Chapter 1:

Introduction &

Literature Review

1.1 Parkinson's Disease - Global ageing related disorder and its prevalence

Parkinson's disease (PD) is a chronic, slowly progressing, neurodegenerative disorder which involves the demise of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the ventral midbrain region of the brain [1]. A decrease in dopamine levels in the basal ganglia results in a movement disorder marked by typical motor symptoms associated with PD [2]. It is the most frequent movement related disorder after Alzheimer's Disease (AD). Classical motor symptoms have been recognized since the 19th century, when James Parkinson gave his essay on 'the shaking palsy', and it has withstood the passage of time. These cardinal symptoms include tremors at rest, gait disturbances, postural impairment, muscle rigidity and bradykinesia [2], [3]. A spectrum of PD patients develops non-motor symptoms, which are succeeded by motor symptoms by decades, including, hyposmia, constipation, fatigue, anxiety, sleep disturbance, hallucinations, cognitive dysfunctions, etc. [4].

The prevalence of PD is observed in about 950 per 100,000 population of people over 65 years of age, which implies that about 349,000 individuals are affected in the US, and data collected from Europe in 2005 indicated that 4.1-4.6 million people above the age of 50 were affected. Meanwhile, comparatively lower prevalence of PD has been observed in populations of Africa, Asia, and South America [5]. The prevalence of PD has clearly been found to be increasing with the age of an individual. Several studies have indicated that the prevalence of PD is more in males than females, possible reasons being toxicant exposure, neuroprotection by oestrogen, X linkage of genetic risk factors, etc. [6]–[8].

Mental and neurological disorders (MNDs) are an increasing health concern in developing countries like India. According to the Global Burden of Disease study in 2016, India was found to contribute to 15% of global burden due to MNDs and was estimated to increase by up to 23% by 2025 [9]. The crude prevalence of Parkinsonism in India was found to vary between 33 to 328 individuals per 100,000 of population, or as a meta-analysis study indicated variation around 0.7 per 1000 individuals [10]. A survey in Bangalore, Karnataka, which is in the southern part of India, indicated a prevalence rate of 76 per 100,000 in 2004 [11]. In contrast, a survey in 2006 in Kolkata, which is in the eastern part of India showed a prevalence of 45.82 per 100,000 [12]. The state of Kashmir in the northern part of India, the prevalence was 14.1 per 100,000 [13], while a recent study in rural Gujarat in the western region of India indicated a crude prevalence of 42.3 per

100,000 [14]. There was an exception in a study where a survey in Parsi community in Mumbai indicated a prevalence of 192 per 100,000 which was substantially high as compared to the rest of the populations [15]. Such wide discrepancy in the prevalence studies may be due to ethnicity, genetic diversity, and environmental factors in various regions of India. There is an urgent need for large-scale and properly designed and conducted epidemiological studies for prevalence of PD in India, which can identify areas of focus and provide appropriate public health policies. In a developing country like India, environmental risk factors such as rural living, farming exposures, pesticides and well drinking water facilities which are described before may play a role in PD [16].

1.2 Neuroanatomical changes in Parkinson's Disease

The pathophysiological changes that occur in PD are complex and are not completely understood till this day. It is essential to comprehend the structural changes that are responsible for PD to offer early diagnosis and treatment plan. The basal ganglia of the brain receives signals from the cortex for performing voluntary movements and plays a role in cognitive functions [17], and it is one of the most affected brain regions in PD. A study reported that PARKIN PD patients showed atrophy in basal ganglia [18], moreover there is observed loss in length of dendritic spines of the spiny neurons in the striatum. The cerebellum region, which receives the dopaminergic projections from basal ganglia in the brain, show significant contraction and decrease in the gray matter volume as well in early-stage PD patients [19], [20]. A recent study illustrated the neuroanatomical changes in 106 PD patients, which were divided into two groups: 66 PD patients with normal cognition, and 40 with mild cognitive impairment. The latter group showed substantial gray matter density reduction in the cerebellum, temporal lobe, and frontal gyrus, indicating frontal and cerebellar atrophy as major neuroanatomical changes occurring in even early-stage PD patients [21]. Brains of PD patients are even affected in terms of brain volume, i.e., atrophy has been observed in cortical and subcortical areas, resulting in lesser brain volume of PD patients [22]. Hence, a vast number of studies has presented the neuroanatomical changes of major brain structures in PD.

1.3 Etiology of Parkinson's Disease

The etiology of PD is complex, and it is said to be a combination of genetic and environmental factors. There is a multifaceted molecular interaction between these various genetic and environmental risk factors associated with PD. There is a high probability that the pathogenesis of PD is a result of multiple etiologies rather than one [23]. The identification of the functions of several PD-associated genes provides hints about the involvement of molecular mechanisms in the pathophysiology, including loss of proteostasis, mitochondrial dysfunction, lysosomal dysfunctions, defective autophagy, oxidative stress, neuroinflammation and inability of the clearance of proteins [24]. Approximately 15% of cases observed are familial PD, which have a family history of the condition, while majority of the cases are sporadic or idiopathic, which have an unknown etiology or no familial connections [25]. At least 19 genes have been identified up till date that present a risk factor for PD, which include 9 autosomal recessive genes and 10 autosomal dominant genes. Various studies have also connected genetic risk loci and variants associated with sporadic or idiopathic PD. There are both genetic risk factors as well as disruption of molecular mechanisms that contribute to the pathophysiology of this neurodegenerative disorder.

1.4 Pathophysiology of Parkinson's Disease - genetic classification

1.4.1 SNCA (α-synuclein) / PARK1/4

SNCA was one of the first mutations recognized to be associated with autosomal dominant form of PD. The protein encoded by SNCA, α-synuclein, is a 140-amino acid protein that consists of three major domains: an acidic, negatively charged carboxy-terminal domain, a central hydrophobic domain, and an amino-terminal region [26]. The exact function of α-synuclein in the neuronal cells is still elusive, although there are some reports which indicate that the protein is a potent inhibitor of the enzyme phospholipase D2, which may influence the synaptic vesicle release form the neurons [27]. Apart from that, α-synuclein is also reported to play a role in synaptic plasticity, learning and dopamine synthesis [28]. α-synuclein gains neurotoxic function because of its aggregation and accumulation into insoluble α-synuclein fibrils, toxic oligomers and protofibrils [29]. This makes α-synuclein a major component of Lewy bodies, where the phosphorylated form of the protein is found in Lewy body inclusions [30]. Hence, research places

α-synuclein at the very centre stage as a pathological molecule associated with familial and sporadic PD.

1.4.2 LRRK2/PARK8

The PARK8 locus which codes for LRRK2/Dardarin protein is the most common and frequently occurring mutation and is the most common genetic cause of PD [31]. The mutation operates by a gain-of-function mechanism which involves increased kinase and GTPase activity. The resultant increase in kinase activity impairs the protein stability and leads to enlarged lysosomes with improper degradative capacity [32]. Although the mechanism of action of LRRK2 is not known yet, but it is involved in cellular pathways like lysosomal system, protein synthesis, cytoskeletal functions, etc. [31] which can lead to the death of dopaminergic neurons.

1.4.3 PARKIN/PARK2

The PARK2 encodes for the protein PARKIN, which causes a recessively inherited form of PD, by the loss-of-function mutations. PARKIN is a protein composed of two RING-FINGER domains separated by an in-between RING-FINGER domain, and it functions as an E3 ubiquitin-ligase, which conjugates ubiquitin moiety to proteins to target them for degradation by the ubiquitin-proteasome system (UPS) [33]. There have been various reports which also show that PARKIN plays a role in mitochondrial maintenance and induces the autophagy of damaged mitochondria (mitophagy) [34]–[36]. PARKIN mutations result in the disruption of its E3-ligase activity which impairs proteasome-mediated degradation of cellular toxic proteins that may cause neurotoxicity in the PD-relevant regions, especially the substantia nigra [37].

1.4.4 PINK1/PARK6

Mutations on the PARK6 locus in the phosphatase and tensin homolog (PTEN) – induced putative kinase (PINK1) gene is the second most common for recessive parkinsonism. PINK1 is a 581 amino-acid protein with ubiquitous expression in tissues and a relatively high expression in the brain. It is basically a serine/threonine kinase which is its major functional domain and contains a mitochondrial targeting motif and a carboxy terminal autoregulatory domain [38]. Studies suggest that PINK1 and PARKIN work together by interacting with each other and promotes the selective autophagy of depolarized mitochondria and maintenance of mitochondrial quality [39].

1.4.5 DJ-1/PARK7

DJ-1 is the third gene that is linked to autosomal recessive PD and is detected in the forms of point mutations and missense mutations but is still rather uncommon. DJ-1 is a part of a multiprotein complex that stabilizes the mRNA in the cells and serves as a sensor for oxidative stress [40]. The DJ-1 protein forms a homodimer under normal physiological conditions, and one of the mutations, L166P, shows that it has impaired folding and is unable to form a homodimer, instead heterodimerizes with the wild-type DJ-1 [41], [42]. This results in not only unstable proteins which are promptly degraded by proteasome hereby reducing the antioxidant activity in the cell, but the misfolding of these proteins leads to the cellular degradation systems being overwhelmed and generating neurotoxicity. The resulting overload on the degradation pathways of the cell results in abnormal subcellular localization of the protein, for instance, mitochondria [41].

In addition to the genes that are discussed above, several other genomic loci have been identified that have been linked to inherited PD. There are also some chromosomal loci identified as PD-causative, but the exact gene has not been identified. The identified genes have been majorly grouped as "PARK" genes, which are specific chromosomal locations associated with hereditary forms of PD [43]. It is worthy of note though, that mutations in some PARKs may cause only a minor characteristic of the whole complex phenotype of PD, or even may be an extremely rare occurrence in PD [44]. The PARK genes and other genes related to PD have been summarized in Table 1.1.

