1 **Pharmacological rescue of impaired mitophagy in Parkinson's disease-related LRRK2**

2 **G2019S knock-in mice**

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8 Running Title: LRRK2 regulated mitophagy

9 Key Words: Parkinson's, LRRK2, mitophagy, autophagy, kinase inhibitor, GSK3357679A

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Abstract

12 Parkinson's disease (PD) is a major and progressive neurodegenerative disorder, yet the

13 biological mechanisms involved in its aetiology are poorly understood. Evidence links this

14 disorder with mitochondrial dysfunction and/or impaired lysosomal degradation – key features

15 of the autophagy of mitochondria, known as mitophagy. Here we investigated the role of

16 LRRK2, a protein kinase frequently mutated in PD, on this process *in vivo*. Using mitophagy

17 and autophagy reporter mice, bearing either knockout of LRRK2 or expressing the pathogenic

18 kinase-activating G2019S LRRK2 mutation, we found that basal mitophagy was specifically

19 altered in clinically relevant cells and tissues. Our data show that basal mitophagy inversely

20 correlates with LRRK2 kinase activity *in vivo*. In support of this, use of distinct LRRK2 kinase

21 inhibitors in cells increased basal mitophagy, and a CNS penetrant LRRK2 kinase inhibitor,

22 GSK3357679A, rescued the mitophagy defects observed in LRRK2 G2019S mice. This study

23 provides the first *in vivo* evidence that pathogenic LRRK2 directly impairs basal mitophagy, a

24 process with strong links to idiopathic Parkinson's disease, and demonstrates that

25 pharmacological inhibition of LRRK2 is a rational mitophagy-rescue approach and potential

26 PD therapy.

27

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting 1-2% of the population over 60 years old, and 4% above 85 years of age [1]. The main symptoms of PD are muscle rigidity, bradykinesia, resting tremor, and postural instability and may be accompanied by sleep disorders, anosmia, depression, and dementia [2]. It is characterised by a progressive and selective degeneration of dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc). Currently, there are no treatments available that modify the course of neurodegenerative decline. Although this disease is mostly sporadic, about 15% of cases appear to be inherited and in support of this, 20 genes implicated in PD have been identified from familial genetic studies while ~90 loci have been identified from PD-GWAS [3]. The exact causes of PD are currently unknown but some evidence strongly links impaired mitochondrial and lysosomal function to disease pathology [2].

Mutations in *PARK8*, encoding for LRRK2 (Leucine Rich Repeat Kinase 2), are the most frequently reported cause of PD [4]. The most common mutation associated with PD is the substitution of glycine at position 2019 of LRRK2 to serine (G2019S), representing 4% of familial and 1% of sporadic cases [5]. LRRK2 is a large multidomain protein with two catalytic domains: a Ras of complex (ROC) GTPase domain that is able to bind GTP and hydrolyse it, and a kinase domain that utilises a subset of Rab GTPases as substrates [6]. Importantly, all the segregating mutations associated with PD are located in the catalytic core. A mutation in the ROC/COR domain, such as the R1441C/G/H or the Y1699C mutation, leads to decreased GTPase activity and elevated kinase activity [7]. Mutations in the kinase domain, such as the G2019S or the I2020T, also lead to an elevated kinase activity. Hence, enhanced kinase activity appears to be a common factor in pathogenic LRRK2 mutations. Although the function of

3

51 LRRK2 within cells is currently unknown, mounting evidence implicates a role in membrane

52 trafficking [6,8,9].

53 Macroautophagy is a membrane trafficking pathway that delivers intracellular components to

54 the lysosome for degradation [10]. These components can include whole organelles such as

55 mitochondria. The autophagic turnover of mitochondria is termed mitophagy, which acts as a

56 mitochondrial quality control mechanism that allows the selective degradation of damaged or

57 unnecessary mitochondria [11,12]. Mitophagy itself has strong links to PD following the landmark

58 discoveries that PINK1 and Parkin, two other genes mutated in familial PD, sequentially

59 operate to initiate mitophagy in response to mitochondrial depolarisation in cell lines [13–16].

60 However, when this pathway becomes relevant *in vivo,* and under what physiological

61 conditions, is unclear especially given that PINK1 and Parkin are not required for regulation of

62 mitophagy under normal, or basal, conditions [17–19]. Indeed, our understanding of the detailed

63 mechanisms regulating basal mitophagy remains elusive.

64 In this study, we sought to define the physiological link between mitochondrial turnover and

65 LRRK2 in relation to PD. We utilised our previously published mouse reporter models to study

66 mitophagy (*mito*-QC) and autophagy (*auto*-QC; Fig. 1A, C and [17,20,21]) in either LRRK2

67 knockout mice, or knock-in mice harbouring the pathogenic LRRK2 G2019S mutation. Whilst

68 we found minimal impact of LRRK2 on general autophagy (macroautophagy), we observed

69 that the LRRK2 G2019S activation-mutation was associated with reduced mitophagy in

70 specific tissues, including dopaminergic neurons and microglia within the brain. In contrast,

71 knockout of LRRK2 resulted in increased mitophagy. Taken together, these data imply that

72 LRRK2 kinase activity inversely correlates with basal mitophagy levels. In support of this, we

73 found that that treatment of cells or animals with the potent and selective CNS penetrant

74 LRRK2 kinase inhibitor, GSK3357679A (Ding et al., in preparation), rescued these LRRK2

75   G2019S-associated mitophagy defects and enhanced mitophagy in dopamine neurons and

76   microglia in the brains of genotypically normal mice. Our results identify a physiological role

77   for LRRK2 in the regulation of basal mitophagy *in vivo* and underline the potential value of

78   pharmacological inhibition of LRRK2 as a potential therapeutic strategy to ameliorate aspects

79   of Parkinson's disease driven by mitochondrial dysfunction.

80

Material and methods

Animals

Experiments were performed on mice genetically altered for Leucine-rich repeat kinase 2 (LRRK2), using either wild-type, LRRK2 G2019S [6] mutation knock-in mice, or mice in which LRRK2 has been ablated [22,23] (KO). The mitophagy (*mito*-QC) and the autophagy (*auto*-QC) reporter mouse models used in this study were generated as previously described [17,20].

Primary mouse embryonic fibroblasts culture

Primary mouse embryonic fibroblasts (MEFs) were derived, from time-mated pregnant females at E12.5. Primary MEFs were maintained in DMEM (Gibco, 11960-044) supplemented with 10% FBS, 2 mM L-Glutamine (Gibco, 2503-081), 1% Na-Pyruvate (Gibco, 11360-070), 1% Non-essential amino acids (Gibco, 11140-035), 1% Antibiotics (Penicillin/Streptomycin 100 U/ml penicillin and 100 μg/ml streptomycin; Gibco), and 150μM β-Mercaptoethanol (Gibco, 21985-023) at 37°C under a humidified 5% CO_2 atmosphere.

Primary mouse embryonic fibroblasts treatments

 To assess mitophagy and autophagy upon stimulation, cells were treated for 24 hours with either 1 mM 3-Hydroxy-1,2-dimethyl-4(1H)-pyridone (Deferiprone/DFP, Sigma-Aldrich, 379409), or incubated in Earl's balanced salt solution (EBSS, Gibco, 24010-043). mito-QC MEFs were also treated for 24 hours with LRRK2 kinase activity inhibitors GSK2578215A [24] (250, 500, 1000 nM)), MLi-2 [25] (5, 10, and 20 nM), or GSK3357679A (compound **39** , Ding et al., in prep.) (0.1, 1, 10, 100, 1000 nM). All treatments (apart from EBSS) were in DMEM (Gibco, 11960-044) supplemented with 10% FBS, 2 mM L-Glutamine (Gibco, 2503-081), 1% Non-essential amino acids (Gibco, 11140-035), 1% Antibiotics (Penicillin/Streptomycin 100

103   U/ml penicillin and 100 µg/ml streptomycin; Gibco), and 150µM β-Mercaptoethanol (Gibco,

104   21985-023) at 37ºC under a humidified 5% CO_2 atmosphere. MLi-2 and GSK2578215A were

105   synthesized by Natalia Shapiro (University of Dundee) as described previously [24,25].

