waltham, CA). Anti-GAPDH was used to demonstrate equivalent protein loading.

2.4 | Locomotion assay

Adult flies were aged in vials containing 9–14 flies per vial. At days 1, 4, 7, 10, 13, 16, and 21 of life, flies were transferred to a clean vial (without food) and given 1 min to acclimate to the new vial. The vial was then gently tapped three times to trigger the startle-induced locomotion response, then placed on its side for 15 s. The percentage of flies still in motion was then recorded. Differences between genotypes at specific time-points were measured and statistical significance assessed by two-way ANOVA. The global difference between genotypes was assessed by using linear regression to fit a linear curve to the data and to determine whether the slopes were statistically different (Prism GraphPad, San Diego, CA).

2.5 | Constipation assay

Standard cornmeal-agar *Drosophila* medium was melted by microwaving briefly and then mixed with blue food coloring (AmeriColor, Placentia, CA) at a ratio of approximately 1:10 volumes to create uniformly dark blue food. The same batch of food was used for experimental and control groups. Adult flies were aged to 10 days, then transferred to vials with blue food for 24 hr. Flies were then transferred back to standard food, and the percent of blue excrement to total excrement was measured on an hourly basis for 8 hr. Statistical significance was determined by one-way ANOVA of the area under the curve for each genotype. Additionally, flies were photographed at 0, 2, and 4-hr timepoints to demonstrate delayed gut transit of the blue food.

2.6 | Statistics

All statistical analysis aside from that used for RNAseq data analysis was performed using GraphPad Prism version 7.0a. In cases of multiple comparisons, Tukey's multiple comparisons test was used to determine statistical significance.

2.7 | RNA-Seq

Adult flies were aged to 10 days. Four biological replicates per genotype, each consisting of six fly heads (three male, three female), were used. Fly heads were homogenized in Qiazol (Qiagen, Germantown, MD) and phase separated with chloroform. The aqueous phase was

mixed with 100% ethanol at 1:1 ratio then purified on RNeasy columns (Qiagen) per the manufacturer's protocol. Stranded libraries for next-generation sequencing were prepared in the Harvard Biopolymers core facility by depleting total RNA of ribosomal RNA using the Directional RNA-Seq Wafergen system (Wafergen, Fremont, CA). All RNA samples were run on Agilent 2100 TapeStation D1000 High Sensitivity ScreenTape to assess concentration and size distribution prior to library creation and again after library creation. Libraries were also subjected to qPCR analysis for quality control. Libraries were then paired-end sequenced on an Illumina NextSeq 500 instrument.

2.8 | RNA-Seq data analysis

Computational analysis was performed on the Harvard Medical School O2 High-Performance Research Computing Cluster. The four raw sequence (.fastq) files generated by the NextSeq were concatenated for each library, then analyzed with FastQC (Babraham Bioinformatics, Cambridgeshire, UK). Count matrices were generated in R Studio Version 1.1.423 using the Bioconductor (Huber et al., 2015) package called bcbioRNAseq, an open source python framework that aggregates other best-practice pipelines for RNA-Seq analysis developed by the Harvard Chan Bioinformatics Core in the Harvard Chan School of Public Health (Steinbaugh et al., 2018). Within the bcbioRNAseq package, STAR (Dobin et al., 2013) was used to align the sequence reads to the *Drosophila melanogaster* Release 6 reference genome (BDGP6), and Salmon (Patro, Duggal, Love, Irizarry, & Kingsford, 2017) and featureCounts (Liao, Smyth, & Shi, 2014) were used to generate counts associated with known genes. Gene annotations were obtained from Ensembl. Quality of the RNA-Seq data was assessed with MultiQC (Ewels, Magnusson, Lundin, & Käller, 2016). This quality control includes total reads, mapping rate, genes detected, gene saturation, counts per gene, gene count distributions, principal component analysis (Jolliffe, 2002), and sample similarity. Plots were generated by ggplot2 (Wickham, 2016) and heatmaps were generated by pheatmap (Kolde, 2015). Principle component analysis demonstrated the strongest clustering by genotype (accounting for 43% of the variance). Based on the clustering analysis, one sample each from the Control, Glia, and Neuron conditions was excluded from further analysis, leaving a minimum of three remaining biological replicates per genotype. Transcript quantification files produced by Salmon were imported into the DESeq2 package (Love, Huber, & Anders, 2014) and pair-wise differential expression across conditions was analyzed using a generalized linear model implemented in DESeq2. A pseudo-count of 1 was added to all genes with an expression count of 0. Expression counts for all mapped genes are included in Data File S3. Differentially expressed genes had a fold-change between conditions of ≥2 and FDR <0.05. Gene ontology analysis (release August 9, 2018) (Ashburner et al., 2000; Mi et al., 2017; The Gene Ontology Consortium, 2017) and PANTHER classification (version 13.1; Mi, Muruganujan, & Thomas, 2013; Thomas et al., 2003) was performed to identify enriched terms among differentially expressed genes and annotate genes. Mammalian orthologs for *Drosophila* genes were determined using the Drosophila RNAi Screening Center (DRSC) Integrative Ortholog Prediction Tool (DIOPT) version 7.1 (Hu et al., 2011).

2.9 | qRT-PCR

Selected genes identified as differentially expressed in the RNA-Seq analysis were validated by qRT-PCR. Primers for these genes were chosen from the DRSC FlyPrimerBank (Hu et al., 2013). Twenty brains per genotype were dissected in PBS and pooled. Total RNA was prepared with Qiazol (Qiagen) per the manufacturer's instructions then treated with DNase for 15 min. cDNA was prepared using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), then amplified with SYBR green on a StepOne Plus machine (Applied Biosystems). Relative expression was determined using the ΔΔCt method, with normalization to *RPL32* used as a housekeeping gene.

2.10 | Single cell transcriptome atlas

Selected genes identified as differentially expressed in the RNA-Seq analysis were mapped to a recently published *Drosophila* single-cell transcription atlas (Davie et al., 2018) using a publicly available tool: scope.aertslab.org. Marker lists for glial subpopulations were downloaded from this tool and used to annotate gene lists.

2.11 | Data availability

Raw and final RNA-Seq data is available through Gene Expression Omnibus (GEO), accession number GSE128120. All other data that support the findings of this study are available from the corresponding author upon reasonable request (Olsen et al., 2019).

3 | RESULTS

3.1 | Independent expression of α-synuclein in neurons and glia

We have recently published a *Drosophila* model of neuronal α-synucleinopathy in which wild-type human α-synuclein is expressed under the control of a pan-neuronal driver, *Syb-QF2*, using the Q binary expression system (C. J. Potter et al., 2010; Riabinina et al., 2015). These flies have widespread neurodegeneration, α-synuclein aggregation, death of dopaminergic neurons, and impaired motor function (Ordonez et al., 2018). To examine the effects of glial α-synuclein expression, we employed the similar GAL4 binary expression system (Brand & Perrimon, 1993) to drive expression of wild type human α-synuclein under the pan-glial driver, *repo-GAL4* (Sepp et al., 2001). These systems are independent of one another, allowing us to express human α-synuclein in neurons (elav positive cells), glia (repo positive cells), or both cell types (Figure S1a,c,d). Of note, when examined at the whole brain protein level by immunoblotting, expression of α-synuclein is not appreciably higher when expressed in neurons and glia as opposed to neurons alone (Figure S1b), which may reflect strong expression driven by *Syb-QF2*.

3.2 | Glial α-synuclein impairs locomotion

Motor symptoms are the defining clinical feature of parkinsonism, and motor dysfunction has been previously demonstrated in *Drosophila* models of Parkinson's disease in the form of impaired climbing (Feany & Bender, 2000; Ordonez et al., 2018), walking (Chen, Wilburn, Hao, & Tully, 2014; Pokrzywa et al., 2017), and proboscis extension (Cording et al., 2017). Using our model of glial α-synucleinopathy, we compared locomotor behavior in

control flies to flies expressing α-synuclein in glia, neurons, or both cell types. Specifically, we developed a novel walking-based locomotion assay. Briefly, flies are transferred to clean, empty vials, allowed to acclimate for 1 min, and then gently tapped three times to evoke the startle-induced locomotion response (Liao, Morin, & Ahmad, 2014; Ma, Stork, Bergles, & Freeman, 2016; Riemensperger et al., 2013; Yamamoto et al., 2008). The percentage of flies still walking after a 15-s delay is recorded, and differences between control and neuronal α-synuclein flies are highly reproducible over time (Figure S2a) and correlate with our previously published climbing assay (Figure S2b).

Using this locomotion assay, we performed a 21-day time course and determined that glial α-synuclein alone causes a locomotor deficit, and it also exacerbates the effect of neuronal α-synuclein (Figure 1). To ensure that this effect is specific to glial α-synuclein and not simply due to overexpression of an exogenous protein in glia, we repeated this experiment at Day 10, expressing green fluorescent protein (GFP) rather than α-synuclein in glia. GFP expression had no effect on locomotion (Figure S3). We then went further, expressing the R79H mutant of glial fibrillary acidic protein (GFAP), which causes the human astrogliopathy Alexander disease. We have previously shown that R79H GFAP expression in *Drosophila* glia causes noncell-autonomous toxicity to glutamatergic and other neurons in an Alexander disease model (L. Wang et al., 2011), but at the 10-day timepoint examined glial R79H GFAP expression did not enhance neuronal α-synuclein toxicity as measured by the locomotion assay (Figure S3), demonstrating specificity of glial α-synuclein in exacerbating the neuronal α-synuclein phenotype.

3.3 | Glial α-synuclein causes constipation

Autonomic nervous system dysfunction is common in all of the α-synucleinopathies, and constipation, in particular, may predate the onset of motor symptoms by many years (Adams-Carr et al., 2016). Nonmotor symptoms, including constipation, are a significant clinical problem, being more highly correlated with impaired patient quality of life than are motor symptoms (Estrada-Bellmann, Camara-Lemarroy, Calderon-Hernandez, Rocha-Anaya, & Villareal-Velazquez, 2016; Müller, Assmus, Herlofson, Larsen, & Tysnes, 2013; Prakash, Nadkarni, Lye, Yong, & Tan, 2016). The innervation of the gut in *Drosophila* is similar to that in mammals in that it is complex, involving efferent and afferent neurons, with contribution from both the central and peripheral nervous system (Cognigni, Bailey, & Miguel-Aliaga, 2011). We assessed whether glial α-synuclein contributes to constipation in *Drosophila*. In these experiments, 10-day-old files were housed in vials with blue food for 24 hr, then transferred to vials with regular food. Photographs of individual representative flies were taken at time 0, 2, and 4 hr (Figure 2a) demonstrating delayed gut transit for α-synuclein expressing flies compared to control, and markedly delayed gut transit for flies expressing α-synuclein in both neurons and glia. Additionally, the ratio of blue excrement to total excrement was measured per hour up to 8 hr (Figure 2b), also demonstrating the same phenomenon at a population level. That is, glial α-synuclein alone induces constipation to a similar extent as neuronal α-synuclein, and it exacerbates that induced by neuronal α-synuclein.

