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MECHANISM OF ACTION OF AUTOSOMAL RECESSIVE JUVENILE PARKINSONISM GENE MUTATIONS

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Cover picture: Differentiated neuroblastoma cells (red: mitochondria; green: β-actin; blue: nuclei)

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ABSTRACT

Parkinson Disease (PD) is the most common neurodegenerative movement disorder. Although PD is a largely sporadic disease, several genes has been linked to familial forms of PD. This thesis focuses on the mechanism of function of the Autosomal Recessively Juvenile Parkinsonism (AR-JP) associated genes parkin, PTEN induced kinase 1 (PINK1) and DJ-1.

In **Paper I** we described a novel interaction between the E3 ubiquitin ligase parkin and phospholipase C-gamma1 (PLCγ1). We further demonstrated that parkin ubiquitinates and regulates PLCγ1 levels.

Impairment in calcium homeostasis has been suggested to be associated to PD related cell death. Since PLCγ1 is an important enzyme for regulating calcium, we continued by studying the downstream consequences of parkin impairment in the context of PLCγ1-mediated signaling. This study resulted in **Paper II** where we established that parkin deficiency leads to increased lipid hydrolysis and cytosolic calcium levels due to altered PLCγ1 activity. When we blocked calcium release from intracellular stores in parkin-mutant cells, the viability after exposure to oxidative stress was increased.

Previous studies suggest that mitochondrial dysfunction is related to neurodegeneration in PD. **In Paper III** and **IV**, we elucidated the roles of DJ-1 and PINK1 in mitochondrial morphology and dynamics. We showed that DJ-1 or PINK1 knock-down (KD) increased mitochondrial fragmentation and that blocking fission could reverse these phenotypes. In DJ-1 KD, we found that fission was related to oxidative stress, whereas in PINK1-deficient cells the mitochondrial abnormality was likely a consequence of a loss of mitochondrial membrane potential and increased calcineurin activity. In **Paper V** we analyzed mitochondrial motility in differentiated cells. By live imaging we demonstrated that KD of either DJ-1 or PINK1 decreased the rate of mitochondrial motility in neurites. Blocking fission eliminated the difference between the control cells and parkinsonism associated KD cells, suggesting that balanced mitochondrial dynamics is important for neuritic motility.

In conclusion, PD is a multi-factorial disorder involving several degenerative processes and signaling pathways. The AR-JP studies presented in this thesis may help to bring light to the understanding of the underlying mechanisms of PD and to develop novel treatment strategies.

SVENSK SAMMANFATTNING

För närmare 200 år sedan identifierade James Parkinson sjukdomen som sedermera kom att kallas Parkinsons sjukdom. Idag är Parkinsons sjukdom den näst vanligaste neurodegenerativa sjukdomen efter Alzheimers sjukdom och kännetecknas av darrningar och stelhet i kroppen samt svårigheter att röra sig. Man vet att den huvudsakliga orsaken till dessa symptom är en selektiv död av de celler som producerar dopamin. Forskningen har tagit stora kliv framåt i förståelsen av sjukdomsförloppet och i utvecklingen av effektiva mediciner, likväl är Parkinson fortfarande en obotlig sjukdom. De mediciner och behandlingsstrategier som används idag verkar genom att minska symptomen och inte till att bromsa eller avstanna sjukdomen och vi vet ännu inte vad det är som orsakar den selektiva celldöden.

Det yttersta målet med de arbeten som ingår i den här avhandlingen är därför att utröna vilka cellulära mekanismer som kan tänkas orsaka Parkinsons sjukdom.
Eftersom det inte är möjligt att undersöka vad som händer i hjärnan i levande människor har vi har valt att använda cellmodeller där vissa gener förändrats så att cellen liknar de sjuka cellerna i hjärnan hos parkinson-patienter.

Det finns två former av Parkinson, där ena beror på mutationer i genmassan och den andra uppstår från hittills okända orsaker från arv och miljö. Av den totala andelen patienter diagnostiserade med Parkinson, beräknar man att ca 10 % har en genetiskt underliggande faktor. Det är sannolikt att det, trots att de två formerna har olika orsaker, kan vara samma sjukliga processer som äger rum i hjärncellerna. Tanken i detta projekt har därför varit att använda dessa kända faktorer (mutationer) som modeller för den vanligare formen av Parkinson där orsaken är okänd.

Från tidigare studier av patienter med Parkinson, samt djur- och cellmodeller för sjukdomen, vet man att vissa processer sätts igång under sjukdomsprocessen. Cellens energiproducerande organell mitokondrien drabbas hårt, fria radikaler bildas och det sker klumpbildning av proteiner som gör att de inte längre fungerar som de ska. Dessutom tros regleringen av cellens nivåer av kalcium vara av stor vikt för cellernas överlevnad. Utifrån den vetskapen har vi formulerat hypoteser och studerat proteinerna parkin, PINK1 och DJ-1 vilka alla är länkade till den genetiska formen av Parkinsons sjukdom.

Studie 1

Parkin är ett enzym som reglerar andra proteiner genom en process kallad ubiquitinering, där små byggstenar (sk ubiquitin-grupper) byggs på proteinet och bildar en slags kedja eller svans. Ubiquitineringen kan leda till att proteinet bryts ner, transporteras någon annanstans i cellen eller aktiveras beroende på hur

ubiquitineringskedjan är utformad. Man har i tidigare studier funnit att parkin interagerar med ett flertal olika protein (summerade i tabell 1, s. 5).

I den första studien identifierade vi ett nytt protein som interagerar med parkin, nämligen fosfolipas C gamma-1 (PLCγ1). Vi visade att parkin binder till PLCγ1 både i celler där parkin fungerar normalt och där det är muterat på samma sätt som hos vissa patienter, samt i hjärnprover från människa. Vidare visade vi att parkin kan bygga på en ubiquitinkedja ("ubiquitinera") på PLCγ1 och att PLCγ1- nivåerna är lägre i celler som har normalt jämfört med genetiskt förändrat parkin. I hjärnprover från en musmodell som saknar parkin såg vi att det fanns mer PLCγ1. Allt detta tyder på att att interaktionen mellan parkin och PLCγ1 leder till att PLCγ1 bryts ner.

Studie 2

Då vi i Studie 1 visar att parkin styr PLCγ1 som är en viktig del i regleringen av kalcium, valde vi att i Studie 2 fokusera på hur parkin påverkar cellens kalcium.

När PLCγ1 aktiveras bildas nya ämnen som cellen använder för att skicka olika signaler om vad som är på gång i cellen. Vi mätte därför nivåerna av ett av dessa signalämnen i celler som hade normalt eller muterat parkin, samt celler med lägre nivåer av parkin. När parkin var muterat eller mängden lägre fanns det mer av signalämnet, vilket alltså tyder på att PLCγ1 är aktiverat i högre utsträckning när parkin inte fungerar som det ska. Då ett av signalämnets effekter är att cellens kalciumnivåer ökas genom att det släpps ut från lager inuti cellen, mätte vi kalcium. Även mängden kalcium var förhöjd i celler med parkinmutationer och detta gjorde att cellerna dog snabbare när vi utsatte dem för ett skadligt ämne. Genom att stänga av en kalciumkanal som transporterar kalcium från cellens lager kunde vi återställa kalciumbalansen hos de parkinmuterade cellerna och göra att cellerna överlevde i större utsträckning. Sammanfattningsvis identifierade vi i Studie I och II en ny mekanism som länkar den genetiska formen av Parkinsons sjukdom till förhöjda nivåer av kalcium vilket orsakade celldöd.

Studie 3 och 4

Mitokondrier är dynamiska strukturer inuti cellen som kan smälta samman och dela upp sig genom olika processer. PINK1 är ett protein som har visats vara viktigt för mitokondriens struktur och DJ-1 skyddar cellen mot fria radikaler. Dessutom flyttar sig DJ-1 från cytoplasman till mitokondrierna när cellen utsätts för stress. I Studie 3 och 4 valde vi därför att fördjupa oss i de dynamiska processer som ligger bakom mitkondrienätverkets möjlighet att ändra form och undersöka ifall PINK1 eller DJ-1 påverkar dynamiken.

Vi fann att om antingen DJ-1 (Studie 3) eller PINK1 (Studie 4) saknas delas mitokondrierna för mycket. När vi blockerade Drp1, som är ett ämne som hjälper till i delningen normaliserades nätverket. Detta beror på att avsaknad av DJ-1 eller PINK1 resulterar i mer aktivt Drp1. Sedermera härledde vi att aktiveringen av Drp1 orsakades av oxidativ stress i celler som saknar DJ-1 (Studie 3) och av aktivering av calcineurin i celler som saknar PINK1 (Studie 3). I Studie 3 och 4 visar vi alltså att den genetiska formen av Parkinsons sjukdom påverkar mitokondrienätverkets form.

Studie 5

Eftersom de cellinjer vi använde i Studie 3 och 4 är omogna nervceller som saknar utskott som är typiska för nervceller, valde vi att i Studie 5 differentiera cellerna till att bli mer nervcellslika. Detta i syfte att komma närmare hjärnans fysiologi. Mitokondriens rörelser i cellen är dessutom väldigt olika från cellkropp och utskott. I cellkroppen verkar det hela det dynamiska nätverk som vi undersökte i Studie 3 och 4, medan mitokondrierna i utskottet transporteras på rad med hjälp av motorprotein.

Efter att ha differentierat de celler som har lägre nivåer av PINK1 eller DJ-1, spelade vi in filmer där vi mätte mitokondriernas rörelser i anterograd (från cellkroppen och utåt) och retrograd riktning (utifrån och in mot cellkroppen). Vi identifierade skillnader mellan cellerna, där både minskade nivåer av PINK1 och DJ-1 ledde till att mitokondrierna rörde sig mindre. Då mängden Drp1, som styr mitokondriernas delning, minskade rörde sig kontrollcellernas mitokondrier mindre, medan celler med lägre PINK1 och DJ-1-nivåer hade mitokondrier som rörde sig mer. De här resultaten visar att de mekanismer vi undersökte i Studie 3 och 4 även är viktiga för mitokondriernas transport i utskotten. Vidare försökte vi identifiera vilka molekylära mekanismer som kan ge minskad mitokondriell rörelse. I celler med lägre nivåer av PINK1 fann vi ökade kalciumnivåer när vi utsatte mitokondrien för stress.

Sammanfattningsvis har jag i den här avhandlingen studerat hur proteinerna parkin, PINK1 och DJ-1 påverkar kalciumnivåerna i cellen, samt det dynamiska mitokondrienätverket. Då både kalciumnivåer och mitokondriefunktion har visats vara viktiga i Parkinsons sjukdom kan man genom att arbeta med modeller med genetiska förändringar som leder till parkinson-lika symptom bidra till att lösa gåtan om hur Parkinsons sjukdom uppkommer.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals.

