Energy metabolism and Protein synthesis in Parkinson's disease and Dementia with Lewy Bodies

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ENERGY METABOLISM AND PROTEIN SYNTHESIS IN PARKINSON'S DISEASE AND DEMENTIA WITH LEWY BODIES

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CERTIFICATE

This is to certify that the thesis entitled ¨**Energy metabolism and Protein synthesis in Parkinson's disease and Dementia with Lewy Bodies**¨ is a record of the bona fide work done by Ms. **Koneti Anusha with DNI No: H6059514 and NIUB No :15522662** under my supervision and guidance. This thesis is submitted to the University of Barcelona, Faculty of Medicine under the Program of Doctorate in Biomedicine in fulfilment of the requirements for the award of the degree of Doctorate in Philosophy during the year 2012-15.

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ABBREVIATIONS

AD	Alzheimer´s disease
ADP	Adenosine di-phosphate
AG	Angular gyrus
Ala	Alanine
ALS	Amyotrophic lateral sclerosis
ANT	Adenine nucleotide translocase
APO E	Apolipoprotein E
APS	Ammonium per sulphate
ATP	Adenosine Triphosphate
ATP2B3	ATPase, Ca++ transporting, plasma membrane 3
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4
ATP4A	ATPase, H+/K+ exchanging, gastric, alpha polypeptide
ATP56V0B	H+ Transporting ATPase, lysosomal Vo subunit B
ATP5A	ATP synthase, H+ transporting, mitochondrial F1 complex
ATP5D	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit);
ATP5G2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2 (subunit 9)
ATP5H	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d
ATP5J	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F6
ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G
ATP6V1H	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H
BDNF	Brain derived neurotrophic factor
cDNA	complementary DNA
CMA	Chaperone mediated autophagy
CNV's	Copy number Variations
COMT	Catechol-O-methyltransferase
COX VIIC	Cytochrome C oxidase subunit VIIC
COX	Cytochrome c oxidase
COX II	Cytochrome C oxidase subunit II
COX7A2L	Cytochrome C oxidase subunit 7A2L
CT	Cycle threshold
Da	Dalton (g/mol)
DA	Dopamine
DAPI	4' 1-diamidino-2phenylindole
DAT	Dopaminetransporter
DHA	Docasahexaenoic acid
DJ 1	Daisuke-Junko 1
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid
Drp1	Dynamin-related protein 1
DTNB	5,5´-dithiobis-(2-nitrobenzoic)acid
DUB	Decyl ubiquinol
EDTA	Ethylene diaminetetracetic acid
EGTA	Ethylene glycol tetracetic acid
EIF 2-α	Elongation initiation factor 2α
ER	Endoplasmic reticulum
ETC	Electron transport chain
FAD	Flavine-adenine dinucleotide
FC	Frontal cortex
Fp	Flavoprotein subunit
GABA	Gamma Aminobutyric acid
GBA	Glucocerebrosidase gene
Glu	Glutamine
Gly	Glycine
GPe	Globus Pallidus para externa
GSH	Glutathione
GSSH	Oxidised Gutathione
GTPase	Guanosine triphosphate (GTP)

GUSβ	Glucuronidase, beta
H2O	Water
H2O2	Hydrogen peroxide
HCl	Hydrochloric acid
HCO3	Bicarbonate
HD	Huntington's disease
HNE	4-hydroxynonenal
HPLC	High-performance liquid chromatography
HRP	Horse Radish Peroxidase
Hsp	Heatshock protein
HSP	Heavy strand promoter
IsoPs	Isoprostanes
KCN	Potassium cyanide
kDa	kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
KSS	Kearns-Sayre syndrome
LB	Lewy body
LDH	Lactate dehydrogenase
L-DOPA	L-3,4-dihydroxyphenylalanine
LHPP	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase
LoxP	Cre recombinase recognition site
LRRK2	Leucine rich repeat kinase 2 or dardarin
LSP	Light-Strand protein
Lys	Lysine
MAO	Monoamine oxidase
MAPT	Microtubule Associated tau protein
MDAL	3,4-methylenedioxy-N-allylamphetamine
Mfn1/2	Mitofusin1/2
MgCl2	Magnesium chloride
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger- ribonucleic acid
MRPL21	Mitochondrial ribosomal protein L21
MRPL22	Mitochondrial ribosomal protein L22
MRPS10	Mitochondrial ribosomal protein S10
mt DNA	Mitochondrial DNA
nACh	Nictonic acetylcholine
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NDP	Nigrostriatal Dopaminergic Pathway
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10
NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7
NDUFB 8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10
NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8
NK	Neuroketal
NMDA	N-methyl –D-aspartate
NOS	Nitric oxide synthase
NP40	Nonidet P40
NPs	Neuroprostanes
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
OH	Origin of heavystrand replication
OL	Origin of light strand replication
Opa1	Optic atrophy protein 1
OxPhos	Oxidative phosphorylation
PBS	Phosphate buffered saline
PC	Precuneus

PCR	Polymerase chain reaction
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PEP	Phospho enol pyruvate
Pi	Inorganic phosphate
PINK1	P-TEN induced putative kinase 1
PK	Pyruvate Kinase
POLRMT	Mitochondrial RNA polymerase
POLγ	Mitochondrial DNA polymerase γ
PRKN	Parkin
Pro	Proline
PrP	Protein prion protein
PTP1P51	Protein tyrosine phosphatase-interacting protein 51
PUFA	Polyunsaturated fatty acids
Q	Ubiquinone (coenzyme Q)
QH2	Ubiquinol (reduced coenzyme Q)
RC	Respiratory chain
RIN	RNA integrity number
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RP	Ribosomal protein
RPL21	Ribosomal Protein L21
RPL22	Ribosomal Protein L22
RPL23A	Ribosomal Protein L23A
RPL26	Ribosomal Protein L26
RPL27	Ribosomal Protein L27
RPL30	Ribosomal Protein L30
RPL31	Ribosomal Protein L31
RPL5	Ribosomal Protein L5
RPL7	Ribosomal Protein L7
Rpm	Rounds per minute
RPS10	Ribosomal Protein S10
RPS13	Ribosomal Protein S13
RPS16	Ribosomal Protein S16
RPS17	Ribosomal Protein S17
RPS20	Ribosomal protein S20
RPS3A	Ribosomal Protein S3A
RPS5	Ribosomal Protein S5
RPS6	Ribosomal Protein S6
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDHB	Succinate dehydrogenase complex, subunit B
SDS	Sodiumdodecylsulfate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error mean
SLC25A31	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide transclocator member 31
SLC6A6	Solute carrier family 6 (neurotransmitter transporter), member 6
SN	Substantia nigra
SNCA	alpha synuclein
SNpc	Substantia nigra pars compacta
SOD2	Superoxide dismutase 2
STN	Subthalamic nucleus
TCA	Tricarboxylic acid
TFAM	Trancriptional factor A mitochondrial
TFAM	Mitochondrial transcription factor A
TFB2M	Mitochondrial transcription factor B2
Thr	Threonine

TOMM 40	Translocase of outer mitochondrial membrane 40
TOMM 70	Translocase of outer mitochondrial membrane 70
tRNA	Transfer RNA´s
UPR	Unfolded protein response
UPS	Ubiquitin Proteasome system
UQCRC2	Ubiquinol-cytochrome c reductase core protein II
VDAC	Voltage-dependent anion channel
ZNF642	zinc finger protein 642

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3. MATERIALS AND METHODS

4. RESULTS

I. MITOCHONDRIA –Objective 1

INTRODUCTION

---The power of brain is supreme and strength comes afterwards.

------Chanakya neethi-----

1. INTRODUCTION

1.1 PARKINSON'S DISEASE AND DEMENTIA WITH LEWY BODIES

Parkinson's disease (PD) is a progressive neurodegenerative disease first characterised by James Parkinson in 1817 in his monograph "Essay on the Shaking Palsy" as characterized by *"Involuntary tremolous motion with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forewords and to pass from a walking to a running pace: the senses and the intellect being injured"* (Parkinson J. 2002). It is clinically defined as a movement disorder, but is often accompanied by cognitive decline (Wüllner et al., 2007; Park and Stacy, 2009; Goldstein et al., 2010). In 1919 Konstantin Tretiakoff reported that the *Substantia nigra* (SN) is the main cerebral part affected in PD and finally in 1958, Arvind Carlsson (Nobel Prize in 2000) discovered that patients showed a massive loss of the neurotransmitter dopamine in the brain and its role in PD and thus motor dysfunction in PD is caused by the selective degeneration of dopaminergic neurons in the *Substantia nigra* (SN).

Clinical characteristics include resting tremor, slowness of movement (bradykinesia). Loss of movement (akinesia) and postural instability may also develop as pathology progresses (Reichmann, 2010). A loss of olfaction (hyposmia) often precedes motor dysfunction (Wattendorf et al., 2009; Politis et al., 2010) and is attributed to defects in cholinergic circuits (Bohnen et al., 2010). Other non-motor symptoms can develop in late stage disease and include dementia, hallucinations, sleep disturbances, autonomic dysfunction and depression (Wüllner et al., 2007; Park and Stacy, 2009; Politis et al., 2010), (Table 1.1). These symptoms associate with proteinaceous intraneural inclusions called Lewy Bodies (LB) (Gómez-Tortosa et al., 2000; Valls-Solé and Valldeoriola, 2002).

Clinically, individuals without parkinsonian symptoms (incidental PD stages 1 or 2) due to fatal concurrent events with disrupted life expectancies are considered as incidental Parkinson's disease (iPD). The extent of Lewy body (LB) pathology correlates with PD development, initially affecting brainstem and subcortical nuclei and then progressing into the neocortex (Braak et al., 2006). The primary site of pathology differentiates PD from Dementia with Lewy bodies (DLB), as the later is associated with early development of LB's in the cortex (Iseki, 2004; Neef and Walling, 2006). The involvement of these lesions in neurodegeneration remains unclear, despite strong correlations between their presence and clinical disease progression (Lees, 2009). Clinical characteristics of DLB include a progressive cognitive decline of sufficient magnitude to interfere with normal social or ocupational function. Prominent or persisitent memory impairment may not necessarily occur in early stages, but is usually evident with progression. Also inlcude recurrent visual hallucinations, repeated falls, syncope or transient loss of conciousness, neuroleptic sensitivity, systemized delusions and non-visual hallucinations (Mc Keith et al., 1996). Due to a lack of specific and accessible biomarkers, PD cannot be definitively diagnosed until LB's are identified in the SN at autopsy. However, a significant proportion of the aged population have a asymptomatic LB pathology upon post-mortem examination (Lippa et al., 2007). Clinical and post-mortem diagnosis are further complicated by concomitant neurodegenerative conditions (Lippa et al., 2007; Morgan et al., 2010), and ill-defined boundaries between PD and DLB pathology (Linazasoro, 2007). Furthermore, several clinicians have proposed that these conditions exist within a single disease entity and stem from a common pathogenic mechanism (Richard et al., 2002; Aarsland et al., 2004; Emre et al.,2007; Linazasoro, 2007; Goldmann Gross et al., 2008; Aarsland et al., 2009; Lees, 2009). This view remains contentious however, and the defined clinical outcome of each disease reflects differences in the affected neuronal populations (Richard et al., 2002). Despite continuing debate, PD and DLB are now recognised as multi-system disorders, characterised by sequential and progressive

neurodegeneration of cortical and mesencephalic neurons (Linazasoro, 2007). The temporal sequence distinguishes DLB from another related condition, Parkinson's disease dementia (PDD). The term DLB is used when dementia develops before, or within one year after, parkinsonism onset. The term PDD is used when dementia appears more than one year after the onset of otherwise typical Parkinson's disease. However, PDD and DLB may be manifestations of the same neurodegenerative disorder, possibly related to the abnormal accumulation of alpha-synuclein. Clinically PDD includes additional deficits in recognition memory, attention processs and visual perception (Pagonabarraga and kulivesky, 2012; Kehagia et al., 2013), as well as visual hallucinations and cognitive fluctuations (Emre, 2003).

Table 1.1 Motor and non-motor symptoms of Parkinson's disease

Motor	Non motor symptoms
Tremor	Mood disorders : Depression, anxiety and apathy
Rigidity	Cognition : Bradyphrenia, dementia
Bradykinesia	Sleep disorders : Sleep fragmentation, REM sleep disorders, excessive daytime sleepiness, altered sleep-wake cycle
Postural instability	Autonomic disorders : Hypotension,constipation,destrusor dyssynergia,sexual dysfunction,seborrhea,sweating

1.1.1 Epidemiology

PD is the second most common age related neurodegenerative disease (six million patients worldwide) after Alzheimer's Disease (AD). Cole et al., 1996; Papapetropoulos and Mash 2005). The prevalence (proportion in a population at a given time) of PD is about 0.3% of the whole population in industrialized countries. It is more common in the elderly and prevalence rises from 1% in those over 60 years of age to 4% of the population over 80 (de Lau and Breteler, 2006). The mean age of onset is around 60 years, although 5-10% of cases, classified as young onset, begin between the ages of 20 and 50 and are probably hereditary. Some studies have proposed that it is more common in men than women, but others failed to detect any differences between the two sexes (de Lau and Breteler, 2006; Martin, 2010; Samii et al., 2004). DLB which is increasingly recognized as a common cause

of dementia in older people. However, its true frequency remains unclear, with studies reporting a prevalence range from zero to 22.8% of all dementia cases. Recent review on DLB acounted for 4.2% of all diagnosed dementias in the community. In secondary care this increased to 7.5%. The incidence of DLB was 3.8% of new dementia cases (Vann Jones and O'Brien 2014). Findings in America have reported that the overall incidence rate of DLB is lower than the rate for Parkinson's disease. DLB risk increases steeply with age and is markedly higher in men. However, this men-to-women difference may suggest different etiologic mechanisms. The study also reported that the incidence of PDD was substantially lower than that of DLB (overall incidence rate of 2.5 versus 3.5 cases per 100,000 person-years) (Rodolfo Savica et al., 2013).

1.1.2 The Nigrostriatal Dopaminergic Pathway

The motor symptoms associated with PD are a direct consequence of decreased dopamine secretion in the Nigrostriatal Dopaminergic Pathway (NDP), caused by selective degeneration of dopaminergic neurons in the SN. The SN innervates the NDP, which is composed of a circuit of connections through the basal ganglia and thalamus, and include the *corpus striatum*, the *nucleus accumbens*, the *ventral striatum* and the *ventral pallidum.* The *corpus striatum* is further divided into the *neostriatum* (consisting of the caudate nucleus and the putamen) and the globus pallidus. (Albin, 2006; Lees, 2009; Lees et al., 2009). A diagrammatic representation of the basal ganglia is given in Figure 1.1.

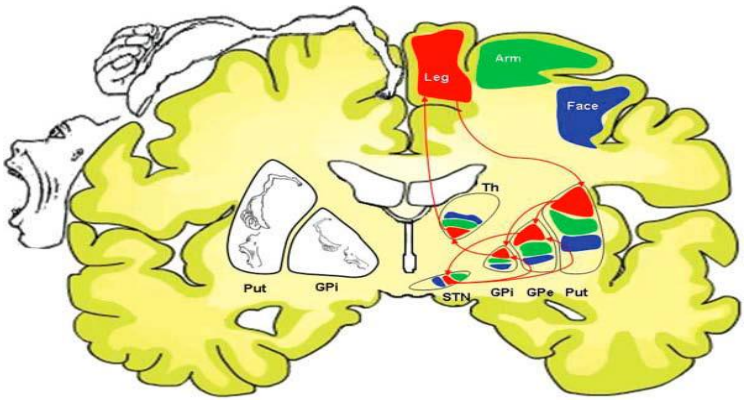


Figure 1.1 Functional organisation of the basal ganglia . Cortical inputs reach the basal ganglia through corticostriatal projections onto medium spiny neurons, which subsequently innervate the globus pallidus pars externa (GPe) and the subthalamic nucleus (STN). The cortical projections exert an opposite (inhibitory/excitatory) effect onto the GPe and globus pallidus pars interna. The GPe projects to the striatum and STN and inhibits their activity. (Adapted from Obeso and colleagues 2006).

The putamen has the most significant role in mediating motor function and is the area most affected by dopamine loss in PD (Crossman, 2000). The striatum is innervated by efferent signals from the cortex and dopaminergic projections from the *Substantia nigra* pars compacta (SNpc), the ventral tegmental area and the retrorubral area (Smith and Kievel, 2000). In turn, efferent circuits from the striatum innervate the thalamus through direct and indirect striatopallidal pathways, which are diagrammatically represented in Figure 1.2.

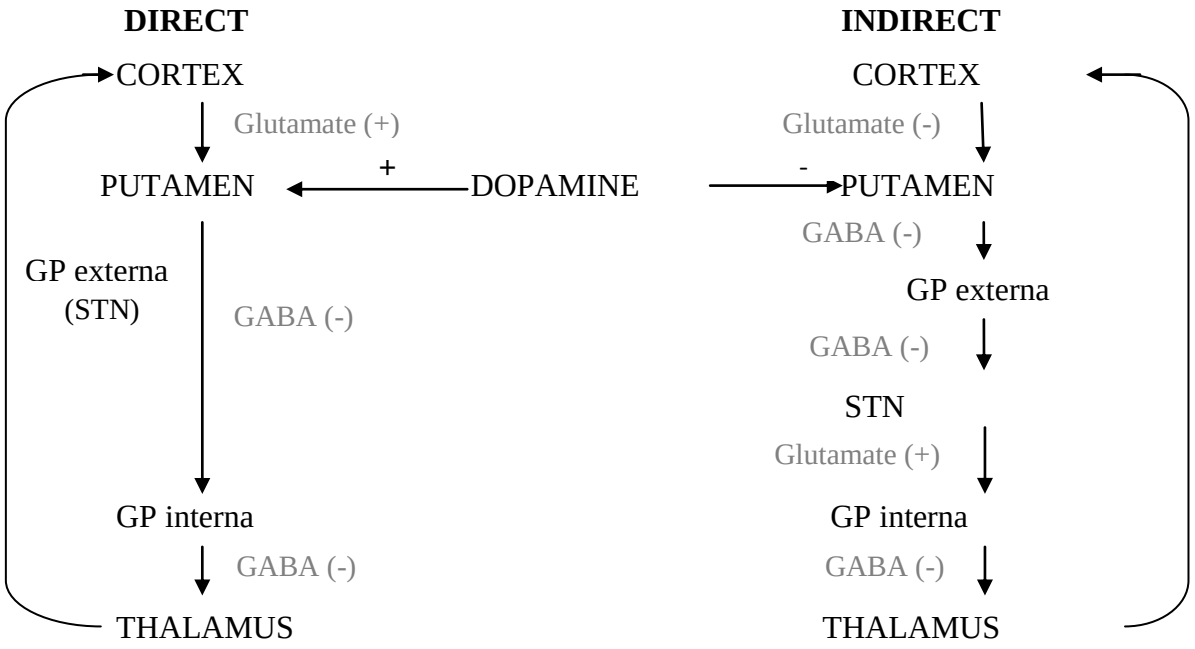


Figure 1.2 The effect of dopamine on the direct and indirect striatopallidal pathways Dopamine release into the putamen regulates movement initiated by the motor cortex via the direct inhibitory, and indirect pathways. Dopamine regulates striatal activity by inhibiting the indirect pathway and facilitating the direct pathway.

Disease-related neurodegeneration decreases dopaminergic input into the dorsolateral putamen and basal ganglia causing an imbalance in the relative activities of the direct and indirect striatopallidal pathways. As a result, output from the putamen is reduced and the excitatory activity of the direct pathway is consequently decreased (Marsden and Obeso,1994; Crossman, 2000; Herrero et al., 2002). In the nigrostriatal circuit, the (SNpc) receives GABAnergic inhibtion from the striatum and feeds back to the striatum with a modulationg dopaminergic input. Dopamine excites striatal neurons, which project to the medial globus pallidus/ SN pars reticularis and thereby releases thalamic

inhibition, maintaining normal speed and tone of movement.The resulting lack of activity of the ventral anterior and ventral lateral thalamic nuclei leads to hypoactivity of the motor cortex and corticospinal tract and consequent inhibition of cortically initiated motor function. These deficits are the direct cause of the motor symptoms of PD, namely akinesia and bradykinesia (Marsden and Obeso, 1994; Crossman, 2000; Galvan and Wichmann, 2008; Reichmann, 2010). The lack of dopamine release from the nigrostriatal pathway prevents activation of neurons within the globus pallidus pars externa (GPe), resulting in dis-inhibition of the STN and hypoactivity of the indirect striatopallidal pathway (Crossman, 2000; Galvan and Wichmann, 2008; Reichmann, 2010).

1.1.3 Neurobiological basis of non-motor symptoms of PD and DLB

The pathology of non-motor symptoms in both PD and DLB correlates with the anatomical localisation of LB's. Specifically, sympathetic denervation of the heart and gastrointestional tract are associated with LB pathology in sympathetic ganglia and the dorsal nucleus of the vagus nerve, respectively (Del Tredici et al., 2002; Orimo et al., 2005; Fujishiro et al., 2008). Psychological symptoms are attributed to deficiencies in noradrenaline and serotonin, which correlate with LB pathology in the *locus coeruleus* and *raphe* (Cheng et al., 1991; Richard et al., 2005). Cholinergic deficits are well documented in PD and DLB and contribute to dementia (Dickson et al., 2009). However, LB pathology remains the most significant correlate of cognitive decline (Maidment et al., 2006; Dickson et al., 2009). LB's are eosinophilic intraneural lesions comprised of multiple proteins and lipids. They were identified by Frederic Lewy in 1912 (Shults, 2006) and their presence within neurons is a pathological characteristic of PD, DLB and neurodegeneration with brain iron accumulation (Hallervorden-Spatz syndrome) (Arawaka et al., 1998; Spillantini et al.,1998; Hardy et al., 2006). These inclusions are usually found within soma but can be localised to the neurites (Lewy neurites) (Shults, 2006). LB's are classified into two major morphological subtypes, namely 'classical' and 'atypical cortical' LB's (Figure 1.3).

Classical LB's are localised to the SN and *locus coeruleus* and comprise a dense granular core surrounded by a fibrillar corona (Figure 1.3A) (Goedert, 1999; Shults, 2006). Atypical cortical LB's are localised to the cortical brain regions (Kosaka et al., 1984), they appear to be entirely composed of fibrillar proteins and are of a uniform density (Figure 1.3B) (Spillantini et al., 1997; Spillantini et al., 1998; Wakabayashi et al., 2007). LB's are composed of a variety of proteins and lipids, although alpha-synuclein comprises the main constituant of protein fibrils (Figure 1.3C) (Spillantini et al., 1998).The second major protein component of LB's is ubiquitin, which associates with proteins

within the fibrilar region and the granular core (Shults, 2006). Some differences in the protein composition of classical and atypical cortical LB's have been described (Wakabayashi et al., 2007). LB's are present prior to overt cell death (Braak et al.,2006), although their causal role in neurodegeneration is undefined.

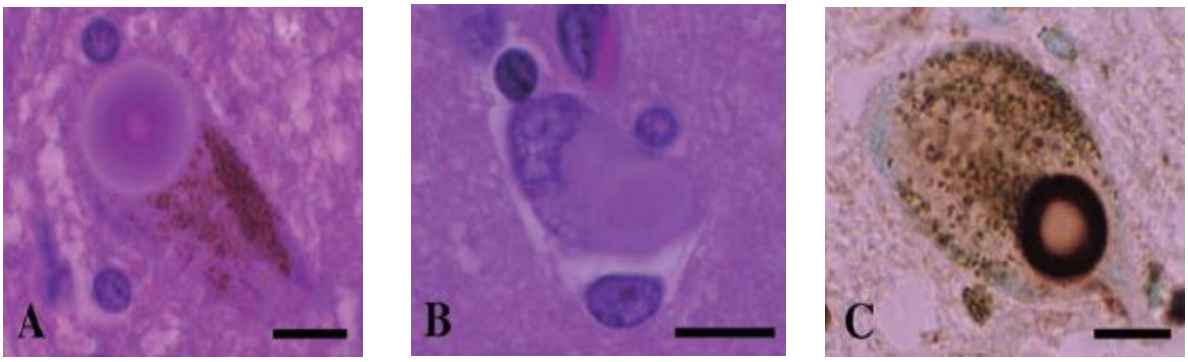


Figure 1.3 Representation of lewy bodies Lewy bodies in tissue from patients with PD (A,C) and DLB (B). A pigmented neuron in the substantia nigra containing a 'classical' LB (A) stained with hematoxylin and eosin. Hematoxylin and eosin staining of an atypical cortical LB (B) in the temporal cortex of a patient with DLB. A typical LB in a pigmented neuron in the SN labeled with anti-α-synuclein (C). Scale bar = 10 μM Images from (Wakabayashi and colleagues, 2007).

1.1.4 Neuropathological staging of PD and DLB

Braak and colleagues (2006) defined six stages of PD progression according to the extent of LB pathology and dopaminergic degeneration. The first two stages of disease are associated with Lewy body development within the vagal nerve of the medulla oblongata, the caudate nucleus, the intermediate reticular zone, the gigantocellular reticular nucleus and the locus coeruleus. These stages do not correlate with symptomatic manifestations of PD (Braak et al., 2006). The SNpc is not effected until stage-three of the disease (Shults, 2006). Stage-four is marked by the formation of Lewy bodies in the cortex (confined to the temporal mesocortex) and the basal forebrain. Their notable loss of melanin-containing dopaminergic neurons of the SN, although clinical symptoms do not manifest until stage-five of the disease when Lewy body pathology advances through the mesocortex. Pathology then begins to affect the neocortex and primary sensory and motor fields of the cerebral cortex (stage-six) (Braak et al., 2006) shown in (Figure 1.4). The SN and the *locus coeruleus* have the highest concentration of LB's in post-mortem tissue (Mezey et al., 1998). Despite

strong acceptance of Braak staging, several recent post-mortem studies have shown differing patterns of LB progression (Burke et al., 2008; Jellinger, 2008).

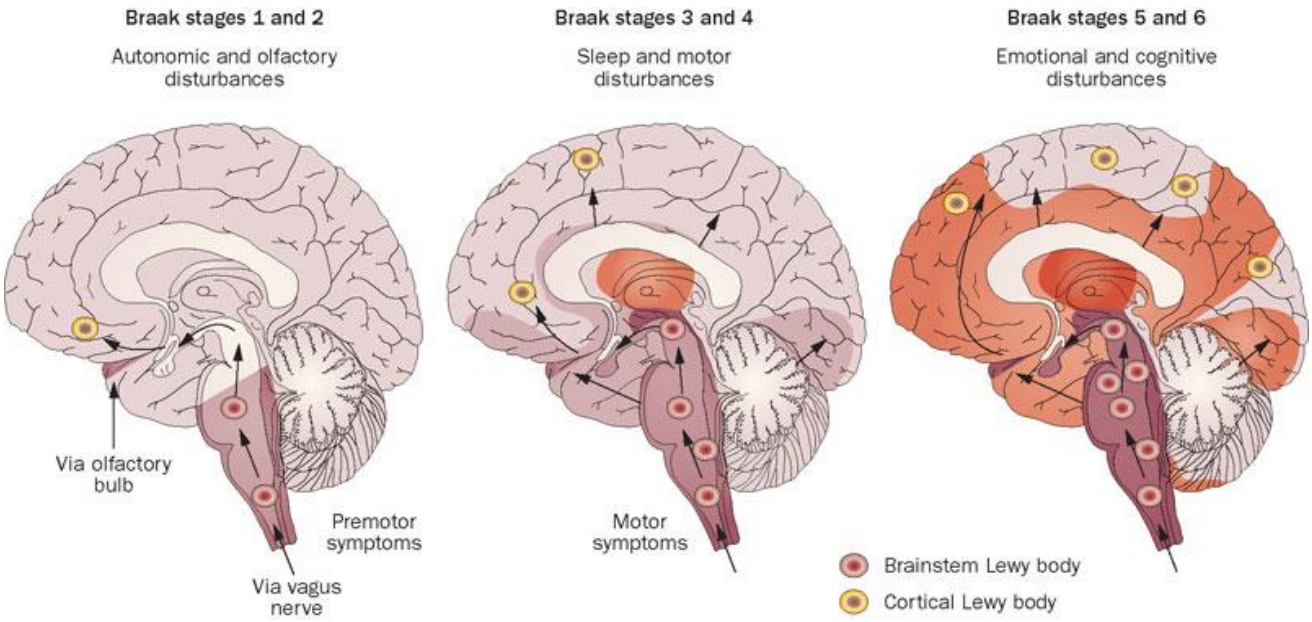


Figure 1.4 Braak staging of brain in Parkinson's disease The Braak staging system of Parkinson disease, showing the initiation sites in the olfactory bulb and the medulla oblongata, through to the later infiltration of Lewy pathology into cortical regions. (Adapted from Richard and colleagues 2012)

The staging of pathology in DLB is less understood. There are three pathological subtypes of the disease, which are determined by the localisation of LB pathology (McKeith et al., 1996). Type-1 DLB primarily affects the subcortical and brainstem regions. Type-2 DLB describes concurrent brainstem and cortical pathology. Type-3 pathology is specifically localised to the cortex, initially in the amygdala then progressing through the limbic regions and the neocortex (McKeith et al., 1996; Yamamoto et al., 2005). Although LB pathology correlates with clinical signs of disease there is little evidence to suggest that it is the primary cause of cell loss. Post-mortem studies have identified LB pathology in the surviving neurons of the SNpc, indicating that these inclusion bodies do not cause acute cell death (Samii et al., 2004). There is some evidence that Lewy body pathology and neurodegeneration are caused by distinct pathogenic mechanisms. Specifically, mutations to the *PRKN* gene cause a Parkinsonism syndrome, which pathologically resembles PD without inclusion bodies, suggesting a lack of association between these processes. Tompkins and Hill (1997), directly compared dopaminergic neurons with and without LB's and indicate inverse correlation between

these inclusions and propensity to undergo apoptosis (Tompkins and Hill, 1997). Contrary to these findings, a gene expression profile study conducted by Lu and colleagues (2005) indicates that genes associated with cell survival were expressed at a higher level in neurons lacking Lewy bodies (Lu et al., 2005).

1.1.5 Current Treatment

Current therapies can aid in controlling the motor symptoms of PD but do not reverse cell loss or prevent further degeneration. Dopamine replacement therapy (levodopa) is the first line of drug treatment for PD patients (Antonini, 2010; Poewe et al., 2010). However, the symptomatic benefits of levodopa decrease over time and fluctuations in motor response occur in the majority of patients after three to five years of treatment. The severity of these fluctuations correlates with the duration of therapy (Abbruzzese, 2008). Levodopa is often used concomitantly with second line therapies such as monoamine oxidase-B inhibitors, dopamine receptor agonists and catechol-*o*-methyl transferase inhibitors. These agents act to augment the effects of levodopa (Singh et al., 2007). Non-pharmacological therapies are recognised as potential methods in PD treatment. Deepbrain stimulation of the subthalamic nucleus is a well established technique of improving motor function (Kopell et al., 2006), although, its use is appropriate for just 5-10% of people with PD due to the strict patient criteria (Pereira and Aziz, 2006). Foetal mesencephalic graft transplantation is an alternative therapy, which offers potential restoration of the NDP and reversal of motor symptoms. Recent clinical trials indicate that these grafts can improve motor function (Olanow et al., 2009), although the success of these grafts is limited and post-mortem analysis indicates LB pathology in dopaminergic neurons of the graft tissue (Snyder and Olanow, 2005; Li et al., 2010). Further development of this technique is hindered by current legal and ethical limitations (Singh et al., 2007). Gene therapy is also undergoing clinical trial in PD patients and some vectors show early indications of success (Feng and Maguire-Zeiss, 2010; Marks et al., 2010).

1.1.6 Etiology

Age is the greatest risk factor associated with PD (Albin, 2006; Fahn, 2010). PD affects approximately 2% of the male population and 1.3% of females over 60 years of age. The incidence increases exponentially past this age to affect up to 5% of the total population over 85 years (de Rijk et al., 1995; Rao et al., 2006). Females are less susceptible owing to the neuroprotective properties of oestrogen. This female sex hormone stimulates the production of brain-derived neurotrophic factor

(BDNF), which facilitates differentiation and survival of dopaminergic neurons (Nishio et al., 1998; Cyr et al., 2002; Sohrabji and Lewis, 2006). BDNF levels are reportedly decreased within the SN in PD patients (Nishio et al., 1998; Mogi et al., 1999; Parain et al., 1999; Howells et al., 2000). The underlying cause of PD and DLB is often attributed to an 'interplay between environmental factors and genetic factors (Horowitz and Greenamyre, 2010), although 90% of cases have no known genetic cause (Vance et al., 2010). It is thought that behavioural factors, specifically high consumption of iron, manganese, lutein, cholesterol and saturated fats, as well as incidences of head trauma, contribute to idiopathic disease pathogenesis (Johnson et al., 1999; Bower et al., 2003; Goldman et al., 2006; Powers et al., 2009). However, these risk factors do not consistently correlate with disease across independent studies (Powers et al., 2009; Spangenberg et al., 2009) and in most incidences the evidence for environmental contributors is weak (Hardy, 2006). Discrepancies within the segregation of PD among identical twins suggest that environmental factors do contribute to disease pathogenesis (Tanner et al., 1999). However, these findings are equally representative of a self propagating disease such as a prion disease (Hardy, 2006). A 'prion-like phenomenon' is currently proposed as an alternative hypothesis for the pathogenesis of neurodegenerative conditions associated with protein aggregation, including PD and DLB (Desplats et al., 2009; Olanow and Prusiner, 2009; Angot et al., 2010; Goedert et al., 2010). As the common molecular pathology invloves the conversion of a normal cellular protein prion protein (PrP) into abnormal isoform.

Reports also say that there is a strong inverse relationship between cigarette smoking and PD, with regular smokers having up to a 50% decreased risk of developing the disease (Tanner et al., 2002). Nicotine acts directly on nicotinic acetylcholine (nACh) receptors on dopaminergic neurons of the nigrostriatal pathway. This binding interaction stimulates the release of DA, increasing dopaminergic innervation of striatal neurons and relief of PD symptoms (Khwaja et al., 2007; Quik et al., 2007; Quik et al., 2009). Clinical manifestations of PD have been linked to exposure to the common pesticides rotenone and paraquat (Langston and Ballard, 1983; Giasson and Lee, 2000; Mccormack, 2002). These agents cause toxicity through induction of mitochondrial and cytosolic free radical production, respectively (Richardson et al., 2005; Ramachandiran et al., 2007). Administration of either paraquat or rotenone directly into the NDP of rodents causes selective degeneration of nigrostriatal dopaminergic neurons (Somayajulu-Nițu et al.,2009; Pan-Montojo et al., 2010). Rotenone toxicity is also linked to the formation of intracellular inclusions which biochemically resemble LB's (Betarbet et al., 2000). There is a strong correlation between familial PD and symptomatic onset prior to the age of fifty years (Fahn, 2010).

Multiple mutations in two distinct genes (*SNCA* and *LRRK2*; Table 1.2) cause dominant Mendelian inheritance of PD. These familial cases are characterised by both neurodegeneration and LB pathology (Hardy et al., 2006; Shulman et al., 2011). Mutations to three genes (*PRKN*; *PINK1* and *DJ-1*; Table 1.2) are associated with recessive inheritance of PD or Parkinsonism, although the occurrence of LB pathology in these conditions is variable (Shulman et al., 2011).

Table 1.2 Genes associated with Mendelian inheritance of PD and Parkinsonism (Adapted from Wray and colleagues 2010)

PARK	Gene	Inheritance	Phenotype	Pathology
1	α-Synuclein	Dominant	Complex mix of Parkinsonism and dementia	Lewy bodies
2	Parkin	Recessive	Juvenile onset Parkinsonism	Nigral cell death, some with Lewy body pathology
6	PINK1	Recessive	Juvenile onset Parkinsonism	One reported case with Lewy bodies
7	DJ1	Recessive	Juvenile onset Parkinsonism	No reported pathology
8	LRRK2	Dominant	Typical Parkinson's disease	Mixed pathology: Lewy bodies tangles and TDP43
9	ATP13A2	Recessive	Juvenile onset Parkinsonism	No reported pathology
14	PLA2G6	Recessive	Juvenile onset Parkinsonism dystonia	Lewy bodies
15	FBXO7	Recessive	Juvenile onset Parkinsonism	No reported pathology
	GBA	Dominant	Typical Parkinson's disease	Lewy bodies
	PANK2	Recessive	Juvenile onset Parkinsonism dystonia	Lewy bodies in NBIA-1 cases
	Tau	Dominant	Frontal temporal dementia with Parkinsonism	Tangles

UCHL1, HTRA2, NR4A2 have all been linked to Parkinson's disease but are of uncertain provenance. The causative genes for PARK 3, 10, 11, 12, 13, 16 remain to be identified.

There is a disproportionately high incidence of glucocerebrosidase gene (*GBA*) mutations in sporadic PD patients (Neumann et al., 2009; Sidransky et al., 2009). It remains undetermined whether these incidences represent a strong susceptibility locus or a Mendelian inheritance pattern with incomplete penetration (Shulman et al., 2011). Other susceptibility loci are found within the *MAPT* gene, encoding the microtubule associated tau protein (Pankratz et al., 2009). Substitution mutations within the *SNCA* gene encoding alpha-synuclein are the most common cause of familial PD and DLB. Four mutations (Ala53Thr (A53T); Ala30Pro (A30P); Glu46Lys (E46K) and Gly209Ala (G204A)), in addition to genomic multiplications of the gene, are associated with dominant disease inheritance (Polymeropoulos et al., 1997; Teive et al., 2001; Seidel et al., 2010). PD caused by mutations to alpha-synuclein has an aggressive phenotype characterised by extensive LB pathology and early age of onset (Polymeropoulos et al., 1997; Spira et al., 2001). The Glu46 Lys mutation segregates with a PD phenotype particularly associated with dementia (Zarranz et al., 2004). Even though several

advances in the understanding of the genetics underlying neurological diseases have been made. The same however has not been true for DLB and the disease etiology still remains mostly elusive, with only a small number of reports suggesting the involvement of *APOE* and *GBA* as risk factors for the development of the disease (Clark et al., 2009; Kobayashi et al., 2011; Pickering-Brown 1994; Hardy et al., 1994). DLB is generally considered a sporadic disorder, but a few cases of familial aggregation have been described. However, even in such cases, the identification of the underlying causal gene has not been a successful endeavor (Bogaerts et al., 2007).

1.1.6.1 PD associated major genes

1.1.6.1.1 Autosomal Dominant PD genes

α-Synuclein

The *SNCA* gene (also known as *PARK1*) was the first causal PD gene identified segregating a pathogenic missense mutation—p.Ala53Thr—in a large Italian family (Polymeropoulos et al., 1996,1997) and was unequivocally associated with familial PD. The 144aa *SNCA* protein encoded by the three different *SNCA* transcripts is typically found as a natively unfolded, soluble protein in the cytoplasm or associated with lipid membranes (Davidson et al., 1998). In addition to three point mutations, several PD families were recently identified as carrying single-allele triplication (initially assigned a separate locus, *PARK4*, (Singleton et al., 2003) but later corrected or duplication events in the wild-type *SNCA* gene (Chartier-Harlin et al., 2004). Interestingly, the severity of the phenotype appears to depend on gene dosage, and patients with *SNCA* duplications bear a closer clinical resemblance to idiopathic PD patients than do patients with triplications. Nevertheless, both MIS-SENSE and multiplication events are extremely rare (Berg et al., 2005). The exact biological function of *SNCA* in brain is still not fully understood, although there is evidence that implicates *SNCA* in neurotransmitter release and vesicle turnover at the presynaptic terminals (Abeliovich et al., 2000; Liu et al., 2004 Vekrellis et al., 2004). Intriguingly, fibril-forming, phosphorylated species of *SNCA* were found to be abundant in insoluble inclusions (Lewy bodies and Lewy neurites), prompting neuropathologists to group several *SNCA*-inclusion-rich diseases together. These 'synucleinopathy disorders' (a term coined by Trojanowski and Lee 2002) primarily encompass sporadic PD, *SNCA*-linked PD, DLB, and multiple-system atrophy, Schlossmacher et al., (2004), but can also be variably found in other neurodegenerative síndromes, Nevertheless, the elucidation of the relevant neurotoxic

SNCA species *in vivo* and the critical steps required for the gain-of-function mechanism remain areas of intense research activity.

Leucine-rich repeat kinase 2 or dardarin

The leucine-rich repeat kinase 2 gene (*LRRK2* also known as *PARK8*) was the second causal gene linked to autosomal dominant inherited PD (Funayama et al., 2002; Paisan-Ruiz et al., 2004; Zimprich et al., 2004a, 2004b). *LRRK2* is a large gene and its transcript contains 51 exons which encodes a 2,527-amino-acid protein named *LRRK2* or Dardarin protein (Paisan-Ruiz et al., 2004). LRRK2 comprises several functional domains suggestive of on the one hand a kinase activity dependent on the GTPase function of the Roc domain and on the other hand a scaffold protein function implied by the multiple protein–protein inter-action regions. Of interest is that *LRRK2* was shown to form dimers under physiological conditions (Greggio et al., 2008). The exact biological function of *LRRK2* remains largely unknown, because no physiological substrates have been identified so far. Numerous working models have been proposed to integrate the complexities of environmental, biochemical, genetic and neuropathological evidence, but a more simplistic model of PD pathogenesis depicts a progressive imbalance between the forces that promote the degeneration of at-risk neurons by increasing mitochondrial dysfunction, oxidative stress, iron accumulation and lipid dysregulation during the aging process, and those that encompass individual or integrated cellular-defense mechanisms.

1.1.6.1.2 Autosomal recessive PD genes

PARK2 or parkin

The first of three recessive PD genes identified is *PARK2* , which was linked with disease in a nuclear Japanese consanguineous family (Kitada et al., 1998). *PARK2* spans approximately 1.38Mb and encodes the protein parkin. The 456 amino acid protein harbors four major functional domains corresponding to its function as an E3 ubiquitin ligase (Imai et al., 2000; Shimura et al., 2001; Zhang et al.,2000). Its role in the ubiquitin proteasome system (UPS) comprises of tagging dysfunctional or excessive proteins for degradation. Further, it was shown that under physiological conditions parkin is involved in mitochondrial maintenance (Deng et al., 2008a; Exner et al., 2007; Park et al., 2009; Poole et al., 2008; Weihofen et al., 2009) and might induce subsequent autophagy of dysfunctional mitochondria (Narendra et al., 2008, 2009). The first mutation reports indicated a wide spectrum of

loss-of-function mutations in *PARK2* including simple mutations like nonsense, missense and splice site mutations, indels, as well as copy number variations (CNVs) of the promoter region and single or multiple exons (Hattori et al., 1998a, b; Kitada et al., 1998). *PARK2* mutations were identified spread across the entire gene in either homozygous, compound heterozygous or heterozygous state in familial and sporadic patients from different ethnicities. Heterozygous *PARK2* variants have also been observed in healthy control individuals, making assessment of pathogenicity for these variants quite complex. Approximately 40% of unrelated mutation carriers were reported to harbor a mutation in *PARK2*. Of these, close to 8% carry both a simple mutation as a CNV, whereas carriers of only simple mutations or CNVs are almost equally common (43.8% vs.47.9%). Investigation of the haplotypes on which frequent *PARK2* mutations reside, showed that most CNVs are independent events, whereas point mutations were more commonly transmitted from common founders (Periquet et al., 2001). This suggests that the high mutation frequency in *PARK2* is only partly due to small founder effects.

