Chapter 5: Summary & Conclusion

5.1 Summary:

Parkinson's disease (PD) stands as the second most prevalent neurodegenerative disorder, exerting a profound impact on an individual's motor functions and, ultimately, contributing to mortality. PD pathogenesis is known to spread different regions of the brain; however, the mechanism still is not well understood. Further, the prognosis/diagnosis is poor and only clinically diagnosed at the late stage of pathogenesis. Exosome-mediated intercellular communication between brain cells is a pivotal factor in the propagation of PD pathology. The miRNA cargo carried by exosomes is particularly critical for this role since miRNAs are post-transcriptional regulators of target mRNA and maintained in normal physiological levels. The transfer of miRNAs via exosomes to neighboring cells significantly impacts the physiological and cellular pathways of the recipient cells, ultimately influencing the pathological fate of brain cells. However, the mechanisms governing the release of exosomes in PD conditions from the source cell remain elusive. Identifying the specific exosomal miRNAs implicated in PD pathology holds promise as a valuable diagnostic and prognostic biomarker for early PD detection. The current study focused on the organellar crosstalk between the mitochondria and lysosomes modulating exosome release within the neurons. Furthermore, differential regulation of exosomal miRNA sorting during PD conditions was characterized and its implication in acute neurotoxic model was also investigated and exosomal miRNAs as potential prognosis/diagnosis markers was also investigated in animal model.

5.1.1 Exosome release is modulated by the organellar crosstalk in PD stress conditions

- Exosome release is enhanced in neuroblastoma cell lines as well as glial cell lines during PD stress conditions. The expression of CD63 (a well-characterized exosome marker) is increased in all the cell lines, and NTA analysis shows an increase in the concentration of exosomes.
- PD stress induces complex-I inhibition, mitochondrial dysfunction, and reduction in ATP levels in both neuronal and glial cells.
- Further, lysosomal dysfunction was confirmed in PD stress conditions by the lysosomal acid phosphatase levels, reduced number of lysosomes per cell, and reduced translocation of TFEB to nucleus.

- PD stress inhibits autophagy flux in neuronal and glial cells, which subsequently enhances the exosome release.
- Rapamycin, inhibitor of mTOR, enhances autophagy and decreases the exosome release in neuronal and glial cells in PD stress conditions.

