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Parkinson's disease risk genes act in glia to control neuronal α -synuclein toxicity

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Abstract

Idiopathic Parkinson's disease is the second most common neurodegenerative disease and is estimated to be approximately 30% heritable. Genome wide association studies have revealed numerous loci associated with risk of development of Parkinson's disease. The majority of genes identified in these studies are expressed in glia at either similar or greater levels than their expression in neurons, suggesting that glia may play a role in Parkinson's disease pathogenesis. The role of individual glial risk genes in Parkinson's disease development or progression is unknown, however. We hypothesized that some Parkinson's disease risk genes exert their effects through glia. We developed a *Drosophila* model of α -synucleinopathy in which we can independently manipulate gene expression in neurons and glia. Human wild type α -synuclein is expressed in all neurons, and these flies develop the hallmarks of Parkinson's disease, including motor impairment, death of dopaminergic and other neurons, and α -synuclein aggregation. In these flies, we performed a candidate genetic screen, using RNAi to knockdown 14 well-validated Parkinson's disease risk genes in glia and measuring the effect on locomotion in order to identify glial modifiers of the α -synuclein phenotype. We identified 4 modifiers: *aux*, *Lrrk*, *Ric*, and *Vps13*, orthologs of the human genes *GAK*, *LRRK2*, *RIT2*, and *VPS13C*, respectively. Knockdown of each gene exacerbated neurodegeneration as measured by total and dopaminergic neuron loss. Knockdown of each modifier also increased α -synuclein oligomerization. These results suggest that some Parkinson's disease risk genes exert their effects in glia and that glia can influence neuronal α -synuclein proteostasis in a non-cell-autonomous fashion. Further, this study provides proof of concept that our novel *Drosophila* α -synucleinopathy model can be used

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Supplementary data

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to study glial modifier genes, paving the way for future large unbiased screens to identify novel glial risk factors that contribute to PD risk and progression.

Keywords

Parkinson's disease; Glia; GWAS; LRRK2; Autophagy

1. Introduction

Parkinson's disease is the second most common neurodegenerative disorder and is defined by α -synuclein aggregation and death of dopaminergic neurons. Approximately 5–10% of Parkinson's disease is monogenic.(Lesage and Brice, 2009) To date, point mutations in 20 genes have been reported as causative of familial Parkinson's disease, (Kim and Alcalay, 2017; Blauwendraat et al., 2020) although of these 20, only *LRRK2* and *GBA* mutations are relatively common. Beyond familial Parkinson's disease, so called "idiopathic" Parkinson's disease is also estimated to be 30% heritable,(Keller et al., 2012) and genome wide association studies (GWAS) have now identified nearly 100 loci associated with risk of idiopathic Parkinson's disease.(Nalls et al., 2019) GWAS for Parkinson's disease demonstrate high face validity, in that several of the same genes that cause monogenic forms of Parkinson's disease when mutated are also GWAS hits, including α -synuclein (*SNCA*), *LRRK2*, *GBA*, and *Vps13C*, suggesting that common variants in these genes influence risk of "idiopathic" Parkinson's disease.

There are many challenges in moving from GWAS results to a biological understanding of disease, however. First, GWAS identify variations in allele frequency in single nucleotide polymorphisms (SNPs), which may or may not regulate expression of nearby genes. Thus, there is a need to combine GWAS with expression quantitative trait loci (eQTL) analysis to prioritize potentially causative genes.(Li et al., 2019; Kia et al., 2019; Grenn et al., 2020) Second, GWAS are agnostic to the cell type of origin of disease and may reflect signals coming from diverse neuronal(Nalls et al., 2019) and non-neuronal(Kia et al., 2019; Reynolds et al., 2019) cell types within the brain, as well as cells outside of the brain. (Reynolds et al., 2019; Pierce and Coetzee, 2017; Coetzee et al., 2016; Gagliano et al., 2016) Although computational analyses are invaluable tools in addressing these questions, even the best approaches available today are dependent on the input data, which may be limited. Thus, there is a strong need for mechanistic studies to validate the role of GWAS nominated genes in disease pathogenesis and to determine in which cell types they are exerting their effects.(Pierce et al., 2020)

Such mechanistic studies rely on model organisms, and emerging studies in primary mouse cells as well as human induced pluripotent stem cell (iPSC) derived models support a pathogenic role for some Parkinson's disease GWAS candidate genes in glia, particularly astrocytes. Eight of the genes implicated in monogenic Parkinson's disease have a known function in astrocytes,(Booth et al., 2017) including *LRRK2* and *GBA*, which are also GWAS hits. *LRRK2 G2019S* expressing iPSC-derived astrocytes have downregulated expression of extracellular matrix proteins,(Booth et al., 2019) accelerated

ER stress,(Lee et al., 2019) and impaired autophagy leading to non-cell-autonomous neurodegeneration.(di Domenico et al., 2019) iPSC-derived astrocytes carrying different neuropathic or nonneuropathic *GBA* mutations have varying degrees of astrogliosis and lysosomal dysfunction,(Aflaki et al., 2020) and *GBA D409V* knockin mouse astrocytes demonstrate lysosomal morphology and functional defects as well as defects in cytokine production, which, interestingly, were normalized by inhibition of *LRRK2* kinase activity, (Sanyal et al., 2020) suggesting potential crosstalk between *LRRK2* and *GBA* within astrocytes. These cell culture studies have provided important mechanistic insight regarding the function of *LRRK2* and *GBA* in astrocytes, but little is known about their role in glia *in vivo*. Additionally, the time-intensive nature of iPSC-based co-culture systems makes a higher throughput approach to glial gene investigation challenging. Finally, little is known about the role of Parkinson's disease GWAS candidate genes in other glial cells beyond astrocytes.

To address these challenges and investigate the role of Parkinson's disease GWAS candidates in glia *in vivo* in a systematic manner, we developed a novel *Drosophila* Parkinson's disease model(Ordonez et al., 2018) in which gene expression can be independently manipulated in neurons and glia(Olsen and Feany, 2019) using the (Potter et al., 2010) and UAS-Gal4 expression systems. (Brand and Perrimon, 1993) In this model, human wild type α -synuclein is expressed in all neurons, and flies develop neurodegeneration, loss of dopaminergic neurons, and motor dysfunction. We performed a candidate screen, knocking down a panel of well-validated GWAS nominated genes in glia in order to identify those that enhanced neuronal α -synuclein toxicity. Specifically, we identified *Drosophila* orthologs of *LRRK2*, *GAK*, *Vps13C*, and *RIT2* as glial enhancers. We confirmed that glial knockdown of these genes exacerbated neurodegeneration and loss of dopaminergic neurons. We then investigated the effects of gene knockdown on proteostasis, impairment in which underlies Parkinson's disease pathology. Interestingly, while all modifiers enhanced neurodegeneration, they had divergent effects on α -synuclein aggregation and impairment of autophagy, suggesting that different Parkinson's disease risk genes effect different cellular processes in glia and providing evidence for multiple mechanisms of non-cell-autonomous neurodegeneration.

