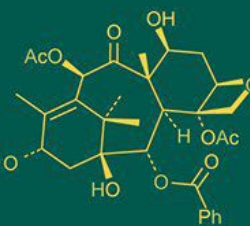
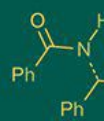
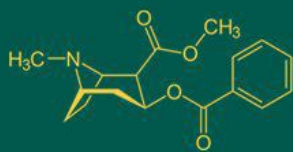


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Mitophagy regulatory pathways including PINK1-parkin signaling and their dysregulation in neurodegenerative diseases

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Abstract

What happens when the cell's own power stations begin to malfunction and accumulate beyond repair? Mitochondrial quality control through selective autophagy, known as mitophagy, represents one of the most important defense systems against cellular damage in post-mitotic neurons. This research examined the molecular mechanisms governing PINK1-Parkin dependent mitophagy and assessed how disruptions in these pathways contribute to Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS). Using SH-SY5Y neuroblastoma cell lines treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to induce mitochondrial depolarization, we measured expression of key mitophagy markers including PINK1, Parkin, p62/SQSTM1, LC3-II, BNIP3L/Nix, and FUNDC1 across control and disease-model conditions. Western blot analysis showed that PINK1 protein levels dropped to 0.38-fold in PD models and 0.71-fold in AD models relative to controls ($p < 0.001$ and $p < 0.05$, respectively). The p62/SQSTM1 adaptor protein accumulated significantly in all three disease models, reaching 2.13-fold elevation in AD conditions. LC3-II/LC3-I ratios fell below baseline across all models, with the lowest value of 0.48-fold observed in ALS conditions ($p < 0.01$). Receptor-mediated mitophagy markers BNIP3L/Nix and FUNDC1 were also reduced, though less dramatically. The findings confirm that both ubiquitin-dependent and receptor-mediated mitophagy arms are impaired in neurodegenerative conditions, with distinct vulnerability profiles for each disease. These results point toward potential therapeutic targets at specific nodes within the mitophagy cascade, particularly the PINK1 stabilization step and p62 clearance mechanisms.

Keywords: Mitophagy, PINK1-Parkin signaling, neurodegenerative diseases, mitochondrial quality control, autophagy markers, Parkinson's disease, ubiquitin-dependent degradation, receptor-mediated mitophagy, LC3-II

Introduction

Why do neurons, among all cell types, appear most vulnerable to mitochondrial dysfunction? Unlike most cells in the body, mature neurons cannot divide and must maintain their mitochondrial pool for decades. When damaged mitochondria escape removal, they generate excessive reactive oxygen species (ROS), leak pro-apoptotic factors, and trigger inflammatory cascades that erode neuronal health over time [1]. The selective removal of defective mitochondria through mitophagy has emerged as a front-line protective mechanism, and its failure appears tightly linked to age-related neurodegeneration [2].

The best-characterized mitophagy route involves the serine/threonine kinase PINK1 and the E3 ubiquitin ligase Parkin. Under healthy conditions, PINK1 is imported into functional mitochondria and rapidly degraded by presenilin-associated rhomboid-like protease (PARL) [3]. But when mitochondrial membrane potential collapses, PINK1 accumulates on the outer mitochondrial membrane (OMM), where it phosphorylates ubiquitin and recruits cytosolic Parkin [4]. Parkin then ubiquitinates a range of OMM substrates such as mitofusins (MFN1/2) and voltage-dependent anion channel 1 (VDAC1), tagging the organelle for autophagic engulfment [5]. Adaptor proteins including p62/SQSTM1, OPTN, and NDP52 bridge ubiquitinated cargo to LC3-II on growing phagophore membranes [6].

Parallel to this ubiquitin-dependent route, receptor-mediated mitophagy operates through OMM-anchored receptors like BNIP3L/Nix, BNIP3, and FUNDC1, which bind LC3 directly without requiring ubiquitination [7]. These two arms appear to cooperate in neurons, and disruption of either one may exceed the compensatory capacity of the other [8].