GENE	PROTEIN	ONSET	INHERITANCE	PREVALENCE
LRRK2/ PARK8	Dardarin	Mid to late onset	Autosomal Dominant	2-40% in different populations[45], [46]
SNCA/ PARK1/4	α - Synuclein	Early onset	Autosomal Dominant	~2.5% of sporadic cases; rather rare[47]
PINK1/ PARK6	PTEN-Induced Putative Kinase1	Early onset	Autosomal Recessive	1-9% across various populations[48]–[51]

PARKIN/ PARK2	Parkin-E3 Ubiquitin Ligase	Early/young onset	Autosomal Recessive	50% of familial cases[52]
DJ-1/ PARK7	DJ-1	Early onset	Autosomal Recessive	<1%; overall rare[53]
UCHL1/ PARK5	Ubiquitin C-terminal hydrolase L1	Early onset, late onset	Autosomal Dominant	Rare among some populations[54], [55]
ATP13A2/ PARK9	ATPase 13A2	Juvenile onset	Autosomal Recessive	~0.2-0.7% in studied populations[56], [57]
GIGYF2/ PARK11	GRB10 interacting GYF protein 2	Late onset	Autosomal Dominant	~5% in familial PD patients[58]
HTRA2/ PARK13	HtrA serine peptidase 2	Late onset	Autosomal Dominant	Extremely rare in particular populations[55], [59], [60]
PLA2G6/ PARK14	Phospholipase A2 group VI	Early onset	Autosomal Recessive	~1.6%; overall rare[56], [61], [62]
FBXO7/ PARK15	F-Box protein 7	Juvenile parkinsonism	Autosomal Recessive	~1.1%; very rare in different populations[56], [63], [64]
VPS35/ PARK17	VPS35 Retromer Complex Component	Late onset	Autosomal Dominant	Rare among various populations[65], [66]
EIF4G1/ PARK18	Eukaryotic translation initiation factor 4 gamma 1	Late onset	Autosomal Dominant	Neither a strong nor common risk factor among different populations[67]–[69]
DNAJC6/ PARK19	DnaJ heat shock protein family (Hsp40) member C6	Early onset	Autosomal Recessive	No study reported among patients
SYNJ1/ PARK20	Synaptojanin 1	Juvenile parkinsonism	Autosomal Recessive	Extremely rare; report in one family[70]
TMEM230/ PARK21	Transmembrane protein 230	Late onset	Autosomal Dominant	Extremely rare and uncommon among various

				populations[71]–[73]
CHCHD2/ PARK22	coiled-coil-helix-coiled-coil-helix domain containing 2	Late onset	Autosomal Dominant	Risk factor among Indian population, but not in other populations[74]–[76]
VPS13C/ PARK23	Vacuolar Protein Sorting 13 homolog C	Early onset	Autosomal Recessive	Identified as a non-risk factor among some populations[77], [78]
GBA	Glucocerebrosidase	Young to middle age onset	Autosomal Recessive	5% among populations[79], [80]

Table 1.1. Details of PD-related genes.

Currently, the proteins that are linked to PD by genetic studies are known to play a role in vesicle trafficking (α-synuclein), the UPS cellular degradation system (PARKIN, UCHL1), mitochondrial functions (PARKIN, PINK1, DJ-1), lysosomal functions (GBA, ATP13A2). All these cellular mechanisms have an overlap to them, ultimately leading to the demise of dopaminergic neurons and which leads to this complex phenotype of PD. Hence, it is important to understand the interactions of the proteins of causative genes, which can disclose the exact molecular mechanisms and the metabolic relationship between these systems which play a role in PD pathophysiology.

1.5 Spread of PD pathogenesis: beyond dopaminergic neurons

Post-mortem brains of PD patients show that α-synuclein lesions and Lewy Bodies (LBs) are not only present in the SNpc, but in other brain regions as well, including the central and peripheral nervous system [81]. This strongly promotes the notion that toxic α-syn in PD may self-oligomerize and undergo self-fibrilization and moreover gradually spread via cell-to-cell transmission to interconnected brain regions. Initially, Braak et al. [82] demonstrated the α-syn presence in distant brain regions like the coeruleus-subcoeruleus complex, the raphe nuclei. Lesions are initially observed in the anterior olfactory nucleus, after which gradually cortical areas and the brain stem is affected in ascending course, stating that PD progresses in six distinct stages. Although, this staging of PD was widely accepted for a long time and was even confirmed by other

groups [83], [84], it was still not accepted in some sporadic PD cases [85]. Moreover, this also does not explain the fact that PD patients' autopsy which have widespread α-syn pathology, still does not manifest clinical symptoms.

Soon after, a new hypothesis was explored by some groups which demonstrated that PD patients which contain a graft of embryonic mesencephalic neurons in their striatum, develop LBs after several years [86]. This suggests a mechanism of host-to-graft transmission of the LB pathology, which showed that α-syn could be released by living or dying cells and spread in a "prion-like" manner into the extracellular environment, and then would be uptaken by the grafted neurons by endocytosis and other pathways.

1.6 Intercellular communication in PD – exosomes are all the hype

In any physiological condition, intricate interactions between neuronal, microglial, and astrocytic cells, along with other dendritic cells and other kinds of cells in the brain are essential for the functioning of the CNS. The glial cell population in the brain (33-66% of brain cell mass) includes astrocytes, microglia, and oligodendrocytes. These serve as major support for neurons and play a role in supply of neurotransmitters, trauma protection, nourishing the blood brain barrier (BBB), pruning of synapses and removal of waste materials [87]–[89]. There needs to be interactive exchanges between different cells of the brain for maintaining neuronal integrity and competence [90]. These interactions may occur in terms of signalling through chemical and electrical synapses, mechanisms of retrograde and anterograde signalling, etc. For instance, neuroinflammation, which is an important pathophysiological feature of PD, is responsible for microglial cell activation, which in turn leads to production of free radicals, which damage many proteins within the dopaminergic neurons [91].

A large pool of studies have described that these exchanges between glia and neurons have been attributed to occur through extracellular vesicles (EVs) (including exosomes and microvesicles), thus playing an important role in neuronal functions, like synaptic strength and plasticity, and neuron survival. There is a myriad role of EVs in maintaining these roles for CNS (central nervous system) homeostasis and optimal functioning of the brain.

There are several convincing evidence for the functional role of EVs in the nervous system, including neuronal EVs, oligodendrocyte EVs, astrocytic EVs and microglia derived EVs. A report has demonstrated that exosomes released from cortical glutamatergic neurons are preferentially endocytosed by hippocampal neurons and these modify synaptic plasticity and mRNA trafficking [92]. Further, another report has shown that glutamatergic neurons produce α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-containing exosomes that can modulate the synaptic plasticity and strength by the receptor transfer to postsynaptic terminals [93]. One study had shown the role of glial cells in synaptic pruning, and how exosomes play a role in modulation of this activity. The study involved stimulation of neuronal differentiation of PC12 cells and induction of neuronal degeneration. Another in vitro model, a mouse microglial cell line (MG6) was co-cultured with PC12 showing the phagocytosis of PC12 neurites. The pre-incubation of MG6 cells with PC12 exosomes accelerated the removal of neurites from degenerating PC12 cells [94]. Astrocyte-derived EVs enhance neuronal survival and play a role in neurite outgrowth, which was evident by the study that showed ectosomes from cortical astrocytes contained pro-inflammatory cytokine, IL-1β, which acts as a potential neuromodulator [95]. Another study showed that Apolipoprotein D (ApoD) is transported from astrocytes to neurons via EVs and promotes their survival by reducing the free radical producing lipid peroxides [96], [97]. Astrocyte-derived EVs also contain Synapsin I, which promotes neurite outgrowth and enhances the survival of hippocampal neurons in mouse model [98]. Oligodendroglial exosomes are reported to improve neuronal survival when the neurons are placed under oxidative stress or nutrient deprivation [99]. Another study with the help of a transwell device demonstrated the exosome communication between cortical neurons and oligodendrocytes. On stimulation of glutamatergic activity in the cortical neurons, the oligodendrocytes release exosomes in a Ca^{2+} dependent manner which contain myelin proteins, oxidative stress inhibitors and proteolipid protein (PLP) [100].

Collectively, all the reports and examples display the vast potential of EV content in synaptic plasticity, oxidative stress protection, neuroprotection, neuronal electrical activity, neurite outgrowth and protection against various cellular stresses. This ability of exosomes to indulge in intercellular communication locally as well as systemically has increased the importance of these vesicles in pathogenesis of diseases, including neurodegenerative disorders. As discussed above, the concept of “prion-like” spread of α-syn in distant brain cells and regions, has led to the speculation that these misfolded proteins might be also moving within EVs [101]. The release of

α-synuclein is calcium-dependent and increases on dysfunction in lysosome acidification, hinting that a non-classical secretion method, the EVs, are involved in the release process [102], [103]. A-syn is reported to be involved in synaptic vesicular processing, and this cellular function of the protein makes it intrinsically linked to exosomes withing the exocytic machinery on the pre-synaptic terminals of neurons [104]. Given the abilities of exosomes in communicating with various cells at distant sites, they are being considered as the major performers in transporting pathological misfolded proteins, like α-synuclein in PD, and hereby spreading the disease throughout the brain.

1.7 Interorganellar crosstalk: role in PD

The overall neuronal homeostasis in the brain is heavily regulated by organelle dynamics and its functions. The maintenance of this organelle network dynamics in the cell is of vital importance in the etiology of various neurodegenerative conditions, including PD [105]. For instance, considering this inter-organelle communication, the contact sites formed between the endoplasmic reticulum (ER) and the mitochondria, occurring at mitochondria-associated membranes (MAMs), are emerging to be fundamental for a variety of functions, including mitochondrial metabolism and dynamics, Ca^{2+} homeostasis, autophagy, and apoptosis, which are altered under the pathological conditions of PD [106]. It has been reported that the ER-mitochondria tethering is modulated in PINK1 and PARKIN genetic forms of PD, which induces a plethora of dysregulated cellular processes leading to neuronal dysfunction and cell death [107], [108]. Similarly, neurons also dynamically form mitochondria-lysosome contact sites throughout the soma, axons, and dendrites, for maintaining homeostasis, and these sites are distinct from the mitophagy pathway. The mitochondria-lysosome contact tethering was found to be perturbed in the GBA1-PD patients which further led to mitochondrial distribution and functions. This contact dynamics was found to be rescued by overexpressing TBC1D15, suggesting the important role of interorganellar crosstalk and communication in neuronal biology of PD [109]. Whether the impaired mitochondria-lysosome contact sites contribute to disease progression or is one of the pathological processes downstream is not known.

