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

**Running title**: A *Drosophila* model of glial α-synucleinopathy

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**Acknowledgements**
The authors would like to thank Dr. John Hutchinson of the Harvard Chan Bioinformatics Core, Harvard T.H. Chan School of Public Health, Boston, MA for assistance with the RNAseq analysis. The project was conducted with the support of Harvard Catalyst and the Harvard Clinical and Translational Science Center (NIH award #UL1 RR 025758 and financial contributions from participating institutions). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health. We also thank Dr. Joanna DiSpirito for assistance with the RNAseq analysis.

We thank the Neurobiology Department and the Neurobiology Imaging Facility for consultation and instrument availability that supported this work. This facility is supported in part by the Neural Imaging Center as part of an NINDS P30 Core Center grant #NS072030. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/glia.23671

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The elav-7E8A10 monocolonal antibody developed by G.M. Rubin of HHMI/Janelia Farm Research and the repo-8D12 monoloclonal antibody developed by C. Goodman of the University of California, Berkeley were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

The GFP N86/8 antibody was from the UC Davis/NIH NeuroMab Facility.

*Drosophila* stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study.

**Word count**
15,275

**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**
Glia, α-synuclein, Parkinson's disease, Multiple System Atrophy, *Drosophila*

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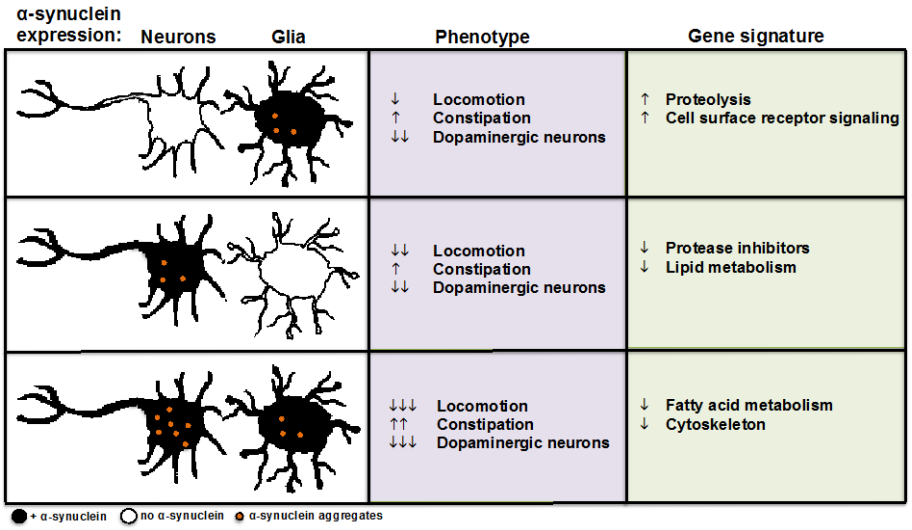


## Main Points

- Glial α-synuclein causes neurodegeneration.
- Glial α-synuclein increases α-synuclein aggregation and death of dopaminergic neurons in a non-cell-autonomous manner.
- Glial and neuronal α-synuclein induce distinct transcriptional programs.



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## 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 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 (L. 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



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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.

## Methods

*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: 1. Control = *Syb-QF2, repo-GAL4/+*, 2. Glia = *Syb-QF2, repo-GAL4/UAS-α-synuclein*, 3. Neurons = *Syb-QF2, repo-GAL4, QUAS-α-synuclein/+*, 4. 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.

*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 hour 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), elav (1:5, mouse, Developmental Studies Hybridoma Bank), tyrosine hydroxylase (1:200 to 1:500, mouse, Immunostar), α-synuclein hSA-2 (1:1000, rabbit, provided as a kind gift from Dr. Michael Schlossmacher), α-synuclein clone A17183G (1:1,000 to 1:10,000, rat, Biolegend). α-synuclein hSA-2 recognizes both monomeric and oligomeric α-synuclein by immunoblotting as well as α-synuclein aggregates by immunostaining. α-synuclein clone A17183G 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 hour (1:200, Southern Biotech) followed by avidin-biotin-peroxidase complex (Vectastain Elite) in PBS for 1 hour. Histochemical detection was performed with diaminobenzidine (ImmPACT DAB, Vector). For



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immunofluorescence, slides were incubated with fluorophore-conjugated secondary antibodies in 2% milk in PBS with 0.3% triton X-100 for 1 hour (1:200, Alexa 488 or Alexa 555, Invitrogen) 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.

*Western blotting*

Fly heads were homogenized in 2X Laemmli buffer, boiled for 10 minutes, and centrifuged. SDS-PAGE was performed (Lonza) followed by transfer to nitrocellulose membrane (Bio-Rad) and microwave antigen retrieval in PBS. Membranes were blocked in 2% milk in PBS with 0.05% Tween-20 for 1 hour, 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). 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 hours. Signal was developed with enhanced chemiluminescence (Thermo Scientific). Anti-GAPDH was used to demonstrate equivalent protein loading.

*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 minute 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 seconds. 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).

*Constipation assay*

Standard cornmeal-agar *Drosophila* medium was melted by microwaving briefly and then mixed with blue food coloring (AmeriColor) 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 hours. 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 hours. 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-hour timepoints to demonstrate delayed gut transit of the blue food.



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*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.

*RNA-seq*
Adult flies were aged to 10 days. 4 biological replicates per genotype, each consisting of 6 fly heads (3 male, 3 female), were used. Fly heads were homogenized in Qiazol (Qiagen) 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). 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.

*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). 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 MJ, Pantano L, Kirchner RD, Barrera V, Chapman BA, Piper ME, Mistry M, Khetani RS, Rutherford KD, Hofmann O, Hutchinson JN, Sui SH, 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 (Y. 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, IT, 2002), and sample similarity. Plots were generated by ggplot2 (Wickham, H, 2016) and heatmaps were generated by pheatmap (Kolde R, 2015). Principle component analysis demonstrated strongest clustering by genotype (accounting for 43% of the variance). Based on the clustering analysis, 1 sample each from the Control, Glia, and Neuron conditions was excluded from further analysis, leaving a minimum of 3 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



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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 Supplemental Data File 3. Differentially expressed genes had a fold-change between conditions of ≥2 and FDR <0.05. Gene ontology analysis (release 2018-08-09) (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).

*qRT-PCR*

Selected genes identified as differentially expressed in the RNASeq analysis were validated by qRT-PCR. Primers for these genes were chosen from the DRSC FlyPrimerBank (Hu et al., 2013). 20 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 minutes. cDNA was prepared using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), 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.

*Single cell transcriptome atlas*

Selected genes identified as differentially expressed in the RNASeq 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.

*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.

## Results

*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,



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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 (Supplemental Figure 1a, 1c-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 (Supplemental Figure 1b), which may reflect strong expression driven by *Syb-QF2*.

*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 one minute, and then gently tapped 3 times to evoke the startle-induced locomotion response (J. 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 second delay is recorded, and differences between control and neuronal α-synuclein flies are highly reproducible over time (Supplemental Figure 2a) and correlate with our previously published climbing assay (Supplemental Figure 2b).