106   *Light Microscopy*

107   MEFs were plated on glass coverslips and treated as described in the previous paragraph. At

108   the end of the treatment, cells were washed twice in DPBS (Gibco, 14190-094), and fixed in

109   3.7% Paraformaldehyde (Sigma, P6148), 200 mM HEPES, pH=7.00 for 20 minutes. Cells were

110   washed twice with, and then incubated for 10 minutes with DMEM, 10mM HEPES. After a

111   wash with DPBS, nuclei were stained with Hoechst 33342 (1 µg/mL, Thermo Scientific, 62249)

112   for 5 minutes. Cells were washed in DPBS and mounted on a slide (VWR, Superfrost, 631-

113   0909) with Prolong Diamond (Thermo Fisher Scientific, P36961). Images were acquired using

114   a Nikon Eclipse Ti-S fluorescence microscope with a 63x objective.

115   *Quantitation of Mitophagy and Autophagy in vitro*

116   Quantification of red-only dots was semi-automatized using the *mito*-QC counter plugin on

117   FIJI as previously described [26,27]. Autophagosomes and autolysosomes were quantified using

118   the Autophagy counter plugin on FIJI developed in house, following the same principle as the

119   mito-QC counter [28]. The macro "auto-QC_counter.ijm" ("version 1.0 release", DOI:

120   10.5281/zenodo.4158361) is available from the following github repository:

121   https://github.com/graemeball/auto-QC_counter.

122   *Animal Studies*

123   Experiments were performed on 81 adult mice (9-23 weeks old) of both genders (n=8-12 per

124   group for the mito-QC reporter, and n=9-15 per group for the auto-QC reporter), all

125   homozygous for the corresponding reporter (mitophagy or autophagy).

126   The effect of the CNS penetrant LRRK2 kinase inhibitor GSK3357679A *in vivo* was assessed

127   using 50 adult mice (9-17 weeks old at the end of the study), all homozygous for the mito-QC

128   reporter. Mice of both genders were randomly assigned to the vehicle or to the GSK3357679A

129   treated group (WT-Vehicle: n=10, WT-GSK3357679A: n=10, G2019S-Vehicle: n=10,

130   G2019S-GSK3357679A: n=10, KO-Vehicle: n=5, and KO-GSK3357679A: n=5). Vehicle

131   treated animals were dosed (10 mL/kg) with aqueous methylcellulose (1% w/v, Sigma, M0512)

132   prepared in sterile water (Baxter, UKF7114), or with GSK3357679A (15 mg/kg/dose) prepared

133   in aqueous methylcellulose. Treatment was administered by oral gavage every 12 hours for a

134   total of four times per mouse. Mice were culled 2 hours (+/- 9 minutes) after the last dosing.

135   Animals were housed in sex-matched littermate groups of between two and five animals per

136   cage in neutral temperature environment (21° ± 1 °C), with a relative humidity of 55-65%, on

137   a 12:12 hour photoperiod, and were provided food and water *ad libitum*. All animal studies

138   were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act

139   1986 as well as the GSK Policy on the Care, Welfare and Treatment of Animals, and were

140   performed in agreement with the guidelines from Directive 2010/63/EU of the European

141   Parliament on the protection of animals used for scientific purposes. All animal studies and

142   breeding were approved by the University of Dundee ethical review committee, and further

143   subjected to approved study plans by the Named Veterinary Surgeon and Compliance Officer

144   (Dr. Ngaire Dennison) and performed under a UK Home Office project license in agreement

145   with the Animal Scientific Procedures Act (ASPA, 1986).

146   ***Sample collection***

147   Mice were terminally anesthetised with an intraperitoneal injection of pentobarbital sodium

148   (Euthatal, Merial) then trans-cardially perfused with DPBS (Gibco, 14190-094) to remove

149   blood. Tissues were collected and either snap frozen in liquid nitrogen and stored at -80ºC for

150   later biochemical analyses or processed by overnight immersion in freshly prepared fixative:

151   3.7% Paraformaldehyde (Sigma, P6148), 200 mM HEPES, pH=7.00. The next day, fixed

152   tissues were washed three times in DPBS, and immersed in a sucrose 30% (w/v) solution

153   containing 0.04% sodium azide until they sank at the bottom of the tube. Samples were stored

154   at 4ºC in that sucrose solution until further processing.

155

Immunolabeling of brain free-floating sections

157   The brain was frozen-sectioned axially using a sledge microtome (Leica, SM2010R), and 50

158   microns thick sections were stored in PBS at 4ºC until further treatment. Free-floating sections

159   were permeabilised using DPBS (Gibco, 14190-094) containing 0.3% Triton X-100 (Sigma

160   Aldrich, T8787) 3 times for 5 minutes. Sections were then blocked for one hour in blocking

161   solution (DPBS containing 10% goat serum (Sigma Aldrich, G9023), and 0.3% Triton X-100).

162   Primary antibody incubation was performed overnight in blocking solution containing one of

163   the following antibodies: Anti-Tyrosine Hydroxylase (1/1000, Millipore, AB152), Anti-Iba-1

164   (1/1000, Wako, 019-19741), Anti Calbindin-D28k (1/1000, Swant, CB38), Anti-Glial

165   Fibrillary Acidic Protein (1/1000, Millipore, MAB360). The next day, sections were washed 2

166   times for 8 minutes in DPBS containing 0.3% Triton X100 and then incubated for 1 hour in

167   blocking solution containing the secondary antibody (1/200, Invitrogen P10994 Goat anti-

168   Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Pacific Blue, or Invitrogen P3182

169   Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Pacific Blue). Sections

170   were then washed 2 times for 8 minutes in DPBS containing 0.3% Triton X100 and mounted

171   on slides (Leica Surgipath® X-tra™ Adhesive, 3800202) using Vectashield Antifade Mounting

172   Medium (Vector Laboratories, H-1000) and sealed with nail polish.

Tissue section and immunostaining

174   Tissues were embedded in an O.C.T. compound matrix (Scigen, 4586), frozen and sectioned

175   with a cryostat (Leica CM1860UV). 12 microns sections were placed on slides (Leica

176   Surgipath® X-tra™ Adhesive, 3800202), and then air dried and kept at -80ºC until further

177   processing. Sections were thawed at room temperature and washed 3 times 5 minutes in DPBS

178   (Gibco, 14190-094). Sections were then counterstained for 5 minutes with Hoechst 33342

179   (1µg/mL, Thermo Scientific, 62249). Slides were mounted using Vectashield Antifade

180   Mounting Medium (Vector Laboratories, H-1000) and high precision cover glasses (No. 1.5H,

181   Marienfeld, 0107222) and sealed with transparent nail polish.

Confocal microscopy

183   Confocal micrographs were obtained by uniform random sampling using either a Zeiss

184   LSM880 with Airyscan, or a Zeiss 710 laser scanning confocal microscope (Plan-Apochromat

185   63x/1.4 Oil DIC M27) using the optimal parameters for acquisition (Nyquist). 10-15 images

186   were acquired per sample, depending on the tissue, by an experimenter blind to all conditions.

187   High resolution, representative images were obtained using the Super Resolution mode of the

188   Zeiss LSM880 with Airyscan.

Quantitation of Mitophagy and Autophagy in vivo

190   Quantification of mitophagy and autophagy was carried out on at least 10 pictures per

191   sample. Images were processed with Volocity Software (version 6.3, Perkin-Elmer). Images

192   were first filtered using a fine filter to suppress noise. Tissue was detected by thresholding the

193   Green channel. For the immunolabelings in the brain (TH, Iba1, Calbindin D-28k, and GFAP),

194   each cell population of interest was detected by thresholding the Pacific Blue labelled channel.

195   A ratio image of the Red/Green channels was then created for each image.

196   For the mito-QC reporter, mitolysosomes were then detected by thresholding the ratio channel

197   as objects with a high Red/Green ratio value within the tissue/cell population of interest. The

198   same ratio channel threshold was used per organ/set of experiments. To avoid the detection of

199   unspecific high ratio pixels in the areas of low reporter expression, a second red threshold was

200   applied to these high ratio pixels. This double thresholding method provides a reliable detection

201   of mitolysosomes as structures with a high Red/Green ratio value and a high Red intensity

202   value.

203   For the general autophagy reporter, high intensity red pixels were detected by thresholding the

204   red channel within the tissue/cell population of interest. The same red channel threshold was

205   used per organ/set of experiments. Autophagosomes and autolysosomes were then

206   differentiated by thresholding the high intensity red pixels depending on their Red/Green ratio

207   channel value. Pixels with a low Red/Green ratio were considered as autophagosomes, whereas

208   pixels with a high Red/Green ratio were considered as autolysosomes. The same ratio channel

209   threshold was used per organ/set of experiments.