A recent report has demonstrated that Rab7a late endosomes act as a platform for the translation of mRNAs encoding proteins for mitochondrial maintenance and functions [110]. Above various

reports may hint towards the regulation of mitochondria-lysosome contacts through Rab7 GTP hydrolysis in the endolysosomal system, but this communication between the organelles has not been well elucidated in PD. The Rab family of GTPases are only emerging to be major players in PD pathology (VPS35, CHMP2B, RAB25, PINK1, PARKIN among others) by controlling cellular functions like endocytosis, sorting, and vesicular trafficking [111]–[113]. PD has been identified as a multifactorial process, with primary contributors being the combination of organellar crosstalk between mitochondrial dysfunction, loss of network integrity, toxic protein accumulation, and the perturbed endo-lysosomal system for the cellular degradation of the toxic proteins [114]. The endo-lysosomal system dysfunction finely regulates the release of toxic proteins in the extracellular milieu making exosome release an integrated part of the PD pathology network [115]. While, these organelles contact sites play distinct roles in neurons and their subtypes, their contribution to modulation of non-neuronal cells like astrocytes and microglia is not well understood, and how the difference in modulation correlates to the exosome release from different cell types of brain and differences in their cargo and functions. The dynamics of the crosstalk of mitochondria with lysosomes, and involvement of lysosomes with autophagic clearance pathway is discussed more in detail, and how the interactions between these cellular organelles may modulate the exosome release in PD.

1.8 Mitochondrial dysfunction in Parkinson's Disease

Although having different etiologies, both sporadic and monogenic forms of PD share the same biochemical, clinical, and pathological features. One of the features is mitochondrial dysfunction, which is observed in both forms of PD. Alterations in any of the vital mitochondrial features can potentially lead to a disease state and has been linked to PD pathophysiology.

Mitochondrial complex I inhibition and oxidative stress: The main form of energy utilized by the cell is ATP, and mitochondria is the central producer of this energy source. It is the main site of oxidative phosphorylation (OXPHOS), which is coupled phosphorylation and redox reactions in the inner membrane. Strong evidence suggest that oxidative stress is generated intracellularly by mitochondria, the primary source of reactive oxygen species (ROS) [116]. Complex I and complex III are supposedly the major sites of ROS production in the mitochondria, and the primary ROS produced is the superoxide radical, which is produced as the result of a single transfer of

electron to oxygen in the respiratory chain. The production of superoxide relies on a variety of factors, and it is mostly produced when there is a low ATP production, or a high NADH/NAD^+ ratio [117]. Being the major producer of ROS, complex I leads the mechanism of death of dopaminergic neurons in PD. The earliest reports first discovered that the complex I activity is deficient in the substantia nigra of the post-mortem brains of PD patients [118]. Subsequently mild complex I deficiency was also observed in cortical brain tissue [119], striatum [120], and skeletal muscle [121] of PD patients. Toxins like MPTP, rotenone and paraquat were identified to be strong inhibitors of mitochondrial complex I and correlated to pathology of PD. Infusion of rotenone to rats was reported to produce dopaminergic death in the nigrostriatal pathway [122], and accidental exposure of drug abusers to MPTP showed parkinsonian symptoms [123]. Following the blockage of complex I, the ROS production dramatically increases which creates an oxidative stress in the cells, this was supported by the study that showed the increase in ROS production in brain mitochondria on exposure to MPP^+ and rotenone proportionally to the complex I inhibition [124]. Subsequently, all biological macromolecules are affected by the oxidative stress due to ROS. This was observed in PD patients' post-mortem brain samples where oxidative damage occurred to proteins, DNA, and lipids [125]. Many pathogenic mutations involved with familial PD are directly linked to mitochondrial dysfunctions, for instance, the autosomal recessive PD-related gene, DJ-1, can scavenge mitochondrial ROS, and the loss of this gene in mutant mice increases the mitochondrial ROS levels [126].

Mitochondrial DNA mutations: The mitochondrial genome (mtDNA) is a double-stranded, circular molecule of 16.6kb and has an independent replication from cell cycle or nuclear DNA replication. The mtDNA encodes 13 proteins, which are the subunits of the respiratory chain complex. Additionally, the mtDNA also encodes 22 tRNAs and 2 rRNAs that are important in mitochondrial protein synthesis [127]. In response to increased oxidative stress, the mtDNA integrity or the copy number of mtDNA may be affected in dopaminergic neurons. Once a particular threshold of ROS generation is crossed, the ROS may cause damage to the mtDNA which may initiate a cascade leading to apoptosis of the cells. The lack of proper DNA repair mechanisms and DNA histones makes the mtDNA even more prone to mutations when cells are exposed to ROS molecules [128]. Earlier studies have demonstrated that the incorporation of mtDNA from platelets of PD patients into cells lacking mtDNA shows complex I deficiency and Lewy body inclusions [129], [130]. These studies confirmed that mtDNA contains some

pathogenic mutations that contribute to PD pathogenesis. The mutations in mitochondrial POLG are associated with levodopa-responsive parkinsonism [131]. POLG is the enzyme that synthesizes and proofreads the mtDNA. The individual dopaminergic neurons dissected from the substantia nigra of postmortem brains from idiopathic PD patients showed mtDNA deletions [132]. Surprisingly, mtDNA deletions were not observed in the other neuronal cell types, which demonstrate that mtDNA deletions are specific to nigral neurons. Mitochondrial ROS may be responsible for the somatic mtDNA alterations in dopaminergic neurons. For instance, the increased ROS production in MPTP-injected mice results in oxidative damage occurring to striatal mtDNA [133]. Another study demonstrated that mice having a dysfunction in the Tfam gene, a mitochondrial transcription factor, in the dopaminergic neurons displayed reduced mitochondrial respiration and mtDNA expression and slowly progressive motor deficits associated with nigrostriatal degeneration [134]. It is still unclear whether mtDNA alterations are a primary or secondary event in PD, but mtDNA mutations primarily contribute to the pathogenesis of PD.

Mitochondria and calcium homeostasis: Intracellular calcium (Ca^{2+}) in neurons is the main secondary messenger to transmit the depolarization status and synaptic activity. The neuronal cytosolic concentration of Ca^{2+} is 10,000-fold lower than the extracellular Ca^{2+}, which increases the cytosolic Ca^{2+} dramatically post-depolarization and its regulation is vital in neurons [135]. To maintain the Ca^{2+} homeostasis, it is rapidly sequestered in mitochondria and the ER (endoplasmic reticulum) in neurons. The mitochondria have a fundamental ability to uptake, retain and release Ca^{2+}, uptake occurs through an electrogenic uniporter and efflux occurs through Na^{+}/Ca^{2+} and H^{+}/Ca^{2+} antiporters [136]. The mitochondrial Ca^{2+} accumulation results in activation of OXPHOS and ATP production which helps meet the metabolic demand of neurons for its electrical activity [135]. During normal synaptic activity, the cytosolic Ca^{2+} levels rise transiently and do not affect the neuronal function. The specialty of dopaminergic neurons in the substantia nigra, however, is that they generate slow action potentials consistently even in the absence of a synaptic signal. This large Ca^{2+}-buffering burden created by the dopaminergic neurons can compromise the respiratory chain, generate oxidative stress, and compromise ATP production [137]. A study further showed that isradipine, which is a L-type Ca^{2+} channel antagonist, was able to reverse the dendritic loss in ventral midbrain slices occurring due to rotenone, moreover, even reduced the nigral dopaminergic neurodegeneration in MPTP-injected mice [138], which showed that Ca^{2+} overload in adult dopaminergic neurons may make them vulnerable to PD. There are many additional studies which

show the implication of Ca^{2+} levels alterations in PD pathology, for instance, neurotoxins like rotenone and MPP^{+} show reduced Ca^{2+} uptake in mitochondria and amplified cytosolic levels of Ca^{2+} in cells in vitro [139], [140]. Furthermore, PINK1 regulates Ca^{2+} release from mitochondria via the Na^{+}/Ca^{2+} exchanger. The mutation of PINK1 in dopaminergic neurons in PD conditions leads to impairment in Ca^{2+} efflux from mitochondria and its accumulation, leading to increased ROS, decreased respiration overall leading to cell apoptosis [141].

1.9 Autophagy and lysosomal dysfunction in Parkinson's Disease

Neuronal cells in the brain are post-mitotic, which is why they are unable to dispose of the aggregated proteins via cell division. Hence, the survival and function of neurons is completely dependent on their ability to efficiently clear the excessive proteins for recycling them for metabolic processes or removal of toxic components hereby maintaining the cellular homeostasis. The process of 'self-consumption' of the excessive proteins and other components in the cell is termed autophagy [142]. The process of autophagy involves the sequestration of macromolecules or organelles into double-membraned structures, which are delivered to the lysosomes of the cell to be degraded and generate the raw materials like proteins, lipids, carbohydrates, and nucleic acids to be reused by the cell, hereby removing toxicity from the cell. This process in cells is usually generated in response to either cellular stress, like starvation or oxidative stress, or in some cases as a form of apoptosis [143]. Due to the high metabolic demand of neurons, they exhibit high basal autophagy and any deficit in this process can tip the balance between cell viability and death. One of the most characteristic hallmarks of PD, along with other neurological disorders, is the lack of efficient protein turnover, which is manifested in the form of PD associated Lewy bodies. The accumulation of these oligomeric misfolded peptides is proof of inefficient autophagy.

The final stage of autophagy is the degradation of the macromolecules or organelles through the lysosome, and the cell relies on lysosome to sufficiently remove or breakdown the toxic components for recycling and maintenance of cellular fitness. If the lysosome fails to degrade the macromolecules, abnormal accumulation of the waste material may occur within lysosomes which also leads to lysosome and overall cellular dysfunction. Lysosomes are the destination where autophagic vesicles and extracellular molecules are both delivered to be finally degraded, and the dysfunction in lysosome pathway has also been involved in the etiology of PD [144].

Autophagy in PD pathogenesis: The neurons perform three different forms of autophagy: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy, all of which are implicated in PD. The process of autophagy is a highly conserved and sequential process, including the initiation, elongation of autophagosome membrane, the entrapment of recognized cargo within the autophagosome, and finally the fusion of autophagosome with lysosome and cargo degradation. There are many mechanisms and molecules involved in the key steps of autophagy that may be associated with either the sporadic or genetic form of PD.