I. Nodi Dehvari*, **Anna Sandebring*,** Amilcar Flores-Morales, Laura Mateos, Yin-Choy Chuan, Mark Goldberg, Mark R Cookson, Richard F Cowburn, Angel Cedazo-Minguez . *Parkin mediated ubiquitination regulates phospholipase C-gamma 1.* Journal of Cellular and Molecular Medicine (2008) 12, 1-8

II. **Anna Sandebring***, Nodi Dehvari*, Monica Perez-Manso, Elena Karpilovski, Mark R Cookson, Richard F Cowburn, Angel Cedazo-Minguez. *Parkin deficiency disrupts calcium homeostasis by modulating phospholipase C signaling*. FEBS Journal (2009) 276, 5041-52

III. Jeff Blackinton*, Kelly Jean Thomas*, Melissa Mc Coy*, Alexandra Beilina, Marcel van der Brug, **Anna Sandebring**, David Miller, Angel Cedazo-Minguez, Mark R Cookson. *Increased oxidative stress in DJ-1-deficient cells is associated with multiple mitochondrial abnormalities.* Manuscript

IV. **Anna Sandebring***, Kelly Jean Thomas*, Alexandra Beilina, Marcel van der Brug, Megan M Cleland, Rili Ahmad, David W Miller, Ibardo Zambrano, Richard F Cowburn, Homira Behbahani, Angel Cedazo-Minguez, Mark R Cookson. *Mitochondrial alterations in PINK1-deficient cells are influenced by calcineurin-dependent dehosphorylation of Dynamin related protein 1.* PLoS One (2009) 4, issue 5

V. **Anna Sandebring**, Monica Perez-Manso, Mark R Cookson, Angel Cedazo-Minguez. *PINK1 or DJ-1 deficiencies impair neuritic motility of mitochondria.* Manuscript

* Equal contribution

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LIST OF ABBREVIATIONS

$\Delta\Psi_m$	mitochondrial membrane potential
6-OHDA	6-hydroxydopamine
AR-JP	Autosomal Recessive Juvenile Parkinsonism
BDNF	brain derived neurotrophic factor
Co-IP	co-immunoprecipiation
CSF	cerebrospinal fluid
Drp1	dynamin related protein 1
EGF	epidermal growth factor
ER	endoplasmic reticulum
GEF	guanine nucleotide exchange factor
GSH	glutathione ethyl ester
GSK3β	glycogen synthetase kinase 3β
hFis1	human fission protein 1
IPs	inositol phosphates
KD	knock-down
KO	knock-out
LB	lewy body
L-DOPA	L-3,4-dihydroxyphenylalanine
Mfn1/2	mitofusin 1/2
MPTP	1-methyl-1,2,3,6-tetrahydropyridine
mPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
NF-κB	nuclear factor-κB
Opa1	optic atrophy protein-1
PD	Parkinson Disease
PH	pleckstrine homology
PI	phosphatidyl inositol
PI_3K	phosphatidyl inositol-3 kinase
PIKE	PI_3K enhancer
PINK1	PTEN induced kinase 1
PKC	protein kinase C
PLCγ1	phospholipase-C γ1
PTEN	phosphatase and tensin homologue
RNS	reactive nitrogen species
ROS	reactive oxygen species
RyR	Ryanodine receptor
SH2/3	Src homology 2/3
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase
TEM	transmission electron tomography
Trk	tyrosine kinase
UPR	unfolded protein response
WT	wild type

INTRODUCTION

PARKINSON DISEASE

In 1817, Dr. James Parkinson published his monography entitled, “An essay on the Shaking Palsy,” in which he described six patients to suffer from “*Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured*” (Parkinson 1817). Due to Parkinson’s accurate description, the father of neurology, Jean Martin Charcot, later suggested to name this newly defined disorder *La Maladie de Parkinson,* or Parkinson Disease (PD).

PD is the second most common neurodegenerative disease after Alzheimer disease and is affecting approximately 1.8 % among Europeans above 65 years of age (de Rijk *et al.* 2000). According to a European study of 100 PD patients, the mean age of onset was estimated to be 62.5 years and the mean disease duration was 13 years (Hughes *et al.* 1993). There are no apparent racial differences in prevalence of PD, nevertheless some studies propose that men are more likely to develop PD than women, however this has not been confirmed across studies (Twelves *et al.* 2003).

Symptoms

PD is clinically diagnosed by bradykinesia, rigidity, resting tremor and postural instability, which are typically observed to be assymetrical at onset (Lees *et al.* 2009, Kovari *et al.* 2009). One of the earliest symptoms of PD is loss of smell, however this is commonly noted in retrospect (Doty *et al.* 1995). At later stages, PD patients experience loss of facial expression, flexed posture, monotone speech, constipation and difficulties chewing and swallowing. Also, cognitive changes and dementia are common features of PD pathology (Robottom & Weiner 2009) however it is believed that the degree of dementia correlates with amount of β-amyloid deposition in the brain, which is a neuropathological hallmark of Alzheimer disease (Kalaitzakis *et al.* 2008, Ballard *et al.* 2006).

Neuropathology

The clinical picture described above arises primarily from dopaminergic depletion resulting from the selective loss of dopaminergic, neuromelanin positive neurons of the *substantia nigra pars compacta* (SNpc), which is a nucleus of the basal ganglia. The

basal ganglia participates in the control of movement and specifically SNpc neurons project to the *striatum*, which is another component of the basal ganglia.

Nigral degeneration is a neuropathological hallmark of PD. This is accompanied by cell loss in other neuronal groups, including the noradrenergic locus coerulus, the cholinergic basal nucleus of Meynert, and the mixed, cholinergic–glutaminergic pedunculopontine nucleus (Jellinger 1991). It has been suggested that degeneration of such non-dopaminergic neurons may account for the postural instability that generally affects PD patients (Grimbergen *et al.* 2009).

A second neuropathological hallmark of PD are intracellular inclusions, termed Lewy bodies (LB) named after Friedrich H. Lewy, the neurologist who first described this abnormality in PD brain. LB and Lewy neurites are α-synuclein positive aggregates composed of more than 70 molecules, located in the soma and neurites respectively. LB are found in several brain regions (Wakabayashi *et al.* 2007) and it is an estimated 3-4 % of surviving nigral neurons that contain LB, independent of disease severity (Greffard *et al.* 2008). A 6 stage neuropathological model has been described by determination of the temporal spread of LB in PD (Braak *et al.* 2003).

Treatments

Although new treatment approaches are under investigation, PD is still considered an incurable, chronic neurodegenerative movement disorder of the aging brain. Treatment strategies aim to provide symptomatic relief by compensating for the loss of dopamine.

The most common is to administer L-DOPA (L-3,4-dihydroxyphenylalanine) which is the precursor to the catecholamines, including dopamine. Most patients have a favourable initial response to L-DOPA, however with long term treatment, side effects such as dyskinesia are common (Schapira *et al.* 2009). Enteral administration has shown a reduction in side effects due to a more even drug distribution, although this needs further evaluation (Nyholm 2006). Monoamine oxidase inhibitors restrain the degradation of dopamine and this has been shown to delay disease progression when administered early during the disease course (Parkinsonstudygroup 2002). High frequency deep brain stimulation of the subthalamic nucleus or of the globus pallidus interna is a surgical approach for treating late stage PD (Benabid *et al.* 2009). Placing grafts of embryonic stem cells into the vicinity of the *striatum* is a regenerative therapy which offers promising recovery rates, although its limitations include access to cells, risk of developing tumors and initiating an immune response (Li *et al.* 2008).

FAMILIAL PARKINSONISM

The discovery of genes involved in the development of parkinsonism has contributed immensely to the comprehension of disease pathogenesis. Although PD is mainly described as a sporadic disorder, recent studies have identified predisposing genetic risk factors. Currently 6 *loci* have been reported by several groups to be associated with rare forms of parkinsonism via dominantly or recessively inherited gene mutations (reviewed in (Belin & Westerlund 2008)). New understanding about the genomics of PD has provided important tools for studying PD related mechanisms.

Autosomal Recessive Juvenile Parkinsonism

Mutations in the genes encoding for parkin, DJ-1 or PTEN induced kinase-1 (PINK1) cause Autosomal Recessive Juvenile (or early onset) Parkinsonism (AR-JP). The AR-JP disease pathology is similar to the idiopathic disease, with L-DOPA responsive motoric disabilities. However, the age of onset is earlier compared to in idiopathic PD, ranging from the second to fourth decade of life. The disease course is usually milder in AR-JP as compared to idiopathic cases. The idiopathic patient is typically affected unilaterally, while AR-JP patients show a bi-lateral symptom onset. Neuropathological examinations reveal nigral degeneration in AR-JP, however LB pathology is not observed (Gasser 2009, Rosner *et al.* 2008) however there are exceptions (Sasaki *et al.* 2004, Pramstaller *et al.* 2005). It is believed that mutations in AR-JP associated genes result in a loss of function by poor protein stability or even lack of functional protein depending on the mutation (Henn *et al.* 2005, Miller *et al.* 2003, Moriwaki *et al.* 2008).

Parkin

The most common cause of AR-JP is a mutation in the gene encoding for parkin. Matsumine and associates (Matsumine *et al.* 1997) were the first to identify this gene locus associated with AR-JP. Based on a Japanese pedigree, parkin was later named and described to be a protein of 465 amino acids expressed in several organs, but abundantly in the brain including the SNpc. Gene sequencing revealed that at the C-terminal, two RING (Really Interesting New Gene) domains were flanked by a cysteine rich in-between RINGs domain (IBR) and that a ubiquitin-like domain was localized at the N-terminus (Kitada *et al.* 1998). Later it was confirmed that parkin indeed is an E3 ubiquitin ligase, with auto-ubiquitination properties (Zhang *et al.* 2000).

Ubiquitination is a process involving three classes of enzymes. Of these, the E3 ubiquitin ligases recognize the substrates and occur last in the chain of ubiquitin ligating events. The complex machinery of ubiquitination can lead to proteasomal

degradation, but depending on the mediating ligases and the structure of the formed ubiquitin chain, targeted proteins can also undergo endocytosis and lysosomal degradation, or translocate and participate in signaling (reviewed in (Hershko & Ciechanover 1998, Komander 2009). E3 mediated activity of parkin has been shown to involve the conjugation to the E2 ubiquitin carrier proteins UbcH7, UbcH8 and UbcH13 (Imai *et al.* 2000, Olzmann *et al.* 2007, Shimura *et al.* 2000).

The search for substrates has been extensive since the discovery of parkin. Today there are many known parkin substrates, covering a range of cellular functions (Table 1, p. 5). For example, it has been suggested that parkin interacts with LB in the sporadic disease (Schlossmacher *et al.* 2002, Choi *et al.* 2000). Indeed, the LB components α-synuclein and synphilin-1 are shown to interact with parkin (Choi *et al.* 2001, Chung *et al.* 2001) and furthermore, parkin protects against toxicity from α-synuclein over-expression (Petrucelli *et al.* 2002). Thus, it has been speculated that parkin participates in the formation of LB, which may explain why brains from AR-JP parkin patients generally lack LB pathology (Takahashi *et al.* 1994, Mori *et al.* 1998).