2. Materials and methods

2.1. *Drosophila*

All fly crosses and aging were performed at 25 °C. All experiments were performed at 10 days post-eclosion unless otherwise noted in the figure legends. All experiments include both male and female flies in which wild type human α -synuclein is expressed in neurons using the pan-neuronal driver *neuronal-synaptobrevin (nSyb)-QF2*. Control flies include the driver but lack transgenic human α -synuclein. Additionally, flies contain either the pan-glial driver *repo-Gal4* or the astrocyte-like glia driver *alm-Gal4* for knockdown of genes of interest.

Transgenic RNAi stocks were obtained from the Bloomington *Drosophila* Stock Center and include *UAS-Vps13 RNAi HMS01715* (Vps13 RNAi #1), *UAS-Vps13 RNAi HMS02460* (Vps13 RNAi #2), *UAS-aux RNAi HMS01935* (aux RNAi #1), *UAS-aux RNAi GL00213*

(aux RNAi #2), *UAS-Ric RNAi GL01247* (Ric RNAi #1), *UAS-Ric RNAi JF02670* (Ric RNAi #2), *UAS-fray RNAi HMJ02228* (fray RNAi #1), *UAS-fray RNAi HMC04151* (fray RNAi #2), *UAS-Syt4 RNAi HMS01934* (Syt4 RNAi #1), and *UAS-Syt4 RNAi JF02272* (Syt4 RNAi #2). The *UAS-Lrrk RNAi #22139* (Lrrk RNAi #2) transgenic line was obtained from Vienna *Drosophila* Resource Center. The following *Drosophila* stocks were kindly provided by the indicated investigators: 1. *nSyb-QF2* by Dr. Christopher Potter, 2. *alrm-Gal4* by Dr. Marc Freeman, 3. *UAS-Lrrk RNAi #1-1-37* (Lrrk RNAi #1) by Dr. Bingwei Lu. Most transgenic strains used in the study were created using *w* as a selectable marker and thus carry a *w* mutation on the X chromosome. Transgenic RNAi lines created by the Transgenic RNAi Project (TRiP) at Harvard Medical School were created in a *y¹ v¹* (*HMS01935*, *GL01247*, *JF02670*, *HMJ02228*, *HMS01934*, *JF02272*) or *y¹ sc v¹* (*HMS01715*, *HMS02460*, *GL00213*, *HMC04151*) genetic background.

Two RNAi lines were used to identify locomotor deficits in the genetic screen, and a single RNAi was used for all subsequent confirmatory studies. The RNAi lines used in the confirmatory studies were *UAS-Vps13 RNAi HMS02460*, *UAS-aux RNAi GL00213*, *UAS-Lrrk RNAi #1-1-37*, and *UAS-Ric RNAi JF02670*. Control RNAi lines used to confirm phenotype specificity were *UAS-mCherry RNAi*, *UAS-EGFP RNAi*, and *UAS-Luciferase RNAi* from the Transgenic RNAi Project (TRiP).

2.2. Immunohistochemistry and immunofluorescence

Flies were fixed in formalin and embedded in paraffin. Either 2 or 4 μ m 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 or pressure cooker 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 h 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 tyrosine hydroxylase (1:200 to 1:500, mouse, Immunostar), α -synuclein 5G4 (1:50,000, mouse, Millipore), ref. (Kim and Alcalay, 2017) p/p62 (1:5000 rabbit Sarkar et al., n.d.), Atg8a/LC3 (clone EIJ4E, 1:2000, rabbit, Cell Signaling). For immunohistochemistry, slides were incubated in biotin-conjugated secondary antibodies in 2% milk in PBS with 0.3% triton X-100 for 1 h (1:200, Southern Biotech) followed by avidin-biotin-peroxidase complex (Vectastain Elite) in PBS for 1 h. Histochemical detection was performed with diaminobenzidine (ImmPACT DAB, Vector). For immunofluorescence, slides were incubated with fluorophore-conjugated secondary antibodies in 2% milk in PBS with 0.3% triton X-100 for 1 h (1:200, Alexa 488 or Alexa 555, Invitrogen) then mounted with DAPI-containing Fluoromount medium (Southern Biotech). Immunofluorescence microscopy was performed on a Zeiss LSM 800 confocal microscope. Images were processed using Fiji.

2.3. Quantification of total cell counts

Single slice images of the anterior medulla from hematoxylin-stained formalin-fixed paraffin-embedded tissue were captured at 40 \times magnification using brightfield microscopy. One image (slice) per fly and 6 flies per genotype were used for quantification. The number of nuclei in each tissue section was counted and normalized to the area of the section.

2.4. Quantification of TH+ neuron counts and α -synuclein aggregates

Single slice images of the anterior medulla from immunofluorescence-stained formalin-fixed paraffin-embedded tissue were captured on a confocal microscope at 63 \times magnification. One image (slice) per fly and 6 flies per genotype were used for quantification. The number of TH+ cells or α -synuclein aggregates in each tissue section was counted and normalized to the area of the section.

2.5. Western blotting

Fly heads were dissected then homogenized in 2 \times Laemmli buffer, boiled for 10 min, 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 h, then immunoblotted with appropriate primary antibody in 2% milk in PBS with 0.05% Tween-20 overnight at 4 $^{\circ}$ C. Primary antibodies used include α -synuclein H3C (1:10,000 to 1:100,000, mouse, Developmental Studies Hybridoma Bank), α -synuclein clone 42 (1:5000 mouse, BD Bioscience), and phospho-serine 129 α -synuclein (1:5000, rabbit, Abcam). 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 h. Signal was developed with enhanced chemiluminescence (Thermo Scientific).

2.6. Oligomer assay

20 fly heads per genotype were homogenized in 20 μ l TNE lysis buffer (10 mM Tris HCl, 150 mM NaCl, 5 mM EGTA, 0.5% nonidet-40) supplemented with HALT protease and phosphatase inhibitor (Roche). The homogenate was briefly spun down to remove debris. The remaining supernatant was ultracentrifuged at 100,000 \times g for 1 h at 4C. The supernatant was transferred to a new tube and combined with 2 \times Laemmli buffer at a 1:1 ratio. SDS-PAGE was then performed as above, except without boiling samples and without microwave antigen retrieval.

2.7. Locomotion assay

Adult flies were aged in vials containing 9–14 flies per vial. At day 10 post-eclosion, 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 were measured and statistical significance assessed by one-way ANOVA.

2.8. Statistics

All statistical analysis aside was performed using GraphPad Prism version 7.0a.

2.9. Data availability

The data that support the findings of this study are available from the corresponding author upon request.