P-TEN-induced putative kinase 1

Homozygosity mapping in *PARK2* negative European families led to the identification of the second autosomal recessive gene, P-TEN induced putative kinase 1*(PINK1)* (Valente et al., 2001, 2002, 2004a). The PINK1 protein is a putative serine/threonine kinase involved in mitochondrial response to cellular and oxidative stress (Valente et al., 2004a). This response is likely mediated by regulation of the calcium efflux, influencing processes such as mitochondrial trafficking (Wang and Schwarz, 2009; Weihofen et al., 2009), ROS formation, mitochondrial respiration efficacy (Liu et al., 2009), and opening of the mitochondrial permeability transition pore (Gandhi et al., 2009) as well as by interaction with cell death inhibitors and chaperones (Plun-Favreau et al., 2007; Pridgeon et al., 2007; Wang et al., 2007). In addition, *PINK1* is an important player in the alleged PINK1/parkin pathway, regulating mitochondrial morphology and functionality in response to stressors (Deng et al., 2008a; Exner et al., 2007; Park et al., 2009; Poole et al.,2008; Weihofen et al., 2009). The *PINK1* mutation spectrum involves nonsense and missense mutations, indels, and whole-gene or single/multiple exon CNVs located across the entire gene. Mutation analyses in familial as well as sporadic patients identified homozygous and compound heterozygous mutations. Approximately 6.5% of known mutation carriers carry a mutation in *PINK1*. Again, many putative pathogenic mutations were also observed in heterozygous state in familial and sporadic patients as well as in healthy control individuals (Abou-Sleiman et al., 2006; Bonifati et al., 2005; Brooks et al., 2009; Choi et al., 2008; Djarmati et al., 2006; Fung et al., 2006a; Healy et al., 2004; Klein et al., 2005; Kumazawa et al.,

2008; Mellick et al., 2009; Nuytemans et al., 2009; Rogaeva et al., 2004; Tan et al., 2005, 2006b; Valente et al., 2004b; Weng et al., 2007). With the current available mutation data, it seems that CNVs in *PINK1* are less common than simple loss-of-function mutations. But at this stage we cannot exclude that this observation represents an ascertainment bias because many studies did not perform *PINK1* dosage analyses and therefore might have missed CNVs in their patient groups.

PARK7 or DJ-1

The third autosomal recessive PD gene, *PARK7* (or Daisuke-Junko-1 (DJ-1)) was identified by homozygosity mapping in an extended Dutch family with multiple consanguinity loops (Bonifati et al., 2003; van Duijn et al., 2001). The *DJ-1* protein was found to be H_2O_2 responsive suggesting that *DJ-1* represents a sensor for oxidative stress, for example, dopamine toxicity (Lev et al., 2009), and acts as an antioxidant (Mitsumoto and Nakagawa, 2001). It was further hypothesized that DJ-1 could be part of a novel E3 ligase complex together with parkin and *PINK1* (Xiong et al., 2009). Mutation analyses identified homozygous, compound heterozygous as well as heterozygous (Bonifati et al., 2003; Clark et al., 2004; Hague et al., 2003; Hedrich et al., 2004a; Nuytemans et al., 2009) missense mutations and CNVs. Also for *PARK7*, heterozygous variants were observed in control individuals. Mutations in *PARK7* are reported near 1% of all known mutation carriers. Current mutation data indicates that CNVs in *PARK7* are less frequent than simple mutations. But, because of the rarity of mutations in *PARK7*, most studies have not analysed their PD patient groups, making it highly likely that putative pathogenic mutations have been missed and that the current mutation frequency of *PARK7* is an underestimate.

1.1.7 Mechanisms of neurodegeneration in PD

The studies of toxic PD models, the functions of PD-related genes and the analysis of postmortem brains of PD patients suggest the two major interconnected hypothesis regarding the pathogenesis of the disease besides other pathogenesis: first misfolding and aggregration of proteins and second the mitochondrial dysfunctions (In detail refer section 1.2) and the consequent oxidative stress.

1.1.7.1 Misfolding and aggregation of proteins

The abnormal deposition of protein is observed in brain of patients affected by several age-related neurodegenerative disease, including PD. Alteration of protein folding, ubiquitin-proteasome system (UPS) and autophagy are considered as the main molecular mechanisms in aggregate formation. (Bennett et al., 1999; Webb et al., 2003; Cuervo et al., 2004). Dysfunction of these systems is implicated in the pathogenesis of neurodegenerative diseases including PD and DLB, among others (McNaught and Jenner, 2001; McNaught et al., 2002b; McNaught et al., 2003; Ravikumar and Rubinsztein, 2004, 2006). The UPS maintains cellular homeostasis through the degradation of short-lived cytoplasmic proteins (Rock et al., 1994; Sherman and Goldberg, 2001). It has a major function in acutely controlling levels of regulatory proteins and is the primary means for eliminating mutant, misfolded and oxidatively damaged proteins (Sherman and Goldberg, 2001). Protein accumulation following saturation or impairment of the UPS may therefore catalyse concentration-dependant aggregation of damaged or misfolded proteins. Significantly, pharmacological inhibition of the UPS causes an accumulation of alpha-synuclein aggregates in primary mesencephalic neurons (McNaught et al., 2002; Rideout et al., 2005) and PC12 cells (Rideout et al., 2001). Post-mortem tissue of PD patients provides direct evidence of UPS dysfunction in the SN. McNaught and colleagues (2001) specifically report decreases in the expression of proteasomal subunits in nigral neurons, but not cortical or striatal tissue (McNaught and Jenner, 2001). These data may indicate a role for UPS impairment in the selective vulnerability of nigral neurons (Betarbet et al.,2005). However, they may equally signify that proteasome dysfunction is secondary to PD pathology. Lewy bodies provide direct evidence that mechanisms of protein catabolism are impaired in PD and DLB. The presence of proteasomal subunits within LB's suggests an involvement of alpha-synuclein in UPS dysfunction (Ii et al., 1997; Wakabayashi et al.,2007). This is supported by studies linking alpha-synuclein overexpression with impaired proteasome activity, an effect that is potentiated by mutant or aggregated alpha-synuclein (Stefanis et al., 2001; Snyder et al., 2003). The antagonistic affect of alpha-synuclein on proteasome function is likely to be conferred through an interaction with the S6' subunit of the 19S proteasome cap (Snyder et al., 2003). In addition to proposed involvement of UPS dysfunction in LB formation, inhibition of this system is directly associated with neurodegeneration (McNaught et al., 2004). Significantly, cell death following systemic exposure to proteasome inhibitors preferentially affects neurons of the SNpc *locus coeruleus*, dorsal motor nucleus of the vagus and the nucleus basalis of Meynert, suggesting involvement in PD. Proteasome inhibition is additionally associated with theformation of alpha-synuclein and ubiquitin positive eosinophilic inclusions (Rideout and Stefanis, 2002; Rideout et al., 2005).

Autophagy

In contrast to the UPS, autophagy is the major mechanism for the degradation of long-lived cytoplasmic proteins and is the only mechanism of organelle recycling. It is specifically involved in the catabolism of large membrane proteins and protein complexes, which are physically unable to be degraded within the narrow barrel of the proteosome (Pan et al., 2008). For this reason, autophagy may be critical in the degradation of aberrant proteins aggregates, which characterise multiple neurodegenerative diseases. In the CNS, autophagy impairment causes neurodegeneration with intraneural accumulation of polyubiquitnated proteins (Hara et al., 2006; Komatsu et al., 2006; Massey et al., 2006). Alpha-synuclein is catabolized, at least in part, by chaperone-mediated autophagy (CMA) (Webb et al., 2003; Cuervo et al., 2004). Suppression of autophagy may therefore be a mechanism of both neurodegeneration and LB formation in PD and DLB (Cuervo et al., 2004). There is some evidence that mutant alpha-synuclein inhibits CMA as it is poorly translocated to the lysosomal lumen and accumulates on the membrane surface. Consequently, the clearance of other CMA substrates is suppressed through steric hindrance of their translocation (Cuervo et al., 2004).

The unfolded protein response

The unfolded protein response (UPR) is a universal cellular mechanism that is activated by the accumulation of unfolded proteins within the lumen of the Endoplasmic Reticulum (ER). The major function of the UPR is to restore homeostasis, by temporarily halting protein translation and increasing the concentration of molecular chaperones in order to facilitate protein folding. When these functions do not successfully restore correct protein folding the UPR initiates host defence mechanisms and in long term ER stress can activate apoptotic pathways. The UPR is shown to be active in both PD and DLB and may be initiated by pathogenic mechanisms of these diseases, including oxidative stress (Holtz et al., 2006). The phosphorylation of alpha-synuclein at Ser-129, as well as its accumulation in the ER causes activation of the UPR (Sugeno et al., 2008). Alternatively, the stress induced upregulation of alpha-synuclein may indicate a homeostatic function in the UPR, consistent with its putative role as a molecular chaperone (Souza et al., 2000a).

1.2 MITOCHONDRIA

Two fundamental activities of everyday life, to eat and to breathe, converge at the level of an organelle present in nearly all cells. Mitochondria use oxygen that we inhale to convert energy in the carbohydrates and lipids in the food into adenosine triphosphate (ATP), the energy carrier used to drive almost all energy-dependent processes in the cell. Mitochondria have prokaryotic ancestry and originate from the entry of a proteobacteria species into the cytoplasm of a primitive host cell about 2 billion years ago (Gray, 1999; Andersson et al., 2003) a symbiosis that gave the host cell the evolutionary advantageous capacity for aerobic metabolism. Deepened integration of the proteobacterium in cellular physiology shaped a ubiquitous organelle with several essential functions. In addition to intermediary metabolism and ATP synthesis, mitochondria have important roles in calcium buffering, regulation of apoptosis, reactive oxygen species (ROS) production and the assembly of iron-sulfur cluster proteins (Lill and Muhlenhoff, 2008). As a consequence of their evolutionary origin, mitochondria share several features with prokaryotes such as containing their own bacteria-like genome and translation machinery. Lateral gene transfer from the mitochondrial DNA (mtDNA) to the nuclear genome has reduced gene content, and mammalian mtDNA only contain 13 protein-coding genes. The remaining~1000 mitochondrial proteins (Pagliarini et al., 2008) are encoded by nuclear DNA. Nuclear-encoded mitochondrial proteins are imported into mitochondria by an import machinery following recognition of an N-terminal targeting peptide (Neupert and Herrmann, 2007).

1.2.1 Mitochondrial ultrastructure

Mitochondria consist of two lipid bi-layers that create four separate mitochondrial compartments (Fig.1): the mitochondrial outer membrane, the intermembrane space, the inner membrane and the matrix. The outer membrane is semipermeable due to high amounts of channel proteins of the porin family that allows diffusion of molecules with a molecular weight of less than ~5 kDa across the membrane. Concentrations of small molecules within the intermembrane space are therefore in equilibrium with the cytosol. The inner membrane is very protein-dense and rich in cardiolipin, a specialized diphosphatidylglycerol lipid. Unlike the outer membrane, the inner membrane is impermeable to ions and solutes. Transfer across the inner membrane depends on specialized transporters, for example the adenine nucleotide translocator (ANT) that exchanges ADP and ATP. This impermeability is important for the respiratory chain (RC) complexes, which are embedded in the inner membrane. Invaginations of the inner membrane create subcompartments of the

intermembrane space called *cristae*. Formation of cristae may facilitate metabolism by greatly expanding the surface area between the RC in the inner membrane and the matrix compartment. The morphology of cristae can vary widely between different organisms, tissues and conditions (Zick et al., 2009). The mechanism that shape cristae are incompletely understood but may include dimerization of the ATP synthase (Davies et al., 2011). The matrix harbors the mtDNA and enzymes of the tricarboxylic acid (TCA) cycle, β-oxidation and some of the enzymes involved in steroid biosynthesis, gluconeogenesis and the urea cycle.

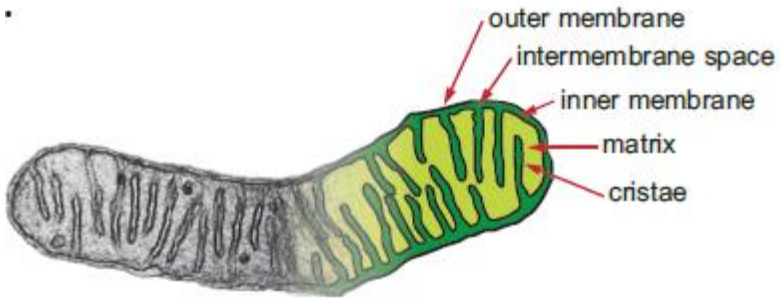


Figure 1.5 Mitochondrial ultrastructure. An electron micrograph shows the Mitochondrial outer and inner membranes that separate the intermembrane space and the mitochondrial matrix. Folding of the inner membrane into the matrix give rise to cristae.

1.2.2 Mitochondrial dynamics

The size and shape of mitochondria varies between cell types and tissues, from being sphere-like to forming long interconnected tubules (Kuznetsov et al., 2009). The morphology is dynamic and mitochondria can move and remodel through fusion of separate mitochondria and division into daughter units. Conserved protein machineries regulate mitochondrial fusion and division (fission) (Detmer and Chan, 2007; Westermann, 2010). The relative activity of these opposing processes dictates the overall shape, which can rapidly change in response to altered conditions.

1.2.2.1 Mitochondrial fusion

Fusion of the outer membrane of mammalian mitochondria is mediated by two closely related proteins, Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) (Chen et al., 2003). Mitofusins are large dynamin related GTPases that are anchored to the outer membrane with both the N and C-terminal

domains facing the cytoplasm. C-terminal heptad repeat domains of mitofusins on adjacent mitochondria can interact in *trans* to form either homotypic (Mfn1-Mfn1 or Mfn2-Mfn2) or heterotypic (Mfn1-Mfn2) complexes that tether the membranes in close proximity (Koshiba et al., 2004). It is believed that subsequent GTPase activity induce a conformational change that fuse the juxtaposed membranes. Loss of the gene for either *Mfn1* or *Mfn2* decreases mitochondrial fusion and leads to fragmentation of the mitochondrial network (Chen et al., 2003). Loss of both genes completely abolishes fusion (Koshiba et al., 2004). Only a single Mfn homologue is found in yeast, and the role for the two separate mitofusins in mammals is not fully understood. Additional functions have been ascribed to Mfn2, including a role in mitochondrial axonal transport (Misko et al., 2010) and the tethering of mitochondria to the endoplasmatic reticulum (de Brito and Scorrano, 2008).

The mechanisms that shape inner membrane morphology are less well understood. The inner membrane large GTPase OPA1 is required for fusion, and loss of *OPA1* results in mitochondrial fragmentation and aberrant cristae formation (Song et al., 2009). Alternative splicing generates multiple OPA1 isoforms. In addition, OPA1 is subject to proteolytic processing by inner membrane proteases to generate shorter isoforms of the protein (Westermann, 2010). Fusion requires both the long and short isoforms (Song et al., 2007). Reduced mitochondrial membrane potential promotes processing and inhibits inner membrane fusion through loss of long OPA1 isoforms (Duvezin--Caubet et al., 2006).

Fusion allows inter-mitochondrial content mixing and is essential to maintain a functional mitochondrial network. Knockout of either *Mfn1* or *Mfn2* in mice results in embryonic lethality (Chen et al., 2003), and tissue-specific knockout of *Mfn2* in cerebellar neurons leads to neurodegeneration and mitochondrial dysfunction in affected neurons (Chen et al., 2007). In humans, mutations in *MFN2* cause Charcot-Marie-Tooth type 2A, a peripheral neuropathy that affects long axons and cause distal weakness and sensory loss in the limbs. Mutations in *OPA1* is a major cause of dominant optic atrophy, a disease that leads to blindness due to degeneration of retinal ganglion cells (Delettre et al., 2002).

1.2.2.2 Mitochondrial fission

Fission of mitochondria depends on the large GTPase dynamin-related protein 1 (*Drp1*). *Drp1* is predominantly located in the cytoplasm with a minor fraction found on punctate spots on the mitochondrial surface. During fission, these sites recruit cytosolic Drp1, which oligomerize to

encircle and constrict the mitochondrial tubule to promote scission of the membranes (Ingerman et al., 2005). The mechanism that dictates future fission sites is not well understood. In yeast, a small outer membrane protein called Fis1 is required for the recruitment of cytosolic Drp1, but the mammalian homologue is dispensable for the process (Lee et al., 2004). Instead, recruitment in mammals appears to depend on mitochondrial fission Factor (Mff) (Otera et al., 2010).

Fission is important for proper segregation and distribution of mitochondria, and Drp1-mediated fission is also an early event during apoptosis. *Drp1* knockout mice are embryonic lethal and have highly connected mitochondria with aberrant morphology (Ishihara et al., 2009; Wakabayashi et al., 2009). *Drp1* knockout neurons fail to distribute mitochondria in neural processes and have impaired synapse formation (Ishihara et al., 2009). The importance of mitochondrial fission in humans has been illustrated by a case with fatal brain malformation due to a spontaneous dominant-negative *DRP1* mutation (Waterham et al., 2007).

1.2.2.3 Mitochondrial motility

Distribution of mitochondria within the cell is essential during cell division and to position mitochondria at sites with high energy demands (Hollenbeck and Saxton, 2005). Neurons are extreme in their need to transport mitochondria over vast distances. In axons of lower motor neuron explants, mitochondria travel at peak velocities of 1.0 μm/s in the anterior direction and 1.4 μm/s in the retrograde direction (Misgeld et al., 2007). Although referred to as fast axonal transport, this corresponds to a minimal transit time of ~10 days from cell body to axon terminal in the longest of human axons. However, mitochondrial transport includes frequent pauses (saltatory movement), and net velocity over a distance is therefore likely slower (Hollenbeck and Saxton, 2005).

Fast axonal transport of mitochondria depends on microtubules (Fig. 2B). The kinesin-1 family of molecular motors drives anterograde transport, i.e. movement from the cell body towards the axon terminal and the microtubule (+)-end. Retrograde transport towards the microtubule (-)-end is driven by dynein (Pilling et al., 2006). Mutational screens in *Drosophila* have revealed two adaptor proteins for mitochondria-specific transport, Milton (Stowers et al., 2002) and Miro (Guo et al., 2005). Both genes have two mammalian orthologues named *TRAK*(1/2) and *MIRO*(1/2), respectively. The RHO family GTPase MIRO is attached to the outer membrane and binds the adaptor protein Milton/TRAK, which in turn interacts with kinesin (Fransson et al., 2006; Wang and Schwarz, 2009). MIRO contains two Ca2+-binding EF-hand motifs that arrest mitochondrial motility upon Ca2+-

binding (Wang and Schwarz, 2009), suggesting a mechanism to direct mitochondria to subcellular regions with high Ca2+ levels. Phosphorylation of MIRO by the kinase PINK1 (Wang et al., 2011) may arrest transport in a membrane potential-dependent manner. Several other proteins have been implied in the regulation of mitochondrial transport (Sheng and Cai, 2012).

1.2.3 Oxidative phosphorylation and the respiratory chain

Catabolism of carbohydrates and lipids by glycolysis, the TCA cycle and β-oxidation generates reduced forms of the electron carriers NADH and $FADH_2$. The mitochondrial RC consists of four enzyme complexes (complexes I-IV) situated in the mitochondrial innermembrane (Figure 1.6). The RC harvests electrons from NADH and $FADH_2$ and transfer them via a series of redox reactions to finally reduce molecular oxygen to water. This series of redox reactions is coupled to the translocation of protons across the inner membrane at complexes I, III and IV to generate an electrochmical gradient of protons acros the innermembrane. This so called proton motive force drives the ATP synthase and couples cellular respiration to ATP production by oxidative phosphorylation (OxPhos) (Mitchell, 1961).

The OxPhos machinery is unique in its dependence on both nuclear and mitochondrial genes. The mtDNA encodes 13 essential subunits of complexes I, III, IV and V, whereas the remaining ~80 subunits of complexes I-V are encoded by nuclear DNA. In addition, correct complex assembly is atleast partly dependent on a number of nuclear-encoded assembly chaperones. Higher order assemblies of RC complexes, called supercomplexes, can be isolated by native gel Electrophoresis (Acin-Perez et al., 2008). Electron microscopy studies have shown that complexes I, III and IV form a supercomplex (in 1:2:1 stoichiometry) that brings the sites for electron transfer between the complexes in close proximity (Vonck and Schafer, 2009; Althoff et al., 2011).

Complex I (NADH: ubiquinone oxidoreductase) couples the oxidation of NADH to the reduction of the membrane-soluble electron carrier coenzyme Q (ubiquinone). Complex I is the largest of the electron transport chain complexes and consists of 45 subunits that assemble into a ~1 MDa structure. The overall architecture is L-shaped with a hydrophobic membrane-embedded arm and a hydrophilic peripheral arm that protrude into the matrix. The peripheral arm contains the redox centers necessary for electron transport, whereas the membrane arm, which contains all of the mtDNA-encoded subunits (ND1-6, ND4L), makes up the proton translocation machinery. Oxidation of NADH takes place in the peripheral arm by the transfer of two electrons to an acceptor flavin mononucleotide

(FMN). These electrons are then sequentially transferred via a series of iron-sulfur clusters (Sazanov and Hinchliffe, 2006) to the proximal part of the peripheral arm, where ubiquinone (Q) accepts the electrons to form first semiquinone (QH•-), then ubiquinol (QH_2).

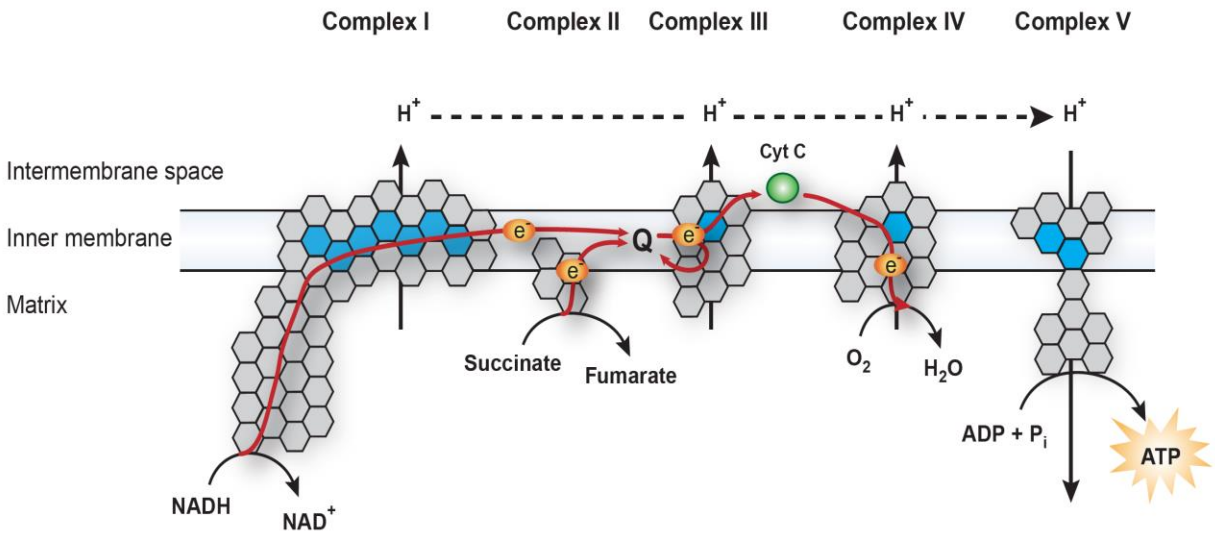


Figure 1.6 The five enzyme complexes of the OxPhos machinery. Complex I (NADH:ubiquinoneoxidoreductase) and complexII (succinatedehydrogenase) transfer electrons(e) from intermediary metabolites to the innermembrane electroncarrier coenzymeQ(Q). Complex III (ubiquinolcytochromecreductase) subsequently transfer electronsf rom reduced coenzyme Q to the electrons shuttling protein cytochromec (CytC). The electron carried by reduced cytochromec is used in the final step to reduce oxygen (O_2) to water (H_2O) by complex IV (cytochromecoxidase). The transfer of electrons (redarrows) is coupled to the translocation of protons (H+) across the inner mitochondrial membrane bycomplexes I, III and IV. This generates the proton gradient that drives the formation of ATP from ADP and inorganic phosphate (Pi) by complex V(ATP synthase). Each hexagon represents a unique subunit, with those encoded by mtDNA shown in blue; adapted from Schapira et al., 2006). Oligomerization of complexV subunits and higher order structure of complexes are not illustrated.

The energy released by the electron transfer is coupled to the translocation of protons across the inner membrane by the membrane arm. Recent crystal structures (Hunte et al., 2010; Efremov and Sazanov, 2011) suggest that a rod-like helix parallel to the membrane arm acts like a piston to transfer conformational energy from the Q reduction site to two separate proton channels in the membrane arm (Brandt, 2011). Each electron transferred drives translocation of one proton at each channel, so that each NADH (2 electrons) in total drives translocation of 4 protons.

Complex II (succinate: ubiquinone reductase or succinate dehydrogenase) is another source of QH_2, but does not directly contribute to the proton motive force. Complex II consists of four subunits and is integrated in the TCA cycle by the SDH-A subunit that catalyzes the succinate to fumarate reaction. In doing so it acquires electrons to the covalently bound cofactor FAD, from which the electrons are further transferred via three iron sulfur clusters in the SDH-B subunit to the final electron acceptor coenzyme Q. Two hydrophobic subunits provide the Q binding site and anchor the complex to the inner membrane. All complex II subunits are nuclear-encoded, and the complex forms a homotrimer. A third source of QH_2 comes from the catabolism of fatty acids. Electrons from $FADH_2$ generated by fatty acyl-CoA dehydrogenas are collected by the electron transfer flavoprotein (ETF) and transferred to the pool of coenzyme Q in the inner membrane by the enzyme ETF: ubiquinone reductase.

Complex III (ubiquinol-cytochrome c reductase) transfers electrons from QH_2 to cytochrome c, a soluble protein in the intermembrane space, and couples this reaction to the translocation of protons across the inner membrane. Complex III consists of eleven subunits and functions as a homodimer. Only three of the subunits, including the only mtDNA-encoded subunit, cytochrome b, contain redox centers and participate in electron transport (Saraste, 1999). The first electron from QH_2 is transferred directly to cytochrome c, which is only a single- electron acceptor. Thus, the second electron cannot be transferred directly to cytochrome c and is instead recycled by a mechanism called the Q cycle. This is achieved by transferring the Second electron via the cytochrome b subunit to another Q molecule at a second binding site to generate semi-ubiquinone. During the next round of cytochrome c reduction, this semi-ubiquinone is further reduced to QH_2. Th net effect of the Q cycle is that every QH_2 results in the reduction of two cytochrome c molecules while translocating four protons (Saraste, 1999).

Complex IV (cytochrome c oxidase) harvests electrons from reduced cytochrome c and delivers them to the final electron acceptor, molecular oxygen. Three of the 13 subunits are mtDNA-encoded

(COXI-III), and together make up the catalytic core. Four molecules of reduced cytochrome c sequentially deliver electrons to heme and copper centers in the oxidase, which in turn passes them on to O_2 to generate two molecules of H_2O (Saraste, 1999). This reaction is coupled to the translocation of four protons.

Complex V (ATP synthase) uses the proton gradient generated by complexes I, III and IV to synthesize ATP. The complex consists of 14 different subunits, two of which are mtDNA encoded, and hetero-oligomerize to form a 600 kDa complex with a membrane-bound part (F0) and a catalytic part (F1) that protrudes into the matrix. Movement of protons through a channel in the F0 part causes rotation of a central "rotor" that connects the F0 and F1 parts. The F1 part is prevented from rotating by a peripheral stalk that anchors it to the static membrane part (Rubinstein et al., 2003). Instead, the rotational energy induces conformational changes that catalyze the formation of ATP from ADP and inorganic phosphate. Under some conditions, activity of the ATP synthase can be reversed so that ATP hydrolysis instead drives proton pumping from the matrix to the intermembrane space. Recent electron microscopy studies has shown that complex V forms dimers situated along cristae ridges and may be responsible for shaping inner membrane curvature (Davies et al., 2011).

1.2.4 Mitochondrial ROS production

Reactive oxygen species (ROS) are important byproducts of the RC and form when an electron escapes from the preferred path. Such electrons often react with oxygen to generate a superoxide anion (O_2•-). Complexes I and III are the main sites for mitochondrial ROS production and it has been estimated that 0.2% of molecular oxygen forms superoxide (Balaban et al., 2005). Mitochondrial superoxide dismutase (SOD_2) converts O_2•- into another oxidant, hydrogen peroxide (H_2O_2). H_2O_2 can be enzymatically reduced to water by catalase or glutathione peroxidase, but can also react with transition metal ions to generate a highly reactive hydroxyl radical (OH•) by the Fenton reaction. ROS in the form of O_2•-, H_2O_2 or OH• can cause oxidative damage to DNA, proteins and lipids. ROS may also play a physiological role in redox signaling (Finkel, 2011; Murphy et al., 2011).

1.2.5 Mitochondrial genetics

The mammalian mitochondrial genome is a circular DNA molecule (Fig. 4) of ~16.5 kb (16.6 kb in humans, 16.3 kb in mice). Each cell contains thousands of copies of mtDNA but it only makes up ~1% of the total cellular DNA content. The mtDNA encodes 13 proteins that are all subunits of the oxidative phosphorylation system as well as 22 tRNAs and two ribosomal RNAs (12S and 16S

rRNAs) (Anderson et al., 1981). All proteins necessary for transcription, replication and maintenance of mtDNA are nuclear-encoded. The two mtDNA strands are called the heavy strand (H-strand) and the light strand (L-strand), based on the difference in molecular weight that results from an imbalance in the content of purines. The sequence is extremely compact with no introns and consists almost exclusively of coding sequence. The major exception is the ~1 kb region of regulatory sequence known as the displacement loop (D-loop).

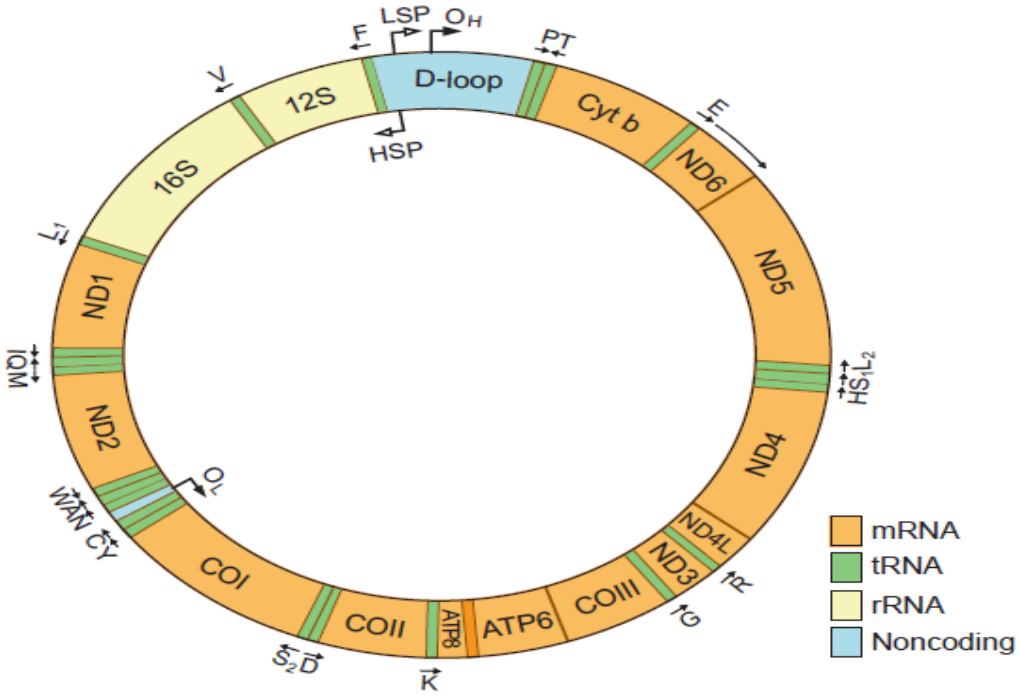


Figure 1.7 Gene organization of mammalian mtDNA. Each gene is represented by a box with tRNA Genes indicated by one letter amino acid codes and direction of transcription indicated by arrows. The D-loop is the only longer non-coding region and contains promoters for transcription of both strands (LSP and HSP) and the origin of leading strand replication (OH). Lagging strand replication is initiated from a site (OL) within a cluster of tRNA genes. The H-strand is transcribed from HSP and encodes 2 rRNAs (12S and 16S rRNA), 12 mRNAs (ND1–5, ND4L, Cyt b, COI–III, ATP6, and ATP8), and 14 tRNAs (F,V, L1, I, M, W, D, K, G, R, H, S1, L2, T). The L-strand is transcribed from LSP and encodes the mRNA for ND6 and 8 tRNAs (P, E, S2, Y, C, N,A, Q). Adapted from (Larsson, 2010).

1.2.5.1 Organization of the mitochondrial genome

The mitochondrial genome is organized by associated protein components to form structures called nucleoids (Bogenhagen, 2011). Each nucleoid has a size of ~100 nm (Kukat et al., 2011) and may be associated with the inner membrane (Wang and Bogenhagen, 2006). The number of mitochondrial genomes contained in each nucleoid is debated (Bogenhagen, 2011), but a recent study combining quantitative PCR with super-resolution light microscopy estimated that each nucleoid on average contains 1.4 mtDNA molecules (Kukat et al., 2011), suggesting that most nucleoids contain only a single mitochondrial genome. The mitochondrial transcription factor A (TFAM) is an abundant mitochondrial protein, present in about 1,000 molecules per mtDNA copy, and it is a major component of the nucleoid structure (Kaufman et al., 2007; Bogenhagen, 2011). TFAM contains two high mobility group (HMG)-box domains that can bind double stranded DNA in both sequence-specific and sequence-unspecific manner. Binding of TFAM bends the DNA helix almost 180° (Ngo et al., 2011; Rubio-Cosials et al., 2011), and binding of increasing amounts of TFAM compacts the DNA (Kaufman et al., 2007). Consistent with a role in DNA packaging, mtDNA copy number co-varies with the levels of TFAM (Larsson et al., 1994; 1998; Ekstrand et al., 2004).

1.2.5.2 Transcription of mt DNA

Both the H-strand and the L-strand are transcribed in a bi-directional fashion from two promoters in the D-loop to create long polycistronic transcripts (Fig. 4). These primary transcripts are post-transcriptionally cleaved to generate individual mRNA, tRNA and rRNA molecules (Ojala et al., 1981). Mitochondrial mRNAs are polyadenylated but are not capped in the 5´-end. Transcription of the H-strand is initiated upstream of tRNAPhe by the H-strand promoter (HSP) and generates a transcript containing the 12S and 16S rRNAs, 14 tRNAs, and all Protein coding genes except ND6. The L-strand promoter (LSP) initiates transcription of the L-strand that contains 8 tRNAs and the gene encoding ND6. A second H-strand promoter (HSP2) just upstream of the 12S rRNA gene has been reported but its existence is debated as it has not been possible to reconstitute *in vitro* (Litonin et al., 2010). The organelle-specific mitochondrial RNA polymerase (POLRMT) mediates mtDNA transcription. POLRMT consists of a single subunit and shares homology with bacteriophage RNA polymerases. TFAM and the mitochondrial transcription factor B2 (TFB2M) are additionally required for transcription initiation and together sufficient to initiate transcription from HSP and LSP templates *in vitro* (Falkenberg et al., 2002). Both promoters contain an upstream recognition sequence to which TFAM can bind with sequence-specificity (Fisher et al., 1989). TFB2M and

POLRMT are recruited by the C-terminal domain of TFAM and cooperatively bind the transcription start site (Sologub et al., 2009).

1.2.5.3 Replication of mtDNA

Replication of mtDNA is under relaxed control, and each mtDNA molecule may replicate several times during the cell cycle or not at all (Bogenhagen and Clayton, 1977). The mitochondrial DNA polymerase-γ (POLγ) consists of a catalytic A subunit and two accessory B subunits. The minimal replication machinery also includes mitochondrial single-stranded DNA-binding protein (mtSSB) and the hexameric mitochondrial DNA helicase TWINKLE (Korhonen et al., 2004). In addition, POLRMT is required for replication initiation. RNA synthesis initiated at LSP is frequently terminated prematurely at a conserved sequence block within the D-loop and thereby creates the primer for H-strand replication (Falkenberg et al., 2007; Wanrooij et al., 2010). DNA synthesis from origin of H-strand replication (OH) is often aborted after ~700 bp. The resulting nascent strand (called 7S DNA) displaces the parental H-strand and makes the D- loop a triple-stranded structure. The model for leading and lagging strand replication is debated. In the *strand- displacement* model (Clayton, 1982), replication of the leading strand alone proceeds around two thirds of the genome until it reaches the origin of L-strand replication (OL). When the OL sequence becomes single-stranded, it adopts a stem-loop structure that facilitates the generation of a short primer by POLRMT to initiate replication of the lagging strand (Fuste et al., 2010). The *strand-synchronous* model instead propose replication of both strands is coupled and resembles that of nuclear DNA replication in that lagging strand replication involves the formation of Okazaki fragments (Yang et al., 2002).

1.2.5.4 Segregation and transmission of mtDNA

MtDNA may acquire both point mutations and rearrangements such as deletions. The mutation rate of mtDNA is many times higher than that of nuclear DNA (Khrapko et al., 1997). Both mutant and wildtype alleles may coexist within a single cell, a state known as heteroplasmy. Homoplasmy refers to the situation when all mtDNA molecules carry the same allele. The pool of mtDNA molecules distribute stochastically to daughter cells during cell division, and such random segregation may lead to differences in the levels of heteroplasmy between different cells and tissues. Even in postmitotic cells, selective replication of a subset of mtDNA molecules may cause random drift and accumulation of certain mtDNA species over time, so called clonal expansion. The mitochondrial genome is maternally inherited by contribution of oocyte mitochondria. The level of a heteroplasmic

mtDNA variant transmitted from mother to offspring can vary significantly. This phenomenon is attributed to a significant reduction in the number of transmitted genomes and referred to as the 'genetic bottleneck' (Shoubridge and Wai, 2007; Carling et al., 2011). Paternal mitochondria in the spermatozoon midpiece, that enter the oocyte during fertilization, are rapidly degraded by autophagy (Rawi et al., 2011). Paternal inheritance of mtDNA has been reported in a patient (Schwartz and Vissing, 2002), but this appears to be a rare exception (Taylor et al., 2003). Escape from degradation is also seen in interspecific crosses between distantly related mouse species (Gyllensten et al., 1991; Kaneda et al., 1995), suggesting the involvement of a specific recognition receptor.

1.2.6 Mitochondrial disease

Mitochondrial disease constitutes a heterogeneous group of inborn errors of metabolism that result from impaired OxPhos capacity. Rolf Luft was the first to establish mitochondrial dysfunction as a cause of disease. Already 50 years ago he and his colleagues identified abnormal uncoupling of cellular respiration as the underlying cause of hypermetabolism (increased perspiration, weakness and cachexia despite high food intake) in a young adult female (Luft et al., 1962). Today, a large number of mitochondrial disorders have been described that collectively affect about 1 per 8,500 individuals (Chinnery and Turnbull, 2001). Mitochondrial disorders may present from infancy to adulthood with symptoms of either single-or multi-organ failure. The central nervous system, heart and skeletal muscle are commonly affected, presumably due to high energy demands. Diagnosis normally involves biopsy for histology and biochemical measurements. Molecular diagnosis became possible following the identification of pathogenic mtDNA deletions (Holt et al., 1988) and point mutations (Wallace et al., 1988). Mutations in a growing list of nuclear-encoded genes can also cause mitochondrial disease. Pathogenic mutations either directly interfere with OxPhos enzyme kinetics or assembly, or interfere with the production of mtDNA- encoded subunits (Debray et al., 2008). Mutations in either genome may underlie a common clinical presentation. Leigh syndrome, for example, a condition with degenerative lesions in the brainstem and basal ganglia that develop during infancy or early childhood, can result from both nuclear DNA and mtDNA mutations (Naess et al., 2009), and the inheritance can thus be either maternal, autosomal recessive or X-linked. Pathogenic mtDNA variants are often heteroplasmic, and the proportion of mutated mtDNA referred to as the mutation load. Wildtype mtDNA can normally complement the mutated mtDNA if present above a certain threshold level (Rossignol et al., 2003). The mutation load required to develop disease depends on the type of mutation and can vary from 60% for deletions (Hayashi et al.,1991) to >90% for some point mutations (Chomyn et al., 1992). An uneven distribution of the mutation load between different tissues contributes to, but cannot fully explain, clinical heterogeneity. An example of this is

leber's hereditary optic neuropathy (LHON), a cause of subacute blindness and sometimes additional symptoms. A majority of LHON patients carry a homoplasmic mutation in ND1, ND4 or ND6, but the penetrance is incomplete and may depend on nuclear gene variants (McFarland and Turnbull, 2009). Some "classical" syndromes are associated with specific mutations (McFarland and Turnbull, 2009). For example the 3243A>G mutation in the tRNALeu (UUR) gene causes MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) (Goto et al., 1990) and the 8344A>G tRNALys mutation causes MERFF (myoclonic epilepsy and ragged red fibers) (Shoffner et al., 1990). However, mutations in other genes may present with a similar phenotype. Conversely, a heteroplasmic mutation in the protein-coding ATP6 gene is associated with NARP (neurogenic weakness, ataxia and retinitis pigmentosa) at high mutation loads and infantile Leigh syndrome at very High mutation loads (Santorelli et al., 1993). Large mtDNA deletions arise spontaneously and are rarely inherited. Disease severity correlates with tissue distribution and mutation load (McFarland and Turnbull, 2009). MtDNA deletion is a common cause of chronic progressive External ophthalmopleiga (PEO), which may present in adults or during childhood in combination with myopathy, pigmentary retinopathy and cardiac conduction defects (Kearns-Sayre syndrome; KSS). High mutation loads may lead to severe bone marrow failure during infancy (Pearson syndrome). Survivors of Pearson syndrome go on to develop KSS (Larsson et al., 1990). Mutations in nuclear genes necessary for mtDNA replication, including ANT1 (Kaukonen et al., 2000), TWINKLE (Spelbrink et al., 2001) and the catalytic subunit of the mitochondrial DNA polymerase (Van Goethem et al., 2001) can also cause PEO and are associated with accumulation of multiple mtDNA deletions.

1.2.7 Mitochondria in ageing and degenerative disease

Mitochondrial dysfunction is implicated in the process of normal ageing (Trifunovic and Larsson, 2008; Larsson, 2010). RC function declines with age (Trounce et al., 1989) and ageing is associated with increasing amounts of somatic mtDNA mutations (Corral-Debrinski et al., 1992; Cortopassi et al., 1992; Soong et al., 1992). Although the total level of mutated mtDNA in a tissue is low, clonal expansion and random segregation can raise the mutation level in some 14 individual cells to exceed the threshold for RC deficiency (Larsson, 2010). A mosaic pattern of RC-deficient cells is indeed found in several aged tissues including heart (Muller-Hocker, 1989), skeletal muscle (Muller-Hocker, 1990) and brain (Cottrell et al., 2001; Bender et al., 2008). Affected cells have accumulation of mtDNA molecules carrying point mutations or large deletions (Fayet et al., 2002; Bender et al., 2006; Kraytsberg et al., 2006). A causative role for such mutations in ageing is strengthened by the finding

that mice that accumulate high levels of somatic mtDNA point mutations due to expression of a proofreading-deficient. POLγ develop a phenotype resembling premature ageing (Trifunovic et al., 2004). Mitochondrial dysfunction is also implicated in the pathophysiology of several neurodegenerative disorders, including Alzheimer's disease (AD) and PD (Schapira, 2006).

