Effect of Neuroinflammation on Mitochondrial Quality Control Pathways in Parkinson's Disease – Affected Neurons

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Abstract

Parkinson's disease is characterized by motor dysfunction, which is attributed to the loss of dopaminergic neurons in the midbrain. It is currently thought that inflammation of the brain (neuroinflammation) and defects in 'mitophagy', a mitochondrial quality control pathway, contribute significantly to the development of the disease. However, the relationship between these processes is poorly understood. Previous studies have shown that the mitophagic regulatory protein, PINK1, is regulated at the transcriptional level by pro-inflammatory NF-kB transcription factors, implying that neuroinflammation could alter PINK1 expression, thereby compromising the normal operation of the mitophagy pathway. To investigate this we measured the effect of inflammatory cytokines on the steady-state levels of essential mitophagic regulatory proteins and the kinetics of mitophagy using a combination of immunoblotting and live cell imaging. Our data provides new insights into the relationship between neuroinflammation and mitophagy in Parkinson's disease.

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List of Terms

1. **Antibody:** a protein produced in response to a specific antigen
2. **ATP:** adenosine triphosphate is an energy transport molecule, often described as the "energy currency" of the cell
3. **Autophagosome:** double-membrane vesicles that deliver cellular components to lysosomes for degradation
4. **Autophagy:** the process by which cellular components are degraded and destroyed
5. **Autosomal recessive:** the inheritance of two recessive copies of a gene
6. **Bradykinesia:** slowed movement
7. **CCCP:** cyanide m-chlorophenyl hydrazine, an inhibitor of mitochondrial oxidative phosphorylation, which can be used to artificially induce mitophagy
8. **Cell line:** a cell culture composed of the same specific type of cell
9. **Compound A:** a chemically modified aurone, which is the flavonoid that provides some ornamental plants with the yellow coloring in their flowers. This compound has been shown by our collaborators to suppress LPS-induced nuclear translocation of NF-kB proteins and is therefore an inhibitor of the NF-kB pathway
10. **Cytokines:** signaling molecules that are involved with immune response and inflammation
11. **Dementia:** deterioration in one's mental abilities due to loss of brain function
12. **Depolarized mitochondria:** a mitochondria that has lost membrane potential and can therefore not perform cellular respiration

13. **Dopaminergic neurons:** neurons that produce the neurotransmitter, dopamine
14. **Fbxo7:** F-box only protein 7 is a mitophagic regulatory protein that is characterized by the 40 amino acid F-box motif
15. **Fluorescent imaging:** using fluorescent tags to visualize molecules, typically within cells
16. **HeLa**: A human cervical carcinoma cell line
17. **IL-1:** interleukin 1 is a pro-inflammatory cytokine
18. **Inflammaging:** increase in inflammation as one ages
19. **KillerRed:** a photosensitizer that can be targeted to the mitochondria
20. **Kinase:** an enzyme that phosphorylates another molecule
21. **Lewy bodies:** protein aggregates composed of α-synuclein
22. **Lipopolysaccharide (LPS):** found in the outer membrane in Gram negative bacteria that can cause inflammation and an immune response
23. **Microglia:** immune cells that are located in the brain
24. **Mitochondria:** a cell organelle that is the site of cellular respiration and therefore is responsible for the production of ATP
25. **Mitofusins:** proteins embedded in the mitochondrial outer membrane that regulate mitochondrial fission and fusion
26. **mCherry:** a red fluorescent protein
27. **Mitophagy:** the process by which dysfunctional mitochondria are autophagocytized
28. **Mutation:** a change in the nucleotide sequence of a specific region of DNA, which may result in an abnormal phenotype

29. **Neurodegenerative:** the damage and loss of neurons
30. **Neuroinflammation:** inflammation in the central nervous system
31. **Neurotoxic:** when a molecule is toxic to neurons
32. **Neuroprotective:** when a molecule protects neurons
33. **NF-κB (Nuclear factor-kappa B):** a family of (usually) pro-inflammatory transcription factors
34. **Parkin (PARK2):** E2/3 ubiquitin ligase protein the regulates mitophagy
35. **Parkinson's disease:** a neurodegenerative disorder that results in difficulty with motor functions
36. **pEYFP:** a plasmid with the gene encoding the enhanced yellow fluorescent protein, which is used to visualize cells using fluorescent microscopy
37. **Photosensitizer:** a substance that initiates a chemical reaction when it absorbs light
38. **PINK1 (PARK6):** PTEN-induced putative kinase I, a highly labile mitochondrial protein kinase, which is involved in regulating mitophagy
39. **PINK1 A:** a plasmid construct that contains the firefly luciferase gene under the control of the core human PINK1 promoter
40. **Plasmids:** a circular piece of DNA
41. **pNF- κB-Luc:** a plasmid construct that contains the firefly luciferase gene under the control of an artificial promoter, which consists of 5 tandom consensus NF-kB binding sites
42. **Protease:** an enzyme that hydrolyzes peptide bonds

43. **pTA-Luc:** a promoter-less plasmid construct that contains the firefly luciferase gene, which is typically used as a negative control plasmid for luciferase assays
44. **Oxidative phosphorylation:** the last step in cellular respiration where during the electron transport chain NADH is oxidized, resulting in ADP being phosphorylated to ATP by the enzyme ATP synthase and water being formed
45. **RAW 2647.7 cells:** a murine macrophage-like cell line
46. **ROS:** reactive oxygen species, a byproduct of the electron transport chain, which at high levels can cause damage to the cell
47. **SDS-PAGE:** sodium dodecyl sulfate polyacrylamide gel electrophoresis is a process used to separate proteins. SDS is used to unfold proteins to allow individual chains to separate and to impart a negative charge on the proteins
48. **SH-SY5Y:** a human dopaminergic, neuronal cell line derived from a neuroblastoma tumor
49. **Substantia nigra pars compacta:** a region in the midbrain whose function is motor control
50. **TNF-α:** tumor necrosis factor alpha is a pro-inflammatory cytokine
51. **Transfection:** the process by which nucleic acids are introduced into mammalian cells, generally using a lipid-based carrier molecule
52. **Translocate:** movement from one place to another
53. **Ubiquitination:** a post-translational modification where single or multiple ubiquitin moieties are added to a protein
54. **Western Blot:** a technique used to detect a certain protein in cells
55. **α-synuclein:** the 140 amino acid protein that composes Lewy bodies

I. Introduction

A. Background

Parkinson's disease (PD) is a neurodegenerative disorder that mainly affects adults over 65 years of age. People with this disease suffer from motor dysfunction, which is attributed to the loss of dopaminergic neurons in the substantia nigra pars compacta. The mechanism that leads to the death of these cells is unclear, however, it is thought that aggregation of misfolded proteins (including α-synuclein), neuroinflammation, and mitochondrial dysfunction are significant contributing factors.

