Characterization of the Autophagic-Lysosomal Pathway in Parkinson's Disease using Patient iPSC-derived LRRK2 G2019S Dopaminergic Neurons

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Abstract

OBJECTIVES: Mechanisms of dopaminergic neuron cell death in Parkinson's Disease (PD) is complex and combinatorial, with impairments in multiple cellular pathways impacting mitochondrial function and endosomal/lysosomal protein degradation. Mutations in leucine-rich repeat kinase 2 (LRRK2) and glucocerebrosidase (GBA), have been shown to both impact kinetics of the autophagic-lysosomal pathway (ALP) and contribute to PD-associated protein accumulation and aggregation. This study uses induced pluripotent stem cells (iPSCs)-derived dopaminergic neurons (iCell® DopaNeurons) generated from apparently healthy normal donors (AHN) and clinically diagnosed PD patients harboring LRRK2 G2019S or GBA1 N370S mutations [derived from iPSCs provided by Parkinson's Progression Markers Initiative (PPMI), part of The Michael J. Fox Foundation (MJFF)] to investigate their utility in evaluating ALP changes associated with PD.

METHODS: Endosome and lysosome protein expression was quantified in iPSC-derived dopaminergic neurons at 7, 14, and 21 days using high throughput imaging of specific antibodies from Cell Signaling Technology (CST). Exogenous modulators of autophagy (Torin 1) and mitochondrial stress (Rotenone) were applied to the LRRK2, GBA and AHN dopaminergic neurons to evaluate the effect of pathway stressors on ALP protein expression. Within all conditions, neural survival and synapse formation were also quantified.

RESULTS: We show both AHN and patient-derived iCell DopaNeurons express dopaminergic (i.e., TH, FOXA1), synaptic (i.e., PSD95, Synapsin-1), and ALP (i.e., LAMP-1, Rab5, LC3, DRP1, Cathepsin, Galectin 3) specific markers via immunocytochemistry. These lines were applied to high content imaging to develop methods to evaluate pathophysiological phenotype differences in synapse development, ALP kinetics, and responses to mitochondrial and autophagy stressors between control and patient-derived PD dopaminergic neurons.

CONCLUSIONS: These data demonstrate the utility of high-throughput immunocytochemistry and patient-derived iPSC dopaminergic neurons for investigating lysosomal, mitochondrial, and neurodegenerative pathway dynamics.

Materials and Methods

- iCell DopaNeurons are generated from human iPSC lines differentiated into midbrain dopaminergic neurons using FUJIFILM Cellular Dynamics proprietary differentiation methods.
- Patient-derived iCell DopaNeurons were generated using iPSC lines accessed through the Parkinson's Progression Markers Initiative (PPMI) and The Michael J. Fox Foundation (MJFF). These cell lines are from clinically symptomatic PD patients carrying known risk-associated gene mutations.
- iCell DopaNeurons were cultured for 14 days, followed by methanol fixation and processing for immuno-cytochemistry.
- Recombinant monoclonal antibodies against various cellular markers and CNS cell types were analyzed in patient iPSC-derived dopaminergic neurons by immunofluorescence utilizing the Cell Signaling Technologies Immunofluorescence Protocol. Antibodies used have previously been validated in mouse brain tissue
- Images were captured at 630X using the Operetta CLS HCA system in confocal mode.
- Image quantification was performed using Harmony high-content analysis software, Mitochondria Analyzer in ImageJ, as well as Cell Profiler.

Product Name	Catalog #	Donor	Genotype
iCell DopaNeurons	R1032	01279	AHN
iCell DopaNeurons LRRK2 G2019S	R1234	11299	LRRK2 G2019S
iCell DopaNeurons LRRK2 G2019S/G mutation-corrected control	R1243	11299	LRRK2 G2019S/G
iCell DopaNeurons GBA N370S	R1231	11344	GBA N370S
iCell DopaNeurons GBA N370S/N mutation-corrected control	Coming Soon	11344	GBA N370S/N

Cell Signaling Technologies Antibodies	Catalog #
β3-Tubulin (E9F3E) Mouse mAb	45058
LAMP1 (D2D11) XP® Rabbit mAb	9091
LC3A/B (D3U4C) XP® Rabbit mAb	12741
Cathepsin B (D1C7Y) XP® Rabbit mAb	31718
COX IV (3E11) Rabbit mAb	4850
Myelin Basic Protein (E9P7U) Mouse mAb	83683
Lamin A/C (4C11) Mouse mAb	4777
GFAP (GA5) Mouse mAb	3670
Additional antibody markers can be found at cellsignal.com	