Over 100 parkin mutations, including exonic rearrangements, pointmutations and small deletions or insertions have been identified, which makes parkin the most common cause of early-onset parkinsonism (Hedrich *et al.* 2004). Also, parkin haploinsufficiency, especially for dosage mutations, has been suggested to increase the risk of early-onset parkinsonism (Pankratz *et al.* 2009). In contrast to Parkin knock-out (KO) mice that fail to mimic parkinsonism (Goldberg *et al.* 2003) the Q311X parkin-mutant mouse model, reveal several of the key PD pathologies (Lu *et al.* 2009). Parkin appears to be a pleiotropic protein important for many cellular functions, including the intricate regulation of mitochondrial morphology. Parkin KO mice (Stichel *et al.* 2007) and particularly the Drosophila parkin knock-down (KD) model shows mitochondrial impairment (Poole *et al.* 2008) (Animal models for AR-JP are summarized in Table 2, p. 9). In this framework, parkin has been suggested to act in the same signaling pathway as PINK1, as described in a section below (p. 8). Parkin also binds to and repairs the nuclear genome (Kao 2009).

Specific and protective, proteasome independent pathways stimulated by parkin involve the upregulation of anti-apoptotic genes through enhancement of nuclear factor κB (NF-κB) transcription (Henn *et al.* 2007), repression of p53 transcription by direct association to its promoter region (da Costa *et al.* 2009), through inhibition of the c-Jun terminal kinase pathway in Drosophila (Cha *et al.* 2005) and further by promoting Akt signaling by inhibiting internalization of the epidermal growth factor receptor (EGF-R) (Fallon *et al.* 2006).

DJ-1

The AR-JP associated gene DJ-1 (Bonifati *et al.* 2003) was originally identified as an ubiquitously expressed novel oncogene induced by growth stimuli that translocates from cytoplasm to nuclei during the synthesis phase of the cell cycle (Nagakubo *et al.* 1997). DJ-1 forms a dimer and cysteine residue at position 46 has been shown to be a crucial residue for linking the monomers together (Ito *et al.* 2006). DJ-1 may function as an antioxidant by reducing ROS levels from H_2O_2 *in vitro* (Taira *et al.* 2004). Others, however, have been unable to reproduce these results, and instead show that DJ-1 functions rather as a redox-regulated molecular chaperone, demonstrated by DJ-1 mediated inhibition of α-synuclein aggregates (Shendelman *et al.* 2004).

Mice and drosophila models lacking functional DJ-1 show no signs of dopaminergic depletion, however, locomotor activity is either basally reduced or severely affected by oxidative stress, but do not show any signs of dopaminergic cell death (Chen *et al.* 2005, Goldberg *et al.* 2005, Kim *et al.* 2005b, Meulener *et al.* 2005, Park *et al.* 2005). However, in aged drosophila, DJ-1 KD gave a decreased number of dopaminergic cells and DJ-1 was also connected to phosphatidyl inositol-3 kinase (PI-3K) signaling (Yang *et al.* 2005). This is in concordance with other studies where it was found that DJ-1 inhibits the PI-3K inhibitor PTEN (Kim *et al.* 2005a). Exposure to oxidative stress or expression of mutated DJ-1 were also shown to bind and exhibit stronger PTEN inhibition than WT DJ-1, resulting in increased PI3-K activity and Akt phosphorylation (Kim *et al.* 2009).

Mutations in DJ-1 are very rare, estimated to account for less than 1 % of the AR-JP cases. However, a small amount of DJ-1 localizes to LB (Bandopadhyay *et al.* 2004), and DJ-1 is increased in both plasma and cerebrospinal fluid (CSF) of sporadic PD patients (Waragai *et al.* 2007, Waragai *et al.* 2006), suggesting an important role for DJ-1 in sporadic PD.

In the brain, DJ-1 appears to be expressed mainly in astrocytes and has indeed been suggested to regulate the astrocytic pro-inflammatory response in mice (Waak *et al.* 2009). DJ-1 KO mice have increased resting levels of muscular cell cytosolic calcium, a

phenotypic expression that could be reversed by adding resveratrol, an antioxidant and stimulator of mitochondrial biogenesis (Shtifman *et al.* 2009).

Under oxidative stress, DJ-1 has been shown to localize to the inner mitochondrial intermembrane space and matrix (Zhang *et al.* 2005) and to the outer mitochondrial membrane (Canet-Aviles *et al.* 2004). Yet, pathogenic DJ-1 mutations do not appear to influence mitochondrial localization (Zhang et al. 2005). Interestingly, a recent study show that DJ-1 promotes cellular respiration by associating to two subunits of complex I in the respiratory chain, ND1 and NDUFA4 (Hayashi *et al.* 2009).

PINK1

Profiling of genes activated by the tumor suppressor phosphatase and tensin homologue (PTEN) resulted in the discovery of the putative serine/threonine kinase PINK1 (Unoki & Nakamura 2001). Three years later, this 581 amino acid protein was identified as the third AR-JP associated gene. The authors from this study predicted that PINK1 contains a N-terminal mitochondrial targeting motif and showed that WT, but not mutant PINK1, stabilized the mitochondrial membrane potential ($\Delta\Psi_m$) and protected cells from apoptosis when exposed to proteasome inhibition (Valente *et al.* 2004). Studies have offered various postulates for anti-apoptotic effects of PINK1, through a direct phosphorylation of the mitochondrial chaperone TRAP1 (Pridgeon *et al.* 2007), by regulating the mitochondrial permeability transition pore (mPTP) (Wang *et al.* 2007), or via some unknown mechanism (Deng *et al.* 2005, Petit *et al.* 2005). Others and we suggest that PINK1 is not protective for apoptosis *per se* (Berger *et al.* 2009, Sandebring *et al.* 2009). Instead, it is suggested that sustained viability from WT PINK1 expression is a downstream consequence from other protective cellular mechanisms. Mutations in PINK1 are estimated to account for 4-5 % of the AR-JP cases and for 1-2 % of the early onset sporadic cases as heterozygous mutations (Marongiu *et al.* 2008). Similar to parkin, PINK1 has been shown to accumulate in LB inclusions in sporadic PD, however, the underlying mechanisms for this have not been elucidated (Murakami *et al.* 2007).

Both WT and mutant PINK1 are cleaved when imported into the mitochondria and localize mainly to the inner mitochondrial membrane and inner mitochondrial space (Gandhi *et al.* 2006, Silvestri *et al.* 2005, Pridgeon et al. 2007, Muqit *et al.* 2006). PINK1 is indeed important for maintaining mitochondrial morphology and $\Delta\Psi_m$ (Exner *et al.* 2007). A portion of the protein may be localized in the outer membrane with its C-terminus facing the cytoplasm (Zhou *et al.* 2008), and several studies suggest full length and cleaved PINK1 are present in the cytosolic compartment (Beilina *et al.* 2005, Weihofen *et al.* 2008, Haque *et al.* 2008). PINK1 has been suggested to mediate phosphorylation of the mitochondrial protease Omi/HtrA2 and

thereby regulate its proteolytic activity that increases the resistance to mitochondrial stress (Plun-Favreau *et al.* 2007). However, these results are contradicted in another study (Yun *et al.* 2008) and further analysis is therefore required to establish whether or not Omi is in the same signaling pathway as PINK1.

Calcium homeostasis is regulated by a complex machinery. Its disruption has been described as an important factor in neurodegenerative disease, including PD (Chan *et al.* 2009, Wojda *et al.* 2008). Mitochondria of PINK1-deficient cells have been shown to accumulate calcium, resulting in calcium overload and mPTP opening (Gandhi *et al.* 2009). Moreover, WT but not mutant PINK1 may protect against α-synuclein-induced increase of cytosolic calcium, which could be reversed by blocking the mitochondrial calcium uptake pore (Marongiu *et al.* 2009). Albeit many different consequences of PINK1 deficiency have been described, it is possible that they are multiple aspects with a common origin. Identifying authentic substrates for PINK1 would indeed help to identify the most upstream event of the degenerative series of effects from PINK1 deficiency.

Pathways or functions where AR-JP genes converge

The outcome from loss of the drosophila PINK1 homologue is indistinguishable from parkin-deficient flies. These exhibit motoric defects due to dopaminergic cell depletion arising from mitochondrial deficits. This PINK1 deficiency phenotype could be rescued by parkin over-expression, but not *vice versa*, leading to the proposal that PINK1 act upstream of parkin in the same molecular pathway (Clark *et al.* 2006, Park *et al.* 2006, Yang *et al.* 2006). Further studies in drosophila showed that loss of parkin or PINK1 results in enlarged and swollen mitochondria. This phenotype could be rescued by over-expressing the mitochondrial fission protein Drp1, which led to the conclusion that WT drosophila parkin is promoting mitochondrial fission (Poole *et al.* 2008, Deng *et al.* 2008). In human cells, however, parkin and PINK1 have instead been suggested to promote mitochondrial fusion. Others and we showed that Drp1 siRNA could rescue the mitochondrial phenotype from PINK1 deficiency (Sandebring et al. 2009) and parkin deficiency (Lutz *et al.* 2009, Dagda *et al.* 2009). Lutz et al (2009) additionally showed that also in drosophila cells, fission is an early effect from parkin or PINK1 KD, but converts into excess fusion with time, which could explain the discrepancy between studies in mammals versus drosophila. A study from our group also showed that mitochondrial fusion is reduced in DJ-1 KO mouse embryonic fibroblast cells (Blackinton *et al.* 2009).

Possibly in concordance with a role for mitochondrial morphology, parkin also has a direct role in mitochondrial turn-over by selectively mediating the degradation of impaired mitochondria (Narendra *et al.* 2008). Recently it was shown that WT but not mutant PINK1 expression is necessary for, and promotes parkin translocation to the mitochondria (Vives-Bauza *et al.* 2009). Thus, it seems like parkin, PINK1 and possibly DJ-1 are important regulators of mitochondrial morphology and turn-over. With regard to mitochondrial dynamics, PINK1 silencing results in decreased mitochondrial DNA (mtDNA) synthesis (Gegg *et al.* 2009) and parkin is binding to mtDNA, which enhances the synthesis in human neuroblastoma cells (Kuroda *et al.* 2006, Rothfuss *et al.* 2009).

In keeping with the oxidative stress hypothesis of PD, parkin has been shown to interact with the molecular chaperones, Hsp70 and Hsc70 (Moore *et al.* 2008). DJ-1 has further been suggested to mediate its antioxidative effects through an upregulation of Hsp70 and to share this downstream mediator with α-synuclein (Batelli *et al.* 2008). Thus different genetic parkinsonism associated gene products may also be co-ordinated in an oxidative stress response pathway.

Direct association between parkin, DJ-1 and PINK1

There is a growing number of shared or related mechanisms between parkin, DJ-1 and PINK1. It has even been suggested that these proteins in fact interact and forms a complex. Xiong and associates (2009) demonstrated that parkin, PINK1 and DJ-1 form a complex in both mitochondrial and cytosolic fractions from human cells. They also suggested that both PINK1 and DJ-1 expressions are crucial for enabling parkin to maintain its ability to ubiquitinate previously known substrates (Xiong et al. 2009). Further PINK1 regulates parkin RING1 domain-dependent translocation to the mitochondria through direct phosphorylation on Tyr175, which is located in the linker region of parkin (Kim *et al.* 2008). It was confirmed later that mitochondrial relocalization of parkin is dependent on PINK1, but not that this involve the phosphorylation of parkin (Vives-Bauza et al. 2009).