3. Results

3.1. Parkinson's disease risk genes are expressed in glia

In the most current Parkinson's disease GWAS meta-analysis, Nalls et al. identify 90 independent loci associated with risk of Parkinson's disease. (Nalls et al., 2019) Using publicly available human bulk RNAseq data, (Zhang et al., 2016) we examined expression of the nearest candidate gene for each locus across neurons, fetal astrocytes, mature astrocytes, microglia, oligodendrocytes and endothelial cells (Fig. 1). In some instances, two loci mapped to the same nearest gene. Further, 2 genes were not in the Zhang et al. (Zhang et al., 2016) database (*GS1-124 K5.11*, *LINC00693*) and an additional 7 genes were in the database but not meaningfully expressed by any of the brain cell populations (*CASC16*, *CD19*, *CRHR1*, *HLA-DRB5*, *SPPL2B*, *TRIM40*, *WNT3*). Among the remaining 78 genes, only 22 genes (28.5% of the total) were most highly expressed in neurons, and of those, for only 6 genes (*SH3GL2*, *INPP5F*, *SNCA*, *ITGA8*, *PAM*, and *DLG2*) was the neuronal expression greater than 50% of the total expression of that gene. Thus, many genes implicated in Parkinson's disease risk are expressed predominantly in glia or somewhat ubiquitously throughout the brain. A similar distribution between neuronal *versus* non-neuronal predominant expression was seen among the genes identified by Nalls et al. (Nalls et al., 2019) as likely expression quantitative loci (indicated by diamond symbol in Fig. 1).

3.2. A *Drosophila* model for identifying glial modifiers

We hypothesized that some Parkinson's disease GWAS candidate genes might exert their effects in glia. To investigate this, we developed a *Drosophila* model of α -synucleinopathy (Ordonez et al., 2018) in which it is possible to independently manipulate gene expression in glia. (Olsen and Feany, 2019) The flies express human α -synuclein in all neurons, and we performed a candidate screen in order to identify glial enhancers of neuronal α -synuclein toxicity. In glia, we knocked down the *Drosophila* orthologs of 14 strong GWAS candidate genes (14 genes with genome wide significance, (Nalls et al., 2014) $p < 1 \times 10^{-8}$, Table 1) and measured the effect on locomotion. *MAPT* and *DDRGK1* were not included in the screen due to having expression largely restricted to neurons and lack of available reagents, respectively.

Six genes were identified as enhancers in the initial screen: *aux*, *fray*, *Lrrk*, *Ric*, *Syt4*, and *Vps13*. Of these, 4 were selected for further analyses based on amplitude of effect and confirmation with a second RNAi line. *Syt4* was excluded because there was only modest (though statistically significant) enhancement of the phenotype, not meeting the predetermined threshold of biological significance of 25% decrement in locomotion (Supplemental Fig. 1A). *fray* was excluded because the second RNAi line resulted in significant toxicity in control flies (Supplemental Fig. 1B). In the case of *aux*, there was significant toxicity in control flies when the gene was knocked down in all glia using the pan-glial *repo* driver (data not shown). Therefore, the astrocyte-like glia driver *alm-Gal4* was used for all subsequent studies. Thus, the final experimental conditions were pan-glial knockdown of *Lrrk*, *Ric*, and *Vps13* (Fig. 2A), and astrocyte-like glia knockdown of *aux* (Fig. 2B). To confirm that the effect on behavior was specific and not due to genetic

background effects, we also expressed multiple control RNAi constructs in glia, finding no significant decrement in locomotion (Supplemental Fig. 1C). Additionally, we validate the knockdown of each gene by qRT-PCR (Supplemental Fig. 2).

3.3. Knockdown of glial genes enhances neurodegeneration through non-cell-autonomous effects on proteostasis

We next sought to confirm that the knockdown of the glial modifiers enhanced locomotor impairment due to enhanced neurodegeneration, rather than through a symptomatic effect on behavior. We quantified both total numbers of cells (Fig. 3A–B) and the number of dopaminergic neurons (Fig. 3C–D) in the anterior medulla, confirming a decrease in both. The anterior medulla is an easily identified, bilateral anatomical structure with robust pathology and many cell bodies, the vast majority of which are neuronal. (Raji and Potter, 2021)

To determine the mechanism of neurodegeneration, we turned to α -synuclein. Interestingly, although knockdown of each glial modifier enhanced neurodegeneration, only *Lrrk* and *Ric* knockdown led to an increase in the numbers of large α -synuclein aggregates, whereas *Vps13* knockdown did not affect the number of aggregates (Fig. 4A, C), and *aux* knockdown led to a decrease in aggregates (Fig. 4B, D). To confirm that none of the modifiers altered expression of the α -synuclein transgene, we measured α -synuclein by immunoblotting at day 1 post-eclosion. Additionally, none of the modifiers affected baseline phosphorylation of α -synuclein at serine 129 (Supplemental Fig. 3).

There has been extensive debate regarding which molecular species of α -synuclein are pathogenic, with some evidence suggesting that α -synuclein oligomers or fibrils, rather than Lewy bodies or large Lewy body-like inclusions, drive pathology (for recent reviews, see (Bengoa-Vergniory et al., 2017; Alam et al., 2019)). We therefore examined high molecular weight species of α -synuclein by immunoblotting, finding that knockdown of each glial modifier led to an increase in α -synuclein oligomers (Fig. 5). Collectively, these data suggest that manipulation of GWAS candidate genes in glia can enhance neurodegeneration in a non-cell-autonomous manner. Further, they suggest that α -synuclein oligomers, rather than large aggregates, are responsible for this effect.

3.4. Different glial modifiers affect neuronal proteostasis through different mechanisms

To further explore the effects of glial modifiers on proteostasis, we examined the lysosomal-autophagy system. As we have previously demonstrated, (Sarkar et al., n.d.) autophagic flux is impaired in the α -synuclein-expressing flies, leading to accumulation of autophagosomes. Autophagic flux and autophagosomes can be measured in *Drosophila* by immunostaining for ref. (Kim and Alcalay, 2017)p and Atg8a, *Drosophila* orthologs of p62 and LC3, respectively. LC3 is involved in phagophore and autophagosome formation as well as selection of targets for degradation, and p62 mediates degradation of ubiquitinated proteins by binding to ubiquitin and LC3. Glial *Lrrk* knockdown led to a further increase in Atg8a (LC3) and ref. (Kim and Alcalay, 2017)p (p62) puncta beyond that induced by neuronal α -synuclein alone. In contrast, knockdown of *Vps13* and *Ric* had no effect on these markers, and knockdown of *aux* decreased them (Fig. 6), suggesting divergent effects of

the modifiers on autophagy. The increase or decrease in Atg8a (LC3) and ref. (Kim and Alcalay, 2017) p (p62) puncta seen with glial *Lrrk* knockdown and glial *aux* knockdown, respectively, appears to be occurring in neurons, as the puncta co-localize with α -synuclein (Supplemental Fig. 4). We have previously demonstrated that knockdown of *Lrrk* directly in neurons exacerbates the impairment in autophagy induced by α -synuclein, (Sarkar et al., n.d.) suggesting that inhibiting *Lrrk* expression causes similar downstream cell biological effects in neurons regardless of whether the inhibition occurs in neurons or glia.