1.2.8 Mitochondrial dysfunction in PD and DLB

Neurons have limited glycolytic capacity and depend on mitochondria for ATP synthesis through oxidative phosphorylation. This series of redox reactions occurs on the innermitochondrial membrane and involves the transfer of electrons down the electron transport chain (ETC). Oxidative phosphorylation is driven by an electrochemical gradient across the inner-mitochondrial membrane ($\Delta\Psi m$), which is maintained by the transport of protons out of the mitochondrial matrix by complex I, III and IV of the ETC. (In Detail explanation of oxidative phosphorylation and the respective subunits is explained under section 1.3). Mitochondria also regulate cellular Ca2+ homeostasis and are the major endogenous generators of ROS (Kudin et al., 2005; Chan et al., 2009). Excitable tissues, such as neurons and muscle, are particularly susceptible to mitochondrial dysfunction due to their high energy demand (Morais and De Strooper, 2010). Impaired mitochondrial function disrupts homeostatic mechanisms causing energy crisis, oxidative stress and calcium influx. Impaired ETC activity is implicated in PD and DLB, specifically there are reports of decreased complex I activity in both the SN and frontal cortex in sporadic PD and DLB (Parker et al., 1989; Parker et al., 2008; Navarro and Boveris, 2009). Keeney and colleagues (2006) have further identified oxidative damage of complex I in PD, which may contribute to its disassembly and functional impairment (Keeney et al., 2006). Several studies also show decreased function of complexes I, II, III and IV in muscle, platelets and lymphocytes (Bindoff et al., 1991; Mann et al., 1992; Cardellach et al., 1993; Blin et al.,1994), although these findings remain contentious (Martin et al., 1996). Selective inhibition of complex I was first associated with Parkinsonism in pethidine (Demerol) and heroin addicts, through contamination of synthetic heroin with MPTP (Langston and Ballard, 1983). MPTP is oxidised to its active metabolite MPP+ bymonoamine oxidase B and selectively taken into cholinergic neurons by the dopaminetransporter (DAT) (Chiba et al., 1984; Javitch et al., 1985). Its toxic effect is primarily mediated through inhibition of the electron and proton transfer functions of complex I. Deficiencies in these functions cause both free-radical production and energy depletion. However, MPP+ treatment also results in massive release of cytoplasmic dopamine, which contributes to oxidative toxicity (Rollema et al., 1986). Neuronal vulnerability to MPP+ correlates with DAT density and mimics the progression of dopaminergic neuronal loss in early PD (Sanghera et al., 1997; Manning-

Bog et al., 2007). Recent studies have confirmed the involvement of mitochondria in PD. Rotenone-induced complex I inhibition reproduces the anatomical stages of cell loss during the disease (Pan-Montojo et al., 2010). Furthermore, cybrid cell lines containing cytoplasm from PD patients have altered mitochondrial morphology and impaired complex I activity (Trimmer et al., 2000; Veech et al., 2000). These results indicate that mitochondrial dysfunction is independent of the nuclear genome and may suggest a role for age-dependant mitochondrial DNA mutations in disease pathogenesis. The involvement of mitochondria may, however, be specific to neurodegeneration and independent of Lewy body formation. Specifically, proteins involved in mitochondrial function are genetically linked to Parkinsonism independent of LB formation (Table 1.2).

1.2.9 Genetics and mitochondrial dysfunction in PD

Several genes linked to recessive Parkinsonism encode proteins, which function in mitochondrial activity. Of these, *PTEN-induced kinase 1, (PINK1)* and *PRKN* are known to have direct associations with the mitochondria. Loss of function of either protein is associated with mitochondrial dysfunction (Greene et al., 2003; Clark et al., 2006; Yang et al., 2006). Single and double gene knockout studies in drosophila provide compelling evidence that *PINK1* and *parkin* are involved in the same biochemical pathways (Clark et al., 2006; Park et al., 2006). This is supported by the indistinguishable phenotype of PD patients carrying either mutation (Ibanez et al., 2006). Gene silencing or mutation of either *PINK1* or *PRKN* sensitises neurons to complex I inhibition and oxidative stress (Casarejos et al., 2006; Wood-Kaczmar et al., 2008; Grunewald et al., 2010). Furthermore, deficiencies in ETC activity are reported in human PD patients with mutations to either protein (Muftuoglu et al., 2004). It is therefore likely that these proteins are involved in energy production through oxidative phosphorylation. This effect may be mediated by the ability of parkin to regulate the transcription of respiratory chain complexes transcribed from mtDNA through association with mitochondrial transcription factor A (Kuroda et al., 2006). Daisuke-Junko-1 (DJ-1) is a redox sensitive molecular chaperone that is involved in the neuronal response to oxidative stress (Shendelman et al., 2004; Meulener et al., 2006). Mutations to *DJ-1* are a cause of early onset PD (Bonifati et al., 2003). Loss of *DJ-1* function sensitises neurons to cytosolic and mitochondrial ROS production (Meulener et al., 2005; Meulener et al., 2006), as well as MPP+ toxicity in dopaminergic neurons (Kim et al., 2005). These studies indicate that the activity of *DJ-1* is not specific to differential cellular compartments. Hao and colleagues have recently identified a direct role of *DJ-1* in mitochondrial function and ATP production. Overexpression of this protein overwrites the phenotypic toxicity of PINK1-deletion in drosophila (Hao et al., 2010). These results indicate that

DJ-1 plays an imperative role in mediating the response to cytosolic ROS production, as well as mitochondrial function.

1.2.10 Oxidative stress

Oxidative damage is a common consequence of aerobic metabolism. It is mediated by free radicals, which accumulate as by-products of cellular redox reactions. These reactions are essential for energy production, some enzymatic activities and signalling pathways (Patten et al., 2010). The mammalian brain is specifically vulnerable to oxidative stress due to its high-energy requirements and relative lack of antioxidant defence mechanisms (Butterfield, 2006; Halliwell, 2006). Neurons demand vast amounts of ATP to maintain ion status and electrochemical potential despite continual membrane depolarisation, which occurs during action potential propagation and neurosecretion. The respiratory quotient for the brain is effectively one, indicating that most neuronal ATP is synthesised through the oxidative metabolism of glucose. Oxidative phosphorylation is the most efficient pathway of ATP production and occurs on the electron transport chain (ETC) on the inner mitochondrial membrane. The ETC is dependant on O_2 as the final electron acceptor. To accommodate for its O_2 demand, the brain (constituting 2% of body weight) utilizes 20% of total body O_2 consumption (equivalent to 1.68 μM/g・min) (Siesjo, 1984; Zilberter,2011). The chemical properties of O_2 facilitate the formation of highly reactive species. Because the unpaired electrons of O_2 exist in parallel spin states, it is predisposed to univalent reduction and necessitates the production of radical intermediates.The mitochondrial ETC is the major generator of free radicals in the CNS (Kudin et al., 2005; Halliwell, 2006). The electrochemical potential of the inter-mitochondrial environment facilitates the production of superoxide anion (O_2・-) through premature 'leaking' of electrons directly onto O_2. In the CNS, complex I (NADH dehydrogenase) contributes to the majority of mitochondrial O_2・- (Herrero and Barja, 2000). Significantly, complex I impairment has been reported in PD and DLB (Navarro and Boveris, 2009). Cytosolic production of O_2・- and other reactive oxygen species (ROS) occurs through metal ion catalysed reactions, advanced glycosylation end products and other redox-dependent enzymatic reactions. In catecholaminergic neurons, monoamine neurotransmitters autooxidize on exposure to O_2, generating O_2・-. As this reaction is catalysed by Fenton chemistry, its rate is dependent on the both the partial pressure of O_2 and the concentration of transition metals. Cells defend themselves from oxidative damage through enzyme-catalysed conversion of reactive species into inert compounds. The superoxide dismutase (SOD) family of enzymes eliminate O_2・- through alternate oxidation and reduction reactions, yielding H_2O_2 and O_2. The elimination of H_2O_2

produced by SOD's and other oxidases is largely dependant on the free radical scavenger glutathione (GSH). The family of glutathione peroxidise enzymes require GSH as a cofactor for the enzyme coupled redox reaction of H_2O_2 to water (H_2O). GSH is an abundant tripeptide (Glu-Cys-Gly) localised to the cytoplasm and mitochondria. Its potent antioxidant capacity is attributed to the thiol group of its cysteine residue (Rabinovic and Hastings, 1998). Two GSH molecules are able to reduce H_2O_2 by forming a disulfide bridge between them. This reaction yields oxidised glutathione (GSSH), which is rapidly recycled to GSH by glutathione reductases. Post-mortem tissue form PD patients have reduced levels of total GSH in the SN, accompanied by a reduction in the GSH:GSSG ratio (Fitzmaurice et al., 2003; Zeevalk et al., 2007). Saturation of antioxidant defences results in the accumulation of reactive species. Cells typically maintain a balance between antioxidants and free radicals in order to limit oxidative damage. However, synthesis of antioxidants is energetically expensive and this equilibrium is rarely perfect. Most aerobic cells therefore experience continual, yet limited, oxidative damage (Halliwell, 2007). Oxidative stress describes an augmented imbalance in the production of oxidative species and their reduction by cellular defence mechanisms.

1.2.11 Lipid oxidation

Oxidative stress in PD is also evidenced by increased by the observation of 1) increased lipid hydroperoxides in SN (Dexter et al., 1986); 2) increased levels of 4-hydroxy-2-nonenal (HNE), an endproduct of lipidperoxidation (Dexter et al., 1986; Yoritaka et al., 1996). Increased 3-nitrotyrosine immunostaining was shown in LB's and in amorphous deposits in intact and degenerating neurons in PD substantia nigra (Good et al., 1998).

The brain has high concentrations of polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA) and arachidonic acid, compared with other organs. Docosahexaenoic acid (DHA) (22:6ω3) is a polyunsaturated fatty acid uniquely enriched in the brain and retina, especially in synaptic membranes and in photoreceptor cells (Anderson et al., 1970; Bazan and scott 1990; O'Brien and Sampson 1965). Astrocytes play an important role in the delivery of DHA to the blood-brain barrier endothelial cells and to neurons (Bernoud et al., 1998; Moore et al., 1991). Although the physiologic basis for why DHA is enriched in the brain and retina remains unclear, reduced levels of DHA are associated with disturbances in visual acuity, behavior, and learning in young animals and humans (Jensen et al.,1996; Makrides et al., 1996 ; werkman and carlson 1996). As these fatty acids are highly unsaturated, oxidative stress makes them susceptible to lipid peroxidation, which is one of the major outcomes of free radical-mediated injury (Montine et al., 2002). Lipid peroxidation results

in structural damage of membranes, compromising their integrity and consequently cell viability. As some of the lipid peroxidation products are chemically reactive, they are believed to be the major effectors of tissue damage (Mattson et al., 1998). In fact, lipid peroxidation can perpetuate in the presence of free reactive iron, since iron can react with lipid hydroperoxides to generate alkoxyl radical, which in turn can react with polyunsaturated fatty acids, the substrate for lipid peroxidation (Montine et al., 2004). Every phospholipid in every cellular membrane contains a unsaturated fatty acid residue. Many of these are polyunsaturated, and the presence of a methylene group between 2 double bonds renders the fatty acid-sensitive to ROS-induced damage, with their sensitivity to oxidation increasing exponentially as a function of the number of double bonds per fatty acid molecule (Bielski et al., 1983). Consequently the high concentration of PUFA's in brain phospholipids not only makes them prime targets for reaction with oxidizing agents, but also enables them to participate in long free radical chain reactions. Lipid peroxidation generates hyperoxides and endoperoxides, which undergo fragmentation to produce a broad range of reactive intermediates, like alakanals, alkenals, hydroxyalkenals, glyoxal and malondialdehyde. These compunds can react primarily with lysine,arginine and cysteine residues, leading to the formation of both nonenzymatic adducts and crosslinks in proteins.

Isoprostanes (IsoPs) are prostaglandin (PG)-like compounds that are generated *in vivo* nonenzymatically as products of free radical-induced peroxidation of arachidonoyl lipids (Morrow et al., 1990; 1994). Their formation proceeds via PGH_2-like bicyclic endoperoxide intermediates, which are reduced to form F-ring IsoPs (F_2-IsoPs) (Morrow et al., 1990) or undergo rearrangement to form D-ring and E-ring IsoPs (Morrow et al., 1994) and isothromboxanes (Morrow et al., 1996). It has been demonstrated the formation of IsoP-like compounds *in vivo* from free radical-catalyzed peroxidation of DHA (Nourooz-Zadeh et a., 1998; Roberts et al., 1998). Because DHA is highly concentrated in nervous system tissue, these compounds are termed as neuroprostanes (NPs) (Roberts et al., 1998). Analogous to the formation of IsoPs, the formation of NPs also proceeds through bicyclic endoperoxide intermediates that not only are reduced to F-ring compounds but also undergo rearrangement *in vivo* to form D- and E-ring NPs (Roberts et al., 1998; Reich et al., 2000). Therefore IsoK-like compounds could also be generated as rearrangement products of the NP pathway, for which the term neuroketals (NKs). The generation of NKs may induce neuronal injury due to their reactivity and could potentially be involved in the formation of protein cross-links, a common feature in neurodegenerative diseases.

Protein nitration by peroxynitrite represents a major mechanism of oxidative modification of proteins in association with atherosclerosis, Beckman et al., 1994; inflammation Kooy et al., 1995 and ischemia /reperfusion injury Malinski et al., 1993. This aberrant addition of a nitrate group to the ortho position of tyrosine represents one outcome of cellular oxidative stress rendering nitrated proteins dysfunctional Ischiropoulos et al., 1992 and killing neurons in culture Lafon-Cazal et al., 1993. Nitrotyrosine formation in proteins has been demonstrated in atherosclerotic lesions Beckman et al., 1994 and has also been proposed for a role in neurodegenerative disease Beckman et al., 1993, 1995. In vitro studies of such a role for protein nitration have identified nitrotyrosine as a product of the peroxynitrite reaction with superoxide dismutase (SOD) (Ischiropoulos et al., 1992 or neurofilament protein Beckman et al., 1995). Peroxynitrite is produced by a reaction between nitric oxide and superoxide radicals. The nitric oxide radical is generated by the enzyme nitric oxide synthase (NOS), activated by Ca+2/calmodulin, secondary to N-methyl-D-aspartate (NMDA) receptor-mediated cytosolic calcium influx Garthwaite et al., 1995. The potential association of NMDA receptor activity and peroxynitrite production thus represents a link between excitotoxicity and oxidative stress.

1.3 PROTEIN SYNTHESIS

The cell is the smallest metabolically functional unit of life; cells are able to sense and respond to external signals, a process that is tightly regulated at the transcriptional and translational levels. To maintain cellular homeostasis, all cells must continually synthesize new proteins. Translation is a highly coordinated multistep process in eukaryotic cells. The hardware of the translational machinery is composed of translation factors and ribosomes. Ribosomes (polyribosomes) are specialized complexes composed of nucelic acids and proteins that are responsible for mediating all protein synthesis. The ribosomes are located in the cytoplasm; in eukaryotes they are also found on endoplasmic reticulum. Ribosomes translate the genetic information encoded in a messenger RNA (mRNA) into the corresponding sequence of amino acids, thereby synthesizing a polypeptide chain or a protein.The biogenesis of eukaryotic ribosomes occurs within the nucleolus and requires the coordinated assembly of four different ribosomal RNAs (rRNAs) and 80RPs (Sollner-Webb and Mougey, 1991; Uechi, et al.,2001; Yang, 1996; Fromont Racine et al., 2003, Han et al., 2003; Tschochner and Hurt, 2003; Granneman and Baserga, 2004).) and tRNA molecules, are essential for ribosomes to translate mRNA into proteins. Mammalian cells contain hundreds of copies of tandemly repeated rRNA genes that are transcribed with high efficiency to keep up with the cellular metabolic activity and demand for ribosomes. A large group of proteins, known as initiation factors, also play an important role in controlling the rate and specificity of ribosome function.

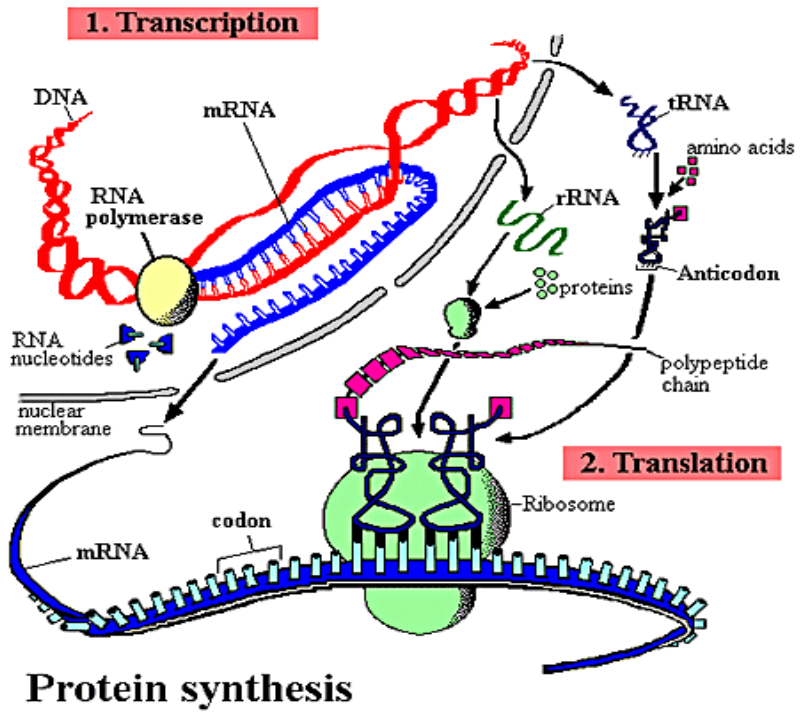


Figure : 1.8 Illustration of Protein synthesis where RNA is transcribed in the nucleus; once completely processed, it is transported to the cytoplasm and translated by the ribosome.

Tandem arrays of ribosomal genes are transcribed by RNA polymerase I in the nucleolous and transcripts are cleaved, modified, and are assembled with r- proteins to form 40S and 60S subunits of a mature eukaryotic ribosome. The 60S subunit, contains 28S, 5.8S and 5S rRNAs and a 40S subunit is composed of 18SrRNA. These rRNAs carry out essential structural and catalytic functions within the ribosomal core. The 18S and 28S genes are transcribed as immature polycistronic precursors by RNA polymerase I (Pol I) in the nucleolus, and the 5S and tRNA genes are synthesized by RNA polymerase III (Pol III) in the nucleoplasm. Ribosomal proteins (RPs) are crucial for ribosome assembly and are synthesized by RNA polymerase II (Pol II). All three classes of RNA polymerases utilize auxiliary transcription factors to recognize specific gene promoters and initiate transcription. These complexes (holoenzymes) contain RNA polymerase, general transcription factors, coactivators, and proteins involved in protein modification, RNA chain elongation, RNA processing and DNA repair. This process is carefully coupled with the synthesis of RPs; after translation, the RPs migrate to the nucleolus for the assembly of 40S and 60S ribosomal subunits. The ribosome structure has revealed that many RPs may function as RNA chaperones during the assembly of ribosomal particles and/or in the stabilization of important domains of the rRNA (Fatica and Tollervey, 2002). Some RPs are involved in the interaction between the 40 and 60S subunits (S13, S15, S19, L2, L5, and L14), make contact with tRNA (S7, S9, S12, S13, L1, and L5) or surround the polypeptide exit channel to stabilize the ribosome (L22, L24, and L29). Assumption was made that most RP genes have a single gene expression control mechanism because they have common promoters and synonymous codon compositions. However, the modern ribosome can be defined as a ribonucleoprotein complex that can neither assemble nor function without its protein complement. Most RPs have been classified into subgroups according to their expression profiles, promoter structures and amino acid and codon compositions. Moreover, the specific expression of certain RP genes is associated with certain human tissues (Bortoluzzi et al., 2001; Ishii et al., 2006). It has been reported that RPs, in addition to their role in stabilizing the ribosome, also exhibit secondary functions that have not yet been fully characterized in other cellular processes such as DNA repair, apoptosis, drug resistance, proliferation, growth inhibition and chemoresistance (Shen et al., 2006; Du et al., 2005). For example, the RPs P0 and S3 possess apurinic/apyrimidinic endonuclease activity that strongly implicates these proteins in DNA repair functions or regulating the formation of specific RNA binding complexes for selected mRNAs (Kim, et al., 1995; Fisher et al., 1990; Poddar et al., 2013). Most of the studies reporting that RPs have extraribosomal functions do not fully demonstrate that the indicated extraribosomal function is unrelated to the ribosome and protein translation.

In addition to antioxidant pathways, cells rely on multiple proteolytic pathways to combat the toxicity of oxidative stress in the brain. These diverse pathways are essential to suppress the elevation in

oxidized macromolecules of the brain and thus eliminate their ability to induce cellular toxicity. Oxidative modifications to proteins can result in altered protein function and structure and potential fragmentation. Oxidative damage to proteins increases the hydrophobicity of the proteins and thereby increases their potential for aggregation (Dasuri et al., 2010; Shringarpure et al., 2001). Interestingly, increased hydrophobicity may serve as an innate target for proteins to undergo proteolysis, highlighting the importance of maintaining proteolysis during conditions of elevated ROS.

1.3.1 Protein synthesis in neurodegeneration

A significant percentage of protein synthesis results in the generation of defective ribosomal products (DRiP's) (Yewdell et al., 1996), occurring as the result of faulty coding and/or transfer within the ribosome complex. The percentage of protein synthesis that results as DRiP's remains debated but ranges from 10 to 50%. Currently the amount of DRiP's that occur in the brain in response to aging and neurodegenerative stressors is unknown, but is a critical issue to address experimentally. This is because even a 10% baseline in protein synthesis resulting in the synthesis of aggregate-prone and highly hydrophobic (misfolded) DRiP's would change the way we look at the role of ''background'' levels of misfolded and aggregate-prone protein in the dysfunction of the brain during aging and neurodegenerative diseases associated with protein aggregation. The removal of DRiP's highlights the importance of maintaining proteasomal-mediated proteolysis as a defense mechanism to reduce the levels of aggregate-prone DRiP's. As DRiP's within the system increase, by definition, there is a corresponding decrease in the synthesis of essential and vital proteins within cells. Studies have demonstrated that there is a significant decrease in ribosomal RNA processing, reduced ribosomal stability, increased oxidative damage to ribosomal complex, and decreased levels of protein synthesis in the Alzheimer's disease brain (Ding et al, 2005, 2006) and impaired protein synthesis at the transcriptional and translational levels during early stages of AD, occurring in cortical regions of AD patients (Ding et al., 2005). Increased levels of RNA oxidation within the ribosome complex were also reported in parts of the brain in patients with mild cognitive impairment and AD (Ding et al., 2006, Shan et al., 2006). Thus, it is highly likely that the levels of DRiP's occur in the brain in response to aging and neurodegenerative disease, with oxidative stress likely to be involved in promoting declines in ribosome function. These data highlight a mechanism for DRiP's to work in concert with the levels of oxidative stress in the brain, to ultimately determine the level of toxicity and/or dysfunction that occurs.

It has been demonstrated that decreased protein synthesis occurs in the aging brain and in age-related neurodegenerative conditions including AD (Sajdel-Sulkowska and Marotta,1984; Langstrom et al.,

1989; Chang et al., 2002; Ferrer, 2002, An et al., 2003; Li et al., 2004; Ding et al., 2007). Protein synthesis has also been shown to play role in other neurodegenerative diseases such as Amyotrophic lateral sclerosis (ALS). For example, reduced protein synthesis along with the enzymatic degradation of the cytoskeleton within the larger dendrites has been found to be involved in neuronal atrophy associated with amyotrophic lateral sclerosis (Kiernan and Hudson 1993). Interestingly, in addition to decreased levels of cytosolic protein synthesis, mitochondrial protein synthesis activity is decreased in many tissues and cellular models (Rattan , 1996; Ryazanov and Nefsky 2002). This decreased protein turnover is associated with physiological consequences such as accumulation of damaged or altered proteins and a corresponding decrease in the synthesis of proteins that are essential to handling damaged protein removal (heat shock proteins, proteases, etc.

In contrast to the above evidences, studies have also suggested that inhibition of protein synthesis could help in preventing the neuronal damage in some neurodegenerative conditions. For example, modest inhibition of protein synthesis by rapamycin has been found to significantly reduce protein aggregate formation in a cellular model of huntington's disease (HD) (King et al., 2008). Attenuation of NMDA (N-methyl-D-aspartate) induced vasodilation was reported after brief exposure to globalischemia orhypoxia (Busija et al., 1996), which may be an indicator of early postischemic neuronal damage. Pretreatment with pharmacological inhibitors of protein synthesis preserved the vascular responses of cerebral arterioles to NMDA, suggesting the involvement of protein synthesis in brain damage following ischemia (Veltkamp et al., 1999). These studies highlight the complexity of the role of protein synthesis and protein degradation in the context of oxidative stress in the brain. Clearly, too much or too-prolonged periods of impaired proteolysis or protein synthesis are deleterious and toxic. Shutting down levels of proteolysis and protein synthesis during the actual injury or initial rise in ROS is probably a very beneficial thing (reducing the amount of damage ROS can do to proteins and proteases), but the ramping up of these pathways after oxidative stressors is essential to successfully survive durations of ROS injury. It is likely that it is the duration of impaired protein synthesis and impaired proteolysis that ultimately contributes to brain aging and neurodegenerative disease, whereby decades are required in the human brain to reach the pathological consequences of lower synthesis and degradation of proteins. The mechanisms responsible for impairments in protein synthesis mediating neurodegeneration are probably based on a decrease in the synthesis of antiapoptotic (Bcl-2, AIF, etc.), prosurvival (growth factors, antioxidant enzymes, heat shock proteins) factors that help to preserve cell homeostasis.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

The hypothesis of this thesis is that if mitochondrial decrease is an early event in PD and DLB and further if mitochondrial dysfunction and alterations of protein synthesis in the PD and DLB pathologies are extended to certain brain areas.

Objective 1- To learn about the mitochondrial alterations in frontal cortex and angular gyrus in PD and DLB post-mortem samples.

Objective 2 - To study the dysregulation of protein synthesis in different regions including the *substantia nigra,* frontal cortex area 8, angular gyrus and precuneus in PD and DLB post-mortem samples.

Materials and Methods

3. MATERIALS AND METHODS

3.1 CASES AND GENERAL PROCESSING

Brain tissues from Substantia nigra (SN), Frontal cortex area 8 (FC), Angular gyrus (AG), Precuneus (PC) were obtained from the Institute of Neuropathology Brain Bank (HUB-ICO-IDIBELL Biobank) and the Biobank of Hospital Clinic-Institute for Biomedical Investigation August Pi i Sunyer (IDIBAPS) following the guidelines both of Spanish legislation on this matter and of the local ethics committee. One hemisphere was immediately cut in 1-cm-thick coronal sections, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags, numbered with water-resistant ink, and stored at -80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphologic studies including neuropathological diagnoses and classification. A summary of the cases and their use in the different methods used is shown in (Table 3.1).

Clinically, cases at stage 1 or 2 were apparently asymptomatic and were identified at the time of neuropathological examination as (incidental iPD), whereas the remaining stage 3 or 4 had suffered from parkinsonism and received treatment for the duration of the disease (usually less than four years) (PD stages 3 or 4). PD-D (PD-D Stages 5 or 6) is used when dementia appears more than one year after the onset of otherwise typical Parkinson's disease. DLB are diagnosed with the presence of lewy bodies in neo cortex (DLB Stages 5 or 6) and appears within or before a year after PD. The causes of death in these cases were unrelated events that had reduced survival expectancies including cardiac infarction, disseminated carcinoma and infectious diseases.

Pioneering studies on human brain tissue have been supported by combined phosphorus and proton magnetic resonance spectroscopic imaging showing mitochondrial dysfunction in midbrain and putamen in PD (Hattingen et al., 2009). Hence the regions angular gyrus and precuneus were selected in the present study which are progressivley affected regions next to *Substantia nigra* (SN) and Frontal cortex (FC). The neuropathologic diagnosis of PD and DLB was based on the classification of Braak (Braak et al., 2006). In the present study 4 regions were examined, **Frontal cortex (FC),** CONTROLS: n =(45) (25 Males and 20 Females Mean age =59 years); iPD CASES: n =(7) (5 Males and 2 Females, Mean age =67.5 years); PD CASES: n=(34) (26 Males and 8 Females, Mean age =60 years); DLB CASES: n =(37) (23 Males and 14 Females, Mean age =80 Years). **Substantia nigra (SN),** CONTROLS: n= (10) (8 Males and 2 Females, Mean age =61 years); PD CASES: n=(6) (5 Males and 1 Female, Mean age =76.5 years); DLB CASES: n=(6) (5 Females and 1 Male, Mean age

=80 years). **Angular gyrus (AG),** CONTROLS: n =(10) (6 Males and 4 Females, Mean age =62 years); PD CASES: n =(8) (7 Males and 1 Female, Mean age =68 years); PDD =(4) (2 Males and 2 Females, Mean age = 77 years). **Precuneus (PC),** CONTROLS: n =(12) (9 Males and 3 Females, Mean age =58 years); PD CASES: n =(14) (10 Males and 4 Females, Mean age =78 years). The post-mortem interval between death and tissue processing was less than 21 h 45 min in all cases.

Table 3.1 Clinical and Pathological Characteristics of Patients and Analysis Methods

Case	Gender	Age	P.M.I	Neu. Diag	Region		WB	Assays	IF	PCR
1	M	71	12h	C	FC		X	X		X
2	M	55	5h 40min	C	FC		X	X		X
3	M	66	5h	C	FC		X	X		X
4	M	59	7h 5min	C	FC		X	X		X
5	M	61	2h 45min	C	FC		X	X		X
6	M	56	5h	C	FC	AG	X	X		X
7	M	59	6h 25min	C	FC		X	X		X
8	M	62	3h	C	FC	AG	X	X		X
9	F	75	3h	C	FC		X	X		X
10	M	53	3h	C	FC		X	X		X
11	M	52	4h 40min	C	FC	AG		X		X
12	M	64	8h 35min	C	FC	AG		X		X
13	F	46	9h 35min	C	FC	AG		X		X
14	M	67	5h	C	FC	AG		X		X
15	F	75	3h	C	FC	AG		X		X
16	F	78	12h	C		AG		X		X
17	M	56	5h	C		AG		X		X
18	F	69	5h 30min	C		AG		X		X
19	M	42	NA	C						
20	F	80	NA	C	FC				X	
21	M	64	NA	C	FC				X	
22	F	79	NA	C	FC				X	
23	F	88	NA	C	FC				X	
24	F	60	NA	C	FC				X	
25	M	42	NA	C	FC				X	
26	F	61	NA	C	FC				X	

(Continued)

Case	Gender	Age	P.M.I	Neu. Diag	Region	WB	Assays	IF	PCR
27	F	51	NA	C	FC				X
28	M	63	NA	C	FC				X
29	M	64	08 h 30m	C	FC				X
30	M	67	14 h 40m	C	FC				X
31	M	56	5h	C	FC				X
32	M	67	5h	C	FC				X
33	M	62	3h	C	FC				X
34	M	52	4h 40m	C	FC				X
35	M	30	4h 10m	C	FC				X
45	F	71	8h 30m	C	FC				X
46	M	59	4h 15m	C	SN				X
47	M	67	14h 40m	C	SN				X
48	M	70	2h	C	SN				X
49	M	61	4h 30m	C	SN				X
50	M	63	8h 5m	C	SN				X
51	M	57	4h 30m	C	SN				X
52	M	60	4h 15m	C	SN				X
53	F	68	4h 30m	C	SN				X
54	F	79	6h 25m	C	SN				X
55	M	43	5h 55 min	C	SN				X
56	M	67	5h	C	PC				X
57	M	30	4h 10m	C	PC				X
58	M	67	11h 45m	C	PC				X
59	F	71	8h 30m	C	PC				X
60	F	86	4h 15m	C	PC				X
61	F	79	6h 25m	C	PC				X
62	M	52	4h 40m	C	PC				X
63	M	61	4h 30m	C	PC				X
64	M	77	6h 55m	C	PC				X
65	M	59	4h 15m	C	PC				X
66	M	76	4h 30m	C	PC				X
67	M	68	4h 45m	C	PC				X
68	M	66	8h	PD 1-2	FC	x			
69	M	68	2h 15min	PD 1-2	FC	x			
70	F	77	3h 15min	PD 1-2	FC	x			
71	M	72	8h 55min	PD 1-2	FC	x			
72	M	57	11h	PD 1-2	FC	x			
73	F	70	10h 50min	PD 1-2	FC	x			
74	M	78	10h 45min	PD 1-2	FC	x			
75	M	47	NA	PD 4	FC			X	

(Continued)

Case	Gender	Age	P.M.I	Neu. Diag	Region		WB	Assays	IF	PCR
76	M	59	NA	PD 4	FC				X	
77	M	35	NA	PD 4	FC				X	
78	M	63	NA	PD 4	FC				X	
79	M	59	NA	PD 4	FC				X	
80	M	79	9h 15min	PD 4	FC		X	X	X	X
81	M	76	4h 30min	PD 4	FC	AG	X	X		X
82	M	69	15h 5min	PD 4	FC	AG	X	X		X
83	F	76	12h	PD 4	FC		X	X		X
84	F	74	10h 15min	PD 3	FC		X	X		X
85	M	68	4h 45min	PD 4	FC	AG	X	X		X
86	M	79	9h 15min	PD 4	FC	AG	X	X		X
87	F	79	6h 20min	PD 4	FC	AG	X	X		X
88	M	57	7h	PD 4	FC	AG	X	X		X
89	M	57	11h	PD 3	FC	AG	X	X		X
90	M	66	5h	PD 4	FC	AG	X	X		X
91	F	70	10h 50min	PD-D	FC	AG		X		X
92	M	78	10h 45min	PD 3	FC			X		X
93	F	54	11h 10min	PD 3	FC			X		X
94	M	85	3h 15min	PD-D	FC	AG		X		X
95	F	70	5h 15min	PD-D	FC	AG		X		X
96	M	69	5h 55min	PD-D	FC	AG		X		X
97	M	66	5h	PD IV	FC					X
98	M	57	11h	PD 3	FC					X
99	M	57	19h	PD 3	FC					X
100	M	76	4h 30m	PD 4	FC					X
101	M	68	4h 45m	PD 4	FC					X
102	M	79	9h 15m	PD 4	FC					X
103	M	69	5h 55m	PD 4	FC					X
104	F	54	11h 10m	PD 3	FC					X
105	F	84	4h 30m	PD 4	FC					X
106	M	68	9h 20m	PD 4	FC					X
107	M	77	12h	PD 4	FC					X
108	M	81	4h 55m	PD 3	FC					X
109	M	75	NA	DLB 5	FC		X	X		
110	M	73	3h 30 min	DLB 5	FC		X	X		X
111	F	77	2h	DLB 5	FC		X	X		X
112	F	84	4h	DLB 5	FC		X	X		X
113	F	88	12h	DLB 5	FC		X	X		X
114	M	81	6h 30 min	DLB 5	FC		X	X		X
115	M	90	5h	DLB 5	FC		X	X		X
116	F	87	4h 15 min	DLB 5	FC		X	X		X
117	F	75	NA	DLB 5	FC		X	X		X
118	M	77	11h	DLB 5	FC		X	X		X

(Continued)

Case	Gender	Age	P.M.I	Neu. Diag	Region	WB	Assays	IF	PCR
119	M	78	7h	DLB 5	FC	X	X		X
120	M	82	4h 30 min	DLB 5	FC		X		X
121	F	73	4h	DLB 5-6	FC		X		X
122	M	78	8h 30min	DLB 5-6	FC		X		X
123	M	80	4h 30min	DLB 5-6	FC		X		
124	M	87	6h	DLB 5-6	FC		X		
125	M	82	11h 30 min	DLB 5-6	FC		X		
126	M	74	6h	DLB 5-6	FC		X		
127	F	75	6h	DLB 5-6	FC		X		
128	M	72	10h	DLB 5-6	FC		X		
129	F	70	4h 30m	DLB 5	FC				X
130	F	77	3h 30m	DLB 5	FC				X
131	F	81	6h 30m	DLB 5	FC				X
132	F	69	4h 30m	DLB 5	FC				X
133	F	79	3h 30m	DLB 5	FC				X
134	M	78	13h 30m	DLB 5	FC				X
135	M	83	14h	DLB 5	FC				X
136	F	77	7h 30m	DLB 5	FC				X
137	M	80	7h 30m	DLB 6	FC				X
138	M	84	16h 30m	DLB 5	FC				X
139	M	79	4h 30m	DLB 5	FC				X
140	M	77	7h 30m	DLB 5	FC				X
141	F	85	6h	DLB 5	FC			X	
142	M	79	NA	DLB 5	FC			X	
143	M	77	9h 30 min	DLB 5	FC			X	
144	M	76	10h	DLB 5	FC			X	
145	M	68	9h 20m	PD 4	SN				X
146	M	80	7h 30m	PD 4	SN				X
147	M	85	11h 45m	PD 4	SN				X
148	M	81	4h 55m	PD 3	SN				X
149	M	84	16h 30m	PD 3	SN				X
150	F	84	4h 30m	PD 4	SN				X
151	M	83	14h	DLB 5	SN				X
152	M	80	7h 30m	DLB 6	SN				X
153	M	76	12h	DLB 5	SN				X
154	F	77	7h 30m	DLB 5	SN				X
155	M	76	21h 45 min	DLB 5	SN			X	
156	M	84	9h	DLB 6	SN			X	
157	M	76	4h 30m	PD 4	PC				X
158	M	68	4h 45m	PD 4	PC				X

(Continued)

Case	Gender	Age	P.M.I	Neuropath. Diag	Region	WB	Assays	IF	PCR
159	M	85	3h 15m	PD 4	PC				X
160	F	70	5h 15m	PD 4	PC				X
161	M	85	11h 45m	PD 4	PC				X
162	F	70	4h 40m	PD 4	PC				X
163	M	83	4h	PD 4	PC				X
164	M	69	15h 5m	PD 4	PC				X
165	M	74	6h 45m	PD 4	PC				X
166	F	88	11h 50m	PD 4	PC				X
167	F	85	6h 15m	DLB 5	PC				X
168	M	84	16h 30m	DLB 5	PC				X
169	M	79	4h 30m	DLB 5	PC				X
170	M	77	7h 30m	DLB 5	PC				X

F = Female; M = Male; NA = Not available; PMI = post-mortem interval; Neu. Diag = Neuropathological Diagnosis PD I-II; PD III-IV according to braak classification; FC = Frontal Cortex; AG = Angular Gyrus; SN = Substantia Nigra; PC = Precuneus; WB = Western Blot; IF = Immunofluorescence; PCR = Polymerase Chain Reaction.

3.2 RNA PURIFICATION

The purification of RNA from the cerebral cortex in frontal cortex (area 8), angular gyrus, other regions of PD samples, and their age-matched controls was undertaken with RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany, DE) following the protocol provided by the supplier. During purification, samples were treated with RNase-free DNase set (Qiagen, DE) for 15 min to avoid extraction and later amplification of genomic DNA. The concentration of each sample was measured at OD 340_{nm} using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachussetts, USA). RNA integrity number (RIN) was verified with the Agilent 2100 Bioanalyzer (Agilent, Santa, Clñara, California, USA).

3.2.1 WHOLE-TRANSCRIPT EXPRESSION ARRAYS

RNA samples from the frontal cortex (area 8) of control (n=7) and PD (n=11) cases were analyzed using the Affymetrix microaaray platform and the Genechip Human Gene 1.1ST Array (Garcia et al., 2013). Microarray Data Normalization and Differential Expression analysis Preprocessing of raw data and statistical analyses were performed using Bioconductor packages in R programming environment (Gentleman 2004) as shown elsewhere (Garcia et al., 2013). The microaaray experiment was deposited in the array Express Database under accession number E-MTAB-1194.

3.2.1.1 Funcitonal Genome Enrichment Analysis (Oxidative Pathway)

mRNA samples from frontal cortex PD (n =11) and age-matched controls (n=7) were hybridized to an Affymetrix Human Gene 1.1 ST Array, that interrogates over 1 million exon clusters within the known and predicted transcribed regions of the entire genome. We found a differential expression of genes belonging to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) pathway "Oxidative phosphorylation" (KEGG:00190). Modification of transcriptional profiles for these oxphos genes in PD was evidenced on the heat map representation for a non-restrictive gene list at non-adjusted probability of $p < 0.1$.

3.2.1.2 Functional Genome Enrichment Analysis (Ribosome Pathway)

To evaluate whether the set of genes of the Ribosome pathway, extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, is enriched in PD smaples we computed the Gene Set Enrichment Analysis from Limma Package (Smyth 2005). It is based on a set of probe-wise t statistics arising for microarray analysis. We computed 3 different tests:1) upregulated genes with positive t statistics, 2) downregulated genes with negative t-statistics; and 3) upregulated or downregualted genes as a whole.

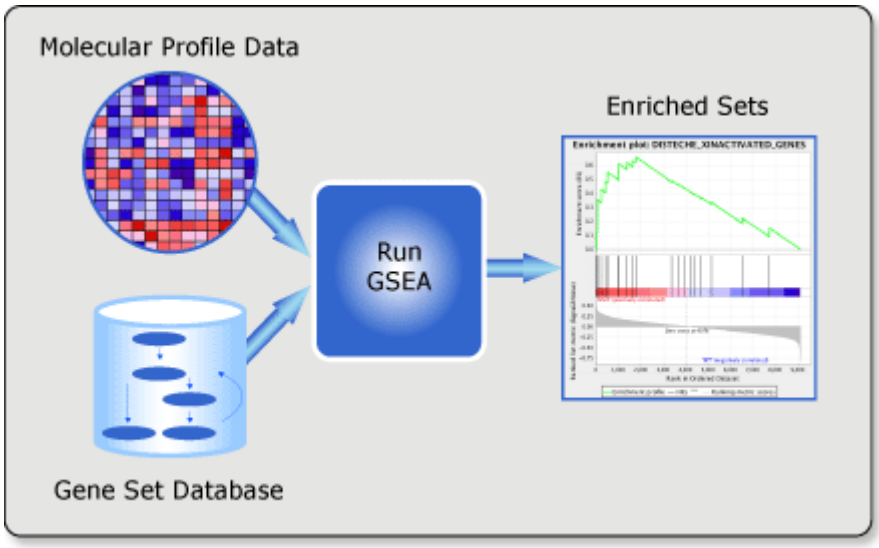


Figure : 3.1 Illustration of Gene Set Enrichment (also called functional enrichment analysis) which indentify classes of genes or proteins that are over-represented in large set of genes or proteins,which uses statistical approach to identify significantly enriched or depleted groups of genes.

3.2.2 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) by using a reverse transcriptase . The cDNA is then used as a template for exponential amplification using PCR.

Retrotranscription reaction

The retrotranscriptase reaction of the RNA samples selected based on their RIN was carried out with the High-Capacity cDNA Archive kit (Applied Biosystems: Foster City, California, USA) following the protocol provided by the manufacturer and is amplified using the Gene Amp® 9700 PCR System thermocycler according the reaction conditions outlined below

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25°C	37°C	85°C	4°C
Time	10min	120min	5min	∞

- **Reaction mixture :** PCR assays were conducted in duplicate on cDNA samples obtained from the retrotranscription reaction and diluted 1:20 for PD and their age-matched controls, in 384-well optical plates (Applied Biosystems) utilizing an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Amplification reactions for each sample were carried out using the 20× TaqMan Gene Expression Assays shown in Table 2.6 and 2.7 (Applied Biosystems) and 2× TaqMan Universal PCR Master Mix (Applied Biosystems). β-Glucuronidase (GUS-β) was used as internal control for normalization. This housekeeping gene was selected because it shows no modifications in several neurodegenerative diseases in human post-mortem brain tissue (Barrachina et al., 2006; Durrenberg et al., 2012).

- **Procedure :** The reactions were performed with the following parameters: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

- **Analysis :** TaqMan PCR data were captured using the Sequence Detector Software (SDS version 2.1, Applied Biosystems). Subsequently, CT data for each sample, human were analyzed with the double delta CT (ΔΔCT) method. Delta CT (ΔCT) values represent the normalized levels of each target gene in relation to endogenous controls (GUS-β), whereas

ΔΔCT values were calculated as the ΔCT of each sample minus the mean ΔCT of the population of control samples (calibrator samples). The fold change was determined using the equation $2^{-\Delta\Delta CT}$. All the data were analyzed by Unpaired Student's *t*-test by using Graphpad prism 5 program. In all experimental procedures the significance level was set at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

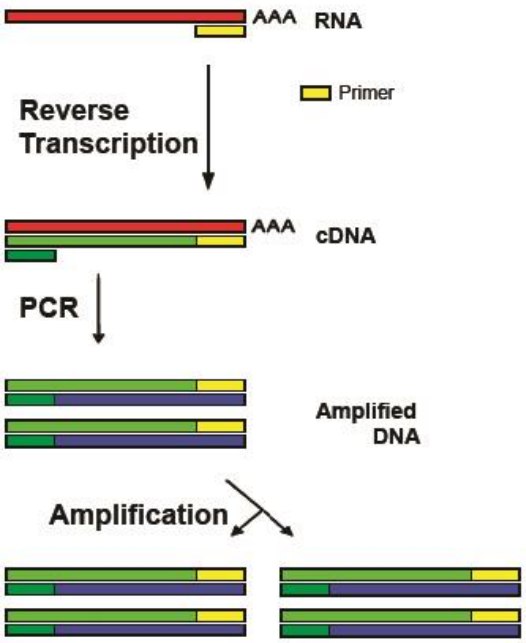


Figure 3.2 : Schematic illustration of RT-PCR where RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR.

