2. REVIEW OF LITERATURE

2.1 Parkinson's disease:

Clinically Parkinson's disease (PD) is characterized by bradykinesia, (slowness of voluntary movement with continuous reduction in speed and amplitude of repetitive actions), akinesias or no movement, rigidity, and tremor. Other characteristics include decreased motor activities and leading to shuffling gait, an absence of arm swing, blinking and lack of facial expression. Tremor will be maximal when the limb is at rest, and slowdown of voluntary movement and is about 4 to 6 hz. Resting tremor is affecting mainly heads, lips, jaw and feet (Jankovic, 2008; Massano and Bhatia, 2012). Patients having PD also symptoms of tightness and stiffness in their joints and muscles and may be accompanied by the cogwheel phenomenon (Findley *et al.*, 1981). Non-motor symptoms include sleep disorders, autonomic dysfunctions, depressions, apathy, fatigue, pain and dementia is also associated with PD (mainly in late PD) (Massano and Bhatia, 2012).

James Parkinson published first report about the clinical case of 6 patients what he called paralysis agitans in his report called "An Essay on the Shaking Palsy" to modern times in 1817. The term "Parkinson's disease" was first described by Charcot and his student in 1872 (Goetz, 2011).

Later in the 1880s, William Gowers describing his personal experience of 80 patients in his "Manual of Diseases of the Nervous System" and known for his colourful prose regarding Parkinsonian tremor (Goetz, 2011),

"The movement of the fingers at the metacarpal-phalangeal joints is similar to that by which Orientals beat their small drums."

Fraedrich Lewy, A German neurologist, described spherical inclusions body that would later bear his name; the Lewy body (LB) in1912 (Holdorff, 2002). Meynert was the first demonstrated the role of basal ganglia in PD (Goetz, 2011). In the 1960s, chemical differences in the brains of Parkinson's patients were identified. The low levels of dopamine were shown to cause the degeneration of nerve cells in part of the brain called the substantia nigra (Goetz, 2011). In the 1960s, the drug Levodopa was first administered to treat the symptoms (Hornykiewicz, 2010) and has since become the "gold standard" in medication by which other anti-parkinsonian drugs would be compared (Hornykiewicz, 2010).

Epidemiological studies show that PD is second most frequently diagnosed neuro-degenerative disease, with a cumulative life-time incidence estimated at 2%. There are relatively few reports about incidence studies of PD, still published figures vary significantly and assessment are often obstruct by a difference in methodology of reporting (Wirdefeldt *et al.*, 2011). Overall, a median standardized incidence rate is 0.014 % in developed countries over the age of 65 or above and 0.16 % in all age groups. The frequency of incidence varies between different countries and also has a gender difference. Several studies have been indicated that a higher prevalence in men than in women (Wirdefeldt *et al.*, 2011) but other report shows no such gender difference in the incidence of PD (Burn, 2007). A most recent study suggested the incidences of PD cases at all ages. Average symptomatic age of PD is 58-60 years (Fahn and Sulzer, 2004) in the USA and very uncommon in people under 40 often caused by an inherited or spontaneous genetic defect.

2.2 Aetiology of Parkinson's disease:

Although major efforts have been initiated to understand the pathogenesis of PD over the last few decades, however the aetiology of PD is still not clearly understood. Without better understanding of the precise mechanisms of PD, it may difficult to develop more effective symptomatic and neuro-protective treatments. It has been proposed for many years that PD is caused by genetic and environmental factors in minority of patients (hereditary and symptomatic PD respectively) (Warner and Schapira, 2003).

2.2.1 Genetic cause of PD:

The familial history of Parkinsonian has been discovered to be ranging around 5-10% in past 2 decades (Lin and Farrer, 2014). Intensive studies are being performed to trace the familial history of identified Parkinsonian traits and the genetic factors involved in various studies (Kitada *et al.*, 1998; Bonifati *et al.*, 2003; Valente *et al.*, 2004). The technological advances such as GWAS, the genome-wide exosomal screening identified 24 genetic loci from the mass screen of 1 lakh controls to 19 thousand parkinsonian patients. These loci stand for more than 8 million single nucleotide polymorphisms which correspond to 30% risk individually (Nalls *et al.*, 2014). The pathological development is rather a combinatorial effect of these genetic alterations by altering various cellular processes. Identification of these novel genetic variations is beginning to illuminate the role of the new modulator and its functional role in a cellular pathway that are involved in PD pathogenesis. The genes implicated in Parkinson's disease are listed in Table no 2.1.

2.2.1.1 α-synuclein (SNCA):

SNCA gene contain six exons which encoding the 140 amino acid cytosolic α-synuclein protein. This was the first gene whose association with autosomal-dominant (AD) mutation Ala53Thr on chromosome 4q22.1 in PD (Polymeropoulos *et al.*, 1997). Afterward, additional pathogenic missense mutations were recognized in SNCA, i.e. Ala30Pro, Glu46Lys (Zarranz *et al.*, 2004), and more recently Gly51Asp and His50Gln (Appel-Cresswell *et al.*, 2013; Lesage *et al.*, 2013; Proukakis *et al.*, 2013) have been described. Duplications and triplications of the entire SNCA gene have been reported (Proukakis *et al.*, 2013) and caused PD by structural SNCA aberrations. SNCA missense mutations or multiplications have been associated with dementia with unusual feature including myoclonus or seizures and pyramidal signs in PD patients. SNCA mutations in PD, it's corresponding protein, α-synuclein form stable β sheets and thus exacerbates the formation of toxic oligomers, protofibrils and fibrils which are main component of Lewy bodies. Wild-type α-synuclein is selectively translocated by autophagic machinery into lysosomes for degradation (Cuervo *et al.*, 2004). The defect in lysosomal enzyme β-glucocerebrosidase leads to altered α-synuclein levels in dopaminergic neurons in PD patients. The latest report shows the bidirectional effect of α-synuclein and β-glucocerebrosidase forms a positive feedback loop that causes accumulation of α-synuclein and lysosomal dysfunction in neurons of idiopathic PD brain (Mazzulli *et al.*, 2011). The normal function of α-synuclein is not well known, but the studies show it's a role in the recycling of synaptic vesicles (Bendor *et al.*, 2013). The current focus is to understanding the mechanisms of α-synuclein toxicity and spread of α-synuclein in neighboring neurons in PD condition (Klein and Westenberger, 2012).

2.2.1.2 LRRK2 (PARK8):

LRRK2 is a large gene, consists of 51 exons that encodes the 2527 amino acid cytoplasmic multi-domain protein with a leucine-rich repeat at the N-terminus of the protein and a kinase domain, protein interaction domain and GTPase domain various conserved domains in between (Klein and Westenberger, 2012). *LRRK2* gene was a second gene for autosomal dominant PD identified in 2004 and is involved in late-onset autosomal-dominant and sporadic PD. The mutation frequency ranging from 2% to 40% of LRRK2 has been reported for different populations (Zimprich *et al.*, 2004). LRRK2 mutations have been described with different clinical and neuropathological nature, often identical with sporadic PD. In 2008, large screening has demonstrated that LRRK2 mutation is the most frequent form of monogenic Parkinsonism worldwide. *LRRK2* mutated PD commonly shows mid-to-late onset without dementia. The pathogenic mechanism leading to PD caused by LRRK2 mutations is still uncertain. However, recently reports show that LRRK2 have a regulatory role in endosome-to-lysosome trafficking, protein translation, mitochondrial function and autophagy (Wang *et al.*, 2012b; Esteves and Cardoso, 2016).