In summary, we identified 4 Parkinson's disease GWAS candidate genes that enhance neuronal α -synuclein induced neurodegeneration in a non-cell-autonomous manner when knocked down in glia. Despite having divergent effects on the accumulation of α -synuclein aggregates, knockdown of each modifier resulted in increased α -synuclein oligomerization, supporting a role for α -synuclein oligomers as drivers of neurotoxicity and suggesting that glia regulate neuronal proteostasis. Further, in the case of *Lrrk* knockdown, we identify impaired autophagy as a potential upstream mechanism, consistent with the known role of *LRRK2* (Giaime et al., 2017; Gómez-Suaga et al., 2012; Orenstein et al., 2013; Plowey et al., 2008; Tong et al., 2012) and with our prior work on neuronal *Lrrk* (Sarkar et al., n.d). Interestingly, knockdown of the other modifiers did not similarly cause an increase in autophagosomes, suggesting that different glial modifiers act through different, specific, mechanisms, rather than simply due to non-specific glial injury or toxicity.

4. Discussion

We and others have previously demonstrated neuronal functions for some Parkinson's disease GWAS candidate genes, including *SNCA*, *LRRK2*, *MAPT*, and *GBA* (Bardai et al., 2018a,b; Blauwendraat et al., 2020; Sarkar et al., 2020; Tran et al., 2020). Here, we suggest that 4 well-validated Parkinson's disease GWAS candidate genes (*LRRK2*, *GAK*, *VPS13C*, *RIT2*) may influence Parkinson's disease pathogenesis at least partially by acting through glia, specifically by influencing glial control of neuronal proteostasis. Proteostasis refers to multiple coordinated homeostatic mechanisms by which cells maintain a healthy and functional proteome. It includes processes that control protein synthesis and folding, disaggregation, and degradation. (Labbadia and Morimoto, 2015) These processes are widely disturbed across the neurodegenerative proteinopathies, (Klaips et al., 2018) likely in part due to a complex feed-forward cycle in which aggregation-prone proteins such as α -synuclein induce proteostatic dysfunction, and this dysfunction in turn exacerbates the pathogenic cascade of α -synuclein misfolding, oligomerization, fibrilization, and aggregation. (Han et al., 2020) The individual steps of this cascade may be controlled by different proteostatic processes, and work in cell culture suggests that different species of α -synuclein may be differentially degraded by the proteasome, macroautophagy, and chaperone mediated autophagy. (Xilouri et al., 2008; Vogiatzi et al., 2008; Gao et al., 2019; Stefanis et al., 2019) Similarly, Prasad et al. found in *Drosophila* that soluble α -synuclein oligomers were removed by the proteasome, (Prasad et al., 2019) whereas insoluble fibrils and aggregates were removed by autophagy, suggesting that different mechanisms are also at play *in vivo*. Here, we find that knockdown of all 4 glial modifiers led to an increase in α -synuclein oligomers, with varying effects on large Lewy-body-like inclusions (Figs. 4, 5, 7). Given that different proteostatic systems (the proteasome, macro-autophagy,

and chaperone-mediated autophagy) are responsible for clearance of different α -synuclein species in neurons, it is possible that individual glial modifiers influence these systems to varying extents.

Non-cell-autonomous control of proteostasis is not well understood. Emerging evidence suggests that glia can alter neuronal autophagy in a variety of diseases, (Kulkarni et al., 2018; Alirezaei et al., 2008; Madill et al., 2017) including in the A53T α -synuclein mouse model of Parkinson's disease. (Gan et al., 2012) This is consistent with our findings for glial *Lrrk* knockdown, which led to an increase in markers of autophagosomes and impaired autophagic flux (Fig. 6, Fig. 7). Our finding that the other glial modifiers increase α -synuclein oligomers without seemingly impairing neuronal autophagy suggests that different modifiers can exert their effects on proteostasis through different upstream mechanisms. Beyond macro-autophagy, there are numerous other cellular processes that affect α -synuclein toxicity, including proteasome function, interactions with chaperones (Burmam et al., 2020) that prevent misfolding or induce chaperone-mediated autophagy, phosphorylation (Zhao et al., 2020; Pinho et al., 2019) and other (Ho et al., 2021) post-translational modifications of α -synuclein, and subcellular localization (Pinho et al., 2019) of α -synuclein. Whether glia can indirectly affect these processes is unknown. Interestingly, one recent study suggests an additional mechanism of non-cell-autonomous control of α -synuclein proteostasis. That is, neuronal α -synuclein aggregation can activate the "immunoproteasome," an inducible form of the proteasome that is assembled in response to inflammatory stimuli, (Driscoll et al., 1993; Seifert et al., 2010) in both neurons and glia. (Ugras et al., 2018) It is therefore possible that changes in proteasome composition or flux in either neurons or glia underlie the effect of other modifiers, particularly in light of the proposed role of the proteasome in clearing oligomers within neurons, as discussed above. (Xilouri et al., 2008; Vogiatzi et al., 2008; Gao et al., 2019; Stefanis et al., 2019; Prasad et al., 2019)

There has been much scientific debate regarding which α -synuclein molecular species are responsible for neurotoxicity. (Lansbury and Lashuel, 2006; Lashuel et al., 2013) Here we find that α -synuclein oligomers, rather than the large Lewy-body-like inclusions, were influenced by glial gene manipulation, and the increase in these species was correlated with increased neurodegeneration. This result is in line with prior work (Chen et al., 2009) using our original *Drosophila* α -synucleinopathy model, (Feany and Bender, 2000) as well as with studies in yeast, where α -synuclein toxicity occurs in the absence of Lewy-body-like inclusions. (Outeiro and Lindquist, 2003; Volles and Lansbury, 2007) α -synuclein mis-folding and oligomerization may be influenced by phosphorylation and other post-translational modifications, as has been shown in some though not all studies. (Oueslati, 2016) While we did not observe a change in baseline serine 129 phosphorylation of α -synuclein in this study, a more comprehensive analysis of post-translational modifications is beyond the scope of this manuscript. Further, a growing body of evidence suggests that not all pathologic α -synuclein conformations are created equal; different α -synuclein strains may result in different α -synucleinopathies (Shahnawaz et al., 2020) or even different prognosis within one disease. (Lau et al., 2020) Whether glia have any non-cell-autonomous influence on post-translational modifications of α -synuclein or differential α -synuclein strain formation in neurons is unknown, though it is interesting that in

multiple system atrophy, defined pathologically by glial cytoplasmic α -synuclein inclusions, oligodendrocytes may be responsible for generating a more pathogenic strain. (Peng et al., 2018) Emerging methods for detecting oligomers in human tissue should shed further light on this critical issue. (Bengoa-Vergniory et al., 2017)