The interaction between DJ-1 and parkin may promote DJ-1 stability, especially for mutant DJ-1 (Moore *et al.* 2005). Hence, a decrease in DJ-1 levels were observed in the detergent insoluble fractions obtained from parkin AR-JP human brain.

Recently, a triple parkin/PINK1/DJ-1 KO mouse model was generated (Kitada *et al.* 2009). However and in concordance with single KO mice models, this triple KO did not show any dopaminergic neurodegeneration either at 3, 12 or 24 months of age, suggesting that AR-JP related proteins are not crucial for maintaing SNpc cell survival during the aging process in mice (Kitada et al. 2009).

PD RELATED FACTORS

Idiopathic PD is believed to be a multifactorial disease involving several important mechanisms that process toward disease onset late in life. In regard to the SNpc neurons, their physiology and function gives clues to which pathways are important to investigate. Firstly, the nuclei is relatively small (containing approximately 550 000 in non-PD patients (Pakkenberg *et al.* 1991)) and projects to a large area of *striatum*. This requires a branched network of neurons and high energetic demands, which puts pressure on mitochondrial ATP-production. Due to the atypical use of calcium channels instead of sodium channels for generating action potentials, calcium buffering is especially important in SNpc neurons. Some studies even suggest that 5-10% of SNpc neurons die for each decade during normal aging (reviewed in (Stark & Pakkenberg 2004)). Further, inclusion bodies of surviving neurons in SNpc of PD patients manifest the importance in investigating endoplasmic reticulum (ER) and oxidative stress.

In the sections below, some of the popular theories regarding potential cause for dopaminergic cell death in PD are discussed.

Mitochondrial impairment

The mitochondrial organelle

The double membrane structure of mitochondria allows a large area of the inner-membrane to be folded inside the boundaries of the outer membrane, forming the cristae, where multiple copies of maternally inherited mtDNA are kept. One of the main functions of mitochondria is to produce energy. The mitochondrial respiratory chain, responsible for ATP production through oxidative phosphorylation, is composed of more than 90 subunits of which 13 are encoded from the mtDNA. Why this small subset of DNA has maintained through evolution is not clear, but may be due to the hydrophobicity of proteins encoded, or a benefit derived from local regulation of ATP production. The respiratory chain is mainly localized in the cristae membranes and is composed of five subunits, which generates an electrochemical gradient by shuttling protons across the inner membrane. A fifth complex generates ATP driven by the achieved proton gradient. The large majority of components important for mitochondrial structure and function are transcribed from the nuclear genome and then imported into the mitochondria. An important function of mitochondria is to mediate apoptosis. This involves the release of apoptotic inducing proteins through the mPTP (Scheffler 1999).

Calcium is mainly stored in the ER, but mitochondria also store calcium, and are especially important at synapses and growth cones at a far distance from the ER. The

main calcium exit from mitochondria is through the outer mitochondrial membrane Na^+/Ca^{2+} exchangers, and to a lesser extent via H^+/Ca^{2+} exchangers. Some calcium is also released through the mPTP, while the uniporter shuttles calcium into the mitochondria (Bernardi 1999).

Exposure to mitochondrial toxicity induces parkinsonism

Although studies have identified hereditary risk factors for PD, there are also environmental risk factors. Indeed, twin studies show that prevalence of PD is not different in monozygotic when compared to dizygotic twins, suggesting that environmental agents play a role (Hatcher *et al.* 2008, Wirdefeldt *et al.* 2008). Notably, exposure to pesticides such as paraquat and rotenone, and metals has been shown to increase the risk of PD for humans (Hancock *et al.* 2008, Dick *et al.* 2007). The herbicide paraquat and the insecticide rotenone exhibit their effects via mitochondrial impairment, suggesting that mitochondrial dysfunction is correlated to disease onset (reviewed by (Hatcher et al. 2008)).

Mitochondrial toxins are commonly used to model PD in cells or in animal models. One such compound is 1-methyl-1,2,3,6-tetrahydropyridine (MPTP), which was discovered from an unfortunate outcome when a group of heroine addicts were self-administrating this mitochondrial complex I inhibitor as contaminating product together with a heroine analogue and hence developed sub-acute chronic parkinsonism, indistinguishable from the sporadic disease (Langston *et al.* 1983). In the brain, glial conversion of MPTP to its toxic metabolite MPP+ leads to an accumulation of the drug in dopaminergic neurons, through direct import via the dopamine transporter, causing subsequent nigral degeneration. In primates, MPTP exposure leads to α-synuclein positive inclusions; however, rodents are apparently resistant to MPTP toxicity (Donnan *et al.* 1986). On the contrary, both the dopamine metabolite 6-hydroxydopamine (6-OHDA), and the complex I inhibitor rotenone, induce nigral degeneration in rodents and is hence readily used in laboratories to produce model animals for PD (Shimohama *et al.* 2003). Chronic rotenone exposure in rats moreover provokes the formation of α-synuclein and ubiquitin inclusions similar to LB (Betarbet *et al.* 2000).

The induction of PD-related features by mitochondrial toxins lends support to the notion that mitochondrial dysfunction is important in PD pathogenesis. Indeed, complex I activity of the mitochondrial respiratory chain has been shown to be decreased in SNpc neurons of idiopathic PD patients (Schapira *et al.* 1989, Janetzky *et al.* 1994).

Mitochondrial dynamics

The rate of dynamics and motility is cell specific, where neurons requires a highly adjustable mitochondrial morphology while for instance cardiomyocyte mitochondria are rather stationary (Kuznetsov *et al.* 2009). Neuronal mitochondria thus undergo constant fusion and fission in order to maintain a healthy and mobile network to support synaptic maintenance, bioenergetics and mtDNA. The mechanisms of fusion and fission involve several mediators, together constituting intricate machinery (reviewed in (Chen & Chan 2005, Knott *et al.* 2008). The key fusion/fission proteins are presented in figure 1, below. Mitochondrial fusion occurs in a two-step process in which the outer membrane fusion is followed by inner membrane fusion. In mammals, the large homologous GTPases Mitofusin1 and 2 (Mfn1/2) control outer membrane fusion whereas inner membranes are fused by Optic atrophy protein-1 (Opa1). Fission is mediated by Dynamin-related protein 1 (Drp1), human Fission protein 1 (hFis1) and endothelin B1. Drp1 is primarily localized in the cytoplasm and translocates to the mitochondrial scission sites where fission is mediated, whereas hFis1 is anchored to the outer mitochondrial membrane via a C-terminal hydrophobic tail and is spread over the membrane surface, thus not only localized at the scission sites (Yoon *et al.* 2003). Endothelin B1 is believed to act downstream of Drp1 and is translocating to the mitochondria during fission (Karbowski *et al.* 2004).

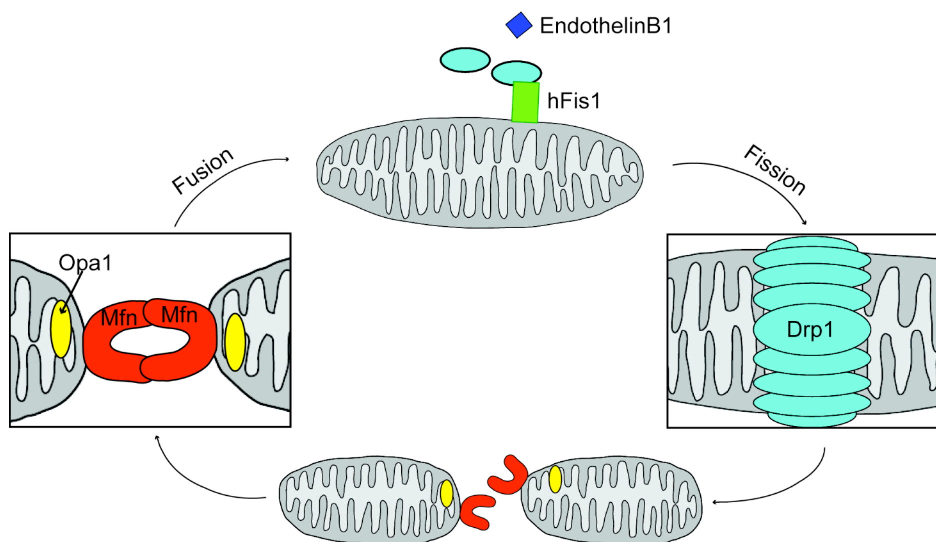

Figure 1. Mitochondrial dynamics

During the fission process, Drp1 is believed to interact with hFis1 and then both outer and inner membranes are divided simultaneously since the scission sites are localized at sites where outer and inner membranes are in contact. Mitochondrial distribution of Drp1 is however not affected by hFis1 KD (Lee *et al.* 2004).

Functional mitochondrial dynamics is important for cell endurance. Down-regulation of Opa1 makes cells extremely vulnerable to apoptotic induction, whereas down-regulation of hFis1 inhibits cell death (Lee et al. 2004). Reducing Drp1 levels by siRNA also increases cellular viability, but to a lesser extent than by Fis1 siRNA. This suggests pro- and anti-apoptotic roles for hFis1 and Opa1, respectively. On the other hand inhibiting fission by expressing dominant negative Drp1 results in loss of synapses and dendritic spines (Li *et al.* 2004). Also influencing cell death and mitochondrial dynamics, the pro-apoptotic regulators BAX and BAK interact with Drp1 and Mfn2 (Karbowski *et al.* 2002, Brooks *et al.* 2007). However, others have demonstrated that mitochondrial fission is not a prerequisite for cell death (Parone *et al.* 2006).

Whether or not and how the fusion and fission processes interact is not clear. However, it has been shown that fusion generates fission and *viceversa* in a cyclical manner. Healthy mitochondria are selectively more prone to fuse as opposed to depolarized mitochondria that are eliminated by autophagy (Twig *et al.* 2008). Thus, mitochondrial segregation by fission is a way for the cell to get rid of malfunctioning mitochondria through lysosomal degradation. As mentioned previously, both of the AR-JP related genes parkin and PINK1 are involved in the process of mitophagy (Narendra et al. 2008, Vives-Bauza et al. 2009).

Mitochondrial motility and distribution

Due to the special morphology of neurons, mitochondria must be readily transported long distances through axons in order to meet ATP demands and accomplish ion buffering where needed in the cell. Mitochondria are typically localized close to ion channels, synapses, growth cones, the nodes of Ranvier and demyelinated interfaces of the axon. Neuritic activity governs the displacement of mitochondria; however, it is not known how mitochondria are led to their destinations. In axons, mitochondria move bidirectional, using the kinesin motor driven machinery for anterograde transport and dynein machinery for retrograde displacement (see figure 2, p.15). Anchor proteins between mitochondria and kinesin involve the Rho GTPases Miro and Milton (Frederick & Shaw 2007). The AR-JP related gene PINK1 was recently shown to interact with both Miro and Milton (Weihofen *et al.* 2009). Also, parkin has been shown to interact with tubulin (Ren et al. 2003) opening the possibility that the mitochondrial transport machinery is involved in AR-JP pathology.