2.2.1.3 Parkin (PARK2*)* and PINK1 (PARK6):

PARK2, which encodes the Parkin protein, was the first identified gene certainly causing an autosomal recessive (AR) form of PD and serve as the frequent known feature identified for early-onset PD (10–20%). Parkin is a 465 amino acid protein, expressed in presynaptic, postsynaptic processes and cell bodies. Parkin mutation is frequently found in the third or fourth decade of the PD patient (Klein and Westenberger, 2012). PD with Parkin mutation is usually gradually developing and shows an excellent counter to dopaminergic treatment. Homozygous mutations in Parkin observed even in childhood and the most often reason of juvenile PD (Lill, 2016). Reported post-mortem study indicate that Parkin muted PD sample frequently shows an absence of Lewy bodies formation, however, neuronal loss and gliosis was observed in SN. The Parkin is believed to function as an E2-enzyme-dependent E3 ubiquitin ligase activity that is involved in the process of UPS (ubiquitin proteasome system) and lysosomal degradation of target proteins and defective mitochondria (Zheng *et al.*, 2010; Chan and Chan, 2011).

PARK6 consists of eight coding exons which encodes the 581 amino acid PINK1 protein is the second gene to be found in early-onset autosomal recessive form of PD (Valente *et al.*, 2002a; Valente *et al.*, 2002b). PINK1 is ubiquitously expressed protein kinase and has mitochondrial targeting motif, a carboxy-terminal autoregulatory domain, and a conserved serine–threonine kinase domain (Klein and Westenberger, 2012). Recent evidence shows that PINK1 and Parkin are function in a common pathway for identified and selectively remove damaged mitochondria from the mitochondrial network (Deas *et al.*, 2009). Low mitochondrial membrane potential leads to PINK1 stabilization on mitochondria and recruits Parkin from the cytosol. Once recruited to mitochondria, Parkin becomes enzymatically active and triggers the autophagic clearance of mitochondria by lysosomes (Pickrell and Youle, 2015).

2.2.1.4 DJ-1 (PARK7):

PARK7 is the third gene associated with AR form of PD and was found a large deletion and missense mutation in Italian and Dutch PD patients in 2003. Twenty-three pathogenic deletion and point mutations were found in patients with PD, and it is mutated in about 1%-2% of EOPD cases (Pankratz *et al.*, 2006). PARK7 gene consists of seven exons which encodes the 189-amino acid, ubiquitously expressed *DJ-1* protein and was initially described as association with oncogenesis in 1997. DJ-1 act as an identifier of cellular oxidative stress (Canet-Aviles *et al.*, 2004; Junn *et al.*, 2005) and forms a dimeric structure under physiologic conditions (Macedo *et al.*, 2003). Its neuro protective function and antioxidant activity is reduced in mutant from of DJ-1 since mutated protein is unstable and rapidly degraded by proteasome (Anderson and Daggett, 2008).

2.2.2 Environmental risk factor cause of PD:

In the cases of sporadic PD, the environmental factor is thought to play a vital role in the root of PD. Several epidemiological studies have been undertaken (de Lau and Breteler, 2006) to identify differences in the experiences of people who develop PD, compared to those who do not to determine which factors may be important. However, it is still difficult to find risk factor and serve only as clues. Here, the following is a list of environmental risk factors for an association of PD which may help us to understand the cause of PD for providing direction for further therapeutic development.

- The single biggest risk factor for Parkinson's disease is increasing age.

- Men have a somewhat higher risk than women.

-Area of residence, occupation and pesticide exposure: Parkinson’s prevalence is higher in the greater dietary intake of persistent organic pollutants such as polychlorinated biphenyls, or PCBs and high levels of industrial emissions of the metal manganese, and possibly copper. Shift work and jobs involving vigorous physical work occupation have lower rates of association with PD. Exposure to toxins like MPTP, pesticide such as an insecticides rotenone and permethrin, organochlorines such as beta-hexachlorocyclohexane, herbicides paraquat and 2,4-dichlorophenoxyacetic acid (2,4-D) have association with high risk of PD (de Lau and Breteler, 2006).

2.2.3 Cellular pathology in pathogenesis of PD:

The most frequently cited cause of PD includes following:

- Neuro-inflammation
- Impaired mitochondrial function
- Defect in " Quality control"
    - Autophagic dysfunction
    - ER stress
    - Proteasomal dysfunction

2.2.3.1 Neuroinflammation and PD:

Neuroinflammation and its association with neurodegenerative diseases are emerging. The association of neuroinflammation in PD was concluded from the presence of reactive microglia within the substantia nigra of PD patients and in the experimental animal studies (McGeer *et al.*, 1988). This was further confirmed by the Positron emission tomography (PET) images of studies of PD brain showing remarkable activation of microglia in various region of the brain (Wu *et al.*, 2013). Microglia plays specialized immune surveillance role in innate immunity against invading pathogens. Recently, neuroinflammation has been suspected as the major cause of the gradual loss of dopaminergic neurons promoted by microglial activation and release of pro-inflammatory cytokines and chemokines. High level of pro-inflammatory cytokines particularly tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and interferon- γ (IFN-γ), is observed in the DAergic nigrostriatal system and other brain regions outside the SN and as well as in CSF and serum in PD patients (Mogi *et al.*, 1994a; Mogi *et al.*, 1994b;

Blum-Degen *et al.*, 1995; Stypula *et al.*, 1996; Dobbs *et al.*, 1999; Reale *et al.*, 2009). These all studies strongly recommend the involvement of immune components in PD pathogenesis.

It has been shown that in the early phase of neurodegeneration process microglia activation play role as beneficial to the host (Cagnin *et al.*, 2006; Hu *et al.*, 2012). However, prolonged activation of microglia significantly expression of a large group of pro-inflammatory cytokines including TNF-α, IL-1β, interleukin-6 (IL-6) and IFN-γ, which activates death signalling and contribute to the acceleration of nigral DA neuron degeneration (Christo, 1980; McCoy *et al.*, 2006). Pathological molecules such as metalloproteinase-3 (MMP-3), α-synuclein, ATP and released from the defective DA neurons leads to further enhance microglia activation, release pro-inflammatory cytokines which amplify the neuroinflammatory responses and result in the driven the neurodegenerative processes (Zhang *et al.*, 2005; Saijo *et al.*, 2009).