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 over-expression 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 (Supplemental Figure 3). 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 non-cell-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 (Supplemental Figure 3), demonstrating specificity of glial α-synuclein in exacerbating the neuronal α-synuclein phenotype.

*Glial α-synuclein causes constipation*



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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). Non-motor 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 hours, then transferred to vials with regular food. Photographs of individual representative flies were taken at time 0, 2, and 4 hours (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 hours (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.

*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, 3d). 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 3d to 3c), 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 (Supplemental



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Figure 4), suggesting that there is not marked loss of this population but rather that glial dysfunction is responsible for the pathogenic effects of glial α-synuclein.

*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 non-cell-autonomous manner. Such non-cell-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).

*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, Supplemental Data File 1). 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, Supplemental Data File 1). 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, Supplemental Data File 1). 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



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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 Supplemental Data File 2 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, 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 (Supplemental Figure 5a). 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 (Supplemental Figure 5b). 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 (Supplemental Figure 5c), and we confirmed a glial expression pattern by immunohistochemistry (Supplemental Figure 5d).

In contrast to glial α-synuclein, neuronal α-synuclein led to downregulation of many transcripts (Figure 5a, 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 4 families of genes: accessory gland protein (Acp), seminal fluid protein (Sfp), serpin (Spn), and odorant binding protein (Obp) families (Supplemental Data File 1). 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



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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 in the Neuron:Control comparison (Figure 5c), and similarly, gene ontology analysis reveals several enriched terms that are mating-related (Supplemental Data File 2). 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 (Supplemental Data File 2). 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.

*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



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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 9 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.

**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 non-dopaminergic 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 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



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(Figure 9). There are 6 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*. Further, 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, Wang, Slone, Yacoubian, & Witt, 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 explain 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 non-cell-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



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increases aggregation of α-synuclein in dopaminergic neurons. This finding could be explained by a non-cell-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 (Supplemental Figure 4). 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.

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[dataset] Olsen AL, Feany MB; 2019; Glial and neuronal alpha synuclein induce unique transcriptional programs; GEO; Accession GSE128120



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## Tables

| 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 |

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| | |
|---|---|
| Tsp42Ed | CD63 |

| Table 3: Differentially expressed genes identified as glial markers in Davie et al. | | |
|---|---|---|
| **Glia:Control** | | |
| **Gene** | **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** | | |
| **Gene** | **Glial population** | **Best human ortholog** |
| CG9507 | Astrocyte like glia, ensheathing glia, cortex glia | KEL |
| Obp44a | Chiasm glia, Astrocyte like glia, Ensheathing glia, Cortex glia | none |
| **Both:Control** | | |
| **Gene** | **Glial population** | **Best human ortholog** |
| 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 |



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| | | |
|---|---|---|
| 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* |
| 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 |
| Lsp1beta | Oxidase, Oxygenase | None |

*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).



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| Table 6: Cytoskeletal genes reduced in Both:Control | | |
|---|---|---|
| **Gene** | **GO Notes** | **Best human ortholog** |
| Mst87F | | None |
| Mst98Cb | | None |
| Tektin-A | Non-motor microtubule binding protein | TEKT4 |
| CG3085 | Non-motor 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 |

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**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-Isorna, & 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, Hernández-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, |



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| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | 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 I 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 C1, which may predispose to α-synuclein pathology (Saito, Suzuki, Hulette, & Murayama, 2004). |
| CG30438 | Glia:Control Neuron:Control Both:Control | Ugt8a (mouse) | UDP galactosyltrnasferase 8A | Schafferer et al | 10 | *Ugta8a* knockout mice have unstable myelin, progressive demyelination, and severe motor coordination deficits (Coetzee et al., 1996). |
| pcl | Glia:Control | **Ctsd (rat), CTSD (human)** | Cathepsin D | Kaji et al (Ctsd), Robak et al (CTSD) | 6* | 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 (Câmara 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 et al., 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 | |



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| | | | | | | |
|---|---|---|---|---|---|---|
| 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 lyosomal 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 et al., 2018). |
| CG15534 | 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 3is 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 | Serpinb1a (mouse) | Serine (or cysteine) peptidase inhibitor, clade B, member 1a | Schafferer et al | 4* | See below for further discussion on Serpin family members. |
| Cyp312a1 | Neuron:Control Both:Control | CYP4F12 (human) | Cytochrome P450 4F12 | Mills et al | 4* | CYP4F12 is a cytochrome P450 family member that localizes to the endoplasmic reticulum and oxidizes arachidonic acid. |
| CG14034 | Neuron:Control Both:Control | **Lpl (mouse)** | Liprotein lipase | Schafferer et al | 2 | Lipoprotein lipases hydrolyze long-chain triglycerides. Lpl knockout mice develop α-synuclein aggregates (Yang et al., 2015). |
| CG18258 | Neuron:Control Both:Control | Lpl (mouse) | Liprotein lipase | Schafferer et al | 1* | |
| Spn77Bb | Neuron:Control Both:Control | SERPINA3 (human), SERPINA1 (human), Serpinb1a (mouse) | Serpin family A member 3, Serpin family A member 1, Serine (or cysteine) peptidase inhibitor, clade B, member 1a | Mills et al (SERPINA3); Schafferer et al (Serpinb1a); Siitonen et al (SERPINA1) | 1* | 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). |



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| | | | | | | |
|---|---|---|---|---|---|---|
| Spn77Bc | Both:Control | SERPINA3 (human), SERPINA1 (human), Serpinb1a (mouse) | Serpin family A member 3, Serpin family A member 1, Serine (or cysteine) peptidase inhibitor, clade B, member 1a | Mills et al (SERPINA3); Schafferer et al (Serpinb1a); Siitonen et al (SERPINA1) | 1* | |
| CG9568 | Glia:Control Both:Control | Cd59a (mouse) | CD59a antigen | Schafferer et al | 1* | 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* | MAGED4 enhances E3 ubiquitin ligase activity. |
| CG9672 | Glia:Control | Prss56 (mouse) | Serine protease 56 | Schafferer et al | 1* | 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* | 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* | PTPRH is a transmembrane phosphatase. Loss of function variants enhance α-synuclein toxicity in *Drosophila* (Jansen et al., 2017). |
| *There are multiple equally ranked orthologs for this gene. | | | | | | |
| Abbreviations: Reactive oxygen species (ROS), hydrogen peroxide ($H_2O_2$) | | | | | | |



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**Figure Legends**

**Fig. 1 Glial α-synuclein impairs locomotion.** Flies were subjected to a gentle tapping stimulus followed by a 15 second delay. The percentage of flies still in motion (% locomotion) following the delay was recorded and averaged over 6 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 4 conditions. *p <0.05, **p<0.01, ***p<0.005. n = minimum of 60 flies per genotype per time point (6 biological replicates of 10 flies each).

**Fig. 2 Glial α-synuclein causes constipation.** Flies were aged to 10 days of life, transferred to blue colored food for 24 hours, then returned to regular food. **a** Photographs were taken at 0, 2, and 4 hours after return to regular food. **b** The % of blue to total fecal matter was counted on an hourly basis for 8 hours after return to regular food (left). Area under the curve is statistically significantly different between conditions as measured by one-way ANOVA (right). ** p<0.01, ****p<0.001. n = minimum 6 biological replicates of 10 flies each.

