Molecular mechanisms of mitochondrial dysfunction in neurodegenerative diseases

Nelson Uchechukwu Osuagwu

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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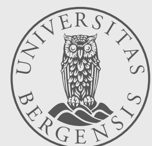
UNIVERSITY OF BERGEN



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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

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I thank God for my life, health, courage and wisdom through this adventure.

Scientific environment

The work presented in this thesis was initiated in February 2018 and was conducted in the Neuromics Research Group, the K.G. Jebsen Center for Translational Research in Parkinson's Disease and Neuro-SysMed Center of Excellence for Clinical Research in Neurological Diseases, at the University of Bergen and Haukeland University Hospital, Norway. The PhD fellowship was funded by grants from the University of Bergen, Bergen, Norway.

The main supervisor was Professor Charalampos Tzoulis MD PhD, and the co-supervisor was Christian Dölle PhD.

List of publications

Paper I

Poly-ADP-ribose assisted protein localization resolves that DJ-1, but not LRRK2 or α-synuclein, is localized to the mitochondrial matrix

Authors: Nelson Osuagwu, Christian Dölle, Charalampos Tzoulis

Publication: PLOS One https://doi.org/10.1371/journal.pone.0219909

Paper II

Long-term mitochondrial ribosomal inhibition induces alpha-synuclein aggregation and modulates Parkinson's disease-associated pathways

Authors: Nelson Osuagwu, Christian Dölle, Charalampos Tzoulis

Publication: manuscript

Paper III

Tau pathology is associated with higher levels of mitochondrial respiratory complexes I and IV

Authors: Nelson Osuagwu, Irene Flønes, Christian Dölle, Charalampos Tzoulis

Publication: manuscript

List of abbreviations

Aβ	amyloid beta
AD	Alzheimer’s disease
ADP	adenosine diphosphate
ALP	autophagy-lysosome pathway
ALS	amyotrophic lateral sclerosis
ANK	ankyrin domain
APOE	apolipoprotein E
APP	amyloid beta precursor protein
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCA	bicinchoninic acid
CI	complex I of the MRC
CII	complex II of the MRC
CIII	complex III of the MRC
CIV	complex IV of the MRC
CV	complex V of the MRC
CA1	cornu ammonis 1 region of the hippocampus
CBD	corticobasal dementia
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CHP	chloramphenicol
CHX	cycloheximide
CJD	Creutzfeld-Jakob disease
COX	cytochrome c oxidase
DA	dopamine
DAPI	4′,6-diamidino-2-phenylindole
DJ-1	protein deglycase DJ-1
DLB	dementia with Lewy bodies
DNA	deoxyribonucleic acid

DTT	dithiothreitol
ECL	enhanced chemiluminescence
$FADH_2$	reduced flavin adenine dinucleotide
FBS	fetal bovine serum
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FFPE	formalin-fixed paraffin-embedded
FTD	frontotemporal dementia
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
IMM	inner mitochondrial membrane
LRRK2	leucine-rich repeat kinase 2
LBs	Lewy bodies
LNs	Lewy neurites
LRR	leucine-rich repeats
MAPT	microtubule-associated protein tau
MM	mitochondrial matrix
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRC	mitochondrial respiratory chain
MSA	multiple system atrophy
mtDNA	mitochondrial DNA
MTS	mitochondrial targeting sequence
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
nDNA	nuclear DNA
NFT	neurofibrillary tangles
NSAID	non-steroidal anti-inflammatory drugs
OCR	oxygen consumption rate
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis

PAR	poly-ADP-ribose
PARAPLAY	poly-ADP-ribose assisted protein localization assay
PARP1cd	catalytic domain of PARP1 enzyme
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson’s disease
PDH	pyruvate dehydrogenase
PFFs	preformed fibrils
P_i	inorganic phosphate
PiD	Pick’s disease
PINK1	PTEN-induced kinase 1
PRKN	Parkin
PRNP	prion protein
PSP	progressive supranuclear palsy
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RPMI1640	Roswell Park Memorial Institute (RPMI) 1640 medium
ROS	reactive oxygen species
RT	room temperature
SDS	sodium dodecyl sulfate
SNCA	alpha-synuclein gene
SN/SNpc	substantia nigra pars compacta
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.1 % TritonX-100
TDP-43	transactive response DNA-binding protein 43
UBL	ubiquitin-like domain
UPS	ubiquitin-proteasome system
VDAC1	voltage-dependent anion channel 1
WT	wild type

ABSTRACT

Background

Neurodegenerative disorders are complex disorders of the central nervous system that are typically characterized by the accumulation of misfolded proteins and progressive degeneration, and death of vulnerable cell populations in the affected brain regions**.**

Mitochondrial dysfunction has been linked to the pathogenesis of several neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease, and is a potential target for novel therapeutic approaches. However, the mechanisms underlying mitochondrial dysfunction and its association with the observed pathological processes in neurodegeneration remain unknown.

Aims

Paper I: To elucidate the subcellular and putative mitochondrial matrix localization of selected Parkinson's disease-related proteins.

Paper II: To establish an *in vitro* cell culture model of chronic, sublethal inhibition of mitochondrial protein translation, in order to analyze the effect of mitochondrial dysfunction on α-synuclein aggregation and other cellular mechanisms implicated in the progression of Parkinson's disease.

Paper III: To elucidate the relationship between the mitochondrial respiratory complexes I and IV and tau aggregation in Alzheimer's disease.

Methods

Paper I: immortalized human cell cultures (HeLa S3 and SH-SY5Y), recombinant DNA transfection, conventional immunocytochemistry and PARAPLAY, fluorescence microscopy.

Paper II: immortalized human cell cultures (SH-SY5Y, transgenic SH-SY5Y cells stably expressing GFP-tagged α-synuclein or GFP alone), SDS-PAGE followed by immunoblotting, transfection of α-synuclein preformed fibrils, *in vitro* toxicity and cell viability assay, Seahorse mitostress assay.

Paper III: quadruple fluorescence immunohistochemistry and microscopy.

Results

Paper I: The Parkinson's disease-related proteins DJ-1, LRRK2 and α-synuclein were analyzed. DJ-1 was detected in the mitochondrial matrix, in addition to the cytosol, under normal and stress conditions. Neither LRRK2 nor α-synuclein (wild-type or Parkinson's disease-related variants) were found in the mitochondrial matrix, under normal or stress conditions.

Paper II: Using chloramphenicol treatment, a chronic, sublethal mitochondrial translation inhibition model was established. Mitochondrial respiratory complex subunit protein levels, mitochondrial respiration, protein degradation pathways and NAD^+-dependent acetylation pathways were severely affected. Chronic chloramphenicol exposure also induced high molecular weight species of α-synuclein protein.

Paper III: There is an association between tau pathology and higher levels of mitochondrial respiratory complex I and complex IV in the CA1 hippocampal region and the entorhinal/trans-entorhinal cortex, in brain samples from individuals with Alzheimer's disease or neurological healthy controls.

Conclusions

Paper I: The sub-organellar localization of Parkinson's disease-related proteins, especially in the mitochondrial matrix, is a challenge and robust methods are needed to precisely localize these proteins. With the PARAPLAY method, the absence of α-synuclein from the mitochondrial matrix was established, suggesting that its previously reported interactions with mitochondrial respiratory chain proteins would take place in the intermembrane space.

Paper II: Using mitochondrial ribosomal inhibition, a model for chronic, sublethal mitochondrial dysfunction was established, which can be applied to investigate the impact of mitochondrial respiratory deficiency on diverse aspects of the disease process in Parkinson's disease, especially abnormal accumulation of α-synuclein, making it a potential *in vitro* disease model.

Paper III: The presence of neuronal tau pathology is associated with higher levels of respiratory complexes I and IV in affected neurons, reflecting a compensatory

upregulation of mitochondrial respiration and/or mitochondrial biogenesis, in response to tau-mediated toxicity.

ABSTRAKT

Bakgrunn

Nevrodegenerative lidelser er komplekse lidelser i sentralnervesystemet som er typisk preget av akkumulering av feilfoldede proteiner og progressiv degenerasjon og død av sårbare cellepopulasjoner i de berørte hjerneområdene.

Mitokondriell dysfunksjon har vært knyttet til patogenesen av flere nevrodegenerative lidelser, inkludert Parkinsons sykdom og Alzheimers sykdom, og er et potensielt mål for nye terapeutiske tilnærminger. Imidlertid forblir mekanismene som ligger til grunn for mitokondriell dysfunksjon og dens assosiasjon med de observerte patologiske prosessene i nevrodegenerasjon ukjente.

Mål

Artikel I: Å belyse den subcellulære og antatte mitokondrielle matriselokaliseringen av utvalgte Parkinsons sykdom-relaterte proteiner.

Artikel II: Å etablere en *in vitro* cellekulturmodell for kronisk, subletal hemming av mitokondriell proteintranslasjon, for å analysere effekten av mitokondriell dysfunksjon på α-synuklein aggregering og andre cellulære mekanismer som er involvert i progresjonen av Parkinsons sykdom.

Artikel III: Å belyse forholdet mellom mitokondrielle respiratoriske komplekser I og IV og tau-aggregering ved Alzheimers sykdom.

Metoder

Artikel I: cellekultur (HeLa S3 og SH-SY5Y), rekombinant DNA-transfeksjon, konvensjonell immuncytokjemi og PARAPLAY, fluorescensmikroskopi.

Artikel II: cellekultur (SH-SY5Y, transgene SH-SY5Y-celler som uttrykker GFP-merket α-synuklein eller GFP alene), SDS-PAGE etterfulgt av immunoblotting, transfeksjon av α-synuklein forhåndsdannede fibriller, *in vitro* toksisitet og celle levedyktighetsanalyse, Seahorse mitostress-analyse.

Artikel III: firedobbel fluorescens immunhistokjemi og mikroskopi.

Resultater

Artikel I: De Parkinsons sykdomsrelaterte proteinene DJ-1, LRRK2 og α-synuklein ble analysert. DJ-1 ble påvist i mitokondriematrisen, i tillegg til cytosolen, under normale forhold og stressforhold. Verken LRRK2 eller α-synuklein (villtype eller Parkinsons sykdom-relaterte varianter) ble funnet i mitokondriematrisen, under normale eller stressforhold.

Artikel II: Ved å bruke kloramfenikol behandling ble det etablert en kronisk, subletal mitokondriell translasjonshemmingsmodell. Mitokondrielle respiratoriske komplekse subenhetsproteinnivåer, mitokondriell respirasjon, proteinnedbrytningsveier og NAD^+-avhengige acetyleringsveier ble alvorlig påvirket. Kronisk eksponering for kloramfenikol induserte også arter av α-synukleinprotein med høy molekylvekt.

Artikel III: Det finnes en assosiasjon mellom tau-patologi og høyere nivåer av mitokondrielt respiratorisk kompleks I og kompleks IV i CA1 hippocampus-regionen og entorhinal/trans-entorhinal cortex, i hjerneprøver fra individer med Alzheimers sykdom eller nevrologiske friske kontroller.

Konklusjoner

Artikel I: Den suborganelle lokaliseringen av Parkinsons sykdom-relaterte proteiner, spesielt i mitokondriematrisen, er en utfordring og robuste metoder er nødvendige for å nøyaktig lokalisere disse proteinene. Med PARAPLAY-metoden ble fraværet av α-synuklein fra den mitokondriellen matriksen etablert, noe som tyder på at dets tidligere rapporterte interaksjoner med mitokondrielle respiratoriske kjedeproteiner ville finne sted i intermembranrommet.

Artikel II: Ved å bruke mitokondriell ribosomal hemming ble det etablert en modell for kronisk, subletal mitokondriell dysfunksjon, som kan brukes til å undersøke virkningen av mitokondriell respirasjonsmangel på ulike aspekter av sykdomsprosessen ved Parkinsons sykdom, spesielt unormal akkumulering av α-synuklein, noe som gjør det til en potensiell *in vitro* sykdomsmodell.

Artikel III: Nevronal tau-patologi er assosiert med høyere nivåer av respiratoriske komplekser I og IV i berørte nevroner, noe som reflekterer en kompenserende

oppregulering av mitokondriell respirasjon og/eller mitokondriell biogenese, som respons på tau-mediert toksisitet.

1. INTRODUCTION

1.1. Neurodegenerative diseases

Neurodegenerative diseases are a group of disorders that are characterized by progressive loss of vulnerable neurons, resulting in a decline of motor and/or a broad spectrum of non-motor functions, including but not limited to cognition, memory, autonomic control, sleep, and neuropsychiatric function. The two most prevalent forms of these disorders are Alzheimer's disease (AD) and Parkinson's disease (PD) (1). The etiology of these diseases remains unknown and, therefore, there is currently no treatment available. The most common risk factor for several of these neurodegenerative diseases is age; therefore, understanding the contribution of ageing to the disease process seems to be crucial to develop an effective treatment (2). Another major feature of neurodegenerative diseases is the involvement of mitochondrial dysfunction. For instance, in PD and AD, mitochondrial DNA defects, mitochondrial respiratory chain impairment, and loss of mitochondrial quality control have been reported (3-7).

Neurodegenerative diseases are usually classified according to their primary clinical phenotype (e.g., movement disorders, cognitive decline, or behavioral disorders), affected anatomical brain regions and pathological phenotypes (8). A histopathological hallmark that is shared among many neurodegenerative diseases and that is used for neuropathological diagnosis is the presence of protein aggregates in brain cells. The most common proteins associated with neurodegenerative diseases include α-synuclein, encoded by the *SNCA* gene, microtubule-associated tau, encoded by the *MAPT* gene, amyloid precursor protein, encoded by the *APP* gene, the transactive response DNA-binding protein 43 (TDP-43), encoded by the *TARDBP* gene and prion protein PrP, encoded by *PRNP* gene (9).

Based on the proteins involved in the disease and their aggregation, neurodegenerative proteinopathies are classified as α-synucleinopathies, tauopathies, TDP-43 proteinopathies, prion disease and amyloidosis (Table 1).

Table 1. Overview of common neurodegenerative diseases. Adapted from (8, 10).

Disease group	Diseases	Aggregated protein	Neuropathology
α-synucleinopathy	PD Dementia with Lewy body (DLB) Multiple system atrophy (MSA)	α-synuclein	Loss of dopaminergic neurons Accumulation of α-synuclein rich Lewy body and Lewy neurites MSA: glial cytoplasmic inclusions
β-amyloidopathy	AD	β-amyloid tau	Presence of β-amyloid plaques and neurofibrillary tangles (NFT) Loss of neurons Neuroinflammation
Tauopathy	Frontotemporal dementia (FTD) NFT dementia Progressive supranuclear palsy (PSP) Pick's disease (PiD) Corticobasal dementia (CBD)	tau	Presence of β-amyloid plaques and neurofibrillary tangles (NFT) Loss of neurons Neuroinflammation PiD: Pick bodies and ballooned neurons
TDP-43 proteinopathy	Frontotemporal lobar degeneration-TDP Amyotrophic lateral sclerosis (ALS)	TDP-43	Neuronal loss Accumulation of TDP-43 aggregates
Prionopathy	Creutzfeldt-Jacob disease (CJD)	PrP	Neuronal loss Accumulation of misfolded prion protein aggregates

Disease-related pathological protein seeds have been shown to be critical for the initiation of intracellular aggregates in several neurodegenerative diseases, process referred to as cell-to-cell transmission or prion-like spreading (11). Recent therapeutic

approaches for treating neurodegenerative diseases involve the inhibition of synthesis of disease-associated proteins, in an effort to reduce their pathological seeding and increase their intracellular clearance (12).
In addition to this, several strategies have been developed for the treatment of neurodegenerative diseases, including gene therapy (13), stem cells therapy (14) and extracellular vesicles derived from mesenchymal stem cells (15), but also strategies aimed at reducing neuroinflammation (16), targeting impaired proteostasis (17), and last but not least passive and active immunotherapies (18, 19), which have shown partial success.

1.1.1. Parkinson's Disease (PD)

Parkinson's disease (PD) is the second most common neurodegenerative disease after AD, with a global prevalence of about 1.8 % of the adult population above the age of 65 and about 3 % among the elderly from 80 years and above (20). Given that ageing is the most crucial risk factor, the number of persons affected by PD is expected to double by the year 2040 (21), resulting in a huge socio-economic burden. PD is characterized by a constellation of motor features, including bradykinesia in combination with tremor or rigidity. In addition, PD patients experience several non-motor symptoms, such as cognitive decline, pain and depression, olfactory decline, constipation, exhaustion, REM sleep disturbances (22-24). Many of the non-motor symptoms appear already many years before disease diagnosis in the prodromal phase, including constipation and olfactory decline. The motor symptoms of PD are usually preceded by the non-motor symptoms that may appear in the prodromal phase of PD, long before the clinical manifestation and diagnosis. While the clinical features of PD can lead to a probable or clinically established diagnosis, there still remains a certain level of uncertainty, since PD and other forms of parkinsonism may show comparable and overlapping clinical symptoms.

PD is a progressive, heterogeneous disease with unknown etiology. Apart from ageing being the most important risk factor for developing PD, an interplay between several genetic and environmental risk factors has been associated with either increased or

lowered risk of developing the disease. A variety of environmental factors, such as exposure to pesticides like rotenone, paraquat, organochlorides and neurotoxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), have been substantially linked to increased risk of developing PD (25). In contrast, other environmental factors like smoking tobacco, and drinking coffee, are associated with a decreased PD risk (26).

The decreased association of smoking with PD has been repeatedly observed in numerous epidemiological studies; however, the factors that contribute to this decreased risk are not well known (27, 28). Nicotine has been demonstrated to be neuroprotective when it activates nicotinic acetylcholine receptors on dopaminergic neurons, thereby triggering the release of dopamine (29, 30). However, it is challenging to determine if smoking prevents PD or whether PD aids in the cessation of cigarette habit. A case-control study suggested that PD patients are more likely to quit smoking compared to healthy individuals (31). Similarly, studies on caffeine, which is believed to be neuroprotective, have also shown an up to 25 % risk reduction of PD (26, 32).

Most PD cases are idiopathic; however, a few monogenic forms of the disease exist (about 5 % of all cases, depending on the population). A greater number of PD cases are sporadic, while about 15 % of cases are known to have a family history (33). Initial reports about monogenic PD involved *SNCA* variants (34, 35). Since then, multiple genetic variants with high penetrance causing monogenic forms of PD have been identified in the following genes: *SNCA, LRRK2, VPS35, UCHL1, DJ-1, PINK1, PRKN, ATP13A2, DNAJC6, VPS13C, FBX07* and *SYNJ1* (36). Selected genes and their respective disease-causing variants are depicted in Figure 1. In addition, about 90 PD-associated risk variants have been identified using genome-wide association studies (37). Interestingly, genetic mutations in glucocerebrosidase (*GBA),* a gene involved in an autosomal-recessive lysosomal storage disorder, have been strongly linked to increased risk of PD, with estimates of about 2.3-9.4 % of PD cases harboring a single *GBA* mutation (38).

One major neuropathological hallmark of PD is the loss of dopaminergic (DA) neurons mainly in the substantia nigra pars compacta (SNpc) projecting to the striatum (39).

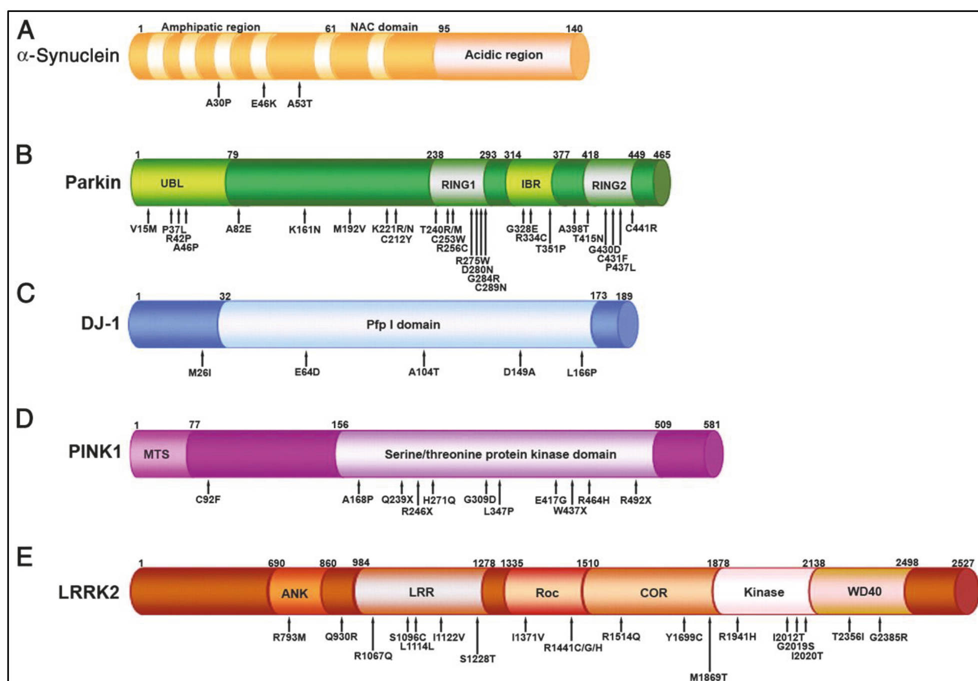


Figure 1. Overview of monogenic Parkinson's disease (PD)-associated proteins and their mutations.

(A) The 140 amino acids long α-synuclein has three domains: the N-terminal amphipathic region, the hydrophobic non-amyloid β component of plaque (NAC) domain and a C-terminal acidic region. This protein is associated with autosomal dominant forms of PD.

(B) Parkin is a 465 amino acids long protein with five domains: an N-terminal ubiquitin-like (UBL), a central linker region, two RING finger (RING1 and RING2) domains and an in-between (IBR) domain. This protein is associated with autosomal recessive forms of PD.

(C) DJ-1 is a 189 amino acids long protein with a highly conserved PfpI domain. This protein is associated with autosomal recessive forms of PD.

(D) PINK1 is a 581 amino acids long protein with an N-terminal mitochondrial targeting sequence (MTS), a transmembrane segment and a serine/threonine protein kinase domain. This protein is associated with autosomal recessive forms of PD.

(E) LRRK2 is a 2527 amino acids multi-domain protein with 6 conserved domains: an ankyrin region (ANK), a leucine-rich repeats (LRR) domain, a GTPase ROC domain, a C-terminal of Roc (COR) domain, a kinase domain and a C-terminal WD40 domain. This protein is associated with autosomal dominant forms of PD. Image adapted from (40).

In addition, widespread neuronal cell loss is found in several subcortical areas, like the locus coeruleus, dorsal motor nucleus of the vagus nerve, the raphe nucleus, the nucleus basalis of Meynert and the olfactory bulb (41). Findings from neuropathological assessment of PD brains suggest that loss of DA neurons within these regions gives rise to the motor symptoms, especially bradykinesia and rigidity (42). Another pathological hallmark of PD is the presence of intracellular Lewy bodies (LBs) in the cell body and Lewy neurites (LNs) in the processes of surviving DA neurons (Figure 2). The major protein component of a Lewy body is misfolded α-synuclein, a protein that is ubiquitously expressed in the presynaptic terminals in the brain and is involved in vesicle trafficking at the synapse (43). Accumulation of α-synuclein occurs in several brain regions, such as substantia nigra and prefrontal cortex, as well as the olfactory bulb, medulla and hippocampus, among others, which may not necessarily coincide with the brain regions affected by extensive neurodegeneration (44, 45).

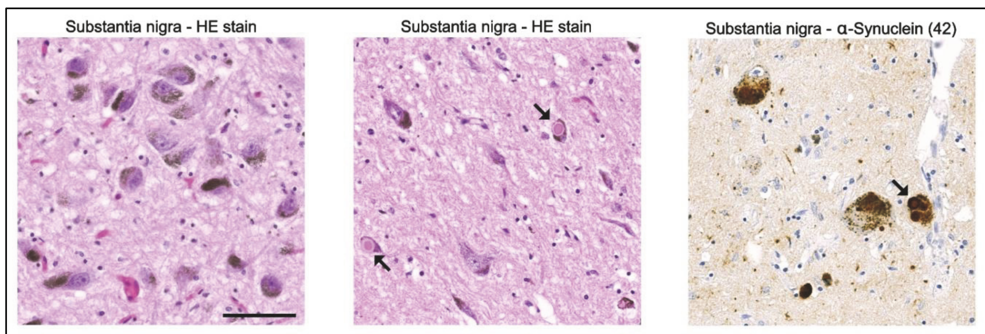


Figure 2. Neuropathological features of Parkinson’s disease (PD).

The figure shows hematoxylin and eosin staining of the substantia nigra (SN) and immunodetection of α-synuclein. Left: SN tissue from a neurological healthy control individual with populated dopaminergic neurons without Lewy pathology. Center: SN tissue from an individual with PD with severe neuronal loss and Lewy body pathology (arrows). Right: Immunoreactivity against α-synuclein shows intraneuronal Lewy body inclusion (arrow) in SN tissue from an individual with PD with severe neuronal loss. Scale bar = 50 μm. Image adapted from (46).

Apart from abnormal α-synuclein aggregation, several mechanisms have been identified to play a key role in the pathogenesis of PD, including mitochondrial dysfunction, abnormal protein clearance, the autophagy-lysosome pathway (ALP) and ubiquitin-proteasome pathway (UPS), and neuroinflammation (47). The UPS and the ALP are responsible for the breakdown and removal of damaged or misfolded proteins within the cell. Monomeric forms of PD-associated α-synuclein are usually processed and cleared by the UPS and ALP, and aberrations in these pathways have been reported to contribute to the accumulation of misfolded α-synuclein protein in PD, facilitating aggregation (48, 49).

Increased levels of microglia activation, T-lymphocytes infiltration, as well as increased levels of pro-inflammatory cytokines in postmortem PD brain tissues compared to healthy individuals suggest that neuroinflammatory responses can contribute to the progression of the disease (50, 51). Furthermore, there is evidence from a PD model that α-synuclein can perturb neuroinflammatory processes and induce microglial activation (52). Nonetheless, it is unclear whether the neuroinflammatory responses exacerbate disease progression or are activated as a response to neuronal damage in PD.

1.1.2. Alzheimer's Disease (AD)

Alzheimer's disease (AD) is the most common neurodegenerative disorder and one of the leading causes of dementia worldwide (53). It is characterized by a progressive loss of cognition and memory decline (54). The increasing number of people living with dementia is expected to triple by the year 2050, as the global population ages, resulting in a huge socio-economic burden to both caregivers and society (55). The greatest risk factor for AD is ageing, with prevalence rates about 3 % of the adult population above the age of 65 and 30 % by age of 85 (56).

The etiology of AD is not fully understood, and both genetics and environmental factors are believed to influence onset and disease progression. The vast majority of cases are idiopathic (57), while the genetic forms of AD are caused by variants in three genes: amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*),

accounting for a small fraction of cases (less than 0.5 %). In addition, several notable genes with AD-associated risk variants include *APOE, BIN1, CLU, PICALM*, *SORL1* and *TREM2* (58-61).