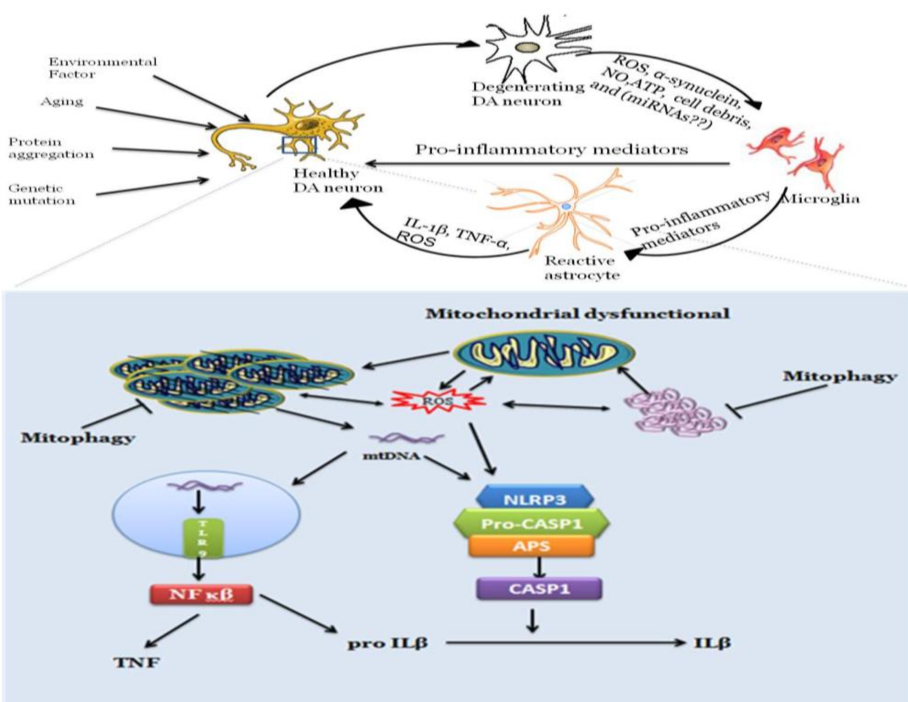

Figure 2.1 Schematic representation of inflammatory mechanisms involved in PD pathogenesis.

Since microglia has two complimentary activation phenotype, Pro-inflammatory (M1 phenotype) and anti-inflammatory (M2 phenotype) are characterized by different arrays of cytokines production depending upon upstream stimulation (Pisanu *et al.*, 2014). Anti-inflammatory factors have neuro-protective and trophic activities and involved in brain repair processes, while pro-inflammatory induces mitochondrial defect, oxidative stress and neuronal cell death.

Emerging evidence shows PD-associated genes have a role in the regulation of immune responses of microglia and astrocytes and immune product in the CNS. The mutated genes, α-synuclein (SCNA) promote distinct microglial activation through the stimulation of toll-like receptor 2 (TLR2)-mediated signaling *in vitro* and in animal model (Su *et al.*, 2008; Chesselet *et al.*, 2012; Watson *et al.*, 2012). The other common mutated LRRK2 gene has direct role LPS induced inflammatory signal. The knockdown of LRRK2 in microglia decreased LPS induced TNF-α and iNOS production and activation of nuclear factor κ B (NF-κB) transcriptional activity (Gillardon *et al.*, 2012; Kim *et al.*, 2012). Emerging reports show that PD-associated genes, like PINK1, PARKIN and DJ1 have been implicated in inflammatory pathways and pathogenesis of PD pathology (Bandopadhyay *et al.*, 2004; Frank-Cannon *et al.*, 2008; Akundi *et al.*, 2011; Tran *et al.*, 2011; Lee and Chung, 2012; Kim *et al.*, 2013a; Trudler *et al.*, 2014). The schematic representation of possible involvement of inflammatory pathways in PD pathogenesis is represented in Figure 2.1 (Wang *et al.*, 2015). However, the precise mechanism of inflammation induced pathogenesis of PD specifically selective neuronal loss is not well understood.

2.2.3.2 Mitochondria and Parkinson's disease:

Now there are established evidence suggesting mitochondrial dysfunction and its association with PD pathogenesis (Schapira, 1993; Lang and Lozano, 1998; Bender *et al.*, 2006). The association of mitochondrial dysfunction with PD was first observed from the toxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), a toxin induced neurotoxicity that shows PD symptoms in drug-abused patients. Its neurotoxicity was confirmed in animal's models where it leads to decreased complex-1 activity, leading to ATP depletion, increased level of reactive oxygen species (ROS) (Burns *et al.*, 1983). Furthermore, the evidences show that inhibition of complex I using rotenone and paraquat leads to loss of DA neurons and induced PD like pathogenesis in animals which has been used to mimic sporadic PD in animals to study the molecular mechanism and exploring the therapeutic strategic. Moreover, a decrease of expression of PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha, co-activator important for mitochondrial gene expressions) was observed in DA neurons in PD (Zheng *et al.*, 2010; Elstner *et al.*, 2011). The analysis of PD brain tissues showed dysfunction of mitochondria and high level of mtDNA depletion (Schapira, 1993; Lang and Lozano, 1998; Bender *et al.*, 2006). Mutations in mitochondrial DNA polymerase (POLG) coding gene show accumulation of multiple mtDNA mutations and develop PD like symptoms (Luoma *et al.*, 2004).

The genes those associated with PD have emerging role in mitochondrial homeostasis. PARKIN mutation is identified as a major cause of PD which is involved in the maintenance of a healthy pool of mitochondria. PARKIN inhibits mitochondrial permeability transition and release of cytochrome c during ceramide treatment (Darios *et al.*, 2003). PARKIN also has a known role in mitochondrial biogenesis (Shin *et al.*, 2011; Gehrke *et al.*, 2015; Stevens *et al.*, 2015; Lee *et al.*, 2017) and protect mtDNA from oxidative damage by indirect stimulate DNA repair. PARKIN bind to mitochondrial transcription factor TFAM to stimulate mitochondrial replication and transcription in proliferating SH-SY5Y cells (Kuroda *et al.*, 2006).

Mutation of PINK1 causes early onset of PD which have a direct role in maintaining of mitochondrial quality control and function. PINK1 deficiency has various effect on mitochondrial function and morphology that include abnormal mitochondrial morphology, comprising complexes I and IV activities, mitochondrial membrane potential, ATP production, mitochondrial import, mitochondrial trafficking, Mitochondrial fusion and fission and mtDNA levels and increases in ROS production (Wilding *et al.*, 1997; Gautier *et al.*, 2008; Wood-Kaczmar *et al.*, 2008; Gandhi *et al.*, 2009; Gegg *et al.*, 2009; Gispert *et al.*, 2009).

Mitochondria play an important role in PD pathogenesis which has emerging association with inflammatory pathways. Mitochondrial dysfunction activates NLRP3 inflammasome activation as well as induces redox-sensitive factor of an NF-κB pathway and increased the level of pro-inflammatory cytokines like TNF and IL1-b (Figure 2.1). The implication of mitochondrial-mediated inflammation and its association with the pathogenesis of PD is not well understood.

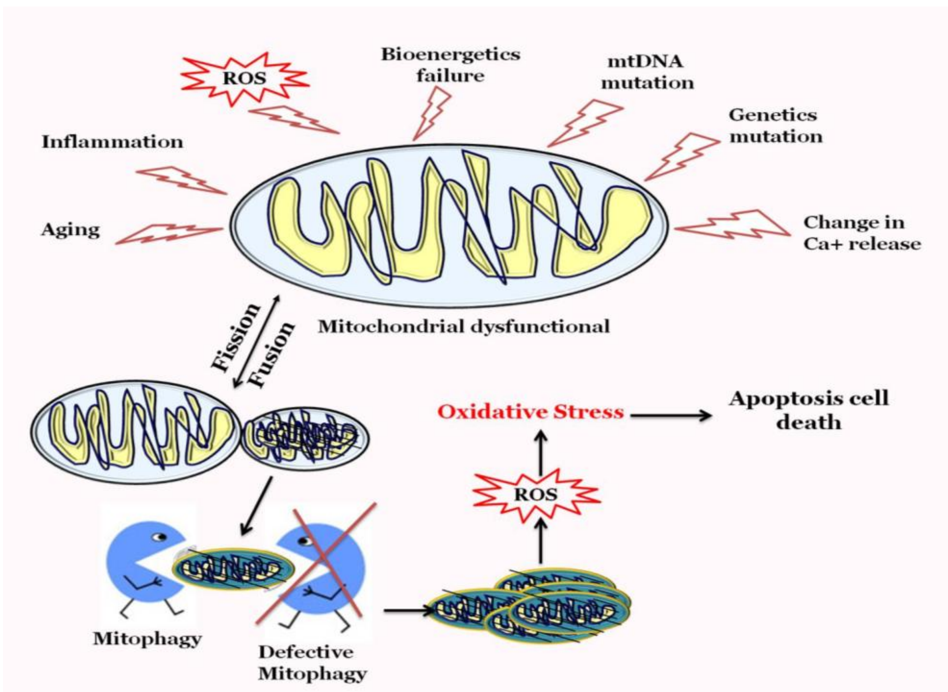

Figure 2.2 Schematic representation of all possible mitochondria alteration in PD pathogenesis.