ATG5 and ATG7 are two genes that are responsible for the membrane elongation in autophagosome formation, and the knockout mice for these genes, although remain viable into adulthood, but have significantly reduced autophagy and exhibit motor dysfunctions and neurodegeneration [145], [146]. Since α-synuclein turnover plays a vital role in development of PD, the wild-type α-synuclein is mostly degraded through macroautophagy [147]. Reports also suggest some degradation of α-synuclein through the ubiquitin-proteasome system (UPS), but that is not suggested to be the main mechanism, since blocking of UPS does not lead to accumulation of α-synuclein, but blocking of CMA does [148]. In case of overexpression of the mutant form of α-synuclein, a cellular autophagy checkpoint molecule, HMGB1 is inhibited, which ultimately reduces the Beclin-1 levels leading to impairment in macroautophagy in PC12 cells [149]. Moreover, analysis of post-mortem nigral samples from PD patients also display decreased levels of CMA markers [150]. The PD-associated gene, LRRK2, is also a CMA-substrate, and mutations in the protein render it a poor binding substrate which obstructs the CMA translocation complex [151]. The skin fibroblasts derived from PD patients containing the G2019S mutation in LRRK2 gene also are found to contain a greater number of autophagic vesicles than that of healthy control skin fibroblasts [152]. The above-mentioned studies indicated that clearance of toxic components was dependent on the autophagy machinery, and dysregulation in this pathway caused due to the genetic mutations related to familial PD, led to the accumulation of α-synuclein hereby contributing to PD pathogenesis.

Apart from mutant genes, PD-relevant neurotoxins contributing to sporadic PD have also demonstrated a role in the autophagy and its alteration leading to neuronal cell death. For instance, mTOR-dependent autophagic machinery enhanced on exposure to 1-methyl-4-phenylpyridinium (MPP+) or induction of dopamine toxicity-induced oxidative stress [153], [154]. Moreover, mild

exposure of MPP+ in neuronal cell line SH-SY5Y inhibited the autophagosome degradation by reducing the cathepsin D activity in the lysosome without altering its acidity. These changes are reversed by the lysosome biogenesis enhancers like trehalose and rapamycin [155].

Lysosomal dysfunction in PD: The last stage of autophagic machinery is the degradation of the macromolecules in the lysosomal compartment of the cells, which are the terminal compartments in this pathway. Lysosomes house various hydrolytic enzymes delivered from the Golgi bodies and contain membrane-based hydrogen pumps (vATPases) which acidify the lumen of the lysosomes. There are several aspects of the functions of the lysosome that can be associated with PD synucleopathies. As mentioned before, the main mechanism of α-synuclein degradation is through CMA, the α-synuclein comprises of a CMA-motif that is recognized by HSC70 chaperone, which then recruits the formed complex to the lysosome, where it interacts with the CMA receptor LAMP-2A, which then leads to α-synuclein being translocated to lysosomal lumen for degradation [147]. However, the A30P and A53T mutated forms of α-synuclein in PD are resistant to degradation by CMA, because these mutant forms can strongly bind to LAMP-2A on the lysosomal membrane, however, are not internalized, hereby not being degraded itself, but as a result, also blocking the degradation of other CMA-dependent substrates [156].

Various genetic studies related to familial PD suggest the involvement of lysosomal dysfunction in PD pathogenesis. For example, rare variants in genes related to lysosomal function, including GBA, LAMP1, ATP13A2, TMEM175, etc. [157]. The GBA gene encodes for the lysosomal enzyme β-glucocerebrosidase (GCase) which breaks down the glucosylceramide to glucose and ceramide. GBA mutations are a very common genetic risk factor in PD, where nigral dopamine neuronal loss is observed along with presence of Lewy bodies and neurites. It manifests an earlier onset, but a greater mortality risk owing to increased cognitive decline and motor symptoms [158]. The mutations in GBA gene result in the disruption of α-synuclein function and induces its aggregation [159]. The resultant accumulation of α-synuclein itself then inhibits GBA function, creating a feed-forward loop of lysosome dysfunction promotion and further accumulation of α-synuclein. The brain regions of PD patients, including substantia nigra, amygdala, striatum and cerebellum are found to be deficient in GCase, irrespective of whether they contain GBA1 mutations, and are shown to have abnormal accumulation of α-synuclein [160], [161]. A recent study has also described that the stem cell derived neuronal PD model consisting of GBA mutation

causes a decline in the cathepsin D levels in the lysosome hereby increasing monomeric α-synuclein levels [162]. Other genes that are relevant to lysosomal functions are ATP13A2, which is a transmembrane ATPase pump on the lysosomes, the levels of which are altered in the dopaminergic nigral neurons of PD patients [163]. Additionally, PARKIN mutations in PD patients display a higher level of lysosomal compartments in the fibroblast cultures, but decreased levels of lysosome function markers like RAB7A, resulting in decreased proteolytic activity [164].

All the studies indicate that the autophagic machinery and lysosomal functions largely contribute to the selective cargo degradation in neuronal cells hereby influencing neuronal health. The mitochondrial turnover through mitophagy is a quality-control mechanism which is responsible for maintaining a healthy mitochondria pool in the neurons, however the implication of dysfunctional mitochondria and its cross talk with different organelle in spread of PD pathogenesis is not clear and needs to be further studied. Owing to the autophagy-lysosomal dysfunction in PD, mitophagy has also been strongly implicated in many neurodegenerative disorders, including PD.

1.10 Mitochondrial turnover – crosstalk with lysosomal functions

A healthy mitochondrial pool and its bioenergetic function is maintained by the balance between biogenesis and mitophagy (Fig 1.1). Any disruption in any one of the pathways may lead to mitochondrial dysfunction leading to pathophysiology of PD. By using the core autophagic machinery, the sequestration of damaged or targeted mitochondria into double-membraned autophagosomes is termed as mitophagy. Subsequently, the autophagosomes fuse with lysosomes where the degradation takes place [165]. Mitophagy is triggered by factors like loss of mitochondrial membrane potential or permeabilization of outer mitochondrial membrane, which may be an attempt to combat the effects of excessive ROS production by mitochondria or the release of mitochondrial pro-apoptotic factors [166]. The vital role of mitophagy in PD pathogenesis is evident from the prevalence of PINK1 and PARKIN induced familial PD. Both these genes normally work together in the same pathway to govern mitochondrial quality control, which solidifies the evidence that mitochondrial damage is vital to PD pathophysiology. When mitochondria are damaged, PINK1 accumulates to its outer membrane, and recruits PARKIN to the dysfunctional mitochondria and activates its E3 ubiquitin ligase activity. Further, PARKIN

ubiquitinates outer mitochondrial membrane proteins like VDAC1 and/or MFN to induce mitochondrial aggregation.

Further, p62 and HDAC6 link the polyubiquitinated mitochondria with LC3, hereby initiating autophagy [167]. Recent genetic studies in *Drosophila* have also shown that PINK1 and PARKIN promotes mitochondrial fission and inhibits fusion. Over-expression of Drp1 or loss of Mfn2/Opa1 can rescue the PD phenotype in PINK1 or PARKIN mutant flies, including flight muscle degeneration, defects in climbing and abnormal wing posture [168]. There is a clear genetic interaction between the PINK1/PARKIN signalling pathway and mitochondrial fidelity and dynamics. There is a specific role of PARKIN in the turnover of mitochondria through mitophagy. PARKIN is specifically recruited to mitochondria which has a low membrane potential, and then these mitochondria are subsequently destroyed through the autophagosome [169], hence PARKIN is like a sensor for mitochondrial integrity. The loss of PINK1 on the other hand, induces mitochondrial fragmentation and accumulation of mitochondrial-autophagosomes in SH-SY5Y neuroblastoma cells [170]. Impaired mitophagy is a hallmark of PD, in fact experimental PD models and post-mortem PD brain samples show the accumulation of dysfunctional mitochondria in the cytosol of insulted neurons [171], [172]. In fact, PD patients containing the PINK1 and/or PARKIN mutations manifest a loss of dopaminergic neurons in the substantia nigra pars compacta region, and the induced pluripotent stem cells (iPSC)-derived dopaminergic neurons derived from these patients show enlarged mitochondria [173].