In addition to highlighting the importance of non-cell-autonomous control of proteostasis in Parkinson's disease pathogenesis, our results have several important implications for the design of future studies. First, they underline the critical need to include investigation of glia in models of Parkinson's disease, as glia may directly contribute to both risk and progression of disease. Second, they suggest that specific glial genes affect Parkinson's disease pathogenesis by influencing specific pathways (*e.g.* *Lrrk* knockdown exacerbating neuronal autophagy impairment, Fig. 7) rather than by a non-specific injury or "reactive" process. Indeed, there may be innumerable different specific reactive states of glia, including both neuroprotective and detrimental states. (Smith et al., 2020; Yun et al., 2018) This additionally has implications for a future of personalized medicine, as different patients may have different disease drivers, both genetically and cellularly (Bandres-Ciga et al., 2020). Our model can serve as an experimental paradigm for further validation of GWAS candidate genes in glia as well as for dissecting these complex glial-neuronal interactions *in vivo*. This represents an essential step in getting from GWAS to an increased understanding of pathogenesis and eventually to creation of novel disease-modifying therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

GBA	Glucosylceramidase beta
GWAS	Genome wide association study
LRRK2	Leucine-rich repeat kinase 2
SNCA	α -synuclein
Vps13C	Vacuolar protein sorting 13 homolog C

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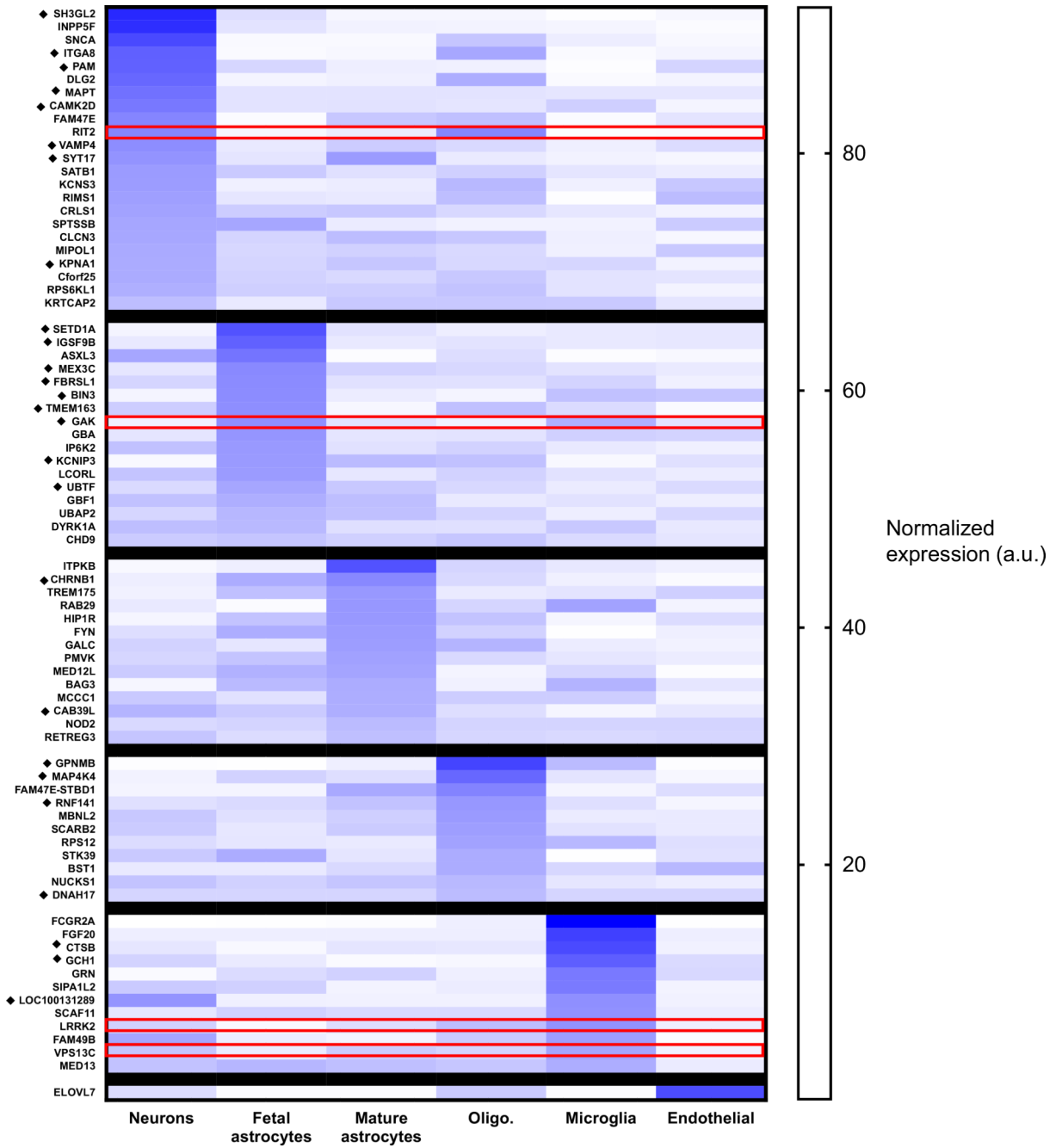


Fig. 1. Parkinson’s disease GWAS candidate genes are expressed in glia. The figure demonstrates the relative expression of Parkinson’s disease GWAS candidate genes across glial subtypes using publicly available RNAseq data: Zhang Y, Sloan SA, Clarke LE, et al. Purification and functional characterization of human astrocytes. *Neuron*. 2016. Oligo. = oligodendrocytes. The diamond symbol (◆) indicates genes identified as likely expression quantitative loci by Nalls et al. Expression pattern for the human orthologs of the *Drosophila* genes further described in this manuscript are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

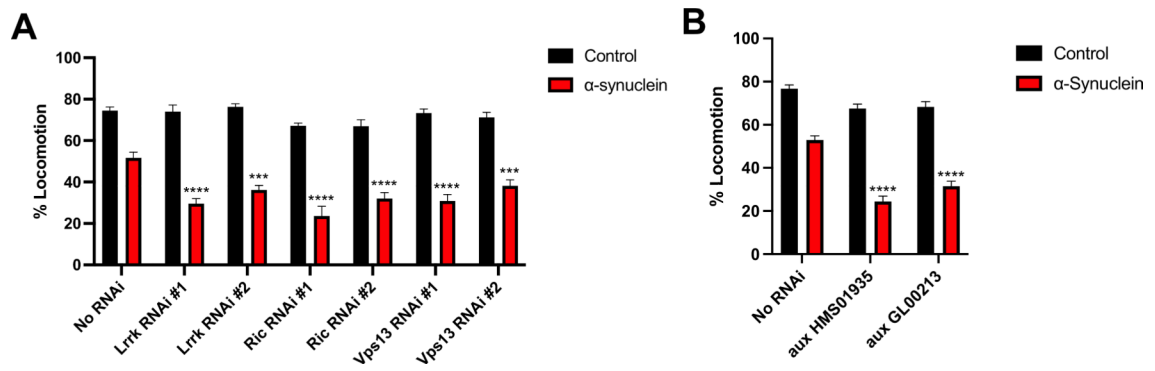


Fig. 2.