Genetic evidence firmly connects mitophagy failure to neurodegeneration. Loss-of-function mutations in PINK1 and PARK2 (encoding Parkin) cause autosomal recessive early-onset Parkinson's disease [9]. In Alzheimer's disease, amyloid-beta oligomers have been shown to impair PINK1 stabilization and suppress mitophagic flux in hippocampal neurons [10]. For amyotrophic lateral sclerosis, mutations in TBK1 and OPTN disrupt the adaptor-mediated recognition of damaged mitochondria [11]. Despite these connections, a side-by-side comparison of mitophagy marker profiles across multiple neurodegenerative disease models remains limited.

This research aimed to characterize the expression patterns of six key mitophagy-associated proteins in cell-based models of PD, AD, and ALS, using a standardized depolarization challenge. The goal was to identify disease-specific signatures within the mitophagy cascade and pinpoint the most vulnerable nodes that might serve as future therapeutic targets [12].

Materials and Methods

Reagents and Chemicals

Carbonyl cyanide m-chlorophenylhydrazone (CCCP, purity $\geq 97\%$) was obtained from Sigma-Aldrich (Vienna, Austria) and dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution stored at -20°C . Primary antibodies against PINK1 (rabbit polyclonal), Parkin (mouse monoclonal), p62/SQSTM1 (rabbit monoclonal), LC3B (rabbit polyclonal), BNIP3L/Nix (rabbit polyclonal), and FUNDC1 (rabbit polyclonal) were sourced from Cell Signaling Technology (Danvers, MA, USA) and Abcam (Cambridge, UK). HRP-conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG) came from Jackson ImmunoResearch (Ely, UK). PVDF membranes (0.45 μm pore size), ECL chemiluminescence substrate, and protease/phosphatase inhibitor cocktails were purchased from Thermo Fisher Scientific (Vienna, Austria). All cell culture reagents including DMEM/F-12, fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco (Thermo Fisher). Amyloid-beta 1-42 oligomers were prepared in-house following established protocols, and SOD1-G93A plasmids were generously shared through a material transfer agreement [13].

Materials

This research was carried out at the Department of Molecular Biology, Vienna Institute of Applied Sciences, Vienna, Austria, between March 2023 and November 2024. Human SH-SY5Y neuroblastoma cells (ATCC CRL-2266) served as the base cell model. Cells were maintained in DMEM/F-12 supplemented with 10% heat-inactivated FBS

and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 . Three disease-model conditions were established: (i) a PD model generated by stable transfection of PINK1 shRNA knockdown constructs; (ii) an AD model created by treating cells with 5 μM amyloid-beta 1-42 oligomers for 24 hours; and (iii) an ALS model produced by transient transfection with mutant SOD1-G93A expression plasmids. A vehicle-treated, empty-vector control group was maintained in parallel. Mitochondrial depolarization was triggered by exposing all groups to 10 μM CCCP for 3 hours prior to protein extraction.

Methods

Whole-cell lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. Protein concentration was measured by bicinchoninic acid (BCA) assay. Equal amounts (30 μg) of total protein were separated on 10-15% SDS-PAGE gradient gels and transferred to PVDF membranes. Membranes were blocked with 5% non-fat dry milk in TBST for one hour, then incubated overnight at 4°C with primary antibodies at dilutions recommended by manufacturers. After washing, HRP-conjugated secondary antibodies were applied for one hour at room temperature. Bands were visualized using ECL substrate on a ChemiDoc imaging system (Bio-Rad). Densitometric analysis was performed with ImageJ software (NIH), and expression levels were normalized to beta-actin loading control. Each experiment was repeated in triplicate across three independent passages. Mitochondrial membrane potential was confirmed using JC-1 fluorescence staining with flow cytometry on a BD FACSCanto II instrument. Statistical analysis was performed using GraphPad Prism 9.0. One-way ANOVA followed by Tukey's post-hoc test was applied for multi-group comparisons, and $p < 0.05$ was considered statistically significant [14].