The classification of AD is based on disease manifestation and whether it is inherited or not. Typical early-onset or sporadic AD manifests before the age of 65, whereas disease manifesting beyond the age of 65 is classified as late-onset AD, accounting for about 95 % of cases in the population (62). In addition to the genetic risk factors, some environmental factors, as well as inflammation (63), cardiovascular disease (64), diabetes (65), stress (66) and exposure to aluminium (67) are also associated with increased risk of developing AD, although the exact mechanisms remain elusive.

The classical neuropathological feature of AD is the extensive loss of neurons and presence of amyloid beta (Aβ)-containing plaques and neurofibrillary tangles (NFT) composed of phosphorylated tau protein in the brain (68) (Figure 3). The presence of Aβ plaques and NFT pathology typically starts in the hippocampus and the entorhinal cortex, before spreading to other frontotemporal cortices (69). Within the hippocampus, the pyramidal neurons of the CA1 region are selectively vulnerable to morphological changes, NFT and plaque formation, and cell loss (70, 71). However, deposition of Aβ has been frequently observed in healthy elderly individuals without cognitive impairment and dementia deposition (72, 73). This observation could suggest that Aβ alone is not sufficient to cause AD, and rather that the presence of both Aβ and tau pathology leads to clinical manifestations and disease diagnosis. The requirement for both Aβ and tau pathology for a neuropathological diagnosis of AD serves as a support for this assumption. On the other hand, some reports strongly indicate a positive correlation between Aβ deposition only, cognitive decline, and disease severity (74, 75). These conflicting reports could be due to interpretations and conclusions drawn from different experimental methodologies and sample sizes.

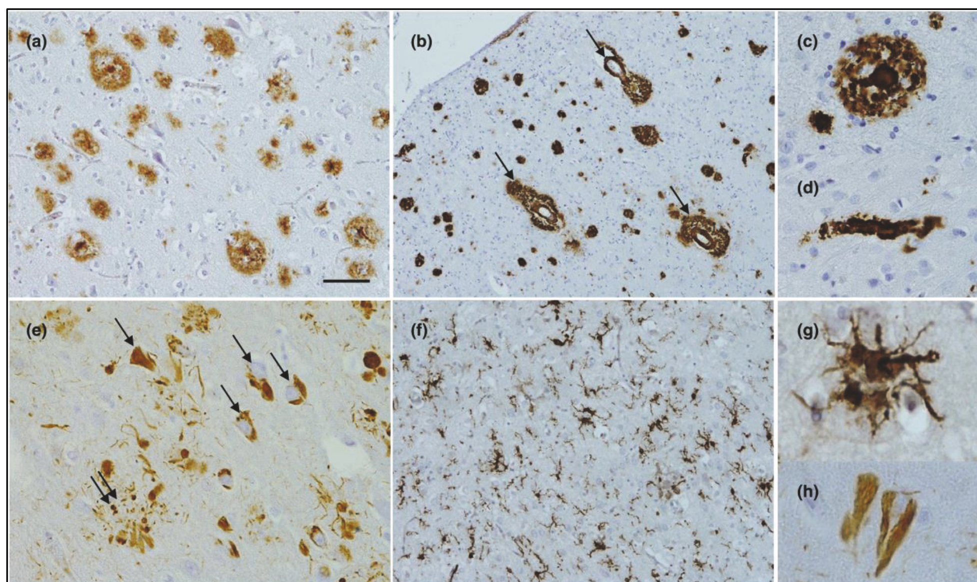


Figure 3. Neuropathological features of Alzheimer's disease (AD).

Immunohistochemistry of amyloid beta (Aβ) plaques and tau in the prefrontal cortex of AD tissue.
(A) Aβ accumulation within blood vessels in the brain. (B) A classical depiction of Aβ plaques at high magnification (arrows). (C) Amyloid β cored plaque at high magnification. (D) Aβ plaque accumulation within capillaries. (E) Immunoreactivity against tau showing NFT (single arrows) and neuritic plaques (double arrows). (F) Prefrontal cortex from AD tissue showing several reactive microglia at low magnification and (G) at high magnification. (H) NFT at high magnification.
Scale bar = 50 μm in A and B, 100 μm in B, 25 μm in C and E, 15 μm in D, G and H. Image adapted from (76).

Numerous studies have also shown that the buildup of NFT, and neuronal and synaptic loss correlate with disease severity and duration, whereas Aβ plaque accumulation typically appears before the onset of deterioration of cognitive ability (77, 78). Although Aβ plaques and NFT are prominent neuropathological features of AD, their mechanistic link and exact role in disease progression, neuronal and synaptic loss are not yet fully understood.

While the pathogenesis of AD remains unclear, there are several notable mechanisms and pathways that play a crucial role in AD pathogenesis, many of which have been implicated also with several other neurodegenerative disorders. Multiple lines of evidence have suggested that mitochondrial dysfunction is one mechanism driving pathological forms of Aβ accumulation and subsequent neuronal and synaptic loss (79). The roles of neuroinflammation and the innate immune system in the pathogenesis of AD have also been recognized as another key driver of AD and other neurodegenerative disorders. Particularly, chronic neuroinflammatory response is believed to be responsible for microglia activation and stimulation of proinflammatory responses that can lead to neuronal cell damage (80).

While the precise interactions between Aβ and tau pathology are still being elucidated, there are indications that Aβ buildup may precede and exacerbate tau pathology. This suggests that targeting Aβ may not only alleviate Aβ plaques but could also have downstream effects on tau pathology. Recent therapeutic strategies include domanezumab, a humanized IgG1 antibody that binds specifically to Aβ. Treatment with donanezumab was shown to be associated with reduced cognitive decline and, more interestingly, with decreased plasma levels of p-tau217, a biomarker of AD pathology (19). Furthermore, AN1792, the first anti-Aβ vaccine clinically tested, was also able to reduce cerebrospinal tau levels in AN1792-treated patients with high anti-AN1792 IgG titers (19).

1.2. Mitochondria

Mitochondria are double membrane-enclosed organelles that have been considered the powerhouses of most eukaryotic cells. Most eukaryotes depend on mitochondria as a major source of cellular energy in the form of adenosine triphosphate (ATP), which is produced from the biochemical conversion of energy from food and nutrients during cellular respiration (81). Apart from their essential role in cellular respiration and ATP synthesis, the mitochondria also take part in, among others, lipid metabolism (e.g., fatty acid oxidation), calcium signaling and stress response (82-84).

A mitochondrion is an organelle with four distinct compartments: a smooth outer mitochondrial membrane (OMM), an inner mitochondrial membrane (IMM), an intermembrane space (IMS) and the mitochondrial matrix (MM) (Figure 4). The OMM forms the boundary between the mitochondrion and the cytosol and allows exchange between the IMM and cytoplasm via channels and pores, while the IMM is impermeable for most passive transport.

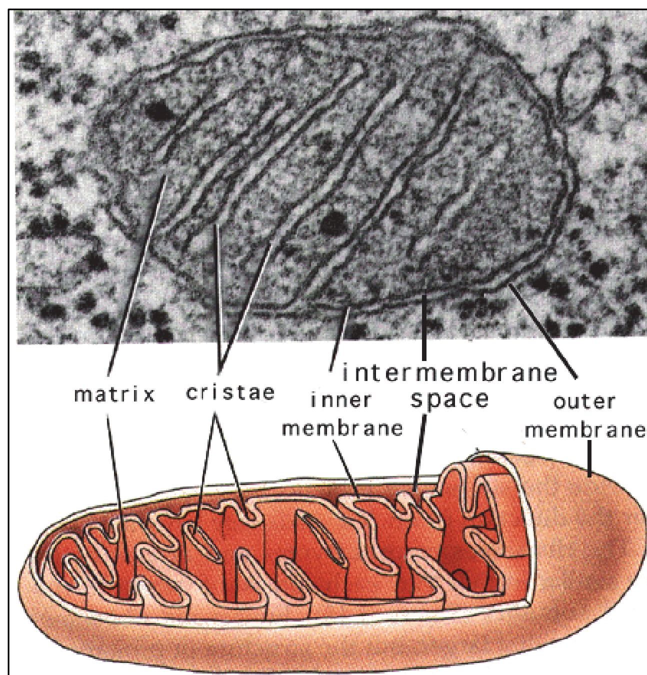


Figure 4. Structure and compartments of a mitochondrion.

The outer mitochondrial membrane is porous and allows passive transport. It surrounds the inner mitochondrial membrane and separates the mitochondrion from the cytoplasm. The inner membrane is tightly closed and only allows active transport via transporter proteins into the mitochondrial matrix, the inner compartment of mitochondria. The inner membrane is differentiated to form cristae, which invaginate into the matrix and are the site for mitochondrial energy conversion.
Image adapted from https://microbewiki.kenyon.edu/index.php/Mitochondria (accessed June 10th, 2023).

The mitochondrial respiratory complexes are located in the IMM and are essential for ATP synthesis via oxidative phosphorylation (OXPHOS). The majority of the mitochondrial metabolic pathways are situated within the matrix (85). A unique feature of the mitochondria is that they have their own genome with a double-stranded circular DNA of 16.569 base pairs. The mitochondrial DNA (mtDNA) is solely inherited from the mother and encodes a total of 37 genes, of which 13 are encoding protein subunits of the respiratory chain, required for OXPHOS, two encode ribosomal RNAs (rRNAs) and 22 are transfer RNA genes (tRNAs) essential for the transcription and translation of mitochondrial proteins (86). The mtDNA and the required mitochondrial ribosomes are also located in the mitochondrial matrix.

The remaining MRC proteins that are essential for mitochondrial OXPHOS are encoded in the nuclear genome, translated by the cytosolic ribosomes and translocated into the mitochondria through the outer and inner translocase protein import machinery (87, 88).

1.2.1. Mitochondrial respiratory chain

The majority of ATP needed for the functioning of the eukaryotic cells is produced within the mitochondria via the process of OXPHOS, carried out by the respiratory chain complexes in the IMM (85). During OXPHOS, electrons are transferred from reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$) to molecular oxygen (O_2) in a series of redox reactions involving several respiratory chain complexes, complexes I-IV (Figure 5). The energy released via the transfer of electrons is used to pump protons from the MM across the IMM, creating a protein gradient in the IMS. The proton motif force across the IMM is coupled to the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) (89). The mitochondrial respiratory chain complexes are composed of both nuclear- and mitochondrial-encoded proteins. A total of 13 mitochondrial- and 76 nuclear-encoded proteins make up the mitochondria respiratory chain complexes from CI-CV (90) (Table 2).

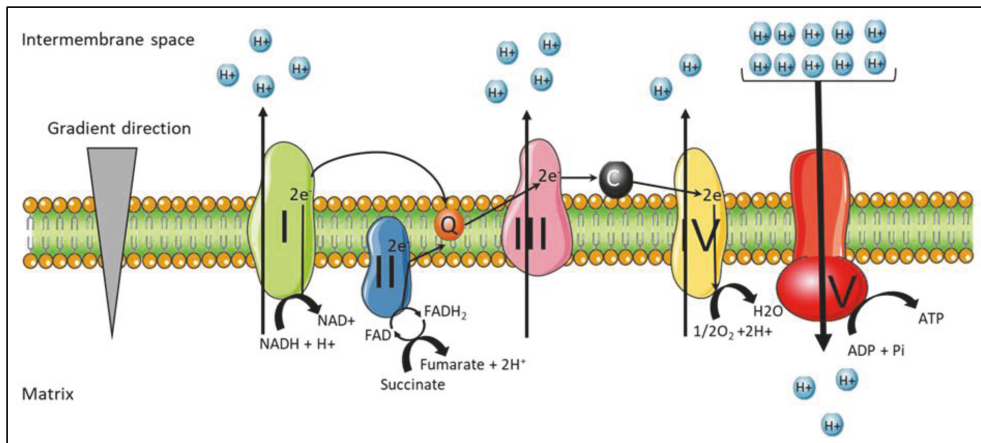


Figure 5. Schematic diagram of the mitochondrial respiratory chain.

The mitochondrial respiratory chain is located within the inner mitochondrial membrane (IMM) and allows ATP synthesis via the process of oxidative phosphorylation (OXPHOS). Electron transfer begins with the oxidation of NADH at complex I (CI) or oxidation of $FADH_2$ at complex II (CII), each transferring electrons to quinone coenzyme Q10 (CoQ10), which in turn transfers the electrons to complex III (CIII). The electrons are passed on from complex III to cytochrome C, and from there further to complex IV. At complex IV, molecular oxygen (O_2) is reduced to water. Complexes I, III and IV act as proton pumps, pumping protons from the matrix into the intermembrane space. About 4 protons pass through complexes I and III respectively, while 2 protons pass through complex IV. This builds up an electrochemical gradient. Complex V (CV) allows the protons to flow back into the matrix, releasing the stored energy, which is coupled to the synthesis of ATP from ADP. Image adapted from (91).

1.2.1.1. Mitochondrial Respiratory Complex I

NADH ubiquinone oxidoreductase, also known as complex I (CI), is the first and largest enzyme of the mitochondrial respiratory chain. It has a total of 45 protein subunits, of which 7 proteins are encoded in the mitochondrial genome and the remaining 38 are in the nuclear genome and are imported into the mitochondria to form a fully functional complex (92).

Table 2. The distribution of nuclear- and mitochondrial-encoded proteins across all mitochondrial respiratory chain complexes.

	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA	7	0	1	3	2
nDNA	38	4	10	10	14
Total	45	4	11	13	16

mtDNA=mitochondrial DNA, nDNA=nuclear DNA

Complex I is also the main electron entry point from fatty acid oxidation and the Krebs cycle into the mitochondrial respiratory chain and is therefore essential for OXPHOS and ATP synthesis (92). Besides its role in the mitochondrial respiratory chain, it is also one of the main sites for ROS production. Overproduction of ROS beyond a certain threshold results in oxidative stress and mitochondrial damage (93). This can lead to complex I deficiency in tissues and organs such as the brain and heart, that rely on energy derived from mitochondrial respiration. Complex I deficiency has also been implicated in several neurodegenerative disorders, including PD (4) and AD (94).

1.2.1.2. Mitochondrial Respiratory Complex II

Complex II, also known as succinate dehydrogenase, is the second enzyme of the mitochondrial respiratory chain. It consists of 4 subunits, all encoded by the nuclear genome. It is the only respiratory chain enzyme without a mitochondrial genome encoded subunit, and the only one that does not pump protons from the MM across the IMM (95). Complex II exerts its functions in both the mitochondrial respiratory chain and the Krebs cycle, where it catalyzes the oxidation of succinate to fumarate resulting in the release of electrons. These electrons are stored in reduced $FADH_2$ and then transferred from complex II to ubiquinone; however, the energy derived from this process is not sufficient to generate hydrogen ions (H^+) across the IMM (89). In addition, complex II is not a membrane-spanning protein, thus protons have no way of crossing

the membrane. Furthermore, since complex II does not contribute to the proton gradient in the IMS as much as complex I does, the ATP yield generated from $FADH_2$ is about 1.5 ATP molecules, compared to 2.5 ATP molecules generated from NADH (94).

1.2.1.3. Mitochondrial Respiratory Complex III

Complex III, also known as cytochrome c reductase, is the third respiratory chain complex and consists of 11 proteins, of which only one protein is encoded in the mitochondrial genome and the remaining 10 proteins are encoded in the nucleus. This complex accepts the electrons from ubiquinol, and transfers them further to cytochrome c, while pumping protons across the inner mitochondrial membrane. (89).

1.2.1.4. Mitochondrial Respiratory Complex IV

Complex IV, also known as cytochrome c oxidase, is composed of 3 mitochondrial-encoded subunits and 10 nuclear-encoded subunits. The enzymatic action of complex IV is the reduction of molecular oxygen (O_2) to H_2O using the electrons derived from cytochrome c, while pumping protons from the MM to the IMS (89).

1.2.1.5. Mitochondrial Respiratory Complex V

ATP synthase, also referred to as complex V, utilizes the proton gradient in the IMS to drive the synthesis of ATP from ADP and P_i. ATP synthase can also work in the reverse way, that is, using ATP hydrolysis to pump protons out of the mitochondrial matrix. This may, for example, be relevant under stress conditions that lead to a reduction of the mitochondrial membrane potential over the inner mitochondrial membrane (96). It consists of 2 mitochondrial-encoded subunits and 16 nuclear-encoded subunits.

1.2.2. Mitochondrial protein synthesis

Mitochondrial ribosomes are the synthesis machinery for proteins encoded by the mitochondrial DNA. They are structurally composed of a large 39S and small 28S subunit (97). The four steps of mitochondrial protein translation include translation

initiation, elongation, termination, and recycling of the ribosome (98). This process is controlled by mitochondrial RNA and regulatory factors and translation activators of mitochondrial protein translation (98, 99). The mitochondrial ribosome is comprised of about 70 % protein and 30 % RNA, whereas the RNA:protein ratio in cytosolic and bacterial ribosomes is about 65 %:35 % (100). The mitochondrial ribosome also differs structurally from other ribosomes because of the absence of an exit site (100).

The mitochondrial protein synthesis machinery is responsible for the synthesis of proteins involved in OXPHOS. However, it involves an interplay between several nuclear and mitochondrial factors (101). An imbalance between mitochondrial protein translation and cytoplasmic translation can have an impact on several physiological and pathological processes, including OXPHOS and fatty acid oxidation (98). Abnormalities in mitochondrial protein translation have been linked to several diseases, including cancer, heart disease, and neurological disorders (97, 101-103), most of which are associated with mtDNA-encoded mitochondrial respiratory chain enzymes dysfunction, reduced ATP synthesis and cellular energy depletion.

Mitochondria have been linked to several aspects of ageing, and mitochondrial dysfunction is considered to play an important role in the pathogenesis of neurodegenerative diseases, despite a clear understanding of its exact role and underlying mechanisms.

1.3. Mitochondrial involvement in PD

Although the mechanisms involved in PD pathogenesis remain poorly understood, mitochondrial dysfunction is considered a critical contributor in the pathogenesis of both monogenic and idiopathic forms of PD (104). In PD, SN dopaminergic neuronal cell loss is highly attributed to their selective vulnerability to mitochondrial dysfunction and oxidative stress, because of their high metabolic demand, complex axonal morphology and high axonal mitochondrial density (105-107). Early evidence linking mitochondrial dysfunction to PD was the exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its metabolite MPP^+ among recreational drug users and the resulting

parkinsonism phenotypes and loss of DA neurons (108). MPTP is a synthetic opioid, that is immediately oxidized to form its toxic metabolite MPP^+ after crossing the blood–brain barrier (BBB). It is then readily absorbed through the dopamine transporter of DA neurons, where it inhibits complex I, leading to decreased ATP synthesis, elevated ROS levels and neuronal death (109, 110). Several toxins like rotenone and paraquat were also observed to inhibit complex I activity and cause Parkinson-like symptoms and neuronal loss (111).

Another major piece of evidence was the identification of complex I deficiency in the SN of postmortem PD brain tissues (112). Several studies have observed similar complex I deficiency and to a lesser extent complex II, III and IV in the SN of PD (113). Mitochondrial dysfunction in the form of complex I dysfunction has also been reported in SN and several other tissues, including skeletal muscle, platelets, and lymphocytes from PD patients (113). In recent years, complex I deficiency in PD has been well-documented, both in PD postmortem brain and in several PD-related disease models. Interestingly, a study investigating complex I deficiency in PD postmortem tissue has shown that complex I deficiency is not restricted to the SN and prefrontal cortex but is rather a global phenomenon that occurs throughout the PD brain (4).

Mitochondrial DNA is more vulnerable than nuclear DNA and accumulates a lot of mutations that are associated with increased risk of several neurodegenerative diseases, including PD. There are more age-related mtDNA deletions linked to mitochondrial dysfunction in DA neurons of the SN in PD compared to age-matched controls (114, 115). More evidence of mtDNA mutations in PD outside the SN also demonstrated high levels of mtDNA deletions, thus suggesting that mitochondrial dysfunction in PD is not limited to a particular tissue or brain region (116). While some studies suggest that somatic mtDNA point mutations are involved in the pathogenesis of neuronal loss in PD (117, 118), others show that mtDNA point mutational load is not elevated in PD (3).

Mitochondrial dysfunction has been associated with genetic and sporadic forms of PD. PD-associated proteins have been shown to reside in the mitochondria or can be recruited to the mitochondria to perform specific functions. Two examples are PINK1 and Parkin, which are key actors in the process of mitophagy (119), and protect cells

against damage caused by defective mitochondria (120). Mitochondrial quality control is typically impaired in PD patients harboring autosomal-recessive genetic mutations in PINK1 and/or Parkin (121, 122). Another example is PD-associated DJ-1, whose role is to ameliorate oxidative stress by protecting against toxins and mitochondrial damage (123, 124). Loss-of-function DJ-1 mutations result in increased ROS levels, increased oxidative stress and mitochondrial dysfunction (125, 126). However, due to the apparent multiple subcellular localization of DJ-1 to several cellular compartments, its mitochondrial, and especially submitochondrial localization remains controversial (127).

The relationship between α-synuclein and mitochondria remains controversial, since α-synuclein is a predominantly cytosolic and nuclear-localized protein. Its key role in the regulation of mitochondrial dynamics and quality control has been shown, α-synuclein influencing processes like mitochondrial transport, fission and mitophagy. Furthermore, a series of evidence suggested that pathological α-synuclein species can impair mitochondrial function. For instance, α-synuclein species interact with the mitochondria by binding to TOM20 receptors and preventing their interaction with their co-receptors (128). This interaction eventually leads to the overproduction of ROS and mitochondrial dysfunction (128). It has also been reported that α-synuclein can be targeted to the mitochondria where it accumulates and binds complex I, thereby decreasing mitochondrial activity and activating mitophagy (129, 130). Conversely, it has also been reported that chronic, systemic inhibition of CI using rotenone in rats leads to the accumulation of α-synuclein cytoplasmic inclusions (131), reproducing features of PD. In PD brain specimens, early stages of LB pathology (as assessed by staining morphology of punctate α-synuclein aggregates) were associated with CI deficiency (132). Lastly, α-synuclein knockout mice present reduced vulnerability to MPTP, suggesting a role for α-synuclein in modulating neurodegenerative phenotypes (133).

All in all, mitochondrial localization of all the mentioned PD-related proteins remains conflicting, especially their potential localization in the mitochondrial matrix. A potential mitochondrial presence or matrix localization of any of these PD-related

proteins could be a strong evidence of how they can modulate mitochondrial function or dysfunction.

1.4. Mitochondrial involvement in AD

The amyloid cascade hypothesis states that early Aβ accumulation or inadequate Aβ clearance are the primary causes of AD (134). This implies that the aggregation of oligomeric forms of Aβ and subsequent initiation of several pathways contribute to the damage of healthy neurons. This mechanism applies in early-onset familial AD, where genetic mutations in genes required for the production and regulation of Aβ have an autosomal dominant pattern of inheritance, but it is believed to also play a major role in idiopathic AD.

Mitochondrial dysfunction is increasingly thought to be associated with AD pathogenesis (135), and several lines of evidence have proposed a mitochondrial cascade hypothesis as the main driver of AD pathogenesis (136). According to the mitochondrial cascade hypothesis, the baseline mitochondrial function of a cell declines gradually at a specific rate; however, when it exceeds a certain threshold, it eventually triggers neuropathologic features that are linked to AD (136).

The mitochondrial hypothesis places mitochondrial dysfunction as the main driver of AD pathogenesis, in contrast to the amyloid hypothesis (137) (Figure 6). However, it does not exclude the involvement of Aβ plaques and tau deposition in AD, it rather places more emphasis on mitochondrial dysfunction.

While it is not yet clear how these two AD pathogenesis hypotheses (amyloid hypothesis or mitochondrial hypothesis) contribute to the initiation and progression of AD, it is obvious that mitochondrial dysfunction is involved.

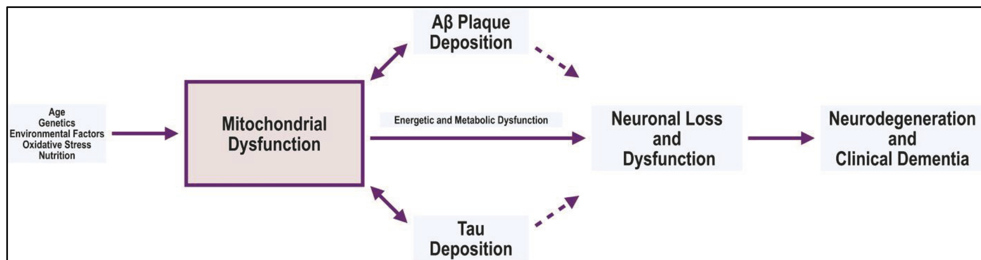


Figure 6. Overview of mitochondrial cascade hypothesis in Alzheimer's disease (AD).

Several risk factors including aging, genetic and environmental factors, oxidative stress and nutrition can contribute to mitochondrial dysfunction. An interplay between mitochondrial dysfunction and AD pathological features (deposition of amyloid beta/Aβ plaques and tau) leads to neuronal loss and subsequently neurodegeneration and AD. Image adapted from (138).

Further evidence of mitochondrial involvement in AD are for example elevated oxidative stress and ROS levels in the AD brain, resulting in neuronal damage (139). Several severe mitochondrial morphological abnormalities, like mitochondrial fragmentation and alteration in size and shape, are caused by oxidative stress. Indicative of decreased mitochondrial functionality, these abnormalities may lead to less efficient ATP synthesis and further increased ROS levels (140-143). Brain regions that are mostly affected by AD pathology, such as the frontal and temporal lobe, are highly susceptible to oxidative stress damage (144). It is possible that distinct ROS-induced damage affects mtDNA differently than nDNA because of its larger quantities of oxidized nucleotide bases (144). Findings from studies using AD cybrid models show that cell lines containing mtDNA from AD patients produce more ROS compared to cell lines with mtDNA from healthy controls (145).

Another evidence of mitochondrial dysfunction in AD is that decreased levels of several mitochondrial enzymes, including pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase complex, have been observed in AD (143, 146).

Moreover, alterations in the activity and protein levels of the mitochondrial respiratory chain enzyme complex IV have been observed in several tissues, including the temporal cortex, hippocampus and platelets of AD patients, where reduced expression of complex IV correlates significantly with the clinical state of AD (146-151). Furthermore, reduced protein levels of complex I, III, IV and V as well as activity of complex IV and V have also been demonstrated in plasma extracellular vesicles in AD compared to healthy controls (152).

Using a novel mitochondrial complex I PET imaging agent, Terada and colleagues detected that mitochondrial complex I impairment is associated with tau pathology in the entorhinal cortex of patients with mild AD compared to healthy controls (94). Their study, however, did not find an association between complex I impairment and Aβ pathology in the brain of individuals with mild AD.

Defective mtDNA maintenance could be one mechanism by which the activity of these enzymes is altered in AD. This assumption is supported by studies which show that mtDNA in AD patients accumulate more deletions compared to healthy controls (153).

Our research group has recently demonstrated that neuronal complex I deficiency is negatively associated with LB pathology (4) and positively associated with early α-synuclein pathology (132). However, in AD, the association between mitochondrial complex I deficiency and the aggregation of pathological tau or lack thereof is inconclusive.