**Fig. 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 <0.05, ** = p <0.01, *** = p<0.005, **** = p<0.001, determined with one-way ANOVA.

**Fig. 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 = 5-6 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.

**Fig. 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 (padjust <0.05 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



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glial and neuronal α-synuclein are present a common set of transcripts are further downregulated as compared to neuronal α-synuclein alone.

**Fig. 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.

**Fig. 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 (Supplemental Data File 2). **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 <0.05.

**Fig. 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 6 that cluster together are those that contribute to the GO terms "myofibril assembly" and "muscle alpha-actinin binding," whereas the lower 4 contribute to the GO term "sperm flagellum assembly."

**Fig. 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 1-14 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 9 pathways of known relevance to human α-synucleinopathies.



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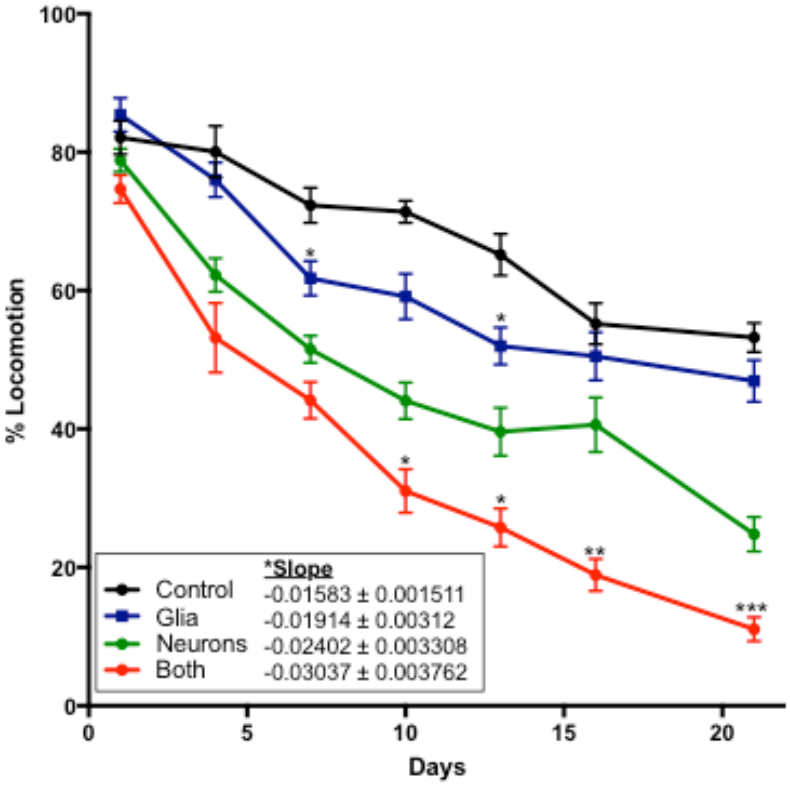


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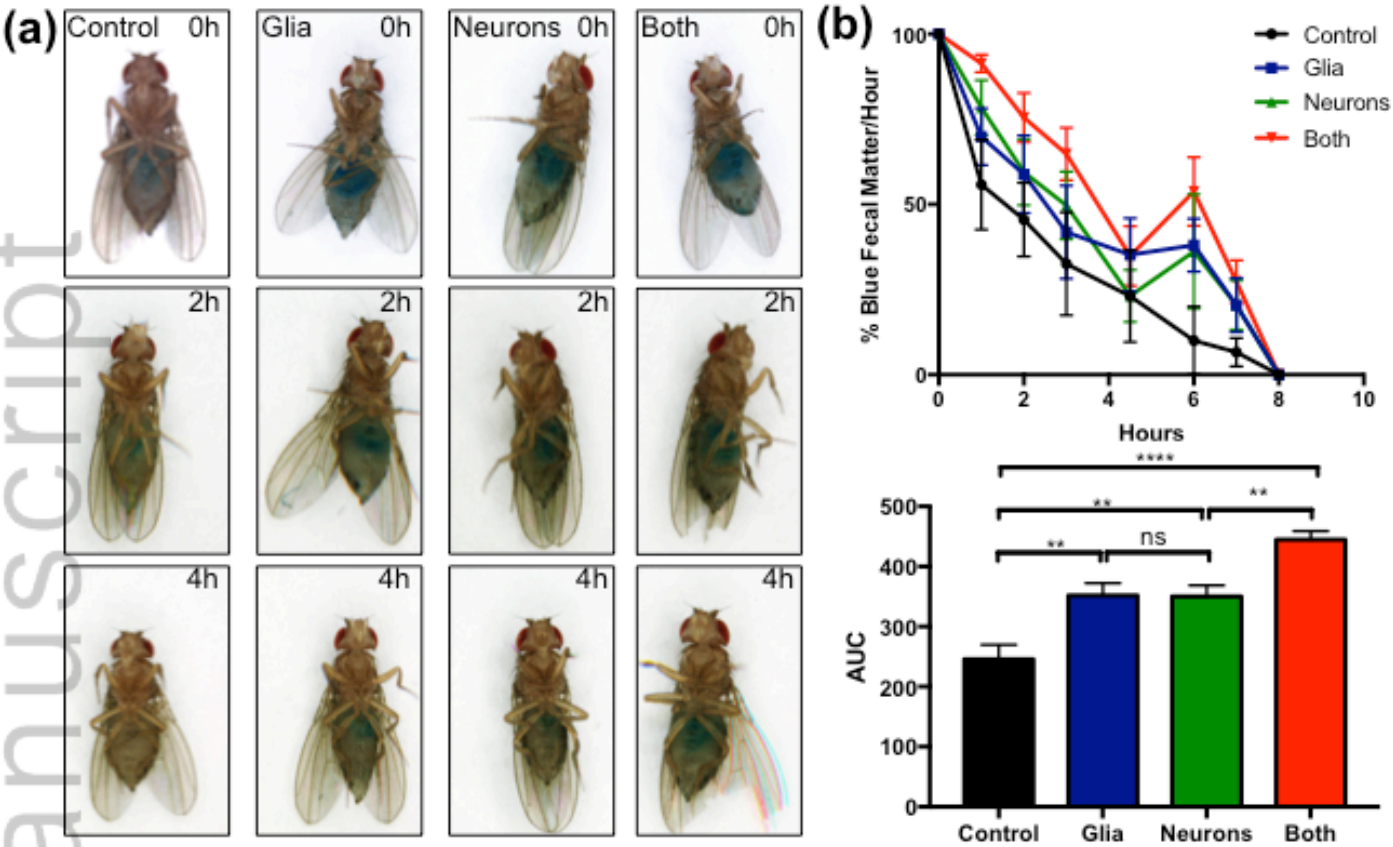