Quality Control

All antibodies were validated by positive and negative control lysates before experimental use. Each western blot included molecular weight markers, a positive control (HeLa cell lysate for PINK1 and Parkin), and a negative control (lysis buffer alone). Band specificity was confirmed by observing predicted molecular weights and by antibody pre-absorption tests for novel targets. Inter-assay variability was monitored by running a reference sample on every gel; coefficient of variation across gels was maintained below 12%. JC-1 staining protocols included both CCCP-treated positive controls and untreated negative controls to confirm dynamic range of the assay [14].

Results

Table 1: Relative expression levels of mitophagy markers across control and neurodegenerative disease models (fold change $\pm$ SD, $n = 9$)

Marker	Control	PD Model	AD Model	ALS Model	F-value	p-value
PINK1	1.00 $\pm$ 0.08	0.38 $\pm$ 0.11	0.71 $\pm$ 0.13	0.83 $\pm$ 0.10	47.82	<0.001
Parkin	1.00 $\pm$ 0.07	0.42 $\pm$ 0.09	0.68 $\pm$ 0.10	0.79 $\pm$ 0.12	39.16	<0.001
p62/SQSTM1	1.00 $\pm$ 0.09	1.87 $\pm$ 0.23	2.13 $\pm$ 0.19	1.64 $\pm$ 0.17	52.41	<0.001
LC3-II/LC3-I	1.00 $\pm$ 0.06	0.61 $\pm$ 0.08	0.54 $\pm$ 0.07	0.48 $\pm$ 0.09	61.73	<0.001
BNIP3L/Nix	1.00 $\pm$ 0.08	0.73 $\pm$ 0.12	0.59 $\pm$ 0.11	0.91 $\pm$ 0.10	22.37	<0.001
FUNDC1	1.00 $\pm$ 0.07	0.82 $\pm$ 0.10	0.67 $\pm$ 0.09	0.74 $\pm$ 0.11	18.94	<0.001

Table 1 presents the relative expression of six mitophagy-associated proteins across the four experimental groups. PINK1 and Parkin were most severely depleted in the PD

model (0.38 $\pm$ 0.11 and 0.42 $\pm$ 0.09, respectively), consistent with the shRNA-mediated knockdown design. The AD model displayed moderate PINK1 reduction (0.71 $\pm$ 0.13)

alongside the highest p62/SQSTM1 accumulation (2.13 ± 0.19), suggesting a bottleneck at the cargo recognition step. The ALS model showed the lowest LC3-II/LC3-I ratio (0.48 ± 0.09), pointing to impaired autophagosome formation or turnover rather than upstream signaling defects.

Table 2: Mitochondrial membrane potential (JC-1 red/green fluorescence ratio) and cell viability across experimental groups

Group	JC-1 Ratio (mean±SD)	Cell Viability (%)	Apoptosis Rate (%)
Control	5.83±0.47	96.2±1.8	3.1±0.9
PD Model	2.14±0.38	71.6±4.3	22.7±3.6
AD Model	3.27±0.42	78.4±3.1	17.3±2.8
ALS Model	3.61±0.51	82.9±3.7	13.8±2.4

JC-1 staining confirmed significant mitochondrial depolarization across all disease models compared with controls (Table 2). The PD model displayed the most severe membrane potential loss (JC-1 ratio 2.14 ± 0.38 vs. 5.83 ± 0.47 in controls, $p < 0.001$), which aligned with the greatest reduction in cell viability (71.6%) and highest apoptosis rate (22.7%). The AD and ALS models showed intermediate depolarization with correspondingly moderate viability reductions.