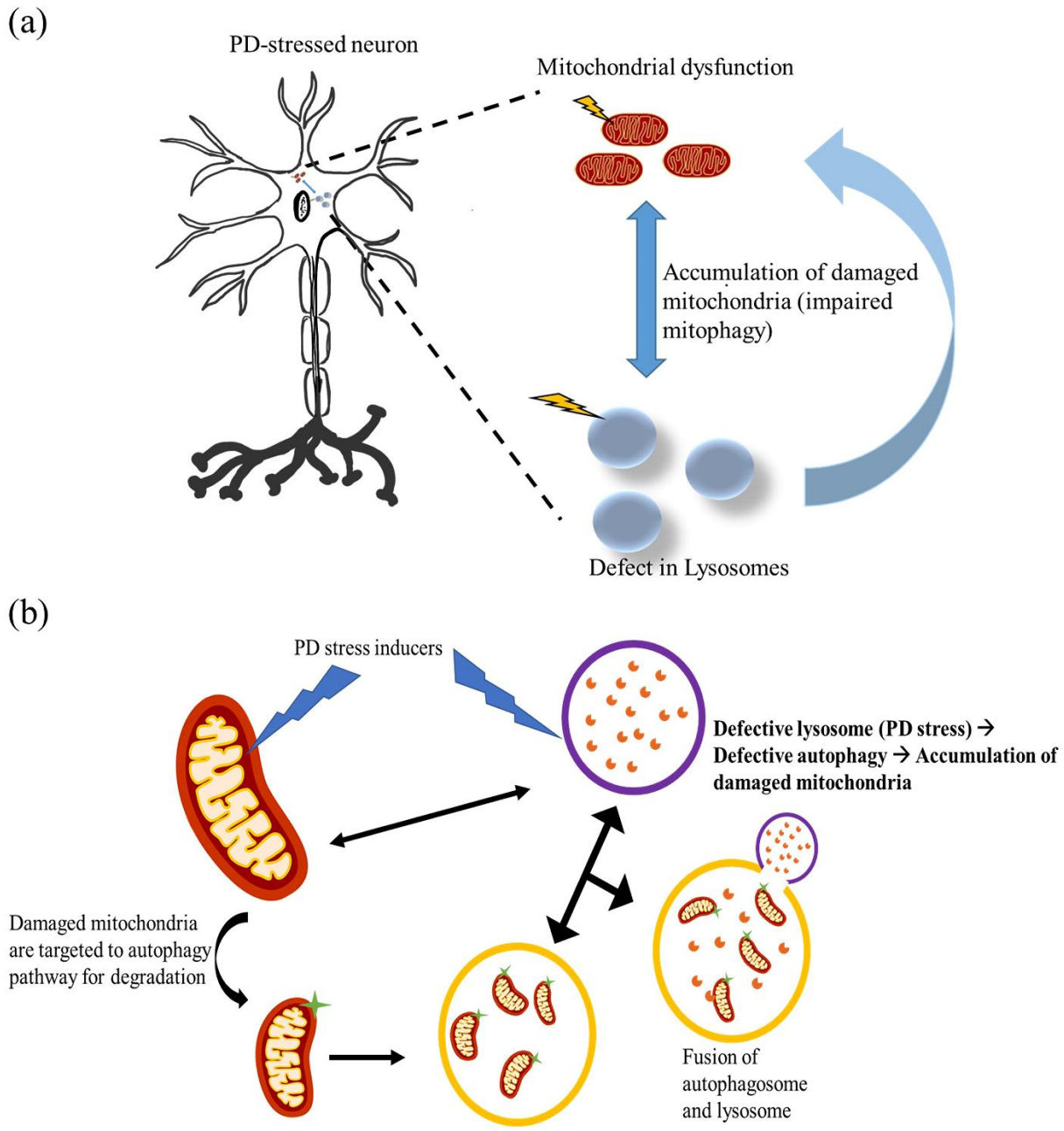

Figure 1.1. Interorganellar crosstalk of mitochondria and lysosome in PD stress conditions. (a) The functions of mitochondria and lysosome are a result of coordination of signalling and cellular metabolism which is vital for neuronal homeostasis. Mitochondrial perturbations in PD affect the lysosomal function since the mutations in mitochondrial genes are often associated with impaired lysosomal or autophagic functions. Similarly, when lysosomes and its biogenesis is defective in PD conditions, the mitochondrial biogenesis is also repressed since the cell signals that the ultimate destination of damaged mitochondria (the lysosomes taking part in mitophagy) are dysfunctional, hence the cell decides to halt the process. (b) The turnover of dysfunctional or depolarized mitochondria through selective process of autophagy called mitophagy is important for healthy organellar function and cell survival.

Mitophagy is the cellular process that links the lysosomes and mitochondria which overall state about the physiological relevance of interorganellar crosstalk in cells. Deficiency in the mitochondrial respiration in PD impairs the formation of autophagosomes and the autophagy flux. And similarly, deficiency in the autophagy flux results in accumulation of depolarized and damaged mitochondria which the cell is unable to clear.

The accumulation of this damaged mitochondria can contribute to apoptosis by enhanced ROS and oxidative stress, and this defective autophagy may also be partly due to a reduction in the number of functional lysosomes. This turns into a vicious cycle where increased ROS from dysfunctional mitochondria leads to oxidative damage to lysosomal membrane, in other words defective autophagy, and in turn this defect further accumulates damaged mitochondria which cannot be degraded efficiently, and the restoration effects of this are observed by rapamycin in MPTP-intoxicated mice [171].

There are other studies which strongly support the mitochondria-lysosomal crosstalk in PD progression. PARK2-PD fibroblasts are associated with mitochondrial damage [164]. Again, familial PD genes, PINK1 induces lysosomal biogenesis, and it is largely dependent on TFEB (transcription factor EB), which is a master regulator of lysosome biogenesis. Further results indicate that PARKIN Q311X mutation can alter the mitochondria quality through the regulation of PGC-1α-TFEB pathway, which is independently restored by rapamycin, rendering it vital for mitochondria-lysosomal homeostasis [174]. More recently, dynamic formation of interorganellar mitochondria-lysosome contact sites have been identified, which are different from the mitophagy sites. The site formation is promoted by active RAB7 lysosomal protein, and these sites allow the regulation of mitochondrial networks by lysosome [175]. This explains a lot about the regulation of dynamics of both organelles and explains why the dysfunction in one organelle affects the other in various human diseases.

1.11 Endolysosomal system: two interlinked pathways

The endolysosomal system of the cell is a complex pathway that consists of a series of membranous organelles that work in sync to maintain the cell homeostasis and responds to stress conditions in pathological conditions. The endolysosomal pathway consists majorly of two component

pathways: exosome biogenesis and release and the autophagy-lysosomal pathway. The two components of this major pathway are intricately linked to each other. There is a coordinated response of the cell according to its physiological state, whether the release of cellular cargo should take place, or lysosomal degradation of the cargo [176] to maintain the overall cellular fitness. Autophagy and exosome biogenesis are cross related because of their shared molecular machinery and organelles, which has important implications in normal physiological state as well as disease states.

1.12 Crosstalk of autophagy and endolysosomal pathways determine the exosome release

The function of autophagy as a degradative pathway is essential for a variety of human diseases, including cancer, microbial infection, and neurodegeneration [177]. The pathway prevents the accumulation of toxic proteins like huntingtin, tau and α-synuclein, which is important in many neurodegenerative diseases. MVB (Multivesicular bodies) production and ESCRT machinery is also involved in neurodegenerative disorders. It has been reported that the dysfunction in ESCRT-III components, by the expression of the mutant CHMP2B protein, resulted in accumulation of autophagosomes in primary cortical neurons [178]. Mutations in CHMP2B are known to be associated with fronto-temporal dementia (FTD3) and amyotrophic lateral sclerosis (ALS) [179]. ESCRT-deficient cells inhibited the autolysosome formation through p62 and Alfy aggregation. Moreover, TDP-43 (major ubiquitinated protein in ALS) clearance requires functional MVBs [180]. These studies clearly suggest that functional MVBs are vital for clearance of abnormal and toxic aggregates of proteins which may ultimately affect neural function leading to neurodegeneration. However, the other pathway that functional MVBs may undergo is the loading and secretion of these harmful aggregates into exosomes which may have a deleterious effect on the bystander cells (Fig 1.2). The exosomes are responsible for spreading of various toxic aggregates related to neurodegenerative disorders like Alzheimer's, Huntington's, and Parkinson's, and toxic aggregates of tau, α-synuclein, TDP-43, etc. have been found in exosomes [181]. This suggests that the autophagic clearance of stress molecules is vital for the health of the cell, and any defect in clearance of protein aggregates may lead to secretion through exosome and may spread to neighboring neurons. Some studies have also shown that the exosome-mediated

transfer of α-synuclein is a mechanism to get rid of the toxic oligomers when the autophagy is hindered [182].

The autophagy machinery is a complex pathway that consists of a set of autophagy related genes (ATGs) and some other accessory proteins that work in sync to degrade cytosolic proteins and organelles by trapping them between double-membraned vesicles and fusing them with the lysosome for degradation. Several candidate proteins of autophagy pathway also play a role in exosome biogenesis suggesting intricate crosstalk. Loss of ATG9 in *Drosophila* altered the autophagy flux and reduced the ILVs, however if ILVs were released as exosomes remained unknown [183]. ATG5 has been shown to dissociate the vacuolar proton pump, which prevents acidification of MVB lumen and reduces its fusion to lysosome, hence increasing the exosome release. Knockout of both ATG5 and ATG16L1 reduces the exosome release and lowers the quantity of LC3B in exosomes [184]. The same study showed that ATG7 did not have any effect on the exosome biogenesis, which means that LC3 lipidation or formation of autophagosomes is not necessary. A previous study similarly reported that the complex ATG12-ATG3 interacts with ALIX, a protein necessary for exosome biogenesis, and regulates exosome release [185]. ALIX knockdown also reduced the basal autophagic flux, which describes the reciprocal regulation of both autophagy and exosome pathways.

Conversely, the failure of exosome release pathway can also lead to redirection of MVBs to lysosome for degradation. The ISGylation of TSG101, and ESCRT accessory protein dramatically reduced the exosome release by promoting protein aggregation and degradation [186]. CD63 knockout increased the autophagic clearance of endocytic compartments, and the exosome release was rescued partially when autophagy was blocked [187].

Neuronal cells often utilize both the pathways to eliminate protein aggregates and reduce proteotoxicity in the cells. In this case, SNCA has been well studied because it is relevant to neurodegeneration in PD and cell-to-cell transmission of α-synuclein is reported which is believed to propagate neurodegeneration [182]. Conversely, when autophagy is inhibited with the help of bafilomycin, it increases the secretion of smaller oligomers which ultimately causes cellular damage, particularly, the highly aggregated α-synuclein is secreted by membrane shedding and low-aggregated molecules are released by exosomes [188]. Loss of the ATPase ion pump ATP13A2 is known to suppress autophagy and cause lysosomal impairment, hereby leading to

accumulation of α-synuclein [189]. The overexpression of ATP13A2 alleviates these effects and lowers levels of α-synuclein, but on the other hand it is associated with autophagosomes and MVBs, which enhances the externalization of α-synuclein through exosomes. In PD models overexpressing SNCA, the inhibition of lysosomal activity by Bafilomycin A1 increases the levels of α-synuclein in the exosomes [190]. Recently it has also been shown that the autophagy-lysosome pathway, not only enhances SNCA in the exosomes and is uptaken by neighboring neurons, but also changes the biochemical profile of EV, and the SNCA is shown to be transferred in an autophagosome-exosome-like vesicle [191]. Exosome release here may be a cellular mechanism to alleviate the proteotoxic stress, but unfortunately, this behaviour might be responsible for further propagation of the disease when bystander neurons internalize these exosomes (Fig 1.2).