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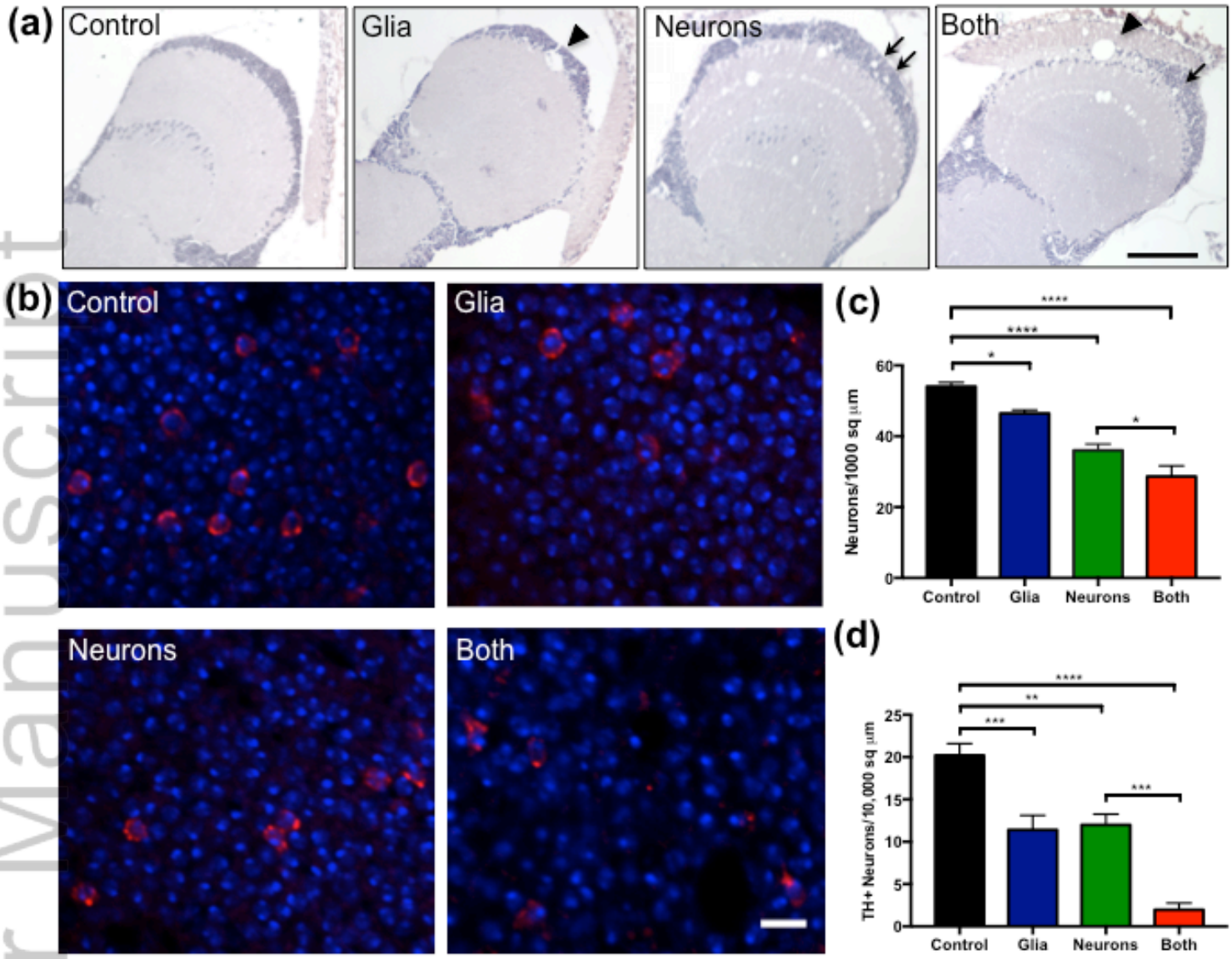


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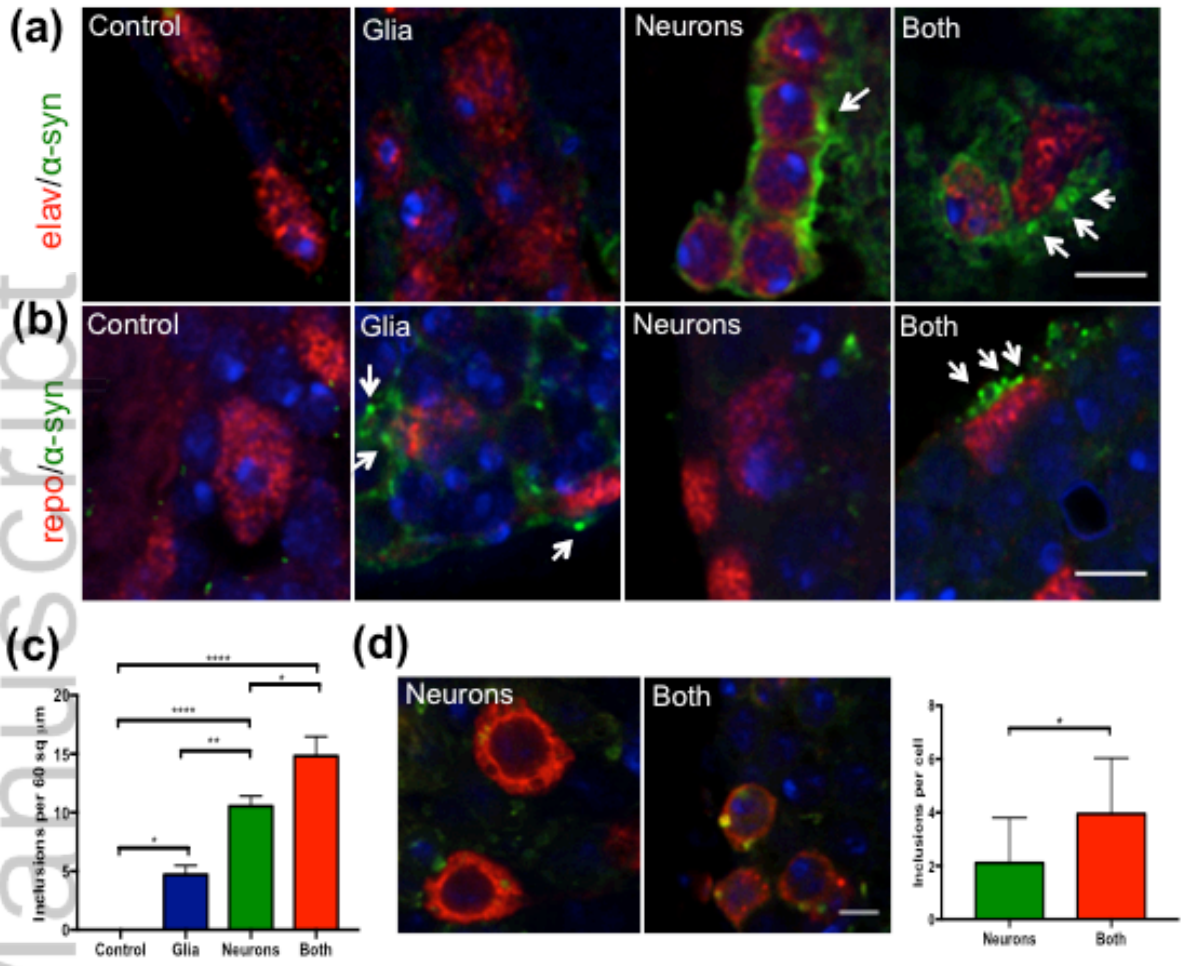