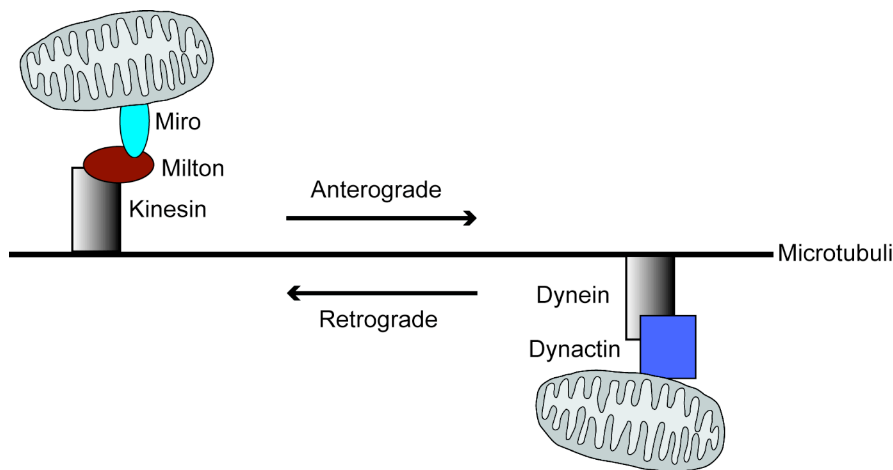

Figure 2. Mitochondrial motility along microtubuli

The Unfolded Protein Response

The presence of LB inclusions in surviving neurons is one of the pathological hallmarks of idiopathic PD. Therefore cellular stress associated with the accumulation of unfolded proteins is believed to be an important aspect of PD. ER stress induces the unfolded protein response (UPR), which is a system involving inhibition of translation, induction of chaperone transcription, as well as degradation of misfolded proteins. Thus, the UPR consists of a protective group of mechanisms involved in PD. When ER stress is prominent and sustained, cells die from apoptosis (reviewed in (Wang & Takahashi 2007)).

The genetic parkinsonism associated proteins α-synuclein, parkin and DJ-1 could be directly associated with the above described UPR mechanisms. Since α-synuclein is a major component of LB, UPR deficiency is believed to be strongly involved in α-synuclein mediated cell death (Hoozemans *et al.* 2007). A direct involvement of parkin in UPR regulation, being an active agent of the ubiquitination system, is perhaps more straightforward to dissect. Not only since some parkin substrates have been shown to accumulate in the absence of parkin (see table 1, p. 5) and thereby induce ER stress, but also because parkin has been shown to interact with and to activate the ER stress protective chaperones Hsp70 and CHIP (Imai *et al.* 2002, Moore et al. 2008). Overexpressing parkin also protects against ER stress and UPR induction is in fact upregulating parkin gene expression (Imai et al. 2000). Thus, it is likely that parkin is involved in the UPR on several different levels.

DJ-1 may also be associated to UPR by upregulating the expression of Hsp70 (Batelli et al. 2008). Moreover, the mitochondrial toxins MPP+, 6-OHDA and rotenone, that were discussed earlier, all induce upregulation of the UPR (Cortopassi *et al.* 2006, Holtz & O'Malley 2003). This association between mitochondrial toxicity and ER stress could be mediated by direct interaction between these two organelles, or by

indirect mechanisms where oxidative stress is involved. Since both mitochondria and ER store calcium, stressing these cellular compartments may also relate to calcium toxicity, as will be discussed further below.

The fact that AR-JP patient brains generally lack LB pathology proposes that PD is rather a syndrome than a unifying disease. In this context, different pathological mechanisms would be important for different pathological features with respect to the genetic background. Nevertheless, the lack of LB in AR-JP cases could also be due to a putative role of parkin, PINK1 or DJ-1 in the process of LB formation. Another possibility is that LB pathology only occurs after long-term cellular stress, as it is noteworthy that idiopathic PD manifests in late stage of life. Conflicting with this latter proposition is the fact that some fetal neuronal grafts implanted in idiopathic PD cases also developed LB pathology after only one decade, thus rather suggesting that inclusions are formed from oxidative stress, neuroinflammation or even prion disease-like related mechanisms (Brundin *et al.* 2008).

Calcium toxicity

When properly regulated, calcium promotes a range of vital neuronal functions, such as neurite outgrowth, synaptogenesis, neurotransmitter release, neuronal plasticity and cell survival (reviewed in (Mattson 2007)). Due to finely tuned calcium homeostasis by channels in the plasma membrane and in the membranes of buffering organelles, normal physiological transient calcium increases are not harmful. However, impairment in this intricate machinery may result in calcium overload, a consequence for several neuronal diseases, as well as in normal aging.

SNpc neurons have atypical calcium channel mediated action potentials in combination with an autonomous, pacemaking activity. This likely increases the demands on adequate calcium handling for survival (Bonci *et al.* 1998, Mercuri *et al.* 1994). Interestingly, it has been shown that neurons of SNpc that express high levels of calcium binding proteins are resistant to cell death in PD (Yamada *et al.* 1990, Mouatt-Prigent *et al.* 1994). This supports calcium toxicity as an important factor in PD pathology.

The gradient between extracellular and intracellular calcium concentrations is estimated to 1:10 000, with approximate concentrations of 50-100 nM inside and 1-2 mM outside of the cell. Thus, the integrity of the plasma membrane is crucial for calcium homeostatic maintenance. Lipid peroxidation can disrupt this intricate machinery by impairing ion-motive ATPases or glutamate and glucose transporters, which depolarize the plasma membrane (Mattson 1998, Arundine & Tymianski 2003). Another possible mechanism leading to toxic calcium concentrations is the impairment of intracellular buffering organelles via ER or mitochondrial stress.

Several systems through which calcium mediates neurotoxicity have been proposed. Calcium activates cysteine proteases, like calpains and caspases, that degrade cytoskeletal components, membrane receptors and enzymes (Chan & Mattson 1999). Moreover, excess calcium promotes oxidative stress and cell death either by having an excess mitochondrial calcium load (Nicholls 2009) or via excitotoxicity induced apoptotic cell death through mitochondrial impairment (Ankarcrona *et al.* 1995).

Intracellular calcium

ER mainly sequesters calcium through the sarco-ER calcium ATPase (SERCA) pumps whereas calcium release from ER to the cytosol is mediated via inositol 1,4,5 triphosphate- (IP_3R) and Ryanodine-receptors (RyR). The binding of IP_3 to IP_3R initiates calcium release which in turn induces calcium induced calcium release from the RyR (Fabiato & Fabiato 1977). IP_3s are generated via the phospholipase C (PLC) dependent hydrolysis of plasma membrane lipids when activated by G-coupled protein or tyrosine kinase (Trk) receptors. There are 13 different PLC isotypes, that are widely expressed in the body. These are subdivided into classes according to their structure and mode of activation, including: PLC-β, -γ, -δ, -ε, -ζ, -η (reviewed in (Rebecchi & Pentyala 2000)).

Two studies in this thesis relate to PLCγ1. PLCγ1 has two catalytic domains separated by two Src homology 2 (SH2) domains binding phosphotyrosine containing sequences, one Src homology 3 (SH3) domain binding to proline-rich sequences and also has a pleckstrine homology (PH) domain, a C2 domain and EF-hand motif.

The SH3 domain mediates the proliferation and mitogenesis promoting functions of PLCγ1 by serving as a guanine nucleotide exchange factor (GEF) for PI_3K enhancer (PIKE), which is a nuclear GTPase stimulating PI_3K activity (Ye *et al.* 2002). PLCγ1 also interacts with Dynamine-1 and by GEF activity stimulates Dynamine-1 mediated EGF-R internalization and upregulates Dynamine-dependent ERK activation, which may be part of the mitogenic properties of PLCγ1 (Choi *et al.* 2004). It has also been shown that PLCγ1 activates mTOR/p70S6-kinase (Markova *et al.* 2009) and is important for agonist induced calcium release independently of its lipase activity (Patterson *et al.* 2002). Thus PLCγ1 clearly has other functions separate from being a lipase.

The PLCγ1 mediated hydrolysis of membrane lipids is generated by SH2 domain mediated complex formation with neurotrophin bound Trk receptor and Trk receptor kinase mediated PLCγ-1 phosphorylation is then promoted (Kaplan *et al.* 1991, Klein *et al.* 1991). PLCγ1 further hydrolyze membrane lipids generating DAG and IP_3 activating Protein Kinase C (PKC) and promoting the release of calcium from ER stores respectively (Kaplan & Miller 2000).

Oxidative stress

Oxidative stress is a consequence of increased levels of reactive oxygen species (ROS) or reactive nitrogen species (RNS). There are several types of radicals, each of which has in common a state of high reactivity to macromolecules of the cell due to an unpaired electron.

Free radical stress is believed to be important in the pathogenesis of PD. Lipid peroxidation and oxidative protein damage are both increased in PD affected regions (Dexter *et al.* 1989, Yoritaka *et al.* 1996). Further, levels of nitratative damage of both α-synuclein and parkin is enhanced in PD (Giasson *et al.* 2000, Yao *et al.* 2004). Moreover mtDNA mutations, which may be generated by oxidative stress, accumulate in SNpc neurons of PD patients (Bender *et al.* 2006).

Superoxide dismutase (SOD) is an important enzyme that eliminates free radicals. Interestingly, while the cytosolic isoform SOD1 is unaltered, the mitochondrial isoform SOD2 is increased in PD patients (Saggu *et al.* 1989), which suggests that mitochondria are the source of oxidative stress in PD. Indeed, malfunctioned mitochondria are believed to generate free radicals due to impaired oxidative phosphorylation. Yet there are other sources of oxidative stress, such as the generation of quinone from dopamine, however dopamine may not be an important source of oxidative stress since tyrosine hydroxylase KO mice are not more sensitive to neurotoxins than their WT littermates (Hasbani *et al.* 2005).

Furthermore, oxidative stress may occur via neuroinflammatory response. Glial activation is present in all affected brain regions of PD, as well as in toxicity PD models (Hirsch & Hunot 2009). Free radicals can also act as signaling molecules. For instance, hydrogen peroxide has been shown to provoke cell death by modulating NFκβ transcription factor and c-Jun kinase (Rhee 2006, Schreck *et al.* 1991). When it comes to AR-JP, DJ-1 has been shown to protect against oxidative stress (Park et al. 2005, Taira et al. 2004). Also, DJ-1 upregulates glutathione synthesis during oxidative stress (Zhou & Freed 2005) and stabilizes the antioxidant regulator Nrf2 (Clements *et al.* 2006).

THESIS AIM

PD is a multifactorial neurodegenerative disorder characterized by protein aggregates, disturbances in calcium homeostasis, mitochondrial dysfunction and oxidative stress. Since the discovery of gene mutations causing familial parkinsonism it has been possible to study what is the underlying cause and relation between PD and specific cellular pathological mechanisms. The aim of this thesis was to investigate the mechanism of action from lack of function of the AR-JP associated gene products parkin, DJ-1 and PINK1.