Table 3.2 Probes used for the study of ribosomal proteins, including probes used for normalization

Probe	Sequence
GUSβ	GCTACTACTTGAAGATGGTGATCGC
RPL5	TGTCTATCCCTCACAGTACCAAACG
RPL7	GAATCAGAGGTATCAATGGAGTGAG
RPL21	GGAGTAGCATTAGTTCTGATGCAGT
RPL22	AGGTGGGAGGTGCCCTGGAAATAGC
RPL23A	AACTCAGAACTTGCCTCCATGGTTG
RPL26	CCGAAGCGGGAGCGGCCAAAATGAA
RPL27	CTAATGCCCACAAGGTACTCTGTGG
RPL30	TATCATTGATCCAGGTGACTCTGAC
RPL31	GGCCAAAGGAATAAGGAATGTGCCA
RPS3A	AGGTGCTAAAGTTGAACGAGCTGAT
RPS5	ACAGGCGAGAACCCTCTGCAGGTCC
RPS6	GGCCCCAAAAGAGCTAGCAGAATCC
RPS10	ATTCCAGTTTAGAGGCGGATTTGGT
RPS13	GACGCAGCGTCCCCACTTGGTTGAA
RPS16	GGTCTTCGGACGCAAGAAGACAGCG
RPS17	GCTGAAGCTTTTGGACTTCGGCAGT

1).GUSβ (glucuronidase, beta); **2). RPL5** (Ribosomal Protein L5); **3). RPL7** (Ribosomal Protein L7); **4). RPL21** (Ribosomal Protein L21); **5). RPL22** (Ribosomal Protein L21); **6). RPL23A** (Ribosomal Protein L23A); **7). RPL26** (Ribosomal Protein L26); **8). RPL27** (Ribosomal Protein L27); **9). RPL30** (Ribosomal Protein L30); **10). RPL31** (Ribosomal Protein L31); **11). RPS3A** (Ribosomal Protein S3A); **12). RPS5** (Ribosomal Protein S5); **13). RPS6** (Ribosomal Protein S6); **14). RPS10** (Ribosomal Protein S10); **15). RPS13** (Ribosomal Protein S13); **16). RPS16** (Ribosomal Protein S16); **17). RPS17** (Ribosomal Protein S17).

Table 3.3 Probes used for the mitochondrial subunit studies, including the probes used for normalization (GUSB) β-glucuronidase.

Probe	Sequence
GUSβ	GCTACTACTTGAAGATGGTGATCGC
NDUFA7	AAGCCAGCAGAGAGCTCTGCTGTAG
NDUFA10	CACTTGCTGACCACAGGACAAGGTG
NDUFB10	GTGACCCTCGTGAGAGAATTTATAG
NDUFS7	ATCCCAGGCTGCCCACCTACGGCCG
NDUFS8	TCTGCCCCGCCCAGGCCATCACCAT
COX7A2L	CAAAAGTTTTTCCAGAAAGCTGATG
ATP2B3	TAAGAAAGGCAAGCAGCAGGATGGG
ATP2B4	CCTGTGGAAGGTCTGTCTGGGAACC
ATP4A	GGCCAGGAGTGGACATTCGGGCAGC
ATP5H	TATGAGAAAGAGATGGAGAAGATGA
ATP5L	CGCTGGTGAACGCTGCTGTGACTTA
ATP5G2	ACTGACAGATGAGAGCCTCAGCAGC
ATP6V0B	TCGTCGCAATTCTTCAGACCTCCAG
ATP6V1H	GATGGGGTAAATTGCATAATGGGAG
SLC25A31	TGGAAAAGGTCCTGAGGAGCGACAA
SLC6A6	CCCTTGTTCTCTGGTATCGGCTATG
LHPP	GGAGGCGGTGGCCAGACTGAAGCGT
PTPIP51	CCCGGACGCCACGTGATGCTCCTGC
ZNF642	GCCCAGGAGATCCCAGTTCAGACTT
RPS20	TGGAAAAGGTGTGTGCTGACTTGAT

1). NDUFS8 (NADH dehydrogenase (ubiquinone) Fe-S protein 8); **2). ATP2B3** (ATPase, Ca++ transporting, plasma membrane 3); **3). ATP2B4** (ATPase, Ca++ transporting, plasma membrane 4); **4). ATP4A** (ATPase, H+/K+ exchanging, gastric, alpha polypeptide); **5). ATP5L** (ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G); **6). ATP5G2** (ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2 (subunit 9)); **7). ATP56V0B** (ATPase, H+ transporting, lysosomal 16kDa, Vo Subunit B) ; **8). ATP6V1H** (ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H); **9). SLC25A31** solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31; **10). SLC6A6** (solute carrier family 6 (neurotransmitter transporter), member 6); **11). LHPP** (phospholysine phosphohistidine inorganic pyrophosphate phosphatase); **12). PTP1P51** (Protein tyrosine phosphatase-interacting protein 51); **13). ZNF642** (zinc finger protein 642); **14). RPS20** (ribosomal protein S20).

3.3 TOTAL PROTEIN EXTRACTION

The samples which are at -80°C with keen care are kept on dry ice and are sliced with an average weight (200mg) per sample related to PD and DLB including age matched control samples. These samples were homogenized using dispersing and mixing motor (Polytron PT-MR1600E , Kinematica, Switzerland) in RIPA buffer (Table 3.2) with inhibitors (Table 3.3) and left for mixing by using orbital shaker (Orbital shaker, J.P.Selecta; S.A., Spain) at 4 °C for 1 hr at 15 rpm and later these are centrifuged at 4°C for 15 min at 13000rpm (Centrifuge 5417R, Eppendorf, Germany). The supernatant obtained after this step is stored at -80°C for further use and the pellet is discarded.

Figure 3.3 For molecular biology and biochemical studies, A) brain tissue from frontal lobe and midbrain were dissected, and subsequently regions of interest were separated. B) For total protein extracts polytron homogenizer was used. C) For mitochondrial extracts a douncetissue grinder was used.

Table 3.4 Ripa lysis buffer

Reagent	Final concentration
Tris HCl (pH 8)	50mM
NaCl	150mM
Nonidet P40	0.1%
Sodium deoxycholate	0.5%
SDS	0.1%

Table 3.5 Inhibitors

Inhibitors	Final concentration
PMSF	1mM
Orthovanadate	1mM
Phosphoglycerophosphate	2.5mM
NaF	1mM

NOTE : Add a half tablet for 5ml of buffer and the inhibitors are mixed just before the use and the RIPA Buffer should be ice-cold.

3.4 ISOLATION OF MITOCHONDRIA

Approximately between 200-300 mg of brain tissue was used for the study. The brain mitochondria were isolated as described (Long et al, 1991). Tissues were minced in ice-cold isolation buffer (Table 3.4), then homogenized and centrifuged at 1000 g for 10 min. The supernatant was decanted and saved. The pellet was washed once with 2 vol of isolation buffer. The supernatant obtained above was then combined and centrifuged at 10,000g for 10min. The mitochondrial pellet was then washed twice with isolation buffer and then finally the mitochondria were resuspended in minimal amount of isolation buffer.

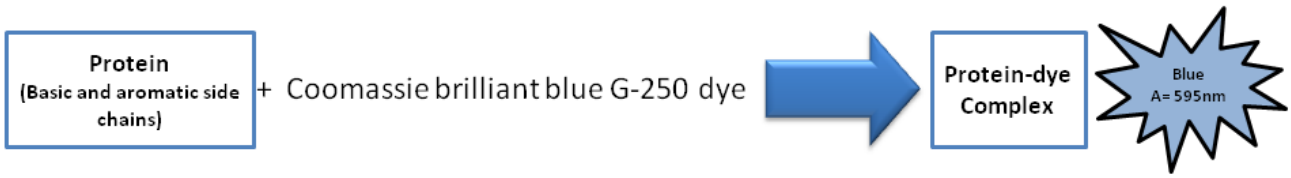
All the above operations were carried out at 4°C. The mitochondrial protein concentration was determined by Bradford Protein Assay Kit (Sigma St. Louis ,USA) using BSA as the standard. (Thermoscientific, Waltham, MA).The mitochondria were aliquoted and stored at -80°C until enzyme analyses were performed. The same aliquots were used for the study of protein quantification through western blotting.

Table 3.6 Isolation Buffer

Reagent	**Final concentration**
Sucrose	0.25M
Tris	10mmol/l
EDTA	0.5mmol/l
pH	7.4

3.5 DETERMINATION OF SOLUBLE PROTEIN CONTENT

The protein content was estimated by the method of Bradford, (1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. The mitochondrial protein concentration was determined by Bradford Protein Assay Kit (Sigma St. Louis ,USA) using BSA as the standard. (Thermoscientific, Waltham, MA).



3.6 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOTTING

Proteins to be separated based on their size was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The procedure for specific detection of proteins using antibodies is western blotting or immunoblotting. The specificty depends on the type and the concentration of antibody used. The stock solutions and steps followed in running electrophoresis and western blotting are described below:

3.6.1. Reagents and Solutions

3.6.1.1. Stock solutions

Acrylamide Bisacrylamide solution

Acrylamide (30.0% w/v) N-N'- Methylene-bisacrylamide (0.8% w/v) (Biorad, Hercules,CA, United states of America) is stored at 4°C in dark.

Tris-HCl, 1.5M (pH 8.8)

1.5 M Tris was prepared by dissolving 93.50 g of Tris in 100.0 ml of MQ-water. The pH of the solution was adjusted to 8.8 using 1N HCl and stored at 4°C.

Tris-HCl, 0.5M (pH 6.8)

0.5 M Tris was prepared by dissolving 18.0 g of Tris in 500.0 ml of double distilled water. The pH of the solution was adjusted to 6.8 using 1N HCl. The solution was stored at 4°C.

Ammonium per sulphate (APS)

APS solution (10%) was prepared by dissolving 0.1 g of APS in 1.0 ml of double distilled water. The solution was stored at 4°C in dark.

Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulphate (10%) was prepared by dissolving 10.0 g of SDS in 100.0 ml of MQ-water. The solution was stored at room temperature.

3.6.2. Preparation of gel monomer

The composition of the resolving and stacking gels was as follows:

Solution	Resolving gel (12%) (ml)	Resolving gel (15%) (ml)	Stacking gel (5%) (ml)
Acrylamide (30%)	4.0	5.0	1.3
Tris, 1.5 M (pH 8.8)	2.50	2.50	_
Tris, 1.0 M (pH 6.8)	-	-	2.5
SDS (10%)	0.05	0.05	0.025
APS	0.1	0.05	0.1
TEMED	0.01	0.01	0.01
Distilled water	4.00	2.4	6.05

Electrode buffer

10X stocks of electrode buffers were prepared which were subsequently diluted before each run. The composition of 10X buffer used was as follows:

Reagent	**Final concentration**
Tris	0.25 M
Glycine	1.92 M
pH	8.50

The 10x buffer is diluted to 1X with the addition of 10 ml of 10% SDS and was stored at 4°C untill before use.

Transfer Buffer (1X)

Reagent	**ml**
Methanol	200 ml
10 X electrode buffer	100 ml
d H_2O	700ml

TBS-T (10X)

Reagent	**Final concentration**
Tris	100 mM
Nacl	1.4 M
pH	7.40

10 ml of Tween-20 is added for every 1 lit of 10X TBS-T.1X TBS-T buffer was diluted from the stock and was stored at 4°C untill before use for washing the membranes.

3.6.3 Preparation of protein samples for SDS-PAGE

Tracking dye (bromophenol blue) 4X sample buffer solution is mixed with β-mercaptoethanol in a ratio of 9:1 were mixed with desired final cocentration of protein to be loaded and was mixed thoroughly. The mixture was heated in dry block thermostat (BIO-TDB-100) for 5 min. Thereafter sample was loaded to each well of the stacking gel.

3.6.4 Polymerization of gels

The SDS-PAGE was carried out with (BIORAD, Dual mini slab gel electrophoreis unit ,Hercules, CA, U.S.A). The resolving gel was poured and immediately layered by 70.0% (v/v) isopropanol to prevent drying and consequent closure of the pores in the gels. The gel was left for complete polymerization, after which the over-layered isopropanol was extensively washed with distilled water. The stacking gel was then poured, with the comb inserted at the top and gel was left undisturbed further to polymerize.

3.6.5 Electrophoresis of gels

The solidified gels were placed in the electrophoretic unit and filled with electrode buffer. Protein samples (10-20µg) and protein marker (BIORAD, hercules, CA, U.S.A) (5.0 µl) were loaded in different wells. A constant current of 6.0 mA/well was applied from a D.C. power supply till the dye front reached the bottom line.

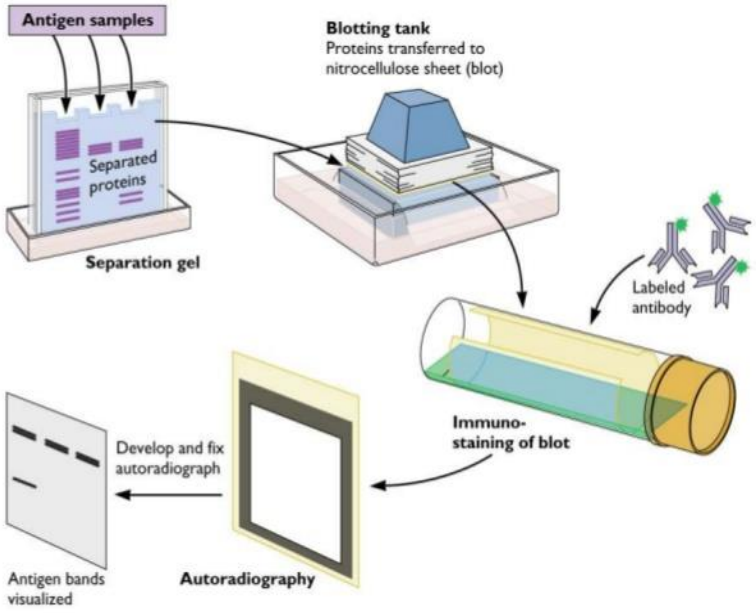


Figure 3.4 : The schematic illustration of gel electrophoresis and western blotting. The bands detected at the end were further used for the quantification of the protein.

3.6.6 Electrophoretic transfer

The electrophoretic transfer is a method of transferring proteins separated on SDS-PAGE gels on to the membranes prior to their detection by immunoblotting.

3.6.6.1 Transfer

Proteins separated in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis are electrophoretically transferred to nitrocellulose membranes (Amersham, GE Healthcare, Buckinghamshire, UK). The membrane was activated in methanol by using transfer buffer. The blot chamber was assembled using two sponges, four sheets of Whatman™ papers, the gels and the membranes which were cut to the exact size of the gel. (400 mA per membrane, 90 minutes). The protein transfer was conducted in an ice-cooled Trans-Blot® Cell at 135V and 1500mA for 3 h. To confirm a good transfer, membranes were stained with ponceau and later washed with TBS-T untill the color disappears.

3.6.6.2 Blocking

Non-specific bindings were blocked by incubation with 5% milk in 1X TBS-T for 1 h at room temperature. After washing, membranes were incubated overnight at 4°C with the antibodies listed in the (Table 2.4). The election of the antibodies was based on their commercial availability and the primary antibodies were diluted accordingly in 1X TBS-T containing 5% albumin.

TABLE 3.7 Characteristics of Primary Antibodies used in the study

Primary antibody	Host	Source	Working dilution	
			IF	WB
Oxphos (Cocktail)	Mouse Polyclonal	Mitosciences (US)		1:6000
Complex I NDUFS3	Mouse Polyclonal	Invitrogen (US)		1:1000
Complex I NDUFS7	Rabbit Polyclonal	Proteintech (UK)	1:100	1:1000
complex I NDUFA7	Rabbit Polyclonal	Proteintech (UK)		1:300
Complex I NDUFA10	Rabbit Polyclonal	Proteintech (UK)		1:500
Complex I NDUFB10	Rabbit Polyclonal	Proteintech (UK)		1:501
Complex II Fp subunit	Mouse Polyclonal	Invitrogen (US)	1:100	1:1000
Complex III Core II	Mouse Polyclonal	Invitrogen (US)	1:100	1:1000
Complex IV Subunit I	Mouse Polyclonal	Invitrogen (US)		1:1000
Complex IV COX 7C	Rabbit Polyclonal	Proteintech (UK)	1:100	1:1000
complex IV COX7A2L	Mouse Polyclonal	Proteintech (UK)		1:1000
Complex V ATP 5D	Rabbit Polyclonal	Proteintech (UK)		1:1000
Complex V ATP 5J	Rabbit Polyclonal	Proteintech (UK)	1:100	1:1000
Complex V ATP 5H	Rabbit Polyclonal	Proteintech (UK)		1:1000
SOD 2	Rabbit Polyclonal	Stressgen (US)		1:1000
TOMM 40	Rabbit Polyclonal	santa Cruz		1:1200
TOMM 70	Rabbit Polyclonal	Proteintech (UK)		1:1000
NRF1	Mouse Polyclonal	santa Cruz		1:500
NRF2	Mouse Polyclonal	santa Cruz		1:500
Tfam	Rabbit Polyclonal	santa Cruz		1:500
Phosphorylated EIF2α	Rabbit Polyclonal	Abcam (UK)		1:500
MRPL21	Rabbit Polyclonal	Proteintech (UK)		1:500
MRPL22	Rabbit Polyclonal	Novus biological		1:500
MRPS10	Rabbit Polyclonal	Proteintech (UK)		1:500
Neuroketal	Goat Polyconal	chemicon (US)		1:5000
MDAL	Rabbit Polyclonal	Academy BioM.cor		1:1000
HNE	Rabbit Polyclonal	Calbiochem (US)		1:1000
VDAC	Rabbit Polyclonal	Abcam (UK)	1:100	1:1000
Beta tubulin	Mouse Polyclonal	Promega		1:5000
Actin	Mouse Polyclonal	Sigma		1:5000

1). Oxphos cocktail consists of **Comlex I NDUFB 8** (NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8), **Complex II SDHB** (Succinate dehydrogenase complex, subunit B), **Complex III UQCRC2** (ubiquinol-cytochrome c reductase core protein II), **Complex IV COX II** (Cytochrome C oxidase subunit II), **Complex V ATP5A** (ATP synthase, H+ transporting, mitochondrial F1 complex); **2). NDUFS3** (NADH dehydrogenase (ubiquinone) Fe-S protein 3); **3). NDUFS7** (NADH dehydrogenase (ubiquinone) Fe-S protein 7); **4) NDUFA7** (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7); **5). NDUFA10** (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10); **6)**. **NDUFB10** (NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10); **7).** Fp (Flavoprotein subunit); **8). COX 7C** (Cytochrome C oxidase subunit VIIC); **9). COX7A2L** (Cytochrome C oxidase subunit VIIA2L); **10). ATP5D** (ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit); **11). ATP5J** (ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F6); **12). ATP5H** (ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d); **13). SOD2** (Superoxide dismutase 2); **14). TOMM 40** (translocase of outer mitochondrial membrane 40); **15). TOMM 70** (translocase of outer mitochondrial

membrane 70); **16). NRF1** (Nuclear respiratory factor 1); **17). NRF2** (Nuclear respiratory factor 2); **18). TFAM** (Trancriptional factor A mitochondrial); **19). EIF 2-α** (Elongation initiation factor 2α); **20). MRPL21** (mitochondrial ribosomal protein L21); **21). MRPL22** (mitochondrial ribosomal protein L22); **22). MRPS10** (mitochondrial ribosomal protein S10); **23). MDAL** (3,4-methylenedioxy-N-allylamphetamine); **24). HNE** (4-hydroxynonenal); **25). VDAC** (Voltage-dependent anion channel).

3.6.6.3 Detection

Membranes were washed thrice for 15 min and then incubated for 1 h at RT in the appropriate HRP conjugated secondary antibodies (1:2000; Dako) and immunocomplexes were revealed by enhanced chemiluminescence reagent (ECL, Amersham, UK).

3.6.6.4 Analysis

Densitometric quantification was carried out with TotalLab v2.01 software (Pharmacia, Uppsala, Sweden). Bands were normalized to VDAC levels for mitochondrial isolation and Beta actin and Beta tubulin were used for normalising for total protein extraction. Data were presented as mean ± standard error of the mean (SEM) in all the experiments. All the data were analyzed by Unpaired Student's *t*-test by using Graphpad prism 5 program. In all experimental procedures the significance level was set at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.7 IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

The immunohistochemical study of complexes was carried out in 4-µm-thick dewaxed paraffin sections. Immunofluorescence was conducted in paraffin sections in 5 controls and 5 PD brains of frontal cortex (Table 3.1).

- **Procedure:** Sections processed for immunofluorescence were pretreated with a saturated solution of Sudan black B (Merck, Darmstadt, Germany) for 15 minutes to block the autofluorescence of lipofuscin granules present in cell bodies and then rinsed in 70% ethanol and washed in distilled water. In all cases, the sections were boiled in citrate buffer (20 minutes) to retrieve antigenicity. All antibodies used are listed in (Table 2.4).

- **Flouorocity:** After incubation with the primary antibody, sections processed for immunofluorescence were incubated with Alexa488, and Alexa 555 (1:400; Molecular Probes, Eugene, OR) fluorescence secondary antibody. Nuclei were stained with DAPI (1:500; Biostatus, Inc., Leicestershire, UK). After washing the sections were mounted in Immuno-Fluore mounting medium (ICN Biomedicals, Irvine, CA), sealed, and dried overnight.

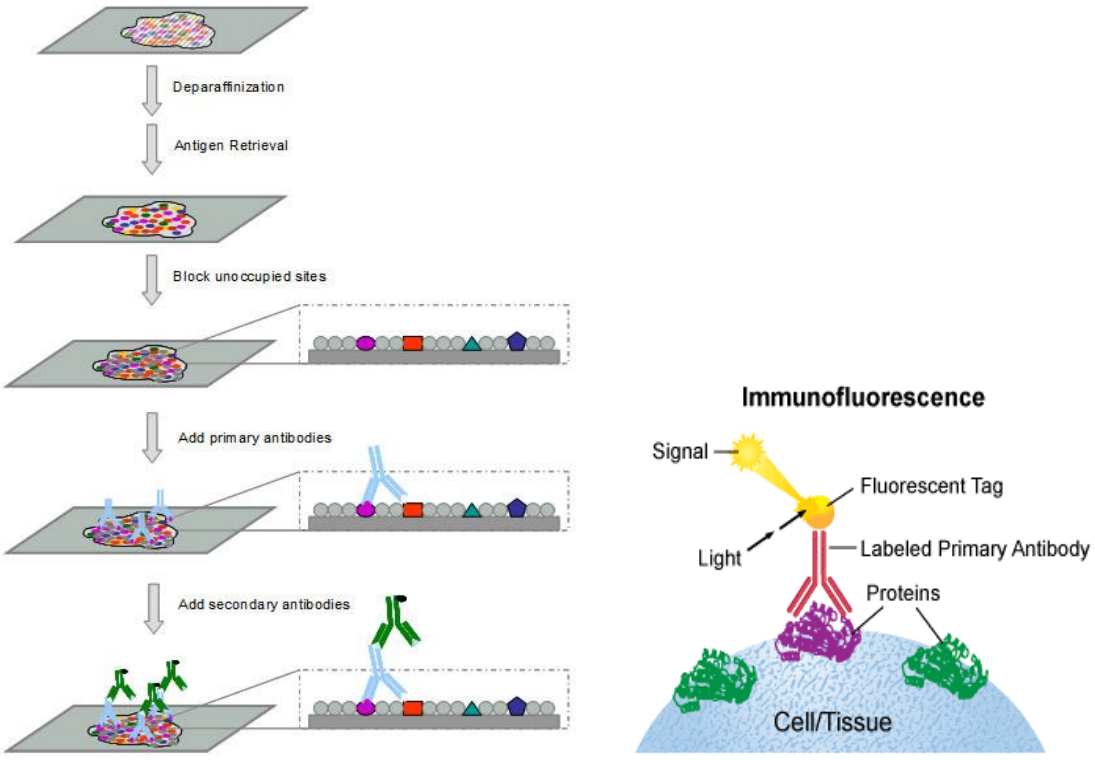


Figure 3.5 : Schematic steps of immunofluorescence staining (Adapted from Protein tech web page).

- **<u>Detection by confocal microscopy :</u>** Sections were examined with a Leica TCS-SL confocal microscope which enables the reconstruction of three-dimensional structures from the obtained images.

- **<u>Densitometric analysis :</u>** To percentage of the complex subunits was calculated by the intensity of the subunit expression in the total area of the neuron . All the images (n=5) in controls and cases the representative pictures were taken from frontal cortex. Per each case 50 neurons on an average were counted to attain the densitometric results. The images were analyzed by using Adobe® Photoshop® CS4.

The specificity of the immunoreaction was tested by incubating sections without the primary antibodies, these sections were negative. Because no antigenic peptides were available for preabsorption control studies. Only antibodies exhibiting specific immunoreactivity in tissue sections were selected for study. Data were presented as mean ± standard error of the mean (SEM) in all the experiments. All the data were analyzed by Student's *t*-test by using prism programme. In all experimental procedures the significance level was set at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.8 MITOCHONDRIAL ENZYME ASSAYS

3.8.1 Sonication of samples

Sonication has been done as described in (Long et al., 2009). The optimum sonication conditions were, 10s 8 times with 10s intervals at 3W for brain mitochondria, possibly increases the activity exposing the enzyme fully to the substrates. Assays for complexes III and V required mitochondrial sonication to express maximal activity, but complexes II and IV were prone to inactivation by excessive mechanical disruption (Zheng et al.,1990).

3.8.2 Spectrophotometric determination of enzyme activities

All the assays were performed in duplicate or triplicate using DU 800 spectrophotometer (Beckaman Coulter, Inc, California,USA), and is blanked with the respective buffer used for each complex and for electroplate readings a Sunrise TM spectrophotometer (TECAN, Switzerland) was used.

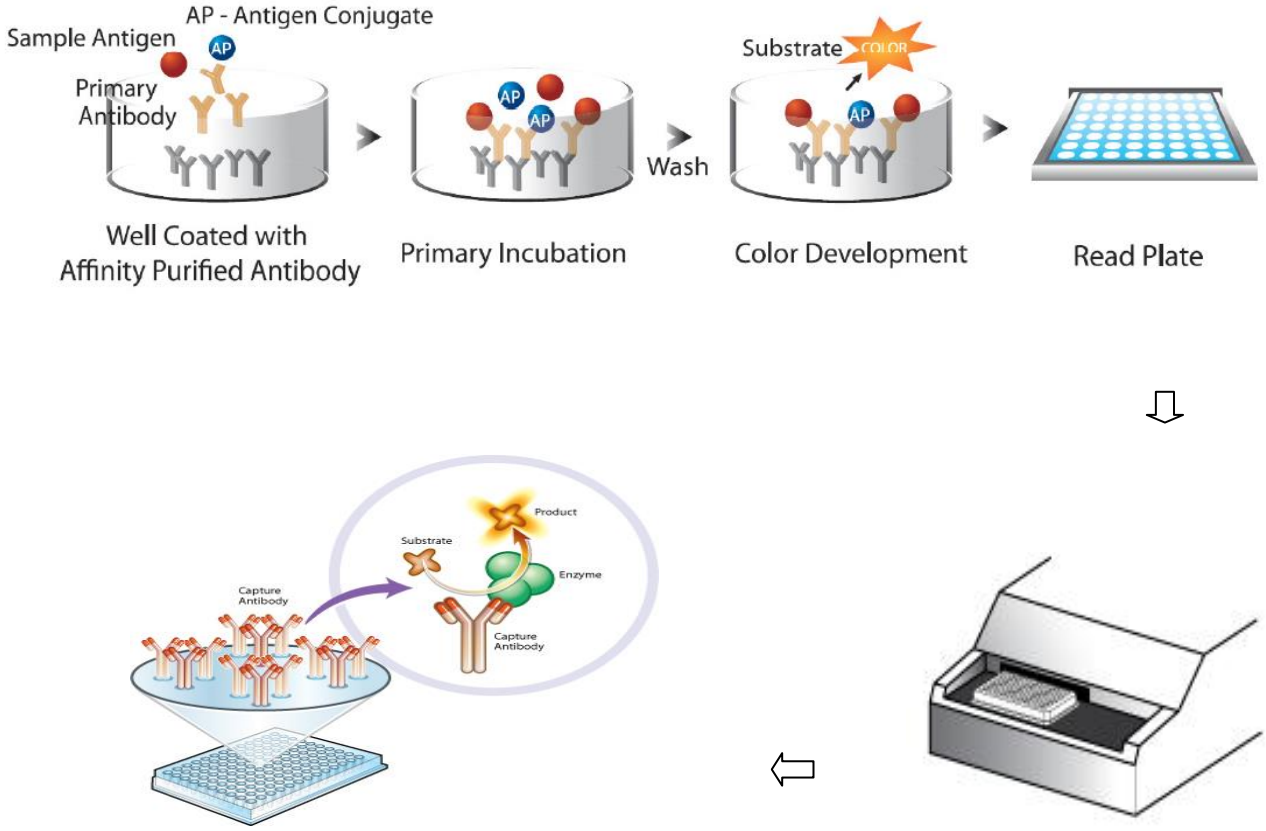
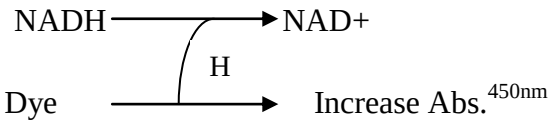


Figure 3.6 : Schematic illustration of enzyme assays. The visible light absorbance determines the activity of the enzyme.

3.8.2.1 NADH : NADH dehyderogenase (Complex I) : Complex I activity is measured by using a kit, Mitochondrial Complex I Activity Kit Novagen, EMD chemicals Inc., San Diego, CA, USA) according to manufacturer´s instructions.

- **Principle :** The enzyme complex is immunocaptured and activity is determined by following the oxidation of NADH to NAD+ and the simultaneous reduction of a dye which leads to increased absorbance at $450_{nm.}$



- **Preparation of substrate :** 20X NADH (40mM) is prepared by adding 1.1 ml of d H_20 and is aliquoted in order to avoid freez thaw cycles and stored at -80^oC until the day of use.

- **Reaction mixture :** The sample is prepared according to manufacturer's instructions, 200.0 µl of diluted smaple is added to each well which is incubated for 3 hrs at room temperature An assay solution of 200.0 µl which contains buffer, 20X NADH, and 100X dye is added to each well. Prior to the addition of assay solution the wells are emptied and washed thrice with 300.0 µl buffer. The plate is read at room temperature and OD 450_{nm} was taken using electroplate spectrophotometer and is read for 30 minutes with an interval of 20-60 seconds.

- **Enzyme blank :** Contained buffer as a null or background reference.

  Complex I activity in each well is proportional to the increase in absorbance at OD 450_{nm} within each well. The specific activity of complex I is calculated and it is expressed as nmol min−1 mg−1 of total proteins (extinction coefficient for NADH 6.2 mM− 1 cm− 1).

3.8.2.2 Succinate- coenzyme Q reductase (Complex II) : *Complex II* activity is measured by using a kit, (Mitochondrial Complex II Activity Kit Novagen ,EMD chemicals Inc., San Diego, CA, USA) according to manufacturer's instructions.

- **Principle :** Complex II catalyzes the electron transfer from succinate to the elctron carier, ubiquinone, but unlike the other four complees it is not a proton pump. The product ubiquinol is utilized by complex III in the repsiratory chain and the product fumarate is necessary to main the TCA cycle.

Succinate+ubiquinone (Q)⟶Fumarate+ubiquinol (QH_2)

The production of ubiquinol by the enzyme is coupled to the reduction of the dye DCPIP (2,6-dichlorophenolindophenol) and a decrease in its absorbance at 600_{nm} which in turn recycles the substrate ubiquinone.

Ubiquinol (QH_2) + DCPIP⟶ Ubiquinone (Q) + $DCPIPH_2$

- **Preparation of substrate :** 50X Succinate solution is prepared by adding 500.0 µl d H_20 water and is aliquoted in order to avoide free thaw cycle and is stored at -80^o C.

- **Reaction mixture :** The sample is prepared according to manufacturer's instructions, 50.0 µl of diluted smaple is added to each well which is incubated for 2 hrs at room temperature. Activity solution of 200.0 µl which contains buffer, ubiquinone 2, succinate and DCPIP is added to each well. Prior to the addition of assay solution the wells are emptied and washed thrice with 300.0 µl of 1X buffer and 40.0 µl of phospholipid solution is added and is incubated for 30 minutes. The plate is read at room temperature and OD 600nm was taken using electroplate spectrophotometer and is read for 60 minutes with an interval of 20-60 seconds.

- **Enzyme blank :** Contained buffer as a null or background reference.

  Complex II activity is measured as the reduction of ubiquione and subsequent reduction of DCPIP is measured as a decrease in absorbance at OD $600_{nm.}$ Complex II activity is expressed as nmol min−1 mg−1 of total proteins (extinction coefficient for DCPIP 19.1 mM−1 cm−1).

3.8.2.3 Decylubiquinol cytochrome C Oxidase (Complex III): (protocol followed in accordance to (M Spinazzi *et al.*,2012 with slight modifications).

- **Preparation of substrate and other reagents : Substrate :** Oxidized cytochrome c solution (1 mM) is prepared by dissolving 12.5 mg in 1 ml of distilled wàter.
  Note : To be prepared fresh or is aliquoted and stored in -20^{o}C to avoid free thaw cycles.
  Antimycin A solution : (10 mg/ml) : Dissolve 25 mg of antimycin A in 2.5ml of ethanol and Store at -20^{o}C.

Decylubiquinol solution (10mM) : Add a few grains of potassium borohydride to 250.0 µl of 10 mM DUB in ethanol. Add 5.0 µl aliquots of 0.1 M HCl until the solution becomes colorless. Briefly spin the solution at 10,000g for 1 min and tranfer the solution into a new 500.0µl tube, avoiding any potassium borohydride crystals. Adjust the pH of the solution between 2-3 with 5.0 µl of aliquots of 1M HCl and keep the solution on ice protected from light. **NOTE :** To be prepared fresh.

- **Reaction mixture :** The reaction mixture contained 50.0 µl of potassium phosphate buffer (0.5M, pH 7.5), 50.0 µl of oxidized cytochrome c, 50.0 µl of KCN (10mM), 20.0 µl of EDTA (5mM, pH 7.5), 10.0 µl of tween 20 (2.5% (vol/vol)) and 10µg of sample of 400.0 µl. A separate cuvette containing the same quantity of reagents and sample with the addition of 10.0 µl of 1 mg/ml antimycin A solution. The cuvette without antimycin A 10.0 µl of absolute ethanol was added. Adjust the volume to 990.0 µl with distilled H2O. Mix by inverting the cuvettes and read the baseline at 550 $_{nm}$ for 2 minutes. The reaction was started by adding 10.0 µl of 10 mM decylubiquinol, mix rapidly by inverting the cuvette using parafilm and then immediately is read OD 550_{nm} for 1-2 minutes.

- **Enzyme blank :** Contained 50.0 µl of potassium phosphate buffer (0.5M, pH 7.5), 50.0 µl of oxidized cytochrome c, 50.0 µl of KCN (10mM), 20.0 µl of EDTA (5mM, pH 7.5), 10.0 µl of tween 20 (2.5% (vol/vol)) and 10µg of sample of 400.0 µl. Adjust the volume to 990 µl with distilled water. The reaction was started by adding 10.0 µl of 10 mM decylubiquinol, mix rapidly by inverting the cuvette using parafilm and then immediately is read OD 550_{nm} for 1-2 minutes.

- **Substrate blank :** Contained 50.0 µl of potassium phosphate buffer (0.5M, pH 7.5), 50.0 µl of oxidized cytochrome c, 50.0 µl of KCN (10mM), 20.0 µl of EDTA (5mM, pH 7.5), 10.0 µl of tween 20 (2.5% (vol/vol)) and 400.0 µl of phosphate buffer (0.5M, pH 7.5). Adjust the volume to 990 µl with distilled water. The reaction was started by adding 10.0 µl of 10 mM decylubiquinol, mix rapidly by inverting the cuvette using parafilm and then immediately is read OD 550_{nm} for 1-2 minutes.

- The specific complex III activity is measured as the antimycin A-sensitive activity. It is calculated by subtracting total complex III activity (without antimycin A) and antimycin A–

resistant activity (with antimycin A), and it is expressed as nmol min−1 mg−1 of total proteins (extinction coeficient for reduced cytochrome c 18.5 mM− 1 cm−1).

3.8.2.4 Cytochrome C Oxidase (Complex IV): *Complex IV* activity is measured by using a kit, (Mitochondrial Complex IVActivity Kit Novagen ,EMD chemicals Inc., San Diego, CA, USA) according to manufacturer's instructions.

- **Principle :** Complex IV activity is determined colorimetrical by following the oxidation of reduced cytochrome c as an absorbance decrease at OD 550_{nm}.

$$4 \text{ cytochrome } c^{-} + 4 H^{+} + 4 H^{+(in)} + O2 \longrightarrow 4 \text{ cytochrome } c^{+} + 2 H_2O + 4 H^{+ (out)}$$

reduced oxidized

↑Abs 550 nm ↓Abs 550 nm

- **Preparation of substrate :** The reduced cytochrome c is prepared by adding 1ml of d H_2O and is aliquoted in order to avoide free thaw cycle and is stored at -80^{o} C.

- **Reaction mixture :** The sample is prepared according to manufacturer's instructions, 200.0 µl of diluted smaple is added to each well which is incubated for 3 hrs at room temperature. Assay solution of 200.0 µl which contains solution I and reduced cytochrome c is added to each well. Prior to the addition of assay solution the wells are emptied and washed thrice with 300.0 µl of solution. The plate is read at 30^{o}C for 120 min at OD 550_{nm} with an interval of 1min using electroplate spectrophotometer.

- **Enzyme blank :** Contained buffer as a null or background reference.

The rate of complex IV activity is always expressed as the initial rate of oxidation of cytochrome c. This oxidation is seen as a decrease in absorbance at OD 550 $_{nm}$. Complex IV activity is expressed as nmol min− 1 mg−1 of total proteins (extinction coefficient for reduced cytochrome C 18.5 mM−1 cm− 1).

3.9.2.5 F1-ATPase (Complex V) : (Protocol followed in accordance with Yan-Yan Ma et al., 2011)

- **Principle :** The assay relies on linking the ATP ase activity. The assay relies on linking the ATPase activity to NADH oxidation via the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase (PK) and then pyruvate to lactate by lactate dehydrogenase (LDH).

- **Preparation of substrate :** 10 mM NADH is prepared by dissolving 7.5 mg of NADH in 1ml of d H_2O. To be prepared fresh.

- **Reaction mixture :** The 1 ml mixture solution contains 0.2 mmol/L NADH, 2.mmol/L PEP, 25µg/mL antimycin A, 50mmol/L MgCl2, 2.5 U /ml LDH, 2 U/mL PK,10mmol/L EGTA, and 40mmol/L Tris HC03,pH 8.0 was equilibrated at 30°C for 10min. ATP was then added to a final concentration of 2.5mmol/L and left for 2 minutes to allow phosphorylation of any adenosine diphosphate (ADP) present in the ATP solution. The reaction was started by adding 25µg of mitochondrial extract. In parallel a separate cuvette containing the same quantity of reagents and sample with the addition of 2µmol/L of oligomycin. The change in the absorbance was recorded over 5 minutes at OD 340_{nm}.

- **Enzyme blank :** Contained 0.2 mmol/L NADH, 2.mmol/L PEP, 25µg/mL antimycin A, 50mmol/L MgCl2, 2.5 U /ml LDH, 2 U/mL PK,10mmol/L EGTA, and 40mmol/L Tris HC03,pH 8.0 was equilibrated at 30°C for 10min. ATP was then added to a final concentration of 2.5mmol/L and left for 2 minutes to allow phosphorylation of any adenosine diphosphate (ADP) present in the ATP solution. The reaction was started by adding 25µg of mitochondrial extract. In parallel a separate cuvette containing the same quantity of reagents and sample with the addition of 2µmol/L of oligomycin. The change in the absorbance was recorded over 5 minutes at OD 340_{nm}.

- **Substrate blank :** Contained 0.2 mmol/L NADH, 2.mmol/L PEP, 25µg/mL antimycin A, 50mmol/L MgCl2, 2.5 U /ml LDH, 2 U/mL PK,10mmol/L EGTA, and 40mmol/L Tris HCO3,pH 8.0 was equilibrated at 30°C for 10min. ATP was then added to a final concentration of 2.5mmol/L and left for 2 minutes to allow phosphorylation of any adenosine diphosphate (ADP) present in the ATP solution. The reaction was started by adding Tris HCO3 pH 8.0. In parallel a separate cuvette containing the same quantity of reagents and

sample with the addition of 2µmol/L of oligomycin. The change in the absorbance was recorded over 5 minutes at OD 340_{nm}.

The specific activity of complex V is calculated and it is expressed as nmol min−1 mg−1 of total proteins (extinction coefficient for NADH 6.2 mM− 1 cm− 1).

3.8.2.6 Citrate Synthase activity **:** protocol followed in accordance to (Spinazzi et al., 2012) with slight modifications.

- **Principle :** Citrate synthase activity was determined as the rate of reduction of 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) to thionitrobenzoic acid at 412nm.

- **Preparation of substrate :** 10 mM oxaloacetic acid solution is prepared by dissolving 6.6 mg of oxaloacetic acid in 5 ml of distilled water. The solution to be prepared fresh.

- **Reaction mixture :** The reaction mixture 1ml contained 500.0 µl of Tris (200mM, pH8.0) with Triton X-100 (0.2% (vol/vol)), 100.0 µl of DTNB, 30.0 µl of Acetyl CoA (10mM) , 10.0 µl of 10µg of sample and final volume is adjusted to that of 950.0 µl with distilled water. Mix by inversion and read the baseline activity at 412 nm for 2 min. The reaction is initiated by adding 50.0 µl of 10mM oxaloacetic acid mixed and then the increase in absorbance is observed at 412 nm for 3 min.

- **Enzyme blank :** 1ml contained 500.0 µl of Tris (200mM, pH8.0) with Triton X-100 (0.2% (vol/vol)), 100.0 µl of DTNB, 30.0 µl of Acetyl CoA (10mM) , 10.0 µl of 10µg of sample and final volume is adjusted to that of 950.0 µl with distilled water. Mix by inversion and read the baseline activity at 412 nm for 2 min. The reaction is initiated by adding 50.0 µl of 10mM oxaloacetic acid mixed and then the increase in absorbance is observed at 412 nm for 3 min.

- **Substrate blank :** 1ml contained 500.0 µl of Tris (200mM, pH 8.0) with Triton X-100 (0.2% (vol/vol)), 100.0 µl of DTNB, 30.0 µl of Acetyl CoA (10mM) , 10.0 µl of Tris (200mM, pH 8.0) and final volume is adjusted to that of 950.0 µl with distilled water. Mix by inversion and read the baseline activity at 412 nm for 2 min. The reaction is initiated by adding 50.0 µl of 10mM oxaloacetic acid mixed and then the increase in absorbance is observed at 412 nm for 3 min.

Citrate synthase activity is expressed as nmol min−1 mg−1 of total proteins (extinction coefficient 13.6 mM−1 cm− 1).