210   ***Filipin staining***

211   Frozen fixed tissue sections were washed in PBS to remove any excess O.C.T compound

212   (Scigen, 4586) excess. Sections were then incubated for 2 hours at room temperature with filipin

213   (200 μg/mL; Sigma-Aldrich, F9765) and then washed twice in PBS. Tissue sections were

214   mounted using Vectashield Antifade Mounting Medium (Vector Laboratories, H-1000) and

215   sealed with nail polish. High resolution, representative images were obtained using the Super

216　Resolution mode of the Zeiss LSM880 with Airyscan (Plan-Apochromat 63x/1.4 Oil DIC

217　M27).

218

219　***Western blotting***

220　Frozen tissue was homogenized with a Cellcrusher™ (Cellcrusher, Cork, Ireland) tissue

221　pulveriser. Approximately 20-30 mg of pulverised tissue were then lysed on ice for 30 min with

222　(10 µL/mg tissue) of RIPA buffer [50 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1%

223　NP-40, 1% Na-deoxycholate, 0.1% SDS, and cOmplete^{TM} protease inhibitor cocktail (Roche,

224　Basel, Switzerland)], phosphatase inhibitor cocktail (1.15 mM sodium molybdate, 4 mM

225　sodium tartrate dihydrate, 10 mM β-glycerophosphoric acid disodium salt pentahydrate, 1 mM

226　sodium fluoride, and 1mM activated sodium orthovanadate), and 10 mM DTT. After lysis, the

227　mixture was vortexed and centrifuged for 10 min at 4 °C at 20,817 G. The supernatant was

228　collected, and the protein concentration determined using the Pierce BCA protein assay kit

229　(ThermoFisher Scientific, Waltham, MA, USA). For each sample, 20-25 µg of protein was

230　separated on a NuPAGE 4–12% Bis-Tris gel (Life technologies, Carlsbad, CA, USA). Proteins

231　were electroblotted to 0.45µm PVDF membranes (Imobilon-P, Merck Millipore, IPVH00010;

232　or Amersham Hybond, GE Healthcare Life Science, 10600023), and immunodetected using

233　primary antibodies directed against phospho-Ser935 LRRK2 rabbit monoclonal (1/1000, MRC

234　PPU Reagents and Services, UDD2), LRRK2 rabbit monoclonal (1/1000, MRC PPU Reagents

235　and Services, UDD3), phospho-Rab10 (Thr73) rabbit monoclonal (1/1000, Abcam, ab230261),

236　Rab10 mouse monoclonal (1/1000, nanoTools 0680-100/Rab10-605B11), α-Tubulin (11H10)

237　Rabbit monoclonal antibody (1/10000, CST, 2125S), and β-Actin mouse monoclonal antibody

238　(1/1000, Proteintech, 60008-1-Ig). All antibodies to LRRK2 were generated by MRC PPU

239　Reagents and Services, University of Dundee (http://mrcppureagents.dundee.ac.uk).

240   *Statistics*

241   Data are represented as means ± SEM. Number of subjects are indicated in the respective figure

242   legends. Statistical analyses were performed using a one-way analysis of variance (ANOVA)

243   or two-way ANOVA followed by a Tukey HSD using RStudio version 1.1.1335 [29]. Statistical

244   significance is displayed as * p< 0.05: ** p < 0.01, *** p<0.001, and **** p<0.0001.

245

246   **Results**

247   ***The pathogenic G2019S LRRK2 mutation impairs basal mitophagy but not autophagy* in**

248   **vitro**

249   To investigate the physiological role of LRRK2 in regulating autophagy we utilised two

250   previously validated and highly similar mouse reporter models [17,20,21]. These transgenic reporter

251   models rely on constitutive expression of a tandem mCherry-GFP tag from the *Rosa26* locus.

252   In the *mito*-QC model, which monitors mitophagy, the tandem tag is localised to mitochondria

253   (by an outer mitochondrial targeting sequence derived from residues 101-152 of the protein

254   FIS1). In the *auto*-QC model, which monitors general (macro)autophagy, the tandem tag is

255   localised to autophagosomes (by conjugation to the N-terminus of MAP1LC3b). For both

256   models, when a mitochondrion or autophagosome is delivered to lysosomes, the low lysosomal

257   luminal pH is sufficient to quench the GFP signal, but not that from mCherry. Hence, the degree

258   of mitophagy or general autophagy can be determined by the appearance of mCherry-only

259   puncta, which represent mito/autolysosomes (Fig. 1A and D). Given that mitophagy is a form

260   of autophagy, the use of both models allows us to monitor the specificity of autophagy *in vivo*.

261   A large disruption of autophagy in general will also influence mitophagy, whereas a block in

262   mitophagy, which likely represents a small fraction of the total autophagy occurring at any one

263   time, will tend to have little influence on the total autophagic levels.

264   To investigate the effect of LRRK2 kinase activity on mitophagy, we first isolated and

265   cultured primary mouse embryonic fibroblasts (MEFs) derived from wild type mice (WT), mice

266   homozygous for the Parkinson's disease-associated LRRK2 G2019S mutant, or mice

267   homozygous for a LRRK2 knockout (KO) variant; all of which were on a homozygous *mito*-

268   QC reporter background (Fig. 1B and C). A small degree of basal mitophagy was evident in all

269   cell lines. However, we observed that LRRK2 G2019S KI mutant cells displayed significantly

270   lower basal mitophagy levels, whereas the absence of LRRK2 (KO) led to an increase of this

271   process. Interestingly, our data suggests that LRRK2 predominantly influences basal mitophagy

272   as deferiprone (DFP), a strong mitophagy inducer [30], increased mitophagy to a similar level

273   across all genotypes (Fig. S1A and B).

274   We next investigated general autophagy using the LRRK2 mouse lines mentioned above

275   on the homozygous *auto*-QC background. In contrast to mitophagy, in isolated primary MEFs

276   we noticed no significant difference in the number of mCherry-only autolysosomes across all

277   the *Lrrk2* genotypes under basal conditions (Fig. 1E and F). We also analysed amino acid

278   starvation-induced autophagy, by incubation in Earls Balanced Salt Solution (EBSS). A robust

279   autophagy response was observed in all cells and as with basal autophagy, the *Lrrk2* genotype

280   failed to significantly alter this large increase in autolysosomes (Fig. S1C).

281   ***LRRK2 kinase inhibitors correct the G2019S mitophagy defects in vitro***

282   Using genetics, our observations show that LRRK2 kinase activity inversely correlates

283   with mitophagy *in vitro*. If this is the case, then pharmacological inhibition of LRRK2 kinase

284   activity should also increase mitophagy. Therefore, we aimed to investigate if the mitophagy

285   deficit observed in the G2019S cells could be rescued with LRRK2-selective kinase inhibitors.

286   To that end, we first studied the effect of two structurally distinct tool LRRK2 kinase inhibitors,

287   GSK2578215A [24] and MLi-2 [25], in primary *mito*-QC MEFs. In the WT group, treatment of cells

288   for 24 h with either compound increased mitophagy to values comparable to what we previously

289   observed in the LRRK2 KO group (Fig. S1D). Although MLi-2 is a more potent LRRK2 kinase

290   inhibitor (IC_{50} 0.76 nM [25]) compared to GSK2578215A (IC_{50} 10nM[24]), at higher concentrations

291   it did not stimulate mitophagy. In the G2019S group, significantly higher concentrations of

292   GSK2578215A were necessary to stimulate mitophagy and this may reflect the increased kinase

293   activity of this mutant [31]. However, with MLi-2 we were not able to fully rescue the mitophagy

15

294 defect. As with the WT cells, treatment with MLi-2 at the highest dose (20 nM) did not stimulate

295 mitophagy. The failure of higher doses of MLi-2 to stimulate mitophagy may be due to an off-

296 target effect, as at 20 nM it also inhibited mitophagy in the LRRK2 KO cells (Fig. S1D).

297 Therefore, we recommend caution when using MLi-2 at high concentrations. Neither

298 compound increased mitophagy in KO cells, demonstrating that their mitophagy-enhancing

299 properties are dependent on LRRK2. Additionally, both compounds inhibited LRRK2 activity,

300 as indicated by loss of LRRK2 S935 phosphorylation (an indirect measure of LRRK2 activity

301 [32], Fig. S1E).