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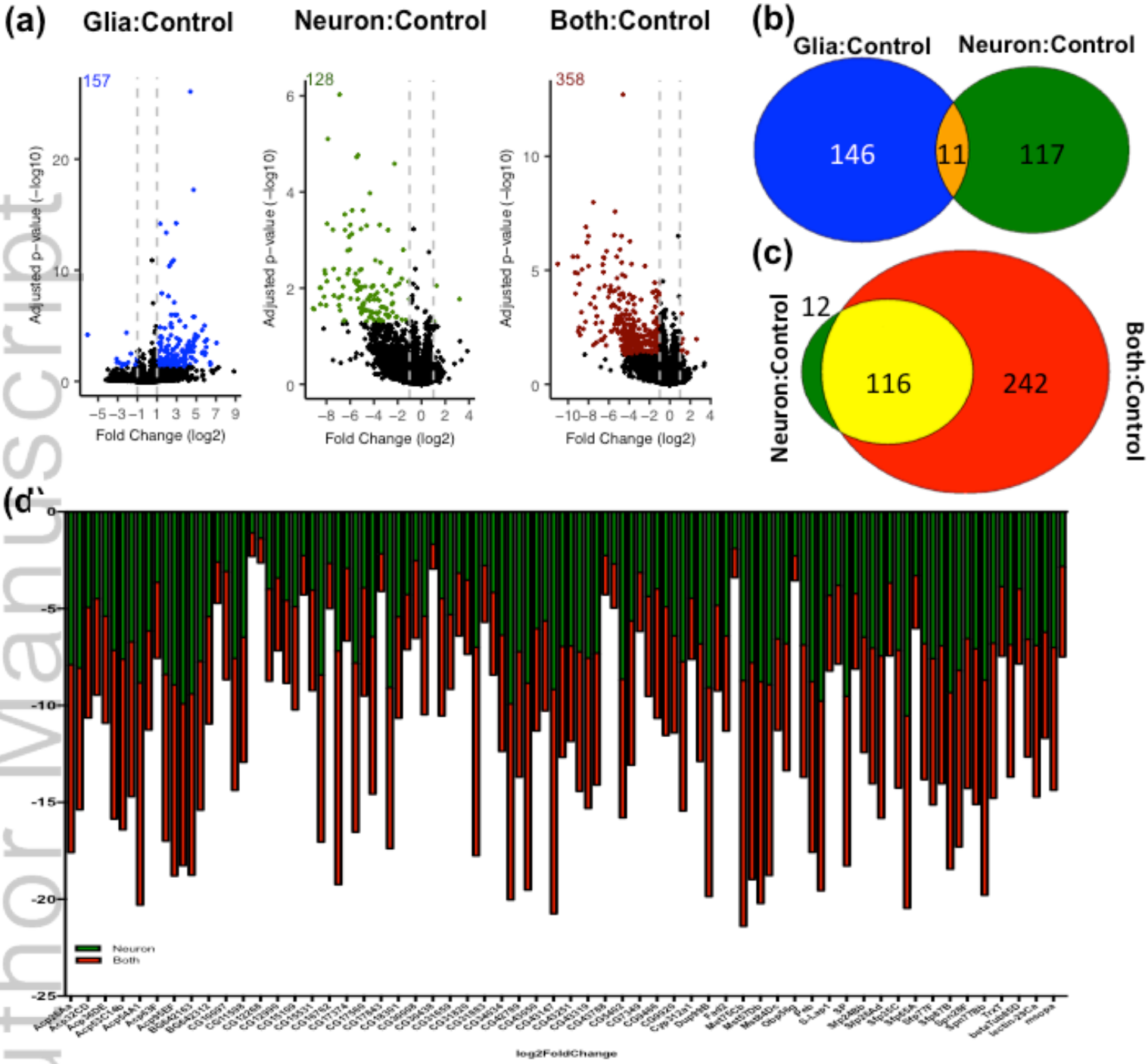


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(a)

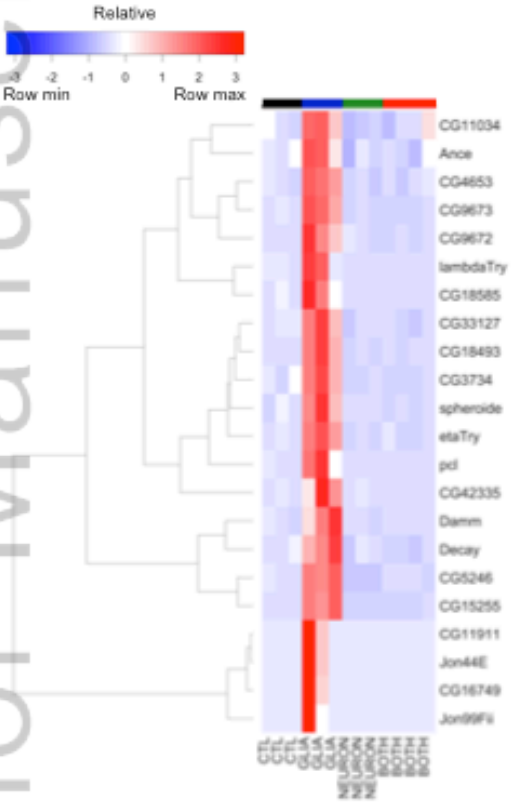
Total=158

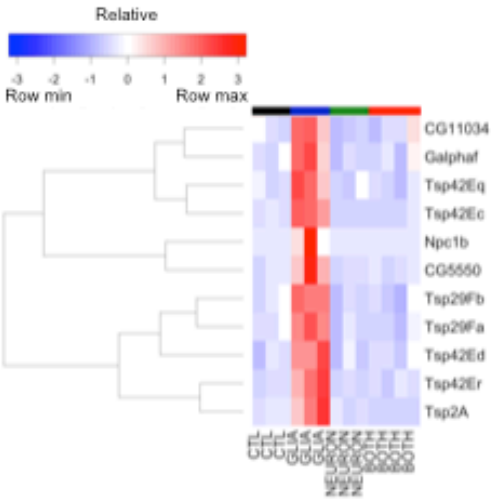
13 Repressed

145 Induced

| 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)

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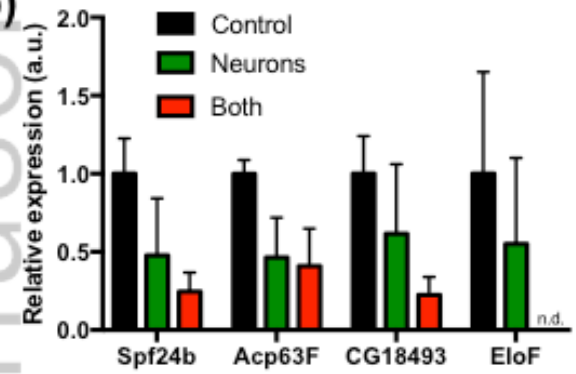
(a)

| 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 |

(b)