Parkinson's Progression Markers Initiative

THE MICHAEL J. FOX FOUNDATION FOR PARKINSON'S RESEARCH

Characterization of iPSC-derived Dopaminergic Neurons

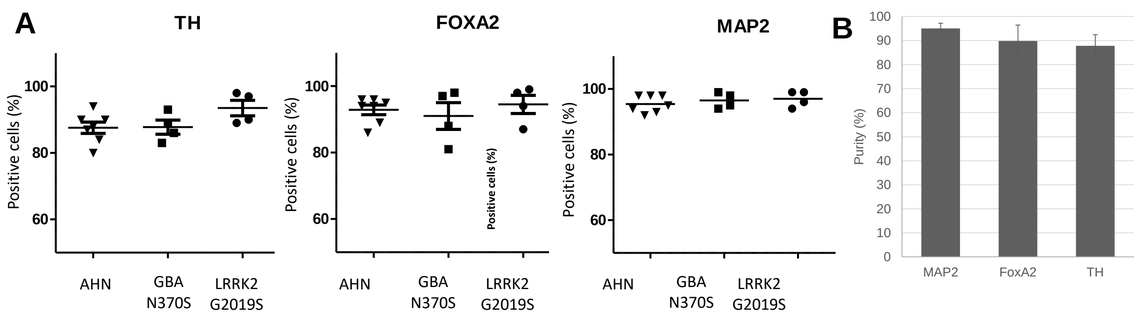

Figure 1: iCell DopaNeurons are a highly pure and consistent population of dopaminergic neurons. (A) Multiple batches of iCell DopaNeurons (GBA N370S, LRRK2 G2019S, and apparently healthy normal (AHN/WT)) were stained for FOXA2, Tyrosine Hydroxylase (TH) and MAP2 antibodies and analyzed via flow cytometry to show purity at day 3 in culture. Similar purity is observed in iCell DopaNeurons from apparently healthy normal (AHN) donors. **(B)** Pooling purity data (n = 27 lots) demonstrates that iCell DopaNeurons are a highly pure population that is consistent across donors and production lots for mutant and normal dopaminergic neurons.

Autophagic-Lysosomal Pathway Gene Expression

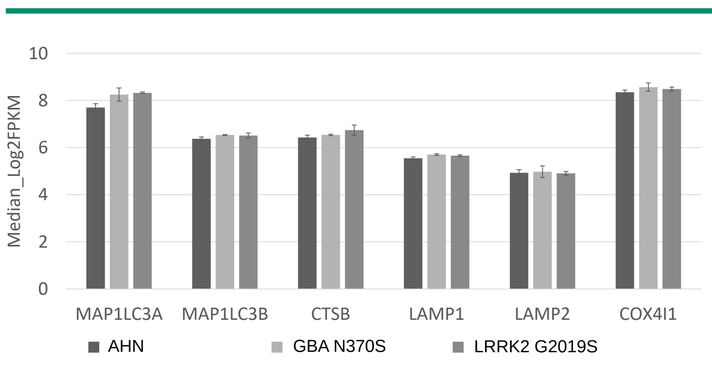

Figure 2: Similar gene expression for autophagic and lysosomal pathways across healthy and PD cells. Bulk RNASeq data from day 14 cultured iCell DopaNeurons (AHN, GBA N370S, and LRRK2 G2019S) show similar levels of expression for autophagic-lysosomal pathway genes, including LC3 (MAP1LC3A, MAP1LC3B, Cathepsin B (CTSB), LAMP1, LAMP2, and COX4I1.