302 With respect to mitophagy, the relatively low potency of GSK2578215A and the off-

303 target effects of MLi2, indicated the need for improved LRRK2 tool inhibitors. For this reason,

304 we turned to GSK3357679A, a novel pyrrolopyrimidine LRRK2 kinase inhibitor that exhibits

305 excellent cellular potency, selectivity, oral bioavailability and PK/PD correlation in animal

306 studies (Ding et al., in prep.). We tested GSK3357679A in primary *mito*-QC MEFs and

307 observed a dose-dependent effect on mitophagy with a maximal stimulation achieved at a

308 concentration of 10 nM in WT cells (Fig. 1G and H). In the G2019S cells we observed a reduced

309 response, with a maximal effect on mitophagy reached at 100 nM. Importantly, contrary to what

310 we observed with MLi-2, GSK3357679A effects on restoration of mitophagy were maintained

311 at the highest concentration used and exhibited no deleterious effects on mitophagy at any

312 concentration in LRRK2 KO cells. Western blotting analysis revealed that GSK3357679A

313 potently inhibited LRRK2 kinase activity in a dose-dependent manner, as indicated by

314 decreased phosphorylation of its substrate Rab10 at threonine 73 [6], as well as reduced LRRK2

315 S935 phosphorylation (Fig. 1H). Thus, genetically and chemically, the data show that LRRK2

316 inhibition enhances basal mitophagy in cells, and GSK3357679A displayed a superior

317 performance compared to other available LRRK2 kinase inhibitors.

318 *Mutation of LRRK2 in vivo alters mitophagy in specific cell populations within the brain*

319   Given the effects of LRRK2 kinase activity on mitophagy *in vitro*, we next sought to

320   use our mouse lines to investigate this *in vivo*. PD is primarily a neurodegenerative disorder, so

321   we first explored mitophagy in the brain. We focussed on four cell populations: two neuronal

322   populations linked to movement - dopaminergic (DA) neurons of the substantia nigra pars

323   compacta (SNpc) and Purkinje neurons of the cerebellum; as well as in two glial cell

324   populations - cortical microglia and cortical astrocytes. In midbrain, we identified SNpc DA

325   neurons using tyrosine hydroxylase (TH) staining and found no difference in the number of DA

326   neurons per field across the *Lrrk2* genotypes (Fig. 2A and B). These cells are the mouse

327   equivalent of the human population of DA neurons that degenerate in PD and we had previously

328   found that they undergo substantial mitophagy [17]. Basal mitophagy was enhanced in the LRRK2

329   KO neurons compared to the WT, and although not statistically significant, mitophagy appeared

330   reduced in DA neurons of LRRK2 G2019S KI mice compared to WT (Fig. 2A and C). This

331   was similar to our earlier observations in MEFs and showed that the presence of LRRK2 can

332   impact mitophagy in this clinically relevant population of neurons within the midbrain. To

333   determine if this effect is typical of neurons in general, we investigated mitophagy in another

334   neuronal population involved in motor control, the Purkinje neurons. These cells were

335   identified in cerebellar sections using immunostaining against the calcium sensor Calbindin-

336   D28k. These cells are rich in mitochondria and as shown previously [20], they also undergo

337   significant mitophagy (Fig. 2D). Contrary to what we observed in SNpc DA neurons, no

338   statistical difference in mitophagy in Purkinje cells was found between any group (Fig. 2E).

339   As we observed neuron-specific alterations of mitophagy, we next examined the effect

340   of *Lrrk2* genotype in two distinct populations glial cells within the cortex. Immune-related

341   microglia were identified by Iba1 (ionized calcium-binding adapter molecule 1, Fig. 2F).

342   Interestingly, we observed an enhanced presence of microglial cells in the cortex of G2019S

343   animals when compared to WT or KO mice (Fig. 2G). We do not yet understand the nature of

344   this increase and further work will be needed to determine if there are simply more microglia

345   in the G2019S mice, or an increased movement of cells to this area of the brain. Regardless,

346   when normalised for cell number, we found a significant decrease in basal mitophagy in

347   G2019S microglia compared to WT, as well as an increase in mitophagy levels in KO cells

348   (Fig. 2H). Thus, as with DA neurons, LRRK2 can impacts basal mitophagy in microglia. In

349   contrast, cortical astrocytes, stained with glial fibrillary acidic protein (GFAP, Fig. 2I), did not

350   show any observable difference in mitophagy across *Lrrk2* genotypes (Fig. 2J).

351   In contrast to the *Lrrk2* genotype effects on mitophagy in DA neurons and microglia,

352   analysis of *auto*-QC mouse brains indicated no change in general macroautophagy in these cell

353   types (Fig. S2). Thus, the LRRK2 G2019S mutation is not causing a major disruption in

354   neuronal autophagy but does influence basal mitophagy levels.

355

356   *Mutation of LRRK2 in vivo also alters mitophagy in peripheral organs with high LRRK2*

357   *expression*

358   We next assessed mitophagy levels in the lungs, a tissue in which the levels of LRRK2

359   are known to be elevated [33,34]. Basal mitophagy across the whole lung was evident in all

360   genotypes and in a similar fashion to MEFs, DA neurons and microglia, mitophagy was reduced

361   in G2019S mice and enhanced (over 2-fold relative to WT) in KO mice (Fig. 3A and B),

362   Consistent with this, mitochondrial content was decreased when comparing KO to G2019S

363   (although no significant increase of this parameter was detected compared to WT, see Fig.

364   S3A). As previously reported [35,36], we observed enlarged type II pneumocytes with large

365   vesicular-like structures in all the animals of the LRRK2 KO group that is attributable to the

366   accumulation of large lamellar bodies, which are secretory lysosomes responsible for surfactant

367   release. We confirmed the nature of these structures as enlarged lamellar bodies by filipin

368   staining lung sections for cholesterol, a component found in surfactant (Fig. S3B).

369   The tissue reported to have the highest LRRK2 expression is the kidney [33,34]. We first

370   investigated mitophagy in the kidney cortex, where we had previously shown the proximal

371   tubules to be a major site of mammalian mitophagy [20]. LRRK2-dependent mitophagy changes

372   in the kidney were much lower in magnitude compared to the lung, yet there was a small

373   decrease in G2019S-expressing tissue (Fig.3C and D). However, we do note that mitophagy is

374   10-fold higher in this region compared to lung, which may mask relatively small changes

375   conferred by *Lrrk2* genotypes.

376   We next studied *in vivo* genotype effects on autophagy, using the same conditions and

377   organs as for the *mito*-QC reporter. Consistent with brain and MEF data, no significant

378   difference in the number of autolysosomes was observed in the lungs of *auto*-QC reporter mice

379   (Fig. 3E and F). Again, enlarged type II pneumocytes were observed in LRRK2 KO animals

380   (Fig. 3E). Likewise, in the kidney cortex we did not detect an effect of *LRRK2* genotype on

381   autolysosomes (Fig. 3G and H). We also note that no major difference was seen in the number

382   of autophagosomes across both lung and kidney (Fig. S3C and D). Taken together, these data

383   suggest that the *Lrrk2* genotype does not majorly affect all autophagy pathways but

384   predominantly impacts basal mitophagy, both *in vitro* and *in vivo*.

385

386   ***GSK3357679A corrects the G2019S mitophagy defect in vivo***

387 We next sought to determine if we could pharmacologically rescue the observed mitophagy

388 defects *in vivo*. For this purpose, we utilised GSK3357679A - the pharmacodynamic

389 characteristics of which have been shown to be suitable for extended oral dosing studies in

390 rodents (Ding et al., in prep). We administered *mito*-QC WT, G2019S, and LRRK2 KO mice

391 with GSK3357679A *via* oral gavage every 12 h for a total of four dosings. During this period,

392 we observed no effect of GSK3357679A on body weight in any genotype (Fig. S4A). Tissues

393 were then harvested 2 h post the final dosing. We focussed our analyses on tissues where our

394 previous analyses of *Lrrk2* genotypic variants suggested a LRRK2-dependent role in

395 mitophagy, namely the lung and brain as well as the kidney.

396 LRRK2 inhibition was confirmed in lung, brain and kidney tissue lysates of GSK3357679A

397 dosed mice by immunoblotting of LRRK2 phopho-S935 and of Rab10 phospho-T73 (Fig. 4A).

398 In the brain, GSK3357679A decreased the phosphorylation of LRRK2 on S935 in both WT and

399 G2019S mice (Fig. 4A and B). However, we were not able to detect Rab10 phosphorylation in

400 this tissue, suggesting Rab phosphorylation is low or tightly regulated (results not shown).