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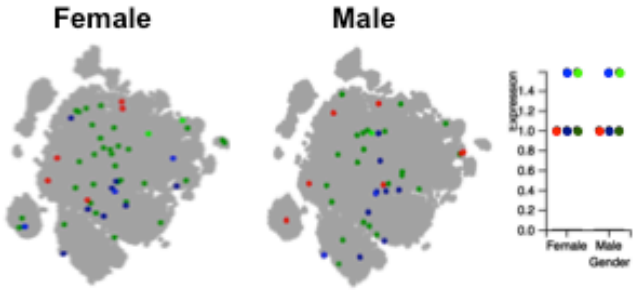
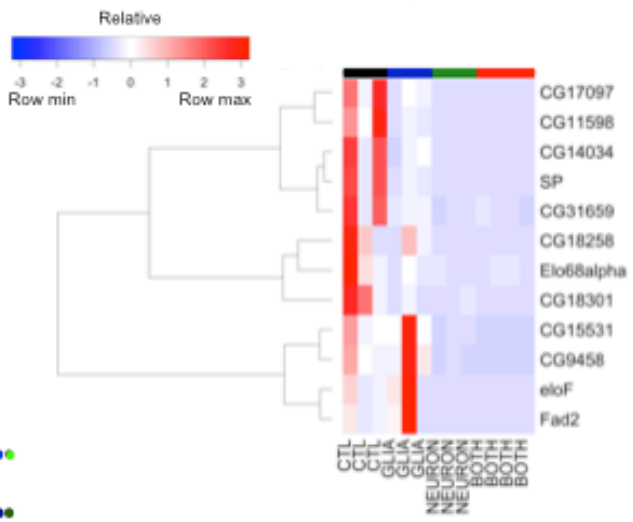



Red: Acp36De
Green: Acp53c14b
Blue: Sfp77f

(d)

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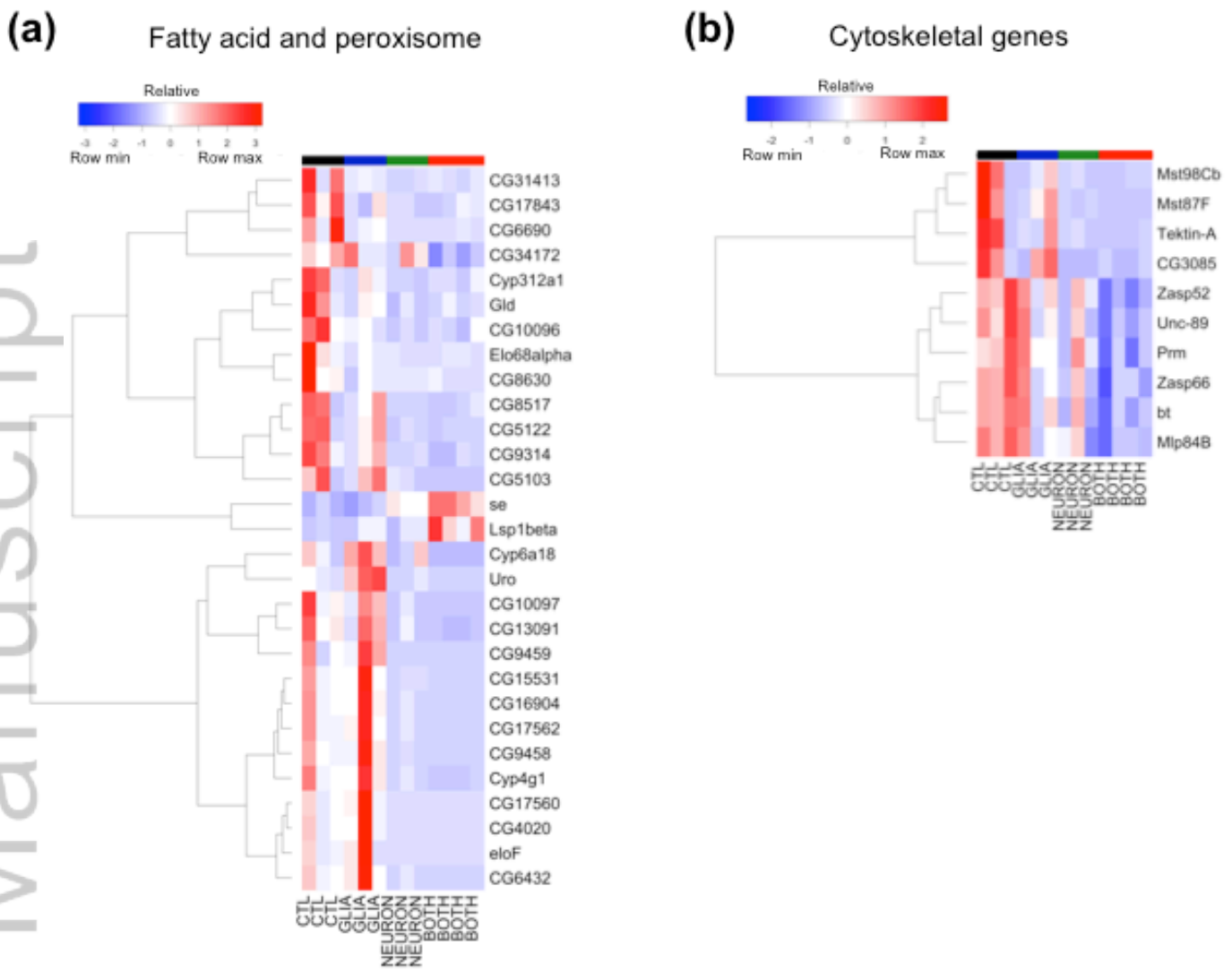