The study of rare familial cases (~5%) has shown us that mutations acquired in an autosomal recessive fashion in Parkin (PARK2) and PTEN-induced putative kinase I (PINK1;PARK6), two genes involved in the regulation of mitophagy, a mitochondrial quality-control pathway, can lead to juvenile onset forms of PD[1-2]. PINK1 recruits Parkin to the damaged mitochondria, which subsequently polyubiquitinates multiple protein targets on the surface of the mitochondria, triggering their destruction by 'macroautophagy', a pathway that degrades damaged or unwanted cellular organelles. Moreover, mutations found in the gene encoding F-box only protein 7 (Fbxo7), another component of the mitophagy pathway, have also been found in severe familial cases of PD[3]. Fbxo7 interacts with both PINK1 and Parkin and helps Parkin translocate to the mitochondria[4]. Because sporadic PD is not associated with mutations in these genes (or any others)**,** it raises the question of whether defects in mitophagy are involved with the development of this condition.

Increases in inflammation throughout the body and the brain is thought to be a normal part of the aging process and is described as 'inflammaging'. Here, the overproduction of cytokines by microglia, the brain's resident immune cells, can lead to neuroinflammation[5], which is also thought to contribute to the development of sporadic PD in old age. A previous study of Parkin gene regulation in mice has shown that in the presence of these cytokines, the nuclear factor-kappa B (NF-κB) pathway is activated, leading to the inhibition of Parkin expression at the transcriptional level[6]. These data would imply that increases in neuroinflammation seen in the aging brain would deplete Parkin levels and possibly reduce the efficiency of the mitochondrial quality control process. *To test this hypothesis, we will use the dopaminergic human cell line, SH-SY5Y cells, exposed to disease-relevant levels of inflammatory cytokines as a model of neuroinflammation in PD. By measuring levels of mitophagy proteins and the kinetics of their association with the mitochondria after damage, we will gain new insights into the effect of neuroinflammation on this process.*

The NF-κB pathway, which is involved with cell survival, differentiation, and proliferation, is a family of transcription factors that regulates many genes, including those for inflammation[7]. RelA (p65) is a ubiquitously expressed member of this family and can be activated by oxidative stress and inflammation, both of which are associated with PD. It has been previously shown that NF-κB is a positive regulator of PINK1[8], raising the interesting prospect that inflammation and NF-κB can affect the function of the mitophagy pathway at both the PINK1 and Parkin level. *We will utilize luciferase-based transcriptional reporter assays (luminometry) to investigate whether PINK1 expression is affected by inflammatory cytokines in an NF-κB-dependent manner in*

human dopaminergic cells. These experiments will further our understanding of the relationship between neuroinflammation and mitochondrial dysfunction in PD.

B. Literature Review

A. Brief history of Parkinson's disease

Parkinson's disease was named after James Parkinson who wrote "An Essay on the Shaking Palsy" in 1817[9]. As indicated by the title of the paper, symptoms of the disease include the loss of motor control, resting tremors, bradykinesia, and oftentimes dementia. In the early 1900's, Friedrich Lewy discovered what are now known as Lewy bodies, which are protein aggregates made from α-synuclein[10]. These are not only characteristic of PD, but also of Lewy body dementia. By 1950, Arvid Carlsson found that the cause of PD is the loss of dopaminergic neurons in the substantia nigra in the brain[11]. Because of this discovery, today PD is treated with a drug that is a precursor to dopamine, levodopa, which helps alleviate some symptoms in the early stages of the disease, including tremors.

B. Mitophagy

Another symptom of PD is dysfunctional mitochondria. In healthy cells, insults that disrupt mitochondrial function result in the depolarization of the inner mitochondrial membrane, triggering mitophagy. This rapidly and specifically removes damaged mitochondria, helping to preserve the overall health of the cell's mitochondrial network. The mitophagy process begins with PINK1 recruiting Parkin to depolarized mitochondria. At healthy mitochondria, PINK1, a serine/threonine

kinase, is constitutively imported across the outer mitochondrial membrane and cleaved by a mitochondrial protease, removing the N-terminal mitochondrial-targeting sequence (MTS) from PINK1. The identity of this protease is currently controversial, but it is suspected to be presenilin-associated rhomboid-like protein (PARL)[12]. Loss of the MTS causes PINK1 proteins to be rapidly shed from the mitochondria and degraded in the cytoplasm. However, dysfunctional mitochondria are not able to import PINK1, and thus the protein is accumulated on the outer membrane of the damaged mitochondria[13]. PINK1 is subsequently able to recruit and activate cytosolic Parkin, a ubiquitin E2/3 ligase, to the mitochondria, leading to the ubiquitination of a broad spectrum of mitochondrial proteins[14]**,** including the mitochondrial fusion proteins, mitofusins (Mfns) 1 and 2[15]. This promotes the fragmentation of damaged regions of the mitochondrial network and their subsequent engulfment by membranous autophagsomes[16]. Fbxo7 helps Parkin recruit to depolarized mitochondria and also interacts with PINK1[4]. Furthermore, Fbxo7 also plays a role in the ubiquitination of Mfns[4]. In this way the mitophagy pathway preserves the integrity of the mitochondrial network by removing damaged regions. It is currently unclear whether the mitochondrial dysfunction observed in sporadic PD, where abnormal mitochondrial function and morphology have been recorded, is a consequence of deficits in the mitophagy system. However, evidence from the study of familial PD cases, where the regulators of this pathway are mutated, is compelling.

C. Measuring mitochondrial dysfunction in live cells.

For the last number of years, the principle method to study mitophagy has relied upon the use of carbonyl cyanide m-chlorophenyl hydrazine (CCCP) to uncouple oxidative phosphorylation in mitochondria, creating artificial mitochondrial dysfunction[17]. Treatment of cells with this compound has been shown to initiate network-wide mitophagy and the near-complete autophagy of all mitochondria within cells[18]. Typically, this is assayed using microscopy of fixed cells stained with fluorescent markers of the mitochondria or other 'end-point' assays. These methods have intrinsically poor temporal resolution, and may not accurately report subtle defects in the kinetics of the process[19]. This is particularly important when studying conditions like PD that develop over a number of decades. For these reasons, I will use live cell fluorescent imaging, a technique that can provide quantitative data at high temporal resolution in individual living cells.

