Fibrillar α-synuclein toxicity depends on functional lysosomes

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Abbreviations

ANOVA: analysis of variance, BSO: buthionine sulfoximine, DFO: deferoxamine, DIV: days in vitro, GSH: reduced glutathione, LAMP-1: lysosome-associated membrane protein 1, PBS: phosphate buffered saline, PD: Parkinson’s disease, PFFs: pre-formed fibrils, SEM: standard error of the mean, WT: wild type.
**Abstract:**

Neurodegeneration in Parkinson’s disease (PD) can be recapitulated in animals by administration of α-synuclein pre-formed fibrils (PFFs) into the brain. However, the mechanism by which these PFFs induce toxicity is unknown. Iron is implicated in PD pathophysiology, so we investigated whether α-synuclein PFFs induce ferroptosis, an iron-dependent cell death pathway. A range of ferroptosis inhibitors were added to a striatal neuron-derived cell line (STHdhQ7/7 cells), a dopaminergic neuron-derived cell line (SN4741 cells) and WT primary cortical neurons, all of which had been intoxicated with α-synuclein PFFs. Viability was not recovered by these inhibitors except for liproxstatin-1, a best-in-class ferroptosis inhibitor, when used at high doses. High dose liproxstatin-1 visibly enlarged the area of a cell that contained acidic vesicles, and elevated the expression of several proteins associated with the autophagy-lysosomal pathway similarly to the known lysosomal inhibitors, chloroquine and bafilomycin A1. Consistent with high dose liproxstatin-1 protecting via a lysosomal mechanism, we further demonstrated that loss of viability induced by α-synuclein PFFs was attenuated by chloroquine and bafilomycin A1 as well as the lysosomal cysteine protease inhibitors, leupeptin, E-64D and Ca-074-Me, but not other autophagy or lysosomal enzyme inhibitors. We confirmed using immunofluorescence microscopy that heparin prevented uptake of α-synuclein PFFs into cells, but that chloroquine did not stop α-synuclein uptake into lysosomes despite impairing lysosomal function and inhibiting α-synuclein toxicity. Together, these data suggested that α-synuclein PFFs are toxic in functional lysosomes in vitro. Therapeutic strategies that prevent α-synuclein fibril uptake into lysosomes may be of benefit in PD.
Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder characterised by death of neurons in the substantia nigra (1), and atrophy in other brain regions (2,3). However, while multiple cell death pathways have been investigated for their potential contribution to neurodegeneration (4), the cell death mechanism involved in neurodegeneration has not been established. While the cause of neuronal death in Parkinson’s disease (PD) is unknown, it likely involves aggregated α-synuclein, since α-synuclein is the major protein component of Lewy bodies, and the α-synuclein-encoding gene, SNCA, is a major genetic risk locus for idiopathic and familial PD (5-7). α-synuclein is found both in the cytosol, and in the extracellular interstitial fluid. Oligomeric α-synuclein is observed in patients’ cerebrospinal fluid (8), which promotes seeding of naïve α-synuclein into aggregated α-synuclein (9,10). Investigating cell death induced by intracellular α-synuclein is limited by the challenge of generating intracellular aggregated species of this protein using in vitro models. The toxic potential of extracellular species, which can be more easily modelled in laboratory settings, is highlighted by the extracellular transmission of Lewy pathology into naïve neurons of PD patients that received neuronal grafts (11,12), which is recapitulated in animals where aggregated α-synuclein injection causes α-synuclein pathology and loss of nigral dopaminergic neurons (13-17). However, it is unknown how α-synuclein causes disease.

Iron accumulation is a feature of the substantia nigra (SN) in PD (18-20), and may contribute to cell death via the newly discovered form of cell death called ferroptosis (4,21). Ferroptosis is an iron-dependent, caspase-independent, regulated necrosis (22). Enzymatic or non-enzymatic iron-induced peroxidation and subsequent rupture of the phospholipid membrane is opposed by glutathione peroxidase 4 (GPX4), which serves as the major checkpoint to ferroptotic cell death (23). GPX4 requires glutathione as a co-factor, and the amino acid cysteine is the rate-limiting factor for glutathione production. Neurons can undergo ferroptosis in vitro and in vivo (24,25), but mature primary neurons are not sensitive to ferroptosis induced by glutamate/erastin (whereas immature neurons are vulnerable (26))—this is likely due to less dependence of primary neurons on the xCT transporter. Ferroptosis can be modelled in vitro using agents that induce ferroptosis by the depletion of cystine (e.g. erastin, which inhibits cystine import through the xCT transporter), or inhibition of glutathione synthesis from cysteine (e.g. buthionine sulfoximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase). Conversely, glutathione (GSH) supplementation, iron chelators such as deferoxamine (DFO), or lipid antioxidants such as liproxstatin-1 and ferrostatin-1 protect against ferroptosis.

Toxin models of PD have been shown to cause cell death via ferroptosis (25), and, apart from iron elevation, additional features of ferroptosis such as depleted glutathione and elevated lipid peroxidation are observed in PD-affected brains post-mortem (27-29). However, it is not known whether extracellular α-synuclein causes cell death via ferroptosis, which we explore here using a cell-based model of toxicity induced by extracellular aggregated α-synuclein.

We show that aggregated α-synuclein does not activate ferroptosis; rather, by unexpectedly discovering a second function of a best-in-class ferroptosis inhibitor, liproxstatin-1, we demonstrate that toxicity is dependent on lysosomal function. These findings are consistent with growing evidence for a pathogenic role of the lysosome in PD, with variants in genes important for endo-lysosomal function, increasing the risk of sporadic and/or familial disease (30-32).

Results:

Characterising α-synuclein toxicity model

We optimised an aggregation protocol utilising constant agitation of recombinant monomeric human α-synuclein for 5 days, and confirmed the presence of amyloid structures at the endpoint by Thioflavin T fluorescence assay (Figure 1a) and electron microscopy (Figure 1b). The sonicated α-synuclein fibrils were similar in appearance to those previously shown to induce PD-like pathology and neurodegeneration in mice (13). Toxicity of PFFs in vitro was first confirmed in SN4741 cells, in which the PFFs caused dose-dependent loss of viability over 24 hours which was not increased by extending exposure to 48 hours (Figure 1c). To test whether toxicity was specific to aggregated α-synuclein in our model, recombinant monomeric α-synuclein was added in parallel with α-synuclein PFFs for 24 hours in SN4741 and STHdhQ7/7 cells. The α-synuclein PFFs were shown to induce loss of viability in both cell types (~10-40% toxicity at 0.005-1µM)
250nM and 500nM erastin were doses that caused mid-ferroptosis preventing them from engaging in further toxicity. Increasing the concentration of soluble wheyl when added at higher concentrations, thus in these cell types, likely reflecting saturation of a specific effect of this protein approximated 40% loss of viability in SN4741 cells were vulnerable to erastin-induced toxicity in dose-optimisation experiments in SN4741 cells, whereas STHdhQ7/7 and SN4741 cells administered erastin showed no impact on α-synuclein-induced loss of viability. While SN4741 and STHdhQ7/7 cells were susceptible to low concentrations of α-synuclein PFFs, the maximum effect of this protein approximated 40% loss of viability in these cell types, likely reflecting saturation of a mechanism critical to toxicity or PFFs becoming less soluble when added at higher concentrations, thus preventing them from engaging in further toxicity.