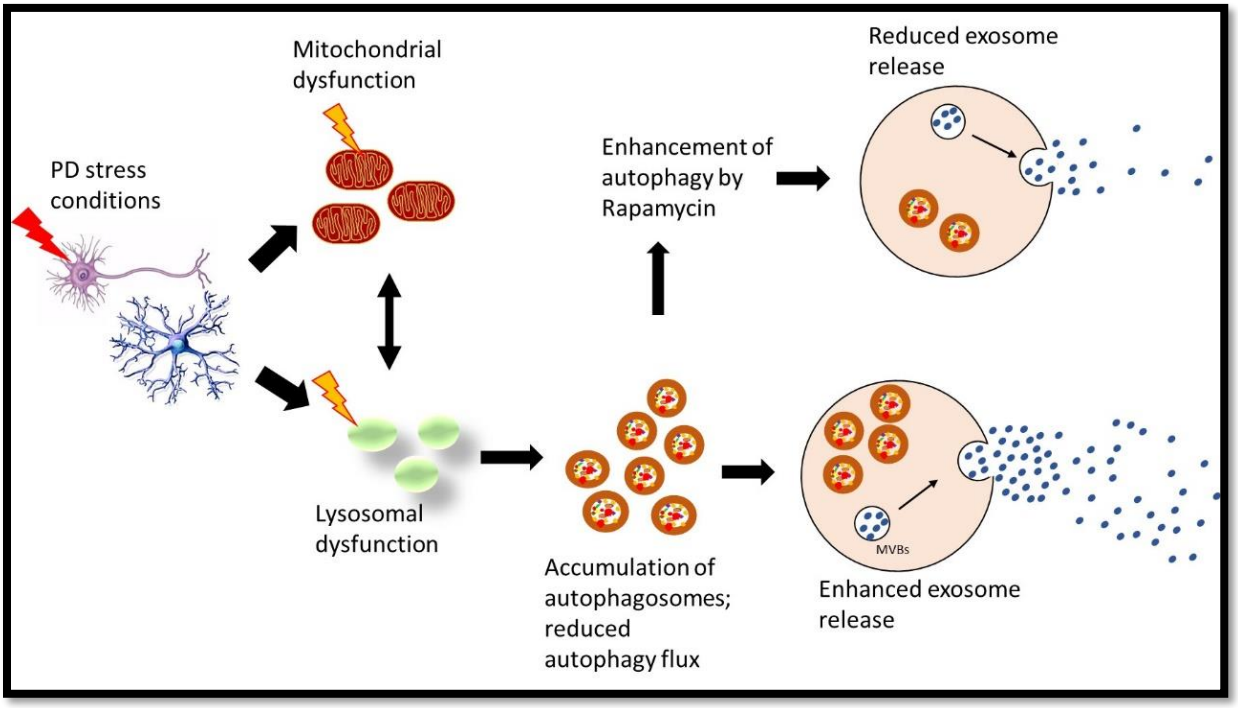

Figure 5.1. Schematic representation of interorganellar crosstalk between mitochondria and lysosome in PD regulating the autophagy pathway, hence, the exosome pathway.

5.1.2 Neuronal exosomes are internalized by recipient cells and modulate the cellular functions

- Exosome-specific dyes used to stain neuronal exosomes are actively uptaken by neuronal and glial recipient cells as observed by confocal microscopy.
- PD-stressed exosomes after internalization show an increased localization to the mitochondria in the recipient cells.

- Neuronal cells treated with exosomes derived from PD stressed neurons enhanced mitochondrial and total cellular ROS levels and decreased mitochondrial membrane potential suggest mitochondrial dysfunction.
- PD-exosomes also induce cell death in recipient neuronal and glial cells as shown by MTT cell viability assay and PARP cleavage.

5.1.3 Exosomal miRNAs are differentially sorted in PD stress conditions and alter mitochondrial functions and cell death in recipient cells

- The isolation of exosomal RNA from PD-stressed neuronal cells followed by NGS analysis confirmed that several miRNAs are altered in exosomes in PD stress conditions.
- The mRNA targets of significantly altered miRNAs were predicted by bioinformatics tools and clustered into important groups using DAVID tool.
- Validation of miRNAs was done by qRT-PCR as well as ddPCR.
- The other critical biological processes modulated by the identified miRNAs were related to apoptosis and ageing, as well as processes related to neuronal differentiation and morphogenesis. The major pathways involved included PI3K-AKT and MAPK signalling pathways, and endocytic pathways.
- Two significant miRNAs were found to be downregulated in PD-stress conditions: hsa-miR-30a-5p and hsa-miR-181c-5p.
- Exogenous expression of the miRNAs in neuronal cells resulted in packaging of the miRNAs into exosomes.
- Mimic-loaded exosomes, when treated to recipient PD-stressed neuronal or glial cells, improved the mitochondrial functions and ameliorated cell death in recipient cells.