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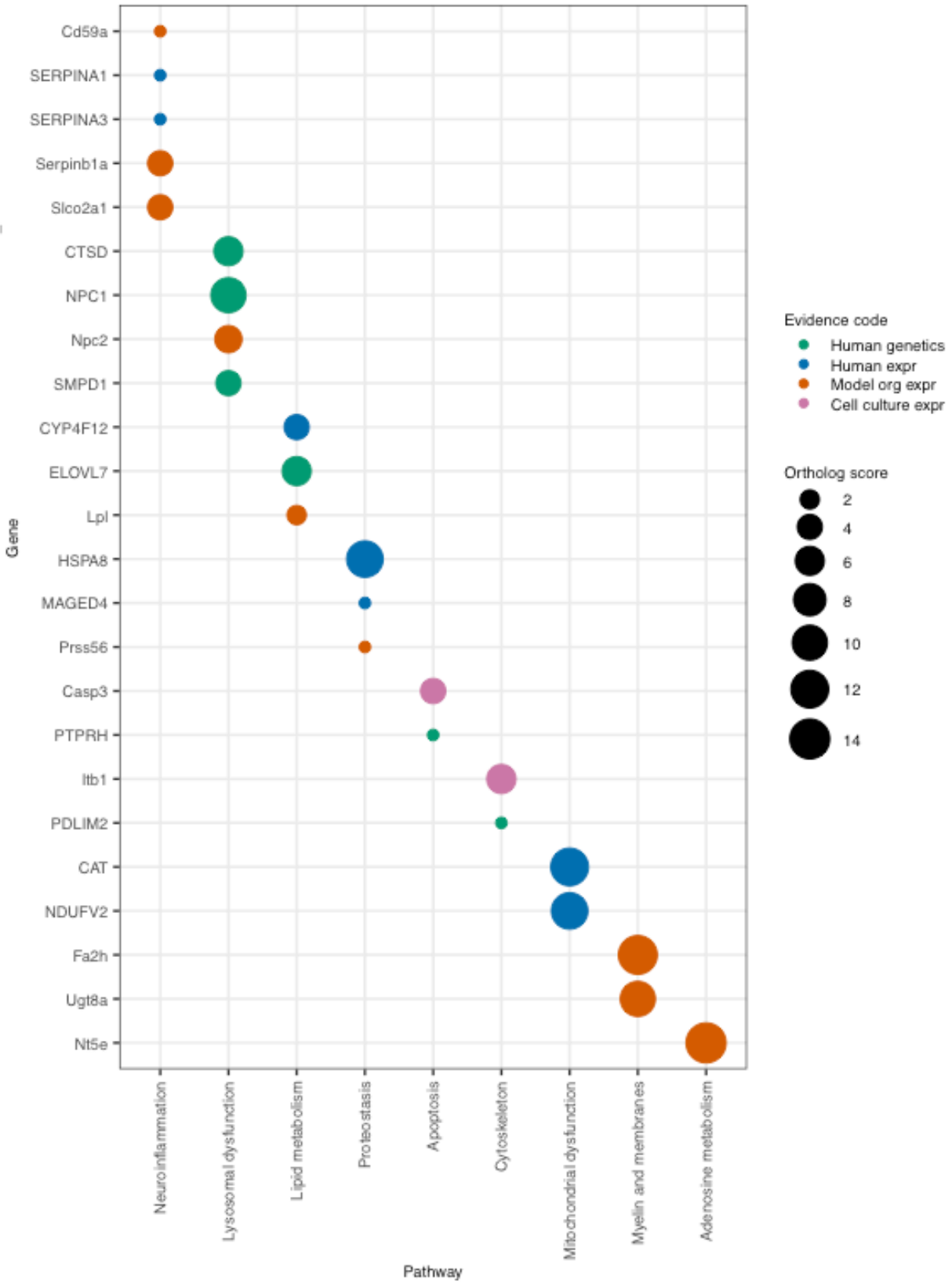


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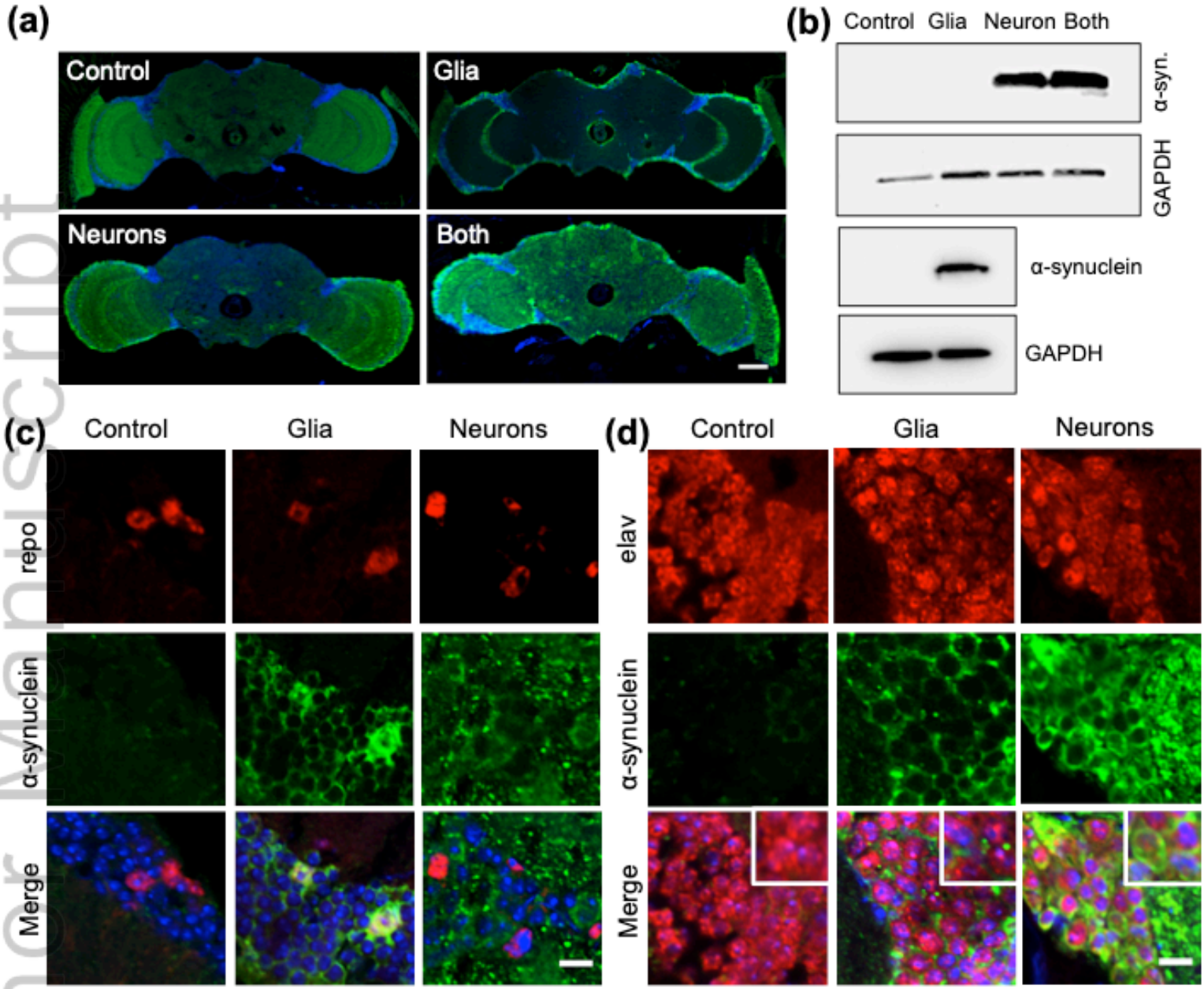


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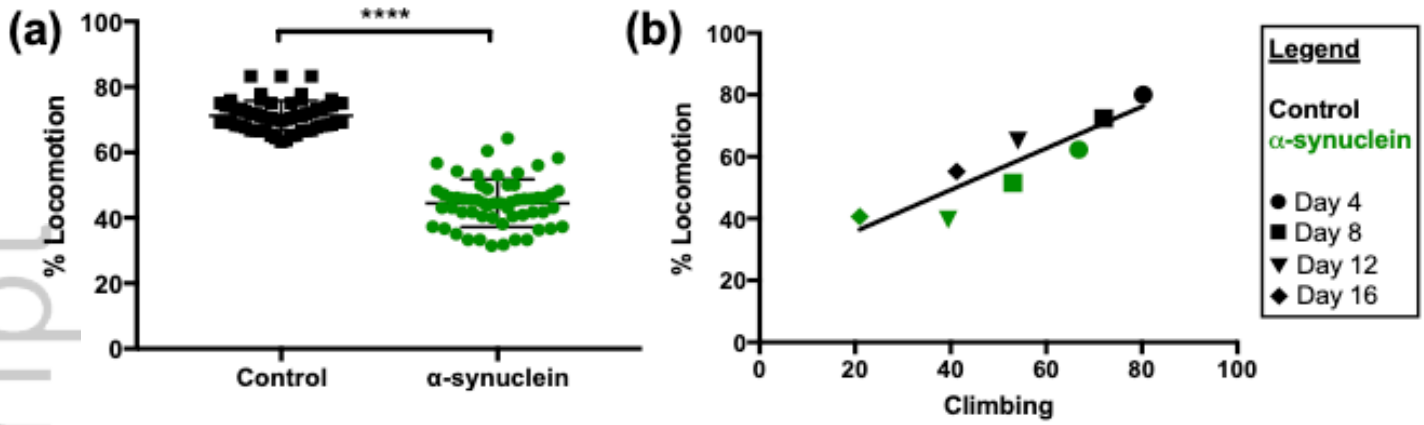