D. Inflammation

In PD patients, there are increased numbers of active microglia cells, which produce inflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1). These particular cytokines can be either neurotoxic or neuroprotective[20], and have been shown to decrease mitochondrial membrane potential and the production of ATP[21]. The NF-κB pathway, the pro-inflammatory signaling system, is activated by these and other pro-inflammatory cytokines and changes gene expression accordingly[22]. As it has been shown that neuroinflammation, and more specifically, NF-κB, may affect the expression of Parkin and PINK1

proteins[6], an increased concentration of inflammatory cytokines in the brain may therefore affect the normal functioning of the mitophagy pathway and mitochondrial health in dopaminergic neurons.

A number of inflammatory diseases, such as Crohn's disease, are treated with inhibitors of NF-κB signaling. This raises the intriguing possibility that PD could also be treated using similar strategies. At MTSU, novel NF-κB inhibitors have been developed from an aurone, which is a plant derived flavonoid responsible for the yellow coloring in some ornamental flowers. This inhibitor, called Compound A, has been shown by our collaborators to inhibit NF-κB activity by suppressing lipopolysaccharide (LPS) induced nuclear translocation of NF-κB proteins.

E. Luminometry

In order to measure the possible effects of NF-κB signaling on PINK1 expression at the transcriptional level, we will measure the effects of over expression of NF-κB transcription factors and the treatment of cells with disease-relevant inducers of NF-κB-activity (i.e. the pro-inflammatory cytokine, TNFα) on the activity of the PINK1 promoter. This will be achieved using a previously described PINK1 promoter luciferase reporter construct[23]. Luciferase is an enzyme found in *Photinus pyralis* (fireflies) that accounts for their bioluminescence. It catalyzes the reaction of D-Luciferin, ATP, and oxygen to produce oxyluciferin, carbon dioxide, AMP, diphosphate, and light, which can then be measured[23].

$$\text{Luciferin} + O_2 + \text{ATP} \rightarrow \text{Oxyluciferin} + CO_2 + \text{AMP} + \text{diphosphate} + \text{LIGHT}$$

This reporter assay is frequently used to quantify NF-κB-dependent regulation of genes in live cells or in the form of end-point assays[24,25]. Using luminometry, we can measure changes in the transcriptional regulation of the PINK1 gene promoter by measuring luminescence.

C. Objectives

Parkinson's disease is a debilitating neurodegenerative disorder that affects approximately ten million people worldwide. Overall this study focuses on how inflammation affects the ability of cells to maintain a 'healthy' mitochondrial network by removing damaged mitochondria by mitophagy. Specifically, this will be examined in two ways. First of all, we will use a combination of luciferase reporter assays and western blotting to determine whether the NF-κB-activating inflammatory cytokine, TNF-α, stimulates a change in expression of essential mitophagic regulators at the transcriptional or protein level. We will then determine whether TNF-α exposure, either by changing the expression of mitophagic regulators or by other means, affects the temporal dynamics of CCCP-induced mitophagy by using time-lapse live cell imaging to observe the kinetics of the process in cells treated with inflammatory stimulus. These experiments will improve our understanding of the possible interactions between neuroinflammation and mitochondrial quality control, two processes which have been implicated in the development of the neurodegenerative disorder, Parkinson's disease.

II. Materials and Methods

A. ***Cell culture*** – SH-SY5Y cells (ATCC, USA), were maintained in 50/50 Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.1% of nonessential amino acids. HeLa cells (ATCC, USA) were maintained in DMEM supplemented with heat-inactivated 10% FBS, 0.1% of penicillin/streptomycin, and 0.1% of nonessential amino acids. RAW 264.7 cells (TBI 71; ATCC, USA) were maintained in DMEM supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, USA), 200 mM L-glutamine, 1% penicillin/streptomycin, and 50 µg/mL gentamicin antibiotic. All cells were kept at 37°C in a humidified atmosphere supplemented with 5% CO_2. All cells were grown to 80% confluency prior to transfection or passaging. For the transfections (scaled for 1 µg of DNA), a solution of PEI (polyethyleneimine) was made up at 1 µg/µL in sterile water, heated, neutralized with HCl to pH 7.2, and filter sterilized using a 0.22 µm filter. 3 µL of PEI solution was added to 100 µL of DMEM and vortexed for 5-10 s. The DNA was added to the solution, incubated at room temp for 15-30 min, and added to the cells. For some of the experiments (e.g. luminometry) Lipofectamine3000 Reagent was used for transfections following the manufacturer's instructions. Unless otherwise indicated, all reagents were obtained from ThermoFisher Scientific (USA).

B. ***Luminometry*** – Cells were transfected with 1.5 μg pNF-κB-Luc (Stratagene, USA) 24 h prior to 6 h treatments with the indicated reagents (e.g. inhibitors and/or activators of the NF-κB pathway). Cells were lysed in 250 μL per well of luminometry lysis buffer (25 mM Tris-phosphate, 1% $_{(w/v)}$ BSA, 0.025% $_{(w/v)}$ dithiothreitol (DTT), 1% Triton X-100, 15% $_{(v/v)}$ glycerol, 0.1 mM EDTA, 8 mM $MgCl_2$, 1x protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride) and incubated on a shaking table at 200 rpm for 15 min. 10 μL of 25 mM ATP was added to each well, and the samples were transferred in duplicates of 100 μL to an opaque-white 96-well plate. 20 μL of 10 mM sodium pyrophosphate was added to each well prior to the addition of 100 μL of 2 mM D-Luciferin. Luminescence was quantified using a SpectraMax M5 plate reader, which was operated with SoftMax Pro 6.3 software (Molecular Devices, Sunnyvale, CA, USA). A value of $*p < 0.05$ was set for significance. Luminescence numeric values of treated groups were compared to control group and results were expressed as mean ± SEM. Statistical significance was analyzed by ANOVA. Specifically, the difference between the treatment groups was analyzed using a multivariate analysis of variance with simple effects in JMP Software.

C. ***Restriction digest*** –To digest plasmid DNA or PCR products, 1 μg of DNA was combined with 2 μL FastDigest Green buffer, 1 μL of the appropriate restriction endonuclease enzyme (e.g. *Hind*III), and 15 μL DI H_2O. This reaction mix was scalable, with all components increased in proportion to one another when it was necessary to digest larger quantities of DNA. After incubating at 37 °C for 30 min,

DNA fragments were separated by agarose gel electrophoresis, typically using a 0.8% agarose gels containing GelRed DNA dye (VWR, USA). DNA bands were visualized under UV illumination.