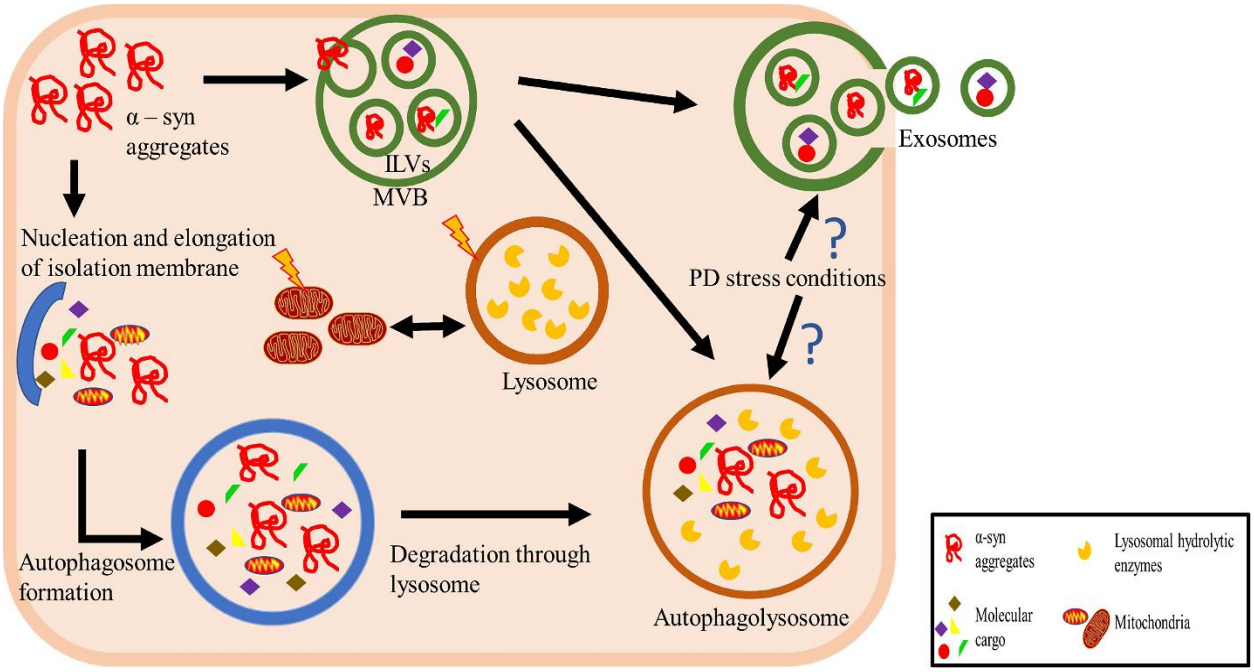

Figure 1.2. An overview of endolysosomal pathway, autophagy, and exosome release pathway in Parkinson's Disease. The packaging of toxic protein aggregates are tagged, recognized, and sequestered in MVBs in form of ILVs. When MVB fuses with the plasma membrane, the ILVs are released in the extracellular milieu in the form of exosomes. On the other hand, protein aggregates can also be sequestered in membrane elongation which form autophagosomes. Autophagosomes contain dysfunctional organelles, molecules tagged for degradation (ubiquitination), and toxic aggregates (α-syn) and other excessive cytoplasmic proteins. Autophagosomes or MVBs both subsequently fuse with the lysosomes to degrade all the encapsulated contents. Lysosomal or autophagic

dysfunction, which is a hallmark of PD, MVBs may release ILVs containing toxic aggregates in the form of exosomes, with the intention of maintaining proteostasis in the cells, but ultimately propagating the toxic aggregates as "seeds" to the bystander cells.

1.13 Exosomes – biogenesis

1.13.1 Canonical pathway

The biogenesis of exosomes is intricately connected with the endolysosomal system of the cell. It begins with the formation of early endosomes, which mature into late endosomes or MVBs. In later stages, the endosomal membrane of MVBs invaginates or buds inwards to form ILVs (intra-luminal vesicles) in the lumen of the MVBs, which are later released in the extracellular milieu as exosomes [192].

The canonical pathway of exosome biogenesis and release involves ESCRT (Endosomal Sorting Complexes Required for Transport) pathway which is the major pathway for exosome biogenesis and release. The components of this pathway work sequentially in an orderly manner to sequestrate the ubiquitinated proteins into the MVBs, and further facilitate the inward budding of the membrane to form ILVs [193] (Fig 1.3). The ESCRT pathway consists of four protein complexes: ESCRT-0, -I, -II, and -III, and some additional accessory proteins, for the whole process of membrane budding and scission to form ILVs and following disassembly of the whole complex. The role of ESCRT-0 is to cluster the ubiquitylated cargo and bind to the phosphatidylinositol-3-phosphate rich membrane regions of the MVB. ESCRT-I and -II are directly implicated in membrane budding and together support the budding process. ESCRT-I is a heterotetramer consisting of the following single subunits: Vps23, Vps28, Vps37 and Mvb12 [194]. The Vps23/TSG101 binds the ubiquitin and the ESCRT-0 in the MVB pathway [195], while the C-terminal helical domain of Vps28 functions in binding to the ESCRT-II components [196]. ALIX/PDCD6IP, which is an accessory protein in the ESCRT pathway, is known to interact with TSG101, and participates in the budding and abscission process [197]. ESCRT-II is also a constitutively assembled heterotetramer of one molecule of Vps22, Vps36 and two subunits of Vps25. The C-terminals of Vps25 bind, recruit, and activate the ESCRT-III component, Vps20 [198]. When the two interacting motifs of both ESCRT-I and -II are docked on the membrane-binding site, a supercomplex is formed and the bud formation is initiated. Both the ESCRT

complexes co-localize to the neck of the bud and seem to act by stabilizing the bud neck [196]. The ESCRT-III is a dynamic polymer and functions in catalysis of membrane neck scission [199]. Coupling of ESCRT-II to Vps20 is essential for MVB biogenesis, and the other subunit Snf7 is required for severing [198]. Vps2 subunit recruits the Vps4, an AAA+ ATPase, which plays a role in the disassembly of ESCRT-III and recycling of its components [200]. The accessory protein ALIX recruits the Snf7 for scission [201]. Reports have demonstrated that overexpression of syntenin increases the ALIX-dependent release of exosomes, while the depletion of syntenin, syndecan or ALIX impaired the exosome release [202]. While in another report, the silencing of ALIX seemed to change the protein composition of exosomes rather than its secretion, which was indicative of the fact that ALIX could affect cargo loading and/or the subtypes of MVBs which are then heterogenous in nature [203].

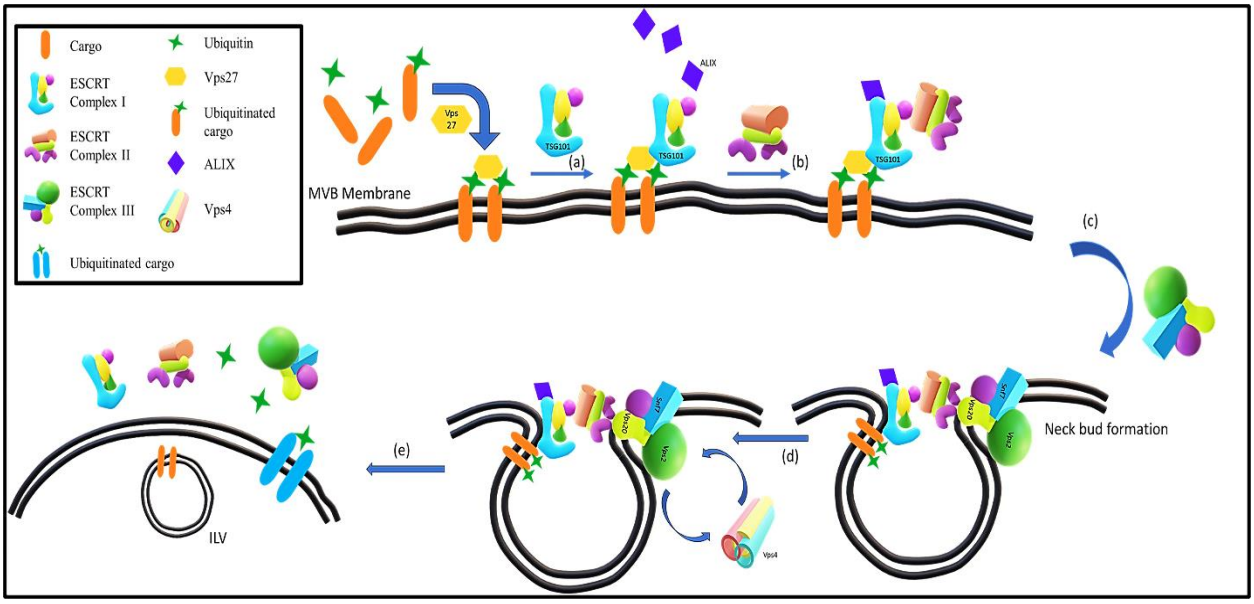

Figure 1.3. ESCRT-dependent exosome biogenesis pathway. The ESCRT-0/Vps27 first initiates the pathway by recognizing ubiquitinated cargo and binding it to the MVB membrane. (a) ESCRT-I is recruited by Vps27 and binds to the TSG101 component of ESCRT-I and ubiquitin. (b) ESCRT-II is recruited, and both the complexes collectively interact with each other to create a bud formation by invaginating the endosomal membrane, with the help of the accessory protein ALIX. (c) ESCRT-III is assembled to the neck of the bud, forming a stable complex which stabilizes the neck bud formation, and Snf7 severs the neck constriction. (d) Vps4 complex then helps to decompose all the subunits of ESCRT-III while the neck scission is completed. (e) All of the ESCRT subunits are detached and undocked from the membrane and ILV formation takes place inside the MVB holding the cargo.

1.13.2 Non-canonical pathway

Even when all the subunits of ESCRT pathway are silenced simultaneously, the exosome release still takes place, which means there are other pathways contributing to the release of exosomes, which are ESCRT-independent [204]. For instance, the ability of lipids to organize in specific membrane domains may play a role in vesicular transport and serve as platform for cell membrane organization, including the MVB membrane. One of the enzymes, nSMase, neutral sphingomyelinase, hydrolyzes sphingomyelin to ceramide, and the inhibition of this enzyme reduces the proteolipid protein (PLP) content in the released exosomes in oligodendroglial cells [205]. Hence, sorting of PLP in exosomes does not occur through ESCRT pathway, but depends on the synthesis of ceramides. However, the ceramide-dependent pathway of exosome release cannot be considered a general one because in some cells, like PC-3 cells, exosome release remained unchanged even on nSNMase inhibition or inhibition of ceramide synthesis [206]. Another lipid involved in exosome biogenesis was phospholipase D, which is enriched on exosomes and its activity was required for exosome release in RBL-2H3 cells [207]. Lysobisphosphatidic acid (LBPA) has been demonstrated to control the formation of ILVs both *in vitro* and *in vivo* through ALIX recruitment [208].