3.4 | Glial α-synuclein causes death of total and dopaminergic neurons, but not glial cells

Neuronal death in varying cortical and subcortical regions occurs to differing extents in all of the α-synucleinopathies, and death of dopaminergic neurons in the substantia nigra pars compacta is a defining pathologic feature of Parkinson's disease. We therefore sought to determine whether glial α-synuclein contributes to neuronal death generally and specifically to death of dopaminergic neurons. We first examined vacuolization, which is seen in patients with dementia with Lewy bodies (Sherzai et al., 2013) and is a common consequence of neurodegeneration in *Drosophila* (Kretzschmar, 2009; Sunderhaus & Kretzschmar, 2016; Wittmann et al., 2001). Glial α-synuclein caused infrequent, large vacuoles, whereas neuronal α-synuclein led to more numerous smaller vacuoles (Figure 3a). Glial α-synuclein alone induced neuron loss (quantified in Figure 3c) and exacerbated the loss of neurons when added to neuronal α-synuclein. More strikingly, glial α-synuclein alone caused loss of dopaminergic neurons and dopaminergic neurons were markedly reduced when both glial and neuronal were present (Figure 3b,d). Interestingly, the degree of loss of dopaminergic neurons due to glial α-synuclein was out of proportion to the total neuron loss (compare degree of change between Glia and Control or Both and Neuron in Figure 3c,d), consistent with differential vulnerability of dopaminergic neurons to glial α-synuclein. In contrast, quantitative examination of repo-stained sections did not reveal a clear difference in the number of glial cells between conditions (Figure S4), suggesting that there is no marked loss of this population but rather that glial dysfunction is responsible for the pathogenic effects of glial α-synuclein.

3.5 | α-Synuclein aggregates in both neurons and glia

α-Synucleinopathies are, by definition, diseases of pathologic α-synuclein aggregation, which occurs to varying extents in neurons and glia depending on the specific disease. We identified α-synuclein aggregates in both neurons and glia in the conditions in which it was expressed in those cell types. Figure 4a demonstrates α-synuclein aggregates in neurons (identified by the marker elav), and Figure 4b demonstrates α-synuclein aggregates in glia (identified by the marker repo). Total α-synuclein aggregates were quantified from low power sections of cortex surrounding the optic lobe (Figure 4c). Interestingly, α-synuclein inclusions in dopaminergic neurons were increased when α-synuclein was present in both neurons and glia as opposed to in neurons alone (Figure 4d), suggesting that the presence of glial α-synuclein is able to perpetuate further α-synuclein aggregation in dopaminergic neurons in a noncell-autonomous manner. Such noncell-autonomous effects have been seen previously in a mouse model of MSA, in which overexpression of human α-synuclein in oligodendrocytes was shown to induce aggregation of endogenous mouse α-synuclein in neurons (Yazawa et al., 2005).

3.6 | α-Synuclein induced transcriptional changes depend on cellular context

Having demonstrated that glial α-synuclein enhances both the clinical phenotype and pathologic hallmarks of the α-synucleinopathies, we next sought to identify whether it altered gene expression. We expressed human wild type α-synuclein in glia, neurons, or both cell types and performed RNA-Seq on whole heads. Differentially expressed genes were identified by pair-wise comparisons of each α-synuclein condition compared to

negative control, hereafter referred to as Glia: Control, Neuron:Control, or Both:Control. Interestingly, the effects of α-synuclein on transcription were markedly different depending on whether the protein was expressed in glia or neurons. Glial α-synuclein resulted in 158 differentially expressed genes compared to control flies lacking α-synuclein (Figure 5a, Data File S1). Nearly all the differentially expressed genes were upregulated (144/157, 92%). In contrast, neuronal α-synuclein resulted in 128 differentially expressed genes compared to control flies, and nearly all of these were downregulated (125/128, 98%; Figure 5A, Data File S1). Furthermore, there is very little overlap between the differentially expressed genes induced by glial versus neuronal α-synuclein (Figure 5b), suggesting that the cellular context of α-synuclein expression matters significantly for gene expression. When α-synuclein was expressed in both neurons and glia, 359 transcripts were differentially expressed (Figure 5a, Data File S1). The majority of these were downregulated (350/358 = 98%) and they include the vast majority of transcripts that were downregulated with neuronal α-synuclein alone, as well as many additional transcripts (Figure 5c). Of the overlapping transcripts that were downregulated with neuronal α-synuclein alone as well as when α-synuclein was present in both neurons and glia, they were downregulated to a greater degree with both glial and neuronal α-synuclein (Figure 5d).

We performed gene ontology analysis for all conditions (see Data File S2 for full gene ontology results). With glial α-synuclein, enriched biological process terms included proteolysis and cell surface receptor signaling (Figure 6a). The proteolysis term is enriched due to expression of many extracellular proteases as well as two caspases (Table 1, Figure 6b). Many of these proteases were not previously known to be expressed in the brain, and their upregulation may reflect a glial response to injury (Purice et al., 2017) or alternatively might represent the senescence-associated secretory phenotype, which may contribute to neurodegeneration (Bussian et al., 2018). The cell surface receptor signaling term is enriched partly due to expression of several tetraspanins (Table 2, Figure 6b), which are of particular interest given that their human orthologs (CD9, CD81, and TSPAN2) are markers of oligodendrocytes or oligodendrocyte precursors (Terada et al., 2002). We confirmed expression of a subset of both the proteases and cell signaling receptor transcripts by qRT-PCR (Figure S5a). Since differentially expressed transcripts could be either neuronal or glial in origin, and we are particularly interested in glial changes, we used a newly created single-cell *Drosophila* brain transcriptome atlas (Davie et al., 2018) to annotate transcripts that were identified in that study as markers of various glial subpopulations (Table 3). In the Glia:Control comparison, we identified 10 upregulated glial markers. We further investigated one of these, *Ance*, which is also one of the proteolysis-related genes (Figure 6b) and is of particular interest as it is the *Drosophila* ortholog of angiotensin-converting enzyme (ACE), and ACE inhibitors have been explored as possible therapeutics in Parkinson's disease (Reardon, Mendelsohn, Chai, & Horne, 2000; Sonsalla et al., 2013). *Ance* expression is enriched in (but not limited to) subperineurial glia, as shown in silico using the single cell transcriptome atlas (Figure S5b). Additionally, we used Ance-GAL4 to drive GFP expression to assess further the pattern of *Ance* expression. These flies demonstrated GFP expression in the head (Figure S5c), and we confirmed a glial expression pattern by immunohistochemistry (Figure S5d).

In contrast to glial α-synuclein, neuronal α-synuclein led to downregulation of many transcripts (Figures 5a and 7a). Gene ontology on these transcripts revealed many terms regulated to mating and hormones (Figure 7a), which is explained due to downregulation of several members of four families of genes: Accessory gland protein (Acp), seminal fluid protein (Sfp), serpin (Spn), and odorant binding protein (Obp) families (Data File S1). Although the Acp and Sfp protein families are named for their expression in the male accessory gland and seminal fluid, respectively, they along with the Spn superfamily are composed mostly of protease inhibitors, raising the possibility of their being repurposed in the brain for protein homeostasis. Indeed, after the mating-related terms, the next enriched term by gene ontology analysis was negative regulation of endopeptidase activity (Figure 7a). We validated expression of several of these genes in the brain either by qRT-PCR (Figure 7b) or in silico analysis using the single cell transcriptome atlas (Figure 7c), where importantly, there were no sex-specific differences in their expression (Figure 7c). The fourth family contributing to the enrichment of mating-related terms is the Obp family. Obp proteins transport odorants to olfactory receptors. This is of some interest given the known olfactory deficits in the α-synucleinopathies, with PD pathology thought to start early in the olfactory bulbs (Del Tredici & Braak, 2016). Following mating-related terms and negative regulation of endopeptidase activity, the third biological process that was over-represented involved genes regulated to lipid metabolism (Table 4, Figure 7d). This finding is consistent with prior studies by our group (Scherzer, Jensen, Gullans, & Feany, 2003) and others (Don et al., 2014; Schafferer et al., 2016) that also identified changes in lipid metabolism related genes due to α-synuclein.

As mentioned above, in the Both:Control comparison, the list of differentially expressed genes contains nearly all of the genes found Control in the Neuron:Control comparison (Figure 5c), and similarly, gene ontology analysis reveals several enriched terms that are mating-related (Data File S2). Beyond these shared genes and terms, however, there are an additional 242 differentially expressed genes in the Both:Control comparison that are not seen in the Neuron:Control comparison (Figure 5c). Additionally, gene ontology analysis in the Both:Control group reveals numerous additional enriched terms related to fatty acid metabolism (Data File S2). Among the fatty acid metabolism-related genes, there are 6 fatty acyl-CoA reductases, 5 fatty acid elongases, 9 genes known to localize to the peroxisome, and several additional enzymes with oxidoreductase activity (Table 5). Of note, there are two orthologs of stearoyl CoA desaturase (SCD), recently implicated as a therapeutic target for PD (Fanning et al., 2018; Vincent et al., 2018). The majority (though not all) of these fatty acid metabolism genes are downregulated, and they are also downregulated in the Neuron:Control comparison (Figure 8a), although only 10/29 reached statistical significance in that condition. In addition to the many terms related to fatty acid metabolism, gene ontology analysis also revealed other enriched terms, including myofibril assembly, muscle α-actinin binding, and sperm flagellum. The component transcripts responsible for these terms being enriched are cytoskeletal genes (Figure 8b, Table 6), which is of interest given our work (Ordonez et al., 2018) as well as the work of others (Chung et al., 2017; Esposito, Dohm, Kermer, Bähr, & Wouters, 2007; Khurana et al., 2017; Sousa et al., 2009) implicating dysfunction of the actin cytoskeleton in α-synuclein neurotoxicity. Similar to the fatty acid metabolism-related genes, the cytoskeletal genes were also downregulated in

Neuron:Control (Figure 8b), though none reached statistical significance in that condition. Collectively, these data suggest that glial α-synuclein both potentiates the transcriptional effects of neuronal α-synuclein and also induces unique transcriptional changes.