To investigate whether toxicity of α-synuclein occurs via ferroptosis, we tested whether several known ferroptosis inhibitors and activators protected and exacerbated cell death, respectively, and compared this to the effect of these compounds on the toxicity induced by the ferroptosis toxin, erastin. Both STHdhQ7/7 and SN4741 cells were vulnerable to erastin-induced ferroptosis (Figure 1g). However, primary cortical neurons were not susceptible (data not shown). Since 250nM and 500nM erastin were doses that caused mid-range toxicity in dose-optimisation experiments in SN4741 and STHdhQ7/7 cells, respectively, these doses were selected for comparing the effect of ferroptosis modulators on PFF- and erastin-treated cells.

To determine whether the toxicity of PFFs had a similar response to known ferroptosis modulators as erastin, BSO, GSH and DFO were administered to erastin- and PFF-treated cells in parallel, and cell viability was assessed. Treatment of STHdhQ7/7 and SN4741 cells with glutamylcysteine synthetase-inhibitor, BSO, which depletes intracellular GSH (33), decreased the baseline viability of cells administered a sub-toxic dose of erastin, as expected (Figure 2a). However, BSO had no impact on α-synuclein PFF toxicity (Figure 2b). Conversely, addition of GSH to treatment media significantly increased the baseline viability of STHdhQ7/7 and SN4741 cells administered erastin (Figure 2c). Yet despite the lack of effect of ferrostatin-1 on PFF toxicity, liproxstatin-1 rescued toxicity caused by α-synuclein PFFs in both cell types (Figure 3d). Liproxstatin-1 has not previously been shown to impact on other cell death pathways such as apoptosis and necroptosis, but to exclude the possibility that liproxstatin-1 rescued PFF toxicity via these mechanisms, we used established inhibitors of these pathways in the PFF assay. Q-VD-OPH, a broad-spectrum caspase inhibitor (34), did not protect cells from α-synuclein toxicity (Figure 4a), nor did 7-Cl-O-Nec-1, which is known to inhibit necroptosis (35,36) (Figure 4b). These results suggested that α-synuclein PFFs did not induce necroptosis or caspase-dependent apoptosis, and liproxstatin-1 rescued PFF toxicity via a different mechanism.

Investigation into additional toxic mechanisms induced by α-synuclein PFFs

Liproxstatin-1 affects lysosomal function

Concentrations of liproxstatin-1 as low as 0.2µM were sufficient to provide full protection against toxic doses of erastin in SN4741 and STHdhQ7/7 cells, whereas micromolar concentrations of liproxstatin-1 were required to reduce the toxicity of α-synuclein PFFs. In combination with the lack of effect of other ferroptosis inhibitors on α-synuclein-induced loss of viability, this observation indicated that liproxstatin-1 likely rescued...
cells from α-synuclein toxicity via a ferroptosis-independent mechanism.

Liproxstatin-1 is a weak base and highly lipophilic (37), properties which are shared with drugs that can become trapped within the lysosome and potentially affect its functions (lysosomotropic drugs) (38,39). As an initial experiment to test the hypothesis that liproxstatin-1 might similarly accumulate within lysosomes and interfere with lysosomal pathways, we imaged the acidic vesicles of SN4741 cells treated with liproxstatin-1 by confocal microscopy. SN4741 cells were administered a dose of liproxstatin-1 only protective against erastin toxicity (0.2µM liproxstatin-1) in addition to doses protective against α-synuclein PFFs (5-10µM liproxstatin-1).

Imaging SN4741 cells following incubation in Lysotracker Red and Hoechst 33342 dyes revealed a visibly larger area of cells were occupied by acidic vesicles when exposed to 5-10µM liproxstatin-1 when compared to vehicle control and 0.2µM liproxstatin-1 (Figure 5a-d). Lysosomal vacuolation, which describes swelling of lysosomes, often occurs in response to lysosomotropic drugs (40). One potential mechanism by which lysosomal vacuolation occurs is that drug accumulation within lysosomes may impair proteolysis and consequently cause an increase in undegraded substrate and enlargement of lysosomes as a result (41). Potentially, high dose liproxstatin-1 induced enlargement of vesicles in our experiments; however, there was insufficient resolution to determine whether it was the quantity or size of acidic vesicles that was increased.

Lysotracker staining in cells administered 5-10µM liproxstatin-1 was consistently duller than that of cells treated with vehicle control and 0.2µM liproxstatin-1. Lysosomotropic compounds have previously been reported to have varied effects on Lysotracker Red DND-99 signal (42), with a decreased signal intensity hypothesised to correspond with increased lysosomal pH. Potentially, micromolar doses of liproxstatin-1 could have induced an increase in lysosomal pH, an event linked to impaired lysosomal degradation, and consequently reduced the signal intensity.

Similar observations of an increase in area of cells occupied by acidic vesicles in response to high dose liproxstatin-1 were also apparent when cells were instead incubated in acridine orange (Figure 5e-h). 10µM liproxstatin-1 treatment again showed indications of a duller intensity of acidic vesicle staining when compared to lower liproxstatin-1 concentrations and vehicle control. These data supported the hypothesis that liproxstatin-1 impacted acidic vesicles at doses sufficient to reduce α-synuclein PFF toxicity, but in excess of that needed to inhibit ferroptosis.

To further investigate the effect of liproxstatin-1 on lysosomal function, we used western blot analysis to quantify levels of several autophagy and lysosomal markers in the lysate of SN4741 cells following 24 hour treatment with liproxstatin-1, and lysosomal inhibitors, chloroquine and bafilomycin A1. Membranes were probed with primary antibodies for proteins commonly associated with autophagosomes, p62 and LC3-II (43), ferritin, which is degraded by the autophagy-lysosomal pathway (44-47), and LAMP-1. When compared to vehicle control, all three drugs had a significant effect on expression levels of ferritin, LAMP-1, p62, and LC3-II (Figure 6). A representative immunoblot shows visible increases in the levels of these markers in response to not only lysosomal inhibitors, chloroquine and bafilomycin A1, but also liproxstatin-1 (Figure 6a).

Ferritin levels were significantly elevated following treatment with 5-10µM liproxstatin-1, 10nM bafilomycin A1 and all doses of chloroquine (Figure 6b). LAMP-1 expression was increased in response to all doses of chloroquine and bafilomycin A1, and the highest liproxstatin-1 dose tested (Figure 6c). Both p62 and LC3-II levels were higher in cells treated with 5-10µM liproxstatin-1, and all chloroquine and bafilomycin A1 concentrations tested (Figure 6d). Similar effects were observed in response to 5-10µM liproxstatin-1 and each lysosomal inhibitor tested in STHdhQ7/7 cells (data not shown). Since only a faint band was detected for LC3-I, only LC3-II bands were quantified relative to β-actin as recommended by the latest guidelines for interpreting autophagy assays (48).

Pharmacological inhibition of lysosomal acidification and cysteine proteases protected against α-synuclein PFF toxicity

Since our data supported a role for high-dose liproxstatin-1 in inhibiting lysosomal function, we investigated whether α-synuclein PFFs may exert their toxicity by entering the cell via the endo-lysosomal pathway. To test whether aggregated α-synuclein caused toxicity via a lysosome-dependent mechanism, we added a range of concentrations of lysosome-targeting drugs to either STHdhQ7/7 cells or SN4741 cells prior to dilution of 1µM α-synuclein PFFs in treatment media. Chloroquine, a drug that accumulates
in lysosomes and inhibits lysosomal acidification (49), produced a dose-dependent rescue of baseline viability when administered to α-synuclein-treated STHdhQ7/7 cells (33-75% protection at 5-20µM) and SN4741 cells (21-56% rescue at 5-20µM) (Figure 7a). Bafilomycin A1, which is a selective inhibitor of vacuolar-H+ ATPase on the lysosomal membrane (50,51), significantly rescued α-synuclein toxicity in STHdhQ7/7 cells (75-118% rescue at 25-50nM) and in SN4741 cells (27-90% rescue at 10-25nM) (Figure 7b). Doses of chloroquine and bafilomycin A1 selected were of a similar range of doses to those previously used in PFF in vitro studies (52,53).