These evidences strongly suggest that the presence of defects in mitochondrial function and biogenesis is the main denominator of sporadic and familial PD. The mitochondrial homeostasis is important for the neuronal health hence different steps of mitochondrial biogenesis to its degradation are tightly regulated in neurons. Any alteration in these steps may have implication in pathogenesis of PD (Figure 2.2).

2.2.3.3 Defect in Quality control:

A neuronal cell has to maintain the quality of proper folding proteins and as well as healthy organelle mainly mitochondria in order to maintain cellular homeostasis. In order to remove defective organelle and misfolded or unfolded proteins, cells have a complex molecular pathway where a defective cargo selectively identified and degraded by lysosome and proteosome. The presence of Lewy body in PD pathogenesis directly suggests a defect in quality control.

2.2.3.3.1 Autophagy-Lysosomal pathways and Parkinson's disease:

The defect in autophagy-lysosomal pathways (ALPs) is observed as a major pathogenic cause of PD and its alterations are seen in genetic and toxin induced animal model of PD as well as in sporadic PD brains. Novel identified genes involved in PD pathogenesis have been shown to be associated with the autophagy pathway. ALPs are one of the proteolytic machinery that recycles defective cargoes to maintain cellular homeostasis. With increasing the age, decreased recycling processes (Cuervo, 2008) have been observed and seem to be a major risk factor of PD. A better understanding of the regulatory mechanism of autophagy in different step during the pathogenesis of PD will help to devise a strategy for the possible for clinical interference.

2.2.3.3.1.1 Autophagy:

The term autophagy (Greek roots: auto, meaning 'self', and phagy, 'to eat') means the process of self-cannibalization through a lysosomal degradation pathway for survival advantages which play a critical role during cell survival, differentiation, development and cellular homeostasis.

Autophagy may help the cell to signal its condition to the surroundings or rapidly modify its protein content to better respond to extrinsic stimuli (Martin and Baehrecke, 2004; Akdemir *et al.*, 2006). The process of autophagy is complicated multi-step process and is tightly regulated. The sequential events are diagrammatic representation in figure 2.3. During autophagy process, an isolated double membrane structure is formed, known as autophagosomal structure, which invaginates and sequesters cytoplasmic constituents including mitochondria, endoplasmic reticulum, peroxisomes, ribosome's and large proteins and protein aggregates. Selection of cytoplasmic constituent is performed by tagging of Ub, where the E3 ligases play a vital role in the process of ubiquitination. The ubiquitinated substrate is recognized by adaptor proteins having UBA domain and preferentially sequestered in autopphagosome. The edges of the membrane fuse to form a double or multi-membranous structure, known as the autophagosome or autophagic vacuole which fuses with lysosomes or vacuoles for degradation of autophagic contents by resident hydrolases (He and Klionsky, 2009).

More than 30 autophagy-related (ATG) genes have been identified in yeast (Cheong *et al.*, 2008; He and Klionsky, 2009). Most of ATG genes identified in lower eukaryotes have orthologs in higher eukaryotes (Cheong *et al.*, 2008; Miller *et al.*, 2008). ATG genes are essential for induction, generation, maturation and recycling of autophagosome. ATG proteins encoded have been categorized into four functional groups viz. a serine/threonine kinase complex (Atg1, Atg13, Atg17), a lipid kinase signaling complex (Atg6, Atg14, Vps34, and Vps15), and two novel ubiquitin-like conjugation system- Atg8 and Atg12 systems and Atg2, Atg9, Atg18 complex (Cheong *et al.*, 2008; Miller *et al.*, 2008). Serine/threonine kinase complex (Atg1, Atg13, Atg17) comprises of initiator proteins and respond to upstream signals of autophagy induction. The lipid kinase signaling complex (Atg6, Atg14, Vps34, and Vps15) involve in vesicle nucleation. Atg8 and Atg12 ubiquitin-like conjugation system involve in vesicle expansion and a recycling system while Atg2, Atg9, Atg18 complex involves in disassembly of Atg proteins from matured autophagosomes (Cheong *et al.*, 2008; Miller *et al.*, 2008).

Recently a novel pathway of autophagy induction has also been discovered which is independent of classical proteins like ATG5 and ATG7 independent (these genes are essential in conventional autophagy system). RAB9, a protein involved in endosomes trafficking, play important role in the formation of autophagosomes in the alternative autophagy system (Nishida *et al.*, 2009). The classical autophagy shows important role in pathogenesis of PD whereas alternate pathway still needs to be elucidated and studied in

Parkinson's disease.

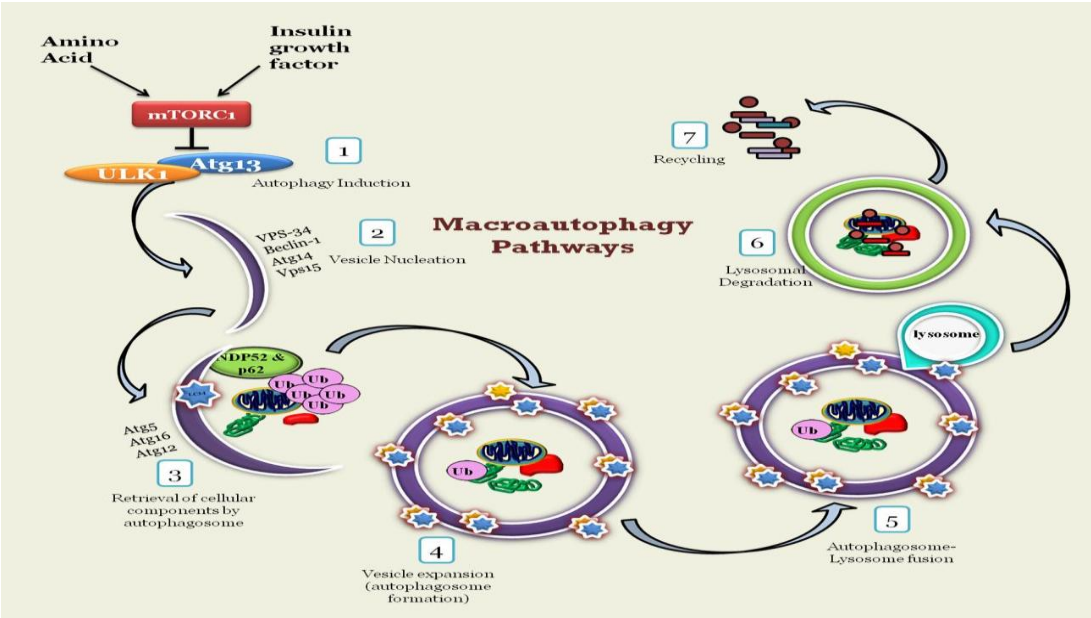

Figure 2.3 The established steps involved in autophagy. *(1) Autophagy induction, (2) Vesicle nucleation, (3) retrieval of cellular component during autophagosome formation,(4) Vesicle elongation and autophagosome formation, (5) Fusion of the autophagosome a lysosome/endosome and (6) Autolysosome cargo degradation*