3.7 | Relevance of the *Drosophila* model for mammalian α-synucleinopathy models and human disease

Drosophila serves as a powerful model organism for investigating human neurodegeneration in large part due to the high conservation of disease-related genes (McGurk, Berson, & Bonini, 2015; Rubin et al., 2000). To explore the relevance of our differentially expressed genes, we identified their rat, mouse, and human orthologs and cross-referenced this list with additional publicly available lists of differentially expressed genes identified in transcriptomic studies of MSA animal models (Kaji et al., 2018; Schafferer et al., 2016) or human post-mortem MSA brains (Langerveld, Mihalko, DeLong, Walburn, & Ide, 2007; Mills, Ward, Kim, Halliday, & Janitz, 2016). We also compared the ortholog list to genes that have been identified as candidate risk genes for any human α-synucleinopathy by examining genome-wide association or whole exome sequencing studies from MSA (X. Gu et al., 2018; Sailer et al., 2016), PD (Chang et al., 2017; Guo et al., 2018; Jansen et al., 2017; Li et al., 2018; Quadri et al., 2015; Robak et al., 2017; Sandor et al., 2017; Schormair et al., 2018; Shulskaya et al., 2018; Siitonen et al., 2017; Ylönen et al., 2017), or DLB (Guerreiro et al., 2018; Keogh et al., 2016; Peuralinna et al., 2015). In total, we found 30 transcripts with a mammalian ortholog that had also been identified in one or more of these studies (Table 7). This list includes orthologs that fell into nine pathways that have been implicated in pathogenesis of human α-synucleinopathies (Figure 9 and Discussion), suggesting that our model can be used to study relevant aspects of human disease pathophysiology.

4 | DISCUSSION

Here we describe a novel *Drosophila* model of glial α-synucleinopathy. We demonstrate that glial α-synuclein forms inclusions, increases aggregation of α-synuclein within dopaminergic neurons, impairs locomotion, causes constipation, and triggers neurodegeneration of both dopaminergic and nondopaminergic neurons. Furthermore, we demonstrate that α-synuclein can induce unique transcriptional programs depending on the cell type in which it is expressed. One striking finding from our RNA-Seq results was how different the transcriptional signatures are between the three conditions, including not only the specific genes that were differentially expressed but also the fact that glial α-synuclein alone led to upregulation of many genes, whereas many genes were downregulated in the other conditions. In fact, only three transcripts were upregulated in the Neuron:Control condition and two of these are known glial markers (Table 3). We have previously reported that nuclear α-synuclein inhibits histone acetylation (Kontopoulos, Parvin, & Feany, 2006), and this inhibition would be expected to decrease transcription. Others have reported direct interactions between α-synuclein and histones (Goers et al., 2003) or DNA (Siddiqui et al., 2012), as well as wide-ranging transcriptional deregulation (Pinho et al., 2019). α-Synuclein-induced transcriptional changes to remain a ripe area for future study, particularly as single cell RNA-Seq becomes more accessible.

Among the differentially expressed genes that we identified across all conditions, we found 30 transcripts that have been reported either as having altered expression in mammalian models of MSA or human patients with MSA or reported as being genetic risk factors for MSA or PD (Table 7). These transcripts are involved in pathways known to be essential for α-synucleinopathy pathogenesis, including mitochondrial function, lysosomal function, myelin synthesis, cytoskeletal function, fatty acid metabolism, apoptosis, and adenosine metabolism (Figure 9). There are six genes meeting the highest level of evidence for a causal role in human disease, having been identified in human GWAS or by WES: *CTSD*, *ELOVL7*, *NPC1*, *SMPD1*, *PTPRH*, and *PDLIM2* (Figure 9). Additionally, there is direct mechanistic evidence in animal models to support a causative role for several genes in α-synucleinopathy pathogenesis. Genes with animal model evidence are bolded in Table 7 and include *CAT*, *HSPA8*, *CTSD*, *Itgb1*, *Casp3*, *Lpl*, and *PTPRH*. Furthermore, while there is not yet direct evidence for *ELOVL7* in α-synucleinopathy pathogenesis, loss of function mutants in yeast orthologs of *ELOVL7* have been shown to exacerbate α-synuclein toxicity (Lee et al., 2011). Beyond these 30 transcripts, we identified an additional novel 298 differentially expressed genes with human orthologs in the Glia:Control or Both:Control conditions, leaving many candidates for future studies.

In addition to being one of very few published transcriptomic studies of glial α-synuclein expression, our model reproduces important α-synuclein induced phenotypes. Autonomic dysfunction is a core clinical feature underlying all of the α-synucleinopathies and includes bladder dysfunction, constipation, cardiovascular abnormalities, sexual dysfunction, sialorrhea, dry eyes, excessive sweating, and altered thermoregulation. These symptoms are a greater contributor to loss of quality of life than are the motor symptoms in PD (Estrada-Bellmann et al., 2016; Martinez-Martin, Rodriguez-Blazquez, Kurtis, Chaudhuri,, & NMSS Validation Group, 2011; Müller et al., 2013; Prakash et al., 2016; Tibar et al., 2018), yet have been challenging to investigate in model organisms. Although common to all of the α-synucleinopathies, autonomic dysfunction is particularly important in MSA (Fanciulli & Wenning, 2015), where it is required to make the diagnosis (Gilman et al., 2008). Only one mouse model of MSA has been reported to have autonomic features (Boudes et al., 2013), and our *Drosophila* model represents a valuable new model further investigating autonomic dysfunction in vivo.

Another interesting pathologic finding in our model is that dopaminergic neurons were lost at a disproportionate rate to total neurons when glial α-synuclein was present, either alone or in conjunction with neuronal α-synuclein. There are several hypotheses as to why dopaminergic neurons are uniquely susceptible to injury in the α-synucleinopathies (Surmeier, Obeso, & Halliday, 2017), including their long, thin, unmyelinated axons with extensive arborization (Matsuda et al., 2009), reliance on calcium homeostasis and susceptibility to oxidative stress (Duda, Pötschke, & Liss, 2016; Tabata et al., 2018), and the inherent toxicity of dopamine (Burbulla et al., 2017; Mor et al., 2017). Additionally, dopaminergic neurons are highly reliant on support from astrocytes (Datta, Ganapathy, Razdan, & Bhonde, 2018; Du, Yu, Chen, Chen, & Yan, 2018; Kuter, Olech, Głowacka, & Paleczna, 2019). However, neither the intrinsic characteristics of dopaminergic neurons nor the general phenomenon of astrocyte dysfunction fully explains why dopaminergic neurons degenerate specifically in α-synucleinopathies, as these features are also present in other

neurodegenerative diseases that do not specifically affect dopaminergic neurons. This raises the possibility of noncell-autonomous toxic effects that are due specifically to glial α-synuclein, as has recently been shown with astrocyte LRRK2 G2019S (di Domenico et al., 2019). In accordance with this, we demonstrate that glial α-synuclein increases aggregation of α-synuclein in dopaminergic neurons. This finding could be explained by a noncell-autonomous effect of glial α-synuclein on neuronal proteostasis or alternatively, by direct spread of α-synuclein from glia to dopaminergic neurons. We have not observed spread of α-synuclein from glia to neurons when it is expressed in glia alone (data not shown). However, we cannot exclude the possibility of spread in the condition in which α-synuclein is expressed in both neurons and glia, as it is possible that neurons must first express α-synuclein themselves in order to be receptive to spread from glia.

In contrast to the marked loss of dopaminergic neurons, glial numbers were not significantly changed between conditions (Figure S4). Prior mammalian studies of glial α-synuclein expression have reported varied results in terms of whether glial α-synuclein expression induces glial cell death. For example, α-synuclein expression in astrocyte cell lines induces apoptosis (M. Liu et al., 2018; N. Stefanova, Klimaschewski, Poewe, Wenning, & Reindl, 2001), whereas a transgenic astrocytic A53T α-synuclein expressing mouse model demonstrated not loss of astrocytes but rather impaired astrocyte function including decreased glutamate transporter expression and blood–brain barrier disruption, leading to marked neurodegeneration (X.-L. Gu et al., 2010). Likewise, of the three transgenic mouse models of oligodendrocyte α-synuclein expression, only one demonstrates loss of oligodendrocytes (Yazawa et al., 2005). The others display mitochondrial abnormalities in oligodendrocytes without frank oligodendrocyte loss (Shults et al., 2005), or absence of oligodendrocyte loss (Kahle et al., 2002) unless the mice are additionally treated with the mitochondrial toxin 3-nitroprinoic acid (Nadia Stefanova et al., 2005). These varied effects of glial α-synuclein expression on glial cell death may stem from different expression levels and reflect the complexity of modeling glial α-synucleinopathy (Bleasel, Halliday, & Kim, 2016).

In summary, our work represents the first report of independent manipulation of gene expression in neurons and glia by combining the Q and GAL4 bipartite expression systems. This is a robust and flexible model that can be used to mechanistically analyze the genetic contributions of neurons and glia to neurodegenerative diseases in vivo at scale. Here we have used these genetic tools to create the first *Drosophila* model of glial α-synucleinopathy, demonstrating in the process that glial α-synuclein induces neurodegeneration and that cell context is critical for α-synuclein induced transcriptional changes. We further demonstrate that this system can be used to identify processes of known relevance to human diseases, including MSA. Beyond investigating the effects of glial α-synuclein, combining the Q and GAL4 expression systems represents a powerful method for dissecting glial and neuronal interactions in vivo in neurodegenerative diseases broadly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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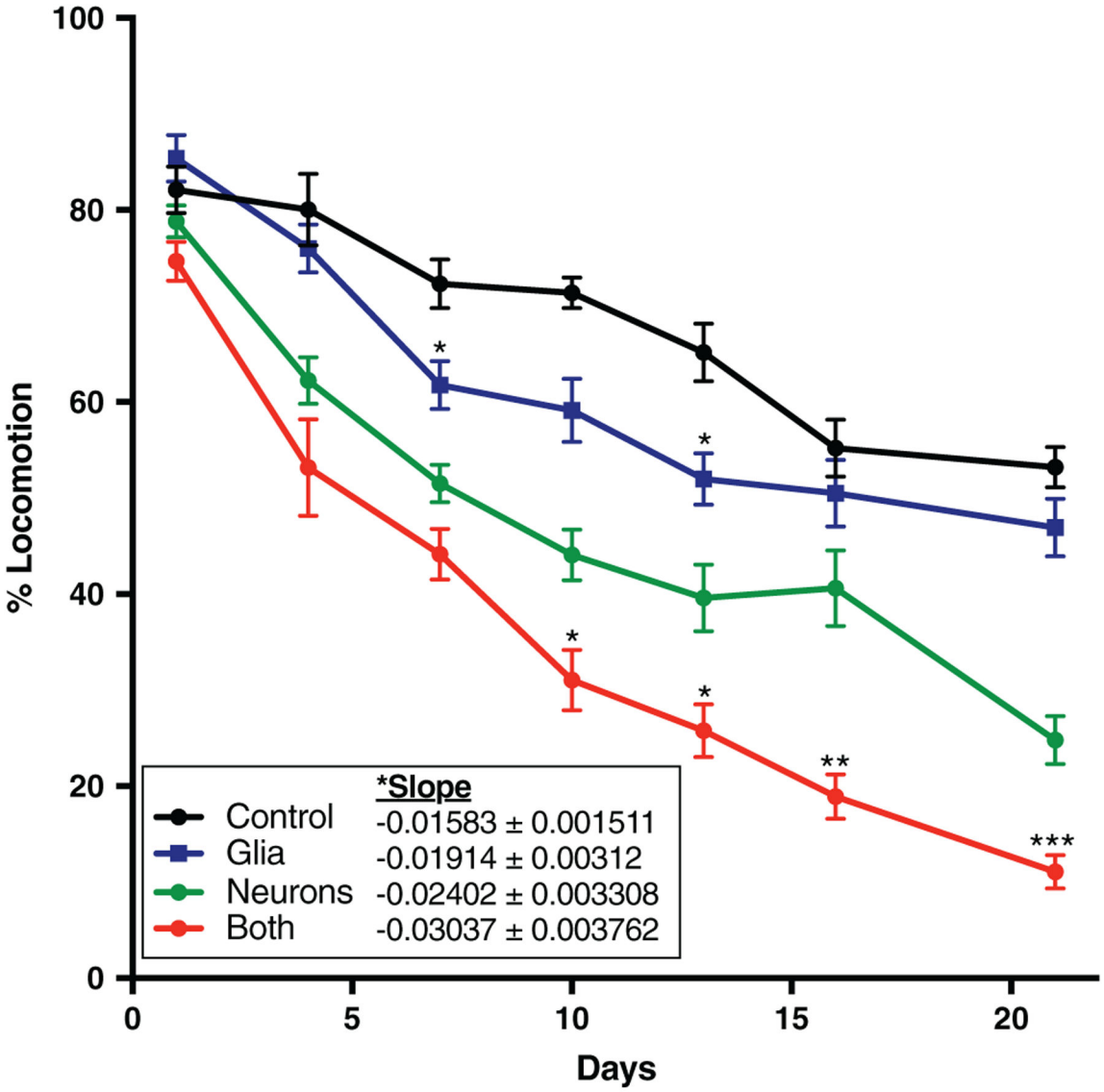