2. AIMS

Mitochondrial dysfunction has been extensively studied in association with ageing and neurodegenerative diseases. However, the precise underlying mechanisms of mitochondrial dysfunction in the pathogenesis of Parkinson's and Alzheimer's diseases remain elusive. The overall goal of this thesis was to further elucidate the causal interplay between mitochondrial dysfunction and abnormal protein aggregation in Parkinson's and Alzheimer's diseases.

To meet this goal, three projects were conducted with the following objectives:

A. (Paper I) To determine mitochondrial matrix localization of Parkinson's disease-related proteins DJ-1, α-synuclein and LRRK2 under normal and stress conditions.

B. (Paper II) To establish an *in vitro* cell culture model of chronic, sublethal inhibition of mitochondrial protein translation, and to analyze the effect of potentially arising mitochondrial dysfunction on α-synuclein aggregation and other cellular mechanisms implicated in the progression of Parkinson's disease.

C. (Paper III) To elucidate the relationship between mitochondrial respiratory complex I and IV and tau accumulation in Alzheimer's disease.

3. METHODS

3.1. Generation of eukaryotic expression vectors (Paper I)

Human *PARK7* cDNA encoding full-length protein deglycase DJ-1 (UniProt ID: Q99497) was amplified from HEK293 cells. The open reading frames (ORFs) encoding the N-terminal domain of *LRRK2* (amino acids 1–266, (154), encoding leucine rich kinase 2, UniProt ID: Q5S007) and full-length α-synuclein (UniProt ID: P37840) were subcloned from preexisting plasmids (Addgene, pDEST53-LRRK2-WT, #25044, and EGFP-alpha synuclein-WT, #40822, respectively). All ORFs were subcloned into pFLAG-CMV-5a (Sigma-Aldrich, #3762) and pcDNA3.1(+)-PARP1cd vectors (155) (see Appendix 1). ORFs encoding α-synuclein mutants (E46K and A53T) were generated by PCR-based site-directed mutagenesis. All constructs were subsequently verified by DNA sequence analysis.

3.2. Cell culture (Papers I and II)

All cells were incubated in a humified atmosphere with 5 % CO_2 at 37 °C. The maximum cell line passage used was 15.
HeLa S3 cells (ATCC, #CCL-2.2) were cultivated in Ham's F12 Glutamax nutrient growth medium and SH-SY5Y cells (ATCC, #CRL-2266) were cultivated in DMEM/Ham's F12 (1:1) Glutamax medium, both supplemented with 10 % (v/v) fetal bovine serum (FBS) (ThermoFisher Scientific, #31331028), 100 U/ml penicillin and 100 µg/ml streptomycin (ThermoFischer Scientific, #15140122).
GFP-tagged α-synuclein-transduced in SH-SY5Y cells (SH-SY5Y-α-syn, Innoprot, #226-P3070) and GFP-transduced SH-SY5Y cells (SH-SY5Y-GFP, Innoprot, #226-P0103) were maintained in RPMI 1640 medium (ThermoFisher Scientific, #61870010) supplemented with 10 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL G-418 (Roche Diagnostics, #04727878001).

3.3. Cell treatment (Paper I and II)

For pharmacological treatments in paper I, 24 h after transfection cells were incubated with 20 µM CCCP for 6 hours or 1 mM (SH-SY5Y) or 2 mM paraquat (HeLa S3) for 24 hours prior to immunocytochemistry analysis.
For protein translation inhibition in paper II, cells were exposed to 50 µg/mL chloramphenicol (CHP; Sigma-Aldrich, #C0378-100G) or 0.07 µg/mL cycloheximide (CHX; Sigma-Aldrich, #C7698-1G) for up to 21 days.

3.4. Transient transfection (Paper I and II)

Transient transfection in paper I was performed using Effectene reagent (Qiagen, #301425), at 70-80 % confluency, according to the manufacturer's specifications.
For the transfection with preformed fibrils (PFFs) of α-synuclein in paper II, cells were seeded in 12-well and 96-well cell culture plates. The transfection mix consisted of Lipofectamine™ 3000 (ThermoFisher Scientific, #L3000015) and recombinant human α-synuclein PFFs (Novus Biologicals, #NBP2-54789; final concentration 2 µg/mL). Cells were incubated at 37 °C for 48 h, followed by cell viability assessment and immunoblotting.

3.5. Immunocytochemistry (Paper I)

Cells were seeded on 12-mm coverslips. The fixation step was performed at 4℃ for 45 min using 3.7 % (v/v) paraformaldehyde in PBS. Cells were permeabilized with 0.5 % (v/v) Triton X-100 in PBS for 15 min at room temperature (RT). Additionally, some cells were treated with 200 nM Mito Tracker Red CMXRos (Cell Signaling Technologies, #9082) for 30 min at 37℃ prior to fixation.
Unspecific antibody binding sites were blocked with growth medium containing 10 % (v/v) FBS for 1 h at RT, followed by overnight incubation with primary antibodies (Table 3) in growth medium at 4 °C. The cells were thoroughly washed 4 times for 5 min in PBS, followed by a 1 h incubation with secondary antibodies in growth medium at RT. The cells were washed once for 5 min with PBS and the nuclei were stained with

DAPI. After two additional washing steps for 5 min in PBS, the coverslips were mounted with ProLong Diamond Antifade (Invitrogen, #P36965).

3.6. Poly-ADP-ribose assisted protein localization assay (Paper I)

Determining the subcellular localization of a protein is a crucial step in deciphering a protein's function and interaction partners within a cell or an organelle. A wide array of methods has been optimized to determine subcellular localization of proteins, including subcellular fractionation, recombinant protein overexpression in fusion constructs with fluorescent reporters or small peptide tags, organelle isolation followed by protease protection assays and immunogold labeling followed by electron microscopy. Some of these strategies do not precisely differentiate between intra-organellar localization, while others may show false positive results, for example due to fraction contamination. In the case of mitochondria, this is especially difficult, given their sub-compartmentalization and the fact that it is a double-membrane organelle.

In conventional immunocytochemistry, antibodies detect and visualize protein localization in cells. Using a primary antibody to target a specific protein in cells, and a fluorescently conjugated secondary antibody, the subcellular localization of the target protein can be revealed. However, this method cannot separate between intra-organellar localization and association with an organelle. Further, a potential partial localization within an organelle can be easily overlooked if the protein has a non-specific cytosolic distribution. Most proteins that are localized in the cytosol and, for example, mitochondria will appear to be only cytosolic.

To address these problems, a novel protein localization assay that makes use of different metabolic conditions of the subcellular compartments of the cell has been established. The distribution of intracellular NAD^+ is different inside distinct subcellular compartments: a large proportion is pooled to the mitochondria, to provide maximal capacity for OXPHOS (156).

NAD^+ is a signaling molecule and substrate involved in a variety of biological processes, including DNA repair, apoptosis, energy metabolism, and cell survival. NAD^+ is also a

substrate in an enzymatic reaction catalyzed by poly-ADP-ribose polymerase 1 (PARP1). Poly-ADP-ribose (PAR) is the end-product of this catalytic reaction. When a protein of interest is overexpressed in fusion constructs with the catalytic domain of PARP1 (PARP1cd), a constitutively active enzyme, the fusion construct will be directed to the native subcellular location of the protein of interest. If adequate levels of NAD^+ are present in the compartment to which the PARP1cd fusion construct is directed, then PAR will be formed and can be detected with PAR-specific antibodies by immunocytochemistry (155).

In the cytosol, PAR is rapidly degraded by poly-ADP-ribose glycohydrolase (PARG) to ADP-ribose. As a result, PAR formation cannot be observed upon expression of a PARP1cd fusion construct with a cytosolic protein. However, PARG activity is diminished in the mitochondrial matrix, and polymer formation can be detected (155). Furthermore, PAR accumulation occurs specifically in the mitochondrial matrix, because of the high NAD^+ concentration present in this suborganellar compartment.
PARAPLAY is also capable of detecting partial localization of a protein expressed in two different locations within the cell e.g., cytosol and mitochondrial matrix. PAR formation readily occurs from the mitochondrially distributed portion of the protein, thus additional information can be obtained by detecting the protein itself, together with PAR polymer formation. Therefore the "hidden" partial localization to the mitochondria of a seemingly cytosolic protein can be revealed by PAR detection.

All in all, the PARAPLAY protein localization method is suitable to determine whether PD-associated proteins are, partially or fully, localized in the mitochondrial matrix.

3.7. Immunohistochemistry (Paper III)

Quadruple fluorescence immunohistochemistry was performed on 3-μm thick formalin-fixed paraffin-embedded (FFPE) sections with primary antibodies listed in Table 3. Sections were deparaffinized and rehydrated for antigen retrieval in low pH EnVision FLEX Target Retrieval Solution (Agilent, #K8005) at 98 °C for 24 min using the Agilent PT link machine. Permeabilization was performed with Tris-buffered saline (TBS)

containing 1 % Triton X-100 for 15 min, followed by blocking in TBS containing 0.1 % Triton X-100 (TBS-T) with 3 % normalized goat serum (Abcam, #ab138478) for 1 hour at RT. Incubation overnight at 4 °C was performed using primary antibodies diluted in blocking solution. The sections were washed 3 times with TBS-T for 5 min, followed by incubation for 1 hour at RT with Alexa Flour and DyLight conjugated secondary antibodies, also listed in Table 3. Additional 3 washes for 5 min with TBS-T were performed before the sections were incubated in 10 mg/mL Sudan black B (Sigma-Aldrich, #199664) for 10 min to quench autofluorescence. The final steps included 3x5 min washing, air drying, and mounting with ProLong Diamond Antifade mountant (Invitrogen, #P36961).

Table 3. List of commercial antibodies used in immunodetection.

Name	Target	Host/Isotype	Supplier	Catalog number	Working dilution
PAR	poly-ADP-ribose	rabbit	Enzo Life Sciences	#ALX-210-890A-01900	1:3000
PAR	poly-ADP-ribose	mouse	Enzo Life Sciences	#ALX-804-220	1:1000
FLAG	FLAG	Rabbit	Sigma-Aldrich	#F7425	1:1000
FLAG	FLAG	mouse	Sigma-Aldrich	#F3165	1:1000
myc	Myc 9E10	mouse	Origene	#TA150121	1:1000
NDUFB10	NDUFB10	rabbit	Abcam	#ab196019	1:1000
NDUFS1	mitochondrial C-I subunit	rabbit	Abcam	#ab169540	1:100
MTCO1	mitochondrial C-IV subunit	mouse IgG2a	ThermoFisher Scientific	#459600	1:100

VDAC1	mitochondrial mass	mouse	Abcam	#ab14734	1:200
Tau	TAU-5	mouse IgG1	ThermoFisher Scientific	#MA5-12808	1:500
Goat anti-mouse 488	Anti-mouse	goat	Life Technologies	#A11001	1:500
Goat anti-rabbit 647	Anti-rabbit	goat	Life Technologies	#A21245	1:500
Alexa Fluor 488	anti-mouse IgG2a	goat	ThermoFisher Scientific	#A21131	1:100
Alexa Fluor 594	anti-rabbit IgG	goat	ThermoFisher Scientific	#A11012	1:100
Alexa Fluor 647	anti-mouse Ig2b	goat	ThermoFisher Scientific	#A21242	1:100
DyLight 405	anti-mouse IgG1	goat	BioLegend	#409109	1:100

3.8. Microscopy (Papers I and III)

Confocal microscopy images for paper I were acquired using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems) with a 100x oil immersion objective (numerical aperture 1.40).
Fluorescent images for paper III were acquired using the Olympus VS120 S6 slide scanner (Olympus Lifesciences) at 40x magnification with filters for 405 nm, 488 nm, 594 nm, and 647 nm.
Neurons were identified by VDAC1 positivity and cell morphology by two independent observers, in three 1 mm^2-areas each from the hippocampus and entorhinal/trans-entorhinal cortex for each section.

3.9. Cell viability assay (Paper II)

Cells were cultivated in triplicate in 96-well culture plates at 37 °C for 96 h. Cell viability was determined by resazurin-based *In Vitro* Toxicology Assay Kit (Sigma-Aldrich, #TOX8-1KT), according to manufacturer's instructions. Fluorimetry values were measured using a TECAN microplate reader.

3.10. Immunoblotting (Paper II)

Cells were washed 1 time with cold PBS and resuspended in SDS lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.2 % SDS). Subsequently, cells were scraped and lysed using a 25 Gauge syringe before being centrifuged at 12,000 x*g* for 5 min at RT. Total protein concentration of the cell lysates supernatants was determined using Pierce BCA Protein Assay Reagent (ThermoFisher Scientific, #23223).
Cell lysates were diluted using 5x SDS sample buffer (250 mM Tris, pH 6.8, 30 % glycerol, 0.05 % (v/v) bromophenol blue, 10 % SDS (v/v) and 10 mM DTT) and boiled at 96 °C for 5 min before loading (20 µg total protein) on polyacrylamide gels for electrophoresis on 4–20 % Mini-PROTEAN® TGX™ gels (Bio-Rad, #4561094).

Protein transfer to a 0.2 µm PVDF membrane (Cat#1704157, Bio-Rad) was performed using the Trans-Blot Turbo system (Bio-Rad) at 25 mA for 1-1.5 h.
Membrane blocking with 5 % milk in TBST blocking solution (1 X Tris-buffered saline (TBS) with 5 % non-fat dry milk and 0.1 % triton-X was performed for 1 h at RT and primary antibody incubation was done at 4 °C overnight. Following 3 washing steps (5 min each) in 0.1 % TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. Following 3 additional washing steps (5 min each) in 0.1 % TBST, the membranes were incubated for 5 min with enhanced chemiluminescence Clarity ECL detection reagent (Bio-Rad, #1705060) for detection of antibody-labelled proteins.
Images were acquired using Bio-Rad ChemiDoc XRS Gel Imaging Systems and bands were quantified using the Bio-Rad Image Lab Software. After detection, membranes were incubated for 30 min at 37 °C with Restore™ Western Stripping Buffer (ThermoFisher Scientific, #21059) and washed twice (5 min each) at RT, followed by blocking and reprobing with loading controls, GAPDH or β-tubulin antibodies. For the full list of primary and secondary antibodies used, see Table 4.

3.11. Seahorse mitostress test (Paper II)

The cellular oxygen consumption rate (OCR) was determined using the Seahorse XFe96 extracellular flux analyzer (Agilent Technologies). The SH-SY5Y-GFP and SH SY5Y-α-syn cell lines were seeded in triplicates in XF96e cell culture microplates (Agilent Technologies, #101085-004) at a density of 100,000 cells/well and allowed to rest overnight at 37 °C in a cell culture incubator.
The XFe96 sensory cartridge (Agilent Technologies) was activated overnight with 200 µL/well of XF calibration solution (Agilent Technologies, #100840-000) and incubated in a non-CO_2 humified incubator at 37 °C. Before the mitostress test was performed, the cell culture medium was replaced with fresh Seahorse XF DMEM (Agilent Technologies, #103575-100) supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose, pH 7.4.

For mitochondrial respiration determination, the ports of the XF96 biosensor cartridge were loaded with 3 µM oligomycin, 2.5 µM FCCP, 1 µM rotenone and 1 µM antimycin A, respectively. The XFe96 analyzer was operated under the manufacturer's instructions at 37 °C. Upon completion, the plates were collected for protein concentration determination, for normalization purposes, using the Pierce BCA protein assay reagent (ThermoFisher Scientific, #23223), following the manufacturer's protocol.

3.12. Study population (Paper III)

The human brain tissue used in the AD study was provided by the brain bank collection at the Department of Neurology, Haukeland University Hospital, Bergen, Norway. Formalin-fixed paraffin-embedded (FFPE) brain tissue slices of 3 µm thickness were used in serial sections. The patient population consisted of three clinically diagnosed advanced stage Alzheimer's Disease (AD) subjects and three age-matched, neurologically healthy control subjects, having comparable postmortem intervals to the patients.

Prior to inclusion in the study, the clinical examination and neuropathological classification of the present β-amyloid pathology were performed, in accordance with the Thal criteria (157, 158).

3.13. Statistical analysis (Papers II and III)

Statistical analysis of protein band intensity in paper II was performed using GraphPad Prism 9.5.0 software. All data are represented as ± standard error of mean (S.E.M), and experiments were performed at least 3 times independently. Statistical significance in each data set was determined by Student's t-test. The proportions of neurons in paper III were compared between AD and neurologically healthy controls using the Pearson Chi-square test. Statistical analyses of fluorescence intensities were conducted using linear mixed effect models, fit using Restricted Maximum Likelihood (REML), with disease status or NFT status as fixed effects, and individuals as random effects. Analyses where the p-value was less than 0.05 were considered significant.

Table 4. List of commercial antibodies used in immunoblotting experiments.

ANTIBODIES	SOURCE	DILUTION	IDENTIFIER
Mouse anti-ATP5A	Abcam	1:5000	Ab14748
Rabbit anti-α-Synuclein	Abcam	1:4000	Ab212184
Rabbit anti-CLLP	Abcam	1:1000	Ab124822
Rabbit-Cyclin D1	Abcam	1:200	Ab16663
Rabbit anti-LONP1	Abcam	1:500	Ab103809
Mouse anti-LAMP1	Abcam	1:1000	Ab25630
Rabbit anti-LAMP2A	Abcam	1:500	Ab18528
Mouse anti-SDHA	Abcam	1:4000	Ab14715
Mouse anti-VDAC1	Abcam	1:1000	Ab14734
Rabbit anti-NDUFS1	Abcam	1:5000	Ab169540
Rabbit anti-NDUFB8	Abcam	1:1000	Ab110242
Mouse anti-NDUFA9	Abcam	1:1000	Ab14713
Rabbit anti-NDUFA10	Abcam	1:1000	Ab174829
Mouse anti-UQCRC2	Abcam	1:3000	Ab14745
Mouse anti-COXIV	Abcam	1:1000	Ab33985
Rabbit anti-ND5	Abcam	1:500	Ab138136
Rabbit anti-TFAM	Abcam	1:1000	Ab176558
Rabbit anti-PGC1α	Abcam	1:1000	Ab54481
Rabbit anti-SIRT1	Sigma	1:500	S5447
Rabbit anti-SIRT3	Sigma	1:1000	S4072
Rabbit anti-SQSTM1/P62	Abcam	1:1000	Ab109012

Rabbit anti- MT-ND1	Invitrogen	1:1000	PA5-101696
Rabbit anti-MT-CYB	Invitrogen	1:500	PA5-100740
Mouse anti-MT-CO1	Invitrogen	1:500	459600
Rabbit anti-Phospho-SIRT1	Invitrogen	1:1000	PA5-17391
Mouse anti-Beta-Tubulin	Sigma	1:5000	T4026
Rabbit anti- Histone H3	Cell Signaling	1:10000	Ab1791
Rabbit anti-Histone H3 (acetyl K27)	Cell Signaling	1:1000	9733S
Rabbit anti-Histone H3 (tri methyl K27)	Cell Signaling	1:500	8173S
Mouse anti-GAPDH	Santa Cruz	1:20000	SC32233
Rabbit anti-PAR pAb	Enzo	1:3000	ALX210890A0100
Rabbit Anti-Mouse HRP	Dako	1:1000	P0260
Swine Anti-Rabbit HRP	Dako	1:2000	P0217

4. RESULTS

4.1. Paper I

Poly-ADP-ribose assisted protein localization resolves that DJ-1, but not LRRK2 or α-synuclein, is localized to the mitochondrial matrix

In this study, the subcellular and submitochondrial localization of DJ-1, LRRK2 and α-synuclein was analyzed using conventional immunocytochemistry, as well as the poly-ADP-ribose assisted protein localization assay (PARAPLAY).

We overexpressed DJ-1, α-synuclein and the N-terminal domain of LRRK2 (subsequently referred to as "LRRK2") as FLAG-tagged recombinant proteins which were detected by conventional immunocytochemistry. All proteins expressed as FLAG fusion constructs were detected in the cytosol and did not show colocalization with mitochondrial structures detected by the mitochondrial marker NDUFB10.

Next, we performed PAR-assisted protein localization assay, PARAPLAY, using constructs of DJ-1, LRRK2 and α-synuclein C-terminally fused to PARP1cd and a myc epitope for protein detection. The PARP1cd-fusion proteins of DJ-1, α-synuclein and LRRK2 were detected in the cytosol and did not show colocalization with mitochondria, confirming that the fusion to PAPR1cd did not change the subcellular localization of these proteins. However, when transfected cells were subjected to PAR immunocytochemistry, cells that expressed the DJ-1-PARP1cd fusion protein showed positive PAR signals that did not correlate with the dispersed, cytosolic pattern of the detected protein, but were associated with mitochondria. On the other hand, the formation of PAR was not observed for the fusion constructs of α-synuclein or LRRK2. This finding was reproducible in both HeLa S3 and SH-SY5Y cells.

Moreover, it was examined whether induction of cellular stress by pharmacological treatment with the ionophore CCCP, leading to disruption of the mitochondrial membrane potential, or with the chemical toxin paraquat, leading to production of reactive oxygen species, would change the localization of the candidate proteins.

However, these compounds did not affect the partial mitochondrial matrix localization of DJ-1. In addition, these stress conditions did not alter the subcellular localization of LRRK2 and α-synuclein, and no PAR formation was detected for either of the recombinant proteins. The unaltered subcellular localization of these proteins, even under stress conditions, was reproducible in both HeLa S3 and SH-SY5Y cells.

Pathogenic α-synuclein mutations have been associated with mitochondrial dysfunction, and it was speculated that this is due to the mitochondrial relocation of α-synuclein. In our experimental setup, we did not observe PAR formation in cells overexpressing α-synuclein harboring mutations A53T or E46K, fused to the PARP1cd. This finding was similar to the wildtype protein, in both HeLa S3 and SH-SY5Y cells. Furthermore, CCCP or paraquat treatment did not induce subcellular relocation of the mutated proteins towards the mitochondrial matrix.

4.2. Paper II

Long-term mitochondrial ribosomal inhibition induces alpha-synuclein aggregation and modulates Parkinson's disease-associated pathways

We established a model of mitochondrial dysfunction, in order to investigate the role of chronic, sublethal mitochondrial ribosomal inhibition in the pathology of PD.

First, we determined the optimal concentrations of mitochondrial (chloramphenicol, CHP) and cytosolic (cycloheximide, CHX) ribosomal inhibitors, which would still support cell growth and viability over a longer time. We found 50 µg/mL CHP and 0.07 µg/mL CHX to be suitable concentrations for SH-SY5Y cells. Long-term, sublethal inhibition of mitochondrial protein synthesis severely affected the expression of several subunits of the mitochondrial respiratory chain (MRC) complexes, including complex I (NDUFS1, NDUFA9, NDUFA10, NDUFB8 and ND1), complex III (UQCRC2) and complex IV (MTCO1) already after 7 days, which was consistently observed also after 14 and 21 days of CHP treatment.

We further investigated the levels of other mitochondrial proteins. The mitochondrial matrix serine proteases Lon protease (LONP1) and caseinolytic peptidase P (ClpP) protein levels were also significantly decreased following long-term mitochondrial protein translation inhibition with CHP. At the same time, protein subunits of complex II (SDHA), complex V (ATP5A), mitochondrial mass marker VDAC1, mitochondrial transcription protein TFAM and mitochondrial biogenesis marker PGC-1α protein levels remained unchanged.

In contrast, when the cytosolic protein translation machinery was inhibited with CHX, none of the studied proteins were affected, even after 21 days of treatment.

We further investigated the effect of protein translation inhibition and the resulting respiratory chain dysfunction on NAD^+-dependent acetylation pathways. Histone H3 acetylation (H3K27) had been observed to be upregulated in PD (159) and changes in the NAD^+/NADH ratio due to OXPHOS deficiency could lead to an increase in histone acetylation. However, we found that treatment with chloramphenicol decreased the levels of H3K27 acetylation. Moreover, protein levels of SIRT1, the NAD^+-dependent deacetylase responsible for H3K27 deacetylation, were also decreased.

Given that MRC dysfunction impairs lysosomal and autophagic activity in cellular models of neurodegeneration, we next investigated the link between protein translation inhibition and the lysosomal and autophagy pathways. The protein levels of lysosomal markers LAMP1 and LAMP2A, as well as autophagy marker P62/SQSTM1 were significantly increased by long-term mitochondrial protein translation inhibition upon CHP treatment. However, long-term cytosolic protein translation inhibition with CHX only resulted in increased levels of LAMP2A, while the other two markers were not affected.

Furthermore, using Seahorse experiments, we found that, upon long-term mitochondrial protein translation inhibition with CHP, the baseline oxygen consumption rate of SH-SY5Y parental and α-synuclein overexpressing cells was significantly reduced, which indicates impaired mitochondrial respiration. Conversely, following CHX long-term treatment, parental cells did not show any effect and α-synuclein overexpressing cells

even showed increased mitochondrial respiration parameters, including basal respiration, maximal respiration, spare respiratory capacity, and ATP production.

Furthermore, mitochondrial translation inhibition led to high molecular weight species of α-synuclein in a-synuclein overexpressing cells, detected by immunoblotting suggestive of oligomerization and aggregation. Cytosolic protein translation inhibition by CHX, on the other hand, did not affect the oligomerization state of α-synuclein in these cells. Treatment with recombinant preformed fibrils (PFFs) of α-synuclein led to high molecular weight oligomeric α-synuclein detection, which was further enhancedupon mitochondrial ribosome inhibition, but not upon CHX treatment or in untreated control cells.

Lastly, we showed that α-synuclein PFFs induced accumulation of poly-ADP-ribose (PAR), an indicator of PARP1 activity due to oxidative stress and potential DNA damage. However, when mitochondrial protein translation inhibition was performed in the presence of CHP, PAR levels were significantly reduced, compared to CHX or untreated control cells.

4.3. Paper III

Tau pathology is associated with higher levels of mitochondrial respiratory complexes I and IV

Defects of the mitochondrial respiratory chain, including mitochondrial respiratory complex I and IV deficiency, have been associated with AD.

We performed quadruple fluorescence immunohistochemistry for subunits of mitochondrial complex I and IV, tau and VDAC1 (mitochondrial mass marker) in the CA1 hippocampal region and the entorhinal/trans-entorhinal cortex of 3 individuals with AD and 3 neurologically healthy individuals. In total, 1,037 and 2,137 neurons were identified and assessed in the CA1 hippocampal region and the entorhinal/trans-entorhinal cortex, respectively.

The fluorescence signals for CI and CIV were divided by the VDAC1 signal of the same neuron, respectively.

Our study showed that CI/VDAC1 levels in the CA1 region were slightly lower in the AD group compared to the control group, while the CIV/VDAC1 levels were similar between the two groups. In the CA1 region, the proportion of NFT-positive neurons was significantly higher in the AD group compared to controls.

NFT-status and association with the respiratory complexes were statistically assessed in the entire study cohort due to the small sample sizes per group. In NFT-positive neurons, the levels of CI/VDAC1 were significantly higher compared to NFT-deficient neurons, which was even more evident in the controls, compared to AD. The same observation was made for CIV/VDAC1 levels.