Assessment of Lysosomes in Patient-derived Dopaminergic Neurons

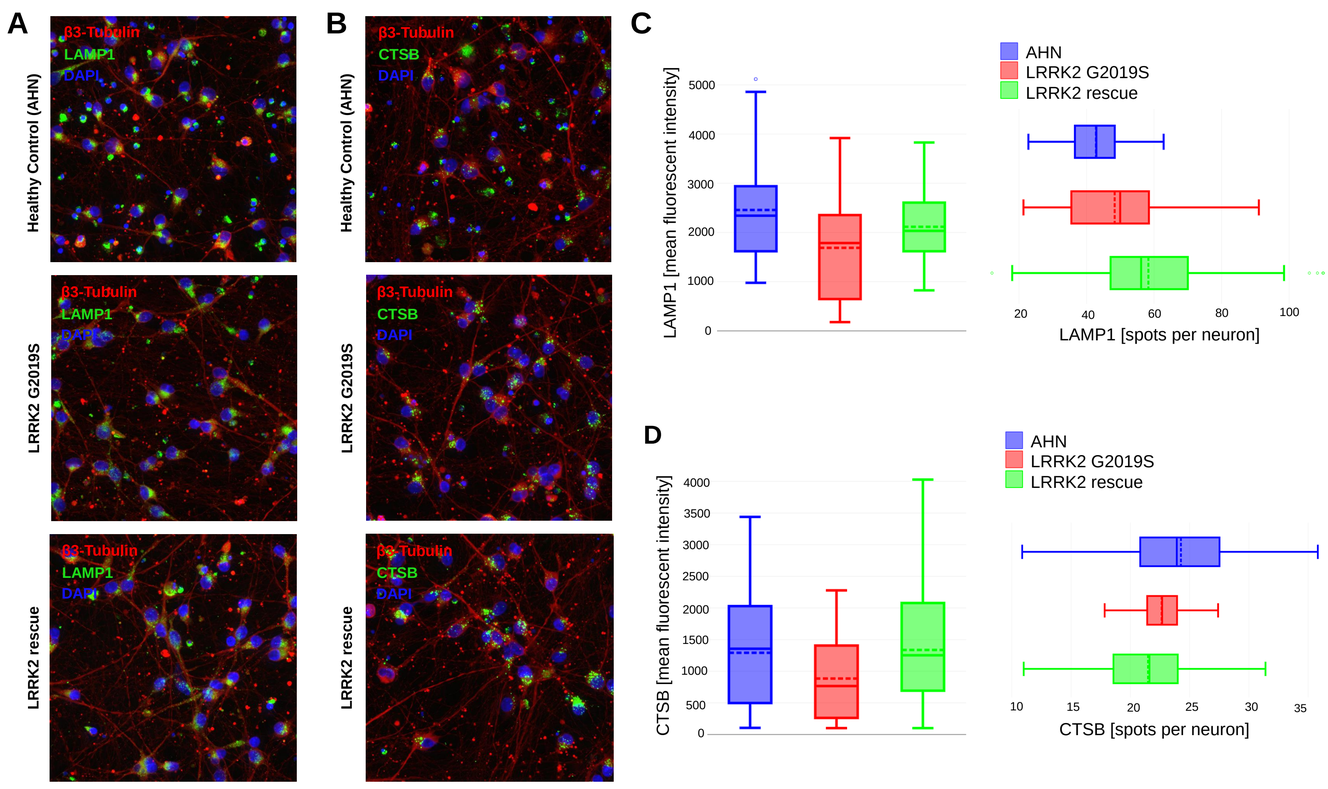

Figure 3: Lysosomal proteins imaged in iCell DopaNeurons. Lysosomal protein LAMP1 **(A)** and Cathepsin B (**B**, CTSB) were imaged in iCell DopaNeurons from healthy control donors, patients harboring a LRRK2 G2019S mutation, and patient-derived neurons with LRRK2 G2019S/G mutation correction (rescue). Neurons were counterstained with BIII-Tubulin (red) and DAPI (blue). **(C, D)** The images were quantified for LAMP1 and CTSB mean fluorescent intensity and spots per neuron. Similar expression profile for LAMP1 and CSTB was observed across cell lines.

LRRK2 G2019S Mutation is Associated with Increase in LC3 Signal

Figure 4: Quantification of LC3. AHN and patient-derived iCell DopaNeurons were cultured for 14 days and stained for LC3, BIII-Tubulin, and DAPI. **(A)** Representative images show healthy neurons. **(B)** Autophagic rate was quantified by measuring LC3 mean fluorescent intensity. Data show that iCell DopaNeurons LRRK2 G2019S cells show increased LC3 staining compared to AHN and the LRRK2 G2019S/G corrected control (rescue).