To investigate whether the mechanism of protection of chloroquine and bafilomycin A1 against α-synuclein PFFs was related to inactivation of lysosomal proteases required for degradation, we tested whether inhibitors of lysosomal proteases similarly exerted protection against α-synuclein toxicity. Leupeptin, an inhibitor of cysteine proteases and serine proteases including cathepsin B, cathepsin L, trypsin and plasmin (54-56), significantly protected against loss of viability induced by 1µM α-synuclein PFFs in STHdhQ7/7 cells (41-51% rescue at 50-250µM) and in SN4741 cells (17-36% rescue at 50-500µM) (Figure 7c). E-64D and Ca-074-Me are specific inhibitors of cysteine proteases such as cathepsins B and L (57-59). E-64D partially rescued toxicity due to 1µM α-synuclein PFFs in STHdhQ7/7 cells (47% rescue at 20µM) and in SN4741 cells (23-42% rescue at 5-25µM) (Figure 7d). Ca-074-Me also protected against PFF-induced loss of viability in SN4741 cells (18-21% rescue at 10-50µM), but did not show significant protection in STHdhQ7/7 cells (Figure 7e).

However, no protective effect was observed with other drugs known to target lysosomal pathways, which could suggest that protection was limited to inhibitors of cysteine proteases. Pepstatin A, an aspartic protease inhibitor (60,61), was not able to rescue toxicity caused by 1µM α-synuclein PFFs in either cell type (Figure 7f). Rapamycin, an inducer of mTOR-dependent autophagy (62), did not significantly affect the extent of α-synuclein PFF-induced toxicity (Figure 7g). Wortmannin and 3-methyladenine, which can suppress autophagy activation via inhibition of class III phosphatidylinositol 3-kinase (63,64), also did not protect against α-synuclein PFF toxicity in either cell type (data for 3-methyladenine exploratory experiment in STHdhQ7/7 cells not shown) (Figure 7h, i).

Heparin prevents uptake and toxicity of α-synuclein PFFs

Since α-synuclein PFFs were administered extracellularly, we hypothesised that PFF internalisation into the endo-lysosomal pathway was important for its toxicity, and therefore we tested a known pharmacological inhibitor of aggregated α-synuclein endocytosis (65,66). Heparin, an inhibitor of heparan sulfate proteoglycan-mediated endocytosis (67), markedly recovered α-synuclein toxicity in STHdhQ7/7 cells (63-103% rescue at 10-100µg/mL) and SN4741 cells (77-84% rescue at 10-100µg/mL) (Figure 7j). Doses of heparin selected were within the range of doses used in previous studies showing an inhibitory effect of heparin on uptake of aggregated α-synuclein (65,66). These data could suggest heparan sulfate proteoglycan-mediated endocytosis is a mechanism by which α-synuclein PFFs are internalised in cells. However, an alternate explanation could be that heparin induced further aggregation of α-synuclein extracellularly to a size too large to enter cells, since heparin has also been shown to induce α-synuclein fibrillisation in vitro (68,69). Regardless, we expect that heparin will have the effect of preventing cellular uptake of PFFs.

We utilised confocal imaging to determine whether internalised α-synuclein PFFs were (1) associated with the endo-lysosomal pathway, and (2) whether this localisation was relevant to α-synuclein-induced neurotoxicity. SN4741 cells plated onto coverslips were administered chloroquine or heparin 1 hour in advance of vehicle control or PFFs, and cells were fixed and permeabilised after treatment with α-synuclein for 24 hours. After incubation in primary and secondary antibodies for LAMP-1 (Alexa Fluor 594 secondary; red) and α-synuclein (Alexa Fluor 488 secondary; green), and a DAPI stain (blue), images were acquired of fixed cells by confocal microscopy.

The α-synuclein signal was barely detectable in controls but clearly visible in cells administered PFFs, with the bulk of α-synuclein accumulating at the extracellular surface of the cell (Figure 8a, b). Quantification of the α-synuclein signal intensity in LAMP-1-rich regions revealed a higher mean α-synuclein signal compared to that of control cells, which suggested that α-synuclein PFFs are present in the endo-lysosomal pathway following their cellular internalisation. A second, higher peak in α-synuclein signal at distances 6-10µm moving
through the Z-stack of PFF-treated cells likely corresponded to membrane associated-PFFs at the top of imaged cells.

100µg/mL heparin caused a pronounced reduction in α-synuclein immunoreactivity when co-administered with α-synuclein PFFs (Figure 8c, d). Apart from several large α-synuclein aggregates that formed extracellularly, there was no obvious difference in the amount of internalised α-synuclein from that of cells treated with the 100µg/mL heparin control. Quantification of LAMP-1 rich regions also showed comparable α-synuclein signal in control and PFF-treated cells, indicating that heparin prevented internalisation of α-synuclein PFFs. The amount of LAMP-1 immunoreactivity was visibly increased by 20µM chloroquine, which is expected since it is an inhibitor of lysosomal proteolysis and causes undegraded substrates to accumulate (Figure 8e). In cells administered both 20µM chloroquine and 1µM α-synuclein PFFs, the α-synuclein signal was visible both intracellularly and associated with the extracellular membrane (Figure 8f). However, in contrast to heparin, chloroquine treatment did not prevent association of α-synuclein PFFs with LAMP-1 rich regions. Lower doses of heparin and chloroquine also replicated these observations, with smaller effect (data not shown).

Discussion:

Aggregated α-synuclein is considered pathogenic in PD, but it is unknown how this protein causes cell death, which may be important for discovering a disease-modifying drug. Studying PFF models in vitro enables a rigorous interrogation of hypothesised mediators of α-synuclein-induced neurodegeneration and provides a platform for testing drugs to potentially target lesions arising from extracellular aggregated α-synuclein in patients. Similar to observations in PFF animal models (13,14,16,17), α-synuclein PFFs were unable to cause full toxicity at the concentrations used, but several of the drugs tested were effective in rescuing the ~40% toxicity induced by the protein. We hypothesised that this cell death was mediated by ferroptosis, since changes consistent with ferroptosis are reported in post-mortem samples of PD patients, and toxin models of PD are reported to cause cell death by ferroptosis. Our hypothesis that α-synuclein PFFs cause ferroptotic cell death was not supported by the results of this study, rather, we provide further support for the endolysosomal pathway in PD pathogenesis.

Despite the majority of our findings demonstrating that α-synuclein PFF toxicity was neither exacerbated nor ameliorated with ferroptosis modulating agents, we found that high doses of liproxstatin-1, a potent anti-ferroptotic agent, was protective against α-synuclein PFFs. Abnormalities in the appearance of acidic vesicles and changes in levels of proteins in the autophagy-lysosomal pathways induced by high-dose liproxstatin-1, a drug with lysosomotropic properties (37), suggest that this drug inhibited lysosomal function.