- **Statistical Analysis**

The enzymatic activities for each mitochondrial complex should be calculated as nmol min − 1 mg −1 of protein according to the following equation (enzyme activity (nmol min − 1 mg − 1) = (Δ Absorbance/min × 1,000)/[(extinction coefficient × volume of sample used in ml) × (sample protein concentration in mg ml − 1)]. Data is presented as mean ± standard error of the mean (SEM) in all the experiments. All the data were analyzed by Unpaired Student's *t*-test by using Graphpad prism 5 program. In all experimental procedures the significance level was set at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3.7 Schematic illustration of the methods in brief

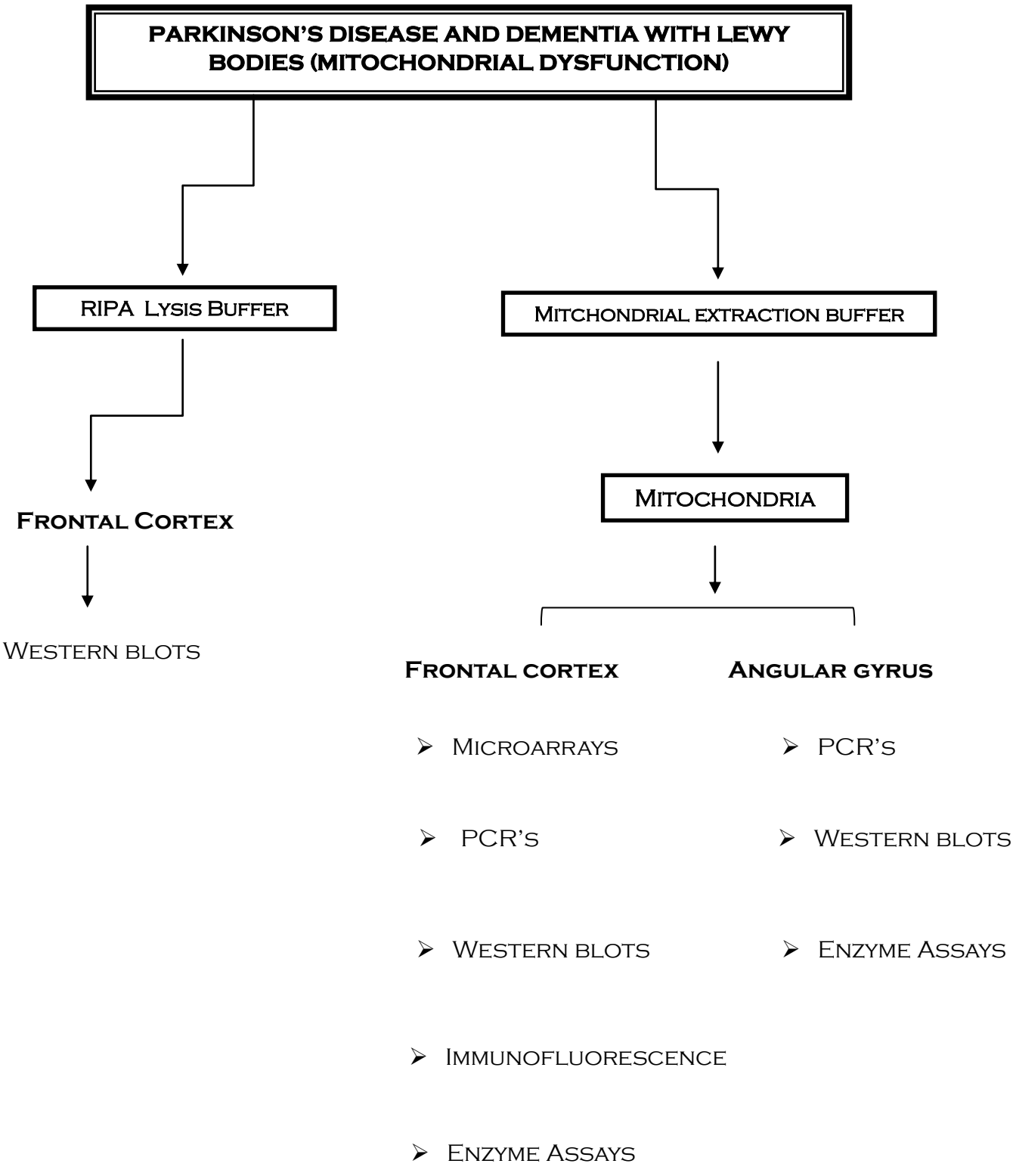
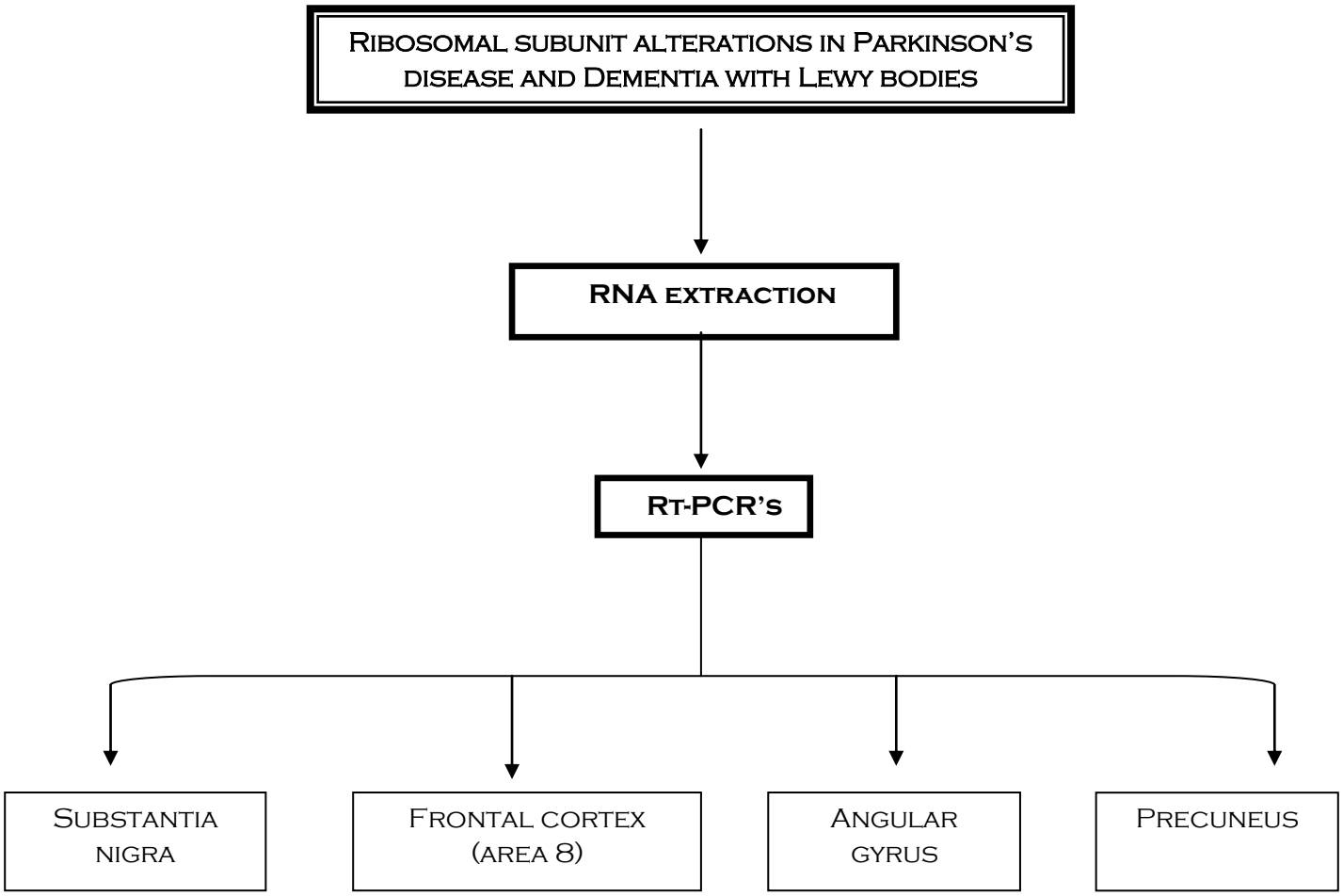


Figure : 3.8 Diagrammatic representation of the methods in brief



RESULTS

Results : Objective 1-Mitochondria

4.1 Global Expression Profiles Identify Dysregulation of Oxidative Phosphorylation Pathway in Frontal cortex (area 8) in PD

Messenger RNA samples from PD (n=11) and age matched controls (n=7) were hybridized to an Affymetrix Human Gene 1.1 ST Array, that interrogates over 1 million exon clusters within the known and predicted transcribed regions of the entire genome. We found a differential expression of genes belonging to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) pathway "Oxidative phosphorylation" (KEGG:00190). Modification of transcriptional profiles for these oxphos genes in PD was evidenced on the heat map representation for a non-restrictive gene list at non-adjusted probability of $p<0.1$. 10 genes have shown the differences in the oxidative pathway NDUFA7 was found to be downregulated. NDUFA7 (prob 0.000384, probadj 0.028881), and the rest of the genes showed no difference when adjusted to $p<0.1$.

NDUFS7 (prob 0.015821, probadj 0.160497)

NDUFA10 (prob 0.019985, probadj 0.178727)

ATP6VoB (prob 0.020167, probadj 0.179778)

LHPP (prob 0.020472, probadj 0.181226)

ATP5G2 (prob 0.020574, probadj 0.181597)

COX7A2L (prob 0.034762, probadj 0.235724)

NDUFB10 (prob 0.036541, probadj 0.242622)

NDUFS8 (prob 0.043787, probadj 0.261596)

ATP6V1H (prob 0.048063, probadj 0.271333).

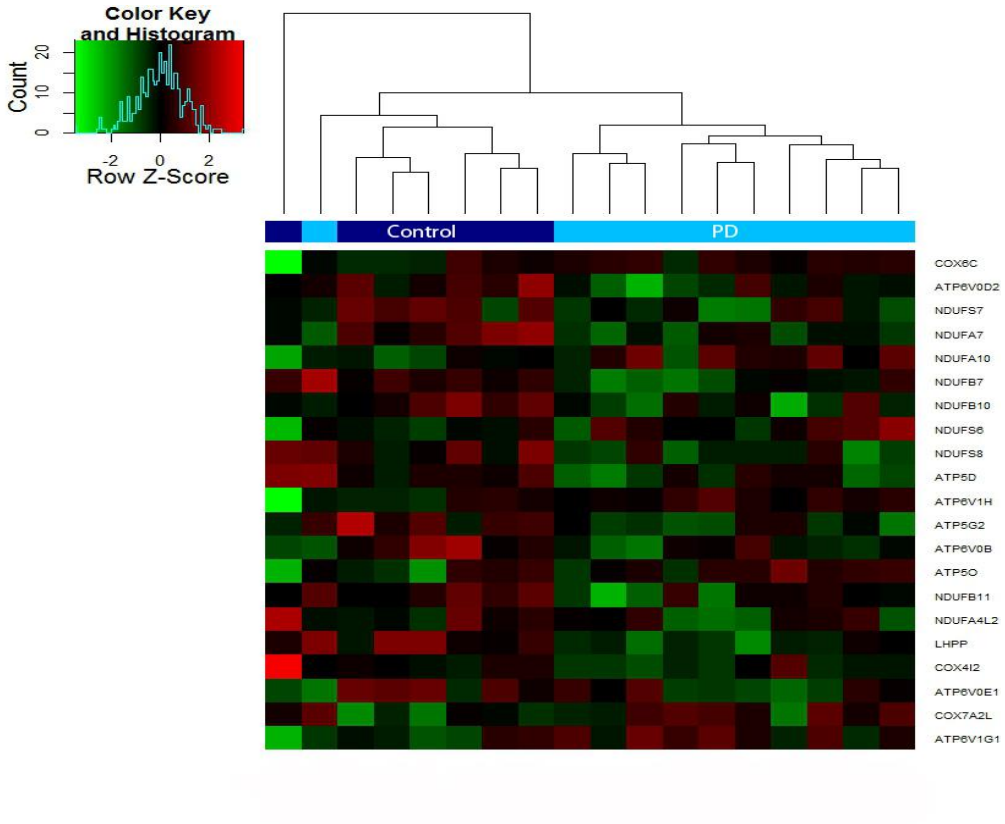


Figure 4.1 Hierarchical clustering heat map of expression intensities of mRNA array transcripts of Oxidative phosphorylation (on the y axis) in Parkinson disease (PD) and controls (on the x axis) at non-adjusted probability of p<0.1. A separation between PD and control profiles indicates modification of the Oxidative phosphorylation profile in PD.

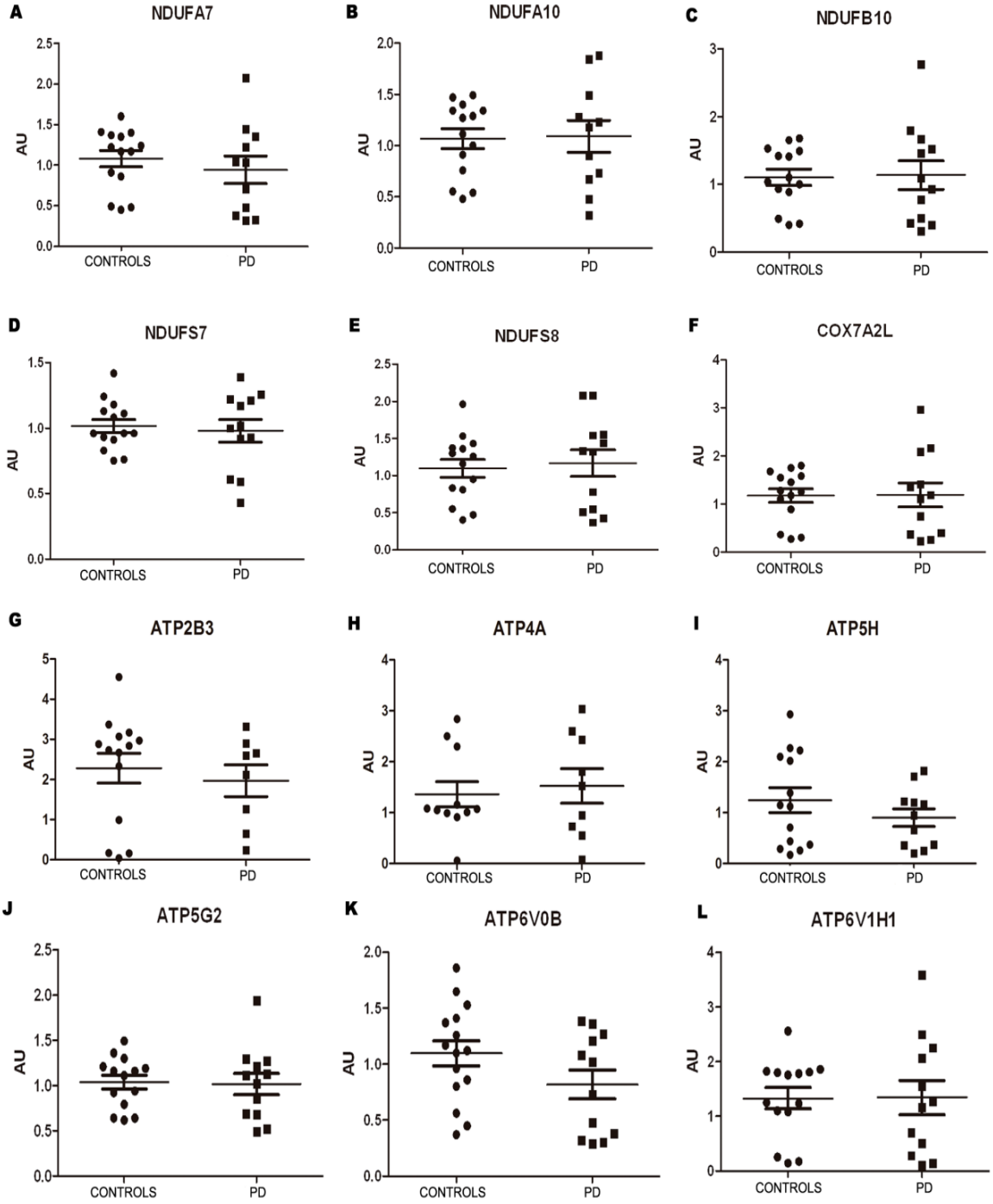
4.2 Validation of dysregulation of mitochondrial subunits using RT-qPCR

Assessment of putative dysregulated genes was conducted using additional cDNAs from PD and DLB with age-matched control cases. This was done to increase robustness of the results obtained in the arrays. The rationale was based on the assumption that identification of dysregulated genes common to different groups of PD cases determined by different methods further strengthens the validity of the observations. Selection of probes was carried out depending on the results of the arrays together with their commercial availability. β-glucoronidase (GUS-β) is used for normalization. This housekeeping gene was selected because it shows no modifications in several neurodegenerative diseases in human post-mortem brain tissue (Barrachina et al., 2006; Durrenberger et al., 2012) and hence is used as a endogenous control.

Eighteen mitochondrial complex subunit genes were selected for RT-qPCR examination : NDUFA7 was the only down regulated gene from the arrays and apart NDUFS7, NDUFA10, ATP6VOB, LHPP, ATP5G2, COX7A2L, NDUFB10, NDUFS8, ATP6V1H1 were additional genes with doubtful significance in the arrays. Apart from these genes we have chosen few more SLC25A31, SLC6A6, PTP1P51 and RPS20 which are important in enzyme activity.

4.2.1 Messenger RNA expression in frontal cortex

4.2.1.1 Messenger RNA expression in PD : Mitochondrial complex subunits mRNA expression levels showed no significant differences in frontal cortex (area 8) in control and diseased cases (Figure 4.2).



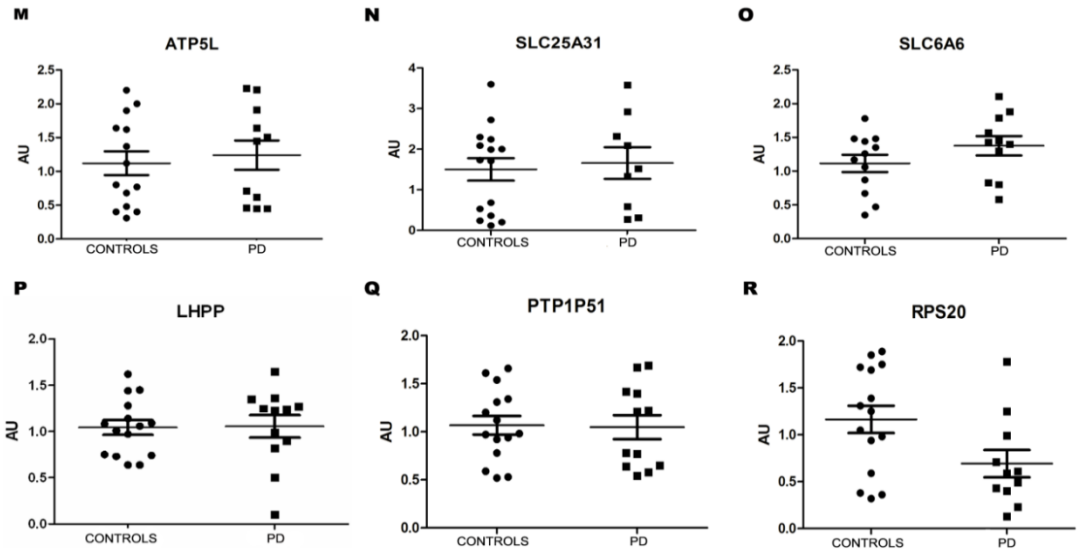
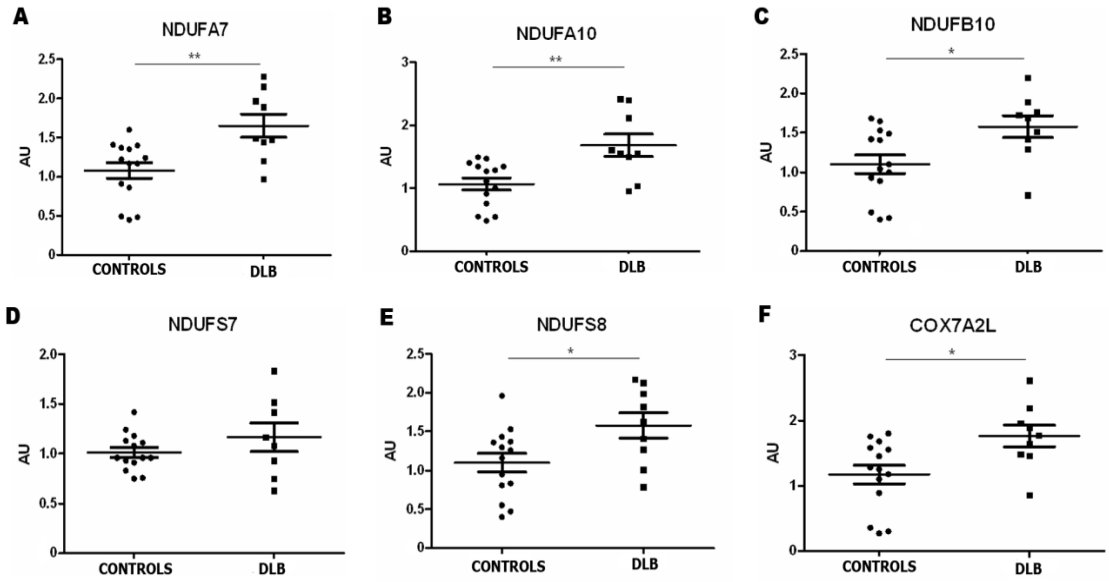


Figure 4.2 Messenger RNA expression levels of selected oxidative phosphorylation pathway in the frontal cortex (area 8) in parkinson's disease (PD) and control cases as determined by taqman PCR assays. No significant differences were seen in the expression levels of probes.

4.2.1.2 Messenger RNA expression in DLB : Mitochondrial complex subunits mRNA expression levels showed significant increase in NDUFA7, NDUFA10 ($p < 0.01$), NDUFB10, NDUFS8, COX7A2L, ATP5G2 and PTP1P51 ($p < 0.05$) (Figure 4.3 A, B, C,E F, J and Q respectively) where as significant decrease was seen in ATP5H ($p < 0.05$) (Figure 4.3 I) in frontal cortex (area 8).



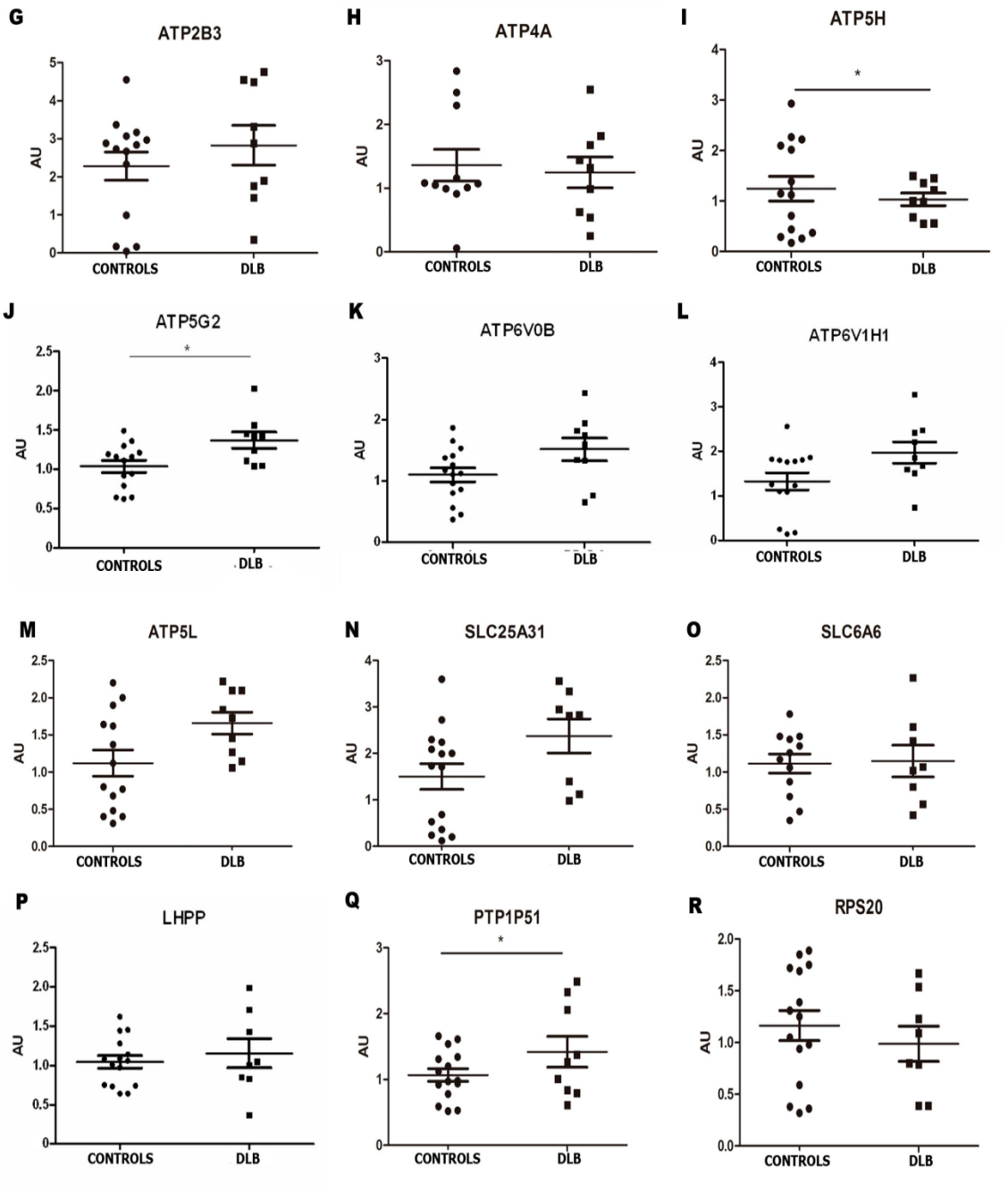


Figure 4.3 Messenger RNA expression levels of selected oxidative phosphorylation pathway in the frontal cortex (area 8) in Parkinson's disease (PD) and control cases as determined by taqman PCR assays. Significant increase was seen in NDUFA7, NDUFA10, NDUFB10, NDUFS8, COX7A2L and ATP5G2, whereas significant decrease was seen in ATP5H. Student's T-test *p< 0.05, **p < 0.01, ***p <0.001.

4.2.2 Messenger RNA expression in angular gyrus

4.2.2.1 Messenger RNA expression in PD : Mitochondrial complex subunits mRNA expression levels in angular gyrus have shown significant increase in ATP5H ($p < 0.05$) whereas the expression levels of the other probes remained unaltered.(Figure 4.4)

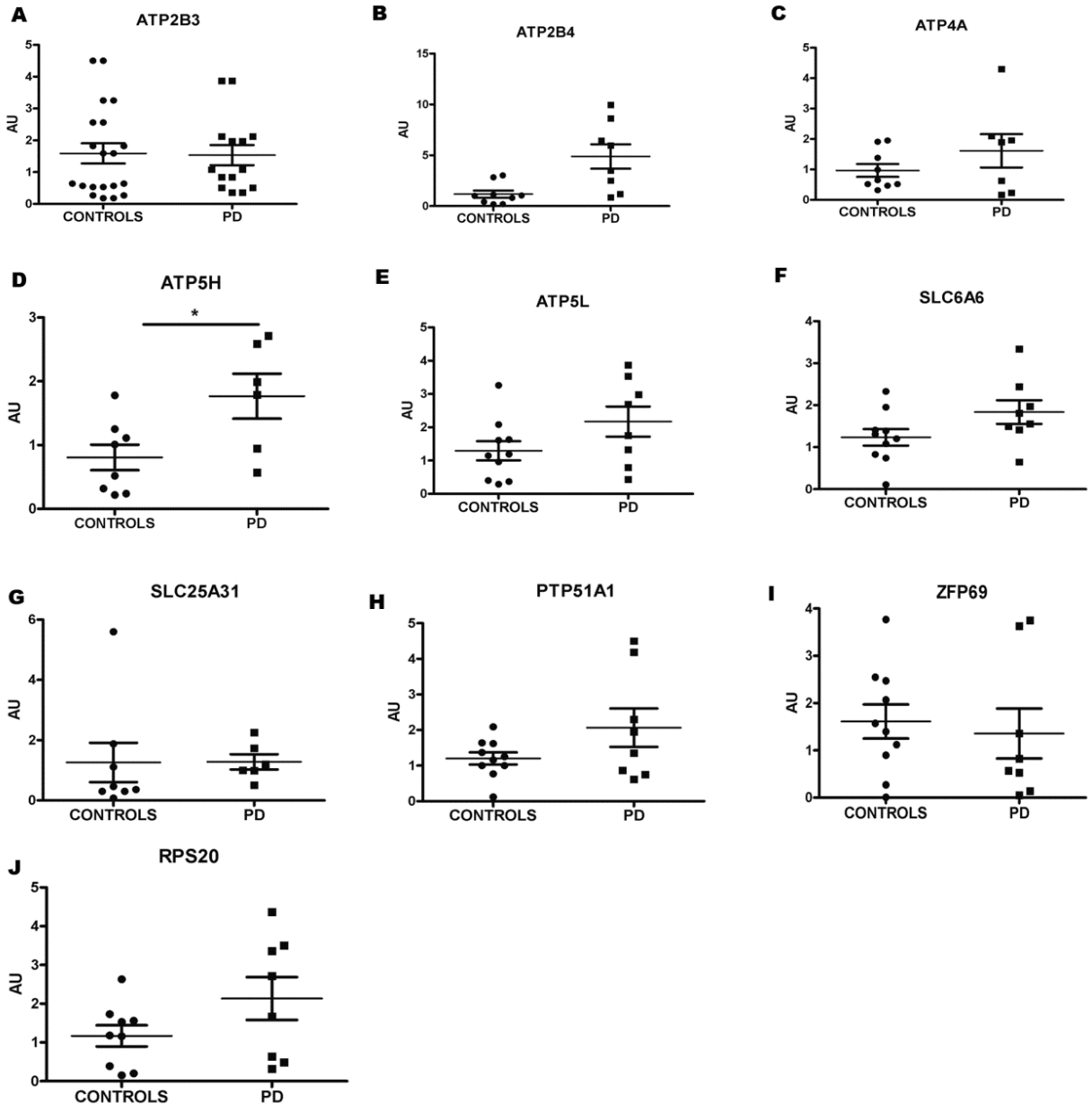


Figure 4.4 Messenger RNA expression levels of selected oxidative phosphorylation pathway in the angular gyrus in Parkinson's disease (PD) and control cases as determined by taqman PCR assays. Significant increase was seen in ATP5H and the other probes remained unaltered. Student's T-test *p < 0.05, **p< 0.01, ***p< 0.001.

4.2.2.2 Messenger RNA expression in PD-Dementia : Mitochondrial complex subunits mRNA expression levels in angular gyrus have shown significant increase in ATP2B4 ($p < 0.05$) whereas the expression levels of the other probes remained unaltered (Figure 4.5).

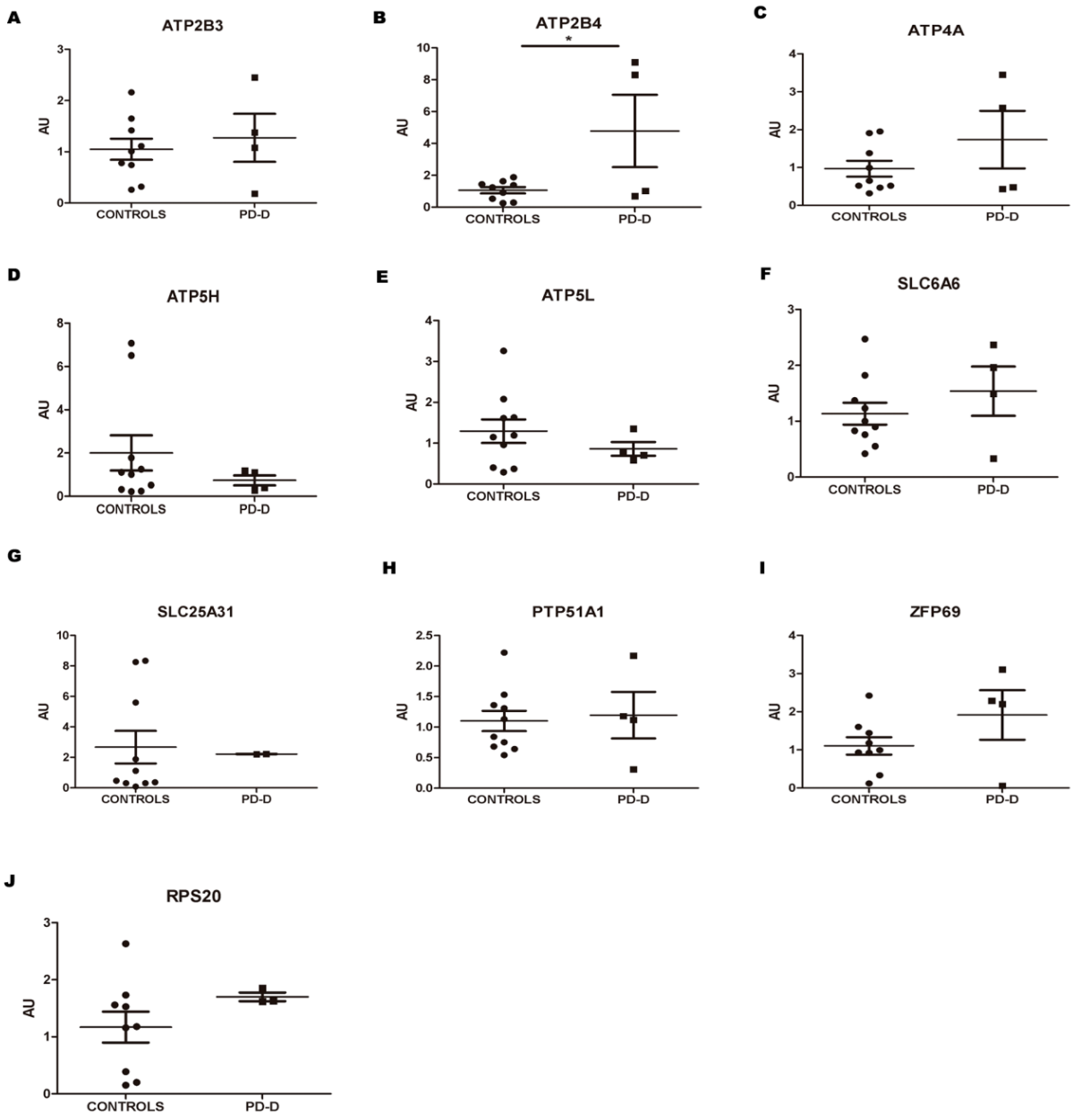


Figure 4.5 Messenger RNA expression levels of selected oxidative phosphorylation pathway in the angular gyrus in Parkinson's disease dementia (PD-D) and control cases as determined by taqman PCR assays. Significant increase was seen in ATP2B4 and the other probes remained unaltered. Student's T-test *p< 0.05, **p< 0.01, ***p< 0.001.

4.3 Gel electrophoresis and western blotting

Western blotting was used to analyze the different mitochondrial complex subunits using two different extraction buffer methods in order to understand the alteration of proteins in mitochondria.

4.3.1 Total protein extraction reveals low levels of VDAC

Mitochondrial voltage-dependent anion channel (VDAC) is a major protein at the crossroads of metabolic and survival pathways and serves as a mitochondrial marker which has also been shown to play a role in apoptosis (Lemasters and Holmuhamedov 2006). To explore the consequences of abundance of mitochondria in the frontal cortex (area 8) cases were subjected to total protein extraction. For this we first analyzed to see the protein expression in Braak stages PD 1-2 which are considered as incidental parkinson´s disease (iPD) (Figure 4.6 A) no changes were seen in VDAC levels at this stage, where as in PD of braak stages 3-4 there was a trend in VDAC levels (Figure 4.6 B). In DLB there was a significant decrease ($p< 0.01$) in VDAC levels. (Figure 4.6 C). This indicates that there is a decrease in VDAC levels as the disease progression occurs.

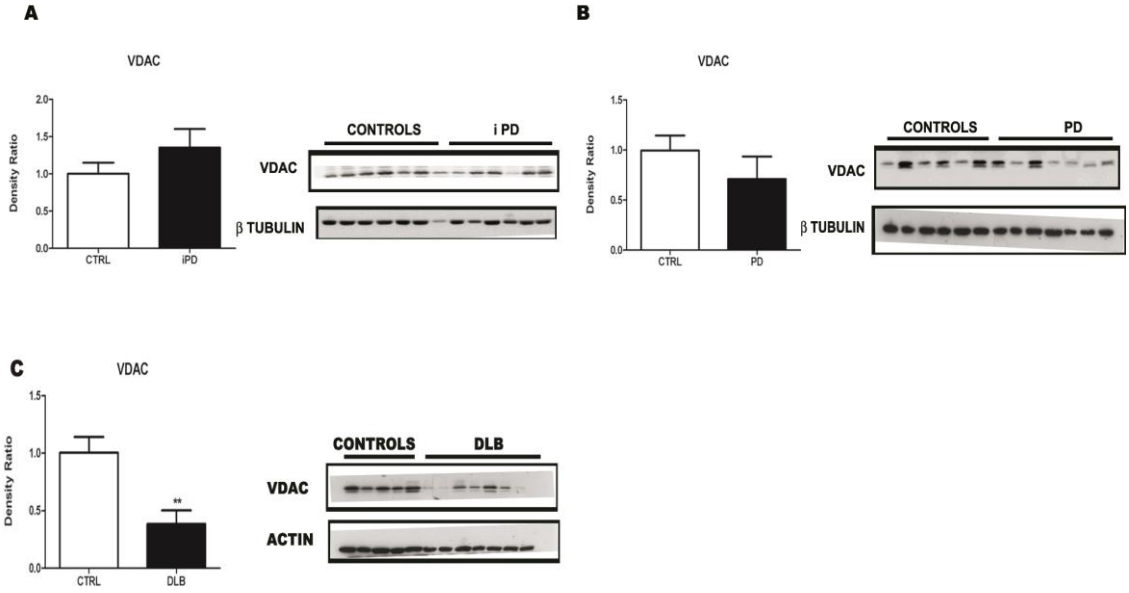


Figure 4.6 Protein expression levels of VDAC in frontal cortex in total protein extracts corrected with beta tubulin and actin levles. (A) VDAC expression in incidental parkinson's disease (iPD) of Braak 1-2 (B) VDAC expression in parkinson's disease (PD) of Braak 3-4 where no significant difference was observed where as (C) VDAC expression in dementia with lewy bodies (DLB) of braak stages 5-6 significant decrease was seen . Student's T-test **p< 0.01.

4.3.1.1 Mitochondrial complex subunits expression is altered in PD and DLB in total protein extraction

To further characterize the involvement of mitochondria in the disease progression, we extended our study to investigate alteration of mitochondrial subunits which may lead to its dysfunction. Oxphos antibody which is a cocktail of different complex subunits was used, in PD (Figure 4.7 A) and in DLB (Figure 4.7 B) no significant differences have been observed.

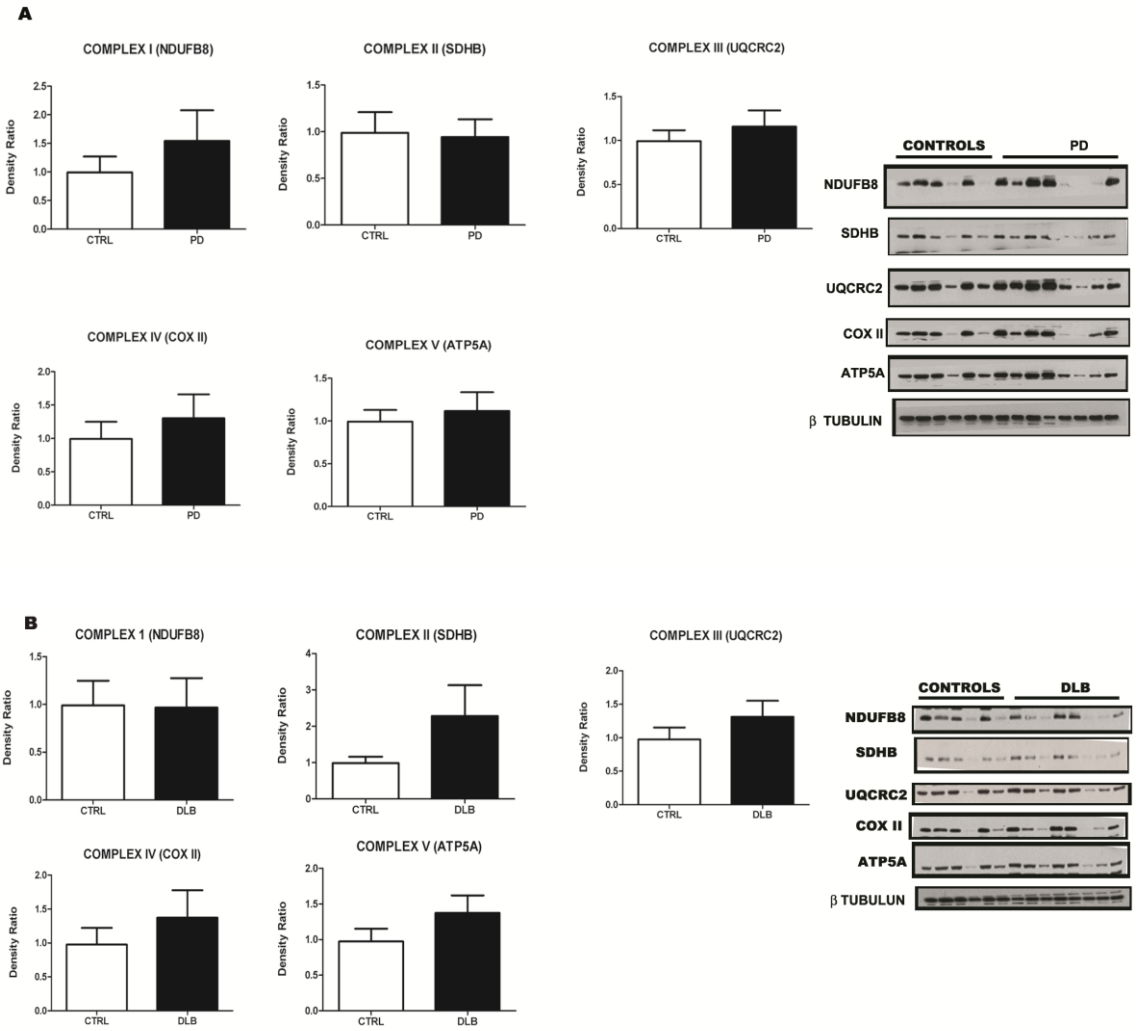


Figure 4.7 Protein expression levels of OXPHOS in frontal cortex in total protein extracts corrected with beta tubulin. (A) OXPHOS expression in PD, (B) OXPHOS expression in DLB. No significant differences were observed.

In PD, superoxide dismutase (SOD2) (Figure 4.8 A), and nuclear respiratory factor 1 (Figure 4.8 B) which is responsible in controlling the free radicals during oxidative stress were found to be significantly down regulated ($p < 0.01$). No significant changes were seen in mitochondrial transcriptional factor A (TFAM) (Figure 4.8 C) important for biogenesis and in eukaryotic translation initiation factor 2-α subunit (EIF2-α) (Figure 4.8 D) which is responsible for protein folding.