FIGURE 1.

Glial α-synuclein impairs locomotion. Flies were subjected to a gentle tapping stimulus followed by a 15-s delay. The percentage of flies still in motion (% locomotion) following the delay was recorded and averaged over six technical replicates. Symbols above the "Glia" and "Both" curves represent statistically significant difference compared to the "Control" and "Neurons" curves, respectively, at a given time point. Slope of the line was determined by linear regression analysis and was also globally statistically significantly different between the four conditions. *$p < .05$, **$p < .01$, ***$p < .005$. n = minimum of 60 flies per genotype per time point (six biological replicates of 10 flies each)

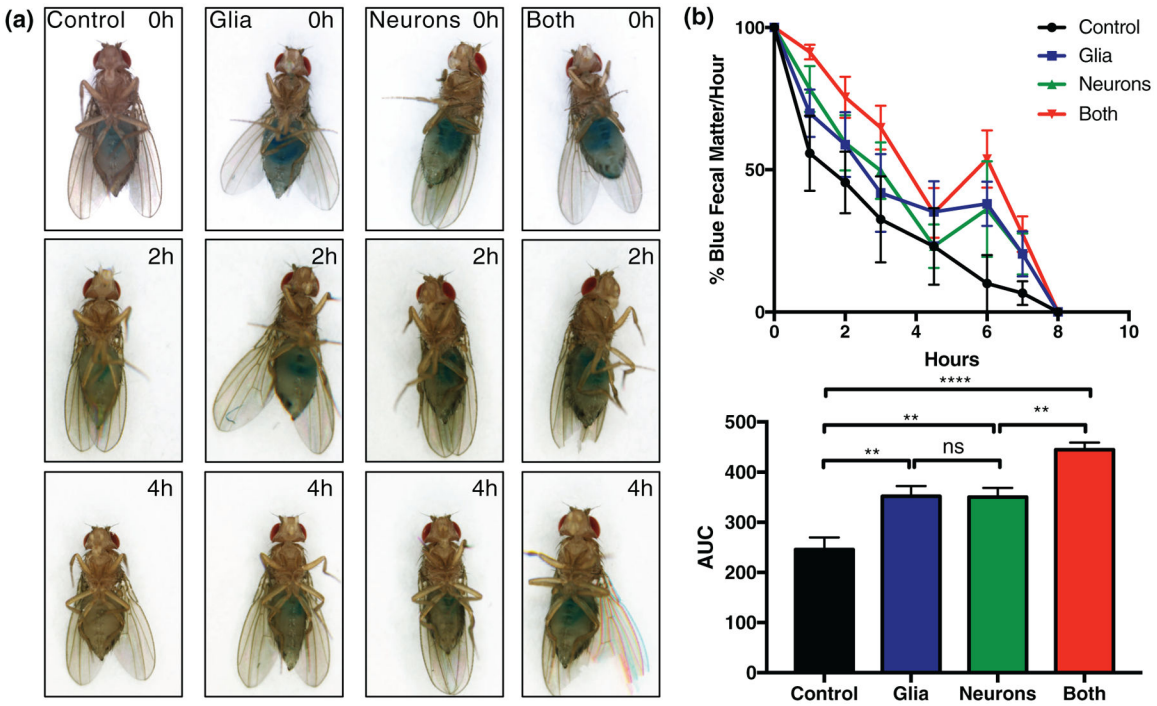


FIGURE 2.

Glial α-synuclein causes constipation. Flies were aged to 10 days of life, transferred to blue colored food for 24 hr, then returned to regular food. (a) Photographs were taken at 0, 2, and 4 hr after return to regular food. (b) The % of blue to total fecal matter was counted on an hourly basis for 8 hr after return to regular food (left). Area under the curve is statistically significantly different between conditions as measured by one-way ANOVA (right). ** $p < .01$, **** $p < .001$. n = minimum six biological replicates of 10 flies each

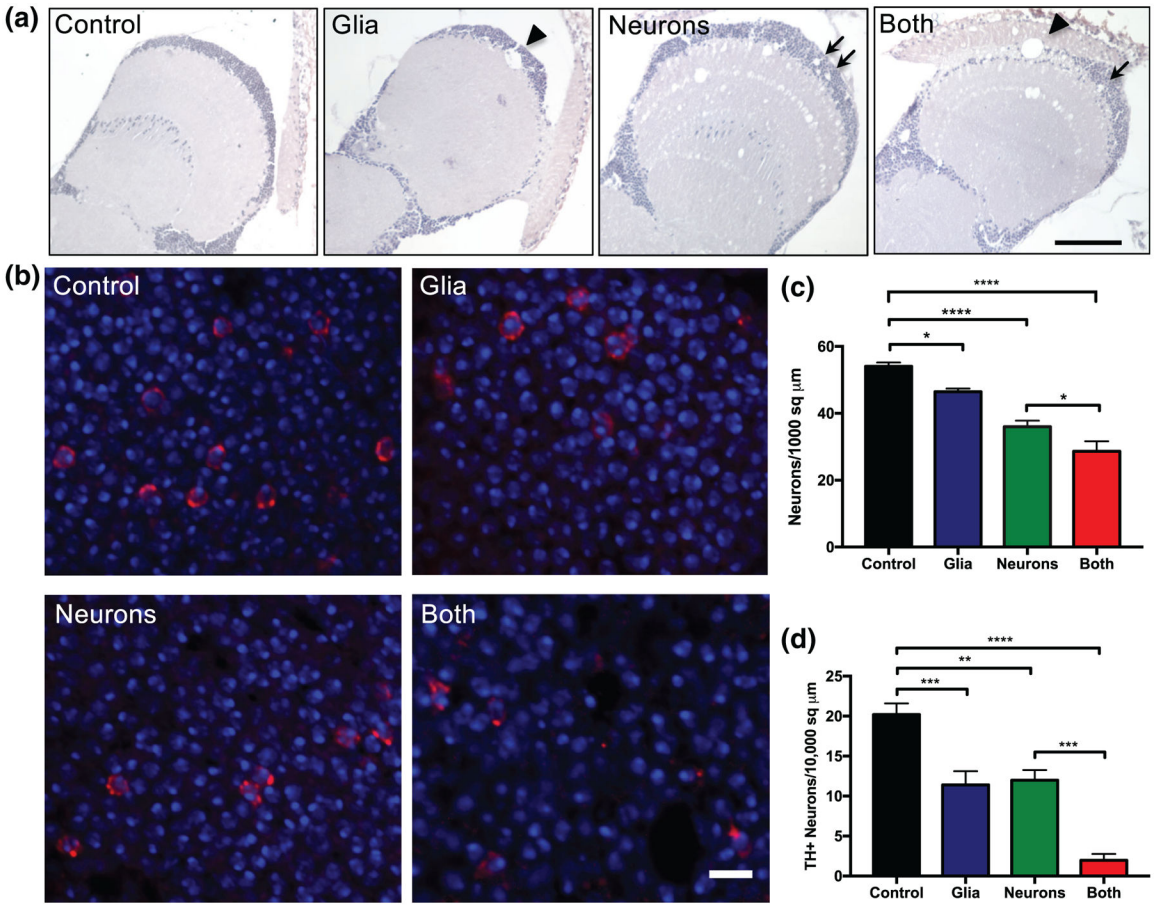


FIGURE 3.