In agreement with this possibility of liproxstatin-1 exerting its protective benefits against α-synuclein PFFs within the lysosome, we observed that α-synuclein PFFs were present in LAMP-1-rich regions, demonstrating that PFFs are internalised by the endo-lysosomal pathway and taken up into lysosomes, similar to previous findings (70). This observation was further supported by our observation that several other drugs targeting the endo-lysosomal pathway (chloroquine, bafilomycin A1, leupeptin, E-64D) were all protective against α-synuclein PFFs. While the effect of drugs targeting the endolysosomal pathway on α-synuclein uptake and spread have been reported (65,66,71,72), the impact of drugs targeting ferroptosis and lysosomal function on the toxicity of extracellular α-synuclein is currently unknown.

While in vitro assays of α-synuclein PFFs often investigate the seeding propensity of this protein, it remains unknown how this pathogenic species of α-synuclein causes toxicity, which is the subject of this study. We show the toxic potential of α-synuclein that may exist independently of endogenous α-synuclein. The observation that toxicity in this model was seemingly independent of endogenous α-synuclein accords with previous work which suggested that neurotoxicity may be differentially affected by the type of α-synuclein oligomer added to cells and reported that oligomers that caused cell death did not induce endogenous α-synuclein aggregate formation (73). In contrast, oligomers that initiated aggregation of endogenous α-synuclein were not neurotoxic, indicating that the recruitment of endogenous α-synuclein may not be essential for cell death induced by extracellular aggregated α-synuclein (73). We extended upon these findings by demonstrating the involvement of the lysosome in the toxic mechanism of α-synuclein.

A popular hypothesis in Parkinson’s disease research is that autophagy activation is beneficial in reducing the deleterious impact of aggregated α-synuclein. Models
utilising α-synuclein over-expression often report that autophagy activation with drugs such as rapamycin activates degradation and reduces levels of WT (74,75), A53T (75,76), and A30P α-synuclein (75). However, relatively few studies have investigated whether autophagy activation translates to protection against α-synuclein-mediated toxicity. Rapamycin has been reported to exacerbate toxicity associated with intracellular α-synuclein aggregates (77) and human over-expression of α-synuclein in yeast cells (78). Therefore, the relationship between autophagy activation and α-synuclein-induced cell death may be complex.

Genetic loci associated with PD such as GBA and LRRK2 have been linked to impaired lysosomal proteolysis in vitro (79,80); however, mutations or deficiency in these genes can have other effects on cell function, including mitochondrial dysfunction (81,82). It is not yet known whether varied expression of these genes increases disease susceptibility by impacting lysosomal proteolysis or another mechanism in patients. Alterations in lysosomal markers are complicated to interpret post-mortem (83,84); given the findings we present here, potentially evidence of lysosomal inhibition could be an adaptive response to protect cells against the toxicity of α-synuclein when it reaches the lysosome.

Lysosomal inhibition has similarly been reported to have varied effects on α-synuclein expression and toxicity, which may depend on the species of α-synuclein, and from what pool it originated from (intracellular or extracellular). In Table 1, we have outlined previous evidence described utilising lysosomal inhibitors effective in our study. Our study provided evidence that lysosomal inhibition reduced the toxicity of extracellular α-synuclein.

Importantly, the lysosome represents a common endpoint for extracellular and intracellular species of α-synuclein, that arrive in the lysosomes via endocytosis and autophagy pathways, respectively (70,75,85,86). Upregulating autophagy-mediated proteolysis, which coalesces cellular content into lysosomes, is often posited to be beneficial in reducing the deleterious impact of aggregated α-synuclein (75,87). However, it is possible that α-synuclein aggregates are toxic to the cell in the lysosome. Indeed, the autophagy inducer rapamycin has been reported to exacerbate toxicity associated with over-expression of human α-synuclein in yeast and mammalian cells (78,88,89). Lysosomal inhibition has shown variable effects on toxicity of α-synuclein of cytosolic origin. For example, in some studies, toxicity induced by over-expression of human α-synuclein was exacerbated by lysosomal inhibitors (90,91), while others showed no effect or the opposite result (79,88). To date, relatively few studies have investigated whether lysosomal modulation translates to protection against α-synuclein-mediated toxicity in models utilising extracellular α-synuclein.

It now becomes a priority to investigate how α-synuclein becomes toxic in the functional lysosome. Prior work has shown that bafilomycin A1 inhibited the extracellular secretion of pro-inflammatory signalling molecule IL-1β, in response to α-synuclein fibril administration in vitro (92). In addition to the activation of inflammatory pathways, aggregated α-synuclein may induce lysosomal permeabilisation (which has previously been reported (52,93)), which is a cellular event that can induce toxicity and cell death (94). It is possible that lysosomal inhibitors re-directed α-synuclein PFFs for secretion in extracellular vesicles, thereby lowering the α-synuclein burden in the cell (95). However, we found that chloroquine treatment, which rescued toxicity, did not decrease levels of α-synuclein in LAMP-1 rich areas. This demonstrates that the toxicity of α-synuclein may be prevented when it is localised in an inhibited lysosome.

In addition to the activation of inflammatory pathways, aggregated α-synuclein may induce toxicity by lysosomal permeabilization (LMP), which has previously been reported (52,93). Toxicity induced by LMP can be rescued by leupeptin and E-64D (96-98). The effect of bafilomycin A1 on this pathway is more variable; Boya et al. (98) indicated bafilomycin A1 was protective, whereas Kanzawa et al. (99) showed that bafilomycin A1 increased susceptibility to LMP. Chloroquine, however, itself causes cell death by LMP (100), so it may be unlikely that chloroquine would also protect against α-synuclein PFFs if they also caused cell death via this mechanism. We found that the benefit of lysosomal inhibitor drugs against PFF toxicity was impressive compared to the potent LMP-inhibitor, Ca-074-Me (101,102), which provided only minimal rescue, also suggesting that LMP is not central to α-synuclein PFF toxicity.

Prevention of α-synuclein uptake into the endolysosomal pathway by heparin may be another strategy for neuroprotection. Heparin has previously been reported to impede uptake of aggregated α-synuclein into cells via heparan-proteoglycan mediated
endocytosis (65,66), but has also been shown to induce α-synuclein aggregation in vitro (68,69), which could also prevent uptake of the protein. Future experiments could further investigate mechanisms of PFF uptake within this model, to determine the mechanism by which PFFs were internalised into cells, and whether other means of targeting endocytosis might be protective. Regardless of the mechanism, we showed that heparin prevented cellular uptake of PFFs, and protected against their toxicity. Our results could suggest that extracellular α-synuclein PFFs may not be inherently toxic, but may become toxic in the functional lysosome.

We used concentrations of the apoptosis (103) and necroptosis (104) inhibitors previously shown to be effective in vitro, and that we confirmed were not toxic in viability assays, however, a limitation of our study was that our data do not confirm they were effective in our conditions. We used sub-toxic doses, and if higher doses are required for effectiveness against α-synuclein pff toxicity, the results could be confounded by cell death induced by these inhibitors.