Glial knockdown of *aux*, *Lrrk*, *Ric*, and *Vps13* exacerbates the locomotor deficit in α -synuclein expressing flies. **A.** *Lrrk*, *Ric*, and *Vps13* were knocked down using the pan-glial driver *repo-Gal4*. The genotypes of control flies (black) and α -synuclein flies (red) are *nSybQF2, repo-Gal4/+*; and *QUAS- α -Syn, nSybQF2, repo-Gal4/+*, respectively. **B.** *aux* was knocked down using the astrocyte-like-glia driver *alm-Gal4*. The genotypes of control flies (black) and α -synuclein flies (red) are *nSybQF2, alm-Gal4/+*; and *QUAS- α -Syn, nSybQF2, alm-Gal4/+*, respectively. Locomotion assay was performed 10 days post-eclosion. N = minimum 60 flies per genotype, 6 biological replicates of 9–14 flies/vial. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

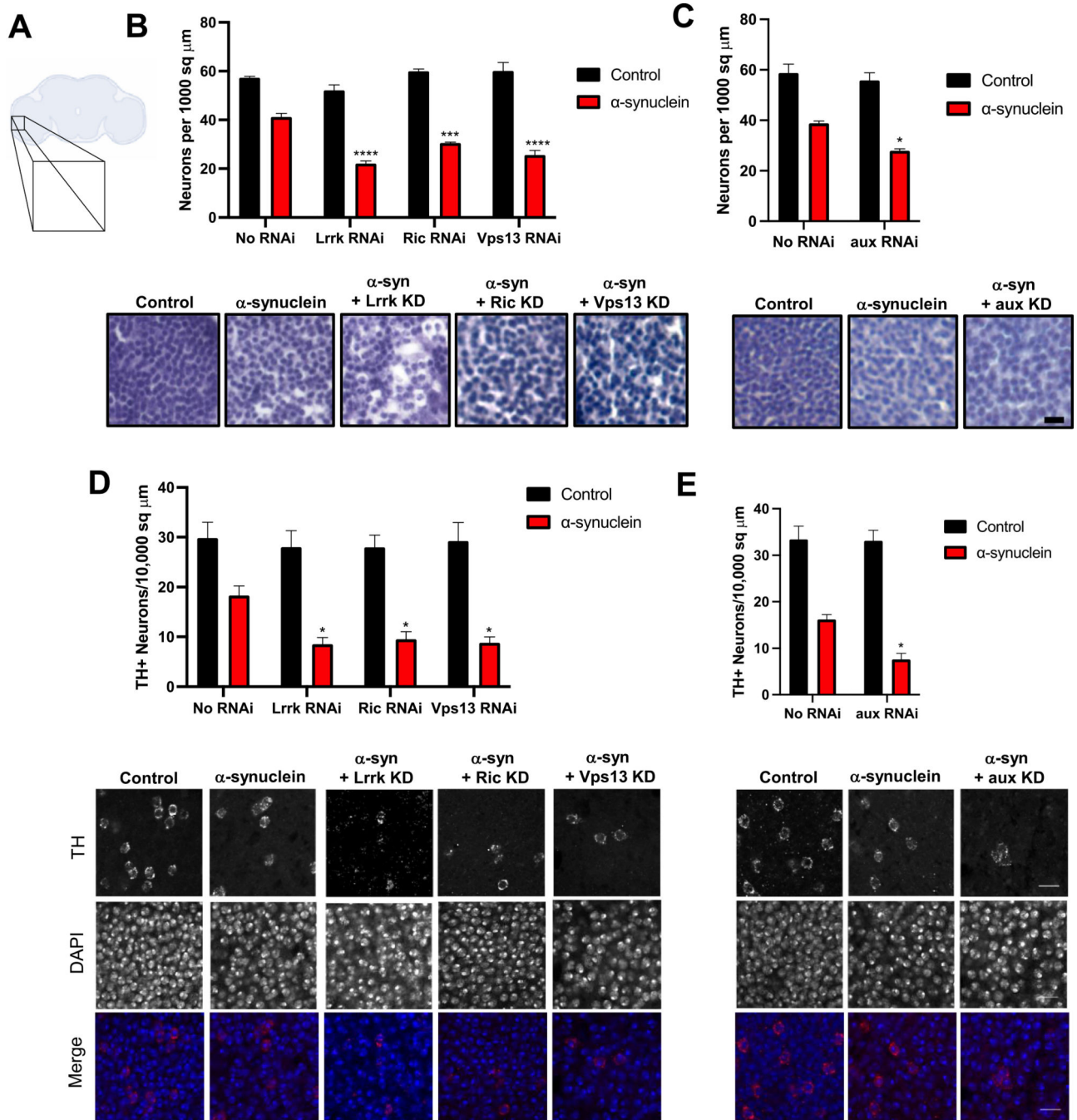
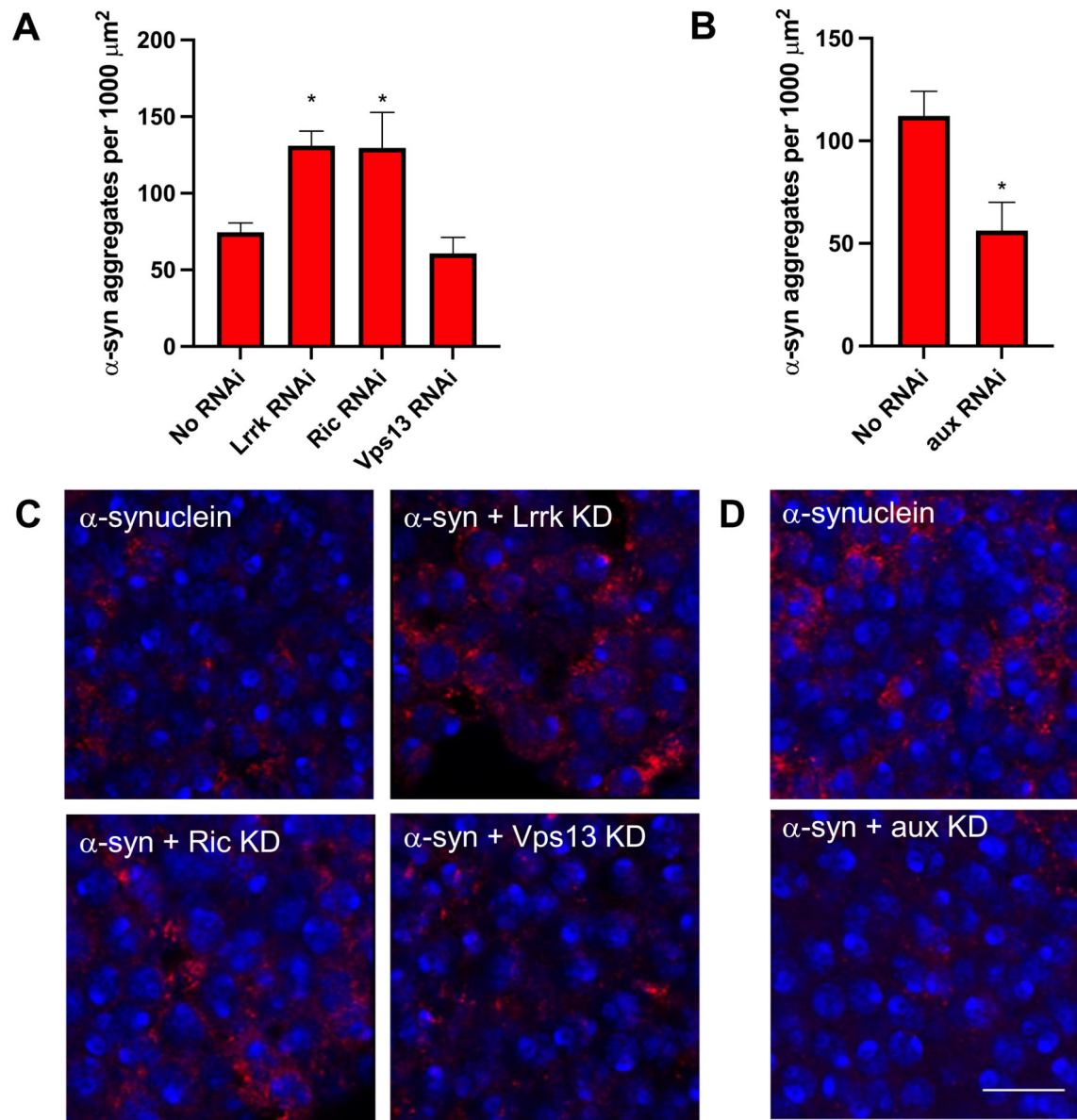