Glial α-synuclein causes neurodegeneration. (a) Optic lobe sections stained with hematoxylin demonstrating vacuolization, an indicator of neurodegeneration. Glial α-synuclein caused infrequent large vacuoles (arrowhead) whereas neuronal α-synuclein caused frequent small vacuoles (arrows). Scale bar = 100 μm. (b) Representative anterior medulla sections stained with DAPI (blue) and tyrosine hydroxylase antibody (red, mouse, 1:200, Immunostar) to indicate dopaminergic neurons. Scale bar = 5 μm. (c) Quantification of total neurons from hematoxylin stained slides of anterior medulla (not shown), $n = 6$ replicates per genotype. (d) Quantification of dopaminergic neurons from anterior medulla, $n = 6$ replicates per genotype. *$p < .05$, **$p < .01$, ***$p < .005$, ****$p < .001$, determined with one-way ANOVA

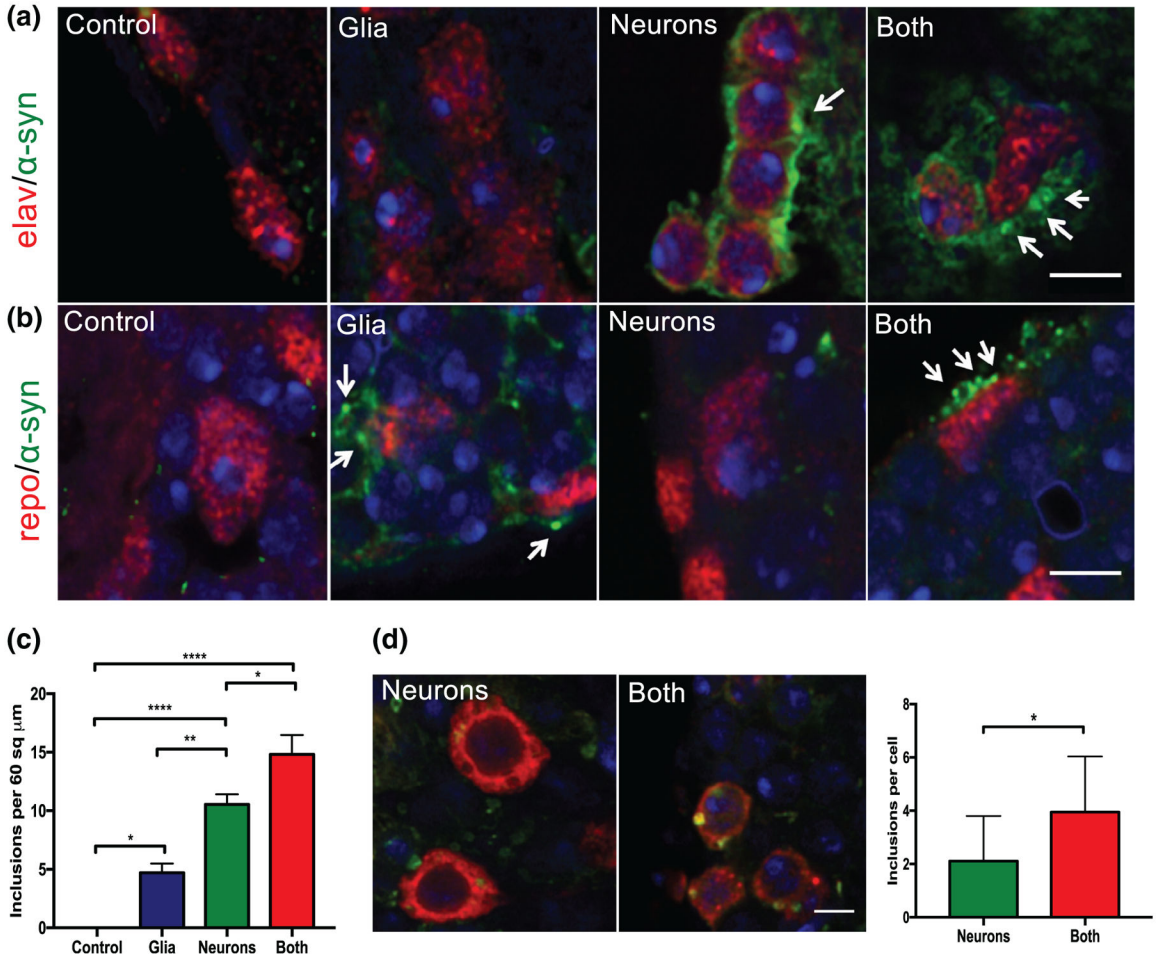


FIGURE 4.

α-Synuclein aggregates in neurons and glia. (a) Immunofluoresence for DAPI (blue), elav (red, mouse 1:5, DSHB), and α-synuclein (green, rabbit 1:1000) by confocal microscopy (3 μm scale). Arrows indicate α-synuclein inclusions in neurons. (b) Immunofluoresence for DAPI (blue), repo (red, mouse 1:5, DSHB), and α-synuclein (green, rabbit 1:1000) by confocal microscopy (3 μm scale). Arrows indicate α-synuclein inclusions in glia. (c) Quantification of total aggregates from optic lobe cortex, $n = 5$–six flies per genotype. (d) Representative immunofluorescence for tyrosine hydroxylase (red, mouse, 1:200, Immunostar), α-synuclein (green, rat, 1:10,000, Biolegend), and DAPI. Scale bar = 5 μm. Inclusions are quantified in the right panel

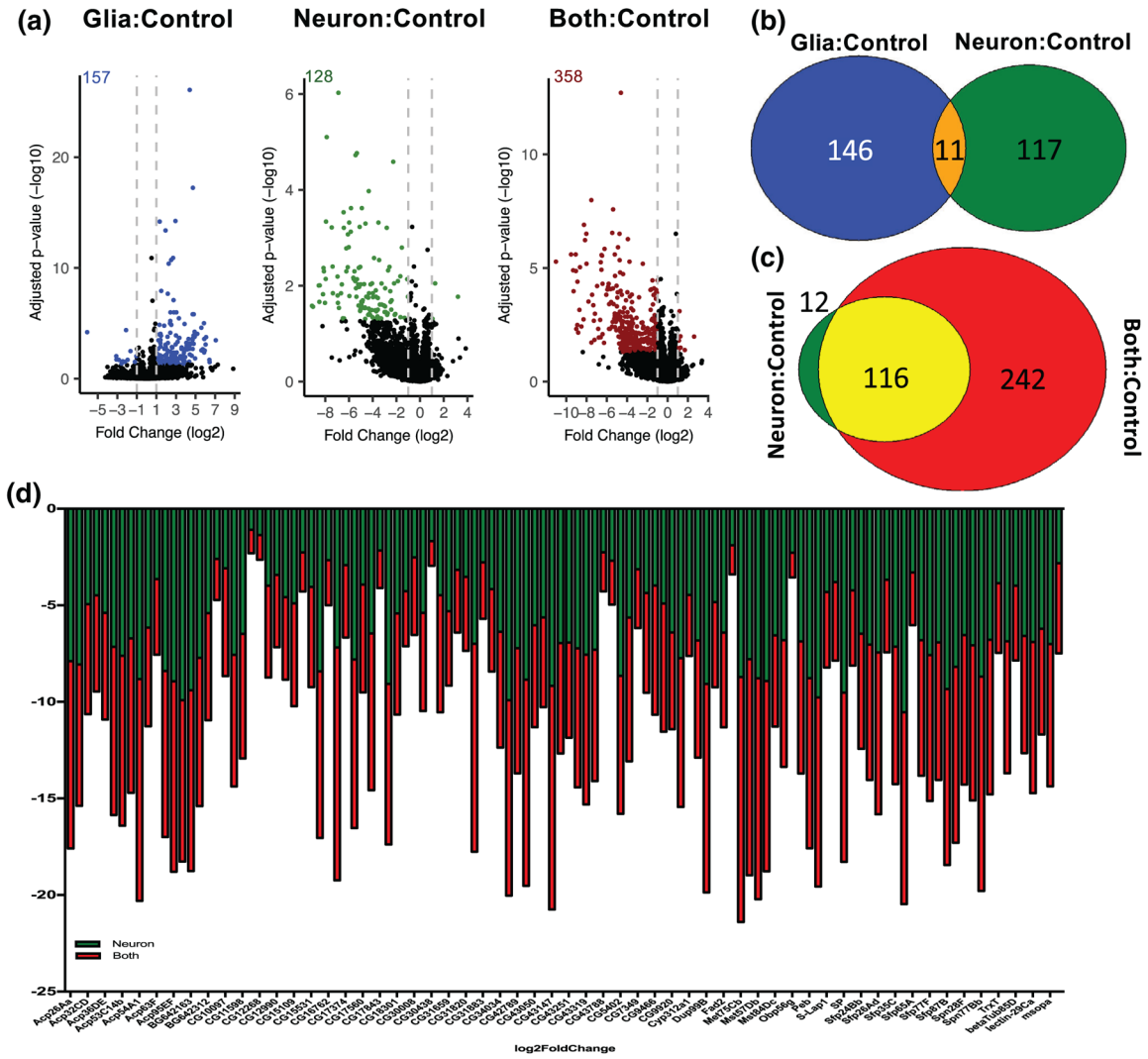
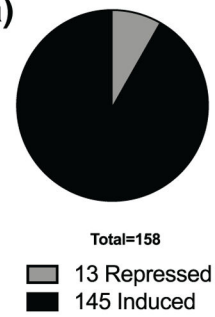


FIGURE 5.

Transcriptional changes induced by α-synuclein depend on its cellular context. Bulk RNA-Seq from whole brains was performed on 10-day old flies. (a) Volcano plots demonstrating transcript expression changes. Colored dots (and numbers) represent statistically significant (p_{adjust} < .05 after correction for multiple comparisons) differentially expressed transcripts with ≥|1| log2fold change. (b) Venn diagram demonstrating little overlap between differentially expressed genes induced by glial and neuronal α-synuclein. (c) Venn diagram demonstrating significant similarity in differentially expressed genes with both glial and neuronal α-synuclein compared to neuronal α-synuclein alone. (d) When both glial and neuronal α-synuclein are present a common set of transcripts are further downregulated as compared to neuronal α-synuclein alone

(a)



Selected Gene Ontology Analysis			
Biological Process	**Fold Enrichment**	**p-value**	**FDR**
Proteolysis	3.34	6.6E-07	2.59E-03
Cell surface receptor signaling	4.81	2.46E-05	5.55E-03

(b)