In the entorhinal cortex, both CI/VDAC1 and CIV/VDAC1 levels were lower in the AD group compared to the control group, but this did not reach statistical significance when accounting for individual in the model. Lastly, the proportion of NFT-positive neurons was significantly higher in the AD group compared to controls.

In all individuals, the relative levels of CI and CIV in the entorhinal cortex were higher in NFT-containing neurons compared to NFT-deficient neurons in the entorhinal cortex, however the effect size was smaller than in the CA1 region. The NFT effects were stronger in the control group for both CI/VDAC1 and CIV/VDAC1 in the entorhinal cortex, as it was in the CA1 region.

5. DISCUSSION

5.1. Paper 1

The localization of a protein inside the cell and/or organelles is fundamental to understanding the protein's function in a physiological context. Furthermore, in the context of deciphering the underlying mechanisms of mitochondrial dysfunction in PD and understanding how PD-linked proteins contribute to mitochondrial dysfunction, determining the precise subcellular localization of these proteins within or around the mitochondria is of paramount importance. In this paper, we reported that DJ-1, but not LRRK2 and α-synuclein, was present in the mitochondrial matrix of human cells under both normal and stress circumstances.

Using the recently established poly-ADP-ribose assisted protein localization assay (PARAPLAY), we identified the subcellular localization of DJ-1 in fusion with the catalytic domain of the PARP1 enzyme. Two aspects of the PARAPLAY method are notable: on the one hand, the localization of the fusion construct is entirely dependent on the protein of interest (DJ-1 in this case). On the other hand, the enzymatic activity of the catalytic domain of PARP1 (PARP1cd) leads to detectable PAR formation only in the lumen of organelles where there is sufficient NAD^+ substrate concentration and a lack of strong PAR-degrading activity. Thus, the overexpression of a PARP1cd-fusion construct with a cytosolic protein would not lead to detectable PAR formation and only proteins that reside in the mitochondrial matrix are able to generate detectable PAR levels in the mitochondria. The association of proteins with the mitochondria from the outside or their intermembrane space localization do not support a robust PAR signal (155).

DJ-1 had previously been reported to localize in the nucleus, cytosol and/or mitochondria (127, 160, 161), and particularly its sub-mitochondrial distribution has been highly disputed. Association with the mitochondrial outer membrane only (160), relocation to the mitochondria upon cellular stress (161), or mitochondrial matrix localization of DJ-1 mutants (127) have all been reported. However, these studies,

though contradictory, have highlighted the significant role of DJ-1 in the mitochondria and the need to determine its exact localization. Using PARAPLAY, we were able to identify the sub-mitochondrial localization of DJ-1, namely in the mitochondrial matrix; however, we cannot fully exclude other compartments (e.g., IMS). Already under normal conditions, DJ-1 is present in the mitochondrial matrix, which allows it to exert its cytoprotective role against potential oxidative damage.
In contrast, LRRK2 and α-synuclein were not detected in the mitochondrial matrix (indicated by the lack of PAR formation). However, the possibility that one or both proteins may still be associated with the mitochondria, outside of the mitochondrial matrix, cannot be eliminated based on these data (for example in Fig 2A, a few yellow dots seemingly indicating colocalization with a mitochondrial marker protein could be observed). This illustrates both the limitations of conventional immunocytochemistry with regard to spatial resolution (close proximity could be mis-interpreted as colocalization) but also its inability to resolve suborganellar localization, which in the case of mitochondria is crucial to elucidate function. In this study, we subcloned only the N-terminal part of the LRRK2 kinase, and therefore cannot eliminate the possibility that full-length LRRK2 may localize in the mitochondrial matrix. However, most mitochondrial proteins have their mitochondrial targeting sequence (MTS) in the N-terminus (162), and their mitochondrial matrix localization is disrupted if the N-terminus of the proteins is omitted, mutated or truncated. Only for a few proteins, an internal mitochondrial targeting signal has been suggested (163, 164). Thus, we are confident that LRRK2 is not mitochondrial matrix localized, based on our conventional and PARAPLAY immunostainings.

Mitochondrial complex I deficiency has been repeatedly observed in PD brains and it is hypothesized to be involved in the pathogenesis of the disease. Further, mitochondrial localization of α-synuclein to mitochondrial-associated ER membranes has been claimed (165). However, a potential mitochondrial matrix localization of α-synuclein was so far unclear and insufficiently explored. As mentioned above, cytosolic localization determined by conventional immunocytochemistry can “hide” a partial mitochondrial localization. Here, PARAPLAY analysis showed no matrix localization

for α-synuclein, determined by the lack of PAR signal. This was the case both under normal and stress conditions and for the PD-related mutants A53T or E46K. However, localization in other mitochondrial compartments, such as the intermembrane space cannot be ruled out, which still would fully allow interaction with complex I or ATP synthase from the outside of the inner mitochondrial membrane. A recent report of α-synuclein interaction with the mitochondrial import receptor TOM20 (128) suggested that α-synuclein may be located on either side of the outer mitochondrial membrane, further supporting our findings that α-synuclein is not found within the mitochondrial matrix.

Further, we examined the subcellular localization of PD-related proteins in toxin-induced models of PD, including CCCP and paraquat treatments. Treatment with the ionophore CCCP led to the collapse of the mitochondrial membrane potential, yet the overexpression of mitoPARP1cd still resulted in PAR formation. This suggests that the CCCP treatment did not interfere with the PARAPLAY detection system. Importantly, DJ-1 localization in the mitochondrial matrix was not affected by CCCP treatment. PARAPLAY analysis is not quantitative, thus, increased overall mitochondrial association as reported earlier for DJ-1 upon oxidative stress (160) could not be assessed. However, its specific mitochondrial matrix localization was still detected, upon oxidative stress treatment, by the robust PAR signal, while the protein itself still appeared cytosolic. Moreover, CCCP treatment did not alter or enhance the localization of α-synuclein or LRRK2, nor did it result in a mitochondrial matrix localization for these proteins.

In conclusion, this work highlighted the challenges of determining the exact subcellular distribution of proteins, especially with conventional methods. One limitation of this study is the exclusive use of transient transfection and expression under a strong promotor. However, if this were to affect protein localization, it would be expected to show additional cytosolic localization of a mitochondrial protein, due to overload of the import machinery, rather than the other way round. Partial mitochondrial localization of DJ-1, reported earlier and confirmed here to be specifically in the mitochondrial matrix,

further indicates that several locations of one full-length protein are possible and should be investigated carefully. Our results strongly point to DJ-1 residing in the mitochondrial matrix and highlight the need for reliable controls and careful interpretation of results.

5.2. Paper II

In this work, we investigated the effects of long-term, sublethal mitochondrial and cytosolic protein translation inhibition on mitochondrial respiratory chain proteins and mitochondrial respiration. We showed that mitochondrial translation inhibition impairs mitochondrial respiration, changes NAD^+-dependent acetylation pathways, and elevates lysosomal and autophagy indicators. Treatment with α-synuclein PFFs led to increased α-synuclein oligomerization upon mitochondrial ribosome inhibition, compared to no or cytosolic ribosome inhibition. Taking all this into account, the sublethal mitochondrial ribosome inhibition represents a potent *in vitro* cell culture model for PD and possibly can be adapted to other diseases.

Previous studies have shown that both low- and high-dose CHP treatment (32 µg/mL or 100 µg/mL) for up to 72 h can reduce protein levels of MRC subunits (166, 167). In keeping with these results, our long-term mitochondrial protein translation inhibition model with a low dosage of CHP (50 µg/mL) dramatically lowered protein levels for both nuclear- and mitochondrial-encoded MRC subunit proteins, however, without an immediate effect on cell viability or proliferation. Furthermore, we found a decrease in the protein expression levels of ClpP and LONP1, as well as a total loss of mitochondrial oxygen consumption rate and respiration. The inhibition of mitochondrial protein translation using CHP has been extensively studied in various cell lines such as HeLa cells, PC12 cells, primary β cells and insulin-producing INS-1E cells (168), and primary fibroblasts (169). These studies have indicated that different cell lines respond differently to CHP treatment: while most cell lines present with reduced levels of MRC proteins and decreased respiration parameters, some cells lines are resistant to CHP affecting the cellular respiration. Respiration was partially resistant to CHP treatment, even when the levels of MRC were markedly reduced, suggesting that MRC protein synthesis may be enhanced in these cells, and a reduction in MRC protein levels must

surpass a certain threshold before the respiratory rates are inhibited (168). While these experiments involved acute treatment with CHP, PD is in fact a chronic disease, requiring chronic, long-term treatment models. In our experiments, the effect of long-term sublethal CHP treatment on MRC proteins was accompanied by an effect on mitochondrial respiration rates in SH-SY5Y cells; however, it did not have any impact on cell viability, at least during the observation period. Despite that, there seemed to be a slowing down in the proliferation of the cells towards the end of the 21 days of treatment with CHP, indicating that the cells were under stress. This may, over long time, contribute to cell death due to the slow and incremental suffering of the cells. It also helps to explain why complex I-deficient neurons are frequently observed in neurodegenerative diseases. This may be surprising given that neurons are supposed to be highly dependent on mitochondrial function, but a slow and sublethal loss of mitochondrial function can apparently be compensated, at least for a period of time.

On the contrary, long-term administration of low-dose CHX (cytosolic protein translation inhibition model) had no impact on mitochondrial OXPHOS protein expression levels or mitochondrial respiration. This is consistent with prior research in which CHX was also demonstrated not to have an impact on the protein levels of several mitochondrial OXPHOS subunits (170).

Given that mitochondrial ribosome inhibition led to mitochondrial dysfunction, we hypothesized that NAD^+/NADH ratio may be affected, thus leading to decreased energy levels within the cells. Moreover, this could affect NAD^+-dependent signaling pathways, including protein acetylation. Acetylation of lysine residues on histone proteins is associated with increased gene expression. We showed that long-term mitochondrial but not cytosolic protein translation inhibition significantly decreased the levels of histone H3 acetylation (H3K27ac), suggesting a link between mitochondrial dysfunction and epigenetic dysregulation of gene expression. Previous studies, however, found elevated acetylation levels of H3K27 at promoters and enhancers associated with mitochondrial impairment, following exposure to the neurotoxic compound rotenone, thus leading to altered gene expression in a PD cell model (171). Others have also shown

hyperacetylated H3K27 status in postmortem PD brain tissues, compared to healthy controls (159).

This apparent discrepancy could be explained by the mechanism of induction of mitochondrial dysfunction or by the duration of toxin exposure (acute short-term treatment with rotenone (171) versus chronic long-term treatment with CHP, as seen in this study). Another possibility could be that the complete failure of mitochondrial respiration also affects other metabolic pathways, such as the Krebs cycle or the PDH pathway, which could in turn lead to a decrease in the production of acetyl-CoA, the substrate for protein acetylation. This could explain the decrease in protein acetylation as seen for H3.

Given the surprising finding of decreased acetylation levels in our cell model, we also investigated the levels of SIRT1 protein, the NAD^+-dependent protein deacetylase responsible for H3K27 deacetylation. In our *in vitro* cellular models, either long-term mitochondrial or cytosolic protein translation inhibition effectively decreased SIRT1 protein levels, independent of mitochondrial dysfunction status. Thus, SIRT1 induction could not be a reason for decreased H3K27 acetylation. However, a decline of SIRT1 activity in aging has been linked to a decrease in NAD^+ levels (172), indicating that NAD^+ supplementation could be a potential therapeutic strategy. One possibility explaining the reduction in SIRT1 protein levels could be a decline in NAD^+/NADH ratio, as a result of MRC deficiency. Alternatively, mitochondrial dysfunction decreases energy levels, affecting other compartments and processes that are energy-dependent, in this case, cytosolic protein translation.

Mitochondrial dysfunction and respiratory failure are frequently associated with changes in autophagic flux and lysosomal function (173). In our work, CHP-induced mitochondrial translation inhibition resulted in an increase of the levels of lysosomal marker proteins and autophagic flux proteins. One explanation for this could be the induction of lysosomal degradation and autophagic flux, in an attempt to compensate for energy deprivation. Moreover, persistent mitochondrial dysfunction should prompt the cell to remove damaged mitochondria, usually facilitated via mitophagy, as indicated

by the observed reduced protein levels of VDAC1, a mitochondrial outer membrane marker.

Another explanation could be reduced autophagic flux and lysosomal degradation capacity. These cargo proteins, shuffling substrates to autophagosomes and lysosomes, are usually degraded with their cargo, and thus their accumulation observed here may indicate a reduced degradation through these pathways. An illustration of this is the increased p62 levels, given that p62 is normally degraded within the lysosomes.

Furthermore, protein degradation and clearance processes are energy-dependent processes e.g., lysosomal acidification depends on ATP levels, therefore a change in the intracellular ATP levels due to impaired MRC and OXPHOS could also impair protein degradation pathways. NAD^+ can also mediate lysosomal acidification processes, and if there is a decline in NAD^+/NADH ratios, as a result of MRC deficiency, this would be reflected in impaired protein degradation processes.

PD-related α-synuclein protein can be degraded either by lysosomes via autophagy, or the proteasomes via the UPS (174); however, its pathological oligomeric forms accumulate intracellularly and are cleared via macroautophagy (175). Disruption in these two pathways may result in α-synuclein aggregation. Indeed, long-term mitochondrial protein translation inhibition led to the accumulation of high molecular weight endogenous α-synuclein oligomers. In addition, treatment with α-synuclein PFFs that are known to enhance the polymerization of endogenous α-synuclein and induce Lewy body-like pathology in animal models (176, 177) led to enhanced aggregation of high molecular weight α-synuclein oligomers upon mitochondrial ribosome inhibition.
This indicated that mitochondrial dysfunction increases the possibility of α-synuclein aggregation, therefore suggesting that mitochondrial dysfunction plays a central role in PD pathogenesis, making our cell culture model a suitable *in vitro* model for screening potential disease-modifying candidates.

Due to low concentration of the seeded PFFs and/or the short duration of exposure to the cells, PFF-induced toxicity was not observed. We used low concentrations of PFFs for seeding primarily because the exposed cells were already under chronic long-term

treatment with the protein translation inhibitors CHP or CHX. Prolonged incubation with PFFs in further experiments will likely lead to better observable α-synuclein aggregation also in intracellular detection by immunocytochemistry, and possibly its cytotoxic effects, as reported earlier (178). Such a model, using prolonged incubation with PFFs, can be used to identify factors or mechanisms that could prevent, delay, or reverse the aggregation of α-synuclein.

PARP1 and PAR metabolism have recently been demonstrated to be involved in the aberrant aggregation of α-synuclein, PFF-induced neurotoxicity, and dopaminergic neuronal death (178): pathologic α-synuclein was found to activate nitric oxide synthase (NOS), causing DNA damage and PARP-1 activation, leading to PAR-dependent cell death. Here, α-synuclein PFFs increased PAR levels, and these were considerably lower in long-term CHP exposed cells, but not in long-term CHX exposed cells. PAR formation is dependent on NAD^+ levels, therefore energy deprivation and lack of NAD^+ as a substrate could explain the lack of PAR formation in CHP-exposed cells. Alternatively, the reduced levels of PAR could be an indication of lack of PARP1 activation, suggesting that α-synuclein aggregation in our setup is independent of PARP1 activation. This observation is contrary to previous findings, where α-synuclein PFFs accelerated α-synuclein fibrillization, induced PARP1 activity and caused neurotoxicity (178).

To summarize, in this work we developed and optimized a chronic, sublethal mitochondrial protein translation inhibition model, that induces mitochondrial dysfunction. Mitochondrial respiration and mitochondrial protein levels (complex I: NDUFS1, NDUFA9, NDUFA10, NDUFB8 and ND1; complex III: UQCRC2; complex IV: MTCO1) were significantly reduced, whereas protein degradation pathways were induced in this cell model. Additionally, high molecular weight species of α-synuclein are induced as a consequence of chronic CHP exposure, and thus this could be applied as a relevant *in vitro* model for PD. We propose that for PD *in vitro* cell models, long-term chronic treatments that capture several aspects of the disease process, especially abnormal α-synuclein protein aggregation, are preferred to acute and short-term treatments.

5.3. Paper III

Mitochondrial dysfunction has been linked to the onset and progression of AD; however, the exact molecular mechanisms are not yet understood. In the case of PD, mitochondrial CI deficiency was shown to be inversely or positively associated with α-synuclein aggregation, depending on the aggregation status (4, 160). In AD, the association between mitochondrial CI/CIV deficiency and pathological tau, or lack thereof, remained inconclusive. Here our findings show that the presence of neuronal tau pathology is associated with higher levels of respiratory complexes I and IV in affected neurons. It has been reported that mitochondrial mass can be increased as a compensatory mechanism that is triggered by mitochondrial respiratory chain deficiency in order to improve the energy homeostasis in tissues (179, 180).

Tau aggregation was associated with higher levels of complexes I and IV in individuals with AD and healthy older controls, though it appears to be more apparent in healthy individuals. One possibility is that a compensatory mitochondrial response to tau-mediated toxicity may be hindered in AD, which could contribute to the negative effects of tau aggregation. This differential phenomenon has been observed in a previous study from our research group, involving mtDNA copy number in the in substantia nigra dopaminergic neurons of individuals with Parkinsons's disease and neurologically healthy older controls. It was observed that mtDNA copy number in the dopaminergic substantia nigra neurons of healthy control individuals increases with age, preserving the wild-type mtDNA population despite the accumulation of deletions. However, Parkinson disease patients do not experience this upregulation, which leads to the population of wild-type mtDNA being reduced and potentially contributing to respiratory chain deficiencies in these cells (3).

The statistical significance of the observed association between NFT pathology and changes in the levels of mitochondrial CI and CIV was found to be lower in the entorhinal cortex compared to the CA1 region. This could be attributed to reduced statistical power, because of the lower number of observed NFT-positive neurons in this area. Although the entorhinal cortex commonly displays notable features in AD, it is

also susceptible to neuronal loss particularly at the terminal stages. Consequently, the reduction in the total number of NFT-positive neurons in this brain region is likely a result of selective loss of these neuronal cell types.

Using quadruple immunofluorescence intensity analysis, we were unable to detect a statistically significant difference in the levels of neuronal CI or CIV between the AD and control groups, as previous reports have done (181). Though elegant, the method has limited sensitivity and is manually challenging when large cohorts are analyzed. To exhaustively address MRC deficiencies in AD, further studies should include increased number of cases and controls, immunodetection, as well as functional assays.

In summary, our study suggests that tau pathology and mitochondrial dysfunction are linked in healthy older adults and individuals with AD. We posit that tau aggregation triggers a physiological compensatory response in neurons, i.e. increased expression of MRC complexes or increased mitochondrial biogenesis. If this compensatory effect is diminished in AD brains, this could lead to tau-mediated toxicity and to neurodegenerative processes.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The overall goal of this thesis has been to investigate molecular mechanisms related to neurodegenerative diseases, specifically PD and AD. We established in paper I that PD-related protein DJ-1 is localized in the mitochondrial matrix, while LRRK2 and α-synuclein do not reside in the mitochondrial matrix. Using the PARAPLAY method, we were able to show that a seemingly diffuse cytosolic localization in typical immunofluorescence microscopy analysis concealed a partial mitochondrial matrix localization of DJ-1. This raises the question of the sensitivity of the methods usually employed to determine subcellular and intraorganellar localization of proteins. We urge caution in the interpretation and analysis of protein localization studies, using methods that cannot distinguish between intra-organellar localization and mere association with the organelle from the outside. Further studies may shed more light and will for example be required to confirm the matrix localization of DJ-1 mutants, full length LRRK2 and the potential localization of α-synuclein in other mitochondrial compartments.

In paper II, we aimed to establish an *in vitro* cell culture model for chronic, sublethal mitochondrial protein translation inhibition. Our study indicates that mitochondrial dysfunction may play a central role in the pathogenesis of PD and contribute to specific pathological observations such as α-synuclein aggregation. This cellular model can be explored as a tool for *in vitro* drug screening of potential PD therapeutic candidates and other neurodegenerative diseases, as well as a mitochondrial dysfunction model in neurodegeneration. Further studies may include a prolonged chronic sublethal treatment with higher α-synuclein PFFs concentrations that could provide more insight into causal connections and potential mechanisms that are relevant for PD. Our attempts to understand the underlying mechanisms have focused on lysosomal and autophagy pathways. Proteasomal function or, rather, impairment due to mitochondrial dysfunction and energy deprivation, would of course be another possibility, that was not investigated at the time. In addition, the link between mitochondrial dysfunction and Aβ and tau aggregation could be studied *in vitro* using this model.

Lastly, in paper III, we aimed to investigate the relationship between tau pathology and complex I/IV in AD, compared to healthy controls. Our findings show that there is an association between presence of neuronal tau pathology and increased levels of mitochondrial CI or CIV in affected neurons. Future investigations will benefit from an even bigger study with a large cohort to corroborate our findings. This may also require the use of a method that allows for more than quadruple immunostaining to accommodate a neuronal and other markers to explore these associations in post-mortem AD brain tissues.

7. REFERENCES

1. Lamptey RNL, Chaulagain B, Trivedi R, Gothwal A, Layek B, Singh J. A Review of the Common Neurodegenerative Disorders: Current Therapeutic Approaches and the Potential Role of Nanotherapeutics. Int J Mol Sci. 2022;23(3).
2. Hou Y, Dan X, Babbar M, Wei Y, Hasselbalch SG, Croteau DL, et al. Ageing as a risk factor for neurodegenerative disease. Nat Rev Neurol. 2019;15(10):565-81.
3. Dölle C, Flønes I, Nido GS, Miletic H, Osuagwu N, Kristoffersen S, et al. Defective mitochondrial DNA homeostasis in the substantia nigra in Parkinson disease. Nat Commun. 2016;7:13548.
4. Flønes IH, Fernandez-Vizarra E, Lykouri M, Brakedal B, Skeie GO, Miletic H, et al. Neuronal complex I deficiency occurs throughout the Parkinson's disease brain, but is not associated with neurodegeneration or mitochondrial DNA damage. Acta Neuropathol. 2018;135(3):409-25.
5. Kerr JS, Adriaanse BA, Greig NH, Mattson MP, Cader MZ, Bohr VA, et al. Mitophagy and Alzheimer's Disease: Cellular and Molecular Mechanisms. Trends Neurosci. 2017;40(3):151-66.
6. Sharma C, Kim S, Nam Y, Jung UJ, Kim SR. Mitochondrial Dysfunction as a Driver of Cognitive Impairment in Alzheimer's Disease. Int J Mol Sci. 2021;22(9).
7. Wang W, Zhao F, Ma X, Perry G, Zhu X. Mitochondria dysfunction in the pathogenesis of Alzheimer's disease: recent advances. Mol Neurodegener. 2020;15(1):30.
8. Dugger BN, Dickson DW. Pathology of Neurodegenerative Diseases. Cold Spring Harb Perspect Biol. 2017;9(7).
9. Kovacs GG, Botond G, Budka H. Protein coding of neurodegenerative dementias: the neuropathological basis of biomarker diagnostics. Acta Neuropathologica. 2010;119(4):389-408.
10. Kovacs GG. Molecular Pathological Classification of Neurodegenerative Diseases: Turning towards Precision Medicine. Int J Mol Sci. 2016;17(2).
11. Walker LC, Diamond MI, Duff KE, Hyman BT. Mechanisms of protein seeding in neurodegenerative diseases. JAMA Neurol. 2013;70(3):304-10.
12. Valera E, Spencer B, Masliah E. Immunotherapeutic Approaches Targeting Amyloid-β, α-Synuclein, and Tau for the Treatment of Neurodegenerative Disorders. Neurotherapeutics. 2016;13(1):179-89.
13. Yu J, Li T, Zhu J. Gene Therapy Strategies Targeting Aging-Related Diseases. Aging Dis. 2023;14(2):398-417.
14. Sivandzade F, Cucullo L. Regenerative Stem Cell Therapy for Neurodegenerative Diseases: An Overview. Int J Mol Sci. 2021;22(4).
15. Palanisamy CP, Pei J, Alugoju P, Anthikapalli NVA, Jayaraman S, Veeraraghavan VP, et al. New strategies of neurodegenerative disease treatment with extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs). Theranostics. 2023;13(12):4138-65.
16. Kip E, Parr-Brownlie LC. Reducing neuroinflammation via therapeutic compounds and lifestyle to prevent or delay progression of Parkinson's disease. Ageing Res Rev. 2022;78:101618.
17. Tyagi A, Pugazhenthi S. A Promising Strategy to Treat Neurodegenerative Diseases by SIRT3 Activation. Int J Mol Sci. 2023;24(2).
18. Kwon S, Iba M, Kim C, Masliah E. Immunotherapies for Aging-Related Neurodegenerative Diseases-Emerging Perspectives and New Targets. Neurotherapeutics. 2020;17(3):935-54.
19. Song C, Shi J, Zhang P, Zhang Y, Xu J, Zhao L, et al. Immunotherapy for Alzheimer's disease: targeting β-amyloid and beyond. Transl Neurodegener. 2022;11(1):18.
20. Lee A, Gilbert RM. Epidemiology of Parkinson Disease. Neurologic Clinics. 2016;34(4):955-65.
21. Dorsey ER, Sherer T, Okun MS, Bloem BR. The Emerging Evidence of the Parkinson Pandemic. J Parkinsons Dis. 2018;8(s1):S3-s8.