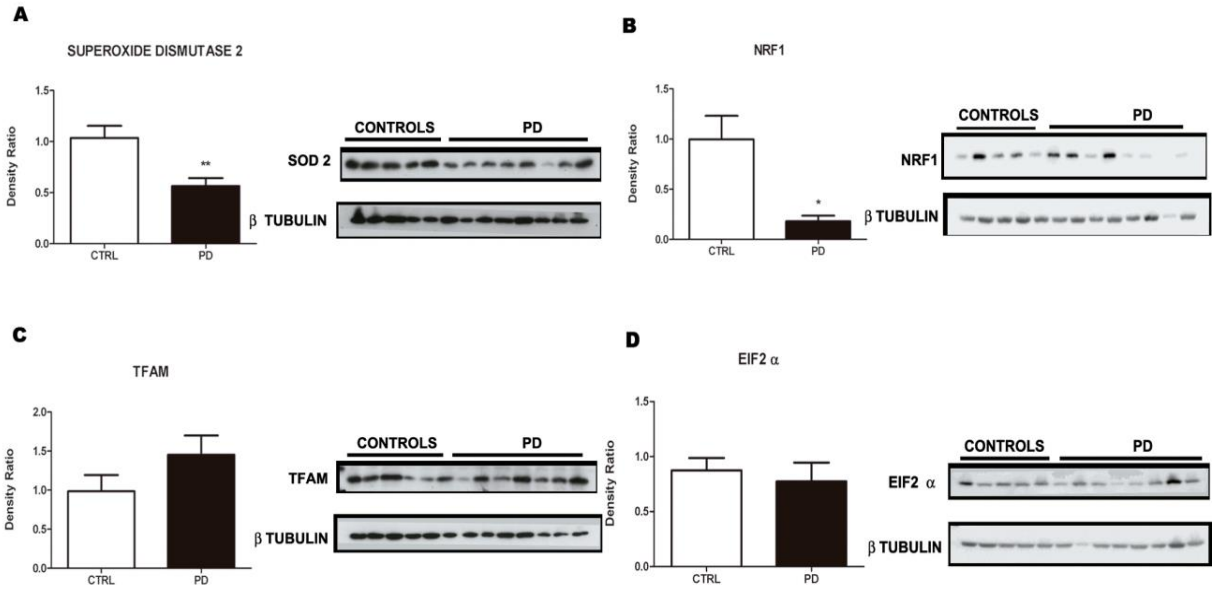


Figure 4.8 Protein expression levels of mitochondrial complex subunits in PD frontal cortex (area 8) in total protein extracts corrected with beta tubulin. Significant down-regulation is seen in SOD2 and NRF1 where as in TFAM and EIF2-α no differences were observed. Student's T-test *p< 0.05 , **p< 0.01.

In DLB, one of the complex I subunits (NDUFS7) (Figure 3.4 A) ($p< 0.001$), TFAM (Figure 4.9 D) and EIF2-α (Figure 4.4 E) ($p< 0.05$) respectively were significantly decreased. No significant differences were seen in NRF1 and SOD2 (Figure 4.4 B).

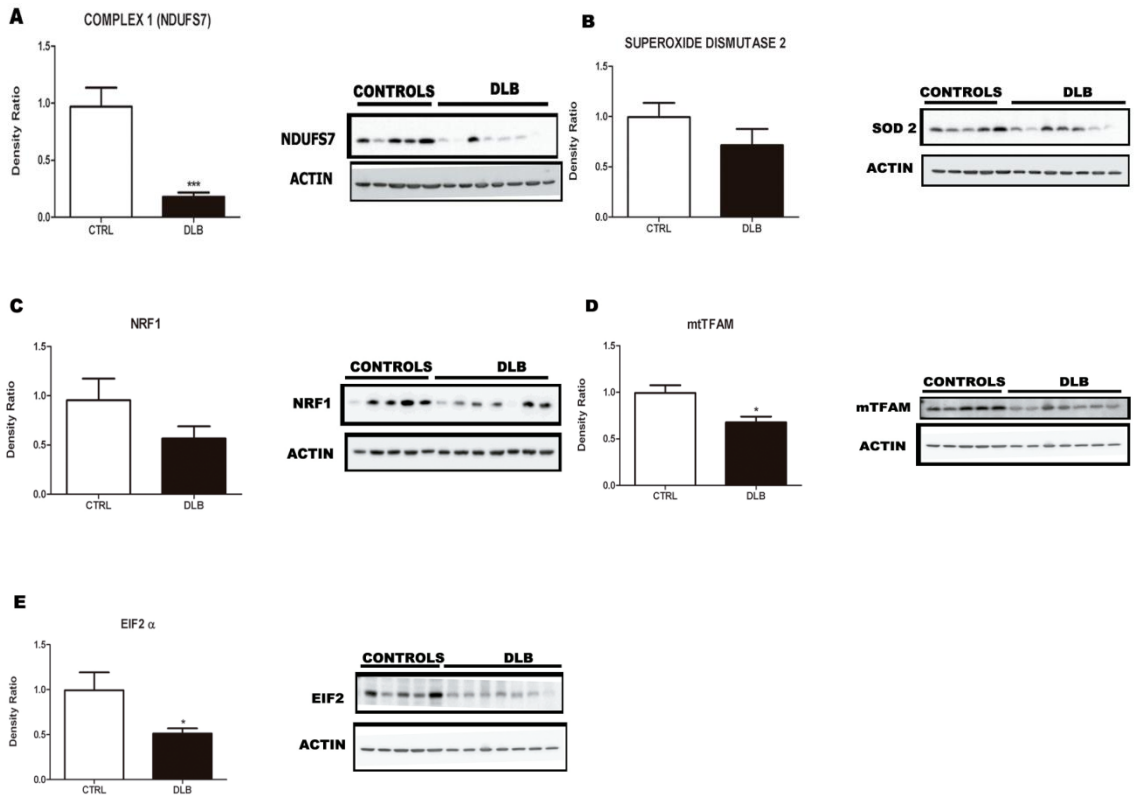


Figure 4.9 Protein expression levels of mitochondrial complex subunits in DLB of frontal cortex (area 8) in total protein extracts corrected with actin. Significant decrease was seen in NDUFS7, TFAM and EIF2-α. Student's T-test *p< 0.05. No differences were seen in SOD2 and NRF2.

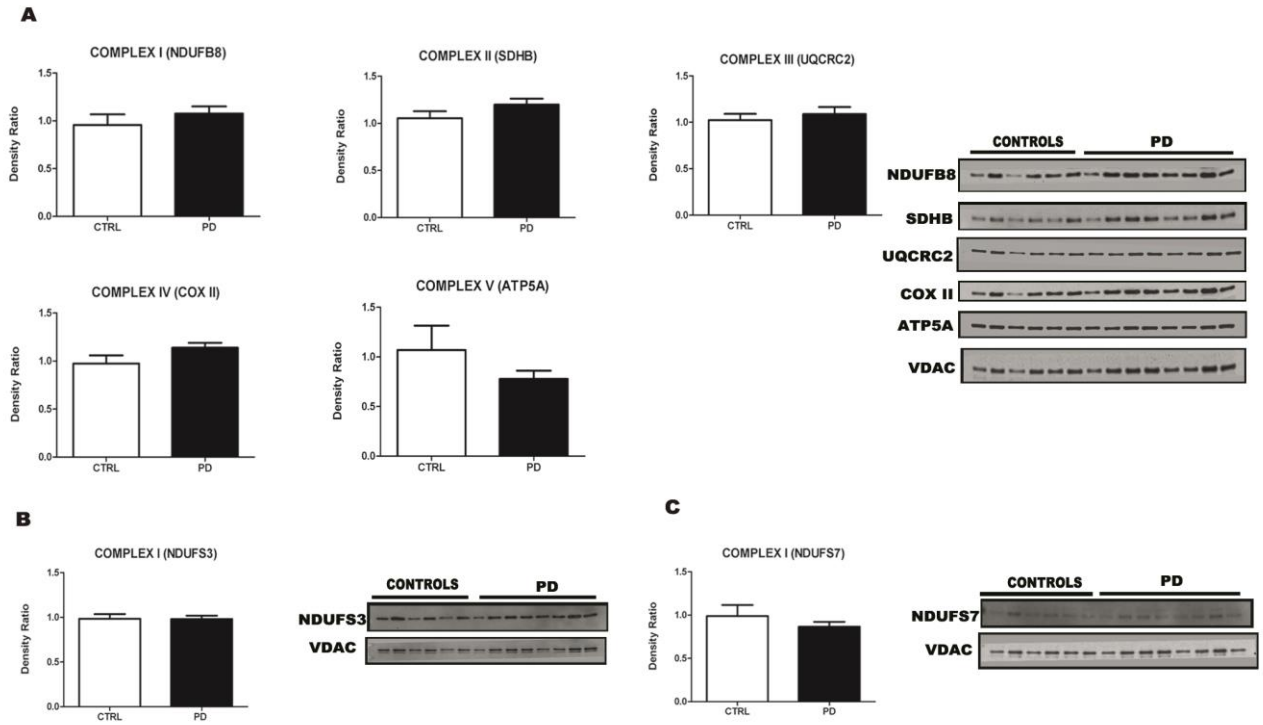
As the objective of the study has been focused on mitochondria, we proceeded to obtain pure mitochondria to better understand the functionality and expression of subunits and oxidative damage of mitochondrial complex subunits.

4.3.2 Assessing mitochondrial dysfunction with mitochondrial isolation

To test whether the complex subunits of mitochondria and other related oxidative and lipidoxidative markers were oxidatively damaged in the frontal cortex and angular gyrus which might lead to the dysfunction of mitochondria, different antibodies were tested.

4.3.2.1 Complex subunits

4.3.2.1.1 Protein expression in PD : Regarding frontal cortex (area 8), no differences were seen in oxphos (Figure 4.10 A) , complex I subunits (NDUFS3), (NDUFS7), (NDUFA7), (NDUFA10), (NDUFB10) (Figure 4.10 B, C, D, E, E, F respectively), complex II subunit (Fp) (Figure 4.10 G), complex IV subunits (subunit I and COX7A2L) (Figure 4.10 J and K), complex V subunit (ATP5H) (Figure 4.10 N). Significant decrease ($p < 0.05$) was seen in complex V (ATPD) and (ATP5J) (Figure 4.10 L and M respectively), where as Complex III (core II) subunit, was significantly increased ($p < 0.05$) (Figure 4.10 I). Regarding angular gyrus in PD, no differences were seen in OXPHOS (Figure 4.11).



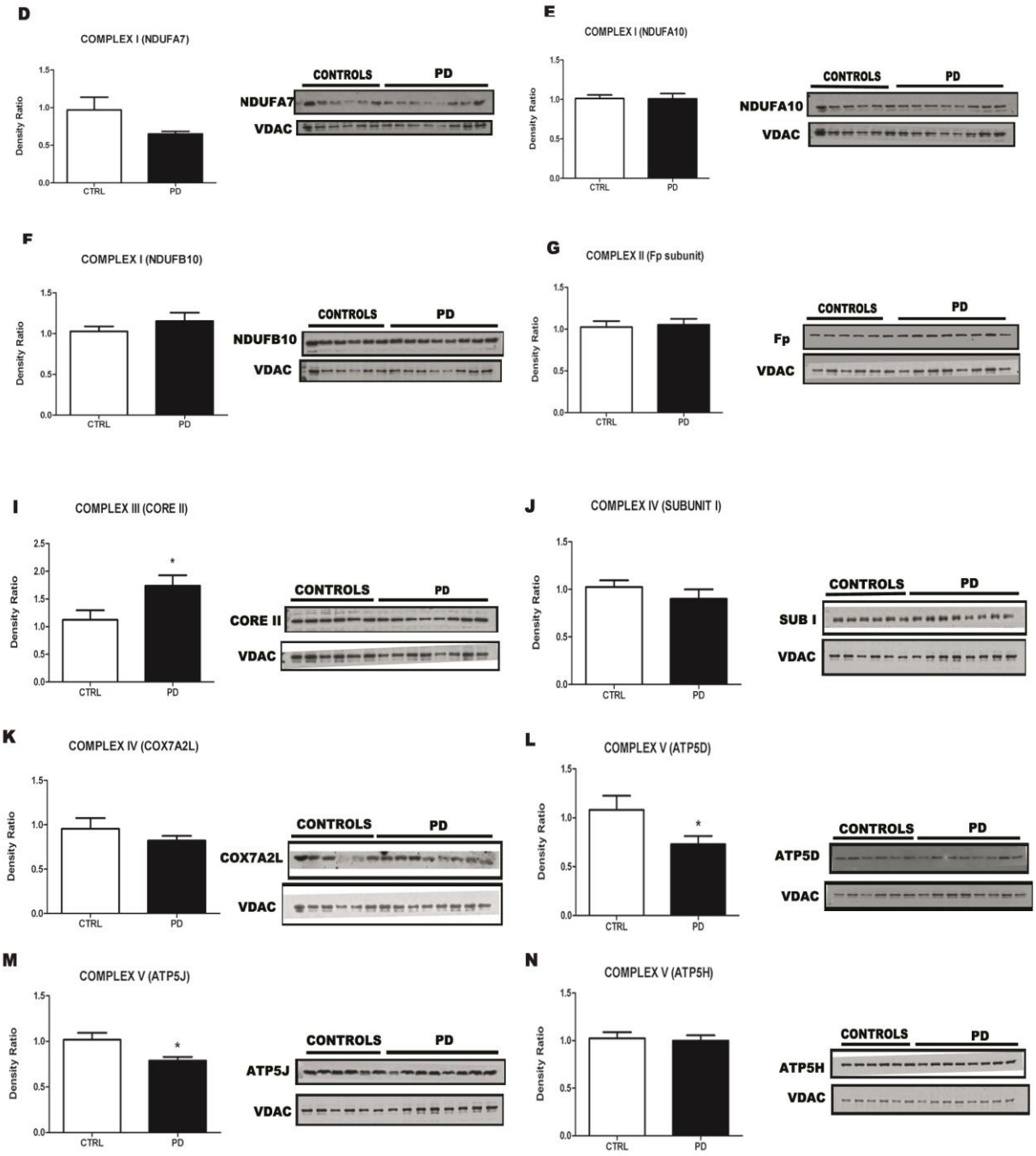


Figure 4.10 Protein expression levels of mitochondrial complex subunits in PD in the frontal cortex (area 8) corrected with VDAC levels as determined by western blots. Significant decrease was seen in complex V subunits of ATP5D, ATP5J where as in complex III core II and TOMM70 upregualtion has been observed. Remaining subunits of complexes remained unaltered. Student's T-test *p< 0.05 .

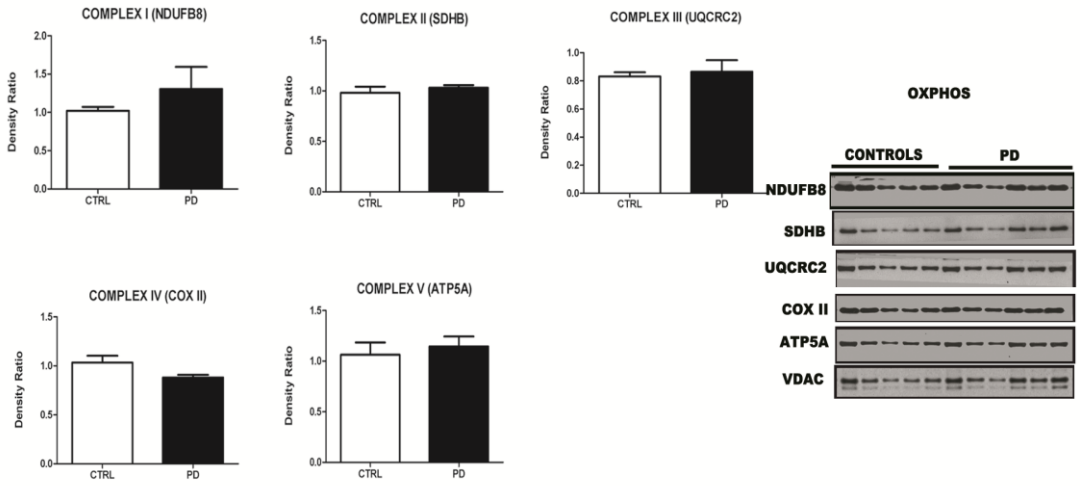
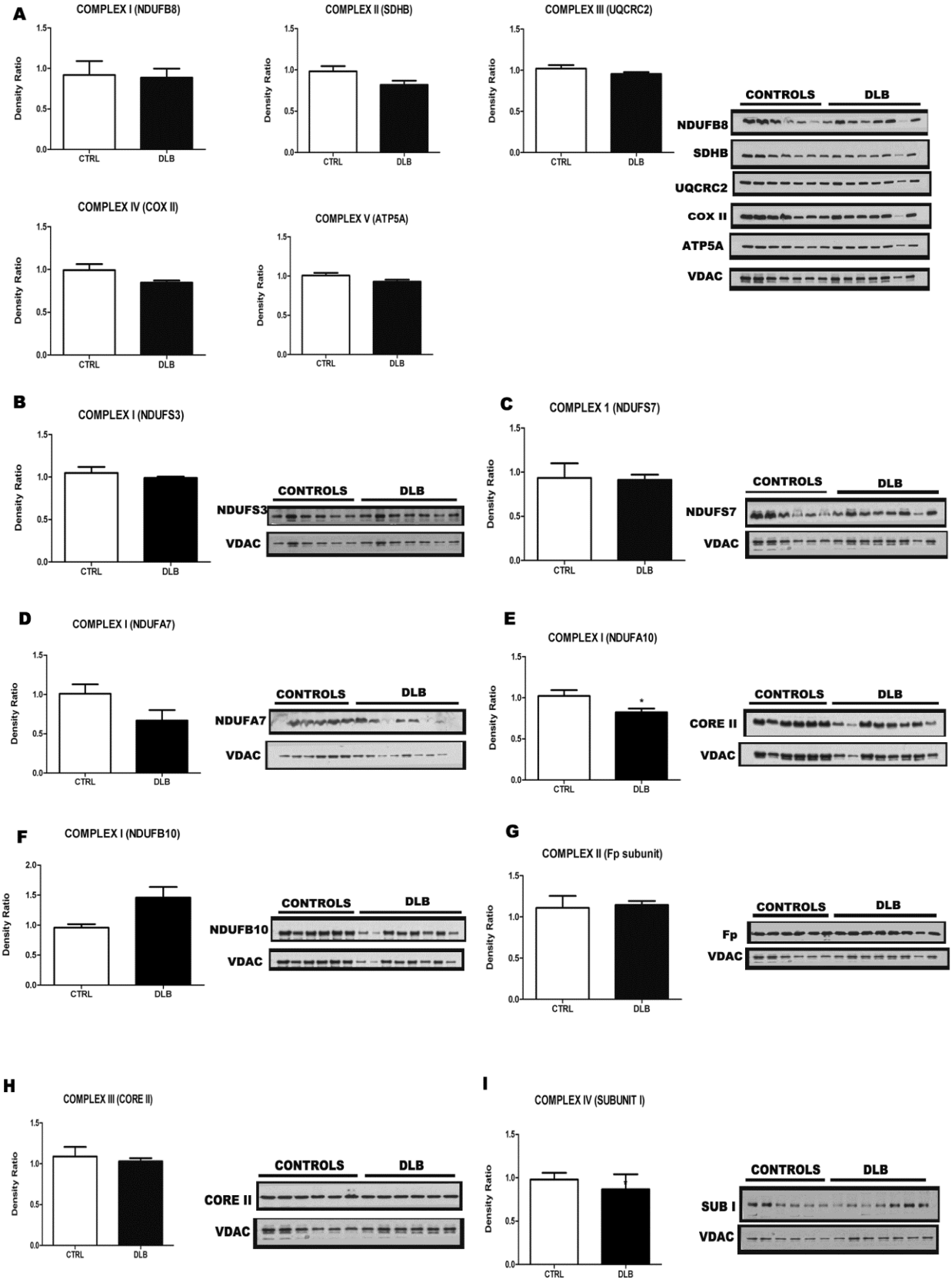


Figure 4.11 Protein expression levels of mitochondrial complex subunits in PD in the angular gyrus and control cases as determined by western blots and corrected with VDAC levels. None of the subunits have shown alteration in protein expression.

4.3.2.1.2 Protein expression in DLB : In frontal cortex (area 8), no differences were seen in Oxphos (Figure 4.12 A), complex I subunits (NDUFS3), (NDUFS7),(NDUFA7), (NDUFB10) (Figure 4.12 B, C, D,F respectively), complex II (Fp) subunit (Figure 4.12 G), complex III (core II) subunit (Figure 4.12 a), complex IV (subunit I) (Figure 4.12 b), complex V subunits (ATP5D), (ATP5H) (Figure 4.12 d and g respectively). Significant decrease ($p<0.05$) was seen in complex I (NDUFA10) subunit (Figure 4.12 E) and complex V (ATP5J) (Figure 4.12 e). On the other hand complex IV (COXVIIC) was found to be significantly increased ($p< 0.01$) (Figure 4.12 c).


A
COMPLEX I (NDUFB8)
COMPLEX II (SDHB)
COMPLEX III (UQCRC2)
COMPLEX IV (COX II)
COMPLEX V (ATP5A)
Density Ratio
CTRL
DLB
CONTROLS
DLB
NDUFB8
SDHB
UQCRC2
COX II
ATP5A
VDAC
B
COMPLEX I (NDUFS3)
NDUFS3
C
COMPLEX 1 (NDUFS7)
NDUFS7
D
COMPLEX I (NDUFA7)
NDUFA7
E
COMPLEX I (NDUFA10)
CORE II
F
COMPLEX I (NDUFB10)
NDUFB10
G
COMPLEX II (Fp subunit)
Fp
H
COMPLEX III (CORE II)
I
COMPLEX IV (SUBUNIT I)
SUB I

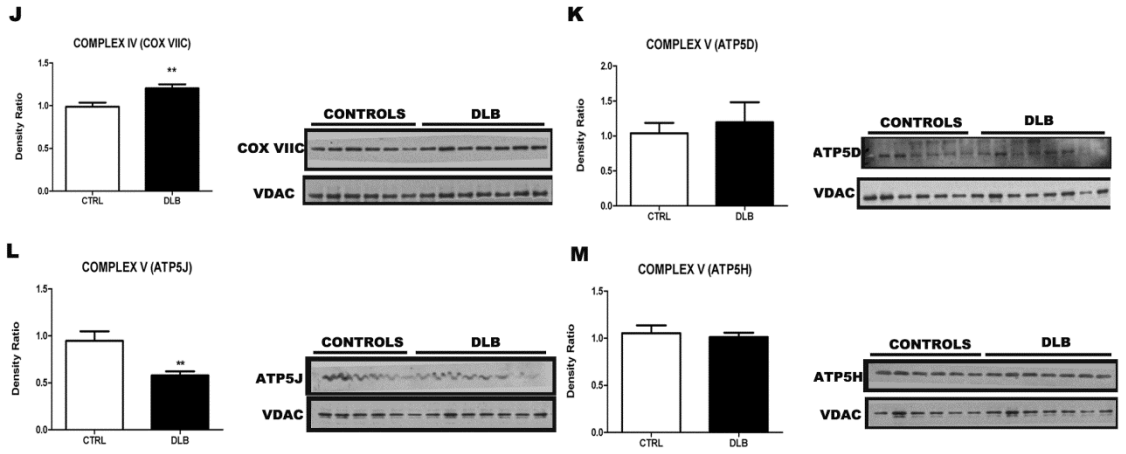
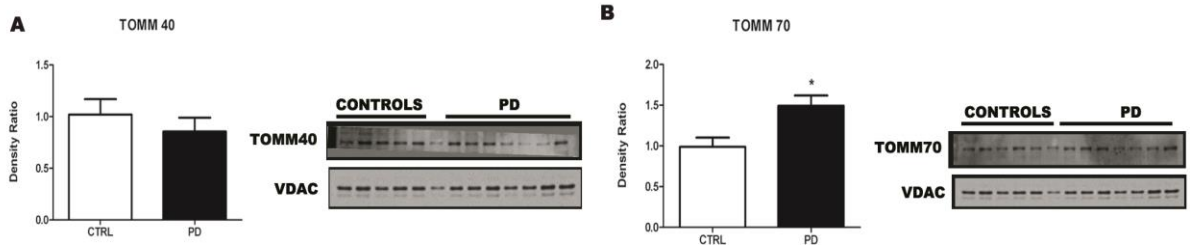


Figure 4.12 Protein expression levels of mitochondrial complex subunits in DLB in the frontal cortex (area 8) corrected with VDAC levels as determined by western blots. Significant decrease was seen in complex I (NDUFA10) subunit complex IV (COXVIIC) and complex V (ATP5J). Other subunits of complexes remained unaltered. Student's T-test *p< 0.05 **p< 0.01.

4.3.2.2 Cellular-localization of mitochondrial markers in PD

To understand further the potential response to oxidative stress, nuclear respiratory factor 1 and 2 (NRF1 and NRF2), stress-dependent expression genes Superoxide dismutase 2 (SOD2), translocase outer membrane (TOMM 40and TOMM 70) of TIM/TOM complex were analyzed.

4.3.2.2.1 Protein expression in PD : No significant differences were seen in the expression levels of TOMM 40 , NRF1 and NRF2 (Figure 4.13 A, C, and D respectively). Significant increase ($p < 0.05$) was seen in TOMM 70 (Figure 4.13 B), whereas SOD2 has shown a significant decrease ($p < 0.05$) (Figure 4.13 E).



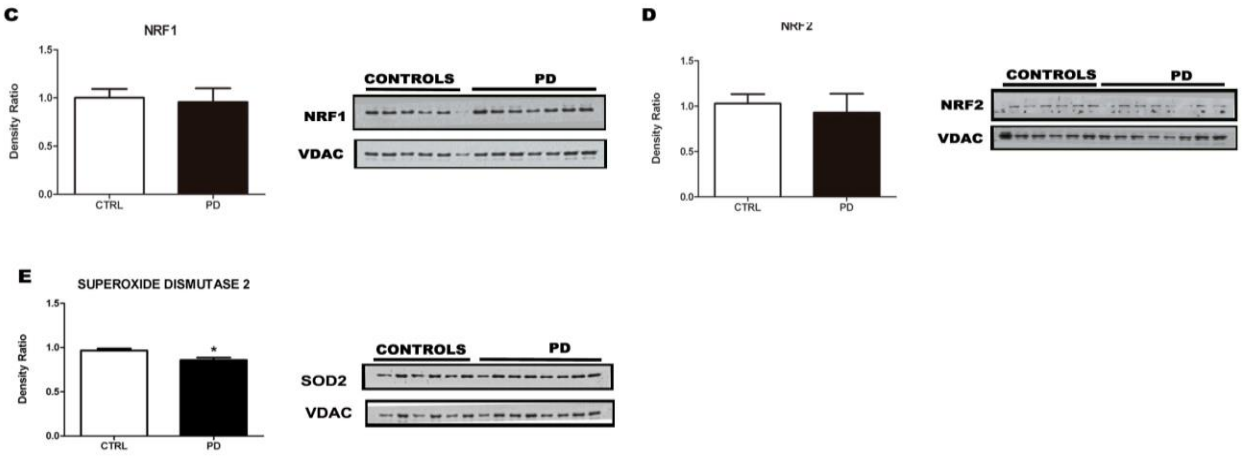


Figure 4.13 Protein expression levels in PD in the frontal cortex (area 8) corrected with VDAC levels as determined by western blots. Significant decrease was seen in SOD2 and significant increase was seen in TOMM70. Other stress markers remained unaltered. Student's T-test *p< 0.05.

4.3.2.2.2 Protein expression in DLB : No significant differences were seen in the expression levels of TOMM70, NRF1 and NRF2 (Figure 4.14 B, C and D respectively), SOD2 (Figure 4.14 E). Significant decrease (p< 0.001) was seen in TOMM40 (Figure 4.14 A).

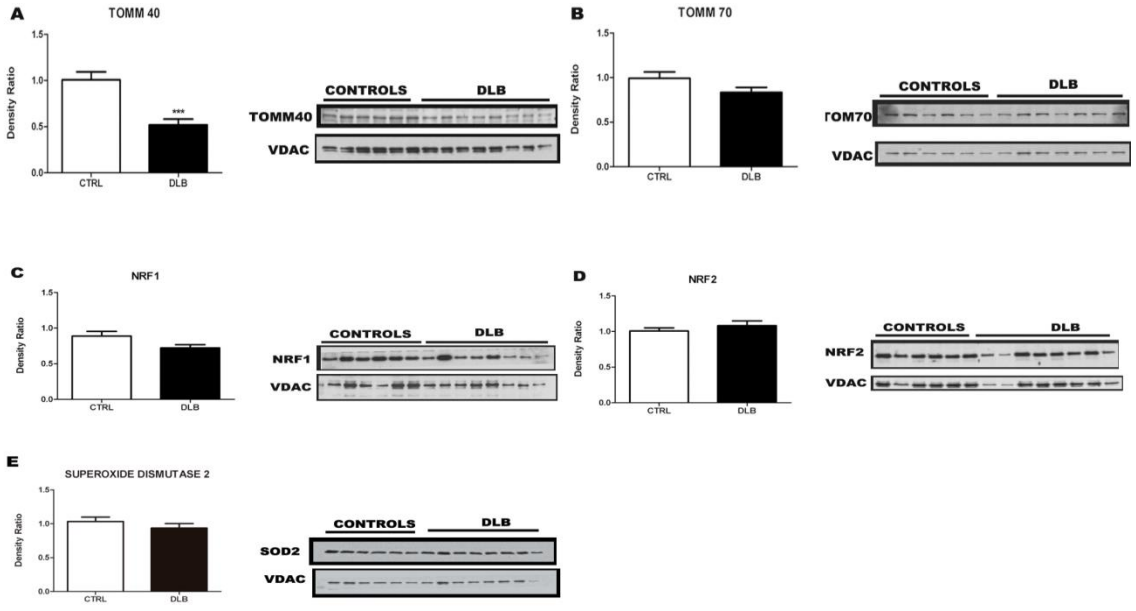


Figure 4.14 Protein expression levels in DLB in the frontal cortex (area 8) corrected with VDAC levels as determined by western blots. Significant decrease was seen in TOMM40. Other stress markers remained unaltered. Student's T-test ***p< 0.001.

4.3.2.3 Unfolded protein response in PD

In order to study protein misfolding which is associated with neurodegenerative diseases, phosphorylated eukaryotic translation initiation factor 2-α subunit (EIF2-α) along with ribosomal protein subunits MRPL21 , MRPL22, MRPS10 which help in mitochondrial protein synthesis were analyzed. To investigate, whether mitochondrial biogenesis was affected in PD, levels of mitochondrial transcriptional factor A (TFAM) was also studied.

4.3.2.3.1 Protein expression in PD : No significant differences were seen in TFAM, EIF2-α, MRPL21, MRPL22, MRPS10 (Figure 4.15 A, B, C, D, E and F respectively).

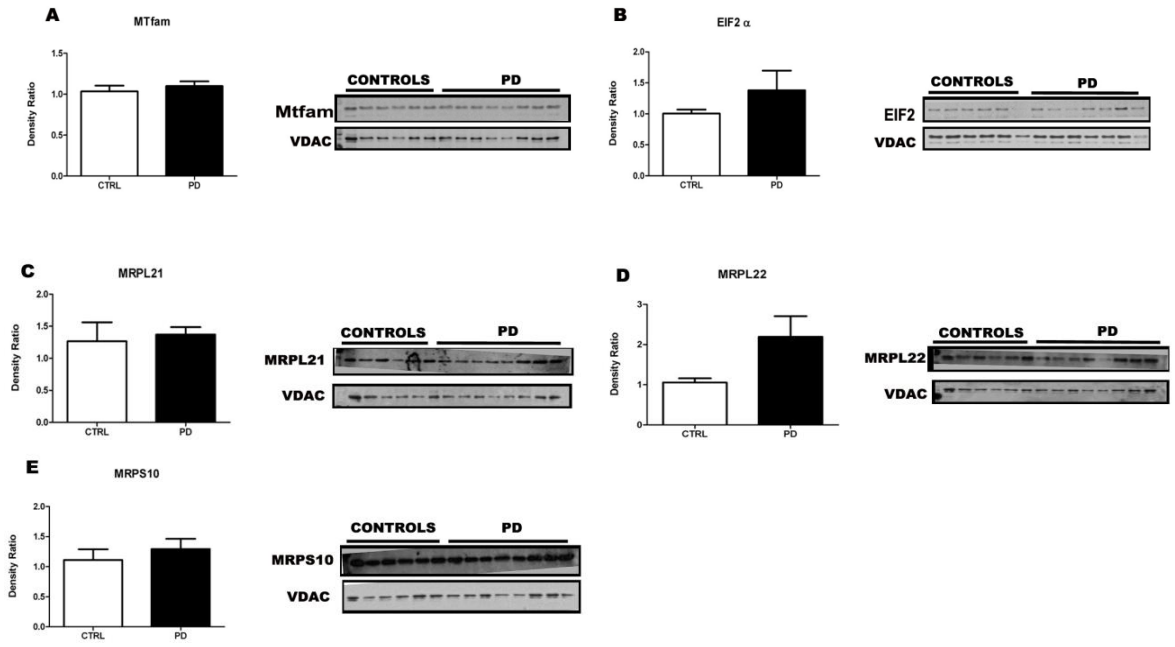


Figure 4.15 Protein expression levels in PD in the frontal cortex (area 8) corrected with VDAC levels as determined by western blots. No significant differences were seen in the transcription factors and ribosomal subunits.

4.3.2.3.2 Protein expression in DLB : No significant differences were seen in TFAM, EIF2-α, MRPL21, MRPL22 (Figure 4.16 A, B, C and D respectively). Significant increase ($p< 0.05$) was seen in MRPS10 (Figure 4.16 E).

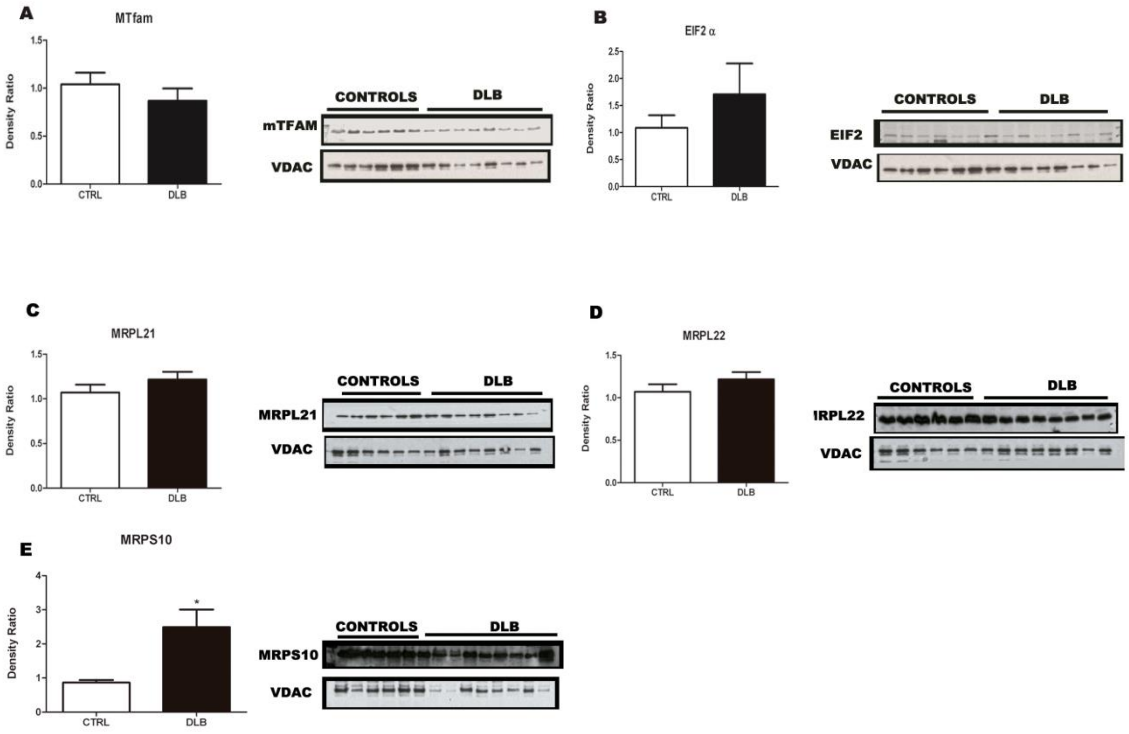


Figure 4.16 Protein expression levels in DLB in the frontal cortex (area 8) corrected with vdac levels as determined by western blots. Significant increase was seen in MRPS10. Other transcriptional factor and ribosomal subunits remained unaltered. Student's T-test *p< 0.05 **p< 0.01, ***p< 0.001.

4.3.2.4 Protein oxidative modifications in PD

The lipidoxidation markers MDAL, hydroxynoninal (HNE), and neuroketal (NKT) were studied to see the oxidative damage on mitochondria.

4.3.2.4.1 Protein expression in PD : No significant differences were observed in HNE and NKT (Figure 4.17 A and C respectively). Significant decrease ($p< 0.01$) was seen in MDAL (Figure 4.17 B) and significant increase was seen in Nitrotyrosine ($p< 0.01$) (Figure 4.17 D).

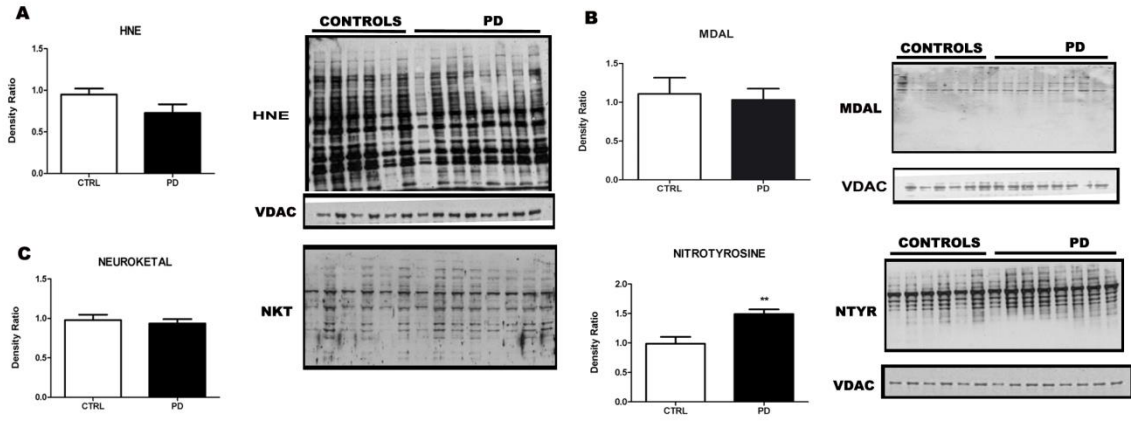


Figure 4.17 Protein expression levels in PD in the frontal cortex (area 8) corrected with vdac levels as determined by western blots. Significant increase was seen in nitrotyrosine. Other oxidative stress markers remained unaltered. Student's T-test *p< 0.05 , **p< 0.01, ***p< 0.001.

4.3.2.4.2 Protein expression in DLB : No significant differences were observed in HNE, MDAL, NKT and nitrotyrosine (Figure 4.18 A, B, C and D respectively).

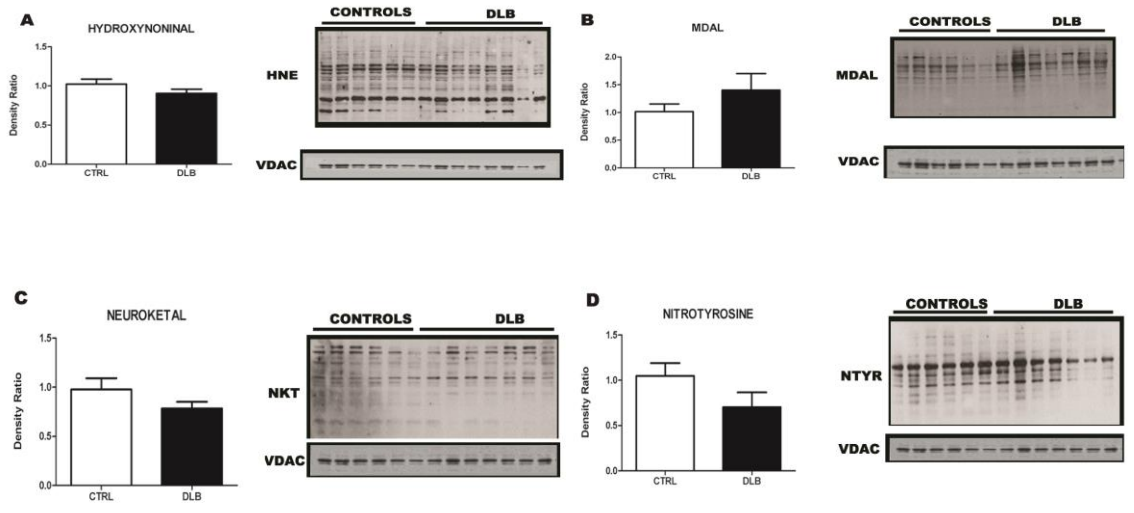
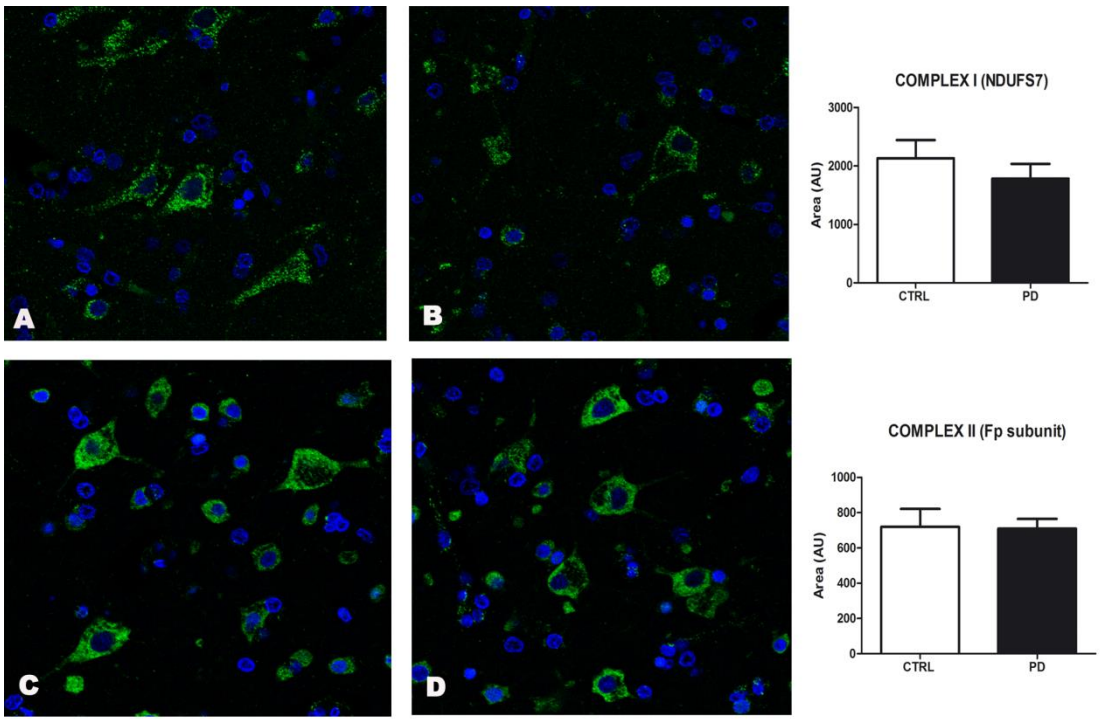


Figure 4.18 Protein expression levels in DLB in the frontal cortex (area 8) corrected with vdac levels as determined by western blots. No differences were seen in the oxidative stress markers.

4.4 Expression of mitochondrial complex subunits in frontal cortex through Immunofluorescence and Confocal microscopy.

Confocal microscopic studies were directed to demonstrate the alteration of subunits in human frontal cortex histologically. Hence to learn whether neurons bearing the mitochondria have altered subunits at protein level immunofluorescence and confocal microscopy to the antibodies stated above (Chapter 2, Table 3.5) were used. This was done to increase the robustness of the results obtained in the western blots. Immunofluorescence was used to detect the presence of selected mitochondrial subunits for which commercial antibodies were available in paraffin sections. Double labelling immunofluorescence and confocal microscopy in frontal cortex was used to study the effect of α-synuclein in the dysfunction of mitochondria in DLB, to this extent double staining of VDAC + α-synculein was used in the present study.