401 Regardless, the loss of S935 phosphorylation on LRRK2 is consistent with kinase inhibition in

402 the brain. LRRK2 kinase inhibition was also observed in the lungs and kidneys of the same

403 animals, with GSK3357679A treated mice showing significant loss of both LRRK2 S935

404 phosphorylation and Rab 10 T73 phosphorylation (Fig. 4A and B).

405 In DA neurons of the SNpc, we found that treatment with GSK3357679A increased

406 mitophagy in both WT and G2019S KI mice. Consistent with earlier results, the vehicle dosed

407 G2019S group displayed significantly lower mitophagy compared to the vehicle- dosed WTs,

408 (Fig. 4C and D, compare with Fig. 2A and C). Importantly, treatment with GSK3357679A

409 restored G2019S mitophagy to base-line WT levels (Fig. 4C and D). No difference was

410   observable in the KO groups in presence of GSK3357679A, showing mitophagy effects are

411   through on-target LRRK2 inhibition.

412   As we had observed differences in cortical microglial mitophagy between genotypes,

413   we next investigated the effect of GSK3357679A treatment on this cell population.

414   GSK3357679A increased mitophagy in the cortical microglia in both WT and G2019S groups

415   (Fig. 4E and F). Consistently, GSK3357679A restored G2019S mitophagy levels to a value

416   similar to that observed in the control group (WT-V). Mitophagy was unaffected by

417   GSK3357679A in the KO groups, confirming GSK3357679A specificity on LRRK2 kinase

418   activity. As seen earlier (Fig. 2G), microglial cell numbers were increased in the cortex of

419   vehicle-treated G2019S mice compared with vehicle-treated WT mice (Fig. S4B). Interestingly,

420   GSK3357679A treatment recovered the increase in number of cortical microglia observed in

421   LRRK2 G2019S KI mice (Fig S4B), suggesting that LRRK2 kinase activity is a key contributor

422   to regulation of microglial numbers in this mouse line.

423   In the lungs we found that GSK3357679A increased mitophagy levels in both WT and G2019S

424   KI animals (Fig. 4C and 4D). Importantly, in the G2019S group, GSK3357679A elevated

425   mitophagy levels to a value similar to the WT Vehicle group, suggesting LRRK2-inhibition can

426   rescue the G2019S-mediated defect in mitophagy in lung also. As observed for other LRRK2

427   inhibitors we observed enlarged lamellar bodies in Type-II pneumocytes in the lungs of mice

428   treated with GSK3357679A, similar to that observed in LRRK2 KO mice (Fig. 3A) and to what

429   has been previously reported in the presence of LRRK2 kinase inhibitors [25,35,37]. GSK3357679A

430   had no effect on mitophagy levels in the lungs of LRRK2 KO mice (Fig.4G). Consistent with

431   the genetics, in the kidney we found that GSK3357679A had a minimal effect on mitophagy

432   despite this organ exhibiting robust LRRK2 inhibition (Fig. S4B and C and Fig 4A). Though

433   as previously mentioned, the very high levels of mitophagy in this tissue could be masking any

434   subtle mitophagy increases.

435   Taken together these results show that a pathogenic mutation of LRRK2 impairs basal

436   mitophagy in cells and tissues. Importantly, this phenotype can be rescued by the use of LRRK2

437   kinase inhibitors.

438   **Discussion**

439   Our work reveals that LRRK2 kinase activity inversely correlates with basal mitophagy levels,

440   both *in vitro* and *in vivo* in specific cells and tissues. Strikingly, the mitophagy defects seemed

441   to be specific to certain cell types. Indeed, we observed different effects in two different

442   neuronal subpopulations and in two microglial subpopulations. More work is needed to

443   understand why mitophagy is more sensitive to LRRK2 kinase activity in these cells, but it may

444   help explain why DA neurons degenerate in PD. Interestingly, we found a higher mitochondrial

445   content in the soma of Purkinje neurons compared to DA neurons, indicative of a higher

446   oxidative metabolism in the former. In addition, mitophagy levels were much lower in the

447   Purkinje cells, implying an inverse correlation between oxidative metabolism and mitophagy,

448   similar to previous observations in different muscle subtypes [26]. Speculatively, the higher basal

449   level of mitophagy in these DA neurons could be required to maintain oxidative metabolism in

450   light of their lower mitochondrial numbers, thus rendering them susceptible to defects affecting

451   mitophagy. Also of potential relevance, we observed a higher difference in mitophagy levels

452   between genotypes in the cortical microglia than in the DA neurons. Brain resident microglia

453   have been shown to have much higher LRRK2 levels and activity than neuronal populations,

454   which could have implications for disease aetiology [38].

455   The work presented here shows for the first time that pathogenic LRRK2 mutations can alter

456   basal mitophagy in clinically relevant cell populations *in vivo*. However, the extent to which

457   impaired mitophagy drives an individual's Parkinson's disease remains to be determined. We

458   and others have previously found that loss of PINK1 or Parkin activity does not significantly

459   alter basal mitophagy rates *in vivo*, despite a well characterised role for the PINK1/Parkin

460   pathway on depolarisation-induced mitophagy [17,18,39]. This implies that the PINK1/Parkin

461   pathway drives mitophagy under distinct types of stress, in contrast to the basally regulated

462   LRRK2 pathway described here. Regardless, if loss of stress-induced PINK1/Parkin-dependent

463   mitophagy can lead to PD, then it is reasonable to assume that loss of LRRK2-regulated basal

464   mitophagy could also contribute. These data now imply that impaired mitophagy may be a

465   common theme in PD pathology.

466   Recent reports in other models support our conclusion of a role for LRRK2 in regulating

467   mitophagy. *In vitro* assays in patient derived fibroblasts bearing G2019S or R1441C LRRK2

468   variants are consistent with our *in vitro* cell assays and observations *in vivo* [40–43].

469   The mechanism by which LRRK2 kinase activity regulates basal mitophagy is currently

470   unclear. LRRK2 has been shown to phosphorylate a subset of Rab GTPases [6] and, given the

471   roles of Rabs in membrane trafficking, it is tempting to suggest that they may be key in

472   regulating this mitophagy pathway [8,44]. It has been recently shown that lysosomal overload

473   stress induces translocation of Rab7L1 and LRRK2 to lysosomes [45]. This leads to the activation

474   of LRRK2 and the stabilisation of Rab8 and Rab10 through phosphorylation. Another recent

475   study showed that LRRK2 mutations inhibit the mitochondrial localisation of Rab10 [40].

476   We used two highly similar reporter models in primary MEFs and in mice to study general

477   autophagy and mitophagy. The use of both the *mito*-QC and the *auto*-QC reporters, in

478   combination with selective LRRK2 kinase inhibitors, provided evidence that LRRK2 kinase

479 activity affects mitophagy, rather than autophagy in general. The role of LRRK2 kinase activity

480 on autophagy has been previously investigated in several studies with inconclusive or

481 contradictory effects [36,46–51]. However, with our reporter systems, we cannot entirely exclude

482 that other selective autophagy pathways are affected. Additionally, total flux through the

483 autophagy pathway is likely to be much higher than the relative flux attributable to mitophagy,

484 and combined with the observed higher inter-individual variability with our autophagy reporter,

485 this makes it potentially more difficult to pick up small changes. For these reasons, it would be

486 unreasonable to entirely exclude the involvement of LRRK2 in general autophagy, although

487 our results lack support for this.

488 Our results show that three structurally distinct selective LRRK2 kinase inhibitors are active on

489 mitophagy in MEF cells, and that *in vivo* the tool compound GSK3357679A demonstrates

490 similar cell-specific effects in DA neurons and microglia within the brain. Importantly, use of

491 this inhibitor *in vitro* and *in vivo* supported our genetic data in suggesting that LRRK2 kinase

492 activity inversely correlates with the level of basal mitophagy. The fact that we could rescue

493 G2019S-impared mitophagy in PD-relevant cell types, within the brain, provides an exciting

494 prospect that LRRK2 inhibitor-mediated correction of mitophagic defects in Parkinson's

495 patients could have therapeutic utility in the clinic. In addition, LRRK2 kinase activity

496 inhibitors could also provide a way to increase mitophagy in general, which could be beneficial

497 in idiopathic PD, or indeed, in other non-related conditions where increased clearance of

498 mitochondria could be beneficial, such as mitochondrial diseases.