2.2.3.3.1.2 Autophagy: Implication in PD pathogenesis

The pathogenic role of autophagy in PD was demonstrated by the finding that α-synuclein, a major constituent of Lewy body, is degraded by macroautophagy and chaperone-mediated autophagy (CMA) (Webb *et al.*, 2003; Cuervo *et al.*, 2004; Vogiatzi *et al.*, 2008). The inhibition of CMA leads to an accumulation of soluble high molecular weight and detergent-insoluble species of α-synuclein. The inhibition of macroautophagy also leads to the accumulation of wild-type α-synuclein, suggesting that CMA and macroautophagy are important pathways for wild-type α-synuclein degradation in neurons (Vogiatzi *et al.*, 2008).

The familial PD-associated molecules, such as the wild-type, A53T and A30P mutants of α-synuclein, DJ-1, Parkin, PINK1 and LRRK2 are known to be involved in the autophagic pathway. The A53T and A30P mutants of the α-synuclein protein is not transported across the lysosomal membrane however it binds more strongly to the LAMP-2A receptor and act as receptor binding inhibitor for preventing other targeted protein for CMA (Martinez-Vicente *et al.*, 2008). The over-expressing Beclin-1 activates autophagy which lead to decreases α-synuclein accumulation and reduces cell death suggesting a direct involvement of autophagy in PD (Spencer *et al.*, 2009).

Parkin and Pink1 involved in selective clearance of defective mitochondria through the mitophagy and now became another area of focus for autophagy and PD research. During mitochondrial membrane depolarization, PINK1 rapidly accumulates on the mitochondrial outer membrane which recruits PARKIN on damaged mitochondria. PARKIN initiates ubiquitination of a large number of mitochondrial proteins (Spencer *et al.*, 2009). Ubiquitinated mitochondria are recognized by adaptor protein, p62/SQSTM1 and sequester to the phagophore (Gegg *et al.*, 2010; Geisler *et al.*, 2010). *PARK2* and *PARK6* mutations showed defective mitophagy and have been implicated in PD pathogenesis (Lee *et al.*, 2010; Matsuda *et al.*, 2010; Narendra *et al.*, 2010) .

Mutation of LRRK2 caused accumulation of the autophagy marker proteins p62 and LC3 suggesting role of LRRK2 in autophagy impairment (Alegre-Abarrategui *et al.*, 2009; Tong *et al.*, 2010). The reduced level of DJ-1 both in Drosophila and human neuroblastoma cells lead to mitochondrial dysfunction and defective autophagy (Eisenberg *et al.*, 1979; Thomas *et al.*, 2011). These evidence strongly suggest the role of autophagy in the pathogenesis of PD.

2.2.3.3.2 ER Stress and Parkinson's disease:

ER stress is one of the less appreciated during PD whereas it may one of the primary causes of PD pathogenesis. The accumulation of misfolded α-synuclein is linked to neurodegeneration in PD and other α-synucleinopathies is observed. It had been observed in A53T mutant human α-synuclein transgenic (A53Tα- synuclein Tg) mouse model of α-synucleinopathy leads to ER stress and UPR. The chronic ER stress may contribute to neurodegeneration in α-synucleinopathy. Moreover, α-synucleinopathy interacts with ER chaperones and over-expression of α-synucleinopathy sensitizes neuronal cells to ER stress induced cell death, suggesting that α-synucleinopathy may have a direct impact on ER function (Colla *et al.*, 2012). The induction of mild ER stress promotes neuroprotection via autophagy activation in Drosophila and mouse model of Parkinson disease (PD) (Fouillet *et al.*, 2012). Emerging studies reveal a clear activation of the UPR where ATF6, XBP1 and CHOP have a vital role in the regulation of dopaminergic neuron survival in neurotoxin-based models of PD in vitro and vivo also (Valdes *et al.*, 2014; Mercado *et al.*, 2016). The role of ER stress and its implication in different forms of PD pathogenesis needs to be further studied.

2.2.3.3.3 Proteasomal function and Parkinson's disease:

Aggregation of misfolded ubiquitinated proteins in Lewy bodies is suggesting a defect in proteasomal clearance of targeted proteins by UPS. Post-mortem studies of sporadic PD brain show low proteasomal activities in the substantia nigra region (McNaught *et al.*, 2003) of PD patients suggesting a role of UPS in PD. Mutation of Parkin, a RING family E3 ligase, has been proposed to represent half of AR early onset PD suggesting the role of UPS pathway with family PD. Parkin mutation is known to lead to dopaminergic selective neurotoxicity due to accumulation of Parkin's putative substrates include synphilin-1 (Chung *et al.*, 2001), α-synuclein (Shimura *et al.*, 2001), p38/JVT-1 (Corti *et al.*, 2003), PaelR (Yang *et al.*, 2003), α/β tubulin (Yang *et al.*, 2003), CDCrel-1 (Zhang *et al.*, 2000), and synaptotagmin IX (Huynh *et al.*, 2003), which is a hallmark of UPS dysfunction in the PD. Inhibition of the proteasome activity in animal model result in formation of proteins aggregates and lose of dopaminergic neurons demonstrate evidence for a role of dysfunctional UPS in PD (McNaught and Olanow, 2003). These evidence suggest the important role of dynamic equilibrium of cellular quality control in maintenance of neuronal health.

2.3 miRNAs: mediator in PD pathogenesis

The common pathways like mitochondrial and protein quality control, autophagy, the ubiquitin-proteasome system (UPS) and apoptosis are now known to be important in the pathogenesis of PD (Hirsch *et al.*, 2013). The dysregulation of these pathways may lead to oxidative stress and cell death which is a major cause of PD. The dynamic regulation of these pathways in neurons is important for their optimal function. The alteration of either in mitochondrial homeostasis and autophagy pathway can lead to initiation accumulation of defective mitochondria, protein aggregation leading to neurodegeneration. Thus, there had been growing interest to identify endogenous modifier of these pathways.

The small non coding RNA called as microRNAs (miRNAs) are emerging regulator of mRNA levels to regulate the level of proteins in physiological level (Sevignani *et al.*, 2006). miRNAs have now been identified as post-transcriptional regulators of gene expression and have been implicated in numerous biological processes. The characterization of miRNAs biogenesis and its expression pattern in different diseases have been the focus of intensive research.

2.3.1 miRNA biogenesis:

Micro RNAs (miRNA) are endogenous regulator of fine tuning of gene expression. miRNAs can be transcribed from genomic DNA by canonical or non-canonical pathways by RNA polymerase II (RNA Pol II) from miRNA genes and intronic sequences in genomic loci. In canonical pathway, RNA polymerase II transcribes ~1000 nt long primary miRNA (pri-miRNA) from miRNA gene as stem-loop structures containing 5’ cap and 3’ tail. The stem loop structure of pri-miRNA is a processed in the nucleus by the RNaseIII enzyme (Drosha) and dsRNA binding partner DGCR8 microprocessor complex to produce ~ 60 -100 nt long precursor miRNA (pre-miRNAs) at 1 helical turn (Figure 2.4A). The pre-miRNA has a ~2-nt overhang at 3’ end which recognized by Exportin-5 and exported out of nuclei in Ran-GTP dependent manner (Sevignani *et al.*, 2006).