LRRK2 G2019S Does Not Change Mitochondria Number or Branching

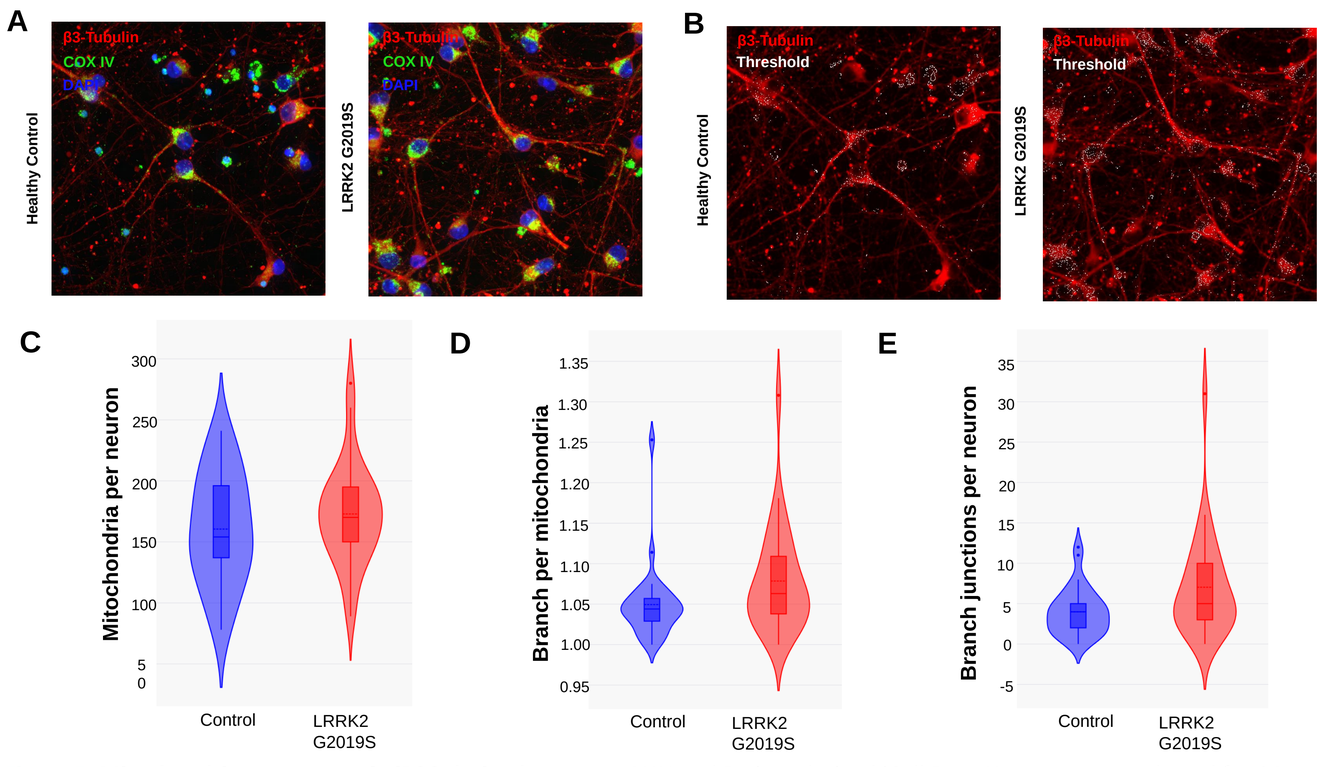

Figure 5: Mitochondria assessment in iPSC-derived neurons. AHN and Patient derived iCell DopaNeurons were cultured for 14 days and stained for COX IV (green), BIII-Tubulin (red), and DAPI (blue). **(A)** Representative images show healthy neurons. **(B)** Threshold was applied to quantify mitochondrial parameters. No change was found for mitochondria per neuron **(C)**, branches per mitochondria **(D)** and branch junctions per neuron **(E)**.

Conclusions

- We show that human iPSC-derived dopaminergic cells are a relevant model for PD and that iCell DopaNeurons are compatible for high-throughput screening and analysis using antibodies against disease-relevant targets.
- CST's portfolio of rabbit and mouse monoclonal antibodies for organelle and CNS markers that can help assess cellular processes in PD. These antibodies can be used for specific labeling of components of the lysosomal and autophagic pathway, allowing quantification of these in iCell DopaNeurons.
- LC3 expression is increased in primary iPSC-derived dopaminergic neurons harboring a LRRK2 G2019S mutation, which can be rescued by WT LRRK2 expression.
- Mitochondria number and gross morphology was not affected by the LRRK2 G2019S mutation.
- Combining a comprehensive portfolio of high-quality monoclonal antibodies with human iPSC-derived dopaminergic neurons is a useful method to characterize disease-associated processes. Understanding changes of cellular protein expression and localization neuronal and glia cells will accelerate new discoveries that lead to a better understanding, diagnosis, and treatment of disease in neurodegenerative diseases, such as PD.

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