22. Armstrong MJ, Okun MS. Diagnosis and Treatment of Parkinson Disease: A Review. Jama. 2020;323(6):548-60.
23. Postuma RB, Aarsland D, Barone P, Burn DJ, Hawkes CH, Oertel W, et al. Identifying prodromal Parkinson's disease: pre-motor disorders in Parkinson's disease. Mov Disord. 2012;27(5):617-26.
24. Berg D, Borghammer P, Fereshtehnejad SM, Heinzel S, Horsager J, Schaeffer E, et al. Prodromal Parkinson disease subtypes - key to understanding heterogeneity. Nat Rev Neurol. 2021;17(6):349-61.
25. Yuan X, Tian Y, Liu C, Zhang Z. Environmental factors in Parkinson's disease: New insights into the molecular mechanisms. Toxicol Lett. 2022;356:1-10.
26. Noyce AJ, Bestwick JP, Silveira-Moriyama L, Hawkes CH, Giovannoni G, Lees AJ, et al. Meta-analysis of early nonmotor features and risk factors for Parkinson disease. Ann Neurol. 2012;72(6):893-901.
27. Paganini-Hill A. Risk factors for parkinson's disease: the leisure world cohort study. Neuroepidemiology. 2001;20(2):118-24.
28. Hernán MA, Zhang SM, Rueda-deCastro AM, Colditz GA, Speizer FE, Ascherio A. Cigarette smoking and the incidence of Parkinson's disease in two prospective studies. Ann Neurol. 2001;50(6):780-6.
29. Bordia T, McGregor M, Papke RL, Decker MW, McIntosh JM, Quik M. The α7 nicotinic receptor agonist ABT-107 protects against nigrostriatal damage in rats with unilateral 6-hydroxydopamine lesions. Exp Neurol. 2015;263:277-84.
30. Srinivasan R, Henley BM, Henderson BJ, Indersmitten T, Cohen BN, Kim CH, et al. Smoking-Relevant Nicotine Concentration Attenuates the Unfolded Protein Response in Dopaminergic Neurons. J Neurosci. 2016;36(1):65-79.
31. Ritz B, Lee PC, Lassen CF, Arah OA. Parkinson disease and smoking revisited: ease of quitting is an early sign of the disease. Neurology. 2014;83(16):1396-402.
32. Chen JF, Xu K, Petzer JP, Staal R, Xu YH, Beilstein M, et al. Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. J Neurosci. 2001;21(10):Rc143.
33. Lesage S, Brice A. Parkinson's disease: from monogenic forms to genetic susceptibility factors. Hum Mol Genet. 2009;18(R1):R48-59.
34. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997;276(5321):2045-7.
35. Nussbaum RL. The Identification of Alpha-Synuclein as the First Parkinson Disease Gene. J Parkinsons Dis. 2017;7(s1):S43-s9.
36. Deng H, Wang P, Jankovic J. The genetics of Parkinson disease. Ageing Res Rev. 2018;42:72-85.
37. Nalls MA, Blauwendraat C, Vallerga CL, Heilbron K, Bandres-Ciga S, Chang D, et al. Identification of novel risk loci, causal insights, and heritable risk for Parkinson's disease: a meta-analysis of genome-wide association studies. Lancet Neurol. 2019;18(12):1091-102.
38. Aflaki E, Westbroek W, Sidransky E. The Complicated Relationship between Gaucher Disease and Parkinsonism: Insights from a Rare Disease. Neuron. 2017;93(4):737-46.
39. Dickson DW. Parkinson's disease and parkinsonism: neuropathology. Cold Spring Harb Perspect Med. 2012;2(8).
40. Mandemakers W, Morais VA, De Strooper B. A cell biological perspective on mitochondrial dysfunction in Parkinson disease and other neurodegenerative diseases. Journal of Cell Science. 2007;120(10):1707-16.
41. Giguère N, Burke Nanni S, Trudeau L-E. On Cell Loss and Selective Vulnerability of Neuronal Populations in Parkinson's Disease. Frontiers in Neurology. 2018;9.
42. Dickson DW, Braak H, Duda JE, Duyckaerts C, Gasser T, Halliday GM, et al. Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria. The Lancet Neurology. 2009;8(12):1150-7.

43. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997;388(6645):839-40.
44. Wiseman JA, Murray HC, Faull R, Dragunow M, Turner CP, Dieriks BV, et al. Aggregate-prone brain regions in Parkinson's disease are rich in unique N-terminus α-synuclein conformers with high proteolysis susceptibility. NPJ Parkinsons Dis. 2024;10(1):1.
45. Guo YJ, Xiong H, Chen K, Zou JJ, Lei P. Brain regions susceptible to alpha-synuclein spreading. Mol Psychiatry. 2022;27(1):758-70.
46. Koeglsperger T, Rumpf S-L, Schließer P, Struebing FL, Brendel M, Levin J, et al. Neuropathology of incidental Lewy body & prodromal Parkinson's disease. Molecular Neurodegeneration. 2023;18(1):32.
47. In: Stoker TB, Greenland JC, editors. Parkinson's Disease: Pathogenesis and Clinical Aspects. Brisbane (AU): Codon Publications; 2018.
48. Xilouri M, Brekk OR, Stefanis L. α-Synuclein and protein degradation systems: a reciprocal relationship. Mol Neurobiol. 2013;47(2):537-51.
49. Lee HJ, Khoshaghideh F, Patel S, Lee SJ. Clearance of alpha-synuclein oligomeric intermediates via the lysosomal degradation pathway. J Neurosci. 2004;24(8):1888-96.
50. Hunot S, Dugas N, Faucheux B, Hartmann A, Tardieu M, Debré P, et al. FcepsilonRII/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-alpha in glial cells. J Neurosci. 1999;19(9):3440-7.
51. Loeffler DA, Camp DM, Conant SB. Complement activation in the Parkinson's disease substantia nigra: an immunocytochemical study. J Neuroinflammation. 2006;3:29.
52. Su X, Maguire-Zeiss KA, Giuliano R, Prifti L, Venkatesh K, Federoff HJ. Synuclein activates microglia in a model of Parkinson's disease. Neurobiol Aging. 2008;29(11):1690-701.
53. Querfurth HW, LaFerla FM. Alzheimer's disease. N Engl J Med. 2010;362(4):329-44.
54. Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol. 1991;30(4):572-80.
55. WHO Guidelines Approved by the Guidelines Review Committee. Risk Reduction of Cognitive Decline and Dementia: WHO Guidelines. Geneva: World Health Organization; 2019.
56. 2019 Alzheimer's disease facts and figures. Alzheimer's & Dementia. 2019;15(3):321-87.
57. Bateman RJ, Aisen PS, De Strooper B, Fox NC, Lemere CA, Ringman JM, et al. Autosomal-dominant Alzheimer's disease: a review and proposal for the prevention of Alzheimer's disease. Alzheimer's Research & Therapy. 2011;3(1):1.
58. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science. 1993;261(5123):921-3.
59. Lee JH, Cheng R, Barral S, Reitz C, Medrano M, Lantigua R, et al. Identification of novel loci for Alzheimer disease and replication of CLU, PICALM, and BIN1 in Caribbean Hispanic individuals. Arch Neurol. 2011;68(3):320-8.
60. Rogaeva E, Meng Y, Lee JH, Gu Y, Kawarai T, Zou F, et al. The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. Nat Genet. 2007;39(2):168-77.
61. Guerreiro R, Wojtas A, Bras J, Carrasquillo M, Rogaeva E, Majounie E, et al. TREM2 variants in Alzheimer's disease. N Engl J Med. 2013;368(2):117-27.
62. Masters CL, Bateman R, Blennow K, Rowe CC, Sperling RA, Cummings JL. Alzheimer's disease. Nature Reviews Disease Primers. 2015;1(1):15056.
63. Kinney JW, Bemiller SM, Murtishaw AS, Leisgang AM, Salazar AM, Lamb BT. Inflammation as a central mechanism in Alzheimer's disease. Alzheimers Dement (N Y). 2018;4:575-90.
64. Stampfer MJ. Cardiovascular disease and Alzheimer's disease: common links. J Intern Med. 2006;260(3):211-23.

65. Butterfield DA, Di Domenico F, Barone E. Elevated risk of type 2 diabetes for development of Alzheimer disease: a key role for oxidative stress in brain. Biochim Biophys Acta. 2014;1842(9):1693-706.
66. Justice NJ. The relationship between stress and Alzheimer's disease. Neurobiol Stress. 2018;8:127-33.
67. Neri L, Hewitt D. Aluminium, Alzheimer's disease, and drinking water. The Lancet. 1991;338(8763):390.
68. Jellinger KA. Neuropathological assessment of the Alzheimer spectrum. Journal of Neural Transmission. 2020;127(9):1229-56.
69. Ridler C. Tau seeding starts early in the entorhinal cortex. Nat Rev Neurol. 2018;14(7):380.
70. Masurkar AV. Towards a circuit-level understanding of hippocampal CA1 dysfunction in Alzheimer's disease across anatomical axes. J Alzheimers Dis Parkinsonism. 2018;8(1).
71. Reilly JF, Games D, Rydel RE, Freedman S, Schenk D, Young WG, et al. Amyloid deposition in the hippocampus and entorhinal cortex: quantitative analysis of a transgenic mouse model. Proc Natl Acad Sci U S A. 2003;100(8):4837-42.
72. Aizenstein HJ, Nebes RD, Saxton JA, Price JC, Mathis CA, Tsopelas ND, et al. Frequent amyloid deposition without significant cognitive impairment among the elderly. Arch Neurol. 2008;65(11):1509-17.
73. Dickson DW, Crystal HA, Mattiace LA, Masur DM, Blau AD, Davies P, et al. Identification of normal and pathological aging in prospectively studied nondemented elderly humans. Neurobiol Aging. 1992;13(1):179-89.
74. Cummings BJ, Cotman CW. Image analysis of beta-amyloid load in Alzheimer's disease and relation to dementia severity. Lancet. 1995;346(8989):1524-8.
75. Esparza TJ, Zhao H, Cirrito JR, Cairns NJ, Bateman RJ, Holtzman DM, et al. Amyloid-β oligomerization in Alzheimer dementia versus high-pathology controls. Ann Neurol. 2013;73(1):104-19.
76. Lane CA, Hardy J, Schott JM. Alzheimer's disease. Eur J Neurol. 2018;25(1):59-70.
77. Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT. Neuropathological alterations in Alzheimer disease. Cold Spring Harb Perspect Med. 2011;1(1):a006189.
78. Ingelsson M, Fukumoto H, Newell KL, Growdon JH, Hedley-Whyte ET, Frosch MP, et al. Early Abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain. Neurology. 2004;62(6):925-31.
79. Du H, Guo L, Yan S, Sosunov AA, McKhann GM, Yan SS. Early deficits in synaptic mitochondria in an Alzheimer's disease mouse model. Proc Natl Acad Sci U S A. 2010;107(43):18670-5.
80. Calsolaro V, Edison P. Neuroinflammation in Alzheimer's disease: Current evidence and future directions. Alzheimers Dement. 2016;12(6):719-32.
81. Focusing on mitochondrial form and function. Nature Cell Biology. 2018;20(7):735-.
82. Rizzuto R, De Stefani D, Raffaello A, Mammucari C. Mitochondria as sensors and regulators of calcium signalling. Nat Rev Mol Cell Biol. 2012;13(9):566-78.
83. Pellegrino MW, Haynes CM. Mitophagy and the mitochondrial unfolded protein response in neurodegeneration and bacterial infection. BMC Biol. 2015;13:22.
84. Nicholls DG. Mitochondrial function and dysfunction in the cell: its relevance to aging and aging-related disease. Int J Biochem Cell Biol. 2002;34(11):1372-81.
85. Kühlbrandt W. Structure and function of mitochondrial membrane protein complexes. BMC Biology. 2015;13(1):89.
86. Rackham O, Filipovska A. Organization and expression of the mammalian mitochondrial genome. Nature Reviews Genetics. 2022;23(10):606-23.
87. Schmidt O, Pfanner N, Meisinger C. Mitochondrial protein import: from proteomics to functional mechanisms. Nat Rev Mol Cell Biol. 2010;11(9):655-67.

88. Harbauer AB, Zahedi RP, Sickmann A, Pfanner N, Meisinger C. The protein import machinery of mitochondria-a regulatory hub in metabolism, stress, and disease. Cell Metab. 2014;19(3):357-72.
89. Deshpande OA, Mohiuddin SS. Biochemistry, Oxidative Phosphorylation. StatPearls. Treasure Island (FL): StatPearls Publishing; 2023.
90. Sousa JS, D'Imprima E, Vonck J. Mitochondrial Respiratory Chain Complexes. Subcell Biochem. 2018;87:167-227.
91. Andrieux P, Chevillard C, Cunha-Neto E, Nunes JP. Mitochondria as a Cellular Hub in Infection and Inflammation. International Journal of Molecular Sciences [Internet]. 2021; 22(21).
92. Sharma LK, Lu J, Bai Y. Mitochondrial respiratory complex I: structure, function and implication in human diseases. Curr Med Chem. 2009;16(10):1266-77.
93. Hirst J, King MS, Pryde KR. The production of reactive oxygen species by complex I. Biochem Soc Trans. 2008;36(Pt 5):976-80.
94. Terada T, Therriault J, Kang MSP, Savard M, Pascoal TA, Lussier F, et al. Mitochondrial complex I abnormalities is associated with tau and clinical symptoms in mild Alzheimer's disease. Molecular Neurodegeneration. 2021;16(1):28.
95. Kluckova K, Bezawork-Geleta A, Rohlena J, Dong L, Neuzil J. Mitochondrial complex II, a novel target for anti-cancer agents. Biochimica et Biophysica Acta (BBA) - Bioenergetics. 2013;1827(5):552-64.
96. Boyer PD. The ATP synthase--a splendid molecular machine. Annu Rev Biochem. 1997;66:717-49.
97. Kummer E, Ban N. Mechanisms and regulation of protein synthesis in mitochondria. Nat Rev Mol Cell Biol. 2021;22(5):307-25.
98. Wang F, Zhang D, Zhang D, Li P, Gao Y. Mitochondrial Protein Translation: Emerging Roles and Clinical Significance in Disease. Front Cell Dev Biol. 2021;9:675465.
99. Mai N, Chrzanowska-Lightowlers ZM, Lightowlers RN. The process of mammalian mitochondrial protein synthesis. Cell Tissue Res. 2017;367(1):5-20.
100. Simcox EM, Reeve AK. An Introduction to Mitochondria, Their Structure and Functions. In: Reeve AK, Simcox EM, Duchen MR, Turnbull DM, editors. Mitochondrial Dysfunction in Neurodegenerative Disorders. Cham: Springer International Publishing; 2016. p. 3-30.
101. Boczonadi V, Horvath R. Mitochondria: impaired mitochondrial translation in human disease. Int J Biochem Cell Biol. 2014;48(100):77-84.
102. De Silva D, Tu YT, Amunts A, Fontanesi F, Barrientos A. Mitochondrial ribosome assembly in health and disease. Cell Cycle. 2015;14(14):2226-50.
103. Ferrari A, Del'Olio S, Barrientos A. The Diseased Mitoribosome. FEBS Lett. 2021;595(8):1025-61.
104. Moon HE, Paek SH. Mitochondrial Dysfunction in Parkinson's Disease. Exp Neurobiol. 2015;24(2):103-16.
105. Haddad D, Nakamura K. Understanding the susceptibility of dopamine neurons to mitochondrial stressors in Parkinson's disease. FEBS Lett. 2015;589(24 Pt A):3702-13.
106. Dias V, Junn E, Mouradian MM. The role of oxidative stress in Parkinson's disease. J Parkinsons Dis. 2013;3(4):461-91.
107. Pacelli C, Giguère N, Bourque M-J, Lévesque M, Slack Ruth S, Trudeau L-É. Elevated Mitochondrial Bioenergetics and Axonal Arborization Size Are Key Contributors to the Vulnerability of Dopamine Neurons. Current Biology. 2015;25(18):2349-60.
108. Ballard PA, Tetrud JW, Langston JW. Permanent human parkinsonism due to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): seven cases. Neurology. 1985;35(7):949-56.
109. Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science. 1983;219(4587):979-80.
110. Martinez TN, Greenamyre JT. Toxin models of mitochondrial dysfunction in Parkinson's disease. Antioxid Redox Signal. 2012;16(9):920-34.

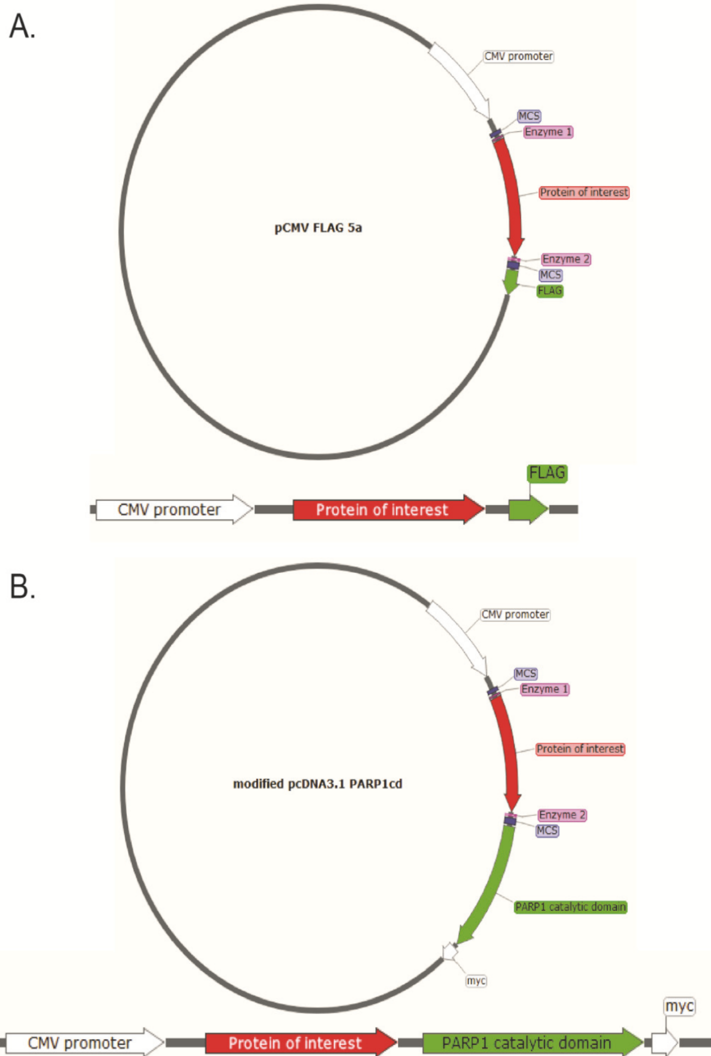
111. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, et al. Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect. 2011;119(6):866-72.
112. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem. 1990;54(3):823-7.
113. Subrahmanian N, LaVoie MJ. Is there a special relationship between complex I activity and nigral neuronal loss in Parkinson's disease? A critical reappraisal. Brain Res. 2021;1767:147434.
114. Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet. 2006;38(5):515-7.
115. Kraytsberg Y, Kudryavtseva E, McKee AC, Geula C, Kowall NW, Khrapko K. Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat Genet. 2006;38(5):518-20.
116. Gu G, Reyes PE, Golden GT, Woltjer RL, Hulette C, Montine TJ, et al. Mitochondrial DNA deletions/rearrangements in parkinson disease and related neurodegenerative disorders. J Neuropathol Exp Neurol. 2002;61(7):634-9.
117. Coxhead J, Kurzawa-Akanbi M, Hussain R, Pyle A, Chinnery P, Hudson G. Somatic mtDNA variation is an important component of Parkinson's disease. Neurobiol Aging. 2016;38:217.e1-.e6.
118. Parker WD, Jr., Parks JK. Mitochondrial ND5 mutations in idiopathic Parkinson's disease. Biochem Biophys Res Commun. 2005;326(3):667-9.
119. Pickrell AM, Youle RJ. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. Neuron. 2015;85(2):257-73.
120. Jin SM, Youle RJ. PINK1- and Parkin-mediated mitophagy at a glance. J Cell Sci. 2012;125(Pt 4):795-9.
121. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998;392(6676):605-8.
122. Valente EM, Bentivoglio AR, Dixon PH, Ferraris A, Ialongo T, Frontali M, et al. Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. Am J Hum Genet. 2001;68(4):895-900.
123. Kahle PJ, Waak J, Gasser T. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. Free Radic Biol Med. 2009;47(10):1354-61.
124. Canet-Avilés RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, et al. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(24):9103-8.
125. Liu L-l, Han Y, Zhang Z-j, Wang Y-q, Hu Y-w, Kaznacheyeva E, et al. Loss of DJ-1 function contributes to Parkinson’s disease pathogenesis in mice via RACK1-mediated PKC activation and MAO-B upregulation. Acta Pharmacologica Sinica. 2023.
126. Dodson MW, Guo M. Pink1, Parkin, DJ-1 and mitochondrial dysfunction in Parkinson's disease. Curr Opin Neurobiol. 2007;17(3):331-7.
127. Kojima W, Kujuro Y, Okatsu K, Bruno Q, Koyano F, Kimura M, et al. Unexpected mitochondrial matrix localization of Parkinson's disease-related DJ-1 mutants but not wild-type DJ-1. Genes Cells. 2016;21(7):772-88.
128. Di Maio R, Barrett PJ, Hoffman EK, Barrett CW, Zharikov A, Borah A, et al. α-Synuclein binds to TOM20 and inhibits mitochondrial protein import in Parkinson's disease. Sci Transl Med. 2016;8(342):342ra78.
129. Devi L, Raghavendran V, Prabhu BM, Avadhani NG, Anandatheerthavarada HK. Mitochondrial import and accumulation of alpha-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. J Biol Chem. 2008;283(14):9089-100.

130. Chinta SJ, Mallajosyula JK, Rane A, Andersen JK. Mitochondrial α-synuclein accumulation impairs complex I function in dopaminergic neurons and results in increased mitophagy in vivo. Neurosci Lett. 2010;486(3):235-9.
131. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci. 2000;3(12):1301-6.
132. Flønes IH, Nyland H, Sandnes DA, Alves GW, Tysnes OB, Tzoulis C. Early Forms of α-Synuclein Pathology Are Associated with Neuronal Complex I Deficiency in the Substantia Nigra of Individuals with Parkinson's Disease. Biomolecules. 2022;12(6).
133. Thomas B, Mandir AS, West N, Liu Y, Andrabi SA, Stirling W, et al. Resistance to MPTP-neurotoxicity in α-synuclein knockout mice is complemented by human α-synuclein and associated with increased β-synuclein and Akt activation. PLoS One. 2011;6(1):e16706.
134. Kametani F, Hasegawa M. Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease. Front Neurosci. 2018;12:25.
135. Parker WD, Jr., Filley CM, Parks JK. Cytochrome oxidase deficiency in Alzheimer's disease. Neurology. 1990;40(8):1302-3.
136. Swerdlow RH. Mitochondria and Mitochondrial Cascades in Alzheimer's Disease. J Alzheimers Dis. 2018;62(3):1403-16.
137. Swerdlow RH, Burns JM, Khan SM. The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives. Biochim Biophys Acta. 2014;1842(8):1219-31.
138. Ashleigh T, Swerdlow RH, Beal MF. The role of mitochondrial dysfunction in Alzheimer's disease pathogenesis. Alzheimers Dement. 2023;19(1):333-42.
139. Bonda DJ, Wang X, Lee H-G, Smith MA, Perry G, Zhu X. Neuronal failure in Alzheimer's disease: a view through the oxidative stress looking-glass. Neuroscience Bulletin. 2014;30(2):243-52.
140. Hirai K, Aliev G, Nunomura A, Fujioka H, Russell RL, Atwood CS, et al. Mitochondrial abnormalities in Alzheimer's disease. J Neurosci. 2001;21(9):3017-23.
141. Bonda DJ, Wang X, Perry G, Smith MA, Zhu X. Mitochondrial dynamics in Alzheimer's disease: opportunities for future treatment strategies. Drugs Aging. 2010;27(3):181-92.
142. Wang X, Su B, Zheng L, Perry G, Smith MA, Zhu X. The role of abnormal mitochondrial dynamics in the pathogenesis of Alzheimer's disease. J Neurochem. 2009;109 Suppl 1(Suppl 1):153-9.
143. Gibson GE, Sheu KF, Blass JP. Abnormalities of mitochondrial enzymes in Alzheimer disease. J Neural Transm (Vienna). 1998;105(8-9):855-70.
144. Wang J, Markesbery WR, Lovell MA. Increased oxidative damage in nuclear and mitochondrial DNA in mild cognitive impairment. J Neurochem. 2006;96(3):825-32.
145. Swerdlow RH, Parks JK, Cassarino DS, Maguire DJ, Maguire RS, Bennett JP, Jr., et al. Cybrids in Alzheimer's disease: a cellular model of the disease? Neurology. 1997;49(4):918-25.
146. Parker WD, Jr., Parks J, Filley CM, Kleinschmidt-DeMasters BK. Electron transport chain defects in Alzheimer's disease brain. Neurology. 1994;44(6):1090-6.
147. Chandrasekaran K, Giordano T, Brady DR, Stoll J, Martin LJ, Rapoport SI. Impairment in mitochondrial cytochrome oxidase gene expression in Alzheimer disease. Brain Res Mol Brain Res. 1994;24(1-4):336-40.
148. Cottrell DA, Blakely EL, Johnson MA, Ince PG, Turnbull DM. Mitochondrial enzyme-deficient hippocampal neurons and choroidal cells in AD. Neurology. 2001;57(2):260-4.
149. Maurer I, Zierz S, Möller HJ. A selective defect of cytochrome c oxidase is present in brain of Alzheimer disease patients. Neurobiology of Aging. 2000;21(3):455-62.
150. Nagy Z, Esiri MM, LeGris M, Matthews PM. Mitochondrial enzyme expression in the hippocampus in relation to Alzheimer-type pathology. Acta Neuropathol. 1999;97(4):346-54.
151. Parker WD, Jr., Mahr NJ, Filley CM, Parks JK, Hughes D, Young DA, et al. Reduced platelet cytochrome c oxidase activity in Alzheimer's disease. Neurology. 1994;44(6):1086-90.

152. Yao PJ, Eren E, Goetzl EJ, Kapogiannis D. Mitochondrial Electron Transport Chain Protein Abnormalities Detected in Plasma Extracellular Vesicles in Alzheimer's Disease. Biomedicines. 2021;9(11).
153. Krishnan KJ, Ratnaike TE, De Gruyter HL, Jaros E, Turnbull DM. Mitochondrial DNA deletions cause the biochemical defect observed in Alzheimer's disease. Neurobiol Aging. 2012;33(9):2210-4.
154. Waschbüsch D, Michels H, Strassheim S, Ossendorf E, Kessler D, Gloeckner CJ, et al. LRRK2 transport is regulated by its novel interacting partner Rab32. PLoS One. 2014;9(10):e111632.
155. Dölle C, Niere M, Lohndal E, Ziegler M. Visualization of subcellular NAD pools and intra-organellar protein localization by poly-ADP-ribose formation. Cell Mol Life Sci. 2010;67(3):433-43.
156. Stein LR, Imai S-i. The dynamic regulation of NAD metabolism in mitochondria. Trends in Endocrinology & Metabolism. 2012;23(9):420-8.
157. Thal DR, Rüb U, Orantes M, Braak H. Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology. 2002;58(12):1791-800.
158. Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del Tredici K. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol. 2006;112(4):389-404.
159. Toker L, Tran GT, Sundaresan J, Tysnes OB, Alves G, Haugarvoll K, et al. Genome-wide histone acetylation analysis reveals altered transcriptional regulation in the Parkinson's disease brain. Mol Neurodegener. 2021;16(1):31.
160. Canet-Avilés RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, et al. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proc Natl Acad Sci U S A. 2004;101(24):9103-8.
161. Junn E, Jang WH, Zhao X, Jeong BS, Mouradian MM. Mitochondrial localization of DJ-1 leads to enhanced neuroprotection. J Neurosci Res. 2009;87(1):123-9.
162. Bacman SR, Gammage PA, Minczuk M, Moraes CT. Chapter 19 - Manipulation of mitochondrial genes and mtDNA heteroplasmy. In: Pon LA, Schon EA, editors. Methods in Cell Biology. 155: Academic Press; 2020. p. 441-87.
163. Backes S, Hess S, Boos F, Woellhaf MW, Gödel S, Jung M, et al. Tom70 enhances mitochondrial preprotein import efficiency by binding to internal targeting sequences. J Cell Biol. 2018;217(4):1369-82.
164. Bayne AN, Dong J, Amiri S, Farhan SMK, Trempe JF. MTSviewer: A database to visualize mitochondrial targeting sequences, cleavage sites, and mutations on protein structures. PLoS One. 2023;18(4):e0284541.
165. Guardia-Laguarta C, Area-Gomez E, Rüb C, Liu Y, Magrané J, Becker D, et al. α-Synuclein Is Localized to Mitochondria-Associated ER Membranes. The Journal of Neuroscience. 2014;34(1):249-59.
166. Škrtić M, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, et al. Inhibition of Mitochondrial Translation as a Therapeutic Strategy for Human Acute Myeloid Leukemia. Cancer Cell. 2011;20(5):674-88.
167. Sasaki K, Uchiumi T, Toshima T, Yagi M, Do Y, Hirai H, et al. Mitochondrial translation inhibition triggers ATF4 activation, leading to integrated stress response but not to mitochondrial unfolded protein response. Biosci Rep. 2020;40(11).
168. Santo-Domingo J, Chareyron I, Broenimann C, Lassueur S, Wiederkehr A. Antibiotics induce mitonuclear protein imbalance but fail to inhibit respiration and nutrient activation in pancreatic β-cells. Experimental Cell Research. 2017;357(2):170-80.
169. Jones CN, Miller C, Tenenbaum A, Spremulli LL, Saada A. Antibiotic effects on mitochondrial translation and in patients with mitochondrial translational defects. Mitochondrion. 2009;9(6):429-37.
170. Pryde KR, Taanman JW, Schapira AH. A LON-ClpP Proteolytic Axis Degrades Complex I to Extinguish ROS Production in Depolarized Mitochondria. Cell Rep. 2016;17(10):2522-31.