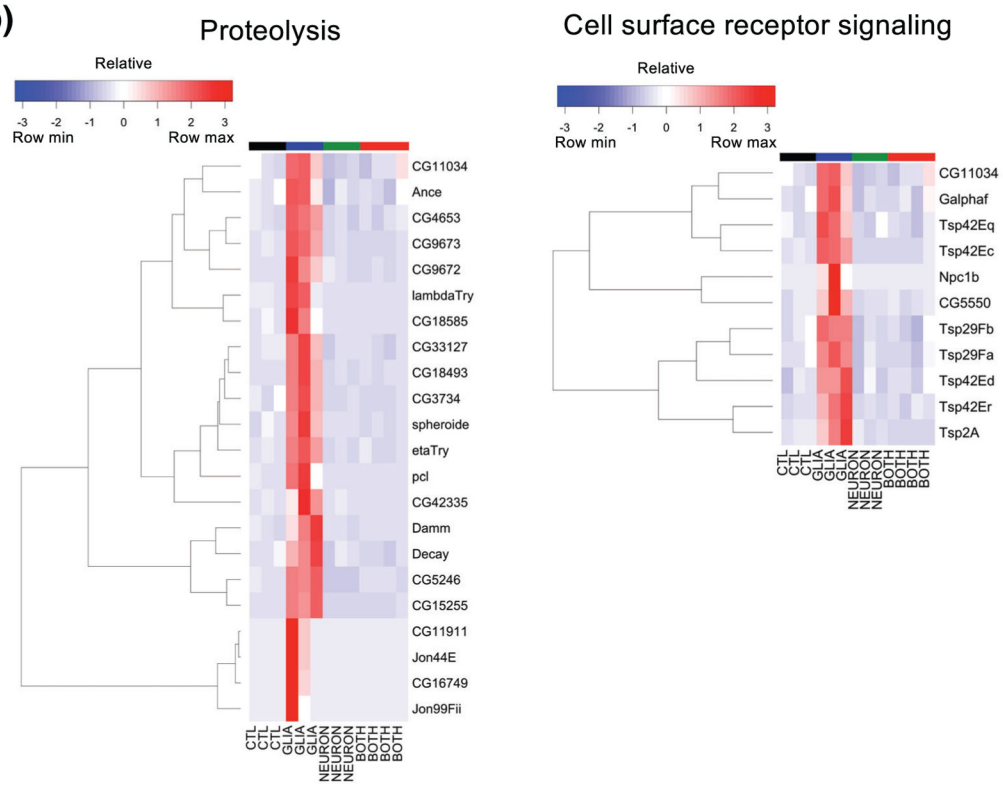
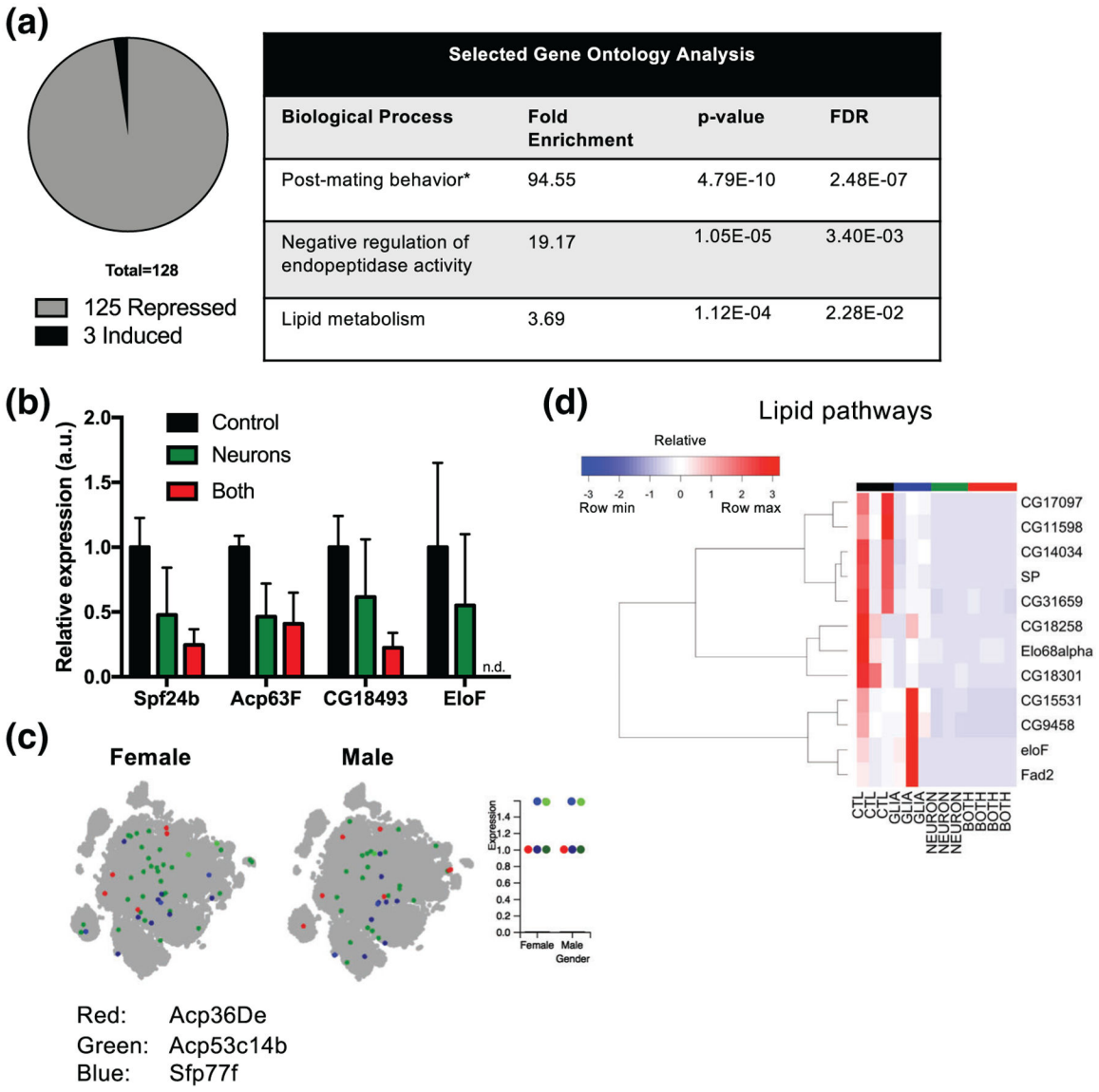


FIGURE 6.

Transcriptional changes induced by glial α-synuclein include upregulation of proteolysis and cell surface receptor signaling. Bulk RNA-Seq from whole brains was performed on 10-day old flies. (a) Gene ontology analysis for upregulated transcripts demonstrates enrichment of the terms “proteolysis” and “cell surface receptor signaling”. (b) Hierarchical clustering of proteolysis and cell surface receptor signaling related transcripts



Selected Gene Ontology Analysis			
Biological Process	**Fold Enrichment**	**p-value**	**FDR**
Post-mating behavior*	94.55	4.79E-10	2.48E-07
Negative regulation of endopeptidase activity	19.17	1.05E-05	3.40E-03
Lipid metabolism	3.69	1.12E-04	2.28E-02

FIGURE 7.

Confirmation of transcriptional changes induced by neuronal α-synuclein. (a) Gene ontology analysis. *Other reproduction-related terms beyond “post-mating behavior” were also enriched (Data file S2). (b) qRT-PCR for male and lipid-related genes. Values in Neuron and Both are normalized to Control. $n = 2–3$ biological replicates. (c) Visualization of selected Acp and Sfp gene expression in single cell transcriptome atlas. The dot plot represents expression. For both Sfp77f and Acp53c14b, there is a high and low expressing population, indicated by dots that are the same color but different intensity. (d) Hierarchical clustering of lipid related genes. All genes were significantly differentially expressed with adjusted p-value < .05

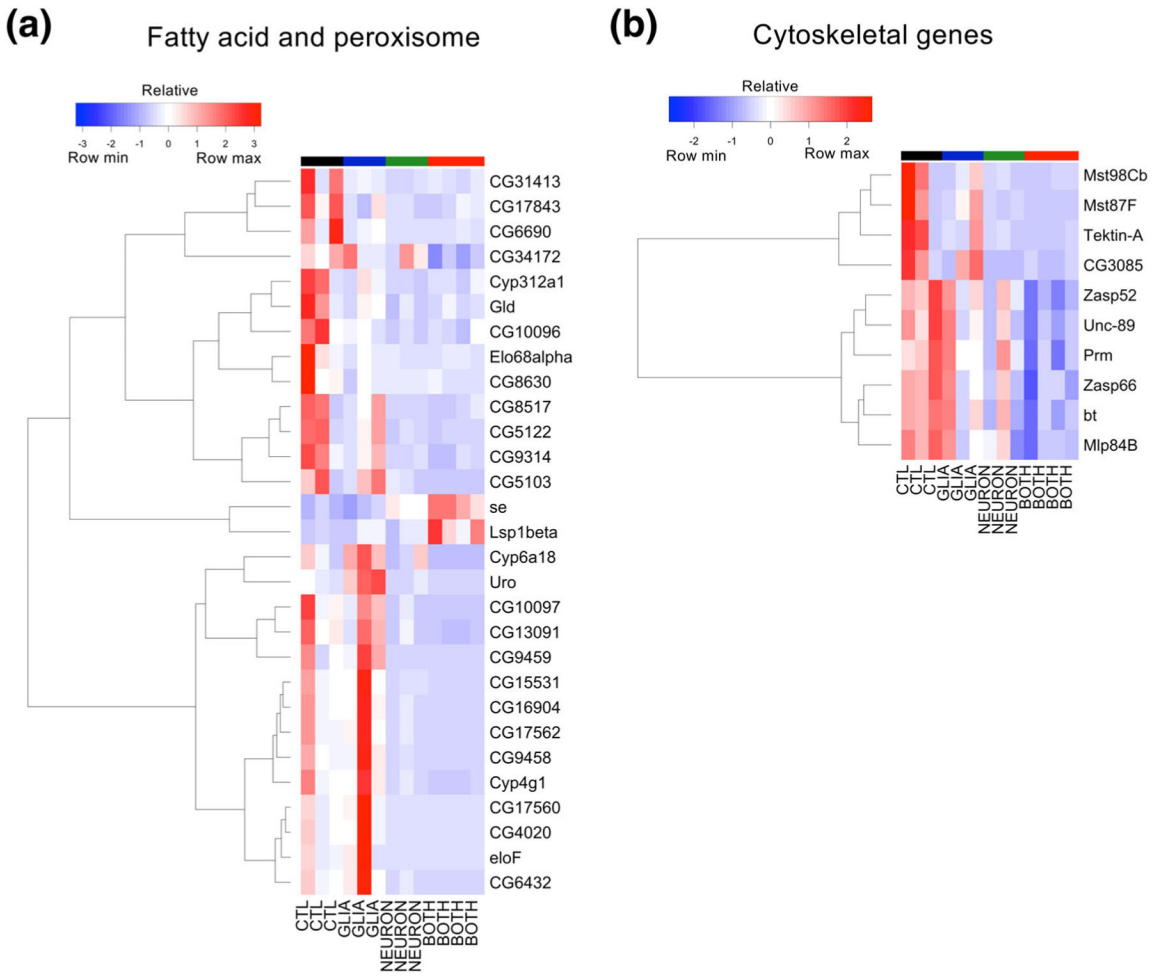


FIGURE 8.