Furthermore, while we used a range of doses of lysosomal-targeting drugs, it is possible that these drugs had additional mechanisms of action that could interfere with our assay. For example, bafilomycin A1 has been shown to act as a potassium ionophore that inhibits mitochondrial function with EC50 ~150nM compared to ~20nM in our study (105). It is unlikely that the potassium ionophore activity of bafilomycin A1 impacted on our assay, since it would be expected to reduce aerobic respiration, as measured by MTT, yet in our assays bafilomycin A1 improved viability. Chloroquine could also potentially work via other mechanisms, for example, chloroquine inhibits thiamine uptake via the SLC19A3 channel at concentrations above 200µM (106), which is 10 times higher than that of the highest dose used in the present study. We cannot exclude the possibility that additional off-target effects of chloroquine, bafilomycin A1, leupeptin and E-64D could explain our findings, the lysosomal interpretation of our findings is strengthened by the fact that there are 4 structurally unrelated compounds which are all known to inhibit the activity of lysosomal enzymes at doses we used in our study.

Targeting lysosomal uptake of α-synuclein could therefore be a potential therapeutic strategy for PD, and potentially other central nervous system disorders in which extracellular α-synuclein is implicated in pathogenesis. Several of the drugs effective in our model are already approved for use in humans, including chloroquine, E-64D and heparin (107-109), and may warrant further development for PD.

Methods:

Preparation of α-synuclein PFFs

Recombinant human α-synuclein was expressed using BL21 (DE3) cells and purified with high performance anion-exchange chromatography and gel filtration by Monash Protein Production Unit before lyophilisation. Preparation of α-synuclein pre-formed fibrils (PFFs) was adapted from the methods utilised by Luk et al. (13). Lyophilised α-synuclein was dissolved in phosphate buffered saline (PBS) (Gibco; pH 7.4), and sonicated in a water-bath (Unisonics model PS-30; 10 minutes) before filtration (Sartorius Stedim Biotech; 0.2µm pore). Protein concentration was determined by bicinchoninic acid assay. An α-synuclein solution (final concentration 5mg/mL) was then added to a 500µL tube, and left to agitate in a Thermoshaker (Multi-Therm Heat-shake; Benchmark; 37°C at 1400rpm for 5 days). The β-sheet structure of α-synuclein was monitored daily using Thioflavin T fluorescence (excitation frequency: 444nm, emission measured at 475nm; Molecular Devices, Flexstation 3 Benchtop Multi-mode Microplate Reader). A sonicator (Branson Digital Sonifier 450; Branson Ultrasonics; amplitude: 20%; 30 seconds) with a microtip probe was used to disrupt α-synuclein fibrils in solution prior to treatment of cells.

The structure of the α-synuclein pre- and post-sonication was assessed by transmission electron microscope (FEI Tecnai F30). Samples were placed onto 300 mesh copper grids and stained with 1% aqueous uranyl acetate (Electron Microscopy Sciences), pH 4.5, for 30 seconds. Air-dried grids were then observed at 300 kV in a FEI Tecnai F30 transmission electron microscope.

Cell culture maintenance

STHdhQ7/7 cells derived from mouse striatal cells (110), and SN4741 cells derived from mouse embryonic nigral dopaminergic neurons (111), were used since these brain regions undergo neurodegeneration in PD (1-3). Each cell type was maintained in Gibco Dulbecco’s Modified Eagle Medium with Glutamax-1 (containing 4.5g/L D-glucose and 25mM 4-(2-
Hydroxyethyl)piperazine-1-ethanesulfonic acid, supplemented with 10% fetal bovine serum and either 1x antibiotic-antimycotic (for STHdhQ7/7 cells; Gibco) or 1x penicillin-streptomycin (for SN4741 cells; Thermo Fisher Scientific). Each cell type was plated in 96-well plates at least 24 hours prior to treatment with α-synuclein.

Primary cortical neurons were harvested from WT (background strains: C57/Bl/6 and C57/Bl6/129sv WT primary cortical neurons), or WT and α-synuclein knockout (KO) mice (background strain: C57Bl6/C3H/HeJ) at embryonic day 14 for α-synuclein PFF dose-response assays. All experiments were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, as described by the National Health and Medical Research Council, and were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee (ethics number: 10-057). The cortices were removed and separated from meninges, chopped into <1mm portions in cold KREBS buffer, and dissociated in 0.016% (w/v) trypsin (Sigma).

The neurons were plated onto poly-D-lysine-coated 96-well plates in Dulbecco’s Modified Eagle Medium (supplemented with 10% FBS, 5% horse serum, 50µg/mL gentamicin (Life Technologies)) and incubated overnight (37°C, 5% CO₂). Plating media was replaced with neurobasal culture media (Gibco) supplemented with 2% B27 supplement (Life Technologies), 0.25% Glutamax (Life Technologies), and 50µg/mL gentamicin (Life Technologies). At 3 days in vitro (DIV), cytosine arabinoside (Sigma) was added to the neurons in Neurobasal media to achieve a final concentration of 2µM (to reduce astrocytic growth). At both 4DIV and 11DIV, 2/3 of the media was replaced with fresh Neurobasal media, before treatment at 14-15DIV. We have confirmed that this protocol yields a prominently neuronal population of cells (>98%).

Treatment of cells with α-synuclein

Monomeric α-synuclein (prepared without Thermoshaker incubation) and PFFs (0-5µM) was administered to cells for 24-48 hours. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (112), with MTT incubation time modified to 3 hours and absorbance wavelength modified to 570nm. For experiments investigating the effect of pharmacological modulators on the toxicity profile of α-synuclein PFFs, the relevant drug was added to cells for 1 hour before α-synuclein PFFs were diluted into the media containing the drug to achieve the desired final concentration (the relevant pharmacological modulator remained in media for the entirety of the assay). For experiments investigating the effect of ferroptosis modulators, 50nM α-synuclein was added to STHdhQ7/7 cells, and 1µM α-synuclein was added to WT primary cortical neurons and SN4741 cells to achieve similar toxicity in each cell type. Doses of 0.5µM and 0.25µM erastin were administered to STHdhQ7/7 cells and SN4741 cells, respectively, due to differing sensitivity to the toxin between cell types. Ferroptosis modulators ferrostatin-1 (10mM stock), liproxstatin-1 (10mM stock) and erastin stocks (10mM stock) were prepared in dimethyl sulfoxide, while DFO, buthionine sulfoximine (BSO) and reduced glutathione (GSH) (Sigma-Aldrich) were solubilised in media.

The following pharmacological inducers and inhibitors of autophagy, lysosomal function and endocytosis were dissolved in dimethyl sulfoxide (Sigma-Aldrich): rapamycin (Abcam; 10mM stock), E-64D (Abcam; 100mM stock), wortmannin (Abcam; 5mM stock), pepstatin A (Abcam; 10mM stock), and Ca-074-Me (Sigma-Aldrich; 50mM stock). Bafilomycin A1 (Sigma-Aldrich) was prepared in 100% ethanol (100µM stock), leupeptin hemisulfate (Abcam) was dissolved in sterile Milli-Q water (50mM stock). Chloroquine (Sigma) and heparin sodium salt (Sigma-Aldrich; approximately 150 USP units/mg), were weighed and dissolved in media prior to dilution for cell culture experiments. Stocks of caspase inhibitor, Q-VD-OPH (Sigma-Aldrich; 10mM stock) and necroptosis inhibitor, 7-Cl-O-Nec-1 (Merck Millipore; 10mM stock), were dissolved in 100% dimethyl sulfoxide. For all viability assays investigating lysosomal mechanisms, a dose of 1µM α-synuclein was used for consistency. The concentrations of drugs used were determined by first performing a toxicity curve, then selecting a range of doses below the threshold that caused loss of viability, and was in the range of prior reports of effective concentration.