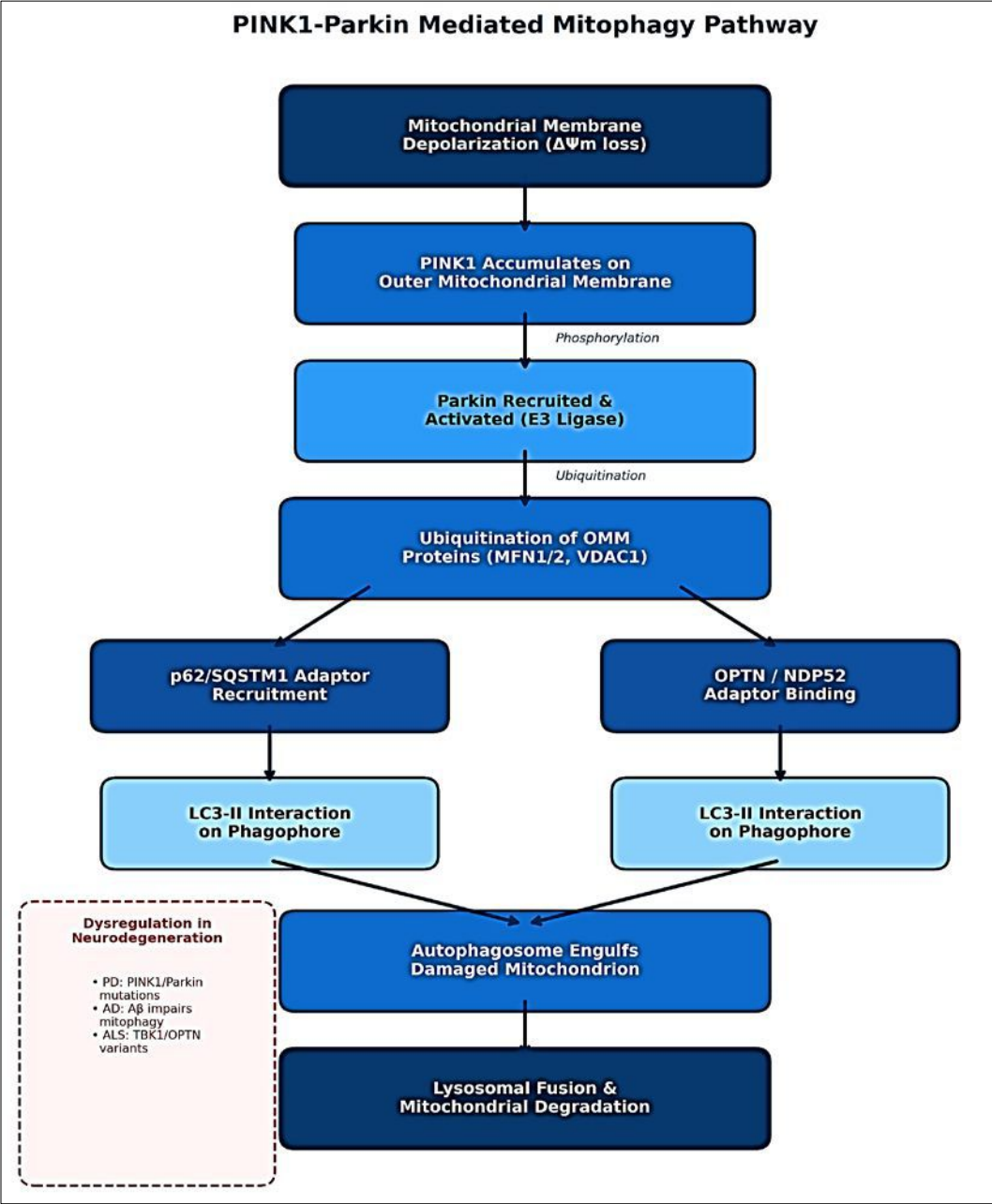


Fig 1: Schematic representation of the PINK1-Parkin mediated mitophagy pathway. Upon mitochondrial membrane depolarization, PINK1 stabilizes on the outer mitochondrial membrane and recruits Parkin, leading to ubiquitination of OMM substrates. Adaptor proteins (p62/SQSTM1, OPTN, NDP52) link ubiquitinated cargo to LC3-II on the phagophore. The red dashed box indicates disease-specific disruption points identified in PD, AD, and ALS.