SPECIFIC AIMS

- To study if parkin interacts with PLCγ1 (Paper I)
- To investigate the effect on calcium homeostasis from parkin and PINK1 deficiencies (Paper II and V)
- To analyze mitochondrial dynamics and motility in DJ-1 or PINK1-deficient cells (Paper III-V)

METHODOLOGY

In this section some of the methods used during this thesis work are discussed and compared. A more detailed description of each method procedure can be found within the papers attached to this thesis book.

MODELS USED IN THE STUDIES

Most of the work in this thesis has been performed using stable transfected human neuroblastoma cell lines. The parental lines SH-SY5Y (used in Paper I and II) and BE(2)-M17 (used in Paper III-V) both express enzymes necessary for dopamine synthesis, making these cell lines suitable and commonly utilized for studying PD related cellular mechanisms.

Neuroblastoma cells (Paper I-V)

An advantage with neuroblastoma cell lines is that introduction of genetic material is not as time consuming as when using animal models. DNA constructs containing WT, familial mutants or KD sequences were introduced into the cells by transfection (SH-SY5Y cells) or lentiviral transduction (M17 cells) and clones expressing similar amounts of protein were selected. Since all cells derive from the same parental line, comparisons between lines usually reflect differences of the genetic material introduced. In Paper I-II and IV, we used stable cell lines over expressing WT and mutant forms of parkin (Fig 3 a) and PINK1 (Fig 3b). The PINK1 'Kinase Dead' cell line expresses an artificial triple mutant (amino acids 216-219, 360-362 and 384-386).

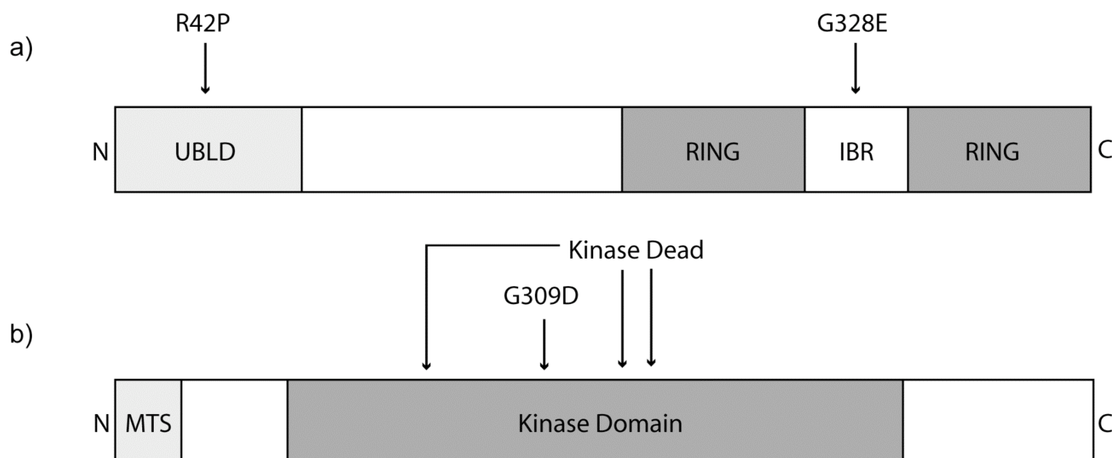

Figure 3. Functional domains of parkin (a) and PINK1 (b). (UBLD: ubiquitin ligating domain; RING: really interesting new gene; IBR: In between RING; MTS: Mitochondrial targeting sequence)

A disadvantage with using neuroblastoma cell lines as a model for neurodegenerative disease is the fact that they are derived from neuronal tumors, and by definition are therefore cancerogenic cells with different features from healthy neurons. However, since our aim of these studies has been to determine the basic functions of genes involved in parkinsonism, we consider the neuroblastoma cells a good model to study mechanisms which are generally well preserved primitive through to more developed cell types.

In Paper V, we used differentiated M17 cells expressing either control, DJ-1 or PINK1 shRNA to study axonal transport of mitochondria. The differentiation was carried out used retinoic acid treatment followed by exposure to brain derived neurotrophic factor (BDNF). After two weeks the cells gained a neuronal phenotype with axonal projections. This model is useful when studying axonal mechanisms.

Human post-mortem brain and transgenic mice brain (Paper I)

In Paper I, we used brain material to validate our findings from SH-SY5Y cell studies. For this purpose, homogenates from Parkin KO mice brain (Goldberg *et al.* 2003) and human post-mortem brain from 3 individuals not diagnosed with PD were used.

One advantage with the use of transgenic mice is that the experimental groups all originate from the same genetic background and have been exposed to an equivalent of environmental influences, resulting in reduced intra-individual variability. Differences between transgenes and their respective controls would therefore be an effect from the genetic alterations. However a major concern with animal models for human disease is how well they reflect the human pathology being studied. For instance, previous characterization of Parkin KO mice reveals no SNpc cell loss or LBs, which are the two neuropathological hallmarks for PD (Goldberg et al. 2003, Itier *et al.* 2003, Von Coelln *et al.* 2004). Thus, the use of Parkin KO mice as model for PD has been questioned (Perez & Palmiter 2005).

Post mortem time is an important factor to take into account when working with human brain material and may account for some of the inter-individual variability. Also the age, lifestyle and cause of death are factors important to take into account when analyzing data obtained from human samples. In Paper I we chose to analyze material from cortex, striatum and *substantia nigra*, thus including both areas directly affected by PD neurodegeneration (*substantia nigra*, which neurons project to *striatum*), as well as an area less affected by PD (cortex).

MITOCHONDRIAL MORPHOLOGY AND DYNAMICS

Microscopy of fixed cells (Paper IV and V)

Due to the complexity and dynamics of the mitochondrial network, a necessary approach for studying this organelle is to use a combination of methods. In Paper III-V we used a range of methods for quantifying mitochondrial network abnormalities at different levels.

In Paper IV we used transmission electron tomography (TEM) to look at individual mitochondria. In this technique, fixed cells can be imaged at very high magnification due to the small de Broglie wavelength of electrons, instead of being limited by the visible light of photons for regular light microscopes. This is a valuable method for evaluating cristae morphology, degree of mitochondrial fusion or fission as well as outer and inner membranous structures and association to lysosomes indicating mitophagy. We have also used confocal microscopy to validate the mitochondrial network using fluorescent labeling of mitochondria in fixed cells. The mitochondria were then quantified according to three categories including: truncated (resulting from an excess of fusion), normal, or fragmented (resulting from an excess of fission). The drawback with this method is that counting is subjective. To reduce subjectivity several different individuals repeated the counting and a mean was presented (Paper IV). Since mitochondria are organized in a large structure, confocal imaging is useful for studying changes in the whole dynamic network, while TEM is valuable for studying individual mitochondria and detailed morphological structures.

Live imaging (Paper III-V)

A more objective method and a good complement to the above described methods is Fluorescence recovery after photobleaching (FRAP) used in Paper III and IV. Live cells are transfected with a mitochondrially targeted GFP construct enabling full visibility of the mitochondrial network. A small area of the mitochondrial network is subsequently exposed to high intensity laser power for complete bleaching of the GFP-signal. Then the time of recovery for the bleached area is measured. The time for GFP intensity to reach the intensity level prior to bleaching is a measure of how well interconnected the mitochondrial network is.

A direct measure of mitochondrial fusion was also employed in Paper IV. Mitochondrially targeted photoactivatable GFP was transfected into cells, allowing for photoactivation of single mitochondria followed by measuring of how fast the photoactivated dye was diluted.

In Paper V we studied mitochondrial anterograde and retrograde transport in axons. We labeled mitochondria fluorescently and imaged the cells live for 20 minutes.

Data processing of this film clip resulted in one kymograph for each neurite. Kymographs gives a graphical representation of spatial position over time. From the kymographs movements of individual mitochondria were traced as lines. Straight lines represent still mitochondria, lines towards the right hand side represent anterograde movements and towards the left retrograde movements. Kymographs were quantified by counting the direction of the lines from each mitochondria. Two different individuals repeated the counting.

An advantage with this method is that the cells that are imaged were not manipulated. The 2-week differentiation process and subsequent imaging was performed in the same Petri dish and were remained in their culture media. A very low laser intensity was employed, to assure that laser induced damage during the recordings was kept at its minimum.

VIABILITY AND MITOCHONDRIAL FUNCTION

Colorimetric assay (Paper II)

MTT experiment is a straight forward , quantitative colorimetric technique in which the yellow tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) is converted to purple formazan crystals by mitochondrial succinic dehydrogenase (Mosmann 1983). The quantity of purple product is measured by absorbance, yielding an estimation of cellular viability. For toxicity studies, MTT is very useful since it is a fast and easy to use method. A drawback is that cells with mitochondrial deficits may be less efficient in converting the salt into crystals, which could then interfere with the result if the aim is to quantify viability independent of mitochondrial function.

Fluorescent probes (Paper III-IV)

Since MTT measures viability and not type of cell death, an accurate investigation of apoptosis or necrosis can instead be done by staining cells with FITC conjugated AnnexinV and the DNA binding agent Propidium Iodide (PI). AnnexinV interacts with Phosphatidyl Serine, which is exposed on the outer cellular surface if the cell is undergoing apoptosis. PI binds to DNA, and thus represents late apoptosis (when co-stained with AnnexinV) or necrosis (single PI staining) if the cell is burst. In Paper IV we validated viability with this staining procedure followed by quantification using flow cytometry.

A way to estimate mitochondrial function is to measure the $\Delta\Psi_m$. The probes appropriate for this application share the property of selectively accumulating in mitochondria according to the $\Delta\Psi_m$ (Scaduto & Grotyohann 1999). We used the

fluorescent rhodamine derivatives tetramethylrhodamine ethyl ester (TMRE; Paper III) and tetramethylrhodamine methyl ester (TMRM; Paper IV and V). The fluorescence was quantified in live cells using flow cytometry or confocal imaging.

PROTEIN-PROTEIN INTERACTION (PAPER I)

In Paper I we utilized two different methods for detecting protein interaction, co-immunoprecipitation (Co-IP) and immunocytochemistry followed by confocal microscopy. For Co-IP, cells were lysed and mixed with primary antibodies targeted for the proteins of interest. This was followed by incubation with sepharose-conjugated beads that bind to the heavy chain of the antibody. Centrifugation resulted in a pellet enriched with beads bound to the antibody and the proteins of interest as well as any interacting proteins. After dissociating the beads, the protein and its putative interactors can be visualized by western blotting. An advantage of Co-IP technique is that the membranes can be stripped off the membranes, which can then be reprobed with several different antibodies. For instance, we used a previously known interactor as control for our experiment while investigating the presence of a novel interactor (Paper I, c-Cbl versus parkin).

The benefit of using confocal imaging for identifying protein interaction is that the proteins are detected *in situ*, compared to Co-IP where the whole cell is lysed. Therefore, one can assume in which cellular organelle the interaction occurs. Also there is a decreased risk of false interactors since the natural compartments are preserved.