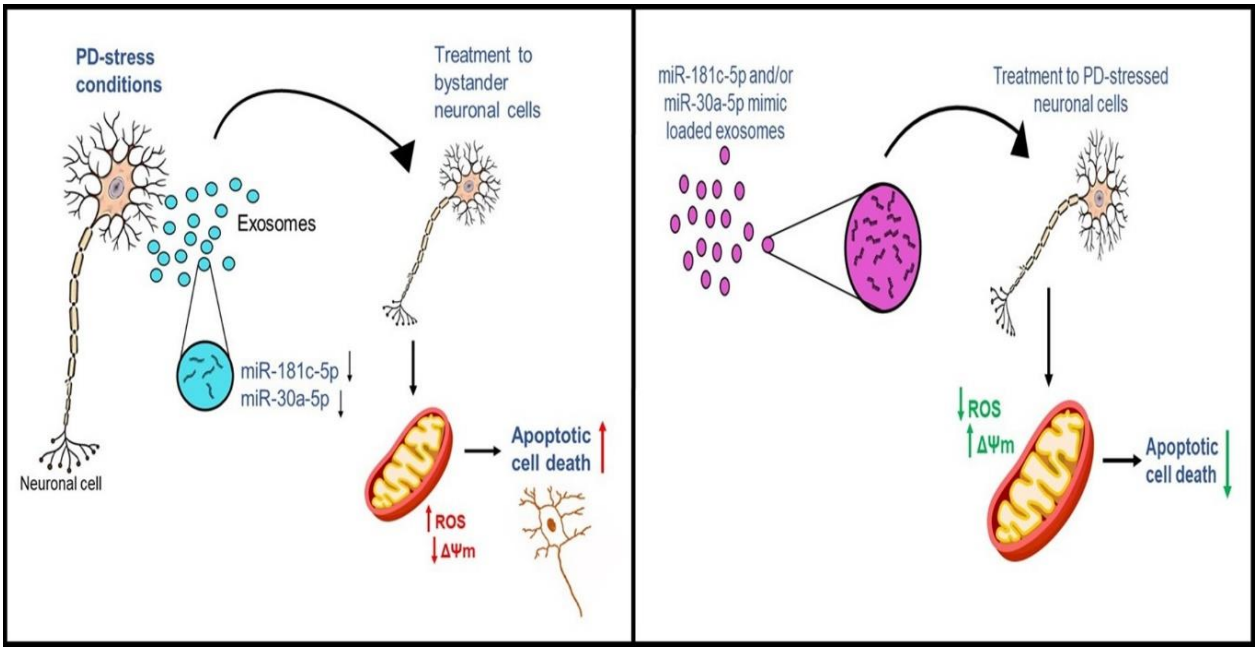

Figure 5.2. Schematic representation of neuronal functions modulated by exosomal miRNAs.

5.1.4 Exosomal cargo from serum of PD-rats affects the cellular functions in primary midbrain neurons

- Concordant with the cell line data, exosomes isolated from the biofluid (serum) of PD-rat models confirms the increased exosome concentration through NTA analysis.
- miRNAs validated in the neuronal cell lines were further validated in the exosomes isolated from primary neurons and the serum and CSF of rotenone-treated rats.
- miR-30a-5p and miR-181c-5p showed differential regulation in CSF and serum exosomes at different time points.
- Serum exosomes induced mitochondrial dysfunction and cell death in primary midbrain neurons, and the cell death is exacerbated in rotenone treated primary neurons.

5.2 Conclusion:

Parkinson's disease (PD) is a neurodegenerative condition characterized by the gradual loss of dopaminergic neurons in the SNpc region of the brain. The primary pathological features include

the presence of α-synuclein cytoplasmic inclusions and mitochondrial dysfunction, observed not only in the SN but also in other parts of the central nervous system, suggesting the propagation of pathology to neighboring neurons. While exosomal inter-neuronal communication is believed to be significant in the disease's spread, the underlying mechanisms remain unclear. Additionally, the understanding of the role of mitochondria in inter-organellar crosstalk with MVBs and lysosomes, and its impact on modulating exosome release in PD, is limited. The study here demonstrates that inter-organellar crosstalk between mitochondria, lysosomes and MVB is now emerging important in PD pathogenesis. The mutation in several proteins regulating mitochondrial dysfunction may in turn inhibit the lysosomal function leading to decreased autophagy flux; hence, increased exosomal release is observed, containing several pathogenic cargo components which may intensify the pathogenesis of PD. The evidence here suggest that the understanding of the inter-organellar crosstalk may provide unique opportunity to modulate the combinatorial strategy to enhance the autophagy flux and prevent exosomal release hereby reducing the spread of PD and helping ameliorate the disease.

Exosomes mediate intercellular communication between neurons, glia, and other cell types throughout PD-relevant brain regions however, the mechanism remains unclear. The current study demonstrates that exosomes are mediators of intercellular communication between neurons during PD stress conditions. Exosomes are actively internalized by recipient neuronal cells and further localize to mitochondria. Profiling of exosomal miRNAs from neuronal cells exposed to 6-OHDA showed that selected miRNAs are differentially sorted into exosomes under PD stress conditions. Exosome-mediated transfer of two identified miRNAs, miR-30a-5p and miR-181c-5p, rescued mitochondrial dysfunction and cell death under PD stress conditions in neuronal cells.