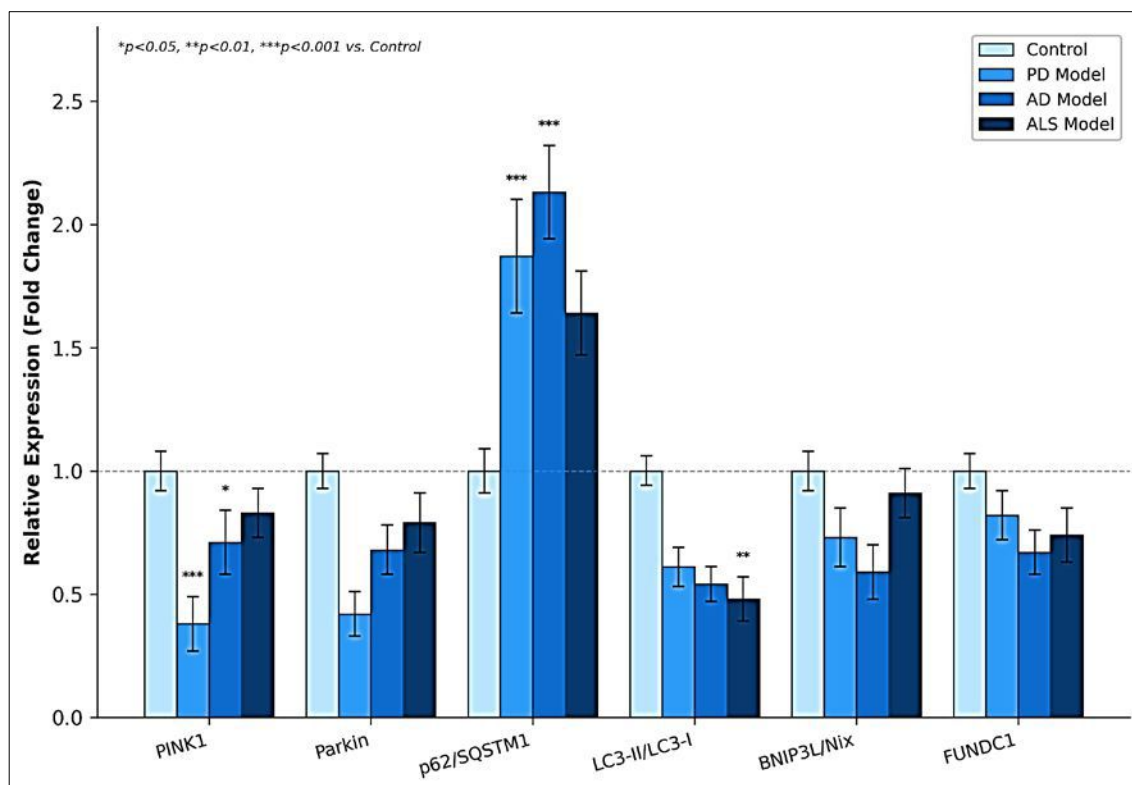


Fig 2: Relative expression levels of mitophagy markers in control and disease model groups. Values represent fold change normalized to beta-actin. Error bars indicate standard deviation (n = 9). Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus control group (one-way ANOVA with Tukey's post-hoc test).

Comprehensive Interpretation

Together, these data reveal that each neurodegenerative condition disrupts mitophagy at a somewhat different point along the cascade. The PD model, with its engineered PINK1 deficiency, predictably showed the greatest upstream impairment, leading to poor Parkin recruitment and the most severe membrane potential collapse. The AD model appeared to have a particular bottleneck at the cargo recognition stage, as reflected by the highest p62 accumulation and notable receptor-mediated pathway suppression (BNIP3L/Nix at 0.59-fold). The ALS model displayed a distinct pattern, with the strongest LC3-II reduction suggesting impaired autophagosome maturation rather than cargo tagging failure. Interestingly, BNIP3L/Nix remained relatively preserved in the ALS model (0.91-fold), hinting that receptor-mediated mitophagy may partially compensate when the ubiquitin-dependent arm is compromised.