In the cytosol, pre-miRNA is further cleaved by the RNaseIII enzyme, Dicer with the help of its dsRNA binding partner, TRBP to produce a ~20 nt miRNA/miRNA* duplex with a 3’ overhang in both the strands mature miRNA duplex. The thermodynamically stable strand called as guide strand associates with Argonaute proteins, AGO 1–4 to form

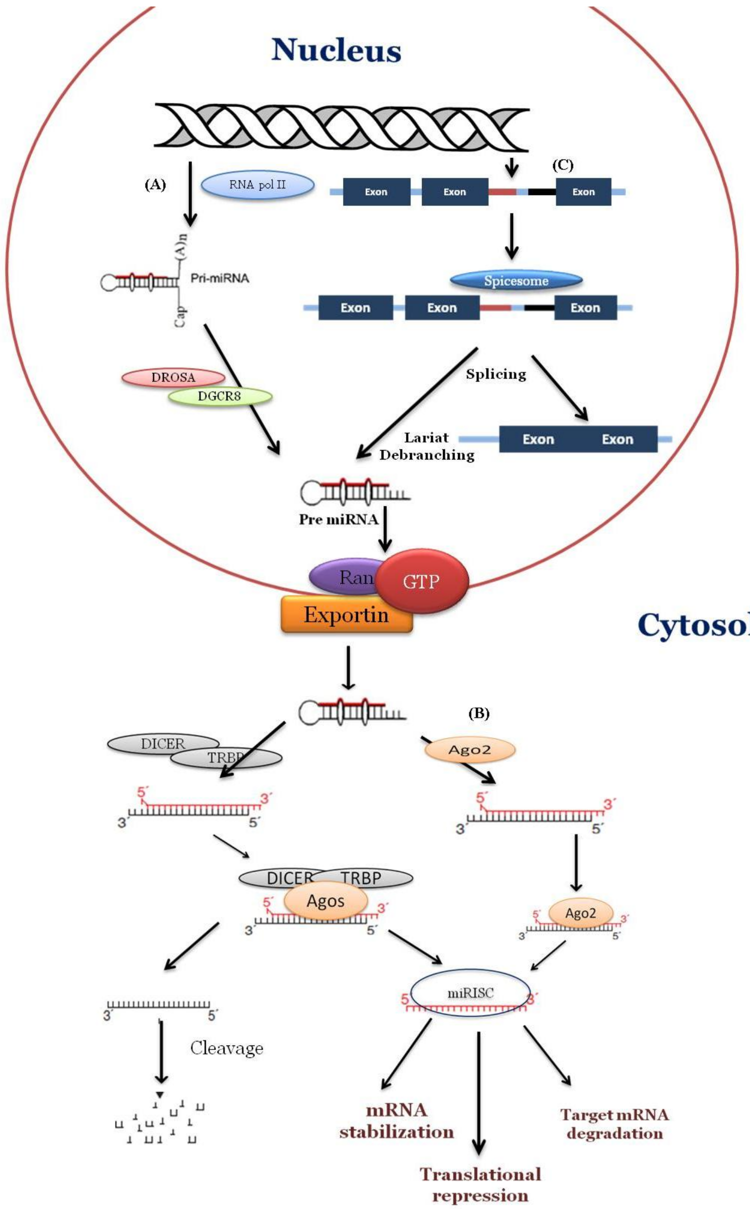

Figure 2.4 Known pathways of miRNAs biogenesis. *(A) The canonical miRNA biogenesis pathway begins with RNA Pol II-mediated transcription of genomic loci containing miRNA genes and Drosha- and Dicer-dependent. (B) Cytoplasmic pri-miRNA undergoes processing by AGO2 in Drosha-dependent/Dicer-independent non-canonical*

pathway. (C) The mirtrons are transcribed in Drosha-independent and Dicer-dependent biogenesis from intronic sequences.

a miRISC (RNA-induced silencing complex) complex. The complimentary strand called passenger strand denoted by miRNA*, was previously thought to be cleaved off however recent evidence suggests that some of these strands may have a biological role. The non-canonical Drosha-dependent/Dicer-independent miRNA biogenesis pathway involves exported pre-miRNA processed by AGO2 in the cytoplasm and produces the mature guide strand for its further action (Figure 2.4B). Other non-canonical pathways, termed Drosha-independent/Dicer-dependent miRNA biogenesis, is referred to as mirtrons. In this pathway, mirtrons are either spliced off from intronic regions, other ncRNAs like tRNAs and snoRNAs or by bypassing the enzymatic cleavage processes. More than one-third of miRNAs are encoded from intronic regions of protein-coding genes forming a lariat structure which is de-branched to form the pre-miRNA hairpin structure Mirtrons are exported to the cytosol and processed similarly to miRNA generated via the canonical pathway (Figure 2.4C) (Westholm and Lai, 2011). The mature miRNA bound Ago2 complex screens complimentary 3' UTR region of target mRNA and most commonly results in down-regulation of gene expression by recruitment of protein complexes causing deadenylation, translational repression and degradation of target mRNA. The binding complementarily of seed sequence and mRNA regulates the fate of mRNA.

2.3.2 Autophagy and miRNAs:

The process of autophagy is a complicated sequential process and is tightly regulated. The sequential events: autophagy induction, vesicle nucleation, vesicle elongation and autophagosome formation, autophagosome retrieval (an intermediate recycling step for the recruitment of lipids and certain regulatory proteins to the growing phagophore), fusion of the autophagosome to a lysosome/endosome and autolysosome formation, and autolysosome cargo degradation (Figure 2.3). According to a physiological condition, autophagy levels are modulated in different pathophysiological conditions. miRNAs are small non-coding RNAs regulate fine tunes of protein as a post-transcriptional regulator and have closely linked to virtually all known fundamental biological process.

The initiation of autophagy pathway is regulated by ULK complex. It had been observed that miR-20a and miR-106b negatively regulate ULK1 expression in during leucine deprivation-induced autophagy in myoblasts (Wu *et al.*, 2012). Similarly, Beclin1-associated PI3KC3 complex is involved in vesicle nucleation of the phagophore. miR-338–5p negatively regulates the expression of PIK3C3 kinase and decreases autophagy that helps in cell migration in colorectal cancer. Beclin-1 is a direct target of miR-30a, miR-17, miR-376b, and miR-216a and these miRNAs negatively regulate Beclin-1 expression and decreases the rate of autophagy (Zhu *et al.*, 2009; Korkmaz *et al.*, 2012; Menghini *et al.*, 2014). miR-374a and miR-630 suppress the level of UVRAG, which is a regulator of the class III PI3K complex (Huang *et al.*, 2012).

ATG proteins are categorized into four functional groups viz. a serine/threonine kinase complex (Atg1, Atg13, Atg17), a lipid kinase signaling complex (Atg6, Atg14, Vps34, and Vps15), and two novel ubiquitin-like conjugation system- Atg7, Atg5, Atg16, Atg10 and Atg12 systems and Atg2, Atg9, Atg18 complex and plays an important role in induction, generation, maturation and recycling of autophagosome. In addition, Atg4 plays a role in LC3 lipidation process.