Fig. 3. Glial knockdown of *aux*, *Lrrk*, *Ric*, and *Vps13* increases α -synuclein induced neurodegeneration. A. schematic of fly brain. Inset corresponds to the medulla. B–C. Total cell counts were measured by staining with hematoxylin. D–E. Immunofluorescence was performed using anti-tyrosine hydroxylase to identify dopaminergic neurons. Nuclei are stained with DAPI. In B and D the genotypes of control flies (black) and α -synuclein flies (red) that lack an RNAi are *nSybQF2, repo-Gal4/+* and *QUAS- α -Syn, nSybQF2, repo-Gal4/+*, respectively. In C and E, the genotypes of control flies (black) and α -synuclein

flies (red) that lack an RNAi are *nSybQF2*, *alm-Gal4/+* and *QUAS- α -Syn*, *nSybQF2*, *alm-Gal4/+*, respectively. $N=6$ flies per genotype. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.001$. Scale bar = 5 μm . All experiments were performed at 10 days post-eclosion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 4.**

Glial modifiers have divergent effects on α -synuclein aggregation. α -synuclein immunofluorescence was performed using an anti- α -synuclein antibody that preferentially recognizes aggregated α -synuclein. Nuclei are stained with DAPI. In A and C the genotype of α -synuclein flies that lack an RNAi is *QUAS- α -Syn, nSybQF2, repo-Gal4/+*. In B and D the genotype of α -synuclein flies that lack an RNAi is *QUAS- α -Syn, nSybQF2, alrm-Gal4/+*. N = 6 flies per genotype. * = $p < 0.05$. Scale bar = 10 μm . All experiments were performed at 10 days post-eclosion.

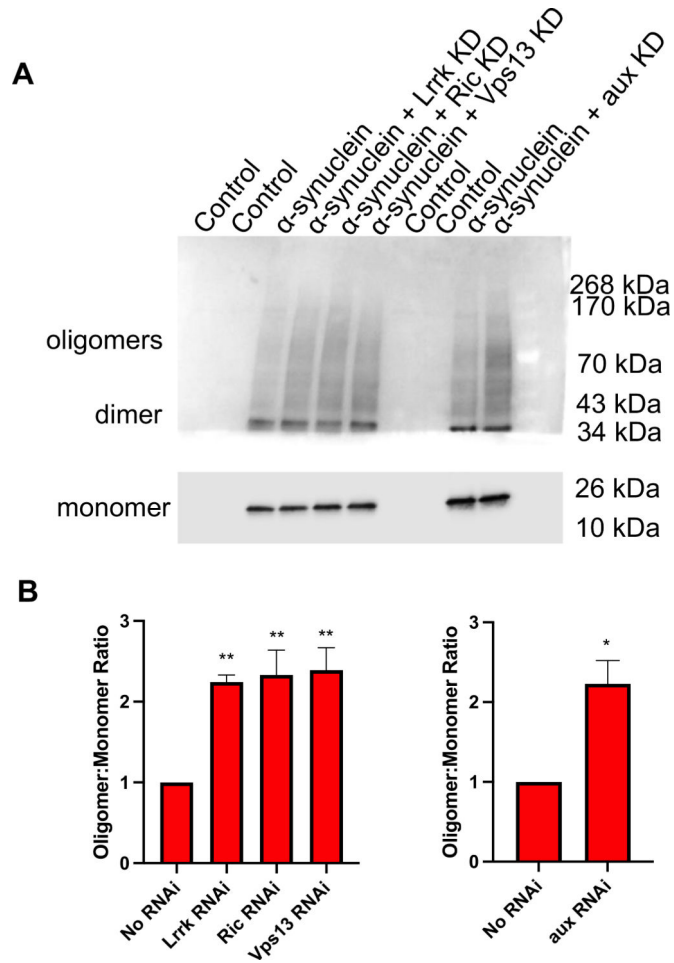


Fig. 5. Glial modifiers increase α -synuclein oligomerization. A. Representative immunoblot using. This immunoblot was cut prior to application of the primary antibody. The top portion demonstrating oligomers and dimers was exposed for 1 min and the bottom portion demonstrating the α -synuclein monomers was exposed for 6 s. B. Quantification from 4 independent experiments. The genotype of control or α -synuclein flies that lack an RNAi is *nSybQF2, repo-Gal4⁺* or *QUAS- α -Syn, nSybQF2, repo-Gal4⁺*, respectively, in the left half of the immunoblot and left panel of B. The genotype of control or α -synuclein flies that lack an RNAi is *nSybQF2, alrm-Gal4⁺* or *QUAS- α -Syn, nSybQF2, alrm-Gal4⁺*, respectively, in the right half of the immunoblot and the right panel of B. All experiments were performed at 10 days post-eclosion using homogenized fly heads.