Fixed cell imaging

SN4741 cells were seeded onto sterile, Poly-D-Lysine-coated (Sigma) glass coverslips and treated with vehicle control or 1µM α-synuclein PFFs for 24 hours.
Coverslips were then gently washed twice in Gibco PBS (500µL per well). Coverslips were first incubated in 4% paraformaldehyde for 15 minutes at room temperature, incubated in 0.1% Triton X-100 for 15 minutes, then immersed in 1% bovine serum albumin blocking buffer for 1 hour. Coverslips were incubated in 50µL primary antibody overnight at 4°C (primary antibodies: anti-LAMP-1 (1D4B, 1:1000, Abcam ab25245) and anti-α-synuclein (MJFR1, 1:500, Abcam ab138501), secondary antibodies for 1 hour at room temperature (LAMP-1 secondary antibody: 1:1000 goat anti-rat IgG H&L Alexa Fluor 594, Abcam ab150168; α-synuclein secondary antibody: 1:500 goat anti-rabbit Alexa Fluor 488, Life Technologies A11008), and 0.5µg/mL 4′,6-Diamidino-2-Phenylindole, dihydrochloride (DAPI; Thermo Fisher Scientific) for 5 minutes. Each step of preparation was followed by four washes of coverslips in PBS.

Coverslips were washed, then mounted onto slides using ProLong Diamond Antifade mountant (Thermo Fisher Scientific). Images were acquired with a Leica SP8 DMi6000 inverted laser scanning microscope, using a 63×1.4 numerical aperture oil objective lens. A Diode 405 laser was used to excite DAPI dye at 405nm and emitted fluorescence was captured by a PMT detector. An argon laser was used to excite Alexa Fluor 488 secondary antibody at 488nm, and a DPSS 561 laser was used to excite the Alexa Fluor 594 secondary antibody at 561nm. Emission from each Alexa Fluor dye was collected by a HyD detector.

To determine the mean intensity of LAMP-1 and α-synuclein signal in LAMP-1-rich regions in control and α-synuclein PFF-treated cells in response to different drug treatments, a rectangular region of interest of equal size was drawn with Fiji image processing software for cells of each condition, and used to quantify signal intensity in each plane of the acquired Z-stack. The signals corresponding to LAMP-1 and α-synuclein were quantified in this region of interest using the Plot Z-axis profile function on Fiji ImageJ (version 2.0.0, ImageJ 1.51w with Java 1.8.0_66; https://fiji.sc/) (113,114).

**Live cell imaging**

SN4741 cells were seeded onto a glass 24-well plate, and treated with various doses of liproxstatin-1. Acridine orange (5µg/mL; BDH Chemicals) was prepared in the treatment media and applied for 15 minutes at 37°C. For other imaging experiments, final concentrations of 50nM Lysotracker Red DND-99 (Life Technologies Australia) and 1:10000 Hoechst 33342 (Thermo Fisher Scientific; 10µg/mL stock) were added to SN4741 cells for 30 minutes in Dulbecco’s Modified Eagle Medium media (supplemented with 10% Fetal Bovine Serum and 1% antibiotic-antimycotic). Cells were washed twice in Hanks’ Balanced Salt Solution (HBSS), and immersed in HBSS for imaging. Fluorescent images of treated cells were acquired on a Leica SP8 DMi6000 inverted laser scanning microscope, using a 63x/1.4 numerical aperture oil objective lens.

For Lysotracker-treated cells, Hoechst 33342 dye was excited by a 405nm laser and fluorescence collected by a PMT detector. Lysotracker Red DND-99 dye was excited by a 561nm laser and fluorescence was captured by a HyD detector. Nuclear material dyed with acridine orange was imaged by excitation with a 488nm laser and fluorescence collected by a PMT detector. Acridine orange dye accumulated in acidic vesicles was excited by a 561nm laser, and fluorescence captured by a HyD detector.

**Western blot**

SN4741 cells were seeded onto a 24-well plate and upon reaching 70-80% confluency, were administered various concentrations of chloroquine diphosphate (Sigma), bafilomycin A1 from *Streptomyces griseus* (Sigma-Aldrich, stock dissolved in 100% ethanol), liproxstatin-1 (Sigma-Aldrich) or vehicle control for 24 hours. Cells were then washed in Gibco PBS and incubated in a lysis buffer for 5 minutes (50mM Tris hydrochloride, 100mM sodium chloride, 0.1% sodium dodecyl sulfate (SDS), 0.1% Triton X-100, 0.1% Tween 20, 1x Roche Complete EDTA-free protease inhibitor cocktail, 1x Roche PhosSTOP, dissolved in Milli-Q water, pH 7.4). Lysed cells were homogenised using a sonicator (Branson Digital Sonifier 450; Branson Ultrasonics; amplitude: 50%; time: 10 seconds) and a microtip probe. Protein quantification of each sample was conducted using a bicinchoninic acid assay, and calculations were performed to determine the volumes needed to ensure equal total protein content in each sample.

1x Bolt lithium dodecyl sulfate sample buffer (Life Technologies) and 1x Bolt Sample Reducing Agent (Life Technologies) was added to each sample, and Milli-Q water was added to ensure equal volumes. Samples were placed on a heat plate set at 90°C for 10-15 minutes to encourage proteins to denature prior to addition to the gel. A NuPage 4-12% Bis-Tris Midi protein gel was loaded into a NuPage cell, which was filled with 1x Bolt MES SDS running buffer. Novaex
Sharp Pre-stained Protein Standard (Life Technologies) was loaded into Lane 1 as a molecular weight marker, and protein samples loaded into the remaining wells. The cell was connected to a Bio-Rad PowerPac and the gel run at settings of 150V, 3A, and 300W. Once loading dye reached the bottom, proteins were transferred from the gel to a polyvinylidene fluoride membrane (PVDF, 0.2µm) using an iBlot 2 Transfer Stack and iBlot 2 Gel transfer device. Upon completion of transfer, the membrane was blocked for one hour in Tris-Buffered Saline and Tween-20 (TBST) containing 5% skim milk, then incubated in the appropriate primary antibody at 4°C overnight (anti-ferritin, 1:1000, Abcam ab75973; anti-p62, 1:1000, Abcam ab56416, anti-LC3B, 1:1000, Sigma-Aldrich L7543; anti-LAMP-1, 1:1000, Abcam ab24170; Anti-β-actin, 1:10000, Sigma-Aldrich A5441). After primary antibody incubation, each membrane was washed in TBST for 50 minutes, before addition of the appropriate secondary antibody (following ferritin, LC3B, LAMP-1 primaries: Dako polyclonal goat anti-rabbit immunoglobulins/horseradish peroxidase, 1:5000, P026002-2 (Agilent Technologies); following p62 and β-actin primaries: Dako polyclonal rabbit anti-mouse immunoglobulins/ horseradish peroxidase, 1:5000, P026002-2 (Agilent Technologies)).

Following incubation in secondary antibodies and subsequent TBST washes, Amersham Enhanced Chemiluminescence western blotting Detection Reagents 1 and 2 (Bio-Strategy) were spread evenly over membranes to allow detection of secondary antibody binding to the membrane. Each membrane was imaged using the ImageReader LAS-4000 program and a LAS-4000 (Fujifilm) image analyser. Densitometry was performed using ImageJ, with the intensity of ferritin, p62, LC3-II and LAMP-1 corrected for a β-actin loading control, and expressed as a percentage of the vehicle control condition.