171. Huang M, Lou D, Charli A, Kong D, Jin H, Zenitsky G, et al. Mitochondrial dysfunction-induced H3K27 hyperacetylation perturbs enhancers in Parkinson's disease. JCI Insight [Internet]. 2021; 6(17).
172. Gomes Ana P, Price Nathan L, Ling Alvin JY, Moslehi Javid J, Montgomery MK, Rajman L, et al. Declining NAD+ Induces a Pseudohypoxic State Disrupting Nuclear-Mitochondrial Communication during Aging. Cell. 2013;155(7):1624-38.
173. Baixauli F, Acín-Pérez R, Villarroya-Beltrí C, Mazzeo C, Nuñez-Andrade N, Gabandé-Rodriguez E, et al. Mitochondrial Respiration Controls Lysosomal Function during Inflammatory T Cell Responses. Cell Metab. 2015;22(3):485-98.
174. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem. 2003;278(27):25009-13.
175. Yu WH, Dorado B, Figueroa HY, Wang L, Planel E, Cookson MR, et al. Metabolic activity determines efficacy of macroautophagic clearance of pathological oligomeric alpha-synuclein. Am J Pathol. 2009;175(2):736-47.
176. Volpicelli-Daley LA, Luk KC, Patel TP, Tanik SA, Riddle DM, Stieber A, et al. Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. Neuron. 2011;72(1):57-71.
177. Karpowicz RJ, Jr., Trojanowski JQ, Lee VM. Transmission of α-synuclein seeds in neurodegenerative disease: recent developments. Lab Invest. 2019;99(7):971-81.
178. Kam T-I, Mao X, Park H, Chou S-C, Karuppagounder SS, Umanah GE, et al. Poly(ADP-ribose) drives pathologic α-synuclein neurodegeneration in Parkinson’s disease. Science. 2018;362(6414):eaat8407.
179. Wredenberg A, Wibom R, Wilhelmsson H, Graff C, Wiener HH, Burden SJ, et al. Increased mitochondrial mass in mitochondrial myopathy mice. Proc Natl Acad Sci U S A. 2002;99(23):15066-71.
180. Giordano C, Iommarini L, Giordano L, Maresca A, Pisano A, Valentino ML, et al. Efficient mitochondrial biogenesis drives incomplete penetrance in Leber's hereditary optic neuropathy. Brain. 2014;137(Pt 2):335-53.
181. Bell SM, Barnes K, De Marco M, Shaw PJ, Ferraiuolo L, Blackburn DJ, et al. Mitochondrial Dysfunction in Alzheimer's Disease: A Biomarker of the Future? Biomedicines. 2021;9(1).

8. APPENDIX



Appendix Figure 1. Molecular architecture of the generated FLAG and PARP1cd fusion constructs. A. FLAG-5a fusion construct with the ORF encoding the protein of interest (marked in red) in fusion with the C-terminal FLAG peptide epitope for detection. B. PARP1cd fusion construct containing the ORF of the protein of interest (marked in red) and the ORF encoding the catalytic domain of PARP1 (marked in green) and a C-terminal myc tag (marked in white).

SCIENTIFIC ARTICLES

Paper I

Poly-ADP-ribose assisted protein localization resolves that DJ-1, but not LRRK2 or α-synuclein, is localized to the mitochondrial matrix

Nelson Osuagwu, Christian Dölle, Charalampos Tzoulis

PLoS ONE (2019)

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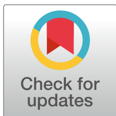

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RESEARCH ARTICLE

Poly-ADP-ribose assisted protein localization resolves that DJ-1, but not LRRK2 or α-synuclein, is localized to the mitochondrial matrix

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Abstract

Several proteins linked to familial Parkinson disease have been associated with mitochondrial (dys-)function and have been described to reside within mitochondria. The putative mitochondrial and sub-mitochondrial localization of these proteins remains disputed, however, potentially due to conflicting results obtained by diverging technical approaches. Using the high-resolution poly-ADP-ribose assisted protein localization assay that also allows for detection of low level and even partial mitochondrial matrix localization, we demonstrate here that DJ-1, but not LRRK2 or α-synuclein, resides in the mitochondrial matrix. The localization of the proteins was not changed in cellular stress models of Parkinson disease and, in case of α-synuclein, not affected by pathological mutations.

Our results verify the ability of DJ-1 to carry out its role also from within mitochondria and suggest that LRRK2 and α-synuclein may interact with and affect mitochondria from outside the mitochondrial matrix.

Introduction

Parkinson disease (PD) is a complex disorder influenced by both genetic and environmental factors [1–4]. However, only about 10% of all cases can be linked to genetic causes [5], thus the majority of cases are sporadic with unknown aetiology. While disease mechanisms still remain largely unclear, it is now well established that mitochondrial dysfunction plays a central role in both familial and sporadic PD [6]. This includes, among others, changes in mitochondrial quality control pathways such as mitochondrial DNA homeostasis and mitophagy [7,8], as well as metabolic changes such as complex I deficiency of the mitochondrial respiratory chain [9,10]. Interestingly, several proteins that have been genetically linked to PD are involved in mitochondrial homeostasis and quality control, including PTEN-induced kinase 1 (PINK1), Parkin, DJ-1 and Leucine-rich repeat kinase 2 (LRRK2) [11–13]. PINK1 and Parkin have been described to protect mitochondria against cellular damage and mediate clearance of damaged mitochondria by mitophagy [14]. LRRK2, a protein kinase, is the most commonly mutated

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protein in familial PD cases [15], while DJ-1 is reported to have neuroprotective function in PD models [16]. α-synuclein, the major component of Lewy bodies found in PD brains, has also been implicated in mitochondrial dysfunction, for example by affecting mitochondrial complex I activity [17].

Several of these proteins have been described to localize partially or entirely to mitochondria, however, in some cases current evidence is conflicting. For example, it is widely established that PINK1 localizes to the mitochondria and is required for Parkin recruitment to the organelles to orchestrate the process of mitophagy [18–20]. DJ-1 has been described to be partially localized to the mitochondria, but its sub-mitochondrial localization remains unclear. Some reports described LRRK2 to be associated with mitochondria [21], while others could not recapitulate these findings [22]. For α-synuclein, few reports suggested mitochondrial localization based on interaction with mitochondrial proteins. Thus, conflicting reports indicate that the mitochondrial localization of DJ-1, LRRK2 and α-synuclein, and particularly the exact sub-mitochondrial localization, which has a direct impact on putative function and interaction, remains to be resolved.

In part, this discrepancy may be due to technical limitations of the most common approaches, such as subcellular fractionation and immunocytochemistry. While subcellular fractionation may present false positive results due to fraction contamination, conventional immunocytochemistry does not distinguish between *intra*-organellar localization and mere association with the organelle from outside.

The recently established *poly-ADP-ribose assisted protein localization assay* (PARAPLAY) resolves this problem of conventional immunocytochemistry and is able to conclusively establish intra-organellar localization [23]. The protein of interest is fused to the catalytic domain of poly-ADP-ribose polymerase 1 (PARP1), termedPARP1cd, which uses NAD^{+} as substrate to generate the immunodetectable biopolymer poly-ADP-ribose (PAR). The subcellular localization of the fusion construct is entirely dependent on the protein of interest. PAR formation is only detectable in the lumen of organelles where sufficient substrate concentration and absence of strong PAR-degrading activity allow for accumulation of PAR [23,24]. Expression of a cytosolic PARP1cd-fusion construct does not lead to detectable PAR formation [23]. Importantly, in mitochondria only proteins that reside in the mitochondrial matrix are able to generate detectable PAR levels, whereas association with the organelle from the outside or intermembrane space localization does not support a robust PAR signal [23]. By combining detection of the recombinant protein itself with the use of PAR formation as readout, intra-organellar (in this study: mitochondrial matrix) localization of the protein is readily established. Importantly, the assay is capable of revealing even partial intra-mitochondrial localization, i.e. when the majority of the protein resides in the cytosol and organellar structures are "hidden" under the cytosolic signal [23].

Here, we investigated three proteins, DJ-1, LRRK2 and α-synuclein, which are all linked to monogenic cases of familial PD, and which have been described to be associated with or located to mitochondria. Using PARAPLAY, we show that DJ-1 is partially localized to the mitochondrial matrix in addition to its cytosolic localization, which remains undiscovered using conventional immunocytochemistry and protein detection alone. In contrast, LRRK2 and α-synuclein do not exhibit intra-mitochondrial localization.

Materials and methods

Chemicals, reagents and media

The following antibodies were used: rabbit and mouse (10H) anti poly-ADP-ribose (ALX-210-890A-01900 and ALX-804-220, respectively, Enzo Life Sciences), rabbit and mouse (M2) anti

FLAG (F7425 and F3165, respectively, Sigma-Aldrich), mouse anti myc (9E10, TA150121, Origene) and rabbit anti NDUFB10 (ab196019, Abcam). Secondary antibodies goat anti mouse 488 (A11001), goat anti rabbit 594 (A11012) and goat anti rabbit 647 (A21245) were from Life Technologies. DNA-modifying and restriction enzymes were from Thermo Fisher Scientific. All cell culture reagents were from Life Technologies, except Carbonyl-cyanide 3-chlorophenyhydrazone (CCCP) and paraquat (Sigma-Aldrich).

Generation of eukaryotic expression vectors

The open reading frame (ORF) encoding full-length DJ-1 was amplified from HEK293 cells cDNA, while the ORFs encoding the N-terminal domain of LRRK2 (amino acids 1–266, [25]) and full-length α-synuclein were amplified from pre-existing plasmids (Addgene: pDEST53-LRRK2-WT #25044 and EGFP-alpha synuclein-WT #40822, respectively). All ORFs were inserted into pFLAG-CMV-5a (Sigma-Aldrich) and pcDNA3.1(+)-PARP1cd [23] vectors. ORFs encoding α-synuclein mutants (E46K and A53T) were generated by PCR-based site-directed mutagenesis. All cloned DNA sequences were verified by DNA sequence analysis.

Cell culture

HeLa S3 cells were cultivated in Ham's F12 Glutamax nutrient growth medium and SH-SY5Y cells were cultivated in DMEM/Ham‘s F12 (1:1) Glutamax medium, both supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37 ˚C in a humidified atmosphere with 5% CO_2. Transient transfection was performed using Effectene reagent (Qiagen) according to the manufacturer's specifications. For pharmacological treatments, 24 h after transfection cells were incubated with 20 μM CCCP for 6 hours or 1 mM (SH-SY5Y) or 2 mM paraquat (HeLa S3) for 24 hours, prior to immunocytochemistry analysis.

Immunocytochemistry

Cells grown on coverslips were fixed with 3.7% (v/v) paraformaldehyde in PBS at 4 ˚C for 45 min, followed by permeabilization with 0.5% (v/v) Triton X-100 in PBS for 15 min at room temperature. In some cases, cells were treated with 200 nM Mito Tracker Red CMXRos (Sigma-Aldrich) for 30 min at 37 ˚C prior to fixation. A blocking step with growth medium containing 10% (v/v) FBS for 1 h at room temperature was followed by overnight incubation with primary antibodies in growth medium at 4 ˚C. After washing 4 times for 5 min in PBS, the cells were further incubated with secondary antibodies in growth medium for 1 h at room temperature. Cells were washed once for 5 min with PBS and the nuclei were stained with DAPI. After washing twice for 5 min in PBS, the coverslips were mounted with ProLong Diamond Antifade (Invitrogen). Images were acquired using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems) with a 100x oil immersion objective (numerical aperture 1.40).

Results

PARAPLAY analysis reveals partial localization of DJ-1 to the mitochondrial matrix

In order to investigate the subcellular localization of the proteins of interest in detail, we first overexpressed DJ-1, α-synuclein and the N-terminal domain of LRRK2 (subsequently referred to as "LRRK2") as FLAG-tagged recombinant proteins which were subsequently detected by conventional immunocytochemistry. DJ-1, LRRK2 and α-synuclein were detected in the

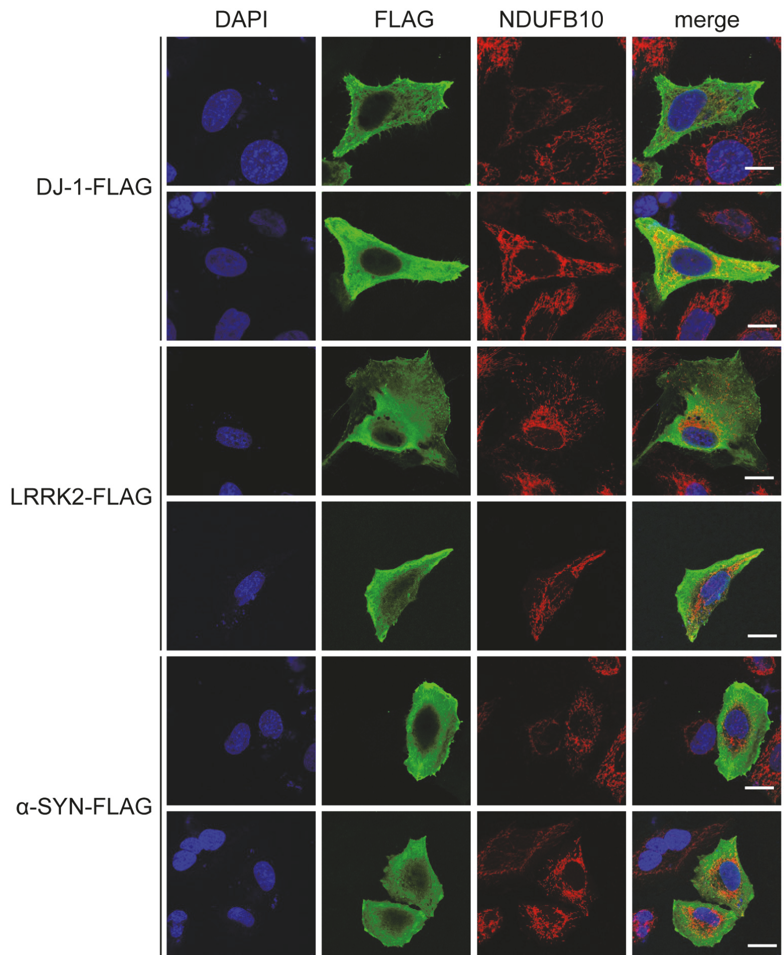

Fig 1. Apparent cytosolic localization of recombinant DJ-1, LRRK2 and α-synuclein. HeLa S3 cells were transiently transfected with constructs encoding C-terminally FLAG-tagged DJ-1, LRRK2 and α-synuclein and subjected to indirect FLAG-immunocytochemistry. Two fluorescence images for each fusion construct are shown, displaying the overexpressed proteins (FLAG), mitochondria (NDUFB10) and the nuclei (DAPI). Scale bar: 10 μm.

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cytosol and did not show any colocalization with mitochondrial structures detected by the mitochondrial marker NDUFB10 (Fig 1).

Next, we performed PAR-assisted protein localization assay, PARAPLAY [23], using constructs of DJ-1, LRRK2 and α-synuclein C-terminally fused to PARP1cd and a myc-epitope for protein detection. Again, when overexpressed in cells, DJ-1, α-synuclein and LRRK2 fusion proteins were detected in the cytosol and did not show colocalization with mitochondria (Fig 2A, S1A Fig). This confirmed that fusion to PAPR1cd did not change subcellular localization.

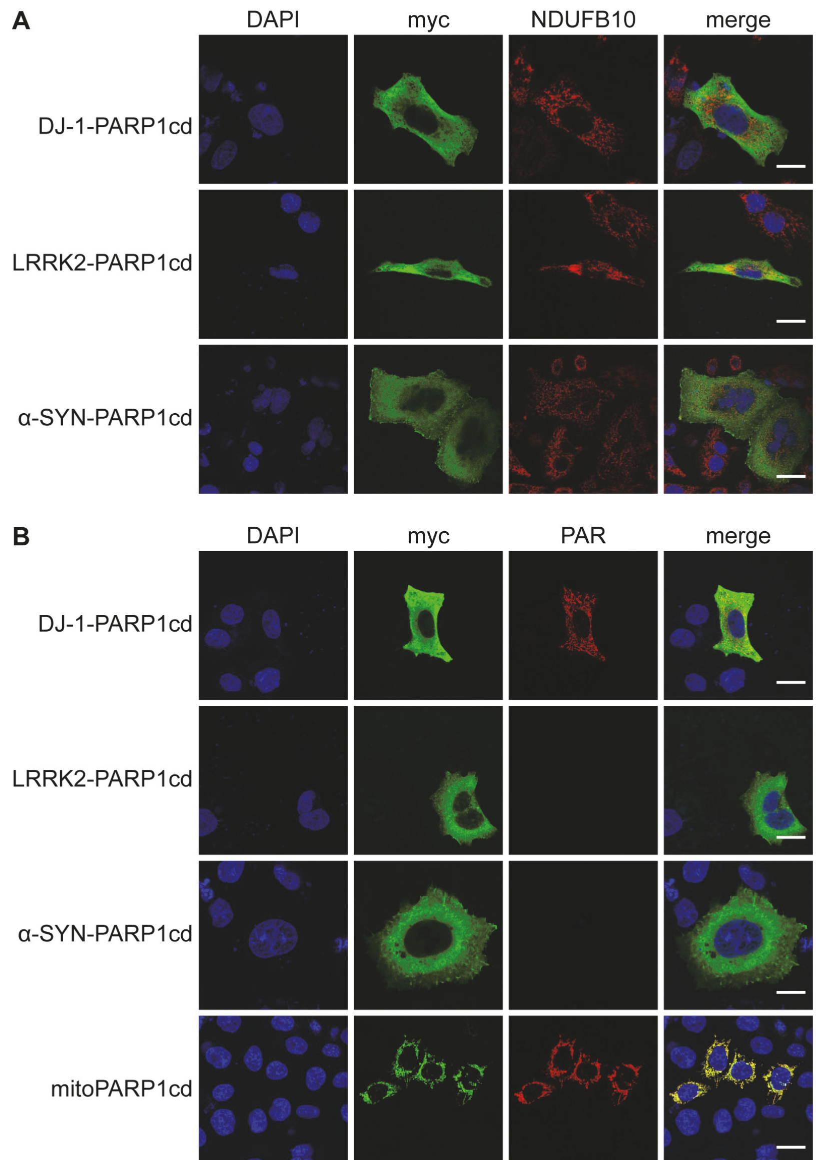

Fig 2. Recombinant DJ-1, but not LRRK2 and α-synuclein, localizes partially to the mitochondrial matrix as revealed by PARAPLAY. HeLa S3 cells were transiently transfected with PARP1cd fusion constructs of DJ-1, LRRK2 and α-synuclein and subjected to indirect immunocytochemistry, detecting the recombinant protein by its myc-epitope and either a mitochondrial marker (A) or PAR accumulation (B). (A) The fluorescent images show the overexpressed proteins (myc), mitochondria (NDUFB10) and the nuclei (DAPI). (B) The fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR) and the nuclei (DAPI). The mitochondrial matrix-targeted fusion protein mitoPARP1cd served as positive control for intra-mitochondrial PAR formation. Scale bar: 10 μm.

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Importantly, when cells were subjected to PAR immunocytochemistry, cells overexpressing the DJ-1-PARP1cd fusion protein showed a positive PAR signal that did not colocalize with the diffuse, cytosolic pattern of the detected protein (Fig 2B, S1B Fig), but in fact colocalized with mitochondria (S2 Fig). Using a mitochondrial matrix-targeted EGFP-PARP1cd-fusion protein, termed mitoPARP1cd [24], we confirmed the specificity of the PAR signal (Fig 2B, S1B Fig). In contrast, PAR formation was not observed for the fusion constructs of α-synuclein or LRRK2 (Fig 2B, S1B Fig). We repeated this experiment using the neuroblastoma SH-SY5Y cell line and confirmed that only DJ-1-PARP1cd expression led to detectable polymer formation, while expression of all other constructs did not (S3 Fig). These results indicated that a portion of DJ-1 localized to the mitochondrial matrix, while α-synuclein and LRRK2 did not.

Subcellular localization is unchanged in toxin-induced models of PD

Previous studies have linked mitochondrial dysfunction and loss of membrane potential to PD. In some cases, these changes of mitochondrial membrane potential could affect protein localization and lead to protein accumulation within mitochondria [18,26]. We therefore tested whether the loss of mitochondrial membrane potential could influence the localization of the proteins of interest in our system. Upon treatment with the ionophore CCCP, the mitochondrial membrane potential collapsed (S4 Fig). Overexpression of mitoPARP1cd still resulted in PAR accumulation, indicating that the treatment did not interfere with the detection system (Fig 3A, S5A Fig). More importantly, while PAR formation was still detected for DJ-1-PARP1cd (Fig 3A, S5A Fig), there was no detectable increase in PAR formation or change in the detection of the protein in the cytosol (S6 Fig). This indicated that the partial mitochondrial matrix localization of DJ-1 protein was not affected and likely not to be dependent on the mitochondrial membrane potential. LRRK2 and α-synuclein still did not reveal any detectable PAR formation, suggesting that also their subcellular localization was unaltered and these proteins were not localized to the mitochondrial matrix under stress conditions (Fig 3A, S5A and S6 Figs).

Paraquat is a chemical toxin that causes a PD-like phenotype in rodents [27] and affects mitochondrial function, leading to production of reactive oxygen species and cell death [28]. As representative for parkinsonism-inducing agents, we next investigated the effect of paraquat on the subcellular localization of these proteins. Pharmacological treatment with paraquat for 24 h did not lead to increased association of the overexpressed proteins with mitochondria (S7 Fig). Moreover, the PAR signal resulting from DJ-1-PARP1cd overexpression was still present, but not increased, while no PAR formation was detectable in case of LRRK2 and α-synuclein (Fig 3B, S5B Fig). Prolonged incubation with paraquat for up to 48 h did not change protein localization or led to detectable PAR formation.

In order to exclude cell type-specific effects we reproduced the experiments under cellular stress conditions in SH-SY5Y cells. Importantly, neither CCCP nor paraquat treatment changed the detectable PAR formation for any of the fusion constructs also in these cells (S8 Fig).

α-synuclein mutations A53T and E46K do not mediate mitochondrial matrix localization

It has been described that pathogenic α-synuclein mutations are associated with mitochondrial dysfunction, such as increased degree of mitochondrial fragmentation [29]. We hypothesized that these effects may be partly mediated by mitochondrial relocation of the mutant

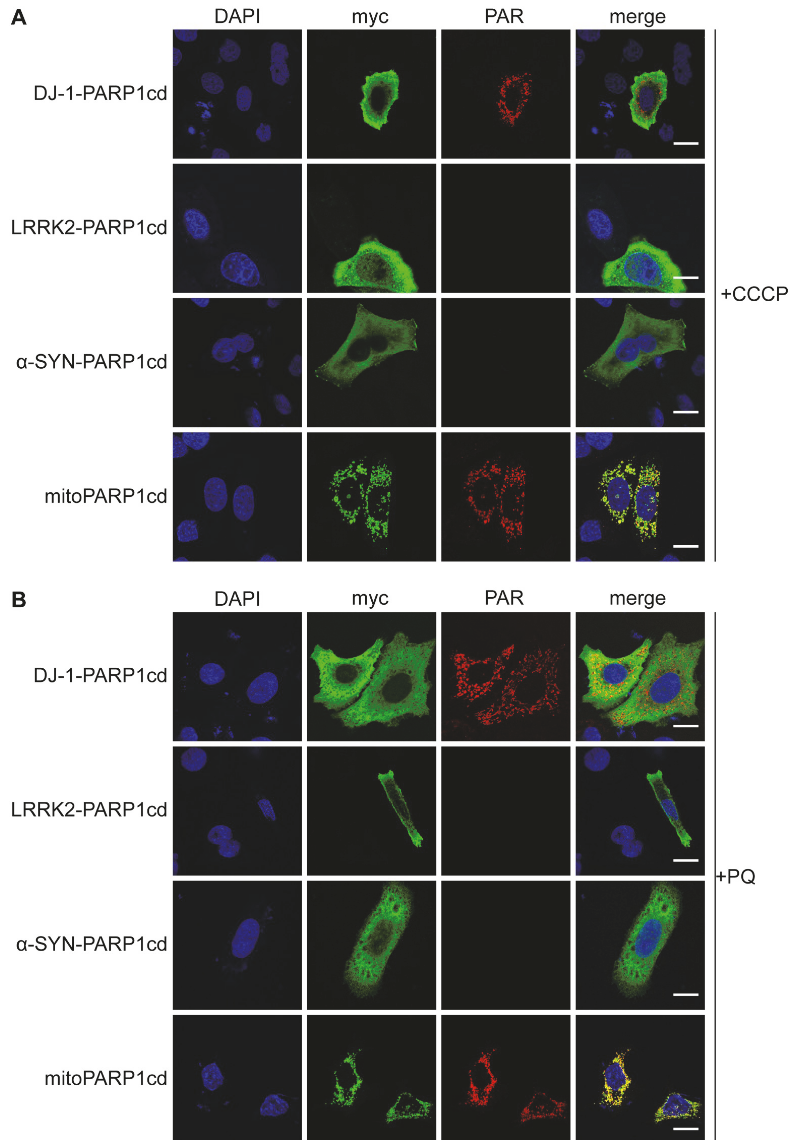

Fig 3. CCCP and paraquat treatment does not affect subcellular localization of recombinant DJ-1, LRRK2 and α-synuclein. Transiently transfected HeLa S3 cells were treated 24 hours after transfection with 20 μM CCCP for 6 hours (A) or 2 mM paraquat (PQ) for 24 hours (B) and subjected to myc and PAR immunocytochemistry. The fluorescent images show overexpressed proteins (myc), PAR accumulation (PAR) and the nuclei (DAPI). MitoPARP1cd served as positive control. Scale bar: 10 μm.