PROTEIN ACTIVITY

In vitro ubiquitin conjugation assay (Paper I)

We utilized an *in vitro* assay for measuring the ubiquitin activity of parkin on PLCγ1. Parkin and PLCγ1 was immuno-precipitated from lysed WT, R42P and G328E parkin over-expressing cells. Levels of parkin and PLCγ1 were then analyzed by immunoblot in order to normalize fractions from all three cell lines and contain similar amounts of each protein. Subsequently, the immunoprecipitates were mixed in a buffer containing reticulocytes, his-tagged ubiquitin, the proteasome inhibitor MG-132 and ATP. The ubiquitin activity was analyzed by immunoblot. Immuno-precipitates are not purely containing parkin and PLCγ1, but also other interactors of these proteins that may interfere with the reaction. On the other hand, this impurity could reflect the physiological situation better than recombinant protein.

Phosphorylation (Paper II-V)

In Paper II-V we looked at protein phosphorylation using western blotting with phosphospecific antibodies (Paper II-V) or using a phospho-enrichment kit followed by western blotting with the protein of interest. We also employed a GTPase assay to measure the activity of the mitochondrial fission protein Drp1 (Paper IV) in which the amount of GTP was measured over time. The GTPase Drp1 was immuno-precipitated and mixed with a buffer containing ^{32}P- GTP. After incubation, chromatography and drying, radioactive bands were detected by autoradiography using a phosphoscreen. GTPase activity of Drp1 was expressed as loss of GTP at each time point.

PI hydrolysis (Paper II)

In Paper II we performed Phosphatidyl Inositol hydrolysis (PI hydrolysis) to estimate the activity of phospholipids. The cells were first incubated with tritium labeled myo-inositols, which are incorporated into the plasma membrane and then hydrolyzed by phospholipids. We then separated the lipids from the soluble phase by Dowex chromatography and measured the amount of soluble compared to the amount of lipid-bound tritium.

Subcellular fractionation (Paper II, IV and V)

Many proteins translocate when they change activation status or are locally activated in some organelles. In order to detect such changes, we have performed subcellular fractionation. We measured translocation of Protein Kinase C isoforms (PKC; Paper II) and Drp1 (Paper IV) and the mitochondrial presence and activity of Glycogen Synthase Kinase 3 β (GSK3β; Paper V). We utilized different purification protocols depending on the localization of the protein investigated, however all protocols were based on a series of centrifugation steps to separate fractions containing different organelles. To ensure fraction purity we performed immunoblotting of specific markers for each compartment.

MEASUREMENTS OF CALCIUM CONCENTRATION (PAPER II AND V)

We have used the chemical calcium indicators Fluo3 probe (Minta *et al.* 1989) (Paper II) and Indo-1 (Paper V) to measure intracellular calcium levels. Cells were loaded with Fluo3-AM or Indo-1AM ester (the AM ester is conjugated to the dye in order to pass readily through the plasma membrane) and mixed with the mild detergent pluronic acid in order to dissolve the dye. Once inside the cell, the AM-groups are

hydrolyzed by endogenous intracellular esterases thus allowing for selective binding to calcium.

The advantage with the ratiometric dye Indo-1 is that there is an internal reference due to dual emissions. Excitation is at 355 nm and light is emitted at two different wave-lengths depending on whether the probe is bound to calcium (emission at 405 nm) or not (emission at 495 nm). The 495 nm emission is thereby used as an internal loading control, such that when the 405 nm emission increases, the 495 nm emission is forcibly reduced (figure 4).

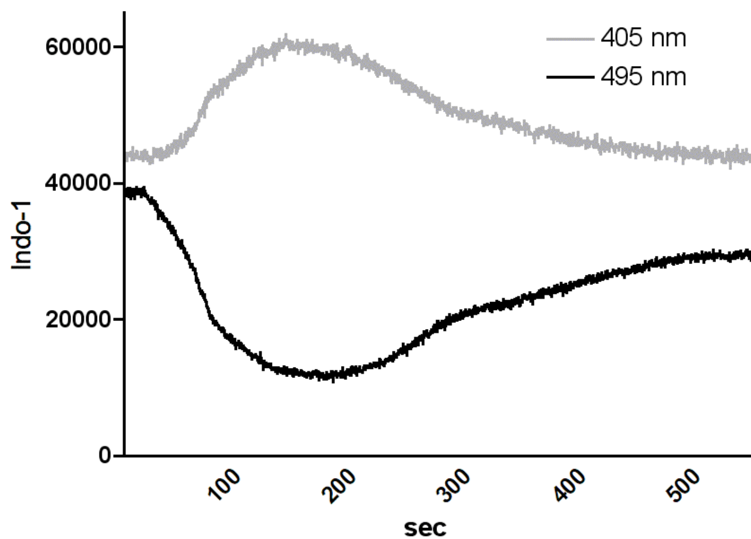

Figure 4: Indo-1 emission. Curve represents treatment with 1 μM CCCP

RESULTS AND DISCUSSION

In this part the main results from each Paper (I-V) are presented and discussed. For more detailed descriptions of specific results or material and methods used for the studies, the reader is referred to the Methodology section or to the Papers I-V.

PARKIN INTERACTS WITH AND UBIQUITINATES PLCγ1

Since parkin is an E3 ubiquitin ligase, it has been suggested that mutations causing AR-JP result in an accumulation of parkin substrates due to impaired proteasomal degradation. The search for new substrates has been extensive and today it is known that parkin is involved in regulating both proteasome dependent and independent pathways. Thus, parkin acts by ubiquitinating specific proteins, but has ubiquitin independent cytoprotective roles too (reviewed in (Moore 2006, Winklhofer 2007)).

In the first paper (Paper I), we identified PLCγ1 as a novel substrate for parkin. Previous studies show that both parkin and PLCγ1 are involved in the same cellular pathway, namely Trk receptor-mediated signaling. Parkin regulates EGF-receptor internalization by proteasome-independent ubiquitination of Eps15, which contains a ubiquitin-interacting motif responsible for EGF-receptor endocytosis (Fallon et al. 2006). Furthermore, PLCγ1 is phosphorylated and thus activated by ligand binding to EGF-receptors and also by the downstream signaling molecule Akt (Kim *et al.* 1991, Wang *et al.* 2006). Phosphorylated PLCγ1 was previously shown to interact with and, more recently, to be mono-ubiquitinated by the RING domain E3 ubiquitin ligase c-Cbl, a member of the same family of E3 ligases as parkin (Singh *et al.* 2007, Tvorogov & Carpenter 2002). Based on the common links between parkin and PLCγ1 described above, we sought to determine whether there could be a direct interaction between these two proteins.

We found that parkin does interact with PLCγ1 in human neuroblastoma cell lines by the use of Co-IP and co-localization visualized by confocal microscopy. This novel interaction was corroborated by Co-IP in brain samples from human cortex, *striatum* and *substantia nigra*.

We next asked whether the interaction between parkin and PLCγ1 would lead to ubiquitination and proteasomal degradation. Using an *in vitro* ubiquitin assay we showed that parkin is able to ubiquitinate PLCγ1, and that parkin mutations R42P and G328E interfere with this ability. Thus the result is a reduction of ubiquitinated PLCγ1. We found that the pattern of parkin-mediated PLCγ1 ubiquitination was not typical poly-ubiquitination. Poly-ubiquitination can be identified on a western blot by a smear

signal of higher molecular weights of the protein of interest. PLCγ1 ubiquitination was instead identified as two major bands on western blot, one corresponding to the full length PLCγ1 and another with an approximate size of 200 kDa. The upper band was equivalent to the ubiquitinated fraction of PLCγ1, a finding that suggested a pattern of multiple mono-ubiquitination. Indeed, parkin has been shown to mono-ubiquitinate other substrates (Corti et al. 2003, Moore et al. 2008). Still, we found a reduction in total PLCγ1 levels when over-expressing parkin in neuroblastoma cells, and further showed a modest accumulation of PLCγ1 in parkin KO mice brain homogenates. Our findings propose that, at least a part of the parkin/PLCγ1 interaction results in degradation of PLCγ1. The subtle effect on degradation may be due to other E3 ligases partially compensating for loss of parkin.

Using confocal imaging we also noted a slight difference in PLCγ1 localization in cells with mutant parkin compared to WT parkin. In the R42P and G328E mutants a vesicular pattern was seen, whereas WT parkin cells primarily showed membrane-associated PLCγ1 staining. Thus it is possible that PLCγ1 localization is governed by parkin ubiquitination, yet this require further investigation.

A regional discrepancy of c-Cbl levels was distinguished in homogenates from human brain, where *substantia nigra* fractions seemingly contained less protein compared to cortex and striatum. Presupposing that parkin and c-Cbl are responsible for regulating PLCγ1, such absence of c-Cbl would make parkin particularly important for nigral regions.

Our data suggests that parkin is regulating PLCγ1 levels by direct association and ubiquitination, which may be of importance for finding novel pathways involved in AR-JP.

PARKIN AND PINK1 DEFICIENCIES DISRUPT CALCIUM HANDLING

Calcium toxicity is involved in the pathogenesis of several neurodegenerative disorders (Surmeier 2007). It is known that neurons in the SNpc rely on calcium channels for mediating action potentials (Bonci et al. 1998). Thus, disruption of calcium homeostasis would theoretically have more devastating consequences in this group of neurons compared to sodium channel-dependent neurons in the rest of the brain. Indeed, nigral cells expressing high levels of calcium binding proteins are spared during sporadic PD nigral cell death (Mouatt-Prigent et al. 1994, Yamada et al. 1990). However, it has not yet been clarified what is the real contribution of impared calcium homeostasis in PD, and which signaling mechanisms could be responsible.

Motivated by our findings that parkin interacts with PLCγ1 (Paper I), which is an upstream component of ER mediated calcium release, and that PINK1 is crucial for mitochondria (discussed below and in Paper III & V), which is an important calcium storage organelle, we explored if AR-JP mutations could result in impairments in calcium managing in neurons.

Parkin regulates calcium levels via PLC signaling

In paper II, we elucidated the PLCγ1 downstream pathway in parkin-mutants and parkin siRNA-treated neuroblastoma cells. We first sought to determine if parkin or parkin mutations have effects on EGF induced PLCγ1 activation. As a measure of PLCγ1 activation, we determined the levels of Tyr783 phosphorylated PLCγ1 (Tyr783-PLCg1) (Wahl *et al.* 1990, Kim *et al.* 1990). Tyr783-PLCγ1 was increased in parkin R42P and G328E mutant cell lines compared to both WT parkin and non-transfected cells after EGF stimulation. From paper I, we knew that parkin mutations interfered with the ability to ubiquitinate PLCγ1, thus opening the possibility that ubiquitination was somehow blocking or regulating the phosphorylation site. Indeed, this is the case for c-Cbl-mediated PLCγ1 ubiquitination (Singh et al. 2007). It has further been shown that over-expressing c-Cbl decreases PI hydrolysis, suggesting that the interaction between PLCγ1 and c-Cbl inhibits PLCγ1 activity (Graham *et al.* 2000). In view of the significance of ubiquitination in governing PLCγ1 activity, identifying the site or sites for parkin mediated PLCγ1 ubiquitination would be of extreme importance toward understanding PLCγ1 function.