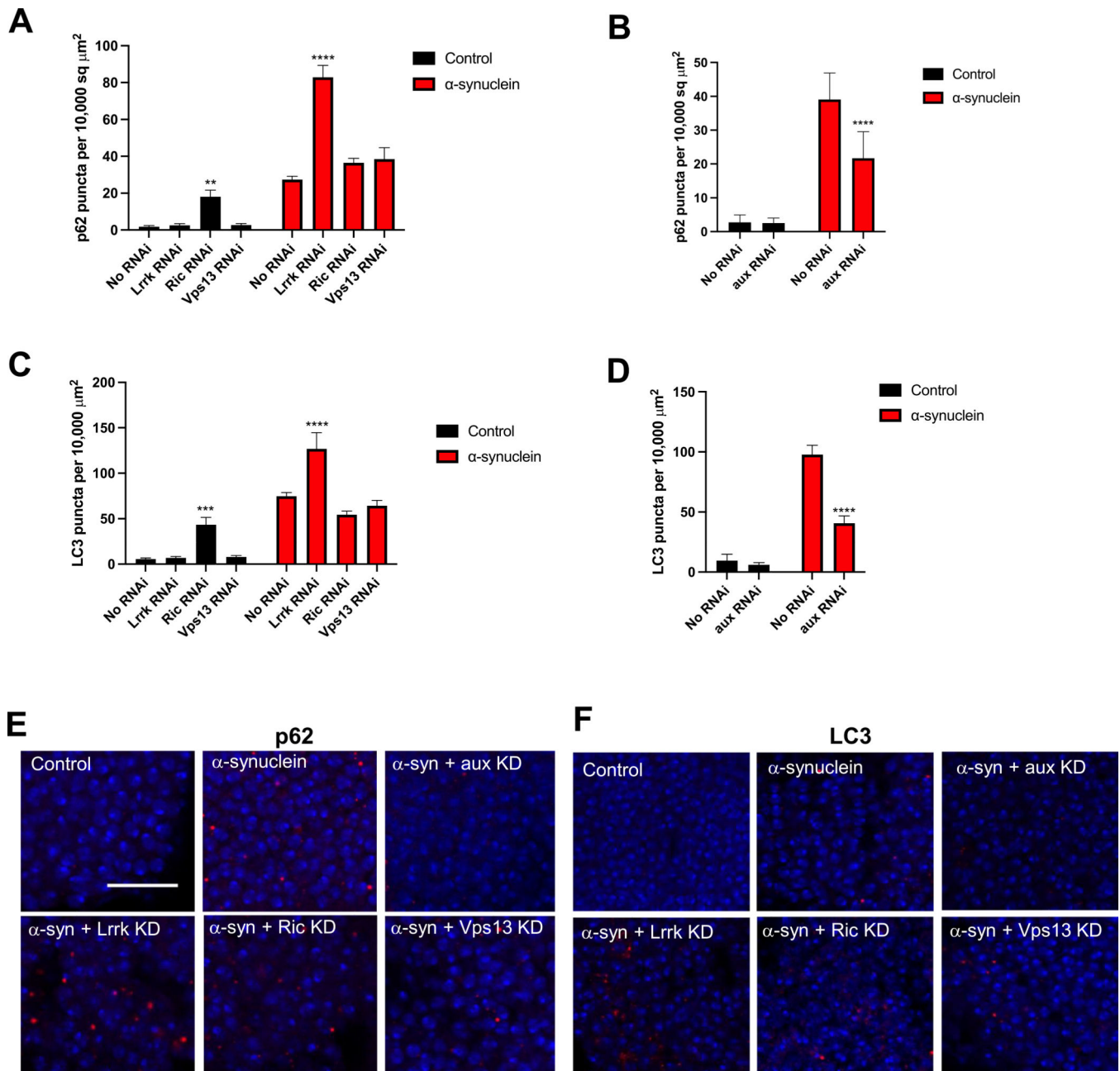


Fig. 6. Glial *Lrrk* knockdown impairs autophagy. p62 (A, C, E) and LC3 (B, D, F) were measured by immunofluorescence. The antibodies used are ref. (Kim and Alcalay, 2017)p/p62, Atg8a/LC3. In A, C, E the genotype of α -synuclein flies that lack an RNAi is *QUAS- α -Syn, nSybQF2, repo-Gal4/+*. In B, D, F the genotype of α -synuclein flies that lack an RNAi is *QUAS- α -Syn, nSybQF2, alrm-Gal4/+*. Nuclei are stained with DAPI. Scale bar = 10 μm . All experiments were performed at 10 days post-eclosion.

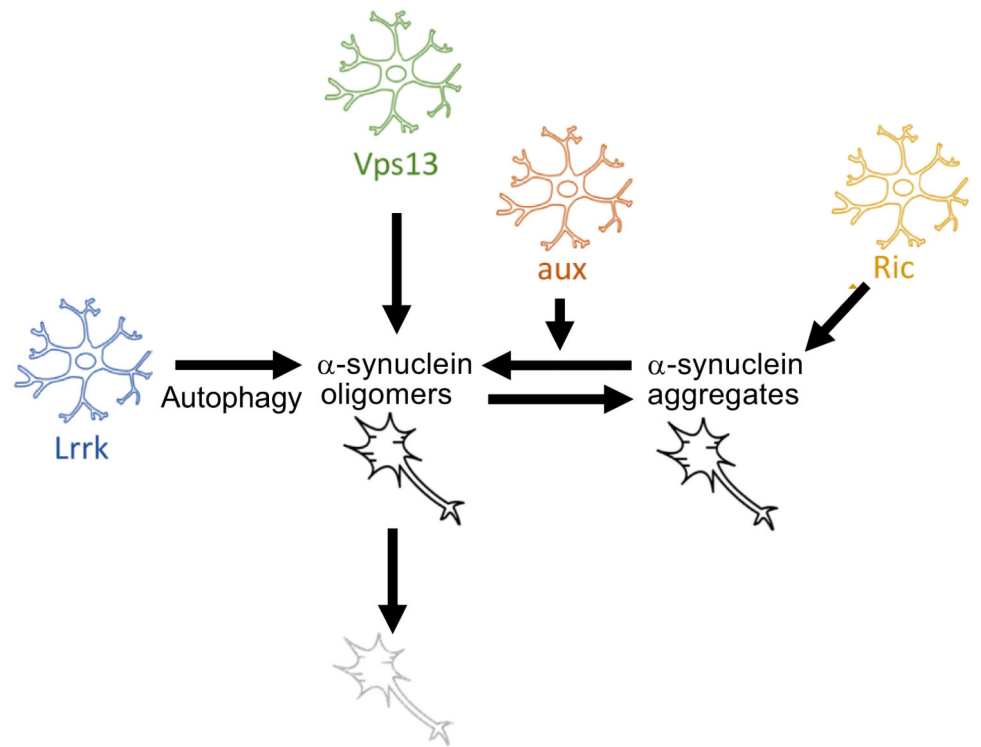


Fig. 7.

Proposed model. All glial modifiers act by increasing α -synuclein oligomerization but have divergent upstream mechanisms. Glial *Lrrk* knockdown impairs neuronal autophagy. Glial *aux* knockdown may favor formation of oligomers as opposed to aggregates. The precise mechanism by which glial *Ric* or *Vps13* knockdown increases α -synuclein oligomerization is unknown.

Table 1:

Genes tested in *Drosophila* screen. The human GWAS nominated gene is shown in the left hand column and the *Drosophila* ortholog(s) on the right.

Genes tested	
GWAS Candidate	<i>Drosophila</i> Ortholog
DGKQ	CG31140
FGF20	bnl
GAK	aux
GBA	CG31148, CG31414
GCH1	Pu
INPP5f	CG7956
LRRK2	Lrrk
MCCC1	CG2118
RIT2	Ric
SCARB2	Emp
STK39	fray
STX1B	Syk1A
SYT11	Syt4
VPS13C	Vps13

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