D. ***Western blotting*** – SH-SY5Y cells were plated at a density of $0.5x10^6$ cells in 60 mm dishes containing 5 mL of culture medium and incubated in the presence of disease-relevant concentrations of TNF-α (Abcam, USA) for 24 h prior to analysis by western blotting. Treated and control cells were lysed in Laemmli lysis buffer (1.25 mL 0.5 M Tris-HCl, pH 6.8; 1 mL glycerol; 2 mL 10 % $_{(w/v)}$ SDS; and 250 μL 0.1% $_{(w/v)}$ Bromophenol Blue and the total volume brought up to 10 mL with DI H_2O) and recovered proteins were separated by SDS-PAGE on 8% gels. Separated proteins were transferred to nitrocellulose membranes, blocked in 5% TBS/T milk (PINK1 blot) or 5% PBS/T milk (actin blot), and western blotted with antibodies raised against PINK1 (1:1000; Cell Signaling, USA) and actin (1:5000; Sigma, USA) overnight. The next day, the nitrocellulose membranes were washed in either TBS/T (PINK1) or PBS/T (actin) and incubated in HRP-conjugate anti-mouse secondary antibodies (1:2000; Cell Signaling, USA) for approximately one hour. Membranes were incubated with HRP substrate in the presence of hydrogen peroxide and the resultant band-luminescence was imaged using a ChemiDoc™ MP Imaging System and Imaging Lab software (Bio-Rad, USA). A two-tailed *t*-test with unequal variance was performed in Mircosoft Excel to determine if results were significant.

E. ***Live cell fluorescent imaging*** – SH-SY5Y cells were plated into 35 mm glass-bottom dishes (Cellvis, USA) and transfected with 1 μg total (50:50 mix) EYFP-Parkin and mito-mCherry. 24 h prior to imaging, cells were treated with 10 ng/mL TNF-α. Cells were imaged using a Nikon Ti-Eclipse widefield fluorescence microscope equipped with a 40x oil-emersion objective, computer-controlled stage, IntensiLight epi-fluorescence illuminator (Nikon, USA), CoolSNAP Myo camera (Photometrics, USA) and a full-enclosure providing temperature, CO_2 and humidity control (InVivo Scientific, USA). The microscope was controlled using Elements software package (Nikon, USA). EYFP (excitation= 524 nm emission= 527 nm) and mCherry (excitation= 587 nm emission= 610 nm) fluorescence were visualized using the appropriate filter sets (Nikon, USA). Immediately after adding CCCP, the cells were imaged once every four minutes for thirty minutes. The time for the initiation of mitophagy was determined based on the first appearance of colocalization between EYFP and mCherry tagged proteins in two consecutive frames[26]. The same procedure was followed with HeLa cells, except the cells were imaged every minute for thirty minutes. A two-tailed *t*-test with unequal variance was performed in Microsoft Excel to determine if there was a significant difference between average time of Parkin colocalization in control cells and those incubated for 24 h in 10 ng/mL TNF-α.

III. Results

TNF-α suppresses the growth of cultured human dopaminergic neurons

TNF-α (ng/mL)	SH-SY5Y cells (x10^5) per mL
10	1.2
5	1.9
2.5	2.1
1.25	2.0
0.5	2.2
0	2.2

Table 1: Measuring how TNF-α compromises the growth of SH-SY5Y cells. *The effect of 24 h treatments of different TNF-α concentrations on SH-SY5Y cells plated at 1 x 10^5 cells/mL 24 h prior to treatment.*

To determine the effects of neuroinflammation on dopaminergic neuron proliferation and viability, SH-SY5Y cells were cultured in disease-relevant concentrations of TNF-α for 24 h. Each plate showed cell debris; however, cells cultured in 10 ng/mL TNF-α had the most cell debris and showed the smallest increase in cell numbers (Table 1). Lower doses had substantially smaller or negligible effects on cell numbers (Table 1). These data suggest that 10 ng/mL TNF-α affects the proliferation of SH-SY5Y cells. As TNF-α is a cytokine, a biological signaling molecule, it can be assumed that its effects on SH-SY5Y at this dose are exerted via TNF receptors (TNFR). Once complexed with TNF-α these receptor are known to induce both pro-apoptotic caspases (possibly generating the cell-death associated debris in TNF-α-treated plates)

and the NF-κB pathway. It is likely that dopaminergic neurons *in vivo* will also respond to similar concentrations of TNF-α. We will therefore use this dose for all subsequent experiments to probe how inflammatory cytokines and neuroinflammation affects the function of the mitophagy pathway.

Exposure of SH-SY5Y cells to TNF-α does not significantly alter the expression of endogenous full-length PINK1

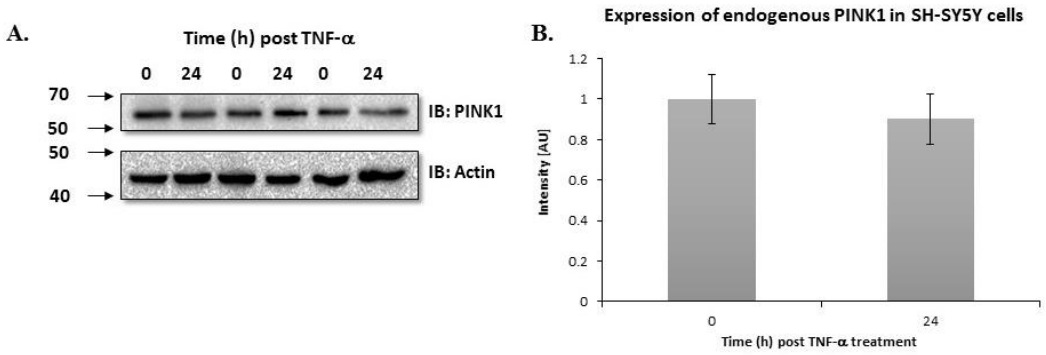

Figure 1: Western blot of endogenous full-length PINK1. *SH-SY5Y cells were cultured in either 0 or 10 ng/mL TNF-α for 24 h and western blotted for PINK1 and actin (A). Expression levels of PINK1 were quantified in triplicate and normalized to actin to control for protein loading (B). Error was calculated as standard error (SE).*

Previous investigations[8] have shown that overexpression of NF-κB proteins increase expression of PINK1 proteins at the transcriptional level in SH-SY5Y cells. As TNF-α signaling via TNFR1+2 activates the NF-κB pathway, we hypothesized that TNF-α treatment would also increase PINK1 expression in SH-SY5Y cells. To test this, SH-SY5Y cells were cultured in 10 ng/mL TNF-α and western blotted for PINK1 proteins. Although quantitative analysis of the western blots suggested that TNF-α produced a small decrease in PINK1 protein expression (Fig. 1B), this was not

statistically significant (two-tailed *t*-test; $p = .002$), indicating that neuroinflammation may not affect the expression of PINK1 proteins.