**Statistical analysis**

A two-way analysis of variance (ANOVA) was used to determine whether there was an interaction between genotype and α-synuclein PFF toxicity for the WT and α-synuclein KO primary cortical neurons, and whether there was an interaction between incubation duration and α-synuclein PFF toxicity in SN4741 cells. In experiments involving treatment of cells with BSO ± α-synuclein were analysed by a two-way independent groups ANOVA to analyse an effect of BSO on α-synuclein-induced loss of viability, as there was significant toxicity associated with the range of BSO concentrations used.

A one-way ANOVA with a Dunnett’s post-hoc test for multiple comparisons was used to compare the cell viability of α-synuclein PFF-treated cells with controls, and to compare the % rescue of α-synuclein PFF + drug treated cells from baseline α-synuclein PFF toxicity. Data from drug co-treatment experiments were expressed as percentage rescue of viability from intoxication caused by α-synuclein PFFs (assigned 0% rescue of viability). The percentage rescue of viability in intoxicated cells co-treated with drugs were calculated based on the viability of intoxicated cells treated with a vehicle (non-intoxicated cells were assigned 100%).
Data availability

Data available upon request. Contact Scott Ayton of the Florey Institute of Neuroscience and Mental Health: scott.ayton@florey.edu.au

Author contributions

Stephanie J. Guiney performed experiments, analysed data, wrote manuscript; Paul A. Adlard supervised experiments, edited manuscript; Peng Lei performed experiments, contributed funding, edited manuscript, scientific concept; Celeste H. Mawal performed experiments; Ashley I. Bush contributed funding, edited manuscript; David I. Finkelstein supervised experiments, contributed scientific concept, edited manuscript; Scott Ayton contributed scientific concept, supervised experiments, funding, edited manuscript.

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Conflict of Interest

Dr. Bush is a shareholder in Alterity Biotechnology Pty Ltd., Cogstate Pty Ltd, Eucalyptus Pty Ltd., Mesoblast Pty Ltd., Brighton Biotech LLC, Nexxtvet Ltd, Grunbiotics Pty Ltd, Collaborative Medicinal Development LLC, and a paid consultant for Collaborative Medicinal Development. Dr. Adlard and Dr. Finkelstein are a shareholders in, and consultants for, Alterity Biotechnology Pty Ltd.

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Table 1 summary of prior findings regarding lysosomal-based drugs in α-synuclein models

| Drug          | Extracellularly administered α-synuclein                                                                 | α-synuclein over-expression models                                                                 |
|---------------|----------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| Chloroquine   | -Chloroquine inhibited α-synuclein degradation in primary neuron-glial cultured cells following administration of recombinant human α-synuclein PFFs (53).  
                          -Chloroquine had no effect on degradation of α-synuclein in PFF-treated cells (77). | -Chloroquine up-regulated the extracellular release of α-synuclein from induced pluripotent stem cells derived from Parkinson’s disease patients with a GBA mutation (115), SH-SY5Y cells induced to over-express WT α-synuclein (72), and from human H4 neuroglioma cells induced to over-express α-synuclein (91) and Syn-T aggregates (116).  
                          -Chloroquine reduced degradation of α-synuclein in SH-SY5Y cells (117).  
                          -Chloroquine did not significantly increase toxicity in human H4 neuroglioma cells induced to over-express α-synuclein (91).  
                          -Chloroquine reduced the extent of α-synuclein aggregation in a SynT cell culture model that induces the formation of intracellular α-synuclein aggregates, but did not affect α-synuclein toxicity (118). |
| Bafilomycin A1| -Bafilomycin A1 inhibited α-synuclein aggregation following addition of mutant S129A α-synuclein PFFs to cells (52).  
                          -Bafilomycin A1 caused α-synuclein accumulation in cultured cells following the addition of recombinant human α-synuclein PFFs (53). | -In mitotic and differentiated PC12 cells induced to over-express WT or A53T α-synuclein, bafilomycin A1 decreased clearance of A53T but not WT α-synuclein (75).  
                          -Bafilomycin A1 reduced α-synuclein aggregation in a SynT cell culture system and exacerbated α-synuclein toxicity (118).  
                          -Following induction of α-synuclein over-expression, bafilomycin A1 increased α-synuclein aggregate formation in differentiated SH-SY5Y cells and monkey fibroblasts (90), and exacerbated loss of viability in differentiated SH-SY5Y cells (90) and human H4 neuroglioma cells (91).  
                          -Bafilomycin A1 increased the release of α-synuclein in extracellular vesicles from α-synuclein-overexpressing SH-SY5Y cells (71), and from induced pluripotent stem cells of Parkinson’s disease patients with a GBA mutation (115), and from human H4 neuroglioma cells that either over-expressed α-synuclein (91,119) or Syn-T aggregates (116). |
| Leupeptin      | -Leupeptin reduced degradation of recombinant α-synuclein that polymerised after addition to cells (120) | -Leupeptin addition to WT mouse primary cortical neurons over-expressing human α-synuclein caused an increase in levels of insoluble |
Leupeptin inhibiting the activity of an enzyme crucial for α-synuclein degradation, and increased α-synuclein aggregate formation in a cell-free aggregation assay (121).

α-synuclein, but did not worsen α-synuclein toxicity (79).