4.4.1 Immunofluorescence and confocal microscopy in PD: The validation by immunofluorescence and confocal microscopy in PD frontal cortex (area 8) of mitochondrial complex subunits have not shown any significant differences but a trend to decrease was seen in VDAC (Figure 4.19 L). Control Cases n=(5) and PD Cases n=(4) were used.



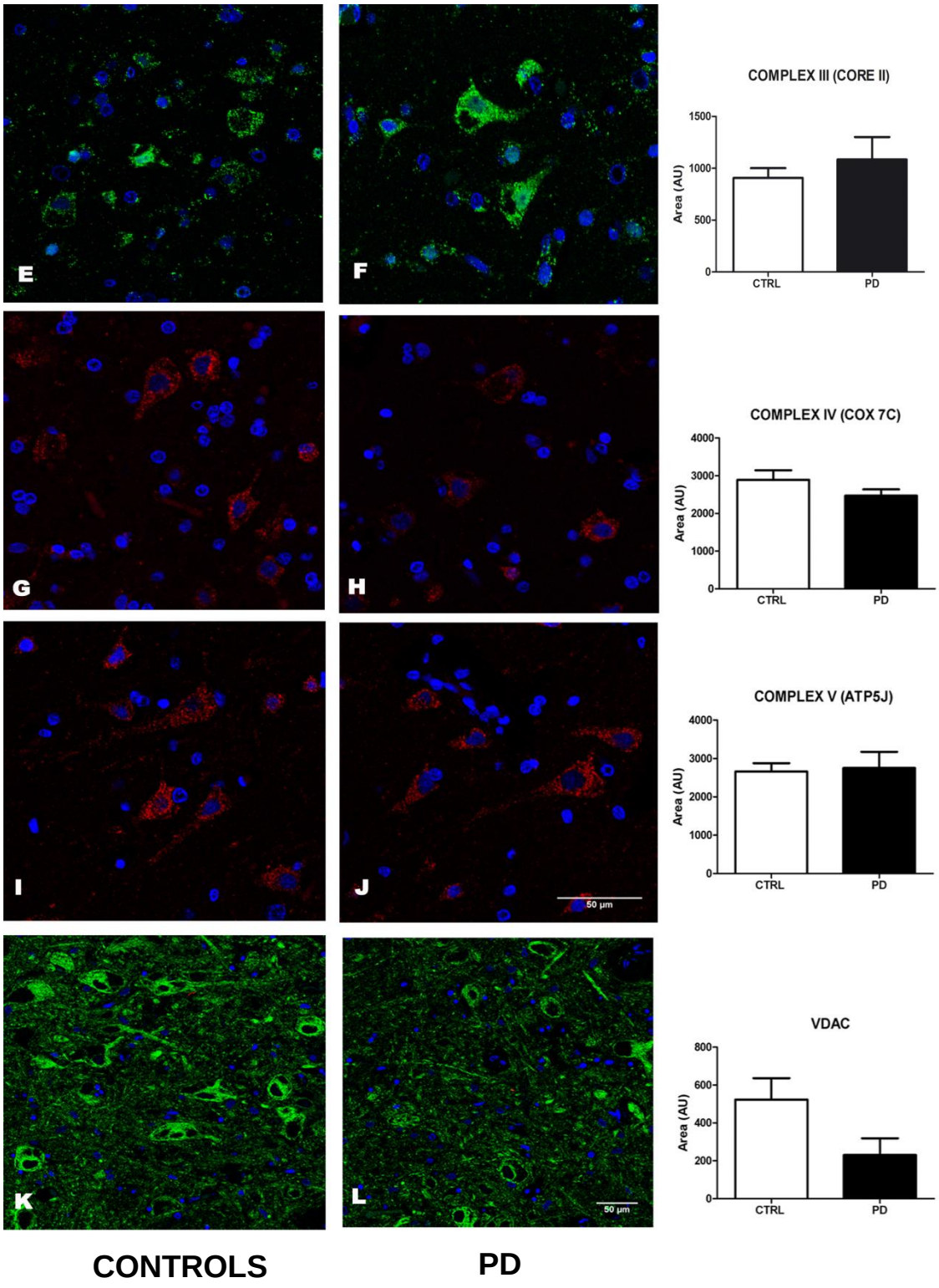
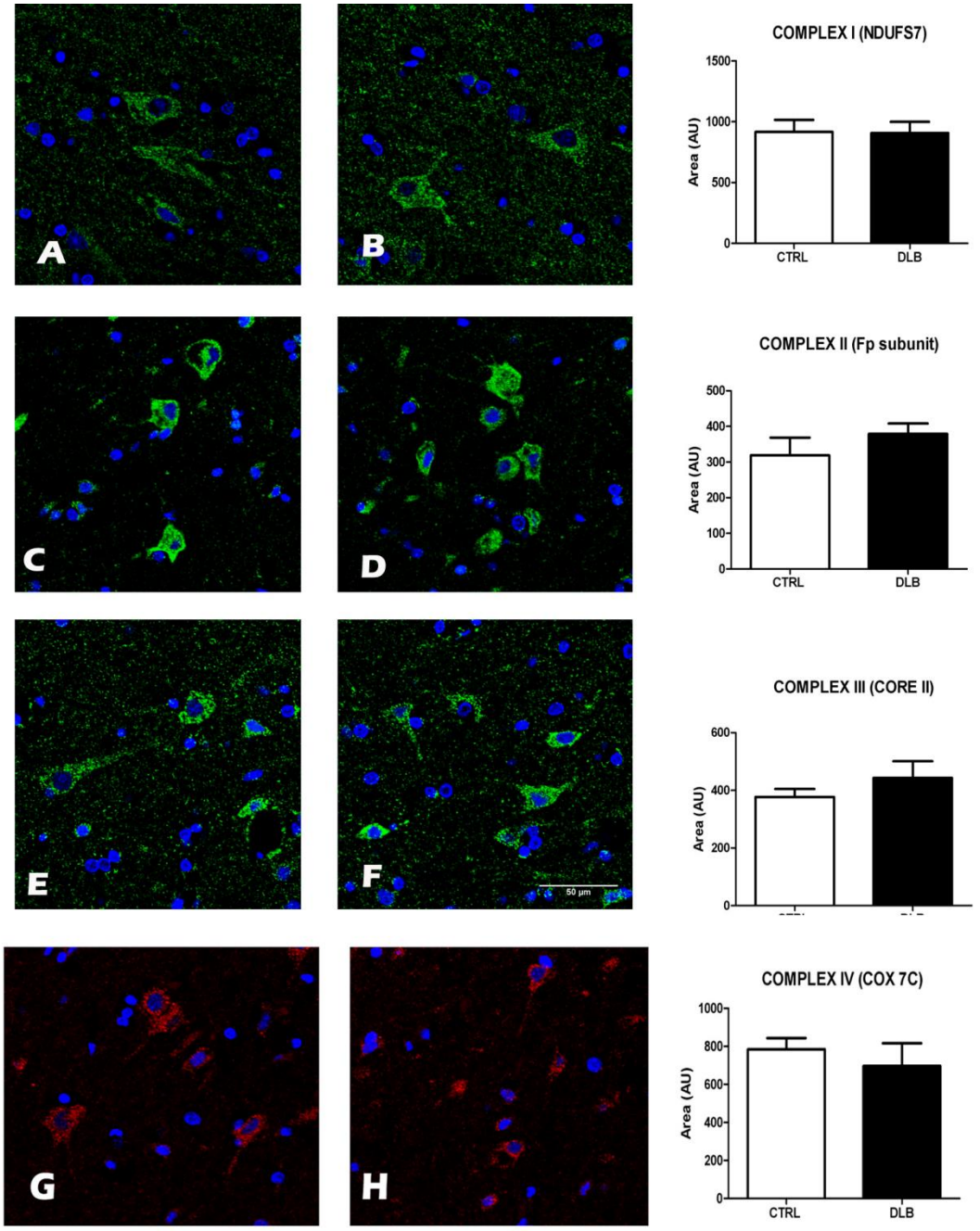


Figure 4.19 Confocal microscopy of complex subuntis in frontal cortex, to the left are controls and to the right are the PD cases, A-J represents the immunoreactivity of mitochondrial complex subunits, K-L represents the immunoreactivity of vdac.Paraffin sections are preincubated with sudan black to block lipofuscin autofluorescence. Nuclei are stained with DRAQ 5. Scale bar = 50 µm.

4.4.2 Immunofluorescence and confocal microscopy in DLB : The validation by double-labeling immunofluorescence and confocal microscopy in frontal cortex of vdac+alpha synuclein has shown that the deposition of alpha-synuclein in the cells reduces the vdac levels in neurons and immunofluorescence and confocal microscopy in frontal cortex (area 8) of mitochondrial complex subunits have not shown any significant differences but a trend to decrease was seen in vdac (Figure 4.20 L).



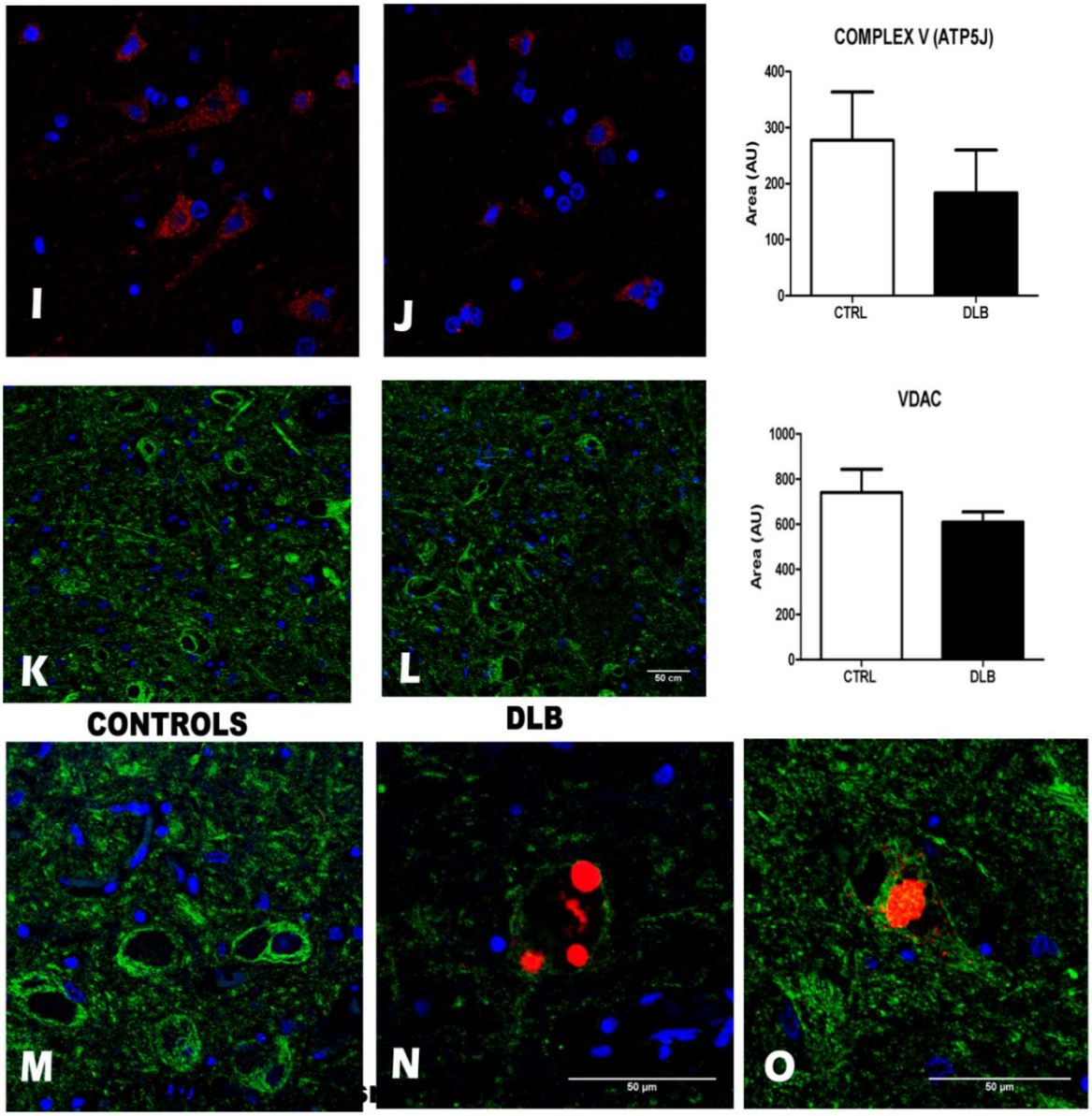


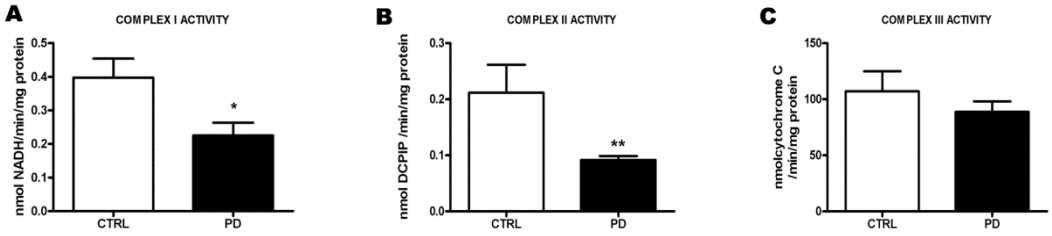
Figure 4.20 Confocal microscopy of complex subunits in the frontal cortex (area 8) to the left are the controls and to the right are the DLB cases. A-J represents the immunoreactivity of mitochondrial complex subunits. K-L represents the immunoreactivity of VDAC. M-O represents the immunoreactivity of VDAC+alpha-synuclein in frontal cortex. Nuclei are stained with DRAQ5. Scale bar = 50µm.

4.5 Validation of mitochondrial enzyme activities as a method to determine the dysfunction of mitochondria in PD and DLB

The mitochondrial respiratory complexes were studied to assess if the mitochondria were functional or not, as protein levels with western blots and pcr's have not shown significant alterations in most of the subunits studied. To this extent we assayed all the five complexes of mitochondria individually in frontal cortex (area 8) and in angular gyrus with and without normalizing to citrate synthase. Citrate synthase (CS), a mitochondrial matrix enzyme, is used as a marker of the abundance of mitochondria within a tissue/cell. This strategy is particularly useful to detect partial respiratory enzymatic dysfunction associated with compensatory mitochondrial proliferation, which might be overlooked by considering only the enzymatic activities normalized to protein content. All the assays were carried out in duplicates or triplicates. We quantified complex III and complex V activity by using inhibitors such as antimycin A and oligomycin respectively which block the flow of electrons within the complex. As a result, the proton pumps are unable to operate, as the gradient becomes too strong for them to overcome. Apart from this, the enzymatic activities were studied in the same individuals of two different regions to study the vulnerability of the regions to the disease in PD.

4.5.1 Mitochondrial complex enzyme activities in frontal cortex

4.5.1.1 Enzyme analysis in PD : The enzyme activities in PD frontal cortex have shown statistically significant decrease in complex I activity ($p < 0.05$) (Figure 4.23 A), complex II activity ($p < 0.01$) (Figure 4.23 B) and complex V activity ($p < 0.01$) (Figure 4.23 E) whereas the other two complexes III and IV remained unaltered (Figure 4.23 C and D respectively) including citrate synthase activity (Figure 4.23 F).



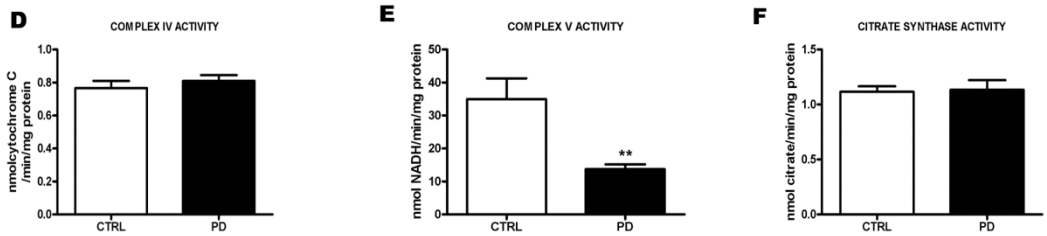


Figure 4.21 (A-E) represents the enzyme activities of respiratory chain complexes in PD of frontal cortex (area 8) and (F) represents the citrate synthase activity. Significant decrease was seen in complex I, complex II and complex V, where as complex III, and complex V including citrate synthase remained unaltered. Student's T-test *p <0.05, **p <0.01, ***p < 0.001.

4.5.1.2 Enzyme analysis in PD corrected with citrate synthase: The enzyme activities in PD frontal cortex have shown statistically significant decrease in complex I activity ($p < 0.05$) (Figure 4.22 G), complex II activity ($p < 0.01$) (Figure 4.22 H) and complex V activity ($p < 0.05$) (Figure 4.22 K) , whereas the other two complexes III and IV remained unaltered (Figure 4.22 I and J respectively) when corrected with citrate synthase activity.

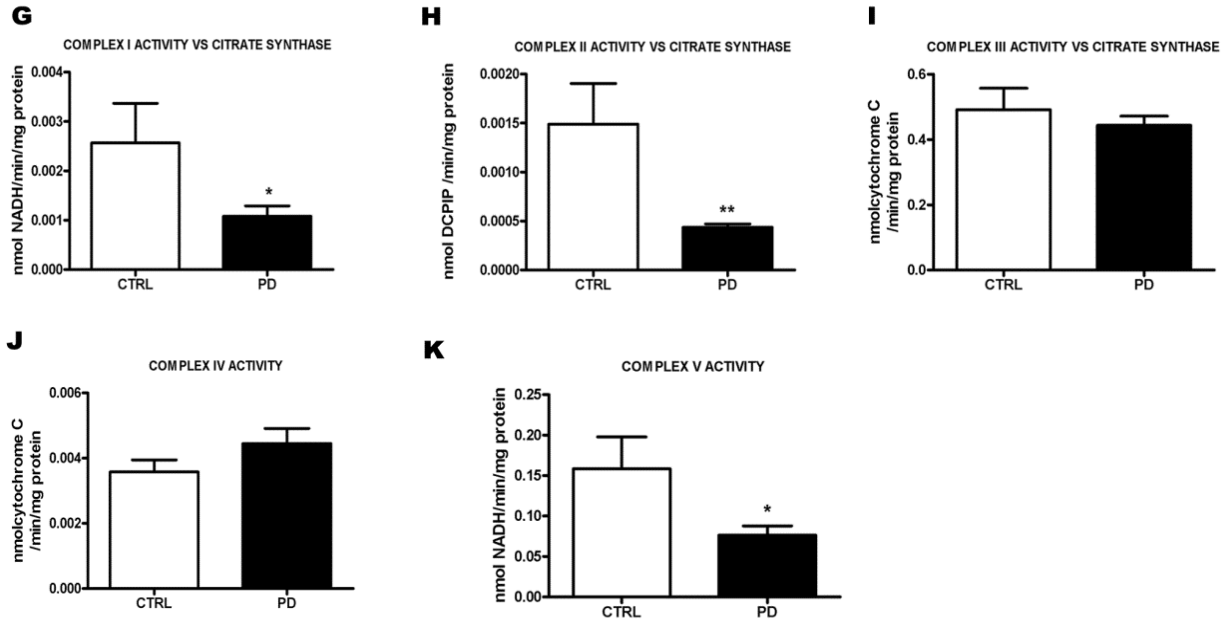


Figure 4.22 (G-K) represents the enzyme activities of respiratory chain complexes in PD of frontal cortex (area 8). Significant decrease was seen in complex I, complex II and complex V, where as complex III, and complex V remained unaltered. Student's T-test *p <0.05, **p <0.01, ***p < 0.001.

4.5.1.3 Enzyme analysis in DLB: The enzyme activities in DLB frontal cortex have shown statistically significant decrease in complex I activity ($p < 0.001$) (Figure 4.23 A), complex III activity ($p < 0.01$) (Figure 4.23 B) and complex V activity ($p < 0.01$) (Figure 4.23 E) and citrate synthase activity. Significant increase was seen in complex IV activity (Figure 4.23 D) whereas the complex II activity remained unaltered (Figure 4.23 B).

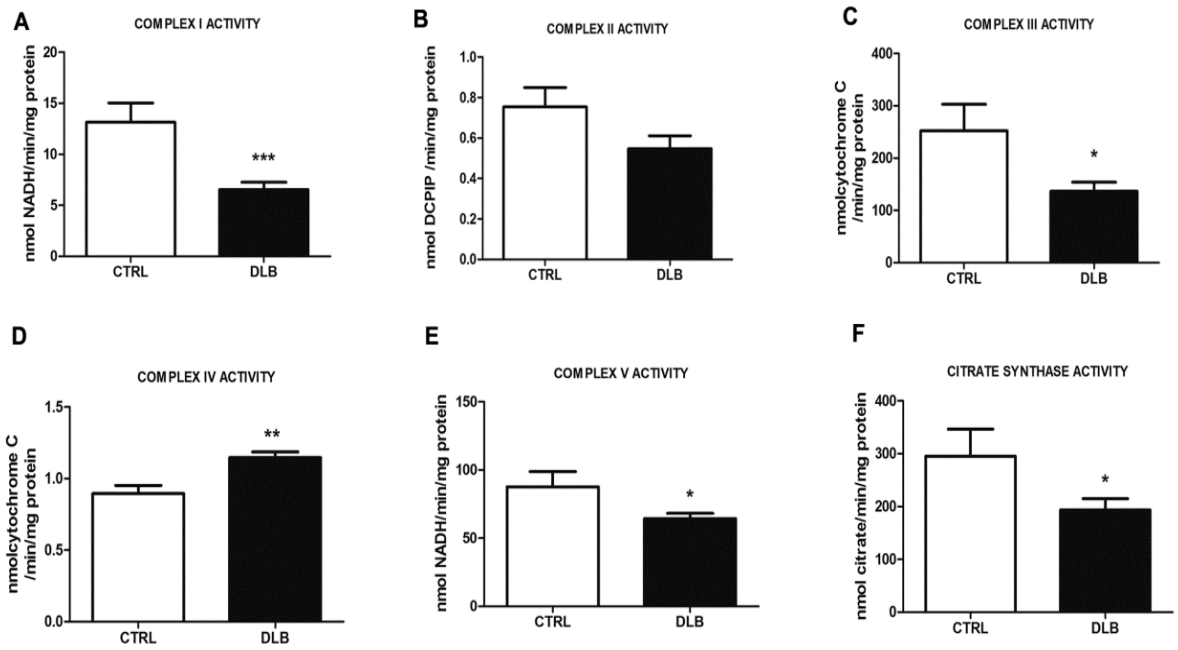


Figure 4.23 (A-E) represents the enzyme activities of respiratory chain complexes in DLB of frontal cortex (area 8) and (F) represents the citrate synthase activity. Significant decrease was seen in complex I, complex III and complex V. Significant increase was seen in complex IV activity, where as complex II activity remained unaltered .Student's T-test *p <0.05, **p <0.01, ***p < 0.001.

4.5.1.4 Enzyme analysis in DLB corrected with citrate synthase : The enzyme activities in DLB when corrected with citrate synthase activity have not shown any significant differences (Figure 4.24) .

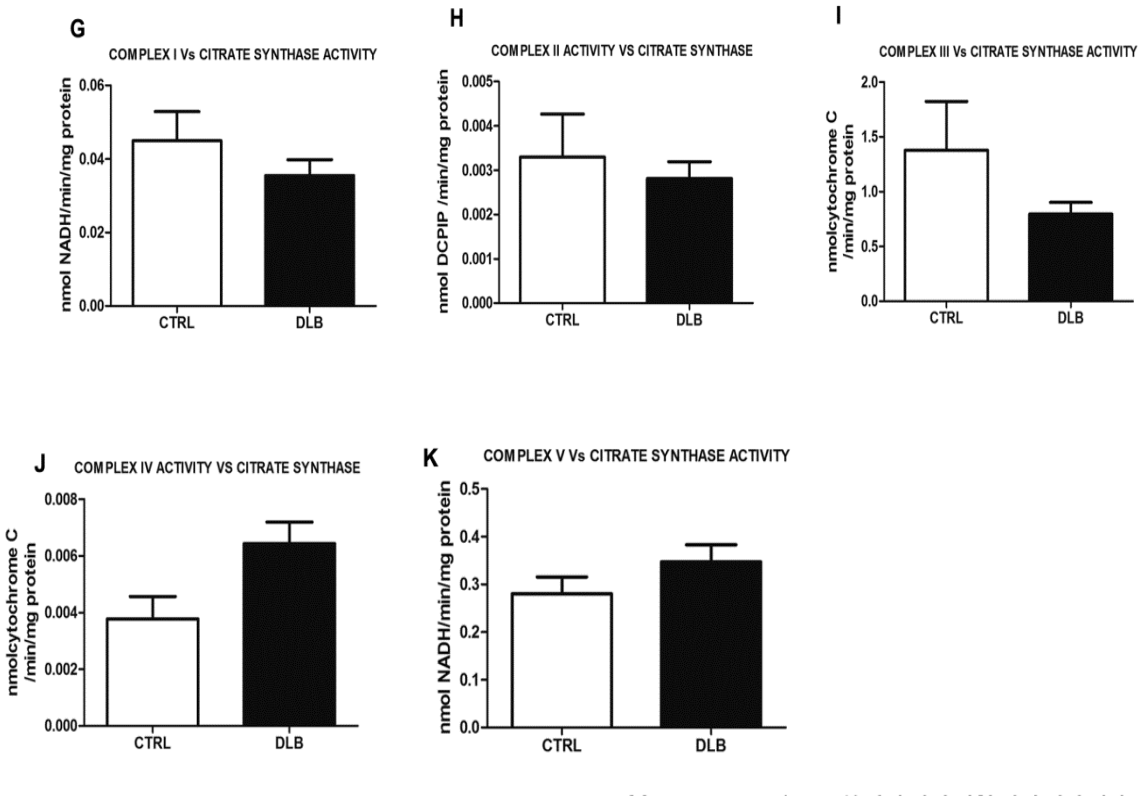


Figure 4.24 (G-K) represents the enzyme activities of respiratory chain complexes in DLB of frontal cortex (area 8). No significant changes were seen in the complex activities when corrected with citrate synthase.

4.5.2 Mitochondrial complex enzyme activities in angular gyrus

4.5.2.1 Enzyme analysis in PD : The enzyme activities in PD angular gyrus have not shown any significant differences in all the complex activities studied including citrates synthase activity (Figure 4.25)

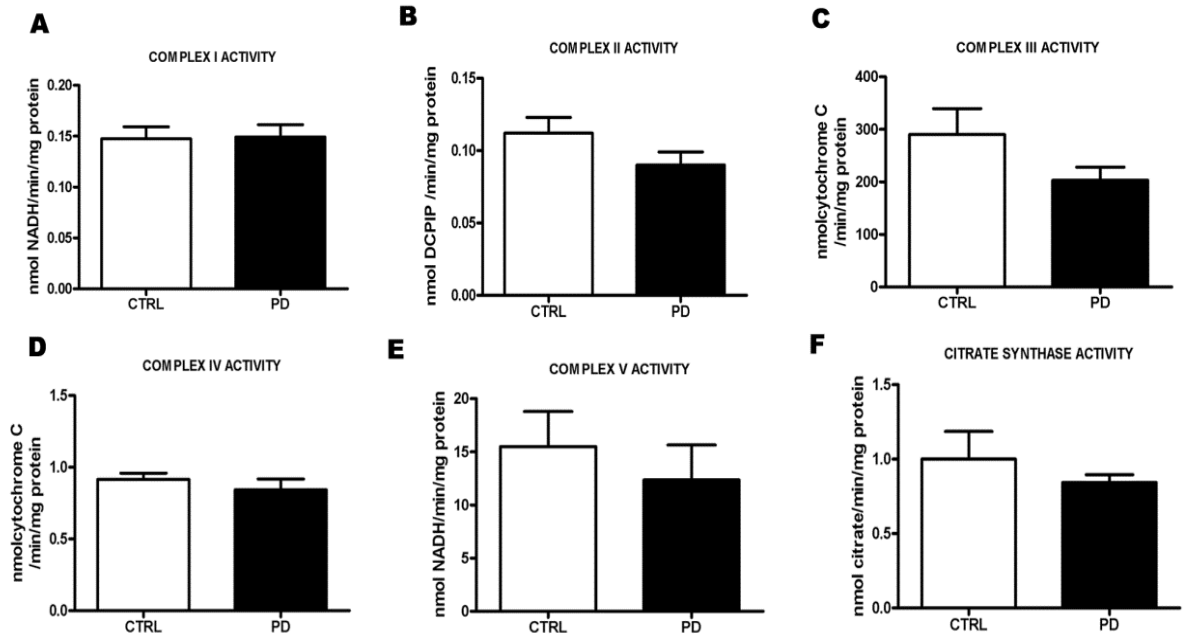


Figure 4.25 (A-E) represents the enzyme activities of respiratory chain complexes in PD of angular gyrus. (F) represents the citrate synthase activity. No significant were seen in the activities in angular gyrus.

4.5.2.2 Enzyme analysis in PD corrected with citrate synthase : The enzyme activities in PD angular gyrus have not shown any significant differences in all the complex activities studied including citrates synthase activity (Figure 4.26) when corrected with citrate synthase activity.

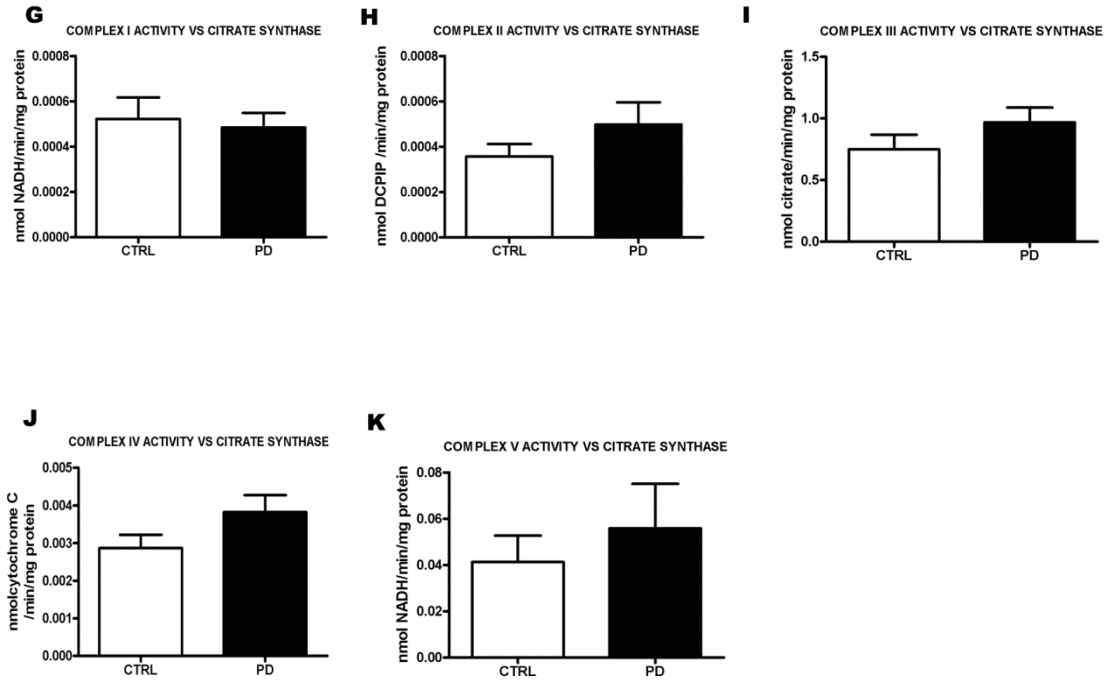


Figure 4.26 (G-K) represents the enzyme activities of respiratory chain complexes in PD of angular gyrus. No significant differences were seen in the activities when corrected with citrate synthase.

4.5.3 Comparative study of enzyme activities of the regions (frontal cortex and angular gyrus) cases obtained from the same individual

4.5.3.1 Enzyme activities in PD : The impairment of respiratory complexes from the above results depicts the pathogenesis of the disease and mitochondrial dysfunction. With this results, it was hypothesized to analyze or make a comparative study in the two regions from which the cases were obtained from the same individual. Significant decrease was seen in complex I, complex II and complex III activity of frontal cortex when compared with angular gyrus.

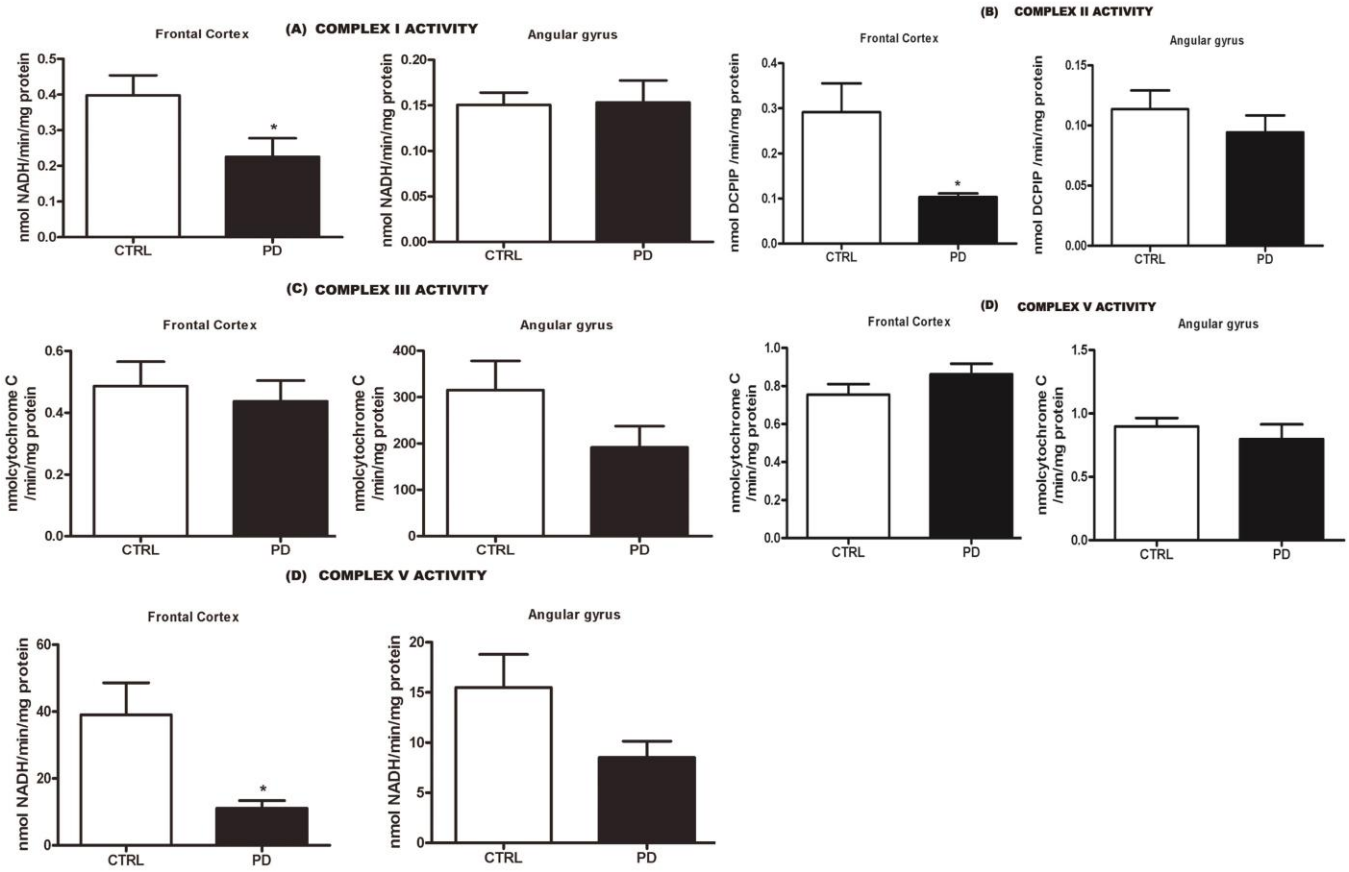


Figure 4.27 (A-D) represents the enzyme activities of comparative study of respiratory chain complexes in PD of frontal cortex and angular gyrus. Significant differences were seen in complex I , complex II and complex V of frontal cortex when compared with angular gyrus. Student's T-test *p< 0.05, **p< 0.01, ***p< 0.001.

Results : Objective 2 – Ribosomal Proteins

4.6 Global Expression Profiles Identify Dysregulation of ribosomal proteins in Frontal cortex (area 8) in PD

We have previously published microarray data of RNA samples from PD (n=4 premotor and 7 with parkinsonism and age-matched controls (n=7) hybridized to an Affymetrix Human Gene 1.1 ST array (Garcia P et al, 2013). To assess whether there are more Ribosome genes deregulated in PD respect to controls than we would expect by chance we analyzed the transcriptomic data derived from these microarrays. We found a significant downregulated gene expression of the Ribosome genes extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000) (KEGG 03010) in PD compared with control samples with a p=5.37E-07. Pathway enrichment was analyzed with Gene set enrichment analysis (GSEA) tests to the t statistics arising for microarray. Deregulation of Ribosome genes occurred in both premotor and parkinsonian stages.

Ribosomal proteins : The basic mechanism of protein synthesis is similar throughout all forms of life, where ribosome plays a crucial role together with its accessories which translate the genetic information encoded in a messenger RNA (mRNA) into the corresponding sequence of amino acids, thereby synthesizing a polypeptide chain or a protein. The present study is designed to identify possible alterations of the ribosomal subunits that commands the protein synthesis in the regions vulnerable to PD and DLB which includes the substantia nigra, frontal cortex (area 8), angular gyrus and precuneus.

4.6.1 mRNA expression of ribosomal subunits in *substantia nigra*

4.6.1.1 The mRNA expression levels in PD : The mRNA analysis of ribosomal subunits have shown significant downregualtion in RPS3A ($p< 0.05$), RPS5 ($p< 0.05$), RPS6 ($p< 0.01$), RPS10 ($p <0.05$), RPS13 ($p< 0.05$), RPS16 ($p< 0.05$) and the remaining subunits remained unaltered (Table 4.1)

Probes	Substantia nigra		
	Control	PD	P Value
RPL5	1.06 ± 0.20	0.49 ± 0.12	
RPL7	1.01 ± 0.13	0.82 ± 0.07	
RPL21	1.16 ± 0.23	0.59 ± 0.06	
RPL22	1.09 ± 0.25	0.81 ± 0.10	
RPL23A	1.01 ± 0.20	0.51 ± 0.09	
RPL26	1.37 ± 0.39	0.41 ± 0.07	
RPL27	0.88 ± 0.13	0.65 ± 0.11	
RPL30	1.09 ± 0.21	0.77 ± 0.15	
RPL31	1.02 ± 0.18	0.87 ± 0.09	
RPS3A	1.13 ± 0.17	0.40 ± 0.05	↓ *
RPS5	1.07 ± 0.13	0.32 ± 0.02	↓ *
RPS6	0.93 ± 0.09	0.35 ± 0.05	↓ **
RPS10	1.11 ± 0.16	0.23 ± 0.02	↓ *
RPS13	0.92 ± 0.13	0.23 ± 0.06	↓ *
RPS16	0.90 ± 0.13	0.30 ± 0.10	↓ *
RPS17	0.96 ± 0.21	0.17 ± 0.05	

Table 4.1 mRNA expression levels of selected ribosomal probes in control and PD cases in the *substantia nigra* as determined by TaqMan PCR assays using *GUS-β* for normalization. Significant downregulation was seen in RPS3A, RPS5, RPS6, RPS10,RPS13 and RPS16 whereas the remaining subunits remained unaltered. Student's T-test *p< 0.05, **p< 0.01, ***p< 0.001.

4.6.1.2 The mRNA expression levels in DLB : The mRNA analysis of ribosomal subunits have shown significant downregualtion in RPS3A ($p< 0.05$), RPS5 ($p< 0.001$), RPS6 ($p< 0.01$), RPS10 ($p <0.01$), RPS13 ($p< 0.01$), RPS16 ($p< 0.01$) and the remaining subunits remained unaltered (Table 4.2).

Probes	Substantia nigra		
	Control	DLB	P Value
RPL5	1.06 ± 0.20	0.64 ± 0.20	
RPL7	1.01 ± 0.13	0.61 ± 0.10	
RPL21	1.16 ± 0.23	0.58 ± 0.14	
RPL22	1.09 ± 0.25	0.77 ± 0.19	
RPL23A	1.01 ± 0.20	0.40 ± 0.02	
RPL26	1.37 ± 0.39	0.16 ± 0.03	
RPL27	0.88 ± 0.13	0.54 ± 0.10	
RPL30	1.09 ± 0.21	1.05 ± 0.26	
RPL31	1.02 ± 0.18	2.29 ± 0.58	
RPS3A	1.13 ± 0.17	0.31 ± 0.10	↓ *
RPS5	1.07 ± 0.13	0.34 ± 0.13	↓ **
RPS6	0.93 ± 0.09	0.38 ± 0.09	↓ **
RPS10	1.11 ± 0.16	0.18 ± 0.04	↓ **
RPS13	0.92 ± 0.13	0.29 ± 0.06	↓ **
RPS16	0.90 ± 0.13	0.19 ± 0.08	↓ **
RPS17	0.96 ± 0.21	0.43 ± 0.11	

Table 4.2 mRNA expression levels of selected ribosomal probes in control and DLB cases in the *substantia nigra* as determined by TaqMan PCR assays using *GUS-β* for normalization. Significant downregulation was seen in RPS3A, RPS5, RPS6, RPS10,RPS13 and RPS16 whereas the remaining subunits remained unaltered. Student's T-test *p< 0.05, **p< 0.01, ***p< 0.001.

4.6.2 mRNA expression of ribosomal subunits in frontal cortex (area 8)

4.6.2.1 : The mRNA expression levels in PD : The mRNA analysis of selected ribosomal subunits have shown significant downregualtion in RPL5 ($p< 0.05$), RPL7 ($p< 0.001$), RPL22 ($p< 0.001$), RPL31 ($p< 0.01$), RPS3A ($p< 0.01$), RPS6 ($p < 0.05$), RPS10 ($p < 0.01$), RPS13 ($p< 0.01$), RPS17 ($p< 0.01$), (Table 4.3) where as the rest of the subunits remained unaltered.

Probes	Frontal cortex (area 8)		
	Control	PD	P Value
RPL5	1.48 ± 0.34	0.46 ± 0.10	↓ *
RPL7	1.16 ± 0.14	0.11 ± 0.02	↓ ***
RPL21	1.42 ± 0.33	0.72 ± 0.20	
RPL22	1.30 ± 0.22	0.03 ± 0.005	↓ ***
RPL23A	0.09 ± 0.11	0.66 ± 0.18	
RPL26	1.22 ± 0.22	1.38 ± 0.44	
RPL27	1.74 ± 0.63	0.36 ± 0.09	
RPL30	1.23 ± 0.37	1.19 ± 0.33	
RPL31	1.58 ± 0.42	0.16 ± 0.04	↓ **
RPS3A	1.16 ± 0.26	0.21 ± 0.05	↓ **
RPS5	1.37 ± 0.31	1.02 ± 0.33	
RPS6	1.37 ± 0.33	0.46 ± 0.10	↓ *
RPS10	1.46 ± 0.32	0.20 ± 0.04	↓ **
RPS13	1.06 ± 0.11	0.5 ± 0.14	↓ **
RPS16	1.41 ± 0.38	3.22 ± 0.90	
RPS17	1.09 ± 0.14	0.47 ± 0.15	↓ **

Table 4.3 mRNA expression levels of selected ribosomal probes in control and PD cases in the frontal cortex as determined by TaqMan PCR assays using *GUS-β* for normalization. Significant downregulation was seen in RPL5, RPL7, RPL22, RPL31, RPS3A, RPS6, RPS10,RPS13 and RPS17 where as the other subunits remained unaltered. Student's T-test *p< 0.05, **p< 0.01, ***p< 0.001.