Optimization of a transcriptional reporter assay to measure NF-κB-dependent transcription

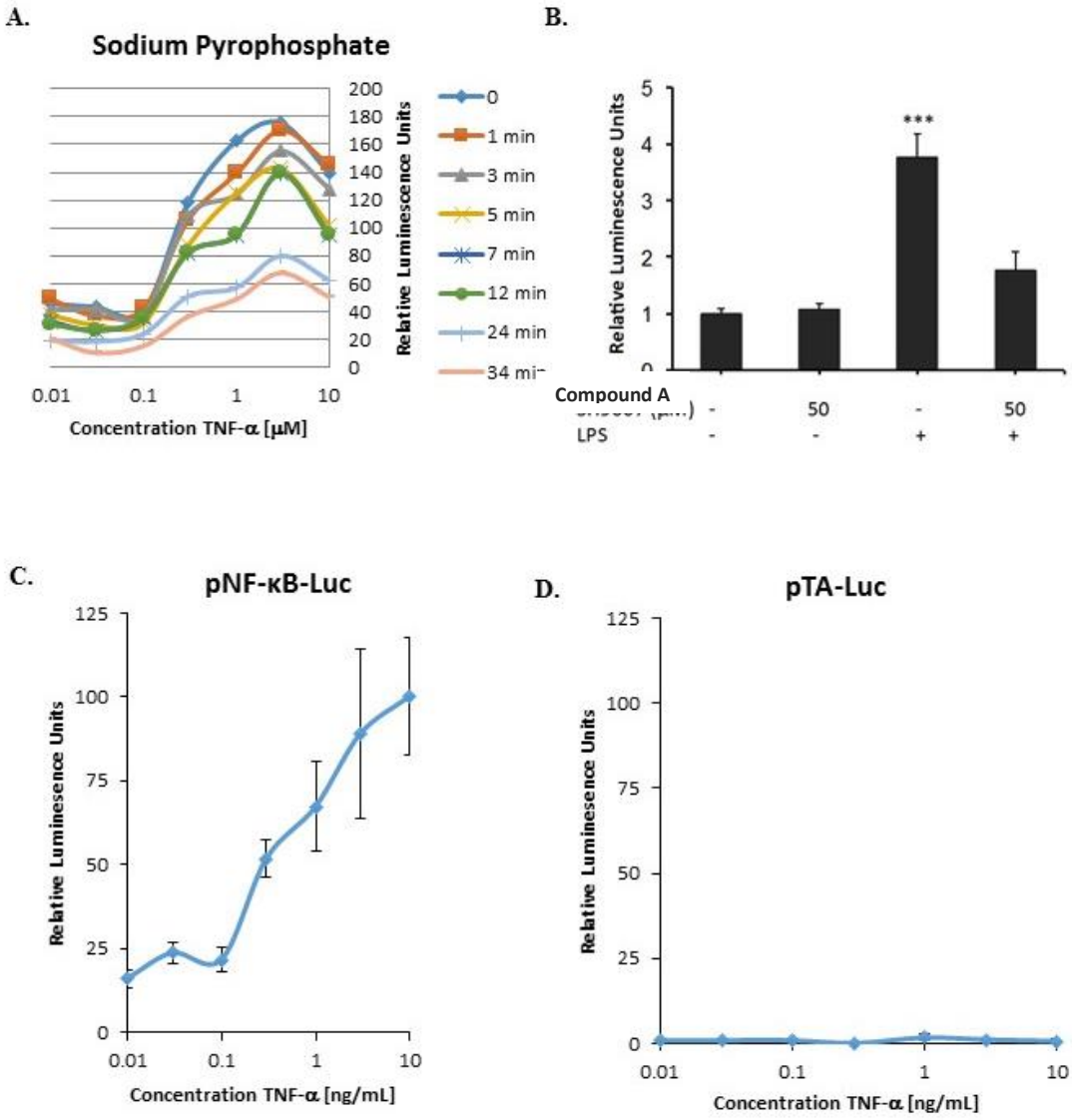

Figure 2: Luminometry optimization experiments*. With the addition of sodium pyrophosphate to the luciferase reaction, luminescence was measured in HeLa cells expressing pNF- κB-Luc and treated with TNF-α 6 h prior to lysis (A). RAW 264.7 cells expressing pNF- κB-Luc were treated with or without Compound A and lipopolysaccharide (20 ng/mL). Significance was determined by ANOVA (See section 2.B; *p<0.05, **p<0.01, ***p<0.001). (B). HeLa cells transfected with either pNF- κB-Luc (C) or pTA-Luc (D) were treated with the indicated TNF-α concentrations 6 h prior to lysis. All conditions were repeated in triplicate with individual samples assayed in duplicate. Data presented as the mean. Numeric values were normalized to control groups. Error was calculated as SE.*

As the PINK1 protein is known to be highly labile, the previous experiment could not exclude the possibility that increases in PINK1 expression at the transcriptional level were not propagated up to the protein level. We therefore designed luminometry-based transcriptional reporter assays to measure transactivation of the PINK1 promoter in the presence of TNF-α. Our initial control experiments utilized the pNF-κB-Luc reporter construct, which is a luciferase expression vector where transcription of the luciferase gene is controlled by 5 tandem consensus NF-κB binding sites. Therefore, this construct reports on the activation of the NF-κB pathway.

Typically, luminometry assays are performed in a 96-well plate format, and expression of the luciferase reporter enzyme in each sample is assayed using a plate reader equipped with an injector. In this way, each sample receives the luciferin substrate that initiates the luciferase light-generating reaction immediately before it is read. As the plate reader that was available to us did not have a substrate injector, we would have to deliver the substrate manually to each sample before reading the entire plate. This can be a slow process. As the light generated by each sample starts to decay rapidly after substrate edition, we had to develop a method to stabilize the reaction, ensuring that accurate readings of luciferase expression could be made despite the potential lag-time between substrate edition and light quantification. To do this, we added sodium pyrophosphate to each sample prior to D-Luciferase. Sodium pyrophosphate is a known stabilizer of the luciferase reaction. Byproducts of the luciferase reaction inhibit the luciferase enzyme, causing the light-generating reaction to effectively end after a few seconds. Sodium pyrophosphate acts as an antagonist to these metabolites, extending the period of time that each sample will produce light after substrate addition.