499 Here we demonstrate, through both genetic manipulation and pharmacology, that the most

500 common mutation in PD impairs basal mitophagy in tissues and cells of clinical relevance. The

501 fact that we can rescue this genetic defect in mitophagy using LRRK2 inhibitors, holds promise

502 for future PD therapeutics.

Acknowledgements

Author contribution

Conception and design were done by F.S., A.D.R. and I.G.G. Experiments were performed by F.S. and A.R.P. Data analysis was carried out by F.S. The autophagy counter plugin for FIJI was developed by G.B. and F.S. Drafting and revision of the manuscript was carried out by F.S., A.D.R. and I.G.G. All authors received and approved the final document.

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635

636   **Figure legends**

637   **Figure 1. LRRK2 kinase activity impairs basal mitophagy *in vitro***

638   (**A**) Schematics of the *mito*-QC reporter in mouse model. (**B**) Representative images of *mito*-

639   QC primary MEF cultures established from LRRK2 WT, LRRK2 G2019S, and LRRK2 KO

640   embryos. Boxed area is magnified on the right and arrowheads indicate examples of

641   mitophagy (mCherry-only mitolysosomes). (**C**) Quantitation of data shown in B from 6-9

642   independent experiments. Below is representative immunoblot showing LRRK2 protein

643   expression. (**D**) Schematics of the *auto*-QC reporter in mouse model. (**E**) Representative

644   images of *auto*-QC primary MEF cultures established from LRRK2 WT, LRRK2 G2019S,

645   and LRRK2 KO embryos. Boxed area is magnified on the right and arrowheads indicate

646   examples of autolysosomes and arrows highlight autophagosomes. (**F**) Quantitation of data

647   shown in E from 4-6 independent experiments. (**G**) Representative images of *mito*-QC

648   primary MEFs treated with control (DMSO) or 100 nM GSK3357679A. Boxed area is

649   magnified on bottom left and arrowheads indicate examples of mitolysosomes. (**H**)

650   Quantitation of mitophagy shown in G from 3-7 independent experiments. Corresponding

651   immunoblot of indicated proteins is shown below. Scale bars, 10 μm. Overall data is

652   represented as mean +/- SEM. Statistical significance is displayed as *$p<0.05$, and

653   ***$p<0.001$.

654   **Figure 2. Mutation of LRRK2 *in vivo* alters brain mitophagy.**

655   (**A**) Representative image of tyrosine hydroxylase (TH) immunolabeled dopaminergic

656   neurons within the substantia nigra pars compacta (SNpc) undergoing basal mitophagy in

657   LRRK2 WT, LRRK2 G2019S, and LRRK2 KO *mito*-QC mice. Arrowheads show examples

658   of mitolysosomes and arrows indicate mitochondria. (**B**) Quantitation of the number of TH

659   positive cells per field of view using a 63x objective. (**C**) Quantitation of basal mitophagy per

660   μm^2 of TH staining, data points represent means from individual mice. (**D**) Representative

661   maximal intensity projection and isosurface render of calbindin immunolabeled Purkinje

662   neurons in *mito*-QC cerebellum sections, undergoing basal mitophagy. (**E**) Quantitation of

663   basal mitophagy per μm^2 of calbindin staining, data points represent means from individual

664   mice. (**F**) Representative image and isosurface renders of Iba1 immunolabeled microglia

665   undergoing basal mitophagy in cortical sections from LRRK2 WT, LRRK2 G2019S, and

666   LRRK2 KO *mito*-QC mice. Arrowheads highlight mitolysosomes. (**G**) Quantitation of the

667   number of Iba-1 positive cells in the brain cortex per field of view using a 63x objective. (**H**)

668   Quantitation of basal mitophagy per Iba1 positive cell body, data points represent means from

669   individual mice. (**I**) Representative maximal intensity projection and isosurface render of

670   GFAP immunolabeled astrocytes undergoing basal mitophagy in *mito*-QC cortical sections.

671   (**J**) Quantitation of basal mitophagy per μm^2 of GFAP staining, data points represent means

672   from individual mice. Scale bars, 10 μm. Overall data is represented as mean +/- SEM.

673   Statistical significance is displayed as **$p < 0.01$, and ***$p < 0.001$.

Figure 3. Effects of mutation of LRRK2 *in vivo* on basal mitophagy and macroautophagy in selected peripheral tissues

676   (**A**) Representative tile scan and images of *mito*-QC lungs from LRRK2 WT, LRRK2

677   G2019S, and LRRK2 KO mice. Arrows on the tile scan highlight Type II pneumocytes (II.),

678   alv. show alveoli. Arrowheads in the higher magnification images indicate examples of

679   mitolysosomes and circled cells correspond to Type II pneumocytes (II.) while I. indicates

680   Type I pneumocytes. (**B**) Quantitation of lung basal mitophagy from data shown in D, data

681   points represent means from individual mice. (**C**) Representative images of *mito*-QC kidney

682   cortex from LRRK2 WT, LRRK2 G2019S, and LRRK2 KO mice. glm. indicates glomeruli

683   and pt. indicates proximal tubule examples. (**D**) Quantitation of kidney basal mitophagy from

684   data shown in F, data points represent means from individual mice. (**E**) Representative

685   images of *auto*-QC lungs from LRRK2 WT, LRRK2 G2019S, and LRRK2 KO mice.

686   Arrowheads highlight autolysosomes, arrows indicate autophagosomes, and II. indicates Type

687   II pneumocytes. (**F**) Quantitation of lung autophagy from data shown in C, data points

688   represent means from individual mice. (**G**) Representative images of *auto*-QC kidney cortex

689   from LRRK2 WT, LRRK2 G2019S, and LRRK2 KO mice. Arrowheads and, arrows as in C,

690   glm. indicates glomeruli, and pt. indicates proximal tubules. (**H**) Quantitation of kidney

691   autophagy from data shown in E, data points represent means from individual mice. Scale

692   bars, Tile scan in A: 100 μm, Other pictures:10 μm. Overall data is represented as mean +/-

693   SEM. Statistical significance is displayed as **$p < 0.01$, and ***$p < 0.001$.

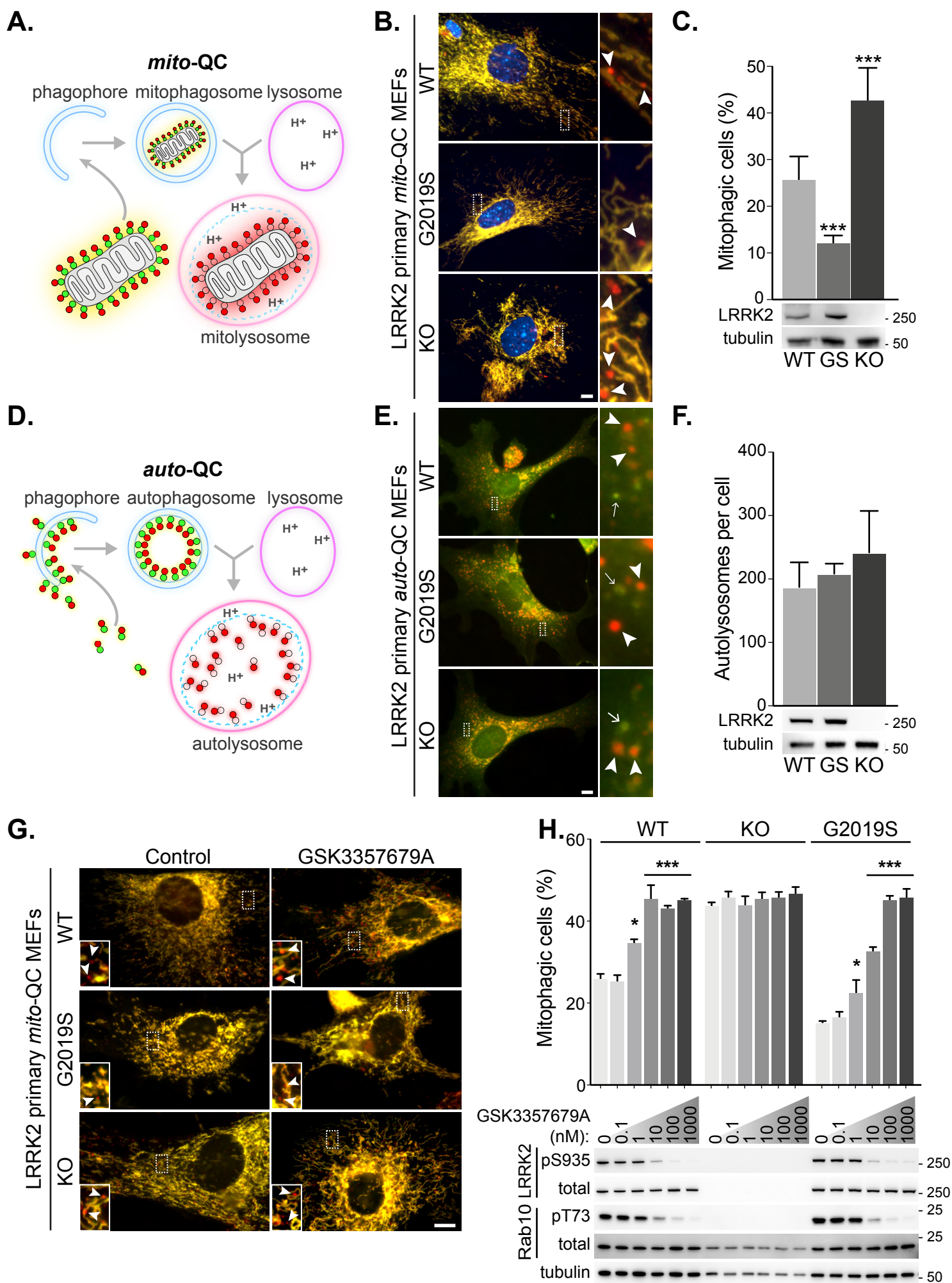694   **Figure 4. Pharmacological rescue of LRRK2-mediated mitophagy defects *in vivo*.**