4.6.2.2 : The mRNA expression levels in DLB : The mRNA analysis of selected ribosomal subunits have shown significant upregualtion in RPL7 ($p< 0.01$), RPL26 ($p< 0.01$), RPL30 ($p< 0.05$), RPL31 ($p< 0.05$), RPS3A ($p < 0.05$), RPS10 ($p < 0.05$), RPS13 ($p< 0.001$), RPS16 ($p< 0.001$), RPS17 ($p< 0.001$), (Table 4.4) where as the rest of the subunits remained unaltered.

Probes	Frontal cortex (area 8)		
	Control	DLB	P Value
RPL5	1.06 ± 0.16	1.23 ± 0.23	
RPL7	1.11 ± 0.22	3.36 ± 0.60	↑ **
RPL21	1.06 ± 0.18	1.13 ± 0.22	
RPL22	1.14 ± 0.16	1.60 ± 0.54	
RPL26	1.03 ± 0.18	3.93 ± 0.51	↑ **
RPL27	1.68 ± 0.76	0.39 ± 0.04	
RPL30	1.63 ± 0.45	3.74 ± 0.91	↑ *
RPL31	1.22 ± 0.24	1.42 ± 0.23	↑ *
RPS3A	1.18 ± 0.19	3.31 ± 0.83	↑ *
RPS5	1.18 ± 0.19	1.11 ± 0.13	
RPS6	1.14 ± 0.15	1.43 ± 0.19	
RPS10	1.08 ± 0.18	3.78 ± 0.54	↑ *
RPS13	1.21 ± 0.23	4.17 ± 0.82	↑ **
RPS16	1.36 ± 0.30	9.55 ± 1.30	↑ ***
RPS17	0.99 ± 0.14	3.48 ± 0.51	↑ ***

Table 4.4 mRNA expression levels of selected ribosomal probes in control and DLB cases in the frontal cortex as determined by TaqMan PCR assays using *GUS-β* for normalization. Significant upregulation was seen in RPL7, RPL26, RPL30, RPL31, RPS3A, RPS10, RPS13 , RPS16 and RPS17 where as the other subunits remained unaltered. Student's T-test *p< 0.05, **p< 0.01,*p< 0.001.**

4.6.3 mRNA expression of ribosomal subunits in angular gyrus

4.6.3.1 : The mRNA expression levels in PD : The mRNA analysis of selected ribosomal subunits have shown significant upregulation RPL7 ($p < 0.05$) and RPS10 ($p < 0.01$) (Table 4.5) where as the rest of the subunits remained unaltered.

Probes	Angular gyrus		
	Control	PD	P Value
RPL5	1.08 ± 0.3	1.49 ± 0.47	
RPL7	1.28 ± 0.26	3.76 ± 1.00	↑ *
RPL21	1.28 ± 0.31	1.80 ± 0.63	
RPL22	0.97 ± 0.22	1.53 ± 0.52	
RPL23A	1.06 ± 0.23	5.23 ± 2.09	
RPL26	1.38 ± 0.33	1.23 ± 0.32	
RPL27	1.18 ± 0.24	1.39 ± 0.46	
RPL30	1.20 ± 0.24	1.05 ± 0.27	
RPL31	1.35 ± 0.37	1.21 ± 0.24	
RPS3A	1.16 ± 0.20	0.91 ± 0.19	
RPS5	1.39 ± 0.40	1.74 ± 0.56	
RPS6	1.02 ± 0.21	1.44 ± 0.32	
RPS10	1.00 ± 0.16	2.04 ± 0.44	↑ *
RPS13	1.10 ± 0.31	2.07 ± 0.48	
RPS16	0.95 ± 0.17	1.90 ± 0.52	
RPS17	1.02 ± 0.20	1.59 ± 0.36	

Table 4.5 mRNA expression levels of selected ribosomal probes in control and PD cases in the angular gyrus as determined by TaqMan PCR assays using *GUS-β* for normalization. Significant upregulation was seen in RPL7 and RPS10 where as the other subunits remained unaltered. Student's T-test *p< 0.05 , **p< 0.01, ***p< 0.001.

4.6.3.2 : The mRNA expression levels in DLB : The mRNA analysis of selected ribosomal subunits have shown significant upregulation in RPL7 ($p< 0.05$), RPL23A ($p< 0.05$) and RPL31 ($p< 0.01$) (Table 4.6) where as the rest of the subunits remained unaltered.

Probes	Angular gyrus		
	Control	DLB	P Value
RPL5	1.08 ± 0.3	1.91 ± 0.28	
RPL7	1.28 ± 0.26	4.11 ± 1.83	↑ *
RPL21	1.28 ± 0.31	1.56 ± 0.46	
RPL22	0.97 ± 0.22	0.70 ± 0.18	
RPL23A	1.06 ± 0.23	2.76 ± 0.93	↑ *
RPL26	1.38 ± 0.33	2.35 ± 1.05	
RPL27	1.18 ± 0.24	1.26 ± 0.37	
RPL30	1.20 ± 0.24	4.15 ± 1.37	
RPL31	1.35 ± 0.37	1.21 ± 0.29	↑ **
RPS3A	1.16 ± 0.20	1.16 ± 0.38	
RPS5	1.39 ± 0.40	1.21 ± 0.37	
RPS6	1.02 ± 0.21	1.34 ± 0.40	
RPS10	1.00 ± 0.16	1.51 ± 0.68	
RPS13	1.10 ± 0.31	0.60 ± 0.17	
RPS16	0.95 ± 0.17	0.85 ± 0.34	
RPS17	1.02 ± 0.20	0.45 ± 0.02	

Table 4.6 mRNA expression levels of selected ribosomal probes in control and DLB cases in the angular gyrus as determined by TaqMan PCR assays using *GUS-β* for normalization. Significant upregulation was seen in RPL7, RPL23A and RPL31 where as the other subunits remained unaltered. Student's T-test *p< 0.05 , **p< 0.01, ***p< 0.001.

4.6.4 mRNA expression of ribosomal subunits in precuneus

4.6.4.1 : The mRNA expression levels in PD : The mRNA analysis of selected ribosomal subunits have shown significant downregulation of RPL5 ($p< 0.01$), RPL27 ($p < 0.05$), RPS16 ($p< 0.05$) and RPS17 ($p< 0.05$) (Table 4.7) where as the rest of the subunits remained unaltered.

Probes	Precuneus		
	Control	PD	P Value
RPL5	1.05 ± 0.1	0.62 ± 0.05	↓ **
RPL7	0.95 ± 0.08	0.67 ± 0.10	
RPL21	1.11 ± 0.12	0.73 ± 0.15	
RPL22	1.18 ± 0.21	0.77 ± 0.09	
RPL23A	1.58 ± 0.38	0.66 ± 0.17	
RPL26	1.58 ± 0.43	1.51 ± 0.41	
RPL27	1.06 ± 0.17	0.57 ± 0.11	↓ *
RPL30	1.24 ± 0.33	1.24 ± 0.34	
RPL31	1.10 ± 0.28	0.76 ± 0.16	
RPS3A	1.21 ± 0.26	0.98 ± 0.18	
RPS5	1.23 ± 0.21	0.95 ± 0.15	
RPS6	1.11 ± 0.14	0.88 ± 0.09	
RPS10	1.22 ± 0.19	0.95 ± 0.16	
RPS13	1.33 ± 0.28	2.05 ± 0.48	
RPS16	1.27 ± 0.23	0.71 ± 0.21	↓ *
RPS17	1.33 ± 0.32	0.43 ± 0.11	↓ *

Table 4.7 mRNA expression levels of selected ribosomal probes in control and PD cases in the precuneus as determined by TaqMan PCR assays using *GUS-β* for normalization. Significant downregualtion was seen in RPL5, RPL27, RPS16 and RPS17 where as the other subunits remained unaltered. Student's T-test *p< 0.05 , **p< 0.01, ***p< 0.001.

4.6.4.2 : The mRNA expression levels in DLB : The mRNA analysis of selected ribosomal subunits have not shown any significant differences of ribosomal subunits studied. (Table 4.8).

Probes	Precuneus		
	Control	DLB	P Value
RPL5	1.05 ± 0.1	1.20 ± 0.19	
RPL7	0.95 ± 0.08	1.24 ± 0.18	
RPL21	1.11 ± 0.12	2.68 ± 1.55	
RPL22	1.18 ± 0.21	1.56 ± 0.59	
RPL23A	1.58 ± 0.38	1.63 ± 0.02	
RPL26	1.58 ± 0.43	1.62 ± 0.51	
RPL27	1.06 ± 0.17	0.84 ± 0.24	
RPL30	1.24 ± 0.33	0.55 ± 0.14	
RPL31	1.10 ± 0.28	2.09 ± 0.76	
RPS3A	1.21 ± 0.26	0.59 ± 0.16	
RPS5	1.23 ± 0.21	0.88 ± 0.26	
RPS6	1.11 ± 0.14	0.95 ± 0.32	
RPS10	1.22 ± 0.19	0.75 ± 0.25	
RPS13	1.33 ± 0.28	1.40 ± 0.41	
RPS16	1.27 ± 0.23	1.33 ± 0.49	
RPS17	1.33 ± 0.32	0.16 ± 0.04	

Table 4.8 mRNA expression levels of selected ribosomal probes in control and DLB cases in the precuneus as determined by TaqMan PCR assays using *GUS-β* for normalization. Significant differences were not seen .

DISCUSSION

--- There is no subtitute for hardwork.

----- Thomas A. Edison-----

5. DISCUSSION

In this thesis, we have reported some remarkable findings, we managed to study the enzyme activities of individual complexes of mitochondria and the alterations in the protein synthesis in PD and DLB post-mortem samples. Different diseases preferentially affect different brain regions and so distinctive clinical patterns of dementia or other related neurodegenerative disorders. Based on the distribution of lewy bodies in different disorders, the present study was focused mainly on PD and DLB as the formation of lewy bodies defines the disease. PD and DLB are considered the paradigms of α-synucleinopathies within the spectrum of neurodegenerative diseases that exhibit α-synuclein in cytosolic protein aggregates (Spillantini et al., 1997, 1998; Baba et al., 1998; Hashimoto and Masliah 1999; Iwatsubo 2003). Even though the major objective of this thesis was to study alterations in PD and DLB, it was important to study initial stage of the disease iPD and extended pathology of PD i.e., PDD. iPD stages 1 or 2 is the situation where pathological lesions of PD are found during post-mortem examination of an individual who had no clinical symptoms. PD stages 3 or 4 is clinically characterized by rigidity, tremor and akinesia and pathologically characterized by loss of nigral neurons in SN. PD stages 5 and 6 include cases with involvement of the neocortex. Some of them, but not all of them can suffer from concomitant cognitive impairment and dementia (PD-D). DLB is clinically characterized by dementia and pathologically always characterized by deposits of cortical lewy bodies corresponding to stages 5 or 6 of PD. However, the temporal sequence distinguishes DLB and PDD. The term DLB is used when dementia develops before, or within one year after, parkinsonism onset. The term PDD is used when dementia appears more than one year after the onset of otherwise typical PD. However, the pathological marks of PD and DLB are lewy bodies which are formed by the deposition of alpha-synuclein.

The studies of toxic induced PD models, the functions of PD-related genes and the analysis of post-mortem brains of PD patients have demonstrated many interconnected pathways that lead to pathogenesis of the disease. However, it has been suggested that besides misfolding and aggregation of proteins , it has been demonstrated that mitochondrial dysfunction also play a role in pathogenesis of PD and DLB. Hence, as there has been a great interest in understanding the involvement of mitochondria and the alterations of proteins which lead to the pathogenesis of PD and DLB. Thus, our work has been focussed towards mitochondrial alterations and ribosomal proteins. The present work in this thesis, began with the results obtained from microarrays and gene set enrichment analysis (GSEA), which demonstrate down-regulation of genes in two pathways i.e., oxidative phosphorylation pathway and in ribosomal proteins.

Neurons depend mostly on mitochondria for their survival. Neurons have highest local demand for mitochondrial ATP production. Although mitochondria might be the key to neuronal integrity and survival, defects in disparate areas of mitochondrial signalling are likely to have deleterious effects that converge due to feedback and feedforward mechanisms. These could include the deregulation of the electron tansport chain, which can cause a dual effect of reduced ATP reduction and increased oxidative stress and is likely to lead to further mitochondrial dysfunction, also leading to pathological activation of pathways involved in aggregation, altered synthesis and apoptosis. Many studies have tried to delineate the mitochondrial pathways involved in PD and DLB to investigate the role of mitochondria in cell pathology. Recent findings have greatly expanded our understanding of the role of mitochondria in the pathogenesis of neurodegenerative diseases like PD and DLB. However, studies in detail regarding the functionality, expression of subunits and oxidative damage to mitochondrial subunits in different regions of brain affected by PD and DLB are yet unclear or less understood.

Besides, pioneering studies on human brain tissue have been supported by combined phosphorous and proton magnetic resonance spectroscopic imaging showing mitochondrial dysfunction in midbrain and putamen in PD (Hattingen et al., 2009). It is also reported that reduced energy metabolism, as revealed with 18 F-fluorodeoxyglucose positron emission tomography, is observed in the frontal cortex and then progresses to the angular gyrus, orbital cortex, posterior cingulus and occipital lobes in patients with mild cognitive impairment (Lyoo et al., 2010; Bohnen et al., 2011; Garcia-García et al., 2012). Further is supported by neuroimaging analyses which suggest that cortical hypometabolism is present even at early stages of PD (Borghammer et al., 2010; Berti et al., 2012). Interestingly, hypometabolism is followed by cortical atrophy in the same areas in PD cases with dementia (Gonzalez-Redondo et al., 2014). Therefore, the hypothetic alteration regarding mitochondrial function could affect the frontal cortex first, with the angular gyrus the least affected at the same time period. Hence the aim of this study was to validate this hypothesis.

A multidisciplinary approach combining molecular biology and biochemistry have been used in the experimental part of this work. To achieve our hypothesis we have divided the work with two objectives. Towards the first objective to study the mitochondrial alterations in PD stages 3-4 and DLB stages 5-6 in frontal cortex and angular gyrus. We first started our study to check the downregulated genes of oxidative phosphorylation pathway based on microarray studies by Real Time quantitative PCR (RT-qPCR). Later, we further continued to do protein quantification through western blots in total protein extraction and mitochondrial extraction by using different antibodies

which includes several mitochondrial complex subunits, cellular localization markers, transcriptional factors, oxidative stress markers and lipid stress markers. In this context, the study of western blots has been done first in total protein extracts to have a general view to what was happening to the above markers and complex subunits. For this, we picked only few markers which represent the specific function. To begin with, to know the levels of mitochondria in iPD stages 1-2 and PD stages 3-4 and DLB stages 5-6 we used VDAC which is a marker of mitochondrial levels. This step was important to know at what stages the mitochondrial levels were altered. Further, with the results obtained we continued to study few markers with specific functions in PD stages 3-4 and DLB stages 5-6. To know the alterations of complexes we used Oxphos antibody which is a cocktail of complex subunits. Besides, we used SOD2 and NRF1 which are oxidative stress markers, TFAM and EIF2-α as transcriptional markers. After obtaining this results from total protein extracts we decided to obtain pure mitochondrial extraction to continue further major experiments. So, the major part of the work done in this thesis for molecular biology and biochemical studies was by mitochondrial extraction. Further, we continued with semiquantitative technique immunofluorescence to complement the studies of western blots and double-labeling confocal microscopy to study the co-localization of protein deposition. Later, we studied enzyme activities of different complexes in frontal cortex and angular gyrus and most importantly enzyme activities also includes a study where same individuals with different regions i.e., frontal cortex and angular gyrus in PD stages 3-4 has been done. However, in angular gyrus, molecular biology studies such as protein quantification through western blots was limited only to few antibodies.

Towards the second objective, to study alteration of protein synthesis occurs in PD stages 3-4 and DLB stages 5-6 and is also region dependent, we tried to validate the downregualted pathway from microarray studies. The protein synthesis is highly important to generate new proteins via ribosomes. It is interesting to see that this ribosomal subunits, which are essential for protein synthesis are impaired in PD and DLB in a region specific-manner. However, the initial RNA studies from GSEA were limited to frontal cortex. In addition we studied, *Substantia nigra,* angular gyrus and precuneus. The important finding of this study is that doing this, it would be possible to compare regional differences in gene regulation in very specific regions differentially vulnerable to PD and DLB.

The results in this thesis are in striking agreement with the hypothesis that the alterations in mitochondria and alterations in protein synthesis occur in PD and DLB and is region dependent.

Detailed explanation of all the studies directed towards mitochondria and protein synthesis will be discussed in the following sections, and all the studies with PD and DLB in this thesis refer to PD stages 3-4 and DLB stages 5-6.

5.1 Mitochondrial dysfunction

5.1.1 Expression of VDAC in PD and DLB

VDAC is a higly conserved mitochondrial outer-membrane protein and serves as a marker of mitochondrial levels. It is traditionally known as a large conductance anion channel important in mitochondrial function, providing the main transmembrane transport of ions, ATP and other metabolites through the outer mitochondrial membrane (Baker et al., 2004). Data within this thesis, show that VDAC levels in total protein extracts of iPD frontal cortex were normal, where as in PD a trend to decrease was observed which clarifies that the alterations of levels of mitochondria is not an early event of the disease and suggest that levels of VDAC expression has been progressively decreased during the advancement of the disease in PD in frontal cortex (area 8). On the other hand in DLB a significant decrease was observed. This might suggest that the decrease in the amounts of VDAC's means a reduced mitochondria load which might contribute to the DLB neuronal dysfucntion. As a result, there might be a decrease in producing ATP and leading to energetic failure and thus, finally contribute to the altered mitochondrial disposal in DLB pathogenesis.

5.1.2 Alterations of mitochondrial subunits

Mitochondrial dysfunction, and specifically impaired activity of the ETC are primary mechanisms of cell death in PD and DLB. Consistent deficits in the subunits and activity of mitochondrial complex I of the electron transport chain in blood platelets and SNpc of PD patients is a prominent phenomenon (Keeney et al., 2006; Beal 2005). Data within this thesis show alterations of complex subunits, oxidative damage and the stress markers, which was observed in few subunits of total and mitochondrial isolation as detailed below. It is important to note that our data do not define exactly which complexes subunits are oxidized. That will require a detailed proteomic analysis of each subunit to identify oxidatively modified amino acids.

Frontal cortex -

We, for the first time, demonstrate a vast study of protein quantification of 18 subunits of different mitochondrial complex subunits which is important in knowing the widespread reduction of multiple members of complexes I-V in PD and DLB of frontal cortex, suggesting that the ETC system is deficient. Complex I is felt to be the major source of superoxide production in the electron transport chain (Kudin et al., 2004; Lambert and Brand, 2004). In the present study, complex I subunits that were immunocaptured have shown significant decrease. On the other hand, only a trend to decrease was observed in few subunits of PD and DLB total and mitochondrial extracts. Data within this thesis indicates no reduction of complex II subunits in either total extracts or mitochondrial isolation of PD and DLB, where as in complex III subunits studied, complex III (core II) subunit in PD have shown significant increase in mitochondrial extracts, but in DLB no significant differences were not observed. It has been demonstrated that defects of the mitochondrial respiratory chain complex II and complex III are extremely rare. Complex IV which is the last but one complex of ETC is responsible to establish a transmembrane difference of proton electrochemical potential that the ATP synthase then uses to synthesize ATP. The present study has shown a significant increase of complex IV (COX VIIC) subunit in mitochondrial extracts of mitochondrial extracts. Complex V is a molecular machine composed of two mechanical rotary motors (F_o and F_1), which interconvert the chemical energy of ATP hydrolysis and H^+ electrochemical potential via a mechanical rotational mechanism (Okuno et al., 2011; Watanabe et al., 2011; Jonckheere et al., 2012a). Altered expression was observed in few subunits of PD and DLB which indicates that neuronal energy metabolism might be modulated by shifting the mode of ATP production.

On the whole, the plausible explanation for the alterations such as increase or decrease, in the complex subunits studied can result in altered functionality of sub-complex assembly, which might contribute to disease progression.

Immunofluorescence and confocal microscopy

The immunofluorescence studies have been directed to complement the studies of the western blots. The subunits were selected based on the availability in immunostaining. Data within this thesis, obtained from the densitometric studies of immunofluorescence and confocal microscopy are in accordance with the western blots in PD and DLB.

The novelity of this study was that by using double-labeling immunofluorescence to VDAC + α-synuclein in DLB, we were able to demonstrate that mitochondria decrease relatively with the deposition of α-synuclein. In PD, it is difficult to find lewy bodies in frontal cortex at stages 3 or 4 and hence the study of double-labeling has not been done.

5.1.3 Damage to the mitochondrial cellular localization markers

Oxidative stress markers

Mitochondria are a major source of free radicals (Pamplona and Bajra 2007). Previous studies on post-mortem tissues have provided some evidence to support the occurrence of oxidative stress in PD. Eukaryotic systems have evolved defenses against damaging moieties, the chief member of which is superoxide dismutase (SOD2), an enzyme that efficiently converts superoxide to the less reactive hydrogen peroxide (H_2O_2), which can freely diffuse cross the mitochondrial membrane. Loss of SOD2 can result in numerous pathological phenotypes in metabolically active tissues; particularly within the central nervous system. In our study of total protein extracts in PD a significant decrease of SOD2 has been observed. This significant downregulation implies that defect in SOD2 may contribute to neurodegeneration in PD. However, where as in DLB only a trend to decrease was observed. Besides, other studies show that mice that lack mitochondrial superoxide dismutase (SOD2 null mice) represent a model of endogenous oxidative stress and develop a severe neurological phenotype (Hinerfeld et al., 2004). However, together, these observations point to impaired mechanism of defense against oxidative stress.

Transcriptional factors

NRF1 and NRF2 target genes include a wide range of genes coding for five mitochondrial respiratory complex subunits, heme biosynthetic enzymes, and regulatory factors involved in the replication and transcription of mtDNA (Scarpulla 2008). NRF1 and NRF2 regulate also the expression of genes involved in oxidative stress response (Motohashi et al., 2002). It has been reported that absence of NRF1 in knockout mice results in lethality late in gestation that is most likely due to abnormal fetal liver erythropoiesis and anemia (Chan et al., 1998). NRF1 is also required for the survival of hepatocytes, and a deficiency in NRF1 in hepatocytes leads to spontaneous development of steatohepatitis and hepatic neoplasia (Chen et al., 2003; Xu et al., 2005). Other studies demonstrate that conditional knockout of NRF1 in the brain leads to proteasome impairment and progressive degeneration in cortical neurons which provides an

evidence that NRF1 is an important transcriptional regulator of proteasome genes (Candy et al ., 2011). We observed that NRF1 was found to be significantly down regulated in PD total protein extracts which indicates as a potential contributor to the pathway for neuronal death, on the other hand data with mitochondrial extracts only a trend to decrease was observed. Apart from this, we observed that NRF2 expression was found to be normal. One of the plausible explanation for the normal levels of NRF2 is when confronted with oxidative stressors, cells must quickly augment their antioxidant capacity to counteract increased ROS production and maintain homeostasis. Under oxidative and electrophilic stresses, the NRF2 signaling pathway is activated to enhance the expression of a multitude of antioxidant and phase II enzymes that restore redox homeostasis. So, a possibility could be that it might not be responding properly to oxidative stress enough to control the oxidative stress in PD and DLB neurons.

Translation initiation factor

The EIF2-α play a key role in the regulation of protein synthesis. Cell signaling in response to an array of diverse stress stimuli converges on the phosphorylation of the alpha-subunit of EIF2. EIF2-alpha curtails general translation in response to a wide array of different cellular stresses while facilitating programs of stress-induced gene expression. This results in severe decline of de novo protein synthesis and also the accumulation of mis-folded proteins. We have observed significant decrease of phosphorylated EIF2- α in total protein extracts of DLB. A number of links between the EIF2-α and apoptosis have been suggested. EIF2-α is cleaved during apoptosis leading to functional changes including loss of activity in translation initiation (Marttila 1988). Though we have not observed any significant changes in mitochondrial extracts, however, collectively it might suggest that the translation process is not occurring so effectively to translate mRNA to proteins, or some proteins accumulate which might support the notion why few of the complex subunits at protein level altered in our study. Besides no differences were observed in PD.

Translocator proteins

TOMM 40 and TOMM 70 are the translocase outer mitochondrial membrane proteins, that forms part of a pore that serves as the import site for cytoplasmic proteins to enter the mitochondrion. Data in this thesis , doesnot show any alterations of TOMM40 in PD of mitochondrial extracts but in contrast we have found that there was a significant decrease in the levels of TOMM 40 in DLB

mitochondrial extracts. (Bender et al., 2013) investigated α-synuclein induced mitochondrial dysfunction and reported that TOMM 40 levels vary reciprocally with α-synuclein levels in brain tissue from human subjects with PD as well as brain tissue from transgenic α-synuclein over expressing mice. So, the plausible explanation for the significant decreased levels of TOMM40 may be due to the α-synculein deposition. Besides this evidence, it is demonstrated that a portion of cellular α-synuclein localizes to mitochondria (Yang et al., 2007) and is imported via the TOMM complex (Devi et al., 2008; McFarland et al., 2008), where it inhibits complex I (Mc Farland., 2008) and possibly other members of the oxidative phosphorylation chain (Devi et al., 2008) and elicits autophagy (Choubey et al., 2011).

On the other hand we have observed that TOMM70 has been significantly increased in PD mitochondrial extracts. It has been shown that, the autosomal recessive gene forms which lead to PD pathology involves interactions with the TOMM complex. *PINK1*, is recruited into mitochondria, enters via the TOMM40 channel, and is degraded in a membrane potential-dependent manner (Matsuda et al., 2010; Jin et al., 2010). If the inner membrane potential collapses resulting in membrane depolarization, *PINK1* associates with TOM via TOMM22 in a high molecular weight complex134. In this form it recruits and activates *PARK2*, leading to the destruction of outer membrane proteins and eventual mitophagy (Matsuda et al., 2010; Derek et al., 2010). Initially *PARK2* is recruited to TOMM70 (or TOMM70A) or a near-by site (Bertolin et al., 2013); at later times it is associated with TOMM22 and subsequently with other outer membrane proteins (Saraff et al., 2013). Pathogenic mutations in either protein disruption (Bertolin et al., 2013; Derek Narendra et al., 2010) result in the accumulation of dysfunctional mitochondria and possible explanation is that, changes in the activity or abundance of any of the other members of the TOMM complex could contribute to disease etiology.

Mitobiogenesis

A decreased mitochondrial biogenesis has been reported in many neurodegenerative diseases (Austin and St-Pierre 2012). The mitochondrial transcription factor A (TFAM) is one of the central regulatory components of mitochondrial nucleoids (Spelbrink, 2010). In particular, it has been documented that the problem in PD frontal cortex does not lie with mitobiogenesis signaling which appears to be preserved (Ravinder et al., 2012). In agreement to this, data in this thesis have shown that TFAM has not been altered in PD total extracts but in DLB total extracts a significant decrease

has been observed. This reduction might suggest that there is the impartment of mitochondrial biogenesis which is likely contributing to mitochondria dysfunction in DLB.

Mitochondrial ribosomal protein subunit damage

The traditional roles of the small and large ribosomal subunits include binding and decoding mRNAs as well as catalysis of the peptide bond formation (Nissen et al., 2000; Carter et al., 2001; Yusupov et al., 2001; Yusupova et al., 2001). Mitochondria-specific ribosomal proteins have key roles in disease, however, their functions within mitochondria are not known. In mitochondria, however, some of the ribosomal proteins have acquired additional functions, linking this organelle to apoptosis, cancer and disease states, as well as the aging process and virus integration (Ogawa et al., 2003; Kashuba et al., 2008). For example, the small subunit proteins DAP3 (MRPS29) and MRPS30, and the large subunit proteins MRPL37 and MRPL41 are apoptotic proteins (Kissil et al., 1995; Koc et al., 2001c; Levshenkova et al., 2004; Chintharlapalli et al., 2005). With this support we tried to see if there was any alteration of mitoribosomes and we observed significant increase only in MRPS10 of DLB mitochondrial extracts. The significant increase of this protein might suggest that it should be compensating mechanism in response to balancing the large subunits over small subunits for the effective protein synthesis. Besides no differences were found in PD. The novelity of this study is that we for the first time demonstrate a study related to mitoribosomes in PD and DLB.

Lipid oxidation damage

The density of the neural proteins is altered (increased or decreased) following lenghty PMI due to oxidation and/or nitration. Several antibodies are used to detect oxidation and nitration of lipids, proteins and carbohydrates. Common lipidoxidation markers include MDAL, HNE, NKT and nitration marker N-TYR. Oxidative/nitrosative stress is well documented in *Substantia nigra,* as evidenced by increased protein carbonyls (Alam et al., 1997; Floor et al., 1998, Dalfo et al., 2005). Despite the previous observations focused on *Substantia nigra,* little is known about the lipidoxidation in mitochondria in cerebral cortex in PD and DLB. Data within this thesis, have intended to increase the understanding of lipidoxidative damage in cerebral cortex of PD and DLB in mitochondrial extracts.

HNE is a highly reactive lipophilic alpha, beta-alkenal, which can form stable adducts with thiol or amine groups on proteins. HNE can also activate members of the caspase family and cause DNA fragmentation, leading to apoptosis (Liu et al., 2000). HNE adducts are found to be increased in nigral neurons of PD (Yoritaka et al., 1996). Modification of proteins by 4-HNE impairs the function of neuronal glucose transporter GLUT-3 (Mark et al., 1997) and the astrocytic glutamate transporter GLT-1 (Blanc et al., 1998) and causes disruption of neuronal microtubules (Neely et al., 1998). Dalfo et al., (2005) have shown that additional lipid factors can also play a role in the lipidoxation in PD and DLB. Another relative lipid aldehyde studied was MDAL. MDAL made up of PUFA residues are extremely sensitive to oxidation. These aldehydes which are derived from lipid peroxidation have been suggested to play a key role in the pathogenesis of neurodegenerative processes (Knight 1997; Marksberry 1997; Simonian and Coyle 1996; Perry et al., 2000). Usually, when lipid proteins are altered they show a significant increase in response to lipidoxidative stress. Data within this thesis have shown no difference in the expression levels of HNE in frontal cortex of PD and DLB. On the other hand, MDAL levels either have not shown any difference in PD. However, on contrary in DLB the levels of MDAL were found to have a trend to increase but not significant, which suggests that, the high propensity of PUFA's for peroxidation under oxidative stress conditions can increase and result in neuronal damage which may contribute to DLB progression. Apart from this, the results within this thesis have not shown any significant differences in NKT's levels both in PD and DLB. Free 3-nitrotyrosine is formed *in vivo* by the reaction of tyrosine with nitrating oxidants and potentially by the degradation of nitrated proteins by the proteasome or other proteolytic enzymes (Souza et al., 2000). Until recently, the detection of free 3-nitrotyrosine under physiological or pathological conditions has been considered as a marker of oxidative and nitrative stress (Ohshima et al., 1990; Greenacre and Ischiropoulos, 2001; Turko and Murad, 2002). The data in this thesis have shown significant increase in PD, where as in DLB no changes were observed. Collectively, the data suggest that in mitochondria there might be less lipid peroxidation stress or might only appear during the advancement of the disease.

5.1.4 Consequences of mosaic respiratory chain deficiency in the brain

Impaired function of oxidative phosphorylation (OXPHOS) may cause disturbances of energy metabolism. Impaired energy metabolism results in decreased respiratory control ratio as well as ATP levels (Rhein et al., 2009). Mitochondrial dysfunction is also a characteristic feature of encephalopathies caused by pathogenic mtDNA mutations. RC-deficient neurons are found in the SNpc and other brain regions in aged humans. Several studies have shown mitochondrial

dysfunction and reduced activity of mitochondrial complex I in SN (Schapira et al., 1990a; Schapira et al., 1990b; Schapira 2008) and in frontal cortex (Parker et al., 2008; Navarro et al., 2009; Rajeshwara Babu et al., 2011). A loss of activity of complex I of the mitochondrial electron transport chain has been observed in many different PD tissues including platelets, brain, muscle and lymphocytes (Benecke et al., 1993; Haas et al., 1995; Mann et al., 1992., Shoffner et al., 1991; Yoshino et al., 1992). Immunoblot studies of complex I suggested abnormalities in PD striatum (Mizuno et al., 1989). Impaired pyruvate oxidation, consistent with an electron transport chain defect is present in PD fibroblasts (Mytilineau et al., 1994). A loss of complex I activity in cerebral cortex has been documented (Navarro et al., 2009) suggesting a systemic ETC impairment. However, the present findings demonstrate reduced enzymatic activity of mitochondrial complex I in the frontal cortex of PD. Thus, showing that mitochondrial dysfunction occurs at relatively early stages of PD at a time in which no apparent clinical cortical dysfunction, including cognitive impairment, is detected in these patients. A plausible explanation is also that impairment of its internal electron transfer function, results in the impairment of the function of the catalytic core initiates internal ROS production leading oxidative damage and more impairment of catalytic core, in a feed forward paradigm. Probably, this could be the reason that we see some of the subunits of complex I in western blots have been altered. However, the complex I defect in PD appears to be systemic, affecting tissues outside the brain.

Complex I activity has been described as being selectively reduced in the frontal cortex in PD, whereas activities of complexes II, III and IV are comparable in PD, DLB and controls (Keeney et al., 2006; Parker et al., 2008; Navarro et al., 2009; Navarro and Boveris, 2009). Inspite of all the above reports, the present findings show additional dysfunction of complexes II and V in PD and complexes III, IV and V in DLB. Methodological differences may account for these discrepancies (Parker et al., 2008). Complex II defects in the heart and skeletal muscle (Melov et al.,1999) have been previously detected. Other studies in mice models suggest that complex II is one of the most sensitive enzymes to the loss of SOD2 activity (Hinerfeld et al., 2004). Our studies also report the impairment of complex II enzyme activity in PD. This impairment might be due to the SOD2 levels where we observed significant down regulation in our studies. Our studies report the significant decrease of complex V activity in PD and DLB. This decrease of complex V activity explains that there is a decrease in the supply of ATP to the cells which finally may be a contributing factor for neuronal death. But the complexes III and IV activities remained insensitive in PD, and whereas in DLB complexes III and IV were altered which implies that not all enzymes are sensitive to impairment in the mitochondria. Besides it was reported that, reduced mitochondrial activity in the

frontal cortex area 8 has been reported at advanced stages of PD (Keeney et al., 2006; Parker et al., 2008; Navarro et al., 2009; Navarro and Boveris, 2009). Studies related to complex IV activity in PD are unclear. However in AD brains reduced COX (complex IV) activity has been seen (Mutisya et al., 1994; Maurer et al., 2000; Parker et al., 1990,1994).

Besides, citrate synthase activity a mitochondrial matrix enzyme was measured and, used as a marker of the abundance of mitochondria within a tissue/cell. The enzyme rates of citrate synthase were measured to provide an indication of the integrity of the mitochondrial preparation. Therefore each complex activity is expressed as both as a rate and as ratio of the rate of citrate synthase.

Data within this thesis have shown that the normalization of the activities of the complexes to citrate synthase in PD have shown significant decrease. This might explain that even though the levels of mitochondria are normal on the whole the complexes are significantly impaired. But, on the contrary in DLB the activities of the complexes have shown no impairment. This might suggest that though there are few mitochondria in DLB they appear to be functional. But, however, with the impairment of complexes, globally there is a energy metabolic failure which might lead to the overproduction of ROS, and might lead to apoptosis and finally cell death.

Angular gyrus –

The angular gyrus is affected at later stages of the disease and its involvement has important implications in the development of dementia in PD (García-García et al., 2012; Gonzalez-Redondo et al., 2014). Interestingly, no alterations in the enzyme activities of complexes I, II, III, IV and V were observed in the angular gyrus in PD and PD-D but up-regulation in the expression of 2 of nine mRNAs which may transcribe several subunits of the distinct respiratory complexes was seen. The protein quantification using western blots also has not shown any differences. Taken together, these observations support the concept that adaptive responses in the mitochondrial machinery occur in the angular gyrus preventing damage until certain thresholds are surpassed at more advanced stages of the disease.

Apart from the above study, we have made a comparative study of enzyme activities of the regions (frontal and angular) cases obtained from the same individual. This study is helpful to analyze what could be happening in the different regions of the brain of same individual who is affected with the PD. This study was only made to have an understanding of impairment of activities and were not

normalized to citrate synthase activities. Data within this thesis have shown that there was a significant decrease in complexes I, II and V activities of frontal cortex (area 8), whereas on the other hand the angular gyrus have not shown any significant impairment in the complexes. The comparative study in DLB wasn't made due to the unavailability of the cases.

The novelity of our study is that we for the first time demonstrate a comparative study of two regions obtained from same individual in PD and as a whole we have studied the possible alterations of individual complex activities in PD and DLB, in different brain regions.

5.1.5 mRNA expression of mitochondrial genes

Curiously, all the above alterations observed in frontal cortex at protein levels and enzyme impairment are not associated with differences in the expression of selected genes encoding mitochondrial proteins at these stages of the disease although protein levels of certain subunits are altered in PD. These findings do not contradict the observation of marked impairment of mitochondrial biogenesis, altered mRNA expression, dysregulation of several microRNAs predicted to interact with complex I regulators and alterations of protein levels, on the contrary they are altered in the advanced stages of the disease in DLB and PDD in frontal cortex and angular gyrus respectively. This reduction of mRNA expression at advanced stages of PD is supported by Thomas et al., 2012. However, the data within in this thesis is consistent with observations indicating the reduced protein subunits, rather than reduced protein expression levels, can jeopardize enzymatic activity of the corresponding mitochondrial complexes (Keeney et al., 2006; Terni et al., 2010).

5.2 Altered expression of ribosomal protein subunits

Ribosomes are cytoplasmic structures measuring 25-30nm composed of 65% RNA's and 35% ribosomal proteins that form a smaller subunit (40S) which binds to mRNA and a larger subunit (60S) which binds to tRNA's and aminoacids. In eukaryotes, the smaller subunit is made of an 18S RNA and 33 proteins whereas the larger subunit is formed by a 5S RNA, a 28S RNA and 46 ribosomal proteins (Doudna and Rath 2002; Granneman and Baserga 2004; Ben-Shem et al., 2011; Rabl et al., 2011; Klinge et al., 2011).

Ribosomal biogenesis is very complex as it involves, in addition to rRNAs, 79 ribosomal proteins, more than 200 non-ribosomal proteins and 75 small nucleolar RNA's (Freed et al., 2010; Kressler et al., 2010; Hetman and Pietzrak 2012). Although the exact function and roles of all ribosomal

proteins in eukaryotes is not known, most of them are involved in structure assembly throughout a variety of protein-protein and protein-RNA interactions with high capacity of remodeling from the inactive status to the process of subunit assembly during protein synthesis (Klein et al., 2004; Henras et al., 2008; Korobeinikova et al., 2012). Ribosomal proteins also participate in protein synthesis initiation and elongation, and they can regulate their own synthesis at the translational level (Stezl et al., 2001; Gluck and Wool, 2002; Wilson et al., 2002; Connell et al., 2002; Dresios et al., 2006; Yu et al., 2009).

Langstrom et al., (1989) reported that polysomes purified from the AD frontal cortices showed 50% reduction of the translational activity per unit polysome than that of control and there are studies demonstrating increased oxidative damage to RNA occurring in late stage AD (Nunomura et al., 2004; Honda et al., 2005). Reduction of protein synthesis was demonstrated in AD (Mann et al., 1991; Sajdel-Sulkowska et al., 1984; Ding et al., 2005). However, no studies have reported the impairment of protein synthesis in PD and DLB. Hence, the objective was to evaluate alterations of ribosomal protein subunits by the evidences of the arrays, that may impair protein synthesis. Thus, this ribosomal dysfunction may contribute to impaired neuronal function and the development of neuropathology in PD and DLB. The present study includes 9 large subunits and 7 small subunits based on some genome enrichment studies described elsewhere (Garcia et al., 2013). Data within this thesis have shown significant decrease in gene expression of 6 small subunits of 7 genes whereas large subunits have shown no alterations of examined ribosomal protein mRNA's in the SN in PD and DLB. Altered ribosomal protein gene expression also occurs in cerebral cortex in PD and DLB but with stage and region peculiarities: 4 of 9 large subunit genes were significantly decreased and 5 of 7 small subunit genes in PD , on the other hand 4 of 9 large subunit genes and 5 of 7 small subunit genes were up-regulated in DLB. This impairment of protein synthesis alternatively, cause the alterations in tRNA that may promote the generation of translational errors or cause an early termination of translation (Han et al., 2003). In either case, potentially deleterious protein species could be generated and contribute to both protein aggregation and neurotoxicity (Wang et al., 1994).

On the other hand 1 of 9 large subunit genes and 1 of 7 small subunit genes in PD where as 3 of 9 large subunit genes in DLB have shown significant up-regulation in angular gyrus. In precuneus 2 of 9 large subunit genes and 2 of 7 small subunit genes have shown significant decrease in PD and where as in DLB no modifications in the expression of genes was seen. This finding suggests that there is no regulatory cross-talk monitoring the levels of the large and small subunit proteins,

instead there seems to be a compensatory mechanism that increases the level of large ribosomal proteins in response to loss of small ribosomal proteins. Therefore, the present findings show marked stage and region dependent differences in ribosomal protein gene expression. Down-regulation in the *Substantia nigra* is consistent with the higher vulnerability of this nucleus when compared to the other analyzed brain regions and gives support to the hypothesis that ribosomal structure and function is severely impaired in the SN in PD. Altered mRNA expression of several ribosomal proteins in the cerebral cortex appears to be a more plastic process depending on the cortical region and stage of the disease. Decreased expression of a few mRNA's of PD followed by up-regulation of a few different ribosomal proteins mRNA's of DLB in frontal cortex and angular gyrus suggests modifications in the structure and functional capacities of cortical ribosomes with disease progression.

6. CONCLUSIONS

Mitochondrial alterations in PD and DLB

6.1 The decrease in the amount of mitochondria is not an early event in the frontal cortex area 8 in PD, as the amount of VDAC levels in iPD (stages 1 or 2) did not differ from control cases, whereas in PD (stages 3 or 4) have shown a trend to decrease whereas in DLB, VDAC levels were significantly decreased.

6.2 Impairment in the enzyme activities of complexes I, II and V is found in the frontal cortex area 8 in PD (stages 3 or 4), and of complexes I, IV and V in the frontal cortex in DLB. This was accompanied by alterations of certain protein subunits i.e., complex III core II, N-TYR, TOMM70 were up-regulated whereas complex V subunits (ATP5J, ATP5D), SOD2 were down-regulated of the respiratory chain in the frontal cortex in PD (stages 3 or 4).

6.3 In DLB alterations of protein subunits complex I NDUFA10 and complex V ATP5J were down-regulated, complex IV (COXVIIC), MRPS10 were up-regulated and mRNA expression of certain subunits NDUFA7, NDUFA10, NDUFB10, NDUFS8, COX7A2L, ATPG2, PTP1P51 were up-regulated, ATP5H was down-regulated.

6.4 Double–labeling immunofluorescence to VDAC and alpha-synuclein shows that the expression of VDAC in the frontal cortex of DLB correlates with the deposition of alpha-synuclein.

6.5 The angular gyrus did not show alterations in the enzyme activities of complex I, II, II, IV and V in PD (stages 3-6). However, we found up-regulation in the expression of 2 of 9 mRNAs which transcribe specific subunits of mitochondrial respiratory complexes in PD stages 3 and 4 and in PDD stages 5 and 6.

These results show mitochondrial alterations in a region specific manner in PD and DLB.

Alterations of protein synthesis in PD and DLB

6.6 mRNAs of several ribosomal proteins are down-regulated in the substantia nigra, frontal cortex, angular gyrus and precuneus in PD (stages 3 or 4) and DLB.

6.7 In contrast, mRNAs of different ribosomal proteins are up-regulated in frontal cortex and angular gyrus in DLB.

The present findings suggest disease-dependent differences in ribosomal protein gene expression.

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