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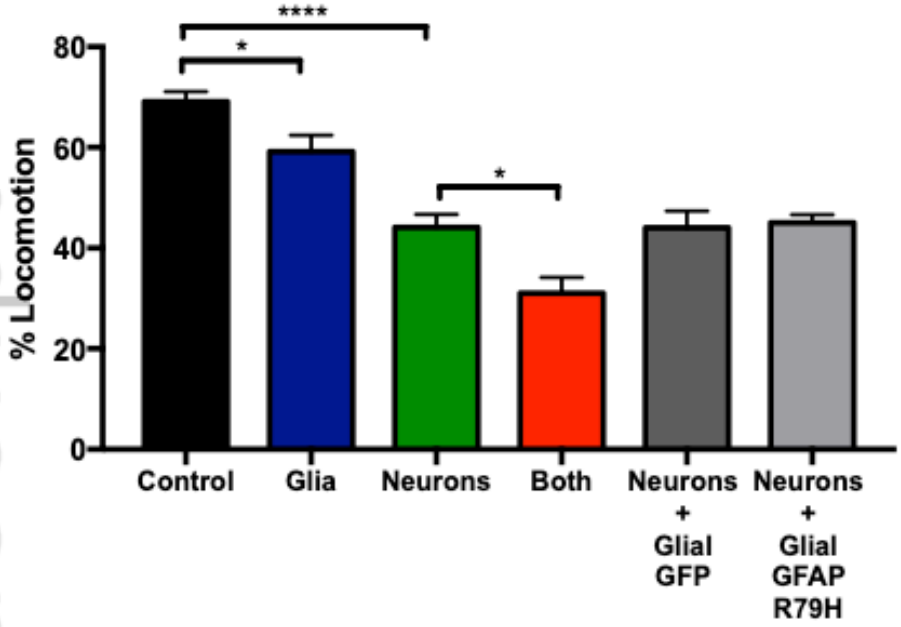


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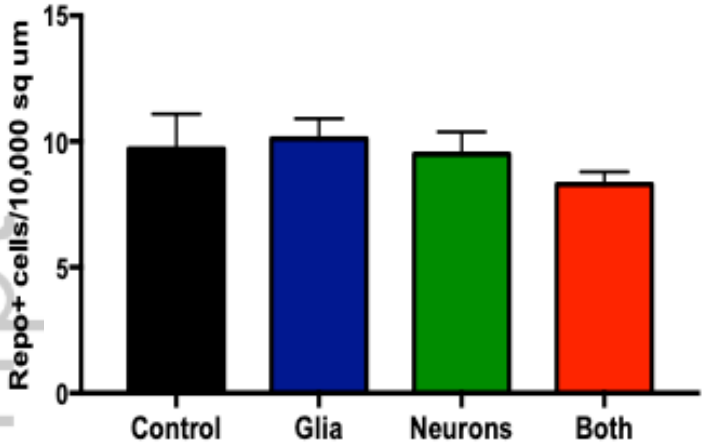


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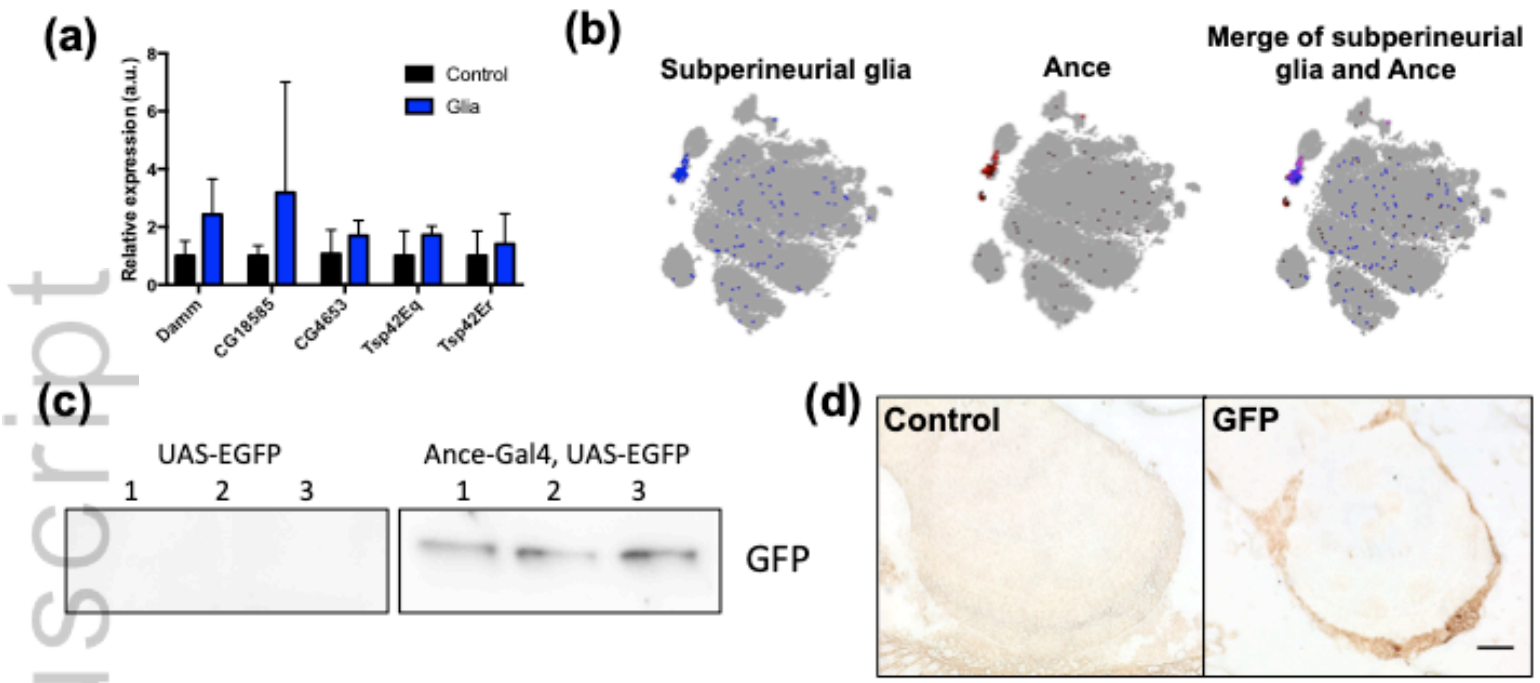


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