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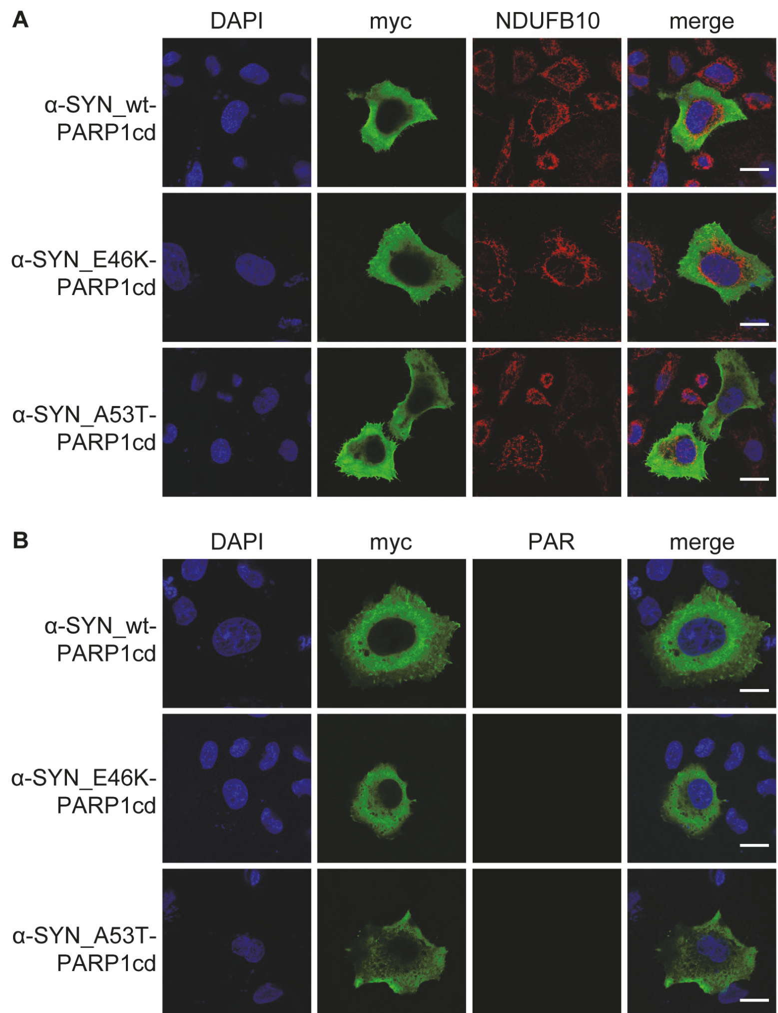

Fig 4. α-synuclein mutant forms are absent from mitochondrial matrix. HeLa S3 cells were transiently transfected with PARP1cd fusion constructs of α-synuclein wild type (wt) and mutants (E46K and A53T) and subsequently subjected to indirect immunocytochemistry detecting the recombinant protein by its myc epitope and either a mitochondrial marker (A) or PAR accumulation (B). (A) The fluorescent images show the overexpressed proteins (myc), mitochondria (NDUFB10) and nuclei (DAPI). (B) The fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR) and the nuclei (DAPI). Scale bar: 10 μm.

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α-synuclein protein. To test this hypothesis, we investigated whether the common α-synuclein mutations A53T and E46K lead to the relocation of the protein to the mitochondrial matrix.

PARAPLAY analysis revealed the absence of the recombinant proteins from mitochondria (Fig 4). An apparent cytosolic localization was detected for the PARP1cd fusion constructs

(Fig 4A, S1A Fig) and PAR formation was not observed for either of the mutated proteins in both HeLa S3 (Fig 4B, S1B Fig) and SH-SY5Y cells (S3 Fig), similar to the wild type protein. Furthermore, cytotoxic stress by loss of mitochondrial membrane potential (S9 Fig) or paraquat treatment (S10 Fig) did not change the subcellular localization of the mutated proteins towards the intra-mitochondrial compartment in both HeLa S3 (S9 and S10 Figs) and SH-S5Y5 cells (S11 Fig).

Discussion

The involvement of mitochondrial dysfunction in PD is widely accepted, yet its exact role and contribution to the progression of the disease remains elusive. The determination of the exact subcellular localizations of PD-linked proteins is one major step to elucidate their contribution to mitochondrial dysfunction and to reveal underlying mechanisms. We report here that DJ-1 is present in the mitochondrial matrix under normal and stress conditions in human cells, while LRRK2 and α-synuclein are absent from the mitochondrial matrix.

The subcellular distribution of DJ-1 has previously been addressed in several studies and it has been reported to localize to the nucleus, the cytosol and the mitochondria. However, especially with regard to its putative sub-mitochondrial distribution, varying and partially conflicting results were reported. Previous studies showed, among others, an association with the mitochondrial outer membrane [30] and that DJ-1 localizes to the mitochondria only in some circumstances or relocates to mitochondria upon cellular stress [31]. Others stated that only mutant DJ-1, but not wild type, localizes to the mitochondrial matrix [32]. Our demonstration that wild type DJ-1 is already present in the mitochondrial matrix under normal conditions further corroborates its important role for mitochondria, enabling it to carry out its cytoprotective function directly at the site of putative oxidative damage and to immediately react to potential insults. Moreover, the loss-of-function mutations found in PD are thus more prone to affect mitochondrial function directly.

In contrast to DJ-1, LRRK2 did not localize to the mitochondrial matrix under any conditions tested in this study. Previously, localization to membranous and vesicular structures in the human brain [33] and a partial association with the outer mitochondrial membrane [21] have been reported for LRRK2, while other studies found LRRK2 mainly in the cytosol and could not recapitulate mitochondrial association [22]. We also detected the recombinant LRRK2 protein rather distributed throughout the cytosol. However, as our results for DJ-1 clearly showed, apparent cytosolic distribution may conceal association with organelles and therefore, we cannot rule out any association with mitochondria or other organelles from the outside. Moreover, we cannot completely rule out the possibility that the full length LRRK2 protein still may localize to mitochondria, although it is in the N-terminal domain where a putative mitochondrial targeting sequence is most often located. However, the absence of our fusion protein from the mitochondrial matrix demonstrated by the lack of PAR signal is consistent with the proposed role of LRRK2 in mitochondrial dynamics, for example by functional interaction with proteins regulating mitochondrial fission (Drp1) and fusion (mitofusins and OPA1) [34].

The endogenous function of α-synuclein has yet to be fully elucidated and defining its subcellular distribution is of great importance in this regard. While it was originally described to localize to the nucleus and the cytosol, more recent findings indicated also localization to mitochondria [35] and mitochondria-associated ER membranes [29]. Especially its described interaction with complex I of the mitochondrial respiratory chain and ATP synthase, both localized to the inner mitochondrial membrane, raised the question whether this interaction results from binding from the outside or inside of the organelle.

Our results strongly suggest that α-synuclein is absent from the mitochondrial matrix, both under normal and stress conditions. Moreover, PD-causing mutations were also not localized to the mitochondrial matrix. This does not contradict the putative role of α-synuclein in mitochondrial dysfunction and interaction with complexes of the mitochondrial respiratory chain, but rather reveals that possible interaction would necessarily take place on the intermembrane space facing part of these complexes. This possibility is further supported by the recent report of protein interaction between α-synuclein and TOM20, a transport protein localized in the outer mitochondrial membrane [36].

Conclusion

Our study confirms that the exact determination of subcellular protein distribution in general is challenging and that conclusions need to be drawn with care, based on reliable and well-controlled results. This is particularly true for mitochondria, which contain multiple closely connected sub-organellar compartments. On the other hand, while we here report the *lack* of intra-mitochondrial localization for two of the investigated proteins, our results also suggest that there may be (in fact, many) more proteins *partially* localized to mitochondria and especially the mitochondrial matrix, which would open a whole new spectrum of interaction partners, substrates and functional impact for those candidates.

We therefore suggest that reported intra-mitochondrial protein localization, or lack thereof, based on conventional immunodetection is evaluated with care, as partial intra-organellar localization is easily missed. We recommend the straight-forward PARAPLAY approach as additional detection system in case of suspected organellar association and impact of proteins of interest on organellar, especially mitochondrial function.

Supporting information

S1 Fig. Recombinant DJ-1, but not LRRK2 and wildtype or mutant α-synuclein, localizes partially to the mitochondrial matrix as revealed by PARAPLAY in HeLa S3 cells. Additional images of HeLa S3 cells transiently transfected with PARP1cd fusion constructs of DJ-1, LRRK2 and α-synuclein wild-type or PD-relevant mutants and subjected to indirect immunocytochemistry, detecting the recombinant protein by its myc-epitope and either a mitochondrial marker (A) or PAR accumulation (B) are shown. (A) The fluorescent images show the overexpressed proteins (myc), mitochondria (NDUFB10) and the nuclei (DAPI). (B) The fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR) and the nuclei (DAPI). The mitochondrial matrix-targeted fusion protein mitoPARP1cd served as positive control for intra-mitochondrial PAR formation. Scale bar: 10 μm.
(TIF)

S2 Fig. DJ-1-PARP1cd dependent PAR formation localizes to mitochondria. HeLa S3 cells were transiently transfected with DJ-1-PARP1cd fusion construct and subjected to immunocytochemical analysis. The fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR), mitochondria (NDUFB10) and nuclei (DAPI). Scale bar: 10 μm.
(TIF)

S3 Fig. Recombinant DJ-1, but not LRRK2 and wildtype or mutant α-synuclein, localizes partially to the mitochondrial matrix in SH-SY5Y cells. Neuroblastoma SH-SY5Y cells were transiently transfected with PARP1cd-fusion constructs of DJ1, LRRK2, α -synuclein wild type and PD-relevant α-synuclein mutants and subjected to indirect immunocytochemistry detecting the recombinant protein by its myc-epitope and PAR accumulation. The fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR) and the nuclei

(DAPI). The mitochondrial matrix-targeted fusion protein mitoPARP1cd served as positive control for intra-mitochondrial PAR formation. Scale bar: 10 μm.
(TIF)

S4 Fig. Loss of mitochondrial membrane potential upon CCCP treatment. HeLa S3 cells were stained with membrane potential dependent MitoTracker Red CMXRos (MT) after incubation in absence or presence of 20 μM CCCP. DAPI staining of nuclei is shown in blue. Scale bar: 10 μm.
(TIF)

S5 Fig. CCCP and paraquat treatment does not affect subcellular localization of recombinant DJ-1, LRRK2 and α-synuclein in HeLa S3 cells. Additional images of transiently transfected HeLa S3 cells treated 24 hours after transfection with 20 μM CCCP for 6 hours (A) or 2 mM paraquat (PQ) for 24 hours (B) and subjected to myc and PAR immunocytochemistry are shown. The fluorescent images show overexpressed proteins (myc), PAR accumulation (PAR) and the nuclei (DAPI). MitoPARP1cd served as positive control. Scale bar: 10 μm.
(TIF)

S6 Fig. Loss of mitochondrial membrane potential does not affect subcellular localization of recombinant DJ-1, LRRK2 and α-synuclein. HeLa S3 cells were transiently transfected with either FLAG-tagged (A) or PARP1cd (B) fusion constructs of DJ-1, LRRK2 and α-synuclein and treated 24 hours after transfection with 20 μM CCCP for 6 hours followed by indirect immunocytochemistry. The fluorescent images show the overexpressed proteins (FLAG (A) or myc (B)), mitochondria (NDUFB10) and the nuclei (DAPI). Scale bar: 10 μm.
(TIF)

S7 Fig. Paraquat treatment does not affect subcellular localization of recombinant DJ-1, LRRK2 and α-synuclein. HeLa S3 cells transiently transfected with either FLAG-tagged (A) or PARP1cd (B) fusion constructs of DJ-1, LRRK2 and α-synuclein were treated 24 hours after transfection with 2 mM paraquat for 24 hours followed by indirect immunocytochemistry. The fluorescent images show the overexpressed proteins (FLAG (A) or myc (B)), mitochondria (NDUFB10) and the nuclei (DAPI). Scale bar: 10 μm.
(TIF)

S8 Fig. CCCP and paraquat treatment does not alter subcellular localization of DJ-1, LRRK2 and α -synuclein in SH-SY5Y cells. SH-S5Y5 cells, transiently transfected with PARP1cd fusion constructs of DJ-1, LRRK2 or α-synuclein, were treated 24 hours after transfection with 20 μM CCCP for 6 hours (A) or 1 mM paraquat for 24 hours (B) and subsequently subjected to indirect immunocytochemistry, detecting the recombinant protein and PAR accumulation (B). The fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR) and nuclei (DAPI). Scale bar: 10 μm.
(TIF)

S9 Fig. Loss of mitochondrial membrane potential does not affect subcellular localization of α-synuclein mutants. HeLa S3 cells, transiently transfected with PARP1cd fusion constructs of α-synuclein wt and mutants (E46K and A53T), were treated 24 hours after transfection with 20 μM CCCP for 6 hours and subsequently subjected to indirect immunocytochemistry, detecting the recombinant protein by its myc epitope and either a mitochondrial marker (A) or PAR accumulation (B). A) The fluorescent images show the overexpressed proteins (myc), mitochondria (NDUFB10) and nuclei (DAPI). Scale bar: 10 μm. (B) The

fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR) and the nuclei (DAPI). Scale bar: 10 μm.
(TIF)

S10 Fig. Paraquat treatment does not affect subcellular localization of α-synuclein mutants. HeLa S3 cells, transiently transfected with PARP1cd fusion constructs of α-synuclein wt and mutants (E46K and A53T), were treated 24 hours after transfection with 2 mM paraquat for 24 hours and subsequently subjected to indirect immunocytochemistry detecting the recombinant protein by its myc epitope and either a mitochondrial marker (A) or PAR accumulation (B). (A) The fluorescent images show the overexpressed proteins (myc), mitochondria (NDUFB10) and nuclei (DAPI). Scale bar: 10 μm. (B) The fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR) and the nuclei (DAPI). Scale bar: 10 μm.
(TIF)

S11 Fig. CCCP and paraquat treatment does not affect subcellular localization of α-synuclein mutants in SH-SY5Y cells. SH-SY5Y cells transiently transfected with PARP1cd-fusion constructs of α-synuclein wt and mutants (E46K and A53T), were treated 24 hours after transfection with 20 μM CCCP for 6 hours (A) or 1 mM paraquat (PQ) for 24 hours (B) and subsequently subjected to indirect immunocytochemistry detecting the recombinant protein and PAR accumulation. The fluorescent images show the overexpressed proteins (myc), PAR accumulation (PAR) and nuclei (DAPI). Scale bar: 10 μm.
(TIF)

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References

1. Klein C, Westenberger A. Genetics of Parkinson's Disease. Cold Spring Harbor Perspectives in Medicine. 2012; 2(1). https://doi.org/10.1101/cshperspect.a008888 PMID: 22315721.
2. Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science. 1983; 219(4587):979–80. Epub 1983/02/25. https://doi.org/10.1126/science.6823561 PMID: 6823561.

3. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, et al. Rotenone, paraquat, and Parkinson's disease. Environmental health perspectives. 2011; 119(6):866–72. Epub 2011/01/29. https://doi.org/10.1289/ehp.1002839 PMID: 21269927.
4. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nature neuroscience. 2000; 3 (12):1301–6. Epub 2000/12/02. https://doi.org/10.1038/81834 PMID: 11100151.
5. Papapetropoulos S, Adi N, Ellul J, Argyriou AA, Chroni E. A prospective study of familial versus sporadic Parkinson's disease. Neuro-degenerative diseases. 2007; 4(6):424–7. Epub 2007/10/16. https://doi.org/10.1159/000107702 PMID: 17934325.
6. Hu Q, Wang G. Mitochondrial dysfunction in Parkinson's disease. Translational neurodegeneration. 2016; 5:14-. https://doi.org/10.1186/s40035-016-0060-6 PMID: 27453777.
7. Dölle C, Flønes I, Nido GS, Miletic H, Osuagwu N, Kristoffersen S, et al. Defective mitochondrial DNA homeostasis in the substantia nigra in Parkinson disease. Nature communications. 2016; 7:13548-. https://doi.org/10.1038/ncomms13548 PMID: 27874000.
8. Pickrell AM, Youle RJ. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. Neuron. 2015; 85(2):257–73. https://doi.org/10.1016/j.neuron.2014.12.007 PMID: 25611507.
9. Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. Lancet (London, England). 1989; 1(8649):1269. Epub 1989/06/03. https://doi.org/10.1016/s0140-6736(89)92366-0 PMID: 2566813.
10. Flønes IH, Fernandez-Vizarra E, Lykouri M, Brakedal B, Skeie GO, Miletic H, et al. Neuronal complex I deficiency occurs throughout the Parkinson's disease brain, but is not associated with neurodegeneration or mitochondrial DNA damage. Acta neuropathologica. 2018; 135(3):409–25. https://doi.org/10.1007/s00401-017-1794-7 PMID: 29270838
11. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nature cell biology. 2010; 12(2):119–31. Epub 2010/01/26. https://doi.org/10.1038/ncb2012 PMID: 20098416.
12. McCoy MK, Cookson MR. DJ-1 regulation of mitochondrial function and autophagy through oxidative stress. Autophagy. 2011; 7(5):531–2. Epub 2011/02/15. https://doi.org/10.4161/auto.7.5.14684 PMID: 21317550.
13. Wang X, Yan MH, Fujioka H, Liu J, Wilson-Delfosse A, Chen SG, et al. LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1. Human Molecular Genetics. 2012; 21 (9):1931–44. https://doi.org/10.1093/hmg/dds003 PMID: 22228096
14. Jin SM, Youle RJ. PINK1- and Parkin-mediated mitophagy at a glance. Journal of Cell Science. 2012; 125(4):795–9. https://doi.org/10.1242/jcs.093849 PMID: 22448035
15. Kumari U, Tan EK. LRRK2 in Parkinson's disease: genetic and clinical studies from patients. Febs j. 2009; 276(22):6455–63. Epub 2009/10/07. https://doi.org/10.1111/j.1742-4658.2009.07344.x PMID: 19804413.
16. Ariga H, Takahashi-Niki K, Kato I, Maita H, Niki T, Iguchi-Ariga SMM. Neuroprotective function of DJ-1 in Parkinson's disease. Oxidative medicine and cellular longevity. 2013; 2013:683920-. Epub 05/16. https://doi.org/10.1155/2013/683920 PMID: 23766857.
17. Chinta SJ, Mallajosyula JK, Rane A, Andersen JK. Mitochondrial alpha-synuclein accumulation impairs complex I function in dopaminergic neurons and results in increased mitophagy in vivo. Neuroscience letters. 2010; 486(3):235–9. Epub 2010/10/05. https://doi.org/10.1016/j.neulet.2010.09.061 PMID: 20887775.
18. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. The Journal of cell biology. 2008; 183(5):795–803. Epub 2008/11/26. https://doi.org/10.1083/jcb.200809125 PMID: 19029340.
19. Narendra DP, Jin SM, Tanaka A, Suen D-F, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS biology. 2010; 8(1):e1000298-e. https://doi.org/10.1371/journal.pbio.1000298 PMID: 20126261.
20. Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. The Journal of Cell Biology. 2010; 189(2):211–21. https://doi.org/10.1083/jcb.200910140 PMID: 20404107
21. West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW, Ross CA, et al. Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102(46):16842–7. https://doi.org/10.1073/pnas.0507360102 PMID: 16269541
22. Greggio E, Lewis PA, van der Brug MP, Ahmad R, Kaganovich A, Ding J, et al. Mutations in LRRK2/dardarin associated with Parkinson disease are more toxic than equivalent mutations in the homologous

kinase LRRK1. Journal of neurochemistry. 2007; 102(1):93–102. Epub 2007/03/31. https://doi.org/10.1111/j.1471-4159.2007.04523.x PMID: 17394548.

23. Dölle C, Niere M, Lohndal E, Ziegler M. Visualization of subcellular NAD pools and intra-organellar protein localization by poly-ADP-ribose formation. Cellular and Molecular Life Sciences. 2010; 67(3):433–43. https://doi.org/10.1007/s00018-009-0190-4 PMID: 19902144

24. Niere M, Mashimo M, Agledal L, Dölle C, Kasamatsu A, Kato J, et al. ADP-ribosylhydrolase 3 (ARH3), Not Poly(ADP-ribose) Glycohydrolase (PARG) Isoforms, Is Responsible for Degradation of Mitochondrial Matrix-associated Poly(ADP-ribose). Journal of Biological Chemistry. 2012; 287(20):16088–102. https://doi.org/10.1074/jbc.M112.349183 PMID: 22433848

25. Waschbüsch D, Michels H, Strassheim S, Ossendorf E, Kessler D, Gloeckner CJ, et al. LRRK2 Transport Is Regulated by Its Novel Interacting Partner Rab32. PLOS ONE. 2014; 9(10):e111632. https://doi.org/10.1371/journal.pone.0111632 PMID: 25360523

26. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS biology. 2010; 8(1):e1000298. Epub 2010/02/04. https://doi.org/10.1371/journal.pbio.1000298 PMID: 20126261.

27. Thiruchelvam M, Richfield EK, Baggs RB, Tank AW, Cory-Slechta DA. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2000; 20(24):9207–14. Epub 2000/01/11. PMID: 11124998.

28. Jang YJ, Won JH, Back MJ, Fu Z, Jang JM, Ha HC, et al. Paraquat Induces Apoptosis through a Mitochondria-Dependent Pathway in RAW264.7 Cells. Biomolecules & Therapeutics. 2015; 23(5):407–13. https://doi.org/10.4062/biomolther.2015.075 PMID: 26336579

29. Guardia-Laguarta C, Area-Gomez E, Rub C, Liu Y, Magrane J, Becker D, et al. alpha-Synuclein is localized to mitochondria-associated ER membranes. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2014; 34(1):249–59. Epub 2014/01/02. https://doi.org/10.1523/jneurosci.2507-13.2014 PMID: 24381286.

30. Canet-Aviles RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, et al. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proc Natl Acad Sci U S A. 2004; 101(24):9103–8. Epub 2004/06/08. https://doi.org/10.1073/pnas.0402959101 PMID: 15181200.

31. Junn E, Jang WH, Zhao X, Jeong BS, Mouradian MM. Mitochondrial Localization of DJ-1 Leads to Enhanced Neuroprotection. Journal of neuroscience research. 2009; 87(1):123–9. https://doi.org/10.1002/jnr.21831 PMID: 18711745

32. Kojima W, Kujuro Y, Okatsu K, Bruno Q, Koyano F, Kimura M, et al. Unexpected mitochondrial matrix localization of Parkinson's disease-related DJ-1 mutants but not wild-type DJ-1. Genes to cells: devoted to molecular & cellular mechanisms. 2016; 21(7):772–88. Epub 2016/06/09. https://doi.org/10.1111/gtc.12382 PMID: 27270837.

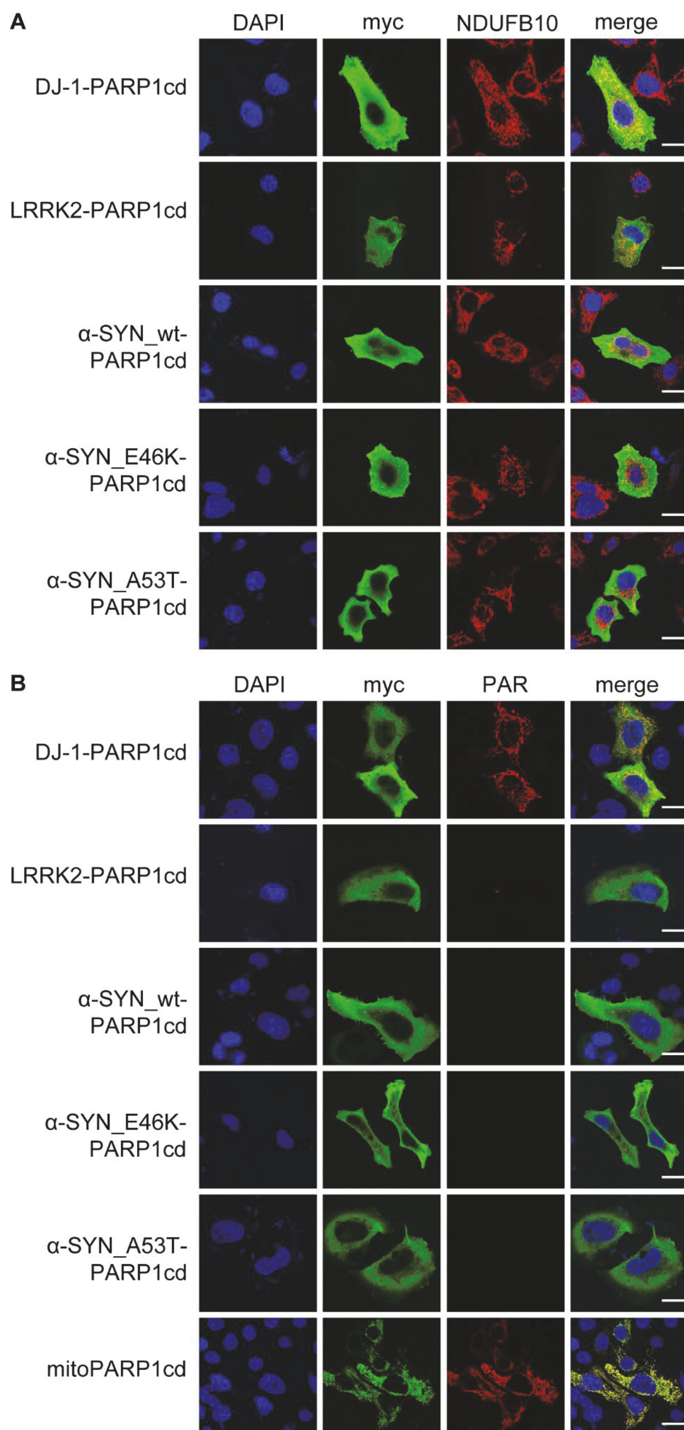
33. Biskup S, Moore DJ, Celsi F, Higashi S, West AB, Andrabi SA, et al. Localization of LRRK2 to membranous and vesicular structures in mammalian brain. Ann Neurol. 2006; 60(5):557–69. Epub 2006/11/23. https://doi.org/10.1002/ana.21019 PMID: 17120249.

34. Stafa K, Tsika E, Moser R, Musso A, Glauser L, Jones A, et al. Functional interaction of Parkinson's disease-associated LRRK2 with members of the dynamin GTPase superfamily. Human molecular genetics. 2014; 23(8):2055–77. Epub 2013/11/28. https://doi.org/10.1093/hmg/ddt600 PMID: 24282027.

35. Robotta M, Gerding HR, Vogel A, Hauser K, Schildknecht S, Karreman C, et al. Alpha-synuclein binds to the inner membrane of mitochondria in an alpha-helical conformation. Chembiochem: a European journal of chemical biology. 2014; 15(17):2499–502. Epub 2014/09/12. https://doi.org/10.1002/cbic.201402281 PMID: 25209675.