To test whether sodium pyrophosphate stabilized our luciferase reactions long enough for us to obtain viable data from our experiments, we treated pNF-κB-Luc transfected HeLa cells with different concentrations of TNF-α and then performed the luciferase assay in the presence of sodium pyrophosphate 8 h post TNF-α addition. The luminescence produced by each sample were repeatedly assayed at the indicated times post luciferin addition (Fig. 2A). Our data show that similar result were obtained for approximately 5-12 min post luciferin addition, suggesting that our reactions were successfully stabilized by pyrophosphate.

We then appraised the ability of this assay to discriminate accurately between different levels of activation of the NF-κB pathway. This was done by repeating the previous assay in triplicate and exposing pNF-κB-Luc transfected HeLa cells to increasing concentrations of TNF-α (Fig. 2C). We also exposed a second set of HeLa cells transfected with pTA-Luc, a promoter-less control vector that should not be responsive to NF-κB activity, to the same concentration series of TNF-α (Fig. 2D). We observed that we could accurately measure different levels of NF-κB pathway activity, as triggered by increasing concentrations of the NF-κB activator, TNF-α. By also assaying for luciferase expression in cells containing a luciferase expression vector without NF-κB binding sites, we also show that our assay can specifically measure NF-κB -dependent gene transcription.

As well as being able to measure increases in NF-κB-dependent transcription, we also tested whether our assay could successfully measure inhibition of NF-κB-dependent transcription. This was performed by treating pNF-κB-Luc transfected RAW 264.7 murine macrophage cells with LPS, a bacteria-derived polysaccharide that stimulates NF-

κB activity in macrophages, in combination with an inhibitor of the NF-κB pathway. This inhibitor, which we will refer to as 'compound A', is a modified aurone, a plant-derived flavonoid. It has previously been shown to inhibit LPS-induced expression of NF-κB target genes in macrophages (Manuscript in preparation, Handy, Nelson, and Farone Labs, MTSU). We found that exposure of pNF-κB-Luc transfected RAW264.7 macrophages to compound A could largely abrogate LPS-induced luciferase expression (Fig. 2B) and this was statistically significant.

Assaying the validity of a previously described luciferase reporter of PINK1 promoter activation

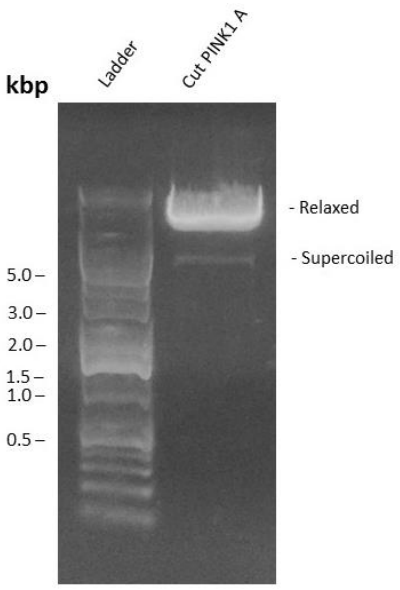

Figure 3: Restriction Digest of pPINK1-A. *pPINK1-A was cut using the restriction enzyme, HINDIII.*

Having optimized the luminometry assay, we next sought to determine the effect of TNF-α on PINK1 at the transcriptional level using this technique. In order to do this,

we obtained pPINK1-A, a plasmid vector containing the luciferase gene under the control of the human PINK1 promoter (previously described by Duan *et al.*[8]). To verify the identity of this plasmid, a diagnostic restriction digest using *Hin*dIII restriction endonuclease was performed. Based on the known sequence of the plasmid, this enzyme would cut within the PINK1 promoter sequence and the vector backbone, releasing a 2 kilobase pair (kbp) fragment from the ~7 kbp plasmid. Therefore, it was expected to see two bands after electrophoresing the cut DNA on a 0.8% agarose gel at around 2.0 and 5.0 kbp. However, post digestion, we observed two bands, both larger than 5 kbp (Fig. 3). The larger band is relaxed plasmid DNA, which is 'nicked' at one site on one strand of the double helix, causing it to run at a slower rate on the gel. The other band is supercoiled DNA, which is uncut and not nicked. Thus, remaining compact, it runs at a faster rate on the gel. These data suggest that the plasmid is not pPINK1-A. As these results are not what we expected, we suspect that the vector is not the plasmid that we think it is; therefore, the luminometry experiment cannot be performed at this time.