A study performed in human melanoma cells proved that depletion of nSNMase did not impair the MVB biogenesis process [209]. Instead, a CD-63 dependent mechanism existed, which was responsible for the sorting of luminal domain of pigment cell-specific protein 17 into the ILVs during melanogenesis. This study demonstrated that tetraspanins, which are transmembrane proteins, can be responsible for ESCRT-independent exosome release. Another supportive study for tetraspanin-dependent exosome release was shown in HEK293 cells where the packaging of β-catenin in exosomes was dependent on CD9 or CD82 [210]. The same study also demonstrated that bone marrow dendritic cells from CD9-knockout mice secreted less amount of flotillin-1 in exosomes.

1.14 Exosome biogenesis machinery: implications in PD

There are multiple reports which demonstrate the role of ESCRT machinery and its proteins in PD. One study validated that α-synuclein is found in the endosomal compartments of the cell.

Moreover, knockdown of Vps4 not only disrupted the MVB biogenesis, but inhibited the lysosomal targeting of α-synuclein, hereby leading it into the extracellular milieu [211]. The role of the MVB pathway has also been studied in the cell-to-cell transmission of α-syn; extracellular α-syn is targeted to the endolysosomal system by the ESCRT pathway where it interacts with CHMP2B and degrades it, this was validated in patients as well as α-syn transgenic mice. The resulting degradation of CHMP4B creates a roadblock for the endocytosed α-syn, which does not get degraded and leads to interneuronal propagation [212].

nSMase2 is sensitive to oxidative stress, and hypoxia conditions reduced the levels of nSMase2, but did not alter its enzymatic activity. The study for the first time demonstrated that nSMase2 reduced the transfer of oligomeric α-syn between neurons by altering the sphingolipids [213]. Another study positively validated in Thy1-αsyn PD mouse models that the inhibition of nSMase2 by acute exposure of the inhibitor reduced the EV biogenesis and brain exosome content, whereas a chronic dose of the inhibitor even reduced the α-syn content in the exosomes released which improved the motor functions [214]. These studies offered nSMase2 inhibition as a novel approach to therapeutic development for PD.

Exosome biogenesis may be broadly categorized as ESCRT machinery dependent or independent, but both pathways exist synergistically in any cell, and moreover the pathways are even related. While the ESCRT machinery exists for formation of ILVs in the MVBs and selective recruitment of ubiquitinated cargo for lysosomal degradation [215], [216], the tetraspanins segregate specific membrane domains through interaction with other proteins and lipids. It is not clear why distinct mechanisms for exosome biogenesis exist, but there is a possibility that the heterogenous subpopulations of exosomes depend on these different mechanisms. While most cargo is packaged in the exosomes using any pathway, some cargo needs a specific pathway for enrichment in exosomes [217]. Moreover, the use of the pathways may also be dependent on the cell type and the physiology or homeostasis of the cell.

1.15 Exosomal cargo and sorting

Exosomes are packaged with a unique content of proteins, lipids and nucleic acids that differ according to the cytoplasmic composition of the parent cell from which it has been derived. The

abundance of these biomolecules within exosomes varies depending on factors such as tissue origin, cell type, and pathological conditions, leading to population heterogeneity and exerting unique effects on recipient cells [218]. Extensive research and high throughput analysis has identified the primary constituents of exosomes which can be categorized broadly as membrane proteins, molecular cargo and a composition of lipids [219]. As for membrane proteins, the most frequent occurring exosomal proteins are tetraspanins like CD63, CD9, CD81, CD82, CD45, CD11b and TSG101 (ESCRT-associated protein), glycoproteins such as, LAMP-1 and 2B, annexins (membrane-binding proteins), Rab proteins, and hsp60, 90 and 79 (heat shock proteins) [219]. Additionally, there are cytoskeletal, metabolic, albumin and various signalling and carrier proteins encapsulated within the exosomes. The vesicles also carry enzymatic proteins, like peroxidases, enolase, lipid kinases, etc., basically constituting all protein content that can be found in the cytosol, or endocytic vesicles of the parent cell [220]. The molecular cargo of exosomes constitutes the DNA, mRNA, miRNAs, and other small non-coding RNAs that are packaged inside the exosomes which create an enormous signalling diversity. The nucleic acids can modify target gene expression ultimately altering the biological function of the recipient cells [221]. Apparently, there are also selective mechanisms existing for the specific sorting of RNA into exosomes, depending on the presence of specific RBPs (RNA-binding proteins) or sequence motifs [221]. Although the precise molecular mechanisms governing the selective enrichment of exosomal cargo and its modulation under pathological conditions remain incompletely understood, there are several bioinformatics databases which provide valuable insights into the differential cargo composition of EVs/exosomes, including Vesiclepedia 2019, exoRBase, Mirandola, ExoBCD, EVmiRNA, Expedia and ExoCarta [222].

Exosomal nucleic acid sorting: Numerous studies have successfully validated the presence of DNA and RNA in exosomes derived from cells of various origins. The composition of nucleic acids within exosomes is greatly influenced by cellular homeostasis and exhibits significant alterations under different physiological as well as pathological conditions [223]–[225]. Through next-generation sequencing, researchers have identified diverse RNA species within exosomes, including mRNAs, long non-coding RNAs, tRNAs, rRNAs, miRNAs, small nuclear and nucleolar RNAs (snRNA and snoRNA), vault RNA, Y-RNAs, etc. [226]. Moreover, recent studies have

established the presence of mitochondrial DNA within exosomes [225], [227], [228]. The below described findings have shed light on the selective enrichment of specific classes of DNA/RNA within exosomes, pointing towards their emerging functional significance.

Sorting of mRNAs inside the exosomes is predominantly regulated by specific RBPs. There are some unique motif sequences and associated factors that govern this process and are responsible for packaging of different mRNAs inside the exosomes. For instance, an earlier study showed that glioblastoma-derived exosomal mRNAs contain a 25-nucleotide long zip code sequence at the 3'UTR end that serve as sorting signals for packaging into extracellular vesicles or microvesicles [229]. Similarly, a study done in-vitro demonstrated the presence of distinct structural motifs, namely CAGUGAGC, UAAUCCCA and ACCAGCCU at the 3'UTR of mRNA populations that are recognized by RBPs like RNA cytosine C5-methyltransferase (NSUN2) or cytosolic Y-Box protein (YB-1), leading to their selective enrichment inside exosomes [230]. A structural motif in the 3'UTR stem-loop of the exosomal RNA was recognized that interacts with YB-1 to promote the exosomal sorting. Recent bioinformatics sequence analysis suggests the potential involvement of connexins in facilitating the sorting of nucleic acids into exosomes since these proteins have a nucleic acid binding domain in their structure. Connexins are integral membrane proteins that facilitate cell-to-cell communication via gap junctions and hemichannel mediated communication with the extracellular milieu. The most widespread connexin, Connexin43 (Cx43) has been found to aid in the transfer of exosomal cargo [231]. The mechanisms of RBP-identifying unique mRNA motifs for exosomal sorting is just beginning to gain attention, and many other factors responsible for the specificity of mRNA recognition still need to be identified.

miRNAs are a class of non-coding RNAs, typically around 22-25 nucleotides in length, which regulate gene expression by targeting specific mRNA sequences through a 7-8 nucleotide seed sequence at their 5' end [232]. While exosomes of different origin continue to maintain a diverse and distinct miRNA population, the underlying mechanisms governing their selective sorting remains poorly understood. Like mRNAs, the sorting of miRNAs in exosomes is also chiefly regulated by specific RBPs (Fig 1.4). A major study showed that the SUMOylated form of a RBP, heterogenous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), binds to a unique motif, the EXO motif, present on the 5' seed region of miRNAs, which plays a crucial role in exosomal miRNA sorting in human primary T-lymphocytes [233]. Similarly, there are other RBPs, for instance, the

RBP, hnRNPU, was found to regulate the sorting of miR-30c-5p into extracellular vesicles, on recognition of a sequence motif, AAMRUGCU [234]. RBPs can also negatively regulate exosomal sorting of miRNAs, which was demonstrated in the exosomal transport of miR-503. miR-503 was shown to interact with hnRNPA2B1, and this interaction is disrupted by a chemotherapeutic drug, which then facilitates the transfer of this miRNA into the exosomes [235]. Similarly, another RBP, SYNCRIP, was found to recognize yet another distinct motif (hEXO motif), GGCU, that drove miRNAs into the exosomes in hepatocytes. Interestingly, it was shown that the incorporation of this motif into cytoplasmic miRNAs directed their sorting into exosomes, and any mutation in this motif impeded the SYNCRIP-binding, thereby inhibiting miRNA loading into exosomes [236]. Naturally, more studies recognized other RBPs, such as Y-Box protein 1 (YBX1), which was responsible for selective sorting of miR-133 in exosomes of endothelial progenitor cells [237]. The same YBX1 was also recognized for miRNA sorting in HEK293 derived exosomes, especially the sorting of miR-223 [238]. More recently, a study investigated the packaging of miR-939 into extracellular vesicles under inflammation conditions, and this miRNA was found to contain the EXO motif [239]. Also, under the influence of inflammatory conditions, the RBP Fragile X mental retardation 1 (FMR1) was shown to potentiate exosomal miRNA sorting [240]. Apart from RBP-facilitated exosomal sorting, post-transcriptional modifications were also speculated to be responsible for the sorting of miRNAs into exosomes. The human urine-derived exosomes were shown to have a miRNA population that is enriched in uridylation at their 3’ end (3’UUUU). This supported the notion that maybe the post-transcriptional modification in the form of uridylation is necessary for packaging of miRNAs in human-urine derived exosomes [241]. Additionally, exosomes derived from metastatic breast cancer cells contain an abundant population of uridylated miRNAs, and sorted by interaction with the RBP, Lupus La protein [242]. miRNA sorting into exosomes in distinct cell types and physiological conditions is still an elusive process, and the selective utilization of RBP-dependent or independent sorting mechanisms still needs to be studied in further depth.