36. Di Maio R, Barrett PJ, Hoffman EK, Barrett CW, Zharikov A, Borah A, et al. α-Synuclein binds TOM20 and inhibits mitochondrial protein import in Parkinson's disease. Science translational medicine. 2016; 8(342):342ra78–ra78. https://doi.org/10.1126/scitranslmed.aaf3634 PMID: 27280685

S1 Fig.



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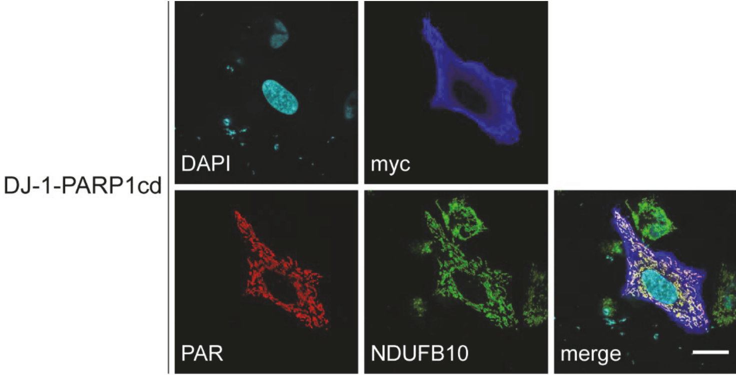

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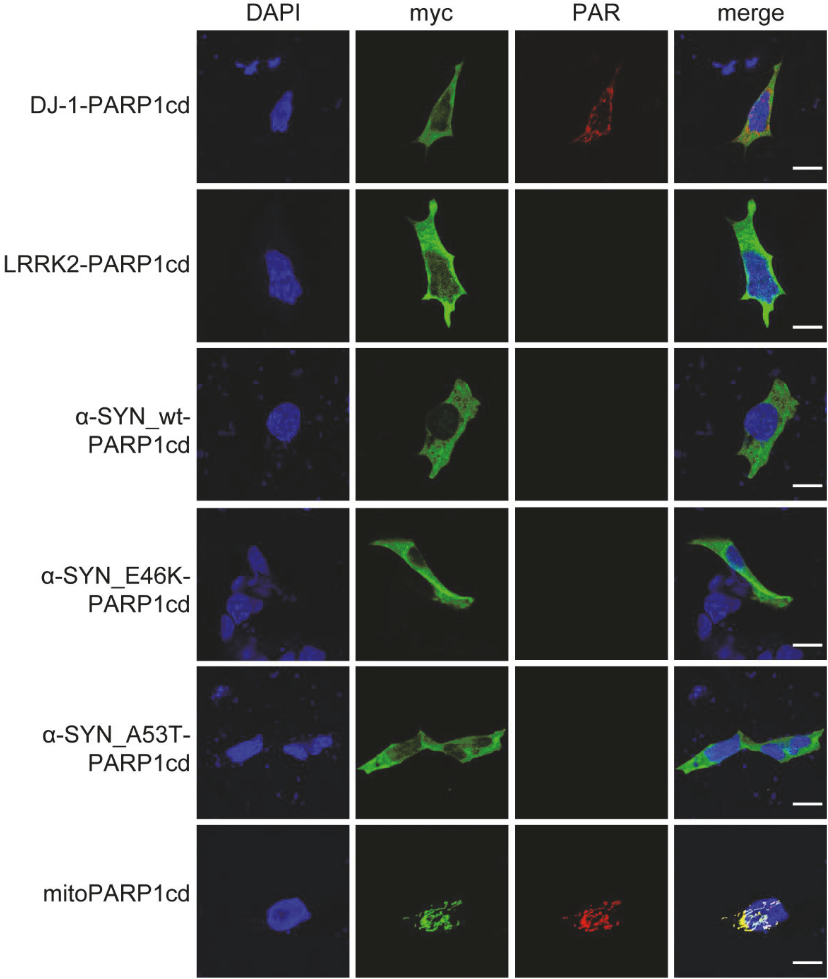

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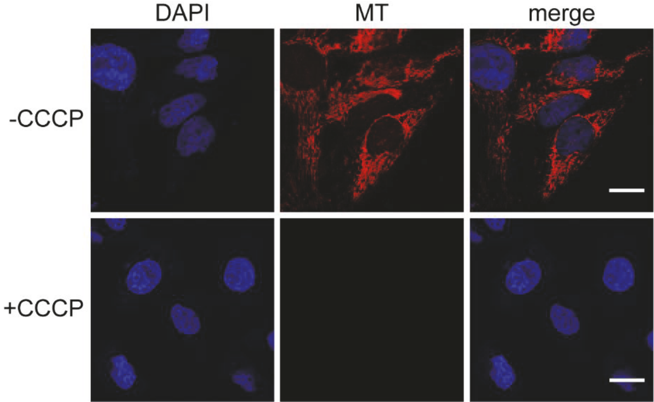

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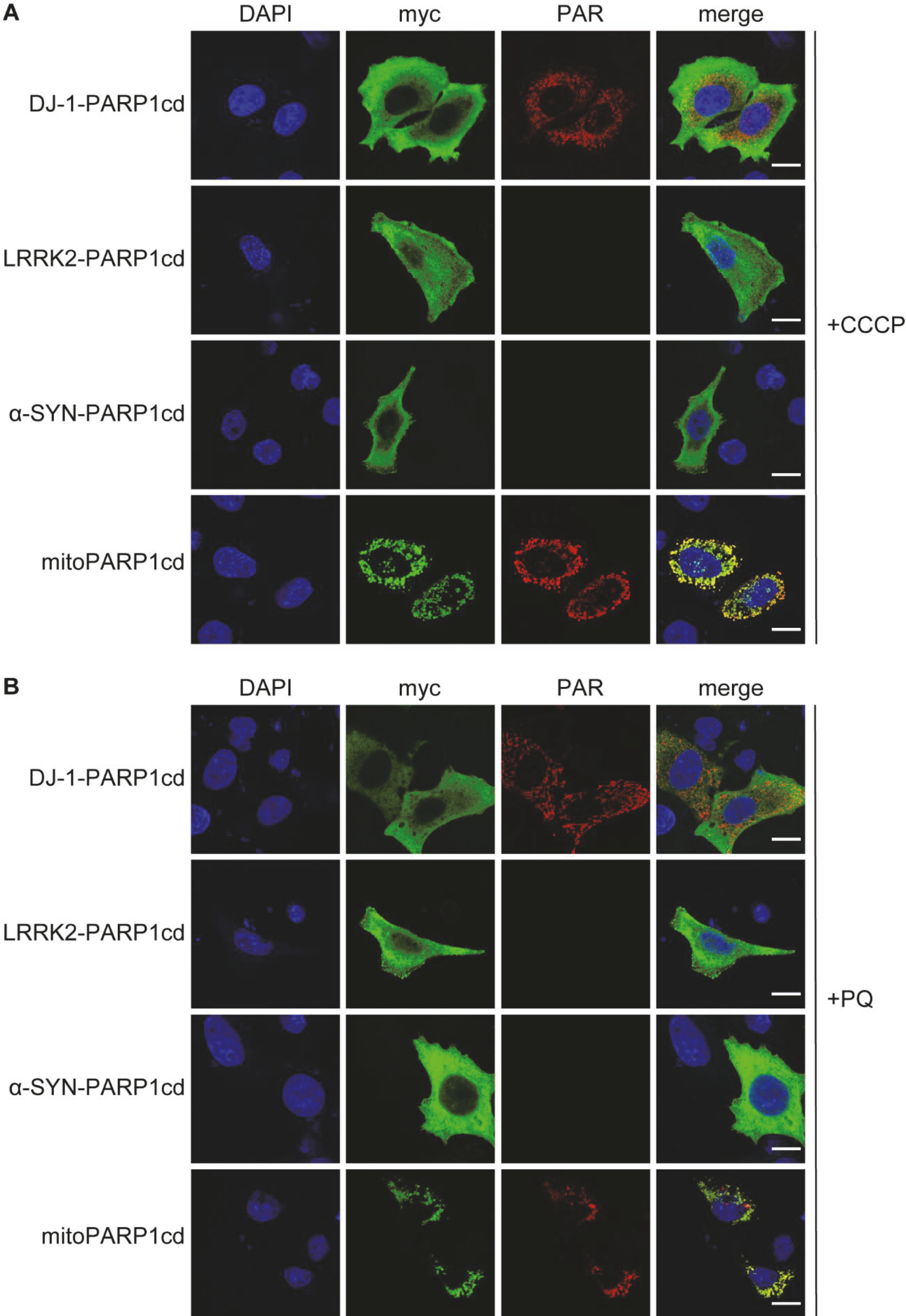

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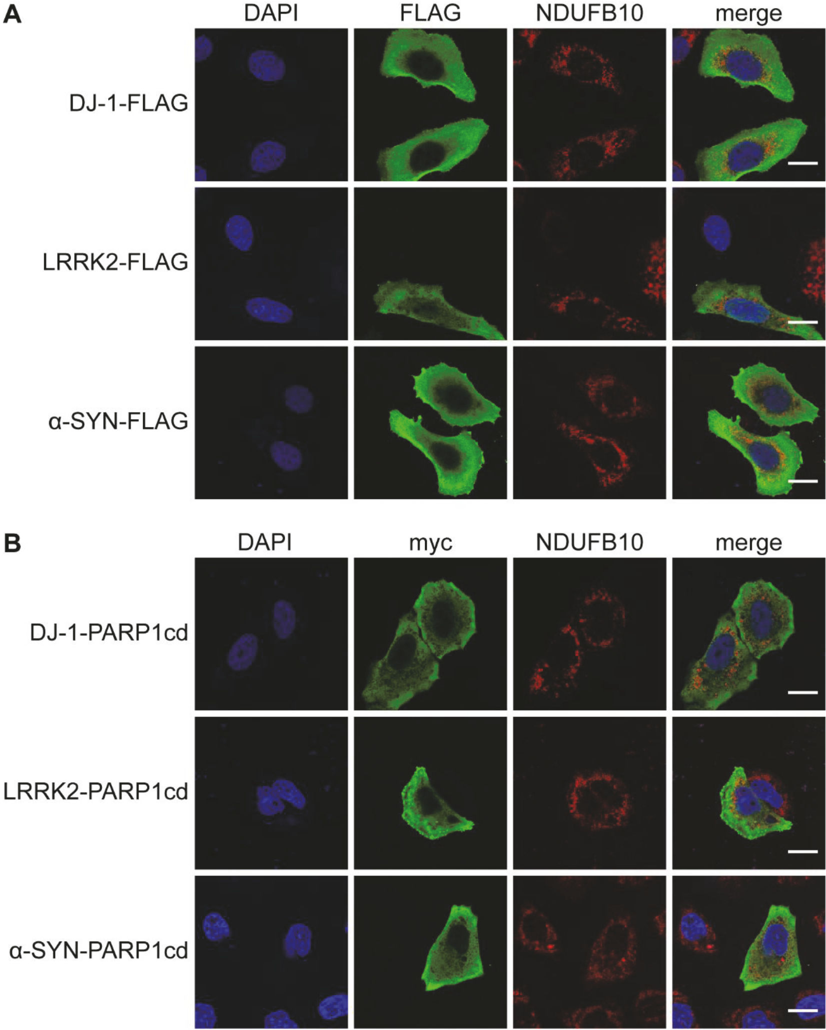
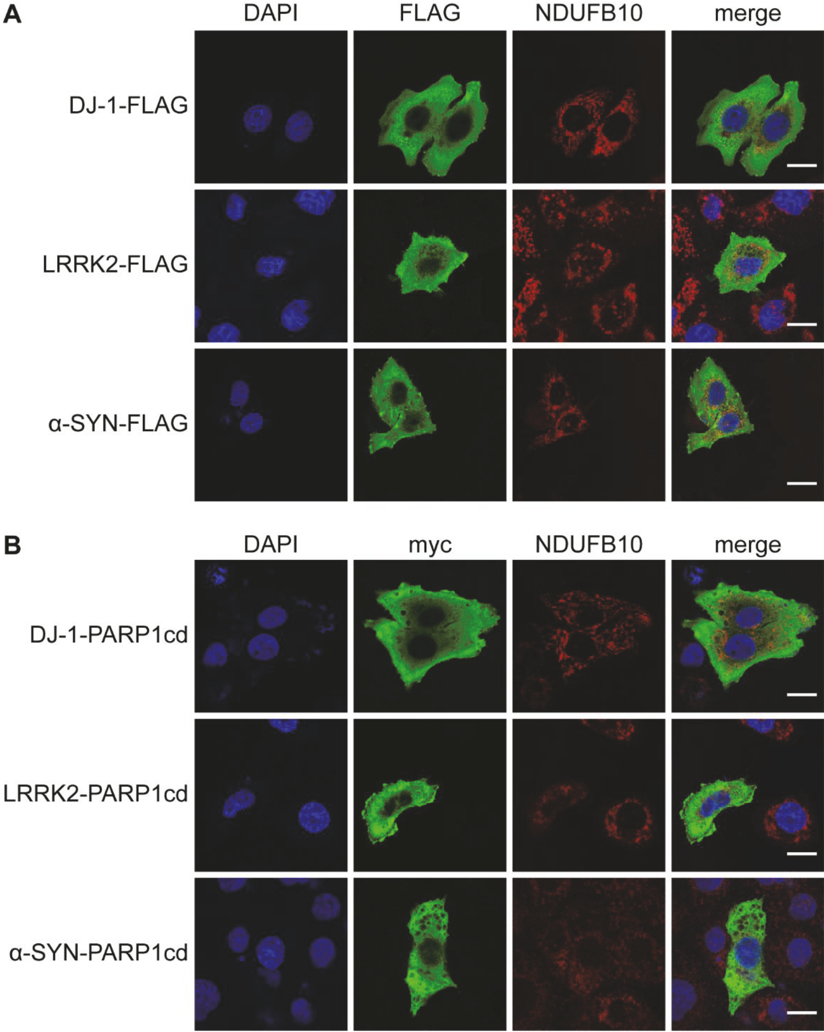


S7 Fig.



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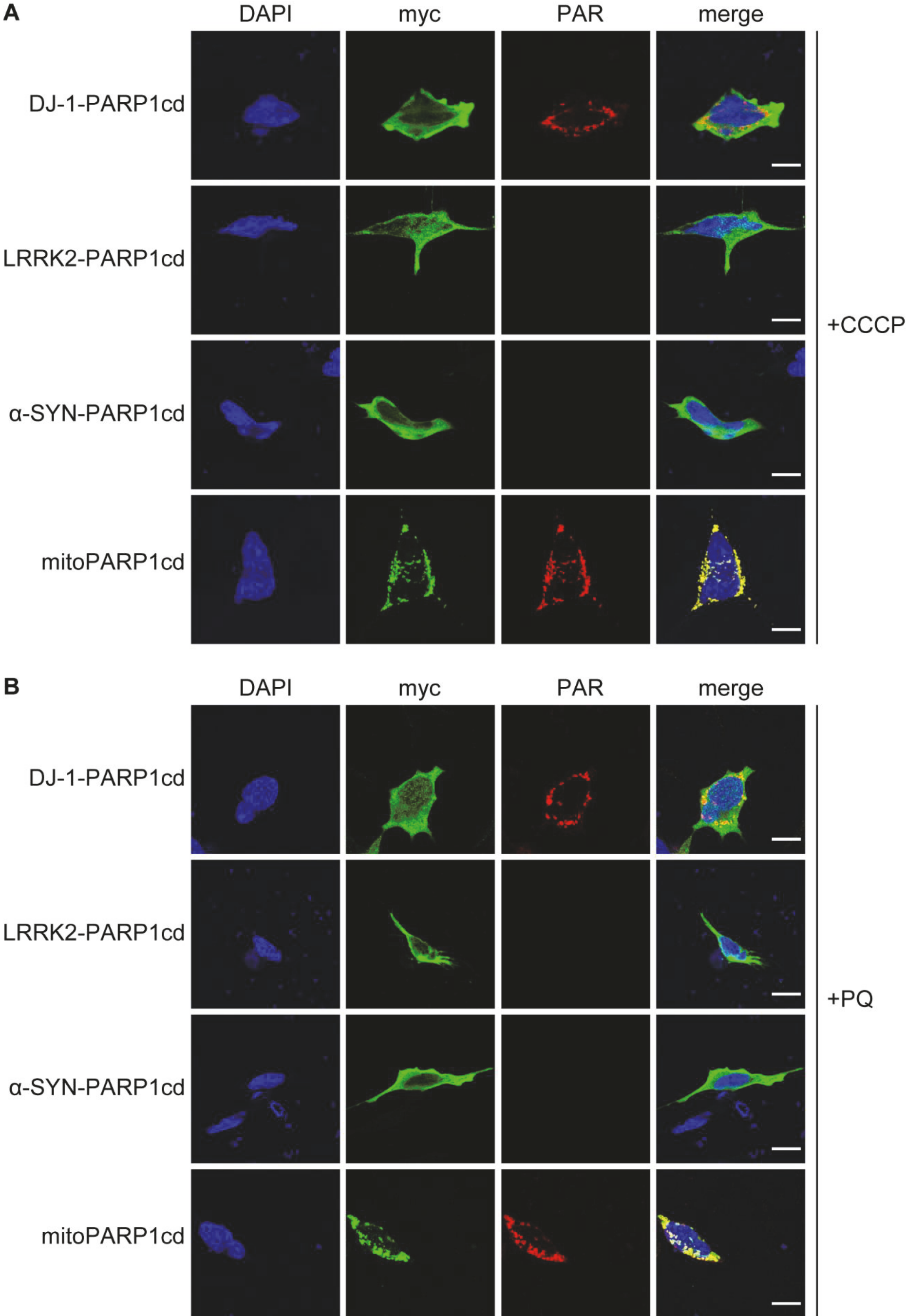

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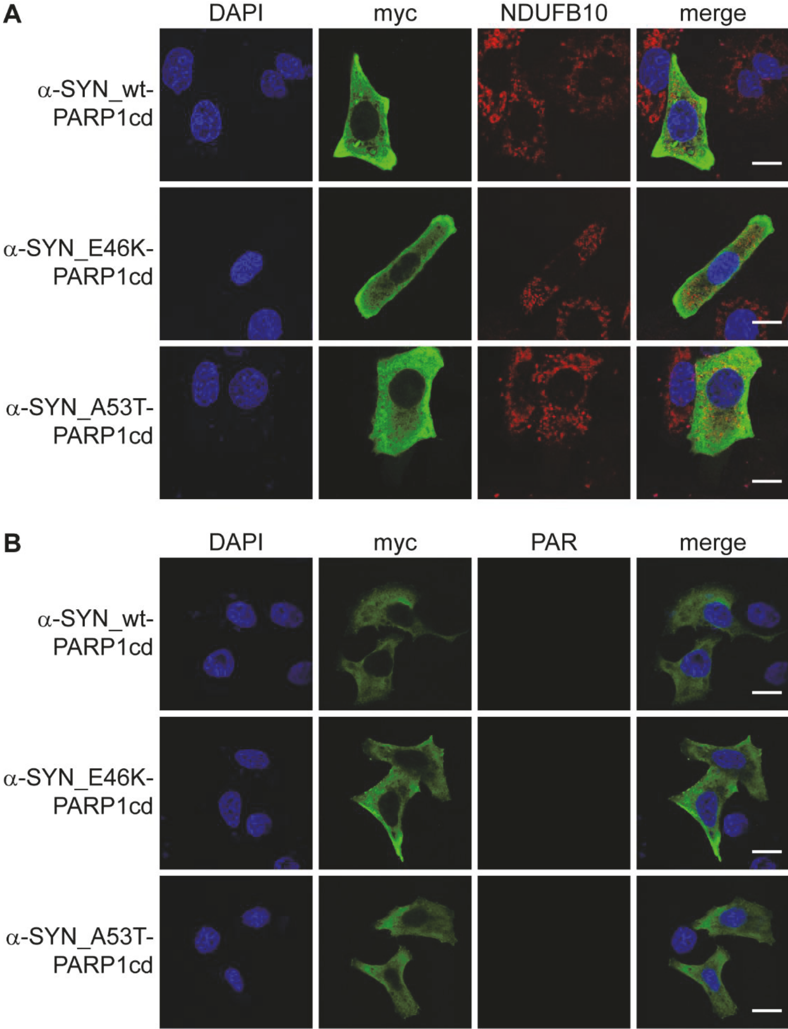
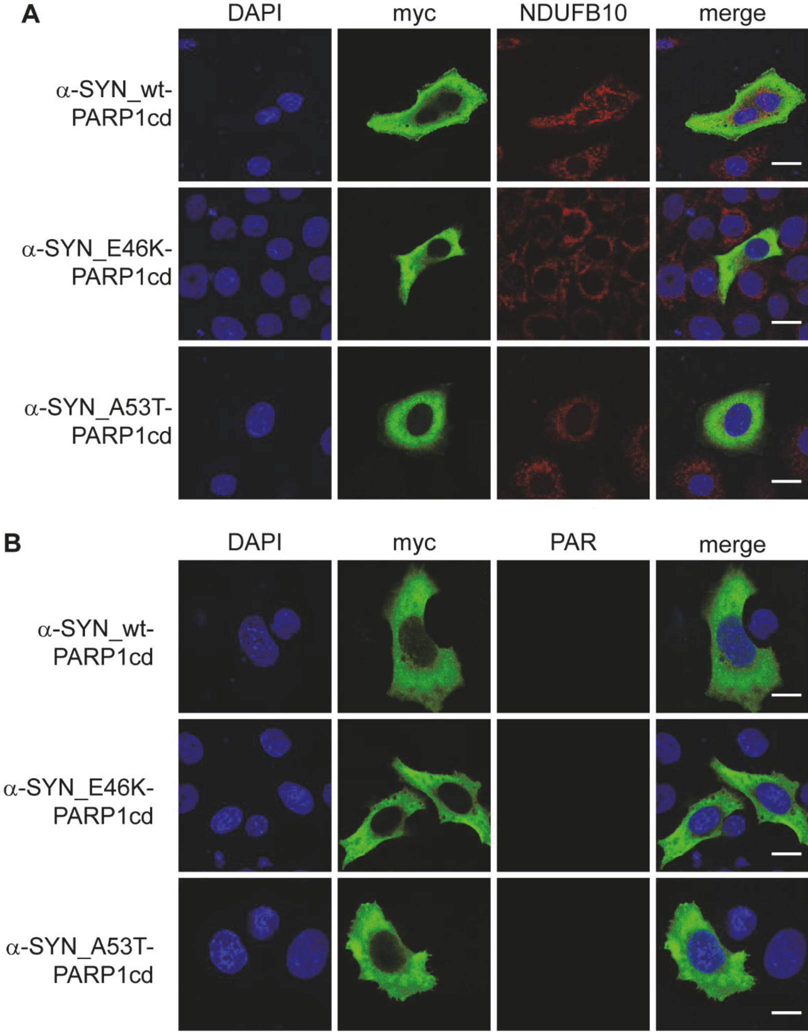
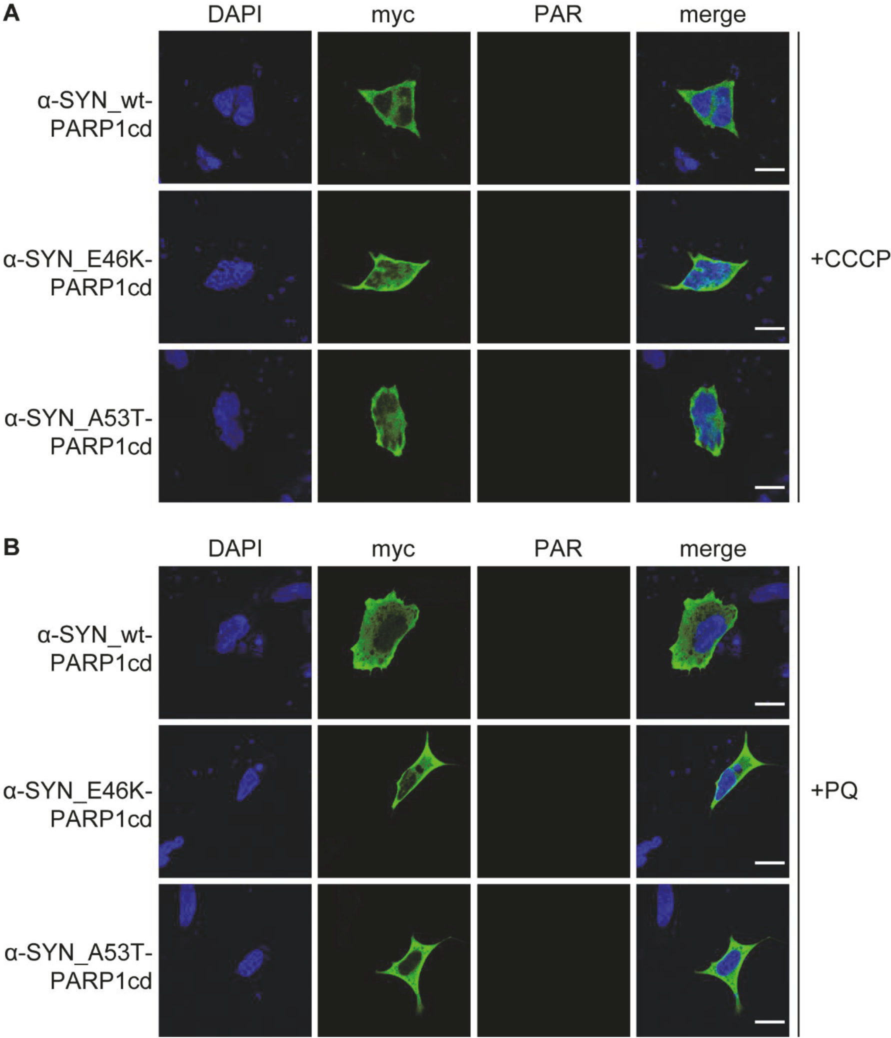


S10 Fig.



S11 Fig.



Paper II

Long-term mitochondrial ribosomal inhibition induces alpha-synuclein aggregation and modulates Parkinson's disease-associated pathways

Nelson Osuagwu, Christian Dölle, Charalampos Tzoulis

Manuscript

Paper III

Tau pathology is associated with higher levels of mitochondrial respiratory complexes I and IV

Nelson Osuagwu, Irene, Flønes, Christian Dölle, Charalampos Tzoulis
Manuscript

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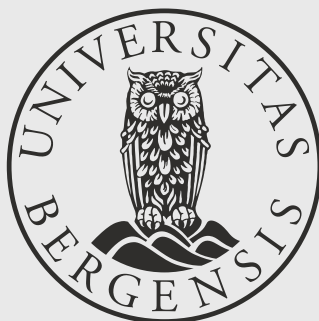

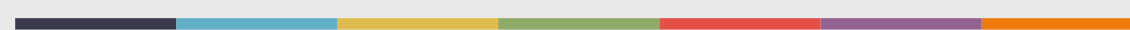

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