695   (**A**) Immunoblots of the indicated proteins from tissue lysates of LRRK2 WT, LRRK2

696   G2019S, and LRRK2 KO *mito*-QC mice treated with vehicle or GSK3357679A. (**B**)

697   Quantitation of phosphorylation data from A (**C**) Representative image of tyrosine

698   hydroxylase (TH) immunolabeled dopaminergic neurons of the substantia nigra pars

699   compacta in LRRK2 WT, LRRK2 G2019S, and LRRK2 KO *mito*-QC mice treated or not

700   (vehicle) with GSK3357679A. Arrowheads indicate mitolysosome examples. (**D**)

701   Quantitation of mitophagy from data shown in C, with the addition of LRRK2 KO. Each data

702   point represents mean value from an individual mouse. (**E**) Representative images of Iba1

703   positive cortical microglia from LRRK2 WT, and LRRK2 G2019S mice treated or not

704   (vehicle) with GSK3357679A. Arrowheads indicate mitolysosomes. (**F**) Quantitation of

705   mitophagy from data shown in E, with the addition of LRRK2 KO. (**G**) Representative

706   images of *mito*-QC lungs from LRRK2 WT, and LRRK2 G2019S mice treated or not

707   (vehicle) with GSK3357679A. Arrowheads highlight mitolysosomes. (**H**) Quantitation of

708   mitophagy from data shown in G, with the addition of LRRK2 KO. For (**B), (D), (F)**, and (**H**),

709   V = vehicle dosed animals, GSK = GSK3357679A dosed animals. Each data point represents

710   mean value from an individual mouse. Scale bars, 10 μm. Overall data is represented as mean

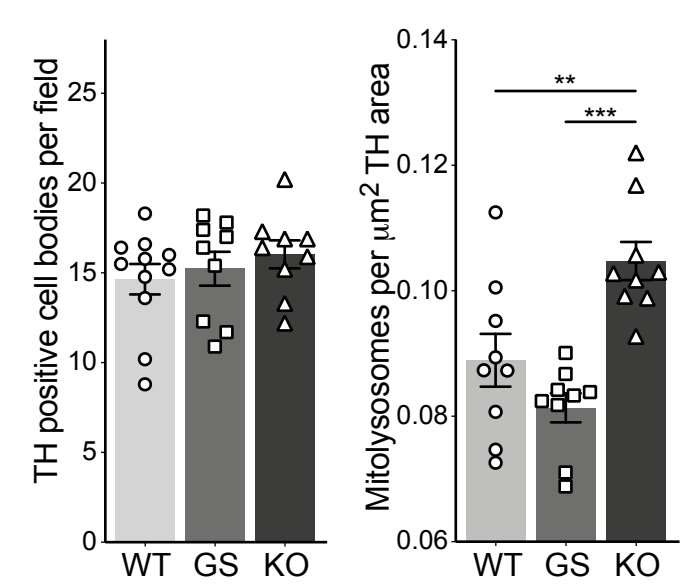711   +/- SEM. Statistical significance is displayed as *p<0.05, **p<0.01, ***p<0.001, and

712   ****p<0.0001.

713   **Figure S1. Stimulated mitophagy and autophagy are unchanged in MEFs**

714   (**A**) Representative images of WT *mito*-QC primary MEF cultures treated with control (CTL)

715   or 1mM deferiprone (DFP) for 24 h to stimulate mitophagy. Arrowheads indicate mitophagy

716   (mCherry-only mitolysosomes). (**B**) Quantitation of basal (CTL) and stimulated (DFP)

717   mitophagy in LRRK2 WT, LRRK2 G2019S, and LRRK2 KO *mito*-QC primary MEFs from

718   3-9 independent experiments. (**C**) Quantitation of *auto*-QC primary MEF cultures in basal

719   (control) and amino acid starvation (EBSS) conditions from 4-6 independent experiments. (**D**)

720   Quantitation of mitophagy in *mito*-QC MEFs, from 3-15 independent experiments treated

721   with increasing concentrations of GSK2578215A and MLi-2. Data is represented as mean +/-

722   SEM. Statistical significance is displayed as ***p<0.001. (**E**) Representative immunoblots

723   MEFs treated as in D. Scale bars, 10 μm. Overall data is represented as mean +/- SEM.

724   Statistical significance is displayed as ***p<0.001.

725

726   **Figure S2. Macroautophagy in the brain is unaltered by *LRRK2* genotype.**

727   (**A**) Representative image of tyrosine hydroxylase (TH) immunolabeled dopaminergic

728   neurons in substantia nigra pars compacta WT *auto*-QC sections. Arrowheads indicate

729   autolysosome examples. (**B**) Quantitation of the number of autolysosomes (left) or

730   autophagosomes (right) per TH area in LRRK2 WT, LRRK2 G2019S, and LRRK2 KO auto-

731   QC mice. Data points are means from individual mice. (**C**) Representative image of Iba1

732   immunolabeled microglia in cortical WT *auto*-QC sections. Arrowheads indicate

34

733 autolysosome examples. (**D**) Quantitation of the number of autolysosomes (left) or

734 autophagosomes (right) per Iba1 positive cell in LRRK2 WT, LRRK2 G2019S, and LRRK2

735 KO *auto*-QC mice. Data points are means from individual mice. Scale bars, 10 μm. Overall

736 data is represented as mean +/- SEM.

737 **Figure S3. Macroautophagy in the lungs and kidney cortex is not changed by LRRK2**

738 **genotype.**

739 (**A**) Quantitation of mitochondrial content, based on total *mito*-QC reporter staining, from

740 lung sections of indicated mice. Data points are means from individual animals. (**B**) Filipin

741 staining of cholesterol in type II pneumocytes within *mito*-QC lungs. Note increased staining

742 in KO. (**C**) Quantitation of basal number of autophagosomes in *auto*-QC lungs from LRRK2

743 WT, LRRK2 G2019S, and LRRK2 KO mice. (**D**) Quantitation of basal number of

744 autophagosomes *auto*-QC kidney cortex from LRRK2 WT, LRRK2 G2019S, and LRRK2

745 KO mice. Scale bars, 10 μm. Data is represented as mean +/- SEM. Statistical significance is

746 displayed as **p<0.01.

747 **Figure S4. Pharmacological inhibition of LRRK2 kinase activity *in vitro* and *in vivo*.**

748 (**A**) Body weights of mice treated over 36 hours with vehicle (V) or GSK3357679A (X).(**B**)

749 Quantitation of Iba-1 positive cells in the brain cortex per field of view (using a 63x

750 objective) in LRRK2 WT, LRRK2 G2019S, and LRRK2 KO *mito*-QC mice treated with

751 vehicle or GSK3357679A. (**C**) Representative images of *mito*-QC kidney cortex from LRRK2

752 WT mice treated with vehicle or GSK3357679A. (**D**) Quantitation of mitophagy from data

753 shown in B. Data points represent the mean value from individual mice. Scale bars, 10 μm.