| E-64/E-64D | -E-64D reduced α-synuclein aggregate formation in response to administration of mutant S129A α-synuclein PFFs to cells (52). |
|------------|----------------------------------------------------------------------------------------------------------------------------------|
|            | -E-64 didn’t cause accumulation of endogenous monomeric or oligomeric α-synuclein in SH-SY5Y cells (122). |
|            | -E-64 increased α-synuclein aggregate formation in differentiated PC12 cells (123) and in monkey fibroblasts induced to express human α-synuclein (90). |
Figure 1. Aggregation optimisation and cell viability profiles of α-synuclein PFFs and erastin. 5mg/mL recombinant WT human monomeric α-synuclein was constantly agitated at 1400rpm and 37°C for 5 days. (a) Thioflavin T assay was used to confirm the presence of fibrillar α-synuclein at an emission wavelength of 475nm (N=5 independent experiments, fluorescence quantified in triplicate at each time point). Values are mean arbitrary Thioflavin T fluorescence ± standard error of the mean (SEM). (b) Electron microscopy was utilised to confirm the presence of fibrillar structures at the endpoint of aggregation, and fibril disruption into smaller fragments following 30 seconds of sonication. Scale bar = 200nm. (c) Loss of viability caused by α-synuclein PFFs did not significantly differ between 24 and 48 hours of treatment in SN4741 cells. (d) SN4741 cells and STHdhQ7/7 cells were susceptible to α-synuclein PFF-induced loss of cell viability, but not monomeric α-synuclein after 24 hours of exposure. (e) WT mouse primary cortical neurons were vulnerable to α-synuclein PFF-induced loss of viability in background strains C57/B16 and C57/B16/129sv. (f) Fibrillar α-synuclein did not differentially affect cell viability in WT and α-synuclein KO mouse primary cortical neurons (N=2 experiments, at least 5 experimental replicates of each concentration. (g) Erastin induced robust toxicity when administered at multiple sub-micromolar concentrations in STHdhQ7/7 cells and SN4741 cells. Unless otherwise specified, all viability assays were performed at least 3 times, with at least 4 experimental replicates of each condition. Cell viability was calculated as a percentage of the control MTT signal, with values shown as mean % control (0µM α-synuclein, PBS vehicle in media) ± SEM. */#/ p < .05, ****/#/#/# p < .0001. n.s. = not significant, mon = monomer.
Figure 2. The effect of pharmacologically targeting GSH and iron on the toxicity of erastin and α-synuclein PFFs. (a) BSO reduced the baseline viability of cells administered 500nM or 250nM erastin in STHdhQ7/7 cells and SN4741, respectively, but did not significantly exacerbate loss of viability caused by 50nM α-synuclein PFFs in STHdhQ7/7 nor 1µM α-synuclein PFFs in SN4741 cells. (c) Treatment of each cell line with 0-1mM GSH increased the viability of cells administered erastin, but did not significantly increase the viability of STHdhQ7/7 cells (N = 2 experiments) or SN4741 cells administered α-synuclein PFFs (d). DFO, an iron chelator, protected against erastin toxicity (e), but did not protect against α-synuclein PFF-induced loss of viability in either cell type (f). At least 3 experimental replicates of each condition were used per experiment. Data shown are the mean % change in baseline viability of cells treated with 250nM or 500nM erastin, 50nM PFFs or 1µM PFFs ± SEM in response to each drug. For STHdhQ7/7 cells: # p < .05, ## p < .01, #### p < .0001; for SN4741 cells: * p < .05, ** p < .01, **** p < .0001. n.s. = not significant.
Figure 3. The effect of lipid peroxidation inhibitors on the toxicity of erastin and α-synuclein PFFs. Ferrostatin-1 protected against erastin toxicity in STHdhQ7/7 and SN4741 cells (a), but did not recover viability of α-synuclein PFF-treated STHdhQ7/7 cells (50nM α-synuclein), SN4741 cells (1µM α-synuclein) nor C57/129sv/B6 WT primary cortical neurons (1µM α-synuclein; N=2 independent experiments (b). Liproxstatin-1 decreased toxicity caused by erastin in STHdhQ7/7 and SN4741 cells (c), and by (d) α-synuclein PFFs in STHdhQ7/7 cells, SN4741 cells (N=2 experiments) and WT primary cortical neurons (N=2 experiments). At least 3 experimental replicates of each condition were used per experiment. Data shown are the mean % change in baseline viability of cells treated with 250nM or 500nM erastin, 50nM PFFs or 1µM PFFs ± SEM in response to each drug. For STHdhQ7/7 cells: ### $p < .001$, #### $p < .0001$; for SN4741 cells: * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$; for WT primary cortical neurons: ^ $p < .05$, ^^ $p < .001$, ^^^ $p < .0001$. 
Figure 4. The effect of known inhibitors of apoptosis and necrosis on α-synuclein-induced loss of viability in SN4741 cells. α-synuclein toxicity was not inhibited with administration of the broad-spectrum caspase inhibitor (a) Q-VD-OPH in SN4741 cells. A necroptosis inhibitor, 7-Cl-O-Nec-1, did not significantly change the extent of α-synuclein PFF-induced loss of viability in (b) SN4741 cells. All viability assays were performed at least 3 times, with at least 4 experimental replicates of each drug concentration ± 1µM α-synuclein. Data shown are mean % increase in baseline viability of cells treated with 1µM α-synuclein PFFs ± SEM.

Figure 5. The effect of liproxstatin-1 on acidic vesicles. Images were acquired of live SN4741 cells in HBSS following 30-minute incubation in 50nM Lysotracker Red DND-99 and 15-minute incubation in 1µg/mL Hoechst 33342 for the following 24-hour treatment conditions: (a) vehicle control, (b) 2.5µM liproxstatin-1, (c) 5µM liproxstatin-1, and (d) 10µM liproxstatin-1. Lysotracker staining and Hoechst staining are indicated by red and blue, respectively. Images were also acquired of live SN4741 cells in HBSS following 15-minute incubation in 5µg/mL acridine orange for each condition (e-h). Acridine orange staining corresponding to acidic vesicles and nuclear material are indicated by red and blue, respectively. The area occupied by acidic vesicle staining was visibly increased for 5-10µM liproxstatin-1 treatments. Images shown are maximum intensity projections representative of the effect observed in repeated experiments (N=3 for Lysotracker staining, N=2 for acridine orange staining).
Figure 6. The effect of liproxstatin-1 and common lysosomal inhibitors on autophagy markers. An immunoblot of each autophagy marker and the loading control, β-actin in response to liproxstatin-1, bafilomycin A1 and chloroquine is shown above (a). Changes in levels of ferritin (b), LAMP-1 (c), p62 (d), and LC3-II (e) in response to liproxstatin-1 (blue), bafilomycin A1 (purple) and chloroquine (green) were analysed relative to β-actin and expressed as mean percentage of vehicle control ± SD for 4 independent experiments (3 experimental replicates per condition). Stars indicate significant difference from control for each drug, data presented on same axis for simplicity. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. 
Figure 7. α-synuclein toxicity was lysosome-dependent in vitro. 1µM α-synuclein PFFs were administered to cultured cells for 24 hours in the presence of lysosome-targeting drugs, and their effect on cell viability was quantified by MTT assay. Loss of viability caused by 1µM α-synuclein PFFs in STHetQ7/7 and SN4741 cells was significantly rescued by (a) chloroquine, (b) bafilomycin A1, (c) leupeptin hemisulfate and (d) E-64D. (e) Ca-074-Me also significantly improved cell viability in SN4741 cells, but not STHetQ7/7 cells administered α-synuclein PFFs. α-synuclein-induced loss of viability was not recovered by (f) pepstatin A, (g) rapamycin, (h) wortmannin or (i) 3-methyladenine. All viability assays were performed at least 3 times, with at least 3 experimental replicates of each drug concentration ± 1µM α-synuclein. (j) Heparin significantly abolished loss of viability caused by 1µM α-synuclein PFFs in STHetQ7/7 cells and SN4741 cells. Data shown are mean % increase in baseline viability of cells treated with 1µM α-synuclein PFFs ± SEM. One-way ANOVA was used to analyse the % increase in baseline viability in each cell type. SN4741 cells- * p < .05, ** p < .01, *** p < .001, **** p < .0001; STHetQ7/7 cells- # p < .05, ### p < .01, #### p < .001, ##### p < .0001. n.s. = not significant.
Figure 8. Intensity of α-synuclein signal in LAMP-1 rich regions of cells administered chloroquine and heparin. Images were acquired of fixed and permeabilised SN4741 cells treated for 24 hours with (a) vehicle control, (b) 1µM α-synuclein, (c) 100µg/mL heparin sodium, (d) 100µg/mL heparin sodium + 1µM α-synuclein, (e) 20µM chloroquine, and (f) 20µM chloroquine + 1µM α-synuclein PFFs. Coverslips were incubated in primary antibodies for human α-synuclein (MJFR-1; anti-rabbit Alexa-Fluor 488 secondary; green), mouse LAMP-1 (1D4B; anti-rat Alexa-Fluor 594 secondary; red), and a DAPI nuclear stain (blue). For each condition, the maximum intensity projection of a Z-stack is shown at the top (scale bar= 50µm), followed by a representative single cell image (scale bar= 5µm), and the corresponding graphs of the mean intensity of α-synuclein signal in LAMP-1-rich regions. Graphs show mean intensity ± SEM at each distance through the Z-plane for each cell. 24 cells were analysed from vehicle control and 1µM α-synuclein, and 10 cells were analysed for all other conditions.
Fibrillar α-synuclein toxicity depends on functional lysosomal
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