CCCP induces mitophagy in HeLa cells

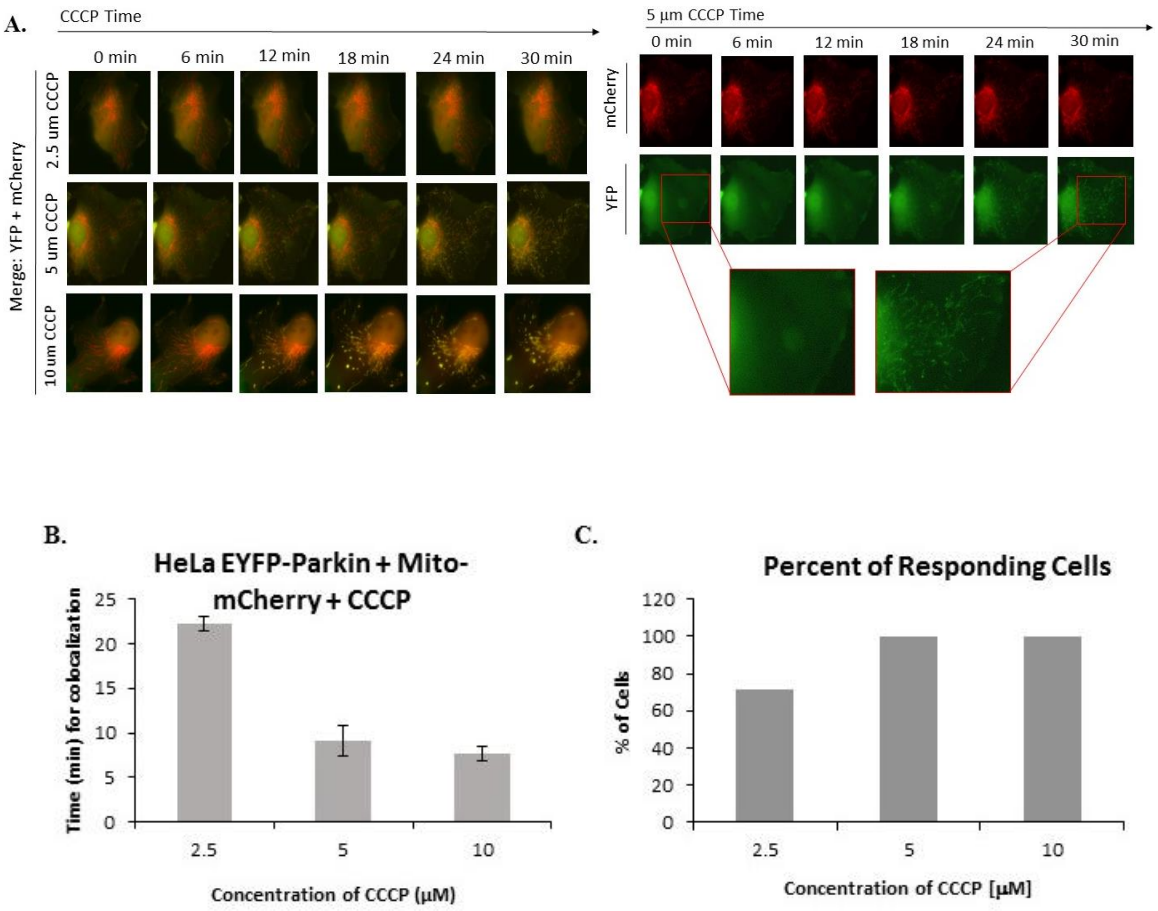

Figure 4: Measuring the effect of different doses of CCCP in live cells. *HeLa cells expressing EYFP-Parkin (Green) and Mito-mCherry (Red), were treated with either 2.5, 5, or 10 µM CCCP (A). The cells were imaged every minute for 30 min post-CCCP. The time it takes for mitophagy to initiate was measured by the first appearance of colocalization between EYFP and mCherry tagged proteins in two consecutive frames[23] (B). The percent of cells responding to CCCP treatment is determined by how many cells initiated mitophagy (C). Error was calculated as SE.*

Although we did not show an effect of TNF-α on the expression of PINK1, an essential mitophagic regulator, other reports have indicated that TNF-α (and NF-κB) can affect other mitophagic regulators and mitochondrial function. It remained possible that TNF-α may still affect the kinetics of mitophagy without altering steady-state PINK1 protein levels. To explore this possibility, I constructed a series of experiments to

measure the dynamics of PINK1 recruitment to damaged mitochondria in living dopaminergic neurons. To optimize this set of experiments and determine the concentration of CCCP needed to induce mitophagy, I utilized the HeLa cervical carcinoma cell line, which is more easily transfected and easier to work with than SH-SY5Y cells. These cells were cotransfected with plasmid constructs to express a red fluorescent mitochondrial marker, 'mito-mCherry', and a yellow fluorescent fusion of Parkin, 'EYFP-Parkin', the mitophagic regulator immediately downstream of PINK1. 24 h post transfection, cells were treated with CCCP immediately before live-cell imaging. It would be expected that as the concentration of CCCP increases, cells would respond increasingly faster to treatment. In cells treated with 10 μM CCCP, EYFP-PINK1 relocalization, and therefore mitophagy, was extremely rapid, occurring faster than in 5 μM CCCP treated cells (Fig. 4B). However, not all cells treated with 2.5 μM CCCP showed relocalization of PINK1-EYFP to the mitochondria, indicating that this concentration is too low to sufficiently depolarize the mitochondrial membrane to induce mitophagy (Fig. 4C). It is highly likely that SH-SY5Y cells will respond similarly to these doses of CCCP[4]. As the 10 μM CCCP dose induced kinetics that were arguably too rapid to record using our equipment, the 5 μM CCCP dose was selected for use for all subsequent live-cell imaging experiments.