Several studies demonstrated the role of miRNAs in the regulation of ATG proteins in different pathological condition and in a different model system. The report shows that ATG14, in a lipid kinase signaling complex is a target by miR-195 (Shi *et al.*, 2013). There were observed in different studies that miR-204 target LC3 beta isoform (Mikhaylova *et al.*, 2012) and miR-101 target ATG4 in LC3 lipidation process. In ATG12 conjugation system, it was observed that miR-375 and miR-17 target ATG7 (Chang *et al.*, 2012; Comincini *et al.*, 2013), miR-181a, miR-374a, and miR-30a target ATG5 (Huang *et al.*, 2012; Yu *et al.*, 2012; Nguyen *et al.*, 2014), miR-23b and miR-630 target ATG12 (Wang *et al.*, 2013), miR-519a target ATG10 (Huang *et al.*, 2012), miR-106b and miR-519a target ATG16L (Huang *et al.*, 2012). In ATG9 conjugation system, miR-34 target ATG9 (Yang *et al.*, 2013a), and miR-143 and miR-130a target ATG2

(Kovaleva *et al.*, 2012; Yang *et al.*, 2013b) (Figure 2.5).

After the maturation, autophagosome fuse with a lysosome to from autophagolysosome and degradation is initiated. During the process of membrane fusion, HOPS complex, Rab-SNARE system and lysosomal membrane proteins such as LAMP1 and LAMP2 plays important role (Figure 2.5). Recently, it was observed that miR-17 suppressed both endocytic and autophagic maturation into the lysosome by targeting TBC1D2/Armus (a

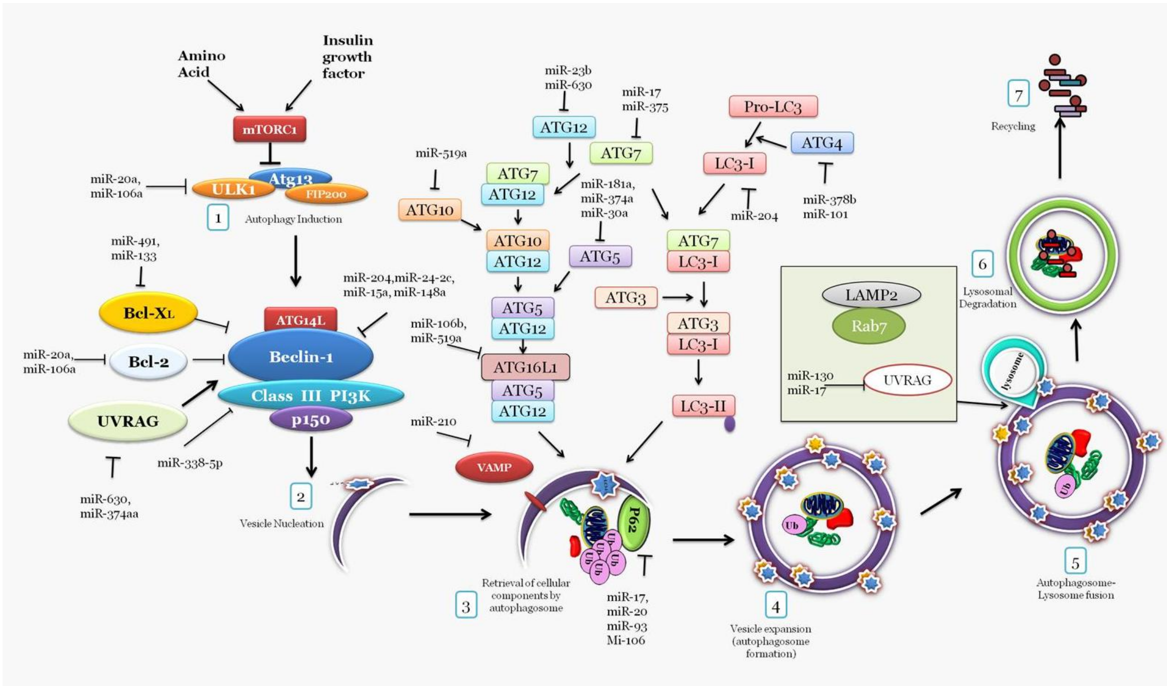

Figure 2.5: The role of miRNAs in regulation of different step of Autophagy.

GAP of the late endosome-related Rab7 GTPase) (Serva *et al.*, 2012). A system biology-based studies identified miR-124, miR-130, miR-204, miR-98 and miR-142 have a regulatory role for the autophagy-lysosomal genes (Jegga *et al.*, 2011). Taken together of all evidence suggest the involvement of different miRNAs in all step of autophagy process suggesting the importance of cross talk of miRNAs and autophagy in normal cellular homeostasis and pathological condition.

2.3.2.1 Autophagy and miRNAs in PD:

In cell base model of PD, Four miRNAs (hsa-miR-21*; hsa-miR-224; hsa-miR-373*; and hsa-miR-379) and three miRNAs (hsa-miR-26b; hsa-miR-106a*; and hsa-miR-301b) decreased luciferase activity associated to *lamp2a* and *hsc70* 3′UTRs respectively. It was also observed that level of these miRNAs significantly increased in PD SNc. Increased expression of these miRNAs decreased the level of hsc70 and LAMP-2A which plays important role in clearing of aggregated *α*-synuclein through autophagy (Alvarez-Erviti *et al.*, 2013).

In a recent study, miR-4487 and miR-595 identified as a target of ULK1 and verified experimentally regulating the ULK1-mediated autophagy in neuroblastoma SH-SY5Y cell (Chen *et al.*, 2015). Transcription factor EB (TFEB) is a master regulator of the ALP and enhancement of TFEB function has been shown to stimulate ALP function (Sardiello *et al.*, 2009). Accumulation of *α*-synuclein oligomers was efficiently cleared by over-expression of TFEB and repression of TFEB by miR-128 exacerbated the vulnerability to α-syn toxicity in DA neuron (Decressac *et al.*, 2013). In MPTP toxin base model of SH-SY5Y, Up-regulation of miR-124 caused autophagosomes (AP) accumulation and lysosomal depletion (Wang *et al.*, 2016) and miR-7 and miR-153 protect cells via upregulation of mTOR pathway (Fragkouli and Doxakis, 2014). These evidences suggest that endogenous miRNAs modulate the dynamics of autophagy in neurons.

2.3.3 Mitochondria and miRNAs:

As discuses above, mitochondrial integrity and homeostasis are essential for PD pathogenesis, hence understanding the endogenous modulators of mitochondrial functions and its homeostasis through autophagy pathway. The mitochondrion is an endosymbiotic organelle and it has its own circular DNA. Human mitochondrial human mitochondrial DNA encodes for twenty-two tRNAs, 16S rRNA and 12S rRNA (Clayton, 2000). Now different proteomics studies has shown that human mitochondria contain more than 1000 proteins, which are transcribed from nuclear DNA, translated in the cytoplasm and then transported to mitochondria for its optimal function (Clayton, 2000). These association and translocation of nuclear genome transcribed encoded mRNA/miRNAs to mitochondria is one of the emerging mechanisms to regulate the mitochondrial functions (Li *et al.*, 2012; Duarte *et al.*, 2014). Our group previously demonstrated the association of non-coding RNA mainly miRNAs with mitochondria (Sripada *et al.*, 2012). Now the studies suggest that miRNAs regulate the mitochondrial homeostasis starting from biogenesis to degradation. The primary transcription factor in mitochondria, TFAM has been identified as a target of miR-200a (Yao *et al.*, 2014) and miR-494 (Yamamoto *et al.*, 2012). miR-696 targets proliferator-activated receptor gamma co activator 1-alpha (PGC-1α) and modulate fatty acid oxidation capacity and mitochondrial biogenesis (Aoi *et al.*, 2010). miRNAs have been recently identified as controller of mitochondrial dynamic: miR-761 regulates the mitochondrial fission factor (MFF) (Long *et al.*, 2013), miR-140 targets Mfn1 (Li *et al.*, 2014b), miR-484 targets mitochondrial fission protein 1 (Fis1) (Wang *et al.*, 2012a), and miR-30 targets Drp-I (Li *et al.*, 2010).