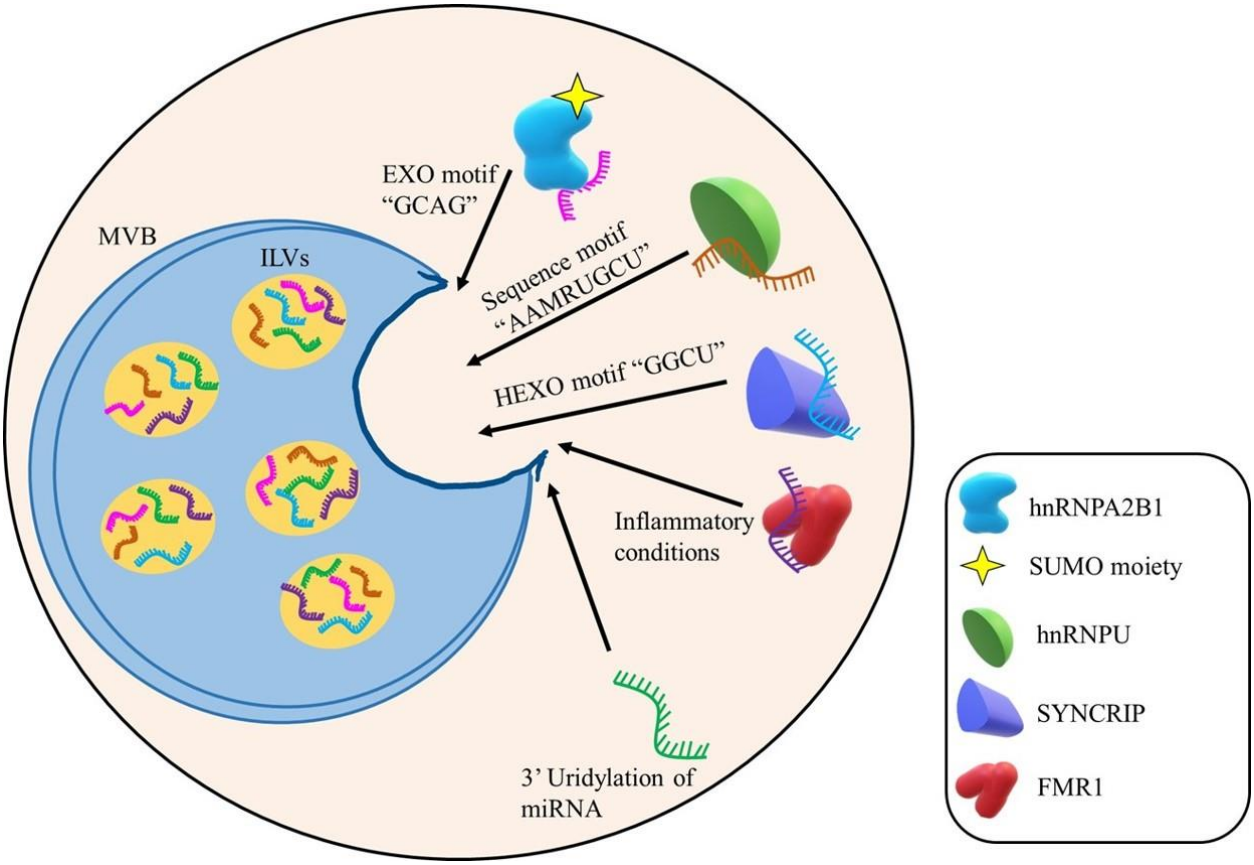

Figure 1.4. MicroRNA sorting into exosomes. Several RNA-binding proteins (RBPs) bind to specific sequence motifs on miRNA and selectively sort them into exosomes**.**

1.16 Exosomal miRNA cargo: role in intercellular communication and PD pathogenesis

A small percentage of exosomes are known to carry miRNAs, which means that only some exosome subtypes carry enough of a miRNA load to affect the recipient cells by gene silencing. Small non-coding RNAs are essential for regulating CNS functions by modulation of post-transcriptional processes and gene silencing [243].

A study showed that miR-124a is packaged in the exosomes originating from the mouse primary cortical neurons, which are then internalized by the astrocytes, resulting in upregulation of GLT1 which facilitates the glutamate uptake in the brain [244]. Moreover, EVs have also been reported to be enriched in miRNAs that can regulate ageing related neuronal processes; miR-219 enriched in EVs isolated from young mice is involved in differentiation of myelinating oligodendrocytes [245]. EVs treated from young animals to adult rats improves myelination in the demyelinated

hippocampal slices [246]. This is because the serum-derived exosomes were enriched in miR-219, which induces production of myelinating oligodendrocytes by inhibiting differentiation regulators.

It's already well established that abnormal expression and aggregation of α-synuclein is vital to PD pathogenesis. A study reported low levels of miR-7 in SNpc of PD patients, and this miRNA plays a role in binding to SNCA mRNA and inhibiting its transcription [247]. Similarly, miR-4639-5p, which is upregulated in blood plasma of PD patients is found to transcriptionally inhibit DJ-1, a well-known PD associated gene [248]. The above and various other studies clearly indicate the important role of the regulatory RNA molecules, miRNAs, in various physiological processes of PD. Although many miRNAs are described to be indicators or modulators of neurodegenerative disorders, their role of being packaged and sorted into exosomes, and modulation of brain functions in neurodegenerative conditions is just beginning to be explored.

A study showed that downregulation of exosomal miR-137 in serum can rescue the oxidative stress in neurons, and promote their cell viability, and moreover inhibit the apoptosis of cells in both cellular and mouse models of PD. The levels of miR-137 were found to be regulated through the OXR1 pathway [249], which activates oxidative stress response genes. A well-studied model of MPP^+ - treated astrocytes showed a significant reduction in exosomal miR-200a-3p through small RNA sequencing. Expression of mir-200a-3p attenuated cell death in MPP^+ - stimulated neuronal cells, SH-SY5Y and exhibited protective effects in primary dopaminergic and hippocampal neuronal cultures through downregulation of MKK4 [250].

These evidence suggest that exosome mediated transfer of miRNAs to the recipient cell types alters its cellular processes, hence it is important to understand the exosome mediated transfer of miRNAs to different cell types under PD stress conditions.

1.17 Exosomal miRNAs – a promising biomarker and therapeutic potential

There has been research striving to identify novel biomarkers for the prognosis, diagnosis, and therapeutic value of neurodegenerative diseases. The myriad roles of exosomal miRNAs in the maintenance of CNS homeostasis is vital for understanding of the neurological disease mechanisms as well as developing novel therapeutics to alleviate neurodegeneration. Exosome populations contain miRNAs that are like the miRNA population of the parent cell from which it

originated. These sets of miRNAs tend to change depending on the physiological conditions of the cell, which means that changes in the levels of miRNAs can define a “molecular signature” that can identify changes in physiological and pathological processes. A pioneer study characterized a whole profile of 24 exosomal miRNAs isolated from the serum of 40 healthy individuals and 109 PD patients. Out of the 24, consistent findings were obtained for 3 miRNAs, where miR-19b was downregulated and miR-195 and miR-24 were significantly upregulated in the serum exosomes of PD patients [251]. The comparative study on the miRNA levels in free plasma versus the miRNA levels in plasma derived EVs in PD patients and healthy controls showed no significant difference in miRNAs was found in free plasma between the groups. The level of miR-331-5p was highly enriched in the plasma EVs of PD patients and miR-505 was significantly downregulated in plasma EVs of PD patients as compared to healthy group [252]. Another group identified miRNAs enriched in the EVs isolated from the CSF of early-stage PD patients. Through machine learning approach, miR-27a-3p and miR-426-5p were present in low levels in PD patients, and the healthy controls demonstrated high levels of miR-125a-5p and low levels of miR-151a-3p [253]. A study was conducted by a group in a large cohort of 139 individuals which were affected by Alzheimer's (AD), Parkinson’s (PD), vascular dementia (VD), vascular parkinsonism (VP) and healthy controls (HC) where the expression of 23 miRNAs was checked in serum derived EVs. The result of many miRNA alterations was observed amongst different pathology groups which served as distinct biomarkers to help identify one condition from the other [254]. A study conducted in 30 PD patients and 30 HC helped identify miR-30c-2-3p, which was significantly higher in the plasma exosomes of PD patients, while miRNAs like, miR-338-3p, miR-138-5p, miR-431-5p, miR-15b-5p and miR106b-3p were all significantly downregulated [255]. Most recently, miR-34a-5p present in plasma EVs was identified as a potential biomarker for PD and has also been reported in other CNS pathologies as well [256].

The ability of EVs and exosomes to facilitate intercellular communication amongst cells at paracrine and at distant sites has inspired the use of these vesicles as therapy carriers and modulators. Moreover, interestingly, exosomes have the innate ability to cross biological barriers, like the blood brain barrier (BBB) with utmost ease, making them cross over into the systemic circulation as well [257]. The encapsulation of miRNAs into the exosomes protected them from dilution in the extracellular milieu or from degradation. Apart from these reasons, there are other multiple advantages which deem exosomes as perfect candidates for gene therapy, including, no

illicit stimulation of an immune response, easy target feasibility by tailoring the exosomal membrane proteins and no endogenous replication. Pioneering studies have successfully demonstrated that exosomes can be specifically modified and engineered to target specific organs and tissues in the body. One such study involved the insertion of brain-specific peptides in LAMP-2B, one of the most widely abundant exosomal membrane proteins. Exosomes containing siRNA for brain-specific knockdown of BACE1 were produced in wild-type mice [258], suggesting that exosomes are useful vehicles for RNAi delivery. A more recent study used the same technique to knockdown α-syn in brains of transgenic mice [259]. Exosomes were modified to express RVG and were loaded with α-syn siRNA, which were then peripherally injected into normal and transgenic mice overexpressing the S129D α-syn. After 7 days of treatment, a significant reduction in α-syn mRNA and protein levels was observed in the brains of both normal and transgenic mice [259]. Another major study involved production of "designer exosomes" in engineered HEK293T cells which were regulated by EXOtic devices, which had the capability to enhance exosome production, and introduce specific miRNA packaging. Therapeutic catalase miRNA from the "designer exosomes" was able to mitigate the neurotoxic effects of 6-OHDA and LPS-induced neuroinflammation in PD mouse models [260]. miR-124 loaded nanoparticles promoted neurogenesis and induced the migration of neurons into the damaged striatum of PD mouse models treated with 6-OHDA, hereby improving the motor performance of the animals [261]. Hence, all the studies indicated that exosomal miRNAs are indeed believed to contain the potential to become tools for prognosis and can be developed as vehicle for targeted drug delivery and develop exosome based therapeutic strategies.