The current study also provides insight into the exploitation of exosomal miRNAs in biofluids as potential diagnostic/prognostic biomarkers for early detection of PD. Preliminary data in primary neurons from rat embryos shows a variety of miRNAs that are differentially expressed in rotenone conditions which can also be detected in in exosomes from the biofluids, CSF and serum, of acute model of rotenone in rats, which demonstrated that some miRNAs, like miR-93-5p is consistently downregulated in CSF, and the levels of miR-30a-5p and miR-484 are downregulated in the serum of rotenone treated rats which display a potential as biomarkers for detection of early-stage PD. Further, serum exosomes from acute model of rotenone can internalized in primary midbrain

neurons displayed mitochondrial defects and neuronal death was exacerbated. In conclusion, the exosomal cargo changes the physiological status of the recipient cell that they are uptaken in, hereby altering the pathological outcome in PD.

5.3 Limitations of the study:

The study provided some interesting conception of the neurobiology of exosomes in PD and the role of exosomal miRNAs in intercellular communication in PD, however there are some limitations which have been summarized below:

1. The study here showed enhanced exosome release in cell lines and biofluids of a rat model, its validation in biofluids derived from PD patients or in dopaminergic induced pluripotent stem cell (iPSC) neurons obtained from individuals with PD would significantly strengthen the credibility of the hypothesis.

2. The study looked into the impact of inter-organellar crosstalk on exosome release, yet the detailed investigation of exosome uptake by recipient cells remains unaddressed. Conducting time-dependent studies to observe the dynamic localization of exosomes with organelles in recipient cells would provide a more proper understanding of their functional implications.

3. To establish certain miRNAs as prognostic and diagnostic biomarkers for PD detection, the analysis of exosomal miRNAs through NGS could have been extended to a large cohort of patient-derived exosomes.

4. Putative target analysis of miRNAs was done through bioinformatics, however validation in recipient cells using Ago-mediated pulldown of targets would enhance the robustness of the findings.

5. In this study, the functional impact of only one exogenously expressed miRNA in exosomes on bystander neuronal and glial cells was examined. However, this may not precisely replicate physiological conditions where multiple exosomal miRNAs simultaneously regulate cellular pathways in the recipient cell.

6. The roles of miR-30a-5p and miR-181c-5p should be validated in iPSC-derived neurons from PD patients to demonstrate their relevance in the context of PD pathology.

7. Exosomes from PD rats injected into healthy rats will provide insights into understanding the functional role of these exosomes in PD pathogenesis under physiological conditions.

5.4 Future perspectives:

This study has addressed interesting questions regarding the regulation of exosome release from source cells, alterations in exosomal miRNA cargo under PD stress conditions, and the role of exosomes in intercellular communication by influencing recipient cell pathways upon uptake. The findings not only shed light on these processes but also provide leads to for therapeutic exploration in combatting PD pathogenesis:

1. PD currently lacks effective treatment hence enhancing mitochondrial and lysosomal functions using antioxidants and organelle-specific drugs may have broad implications for delaying disease progression.

2. Emerging evidence suggest that restoration of mitochondrial functions can also rescue lysosomal functions. NADH/NAD ratio is known to improve the mitochondrial, in turn lysosomal functions hence can be explored in modulation of lysosomal function in genetic/sporadic rodent models of PD.

3. The study reveals an intricate connection between the autophagy pathway and exosome release pathway, with impaired autophagy in PD leading to increased exosome release. Exploring a combinatorial strategy involving autophagy enhancers to mitigate exosome release from dying cells could pave the way for novel PD therapeutics.

4. The distinct pattern of exosomal miRNA sorting in response to various PD stress conditions could serve as a valuable diagnostic and prognostic marker for monitoring PD progression.

5. The study suggests that exosomal miRNAs, influencing cellular functions, have the potential to rescue mitochondrial functions, presenting emerging therapeutic possibilities for PD and other neurodegenerative disorders.

6. The increasing exploration of exosome-based delivery for therapeutic agents opens avenues for targeted therapy in PD. Utilizing antisense oligonucleotide chemistry to downregulate or

overexpress specific miRNAs and packaging them into exosomes ensures precise delivery to affected brain regions.

7. The differential expression of exosomal miRNAs in biofluids emerges as an excellent biomarker for early PD detection. Moreover, the physiological stability of exosomes in biofluids makes them ideal carriers for therapeutic agents, with engineered site-specific receptors facilitating drug delivery to PD-specific brain regions.