Fatty acid metabolism and cytoskeletal genes downregulated by glial and neuronal α-synuclein. (a) Hierarchical clustering of fatty acid metabolism and peroxisome related genes. (b) Hierarchical clustering of cytoskeletal related genes. The top six that cluster together are those that contribute to the GO terms “myofibril assembly” and “muscle alpha-actinin binding,” whereas the lower four contribute to the GO term “sperm flagellum assembly”

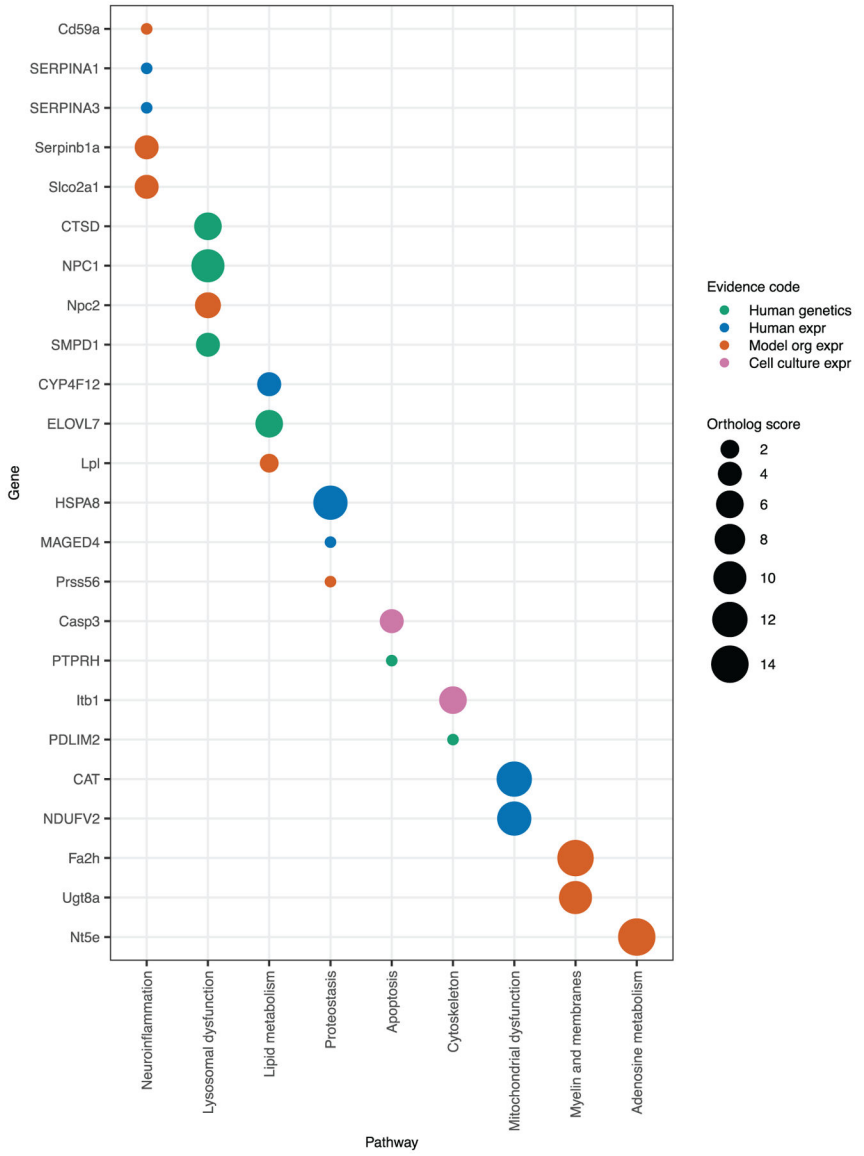


FIGURE 9.

Drosophila RNAseq identifies conserved targets and essential pathways in α-synucleinopathy pathogenesis. Mammalian orthologs of *Drosophila* genes were identified using DRSC Integrative Ortholog Prediction Tool. Orthologs have a ranked score from 1 to 14 indicating the degree of conservation (with 14 being the best). Orthologs that have been previously reported in MSA transcriptomic studies or in human α-synucleinopathy genome wide association studies (GWAS) or whole exome sequencing (WES) studies are shown. The type of evidence is indicated by the color of the circle. Human genetics, human α-synucleinopathy GWAS or WES; Human expr, expression is changed in human MSA patients; Model org expr, expression is changed in a mouse model of MSA; Cell culture expr, expression is changed in a rat oligodendrocyte model of MSA. Orthologs fall into nine pathways of known relevance to human α-synucleinopathies

TABLE 1

Proteolysis related genes upregulated in Glia:Control

Gene	PANTHER protein class	Best human ortholog
Jon44E	Unclassified	C1S, PROC
Jon99Fii	Unclassified	PROC
CG11911	Serine protease	PRSS36, PRSS53
pcl	Aspartic protease	REN, CTSE, CTSD, NAPSA
CG16749	Serine protease	KLK3
CG15255	Metalloprotease	MEP1B, MEP1A
lambdaTry	Serine protease	PRSS53, PRSS36
CG18493	Serine protease	PRSS16
CG42335	Metalloprotease	TRHDE, LVRN, ANPEP
CG18585	Metalloprotease	CPB1
Damm	Cysteine protease (caspase)	CASP3, CASP7, CASP6
CG9672	Serine protease	PRSS56, F10, F9, F7, PROZ, PROC
etaTry	Serine protease	PRSS36, PRSS53
CG5246	Unclassified	KLK14, OVCH1, PRSS3, CFD, OVCH2
CG9673	Serine protease	TPSD1
CG4653	Serine protease	PRSS36, PRSS53
CG3734	Serine protease	PRSS16
Decay	Cysteine protease (caspase)	CASP3
Spheroide	Serine protease	PRSS36, PRSS53, PRSS38
CG33127	Serine protease	None
CG11034	Serine protease	DPP4
Ance	Metalloprotease	ACE

TABLE 2

Cell surface receptor signaling related genes upregulated in Glia:Control

Gene	Best human ortholog
Npc1b	NPC1
Tsp42Ec	TSPAN2, CD81, CD9, TSPAN19, CD63
CG5550	FCN3, FCN1
Tsp2a	UPK1B, CD37, TSPAN8, TSPAN4, UPK1A, CD82, TSPAN18, TSPAN19, TSPAN1, TSPAN9, CD53
Tsp42Eq	CD63
Tsp42Er	TSPAN2, CD81, CD9
Tsp29Fa	CD63
CG11034	DPP4
Galphaf	GNAL
Tsp29Fb	CD63
Tsp42Ed	CD63

TABLE 3

Differentially expressed genes identified as glial markers in Davie et al

Gene Glia:Control	Glial population	Best human ortholog
Pdxk	Chiasm glia, astrocyte like glia, ensheathing glia, cortex glia, perineurial glia	PDXK
Jheh3	Chiasm glia, astrocyte-like glia, ensheathing glia, cortex glia	EPHX1
CG4562	Subperineurial glia	ABCC4
Tsp42Ed	Chiasm glia, astrocyte-like glia, cortex glia, supperineurial glia, perineurial glia	CD63
Ance	Subperineurial glia	ACE
CG8785	Subperineurial glia	SLC36A4
scb	Perineurial glia	ITGA4
Oatp33Ea	Cortex glia	SLCO2A1
CG4301	Subperineurial glia, cortex glia, perineurial glia	ATP11B
CG32368	Ensheathing glia, subperineurial glia, cortex glia, perineurial glia, chiasm glia	None
Neuron:Control		
CG9507	Astrocyte like glia, ensheathing glia, cortex glia	KEL
Obp44a	Chiasm glia, astrocyte like glia, Ensheathing glia, cortex glia	None
Both:Control		
CG31272	Subperineurial glia	SV2A
CG11892	Ensheathing glia	None
Zasp52	Cortex glia	LDB3
CG1208	Subperineurial glia	SLC2A8
CG34423	Astrocyte like glia, chiasm glia, ensheathing glia, subperineurial glia, perineurial glia	ATP5IF1
CG8630	Subperineurial glia	SCD
Lectin-46Cb	Chiasm glia	CLEC9A
CG44243	Ensheathing glia, perineurial glia	LIPT1
CG2765	Ensheathing glia, cortex glia	THRSP
CG1674	Subperineurial glia	SYNPO, SYNPO2, SYNPO2L
Mlp84B	Subperineurial glia	CSRP3
CG14401	Subperineurial glia	None

TABLE 4

Lipid metabolism related genes reduced in Neuron:Control

Gene	PANTHER protein class	Best human ortholog
SP		NONE
CG18258	Esterase, lipase, storage protein	LIPG, LIPL, LIPC
CG17097		LIPK, LIPJ, LIPF, LIPN, LIPA, LIPM
eloF	Acyltransferase	ELOVL1
CG11598	Lipase, serine protease	LIPK, LIPJ, LIPF, LIPN, LIPA, LIPM
Fad2		SCD, SCD5
CG18301		LIPM
CG31659		APOD
Elo68alpha	Acyltransferase	ELOVL4
CG14034		LPL
CG9458	Acyltransferase	ELOVL7
CG15531		SCD

TABLE 5

Fatty acid metabolism and peroxisome genes reduced in Both:Control

Gene	GO notes	Best human ortholog
Cyp6a18	Oxygenase	TBXAS1
eloF	Fatty acid elongation/acyltransferase	ELOVL1
CG16904	Fatty acid elongation/acyltransferase	ELOVL7
Cyp4g1	Oxygenase	CYP4V2
Uro	Uricase	None[a]
CG6690	Oxidase	QSOX2
CG17560	Fatty acyl-CoA reductase, localizes to peroxisome	FAR2
CG10097	Fatty acyl-CoA reductase, localizes to peroxisome	FAR2
CG17562	Fatty acyl-CoA reductase, localizes to peroxisome	FAR2
CG5103		None
CG15531		SCD
CG13091	Fatty acyl-CoA reductase, localizes to peroxisome	FAR2
CG9458	Fatty acid elongation/acyltransferase	ELOVL7
CG5122	Acetyltransferase, acyltransferase, localizes to peroxisome	CRAT
CG6432	Dehydrogenase	ACSS3
CG9459	Fatty acid elongation/acyltransferase	ELOVL7
Elo68alpha	Fatty acid elongation/acyltransferase	ELOVL4
CG8517	Oxidoreductase	None
CG31413	Oxidase	QSOX2
Cyp312a1	Oxygenase	CYP4B1, CYP4F11, CYP4F22, CYP4F12
CG4020	Fatty acyl-CoA reductase, localizes to peroxisome	FAR1
CG10096	Fatty acyl-CoA reductase, localizes to peroxisome	FAR2
CG17843		QSOX2
CG9314	Peroxidase, localizes to peroxisome	CAT
CG34172	Oxidase	COX7A1
Gld		CHDH
CG8630		SCD
Se		GSTO1

Gene	GO notes	Best human ortholog
Lsp1beta	Oxidase, Oxygenase	None

[a]Mouse ortholog is urate oxidase. This gene has been inactivated in humans due to mutation. Lack of the enzyme means that urate is the end product of purine metabolism in humans and represents a major antioxidant, with lower urate levels serving as a potential biomarker of Parkinson's disease (Cipriani, Chen, & Schwarzschild, 2010).