754 Overall data is represented as mean +/- SEM. Statistical significance is displayed as

755 ***p<0.001 and ****p<0.0001.

756

FIGURE 1

FIGURE 2
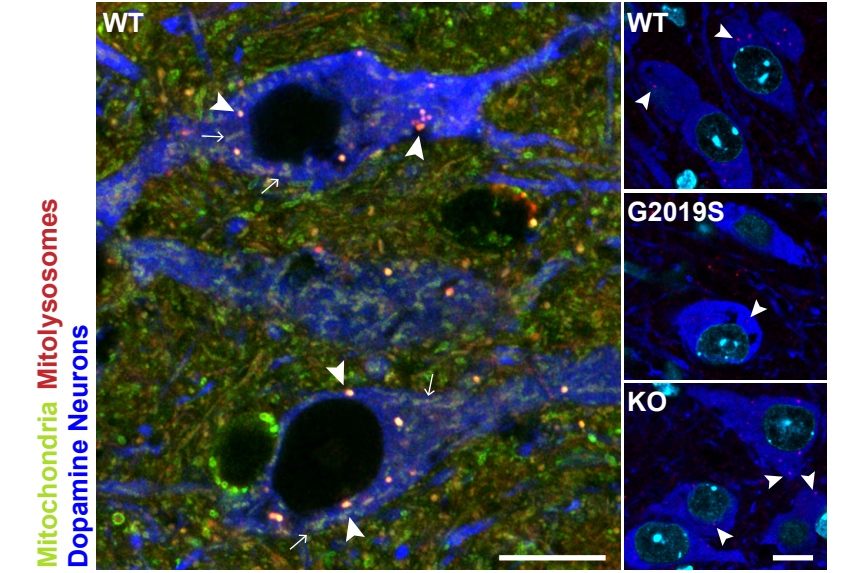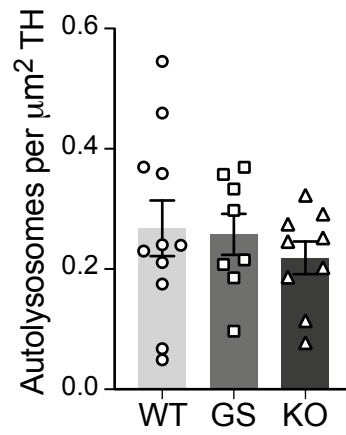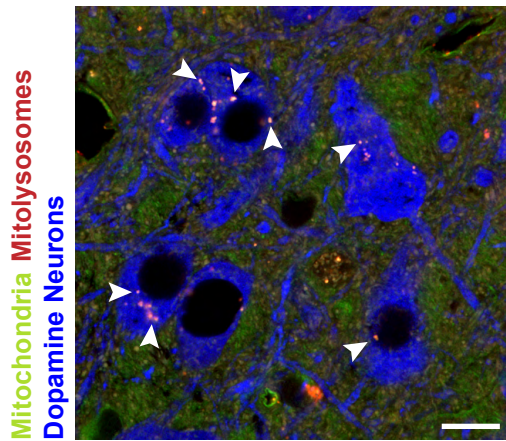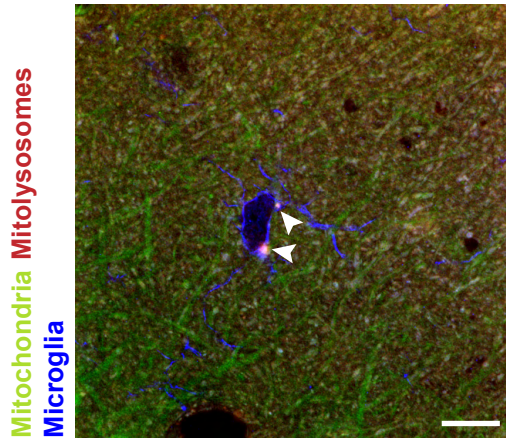
FIGURE 3

FIGURE 4

FIGURE S1

FIGURE S2

A. LRRK2 *auto*-QC Substantia nigra **B.**
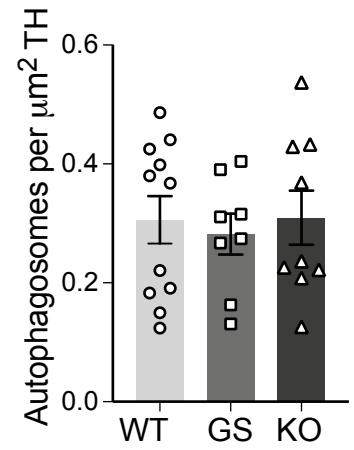
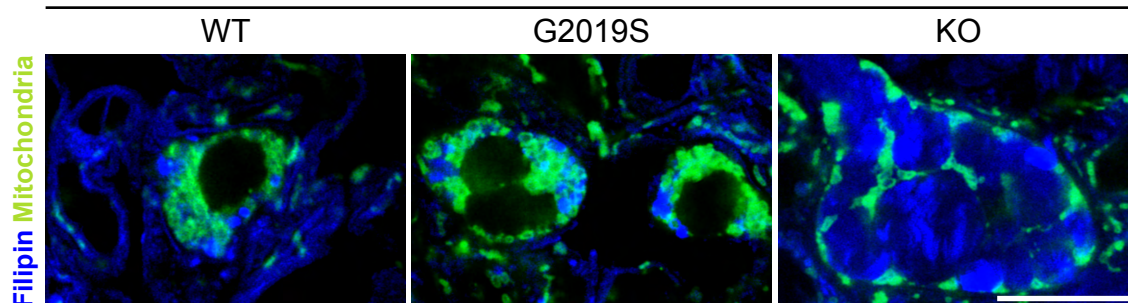**C.** LRRK2 *auto*-QC Cortex **D.**
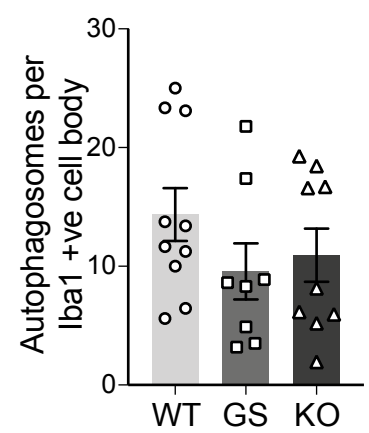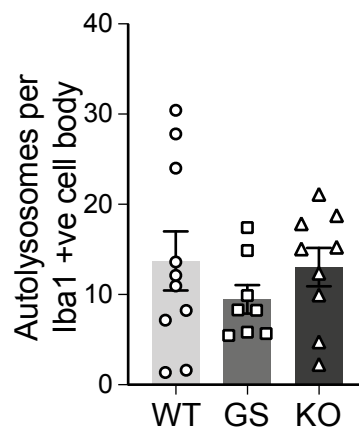
FIGURE S3

A.
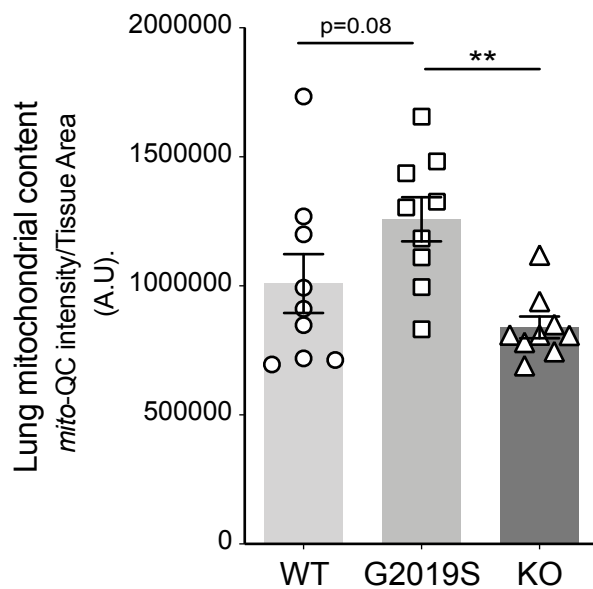
B. LRRK2 *mito*-QC Lung: Type II Pneumocytes

C. LRRK2 *auto*-QC LUNG

D. LRRK2 *auto*-QC KIDNEY

FIGURE S4

A.

B.

C.

WT LRRK2 *mito*-QC Kidney

D.