In addition, miRNAs also have been identified as a regulator of mitochondrial OXPHOS activity. Nuclear encoded the mitochondrial protein, cytochrome *c* oxidase subunit IV plays vital role in ETC and participate in ATP production. miR-338 target cytochrome *c* oxidase subunit IV (COX IV) mRNA in neurons and reduce its protein level (Aschrafi *et al.*, 2008). miR-181c targets electron chain complex IV in cardiomyocytes and control mitochondrial function (Das *et al.*, 2012). Other studies show that miR-181c, miR-210 and miR-338 downregulating Complex IV and miR-141 downregulating Complex V (Bienertova-Vasku *et al.*, 2013) in the mitochondrial ETC. Emerging studies strongly suggest that miRNAs play a vital role mitochondrial homeostasis in normal physiological as the disease condition.

2.3.3.1 Role of miRNAs in mitochondrial homeostasis: In PD

Cross talk of miRNAs and mitochondrial function/ homeostasis in PD pathogenesis is emerging. It has been observed that miR-124 is down regulated in DA neurons of MPTP-induced mice and cell based model of PD and modulate mitochondrial mediated apoptotic pathway (Kanagaraj *et al.*, 2014; Gong *et al.*, 2016). Several studies demonstrate that DJ-1 plays role in mitochondrial complex-I activity by directly binding to its subunits (Hayashi *et al.*, 2009). Knockdown of DJ-1 caused defective mitochondrial morphology and dynamics in neurons (Irrcher *et al.*, 2010). In recent report indicated miR-494 downregulated DJ-1 expression and increases oxidative stress-induced neuronal lesions (Xiong *et al.*, 2014). miR-34b/c also modulate DJ-1 expression indirectly, which regulate mitochondrial function (Minones-Moyano *et al.*, 2011).

In PD brain patient sample, miR-205 expression was significantly down-regulated, which targets LRRK2 (Cho *et al.*, 2013). miR-7 regulate the expression of voltage-dependent anion channel 1 (VDAC1) and stabilize mitochondrial membrane potential in the cellular models of Parkinson disease (Chaudhuri *et al.*, 2016).

All these evidences strongly suggest the role of miRNAs in regulation of mitochondrial function and its implication in PD. However, in literature isolated studies have been initiated however systematic study of miRNAs regulation mitochondrial function, inflammation and cellular quality control system and its implication in pathogenesis of Parkinson disease is lacking.

Table 2.1: List of genes implicated in Parkinson's disease.

Gene Name[1]	Protein Name[2]	Modes of inheritance[3]	Pathways from BioSystems[4]
SNCA	Alpha-synuclein	Autosomal Dominant	• Alpha-synuclein signaling, organism-specific biosystem (from Pathway Interaction Database) • Amyloid fiber formation, organism-specific biosystem (from REACTOME) Ectoderm Differentiation, organism-specific biosystem (from WikiPathways) • Parkin-Ubiquitin Proteasomal System pathway, organism-specific biosystem (from WikiPathways) • Parkinson's disease, organism-specific biosystem (from KEGG) • Parkinsons Disease Pathway, organism-specific biosystem (from WikiPathways)
PINK1	PTEN induced putative kinase 1	Autosomal recessive	• Mitophagy, organism-specific biosystem (from REACTOME) • Mitophagy - animal, organism-specific biosystem (from KEGG) • Mitophagy - animal, conserved biosystem (from KEGG) • Parkinson's disease, organism-specific biosystem (from KEGG) • Parkinsons Disease Pathway, organism-specific biosystem (from WikiPathways) • Pink/Parkin Mediated Mitophagy, organism-specific biosystem (from REACTOME)

PARK2	Parkin	Autosomal recessive	• Deubiquitination, organism-specific biosystem (from REACTOME) • Metabolism of proteins, organism-specific biosystem (from REACTOME) • Mitophagy, organism-specific biosystem (from REACTOME) • Mitophagy - animal, organism-specific biosystem (from KEGG) • Mitophagy - animal, conserved biosystem (from KEGG) • Parkinson's disease, organism-specific biosystem (from KEGG) • Parkinsons Disease Pathway, organism-specific biosystem (from WikiPathways) • Pink/Parkin Mediated Mitophagy, organism-specific biosystem (from REACTOME) • Protein processing in endoplasmic reticulum, conserved biosystem (from KEGG) • Ubiquitin mediated proteolysis, organism-specific biosystem (from KEGG) • Ubiquitin mediated proteolysis, conserved biosystem (from KEGG)
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2	Autosomal Dominant	• Metabolism of proteins, organism-specific biosystem (from REACTOME) • Mitochondrial protein import, organism-specific biosystem (from REACTOME)
LRRK2	Leucine rich repeat kinase 2	Autosomal Dominant	• PTK6 promotes HIF1A stabilization, organism-specific biosystem (from REACTOME) • Parkinson's disease, organism-specific biosystem (from KEGG) • Parkinsons Disease Pathway, organism-specific biosystem (from WikiPathways)
VPS13C	Vacuolar protein sorting 13 homolog C	Autosomal recessive	• Pink/Parkin Mediated Mitophagy
VPS35	Retromer complex	Autosomal Dominant	• Endocytosis, organism-specific biosystem (from KEGG) • Endocytosis, conserved

	component		biosystem (from KEGG) • Signal Transduction, organism-specific biosystem (from REACTOME) • Signaling by Wnt, organism-specific biosystem (from REACTOME)
FBX07	F-box protein 7	Autosomal recessive	• Adaptive Immune System, organism-specific biosystem (from REACTOME) • Antigen processing: Ubiquitination & Proteasome degradation, organism-specific biosystem (from REACTOME) • Class I MHC mediated antigen processing & presentation, organism-specific biosystem (from REACTOME) • Immune System, organism-specific biosystem (from REACTOME)
PARK7	Parkinsonism associated deglycase (DJ 7)	Autosomal recessive	• Alpha-synuclein signaling, organism-specific biosystem (from Pathway Interaction Database) • Parkinson's disease, organism-specific biosystem (from KEGG) • Parkinsons Disease Pathway, organism-specific biosystem (from WikiPathways) • Synaptic Vesicle Pathway, organism-specific biosystem (from WikiPathways)
ATP13A2	ATPase 13A2	Autosomal recessive	• Ion channel transport, organism-specific biosystem (from REACTOME) • Ion transport by P-type ATPases, organism-specific biosystem (from REACTOME) • Transmembrane transport of small molecules, organism-specific biosystem (from REACTOME)

Table 2.1 : List of genes implicated in Parkinson's disease.

[1.] *Name of genes,* [2.] *Name of proteins* [3.] *Modes of inheritance,* [4.] *Pathways from BioSystems*