TABLE 6

Cytoskeletal genes reduced in Both:Control

Gene	GO notes	Best human ortholog
Mst87F		None
Mst98Cb		None
Tektin-A	Nonmotor microtubule binding protein	TEKT4
CG3085	Nonmotor microtubule binding protein	TEKT2
Zasp52	Actin family cytoskeletal protein	LDB3
Zasp66	Alpha-actinin binding	PDLIM1, PDLIM3
Unc-89	Tropomyosin binding, calcium-dependent kinase	SPEG
Prm	Paramyosin	MYH1
bt	Myosin binding	TTN
Mlp84B	Actin family cytoskeletal protein	CSRP3

TABLE 7

Genes implicated in MSA or PD pathogenesis in humans or animal models

Gene	Condition	Relevant ortholog	Gene name	Identified by	Ortholog score	Notes/potential mechanisms
Veil	Glia:Control	Nt5e (mouse)	5′-Nucleotidase Ecto	Schafferer et al.	14	Nt5e converts AMP to adenosine. Caffeine, an adenosine antagonist, is inversely associated with risk of development of PD (Hernán, Takkouche, Caamaño-lsorna, & Gestal-Otero, 2002; Noyce et al., 2012). Adenosine A2A receptor knockout mice are protected in animal models of PD (Kachroo & Schwarzschild, 2012; Xu et al., 2016), and adenosine antagonists are in clinical used for Parkinson's disease in Japan (Kondo, Mizuno,, & Japanese Istradefylline Study Group, 2015). Nt5e expression is altered in PD post-mortem brains (Garcia-Esparcia, Hernandez-Ortega, Ansoleaga, Carmona, & Ferrer, 2015).
Fa2h	Glia:Control	Fa2h (mouse)	Fatty acid 2-hydroxylase	Schafferer et al.	13	Mutations in *FA2H* are associated with neurologic diseases including familial leukodystrophy, levodopa-responsive hereditary spastic paraplegia SPG35, and neurodegeneration with brain iron accumulation (NBIA; Kruer et al., 2010; Scheid et al., 2013; Schneider & Bhatia, 2010; Soehn et al., 2016). The *Fa2h* knockout mouse (K. A. Potter et al., 2011) has demyelination, axon loss, cerebellar abnormalities, and memory deficits.
CG9314	Both:Control	**CAT (human)**	Catalase	Langerveld et al.	12	Catalase protects cells from ROS by metabolizing H_2O_2. It is downregulated in A53T α-synuclein mice (Yakunin et al., 2014). α-synuclein induced H_2O_2 induces microglial migration toward aggregates (S. Wang et al., 2015). PD patients have reduced catalase activity in the substantia nigra (Ambani, Van Woert, & Murphy, 1975), and catalase containing nanoparticle delivery to brain has been explored as a therapeutic strategy in PD animal models (Klyachko et al., 2017).
Hsc70–1	Both:Control	**HSPA8 (human)**	Heat shock cognate 71 kDa protein	Langerveld et al.	11	Hsp70 is a chaperone protein that breaks down α-synuclein fibrils in vitro (Gao et al., 2015) and is upregulated in mouse models of PD (Mak, McCormack, Manning-Bog, Cuervo, & Di Monte, 2010). It may also increase extracellular release of α-synuclein (Fontaine et al., 2016).
CG5703	Both:Control	NDUFV2 (human)	NADH:Ubiquinone Oxidoreductase Core subunit V2	Langerveld et al.	11	NDUFV2 is a subunit of the mitochondrial complex 1 respiratory chain. Rare mutations have been reported as a cause of familial PD (Nishioka et al., 2010), and variants are associated with idiopathic PD in small studies (Hattori, Yoshino, Tanaka, Suzuki, & Mizuno, 1998; Mizuta et al., 2008; Swerdlow et al., 2006). The transcript was downregulated in PD patient CSF (Hossein-Nezhad et al., 2016).
Npc1b	Glia:Control	NPC1 (human)	NPC intracellular cholesterol transporter 1	Shulskaya et al.	10	Mutations in NPC1 cause the lysosomal storage disease Niemman Pick type Cl, which may predispose to α-synuclein pathology (Saito, Suzuki, Hulette, & Murayama, 2004).
CG30438	Glia:Control Neuron:Control Both:Control	Ugt8a (mouse)	UDP galactosyltransferase 8A	Schafferer et al.	10	Ugta8a knockout mice have unstable myelin, progressive demyelination and severe motor coordination deficits (Coetzee et al., 1996).

Gene	Condition	Relevant ortholog	Gene name	Identified by	Ortholog score	Notes/potential mechanisms
Pci	Glia:Control	**CTSD (rat), CTSD (human)**	Cathepsin D	Kaji et al. (Ctsd), Robak et al. (CTSD)	6[a]	Mutations in CTSD cause neuronal ceroid lipofuscinosis (Myllykangas et al., 2005). CTSD cleaves α-synuclein and protects against α-synuclein aggregation and toxicity (Cullen et al., 2009; Kiely et al., 2018; Qiao et al., 2008).
Itgbetanu	Glia:Control	**Itgb1 (rat)**	Integrin beta-1	Kaji et al.	6	Beta 1 integrin is a subunit of many integrin receptors. It promotes microglial migration toward α-synuclein (Kim et al., 2014). It also promotes oligodendrocyte adhesion to fibronectin (Tsuboi et al., 2005), myelin formation (Camara et al., 2009), and dopaminergic neurite outgrowth (Izumi et al., 2017).
CG5278	Glia:Control Neuron:Control Both:Control	ELOVL7 (human)	Elongation of very long chain fatty acids protein 7	Sailer et al., Chang et al.	6	ELOVL7 is a fatty acid elongase. Mutations in yeast orthologs of fatty acid elongases enhance α-synuclein toxicity (Lee, Wang, Slone, Yacoubian, & Witt, 2011). Inhibiting the fatty acid desaturase SCD or its yeast ortholog OLE1 reduces levels of oleic acid and rescues α-synuclein toxicity in model organisms (Fanning et al., 2018; Vincent et al., 2018).
CG16904	Both:Control	ELOVL7 (human)	Elongation of very long chain fatty acids protein 7	Sailer et al., Chang et al.	6	
CG9458	Neuron:Control Both:Control	ELOVL7 (human)	Elongation of very long chain fatty acids protein 7	Sailer et al., Chang et al.	5	
CG30008	Neuron:Control Both:Control	ELOVL7 (human)	Elongation of very long chain fatty acids protein 7	Sailer et al., Chang et al.	5	
CG9459	Both:Control	ELOVL7 (human)	Elongation of very long chain fatty acids protein 7	Sailer et al., Chang et al.	5	
Npc2e	Glia:Control	Npc2 (mouse)	Npc intracellular cholesterol transporter 2	Schafferer et al.	5	Mutations in NPC2 cause the lysosomal storage disease Niemann Pick type C2, but heterozygotes have been reported to have a parkinsonism syndrome (Kluenemann, Nutt, Davis, & Bird, 2013).
Oatp33Ea	Glia:Control	Slco2a1 (mouse)	Solute carrier organic anion transporter family member 2A1	Schafferer et al.	4	Slco2a1 is a prostaglandin receptor expressed on microglia and endothelial cells that may play a role in neuroinflammation (Nakamura etal., 2018).
CGI5534	Glia:Control	SMPD1 (human)	Sphingomyelin phosphodiesterase 1	Robak et al.	4	SMPD1 mutations cause Niemann-Pick disease type A and B. Rare variants have been associated with PD in many small genetic studies prior to Robak et al. (reviewed in [Deng, Xiu, & Jankovic, 2015]).
Decay	Glia:Control	**Casp3 (rat)**	Caspase 3	Kaji et al.	4	Caspases regulate apoptosis. Inhibiting caspase 3 is protective in rat PD models (Y. Liu et al., 2013; Yuan, Ren, Wang, He, & Zhao, 2016).
Damm	Glia:Control	Casp3 (rat)	Caspase 3	Kaji et al.	4	
Spn38F	Neuron:Control Both:Control	Serpinbla (mouse)	Serine (or cysteine) peptidase inhibitor, clade B, member 1a	Schafferer et al.	4[a]	See below for further discussion on Serpin family members.
Cyp312al	Neuron:Control Both:Control	CYP4F12 (human)	Cytochrome P450 4F12	Mills et al.	4[a]	CYP4F12 is a cytochrome P450 family member that localizes to the endoplasmic reticulum and oxidizes arachidonic acid.
CG14034	Neuron:Control	**Lpl (mouse)**	Lipoprotein lipase	Schafferer et al.	2	Lipoprotein lipases hydrolyze long-chain triglycerides. Lpl knockout mice develop α-synuclein aggregates (Yang et al., 2015).

Gene	Condition	Relevant ortholog	Gene name	Identified by	Ortholog score	Notes/potential mechanisms
	Both:Control					
CG18258	Neuron:Control Both:Control	Lpl (mouse)	Liprotein lipase	Schafferer et al.	1[a]	
Spn77Bb	Neuron:Control Both:Control	SERPINA3 (human), SERPINA1 (human), Serpinbla (mouse)	Serpin family A member 3, Serpin family A member 1, serine (or cysteine) peptidase inhibitor, clade B, member 1a	Mills etal. (SERPINA3); Schafferer et al. (Serpinbla); Siitonen et al. (SERPINA1)	1[a]	Serpin family members are protease inhibitors that participate in a wide variety of biological processes including inflammatory signaling cascades. Modified serpinA1 may be a biomarker for PD dementia (Halbgebauer et al., 2016).
Spn77Bc	Both:Control	SERPINA3 (human), SERPINA1 (human), Serpinbla (mouse)	Serpin family A member 3, Serpin family A member 1, serine (or cysteine) peptidase inhibitor, clade B, member 1a	Mills etal. (SERPINA3); Schafferer et al. (Serpinbla); Siitonen et al. (SERPINA1)	1[a]	
CG9568	Glia:Control Both:Control	Cd59a (mouse)	CD59a antigen	Schafferer et al.	1[a]	CD59a is a complement receptor. Complement is used by microglia to prune synapses in development (Schafer 2012) and disease (Hong et al., 2016) and causes formation of neurotoxic astrocytes (Liddelow et al., 2017).
CG15635	Neuron:Control Both:Control	MAGED4 (human)	MAGE family member D4	Langerveld et al.	1[a]	MAGED4 enhances E3 ubiquitin ligase activity.
CG9672	Glia:Control	Prss56 (mouse)	Serine protease 56	Schafferer et al.	1[a]	Prss56 is a serine protease important for eye development.
CG43897	Both:Control	PDLIM2 (human), Pdlim2 (mouse)	PDZ and LIM domain 2	Chang et al. (PDLIM2), Schafferer et al. (Pdlim2),	1[a]	PDLIM2 interacts with the actin cytoskeleton and promotes anchorage-independent growth and cell migration.
Ptp52F	Glia:Control	PTPRH (human)	Protein tyrosine phosphatase, receptor type H	Jansen et al.	1[a]	PTPRH is a transmembrane phosphatase. Loss of function variants enhances α-synuclein toxicity in Drosophila (Jansen et al., 2017).

Abbreviations: H_2O_2: hydrogen peroxide; ROS: reactive oxygen species.

[a]There are multiple equally ranked orthologs for this gene. Those genes with mechanistic evidence supporting a causal role in α-synucleinopathy pathogenesis are indicated in bold.