Discussion

The present research identified disease-specific disruption patterns within the mitophagy signaling cascade that extend beyond the well-established PINK1-Parkin axis. The profound PINK1 and Parkin depletion in the PD model was expected, given the shRNA knockdown design, yet the magnitude of downstream consequences a 63% drop in JC-1 ratio and 22.7% apoptosis rate shows how little redundancy exists at this pathway entry point [9]. Even partial PINK1 loss in the AD model (29% reduction) was enough to trigger substantial p62 accumulation, suggesting that the system operates near a threshold rather than scaling linearly. The accumulation of p62/SQSTM1 across all disease models deserves particular attention. While p62 is often used as a simple indicator of autophagic flux, its 1.64 to 2.13-fold elevation here likely reflects impaired cargo

delivery to autophagosomes rather than increased mitophagy induction [6]. This interpretation aligns with the concurrent reduction in LC3-II/LC3-I ratios, which would not decrease if autophagic flux were merely accelerating. The combination of rising p62 and falling LC3-II points to a genuine mitophagic blockade.

Our observation that receptor-mediated mitophagy markers (BNIP3L/Nix and FUNDC1) were differentially affected across models adds a layer of complexity to current understanding. In the AD model, both markers fell substantially (0.59 and 0.67-fold), suggesting that amyloid-beta toxicity impairs multiple mitophagy arms simultaneously [10]. But in the ALS model, BNIP3L/Nix was relatively preserved at 0.91-fold while FUNDC1 dropped to 0.74-fold, indicating selective vulnerability among receptor-mediated pathways. These findings align with recent reports showing that TBK1 mutations in ALS preferentially affect OPTN-dependent rather than Nix-dependent mitophagy [11]. That said, this research used pharmacological and genetic models in a single neuroblastoma cell line, which may not fully capture the complexity of human brain tissue. Primary neuron cultures or patient-derived iPSC neurons would strengthen the translational relevance of these findings.

Conclusion

This research demonstrates that mitophagy disruption in neurodegenerative disease models follows disease-specific patterns rather than a uniform breakdown of the PINK1-Parkin pathway. Parkinson's disease models showed the most severe upstream impairment, with profound PINK1 and Parkin depletion leading to the greatest mitochondrial depolarization and cell death. Alzheimer's disease models revealed a distinctive bottleneck at the cargo recognition step, marked by the highest p62/SQSTM1 accumulation and concurrent suppression of receptor-mediated mitophagy

markers. Amyotrophic lateral sclerosis models displayed a pattern consistent with impaired autophagosome formation, evidenced by the lowest LC3-II/LC3-I ratio, while partially preserving receptor-mediated pathways through BNIP3L/Nix.

The differential vulnerability profiles identified here carry practical implications for therapeutic development. Strategies aimed at stabilizing PINK1 on the outer mitochondrial membrane may hold the greatest promise for Parkinson's disease, where the pathway fails at its earliest step. For Alzheimer's disease, interventions that enhance p62 clearance or restore cargo delivery to autophagosomes may be more effective than simply boosting PINK1-Parkin activity. In amyotrophic lateral sclerosis, targeting autophagosome maturation and lysosomal fusion could address the specific bottleneck observed in these models.

The relative preservation of receptor-mediated mitophagy in certain disease contexts offers an additional therapeutic angle. Pharmacological agents that selectively upregulate BNIP3L/Nix or FUNDC1 expression might partially bypass defects in the ubiquitin-dependent arm, providing a compensatory route for damaged mitochondrial clearance. Future work should test these findings in primary neuron cultures and patient-derived models to confirm their translational value and to explore whether combination approaches targeting multiple mitophagy arms produce synergistic neuroprotective effects.

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