TNF-α does not significantly alter the kinetics of mitophagy in SH-SY5Y cells

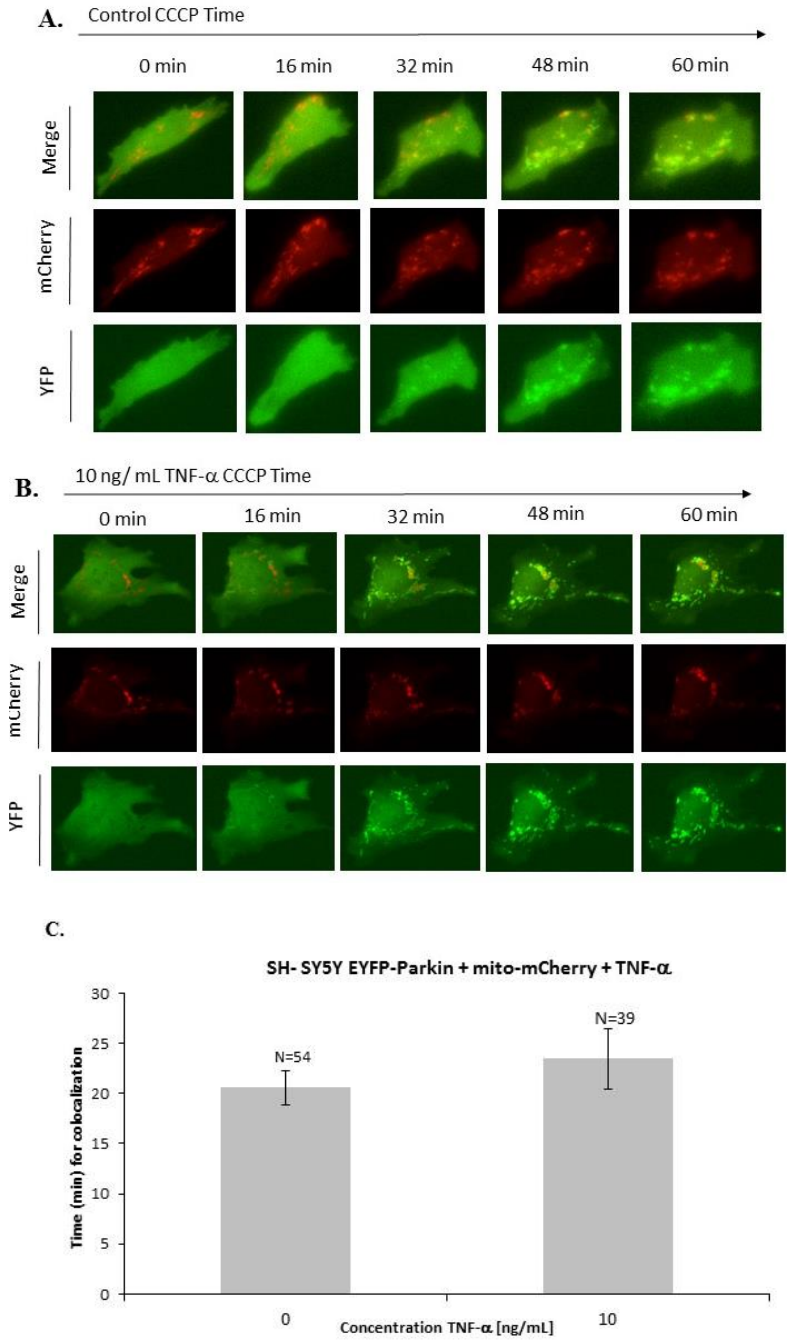

Figure 5: Measuring the effect of inflammatory cytokines on mitophagy in live cells. *SH-SY5Y cells expressing EYFP-Parkin (Green) and Mito-mCherry (Red), were treated with 5 μM CCCP either (A) without or (B) with a 24 h pre-incubation with 10 ng/mL TNF-α. The cells were imaged every 4 minutes for 1 hour post-CCCP. The time it takes mitophagy to occur is measured by the first appearance of colocalization between EYFP and mCherry tagged proteins in two consecutive frames[23]. The number of analyzed cells is displayed above each condition (C). Error was calculated as the SE.*

Using SH-SY5Y dopaminergic neurons, the effect of neuroinflammation on the kinetics of mitophagy was evaluated. Cells were cultured in 10 ng/mL TNF-α for 24 h and treated with 5 μM CCCP immediately before live-cell imaging. While the average time it took for mitophagy to occur was slightly longer in cells incubated in TNF-α (Fig. 5C), this was not statistically significant, (two-tailed *t*-test; $p = 0.080$). This indicates that the presence of neuroinflammation, or at least TNF-α, does not alter the kinetics of mitophagy.

IV. Discussion

The etiology of Parkinson's disease is complex and multifaceted, possibly involving the dysfunction of multiple cellular systems and the over-production of pro-inflammatory cytokines in the brain. A current challenge in the field is to determine whether or not these various changes in the brain are linked. In this particular study, we were interested in whether chronic neuroinflammation was a causative factor in the mitochondrial dysfunction observed in PD. This hypothesis was largely driven by recent studies showing that NF-κB, a transcription factor that is activated as part of the inflammatory response, is a regulator of crucial genes that regulate mitochondrial quality control [6,8]. Specifically, the overexpression of NF-κB proteins was shown to increase expression of PINK1[8] and inflammatory stimuli have been shown to alter the expression of Parkin in an NF-κB-dependent fashion in murine cell lines[6]. These data strongly indicated that increases in neuroinflammation may influence the efficiency of the mitochondrial quality control process.

We investigated the relationship between neuroinflammation and mitophagy, in SH-SY5Y cells, a human cell line model for the dopaminergic neurons lost in PD. We exposed these cells to mitochondrial stressors in the presence of inflammatory cytokines to simulate mitochondrial dysfunction and neuroinflammation and used techniques such as western blotting and live cell fluorescent imaging to survey their effects on the expression of key proteins of the mitophagic pathway and the kinetics of mitophagy. Surprisingly, we found that the incubation of dopaminergic neurons in 10 ng/mL TNF-α 24 h prior to analysis by western blotting did not significantly affect the expression levels

of endogenous full-length PINK1 (Fig. 1). Moreover, in regards to the effect of inflammatory cytokines on the kinetics of mitophagy, treating SH-SY5Y cells with 10 ng/ mL TNF-α 24 h prior to imaging did not significantly alter the average time of Parkin colocalization and thus did not delay the process of mitophagy (Fig. 5).

These results were not in agreement with previous studies, which show that PINK1 and Parkin are regulated by NF-κB[6,8]. However, while the literature suggests that Parkin is negatively transcriptionally regulated by inflammatory cytokines, this study was performed in murine cell lines and used the mouse Parkin promoter[6], which may be regulated differently to the human orthologue. Furthermore, Parkin-null mice, which were also used in the study, do not recapitulate the PD phenotypes observed in human patients that have inherited mutated, inactive copies of the parkin gene in an autosomal recessive fashion[27]. Moreover, the MN9D cell line used, even when differentiated, does not fully recapitulate dopaminergic neurons[28]. This implies that the findings in this study may not be directly applicable to humans, which may explain why our results were not as expected.

Yet, neuroinflammation contributes to dopaminergic cell death. Inflammatory cytokines decrease mitochondrial membrane potential and ATP production, damaging the mitochondria, which causes oxidative stress because reactive oxygen species (ROS) are produced[21]. In these experiments, CCCP treatment was used to induce mitophagy. However, CCCP does not accurately recapitulate the damage and stresses experienced by mitochondria in PD neurons because it does not increase mitochondrial ROS[29]. Additionally, it is unlikely that individual neurons in PD brains would experience spontaneous failure of the entire mitochondrial network as is caused by CCCP. It is more

likely that smaller regions of the mitochondrial network will experience stress or damage at any one moment[18]. Thus, an alternative approach, which may better simulate the mitochondrial stresses experienced in PD, could utilize 'KillerRed', which is a mitochondrially targeted photosensitizer that produces 1000-fold more ROS that enhanced green fluorescent protein (EGFP) when excited with blue light (585 nm)[22]. Using a confocal microscope to apply targeted excitation of KillerRed in specific regions of a cell's mitochondrial network, mitochondrial damage can be created, triggering mitophagic events that are more analogous to those that likely occur in PD neurons.

It should also be noted that the mitophagy process – from initial PINK1 and Parkin translocation to the eventual autophagic destruction of the damaged mitochondria – takes many hours. It is therefore possible that neuroinflammation impacts the mitophagy process downstream of PINK1 and Parkin, which were the focus of this study. Looking at events downstream of PINK1 and Parkin may provide more insight into how these cellular processes are intertwined. Performing longer experiments assaying for the colocalization of autophagy adaptors with the mitochondria and with the overall 'mitochondrial mass' of the cell may provide more definitive answers.

In conclusion, this study indicates that neuroinflammation may not exert a strong influence on the initial stages of the mitochondrial quality control process in dopaminergic neurons but does not exclude the possibility that inflammation may affect later stages of the process or overall mitochondrial health and function in PD.

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