Since the main function of PLCγ1 is to hydrolyze membrane lipids, and thus to generate IPs released to the cytosol, we measured the relative amount of PI hydrolysis in our cell lines. We found that both parkin-mutants and parkin siRNA-treated cells had higher rates of membrane hydrolysis, thus confirming that PLCγ1 is more active when the activity of parkin is reduced. Down-regulating c-Cbl had the same effect as parkin siRNA, suggesting that the increased PI hydrolysis is mainly related to PLCγ1 and not to other parkin-related functions.

IP_3 generated through PI hydrolysis, binds to IP_3 receptors on the ER, instigating calcium release that in turn activates ER RyR which are mediating additional calcium release into the cytosol (Fabiato & Fabiato 1977). Hence, we decided to measure calcium levels and concordantly found that basal cytosolic calcium levels were increased in parkin mutant cells, or in cells treated with siRNA against parkin or c-Cbl. Blocking the RyR by dantrolene reduced calcium levels in parkin-mutant cells to the level of WT and non-transfected cells. Also, blocking of PLC by neomycin could reduce calcium levels in the mutants. In order to distinguish if other sources of calcium

participated, we blocked additional channels and pumps known to regulate intracellular calcium concentrations. Blocking the plasma membrane L or N-type calcium channels with nimodipine or w-conotoxin did not alter the discrepancy between cell lines. Similarly, treatment with thapsigargin, an inhibitor of the calcium ER pump, resulted in a parallel increase of intracellular calcium for all lines. Altogether, these data point out that impaired PLC signaling is, possibly, the major cause for the excess of cytosolic calcium seen in parkin-deficient cells or in AR-JP parkin mutants. If PLCγ1 is solely responsible for the calcium excess or if other PLC-isoforms also participate remains to be investigated.

In Paper I and II we did however show that protein levels and activity of PLCβ remain unaltered in parkin-mutant cell lines. No differences were found between the different cell types when stimulated with the muscarinic acetylcholine receptor agonist, carbachol, indicating that PLCβ is not responsible for parkin deficiency-related increase in calcium. Nevertheless, since an extraordinary high number of channels, organelles and enzymes participate in calcium homeostasis, additional contributions cannot be completely ruled out. We noted that parkin-mutants had a long tail-off effect after stimulation using carbachol.

Apart from ER, mitochondria are also important calcium storage organelles, and parkin has been linked to mitochondrial morphology and is believed to have a specific role in mediating the degradation of uncoupled mitochondria (Narendra et al. 2008, Vives-Bauza et al. 2009). Hence, it is possible that mitochondria in parkin-mutant cells are less effective in taking up calcium resulting in a tail-off effect after high calcium peaks.

PINK1 deficiency increases calcium release provoked by CCCP

In Paper V, we measured calcium after mitochondrial uncoupling in neuroblastoma cell lines and detected an increase of cytosolic calcium in absence of PINK1 when compared to controls. Since the time to re-establish basal calcium levels was similar in cells lacking PINK1 and in controls, we assumed that calcium can be effectively buffered even in absence of PINK1. Yet, further investigation on the relative capacity of mitochondria and ER to store calcium would be required for identifying if and how PINK1 has a role in calcium storage. Indeed, other groups have shown that levels of mitochondrial calcium in PINK1-deficient cells were increased (Gandhi et al. 2009) and also that PINK1 mutant cells exposed to toxic levels of α-synuclein were more prone to release cytosolic calcium (Marongiu et al. 2009). It is thus possible that both parkin and PINK1 have specific roles in regulating mitochondrial calcium and that in AR-JP, disrupted calcium handling acts in concert with the other multiple functions of parkin and PINK1, eventually leading to cell death.

The discrepancy in calcium handling resulting from PINK1 KD uncovered in paper V, differed from that detected in parkin deficiency lines in Paper II. For PINK1 KD, divergence from control cells was unraveled only after mitochondrial uncoupling and not present at basal state as in parkin-mutants. A possible assumption made from this discrepancy is that in spite of a lack of PINK1, ER calcium storage is not dramatically affected. A tempting hypothesis is that instead PINK1 deficiency leads to impairment of tethering between mitochondria and ER as a result of fragmentation of the mitochondrial network. Indeed it has been shown that mitochondrial fusion is important for calcium transfer between mitochondria and ER (de Brito & Scorrano 2008).

PINK1 KD increases the level of GSK3βSer9 in mitochondria

A finding that could potentially explain the discrepant calcium response to mitochondrial uncoupling is that mitochondrial fractions purified from PINK1 KD cells show an increased level of serine 9 phosphorylated GSK3β (GSK3βSer9) (Paper V). GSK3βSer9 has previously been shown to inhibit the mPTP. Since calcium can pass through the mPTP, we hypothesize that either GSK3βSer9 is promoted to compensate for disruption of the $\Delta\Psi_m$ (discussed below, p. 32) or that GSK3βSer9 is a direct effect from PINK1 deficiency upstream of mitochondrial impairment. To validate the latter idea, we treated cells with lithium, known to inhibit GSK3β by enhancing Ser9 phosphorylation, and then purified mitochondria. In concordance with others (Petit-Paitel *et al.* 2009), we found that the inhibition of GSK3β occurs locally in mitochondria, and does not involve a translocation of the already Ser9 phosphorylated cytosolic GSK3β to the mitochondria. The next step is thus to determine if there is a reduction in Ser9 dephosphorylation or an increased Ser9 phosphorylation *in situ* at the mPTP of PINK1 KD mitochondria. A weak GSK3β-mediated phosphorylation of parkin has been suggested (Avraham *et al.* 2007). However, it is as yet undetermined whether or not parkin has a role in PINK1-related alterations in calcium release.

Parkin deficiency alters PKCα levels

PLC activity has two major outputs, the increase in intracellular calcium and the activation of PKC. In Paper II, we sought therefore to determine if parkin deficiency had an effect on the activity of calcium-dependent PKCα and calcium-independent PKCε. Since activated PKC translocates to the membrane, we purified membrane fractions from cells expressing variants of mutant or WT parkin and calculated the ratio between the levels of soluble (not active) and membrane bound (active) PKC, which should reflect PKC activity.

Surprisingly, we found a decrease in the total fraction of calcium-dependent PKCα in parkin-mutant cell lines, yet the net activity was equal to WT parkin cells or non-transfected cells. We hypothesize that this decrease is a compensatory mechanism to retain PKCα activity at normal levels. Indeed overactivity of PKC has previously been shown to result in down-regulation of enzyme levels (Dehvari *et al.* 2007). Another possibility is that parkin directly regulates PKCα transcription or degradation, however this must be determined in future experiments. Neither the protein levels nor activity of the calcium-independent PKCε isoform were altered between cell lines (paper II).

Blocking RyR protected parkin-mutants from 6-OHDA

In Paper II, we further explored how the differences in calcium levels and functional parkin influenced the response to 6-OHDA. 6-OHDA mediates toxicity by oxidative stress and 6-OHDA injection in rat is a commonly used model for PD (reviewed in (Simola *et al.* 2007)).

In agreement with previous findings (Jiang *et al.* 2004), we show that WT but not mutant parkin could protect against 6-OHDA treatment. Pre-treatment with dantrolene could rescue viability to the level of WT parkin expressing cells, which indicates a partial recovery. This suggests that balancing calcium rescue the part of 6-OHDA mediated toxicity for which parkin is protective. However there is an additional mode of toxicity that WT parkin or equilibrating calcium could not suppress. The high dose of oxidative stress imposed by 6-OHDA is likely causing alternate devastating effects, which are not compensated for by parkin.

MITOCHONDRIA ARE AFFECTED BY DJ-1 OR PINK1 KD

To provide an adequate amount of energy for long axonal transports and synaptic transmission, mitochondrial dynamics are particularly important in neurons (reviewed by (Knott et al. 2008)).

It has been proposed that PD pathogenesis involves mitochondrial dysfunction, mainly based on the findings that mitochondrial toxins induce a parkinsonism phenotype in model animals and humans, and that mitochondria of sporadic PD patients contain increased levels of mutated mtDNA and decreased complex I activity (Bender et al. 2006, Janetzky et al. 1994). It is, however, not known what is the underlying mechanism for PD-related mitochondrial dysfunction. In Paper III-V, we sought therefore to elucidate how DJ-1 and PINK1 deficiencies affect mitochondrial morphology, dynamics and motility, by using as model stable DJ-1 and PINK1 KD human neuroblastoma cell lines.

Both DJ-1 and PINK1 KD result in decreased $\Delta\Psi_m$ (Paper III & IV-V, respectively). In PINK1-deficient cells, this finding has been reported beforehand (Exner et al. 2007, Wood-Kaczmar *et al.* 2008).

In Paper IV we confirmed previous reports that WT PINK1, but not G309D familial mutant or artificial kinase-dead triple mutant PINK1 protect against cell death induced by mitochondrial toxicity (Deng et al. 2005, Haque et al. 2008, Petit et al. 2005, Wood-Kaczmar et al. 2008) mediated by the mitochondrial complex I inhibitor rotenone. Since rotenone is known to induce mitochondrial fission, we hypothesized that PINK1 may also be related to mitochondrial dynamics. In order to measure this defect in live cells, we employed fluorescence recovery after photobleaching (FRAP) and found that mitochondria lacking functional DJ-1 or PINK1 had a slower rate of recovery compared to controls, thus implying a deficit in mitochondrial dynamics (Paper III and IV). For PINK1 KD, this has also been shown by others (Dagda et al. 2009, Lutz et al. 2009).

The effect from DJ-1 and PINK1 KD could be reversed by expressing the dominant negative K38A mutant of the mitochondrial fission protein Drp1 or Drp1 siRNA, as well as by expressing the mitochondrial fusion proteins Mitofusin2 and Opa1. In Paper III, we further showed that over-expression of either PINK1, DJ1 or parkin could protect against rotenone-induced deficits in mitochondrial motility (measured by FRAP). We also showed that either parkin or PINK1 could overturn mitochondrial fission provoked by DJ-1 deficiency. This suggests a common phenotypic trait from AR-JP-associated genes that has importance for the maintenance of a dynamic mitochondrial network.

Drp1 activity is increased in DJ-1 and PINK1-deficient cells

The mitochondrial fission protein Drp1 can be activated by calcium (Cribbs & Strack 2007). However while we did not detect any effects on basal calcium levels from PINK1 KD, mitochondrial uncoupling on the other hand resulted in a dramatically increased calcium release (Paper V). We proposed that PINK1-deficient cells may have local calcium alterations surrounding mitochondria, which in turn could activate Drp1.

Indeed the pool of active Drp1 was increased in DJ-1 or PINK1 KD cells (Paper III and IV), but its localization and oligomerization were not altered by PINK1 KD (Paper IV). In Paper IV, we recovered mitochondrial dynamics by blocking calcineurin, a Drp1 phosphatase, with FK-506. Yet the $\Delta\Psi_m$ was not recovered by this calcineurin inhibition, which suggests that the uppermost event in this cascade of devastating events is indeed mitochondrial depolarization.

Fission from DJ-1 KD was reduced by decreasing ROS levels

In concordance with other reports (Andres-Mateos *et al.* 2007, Taira et al. 2004, Takahashi-Niki *et al.* 2004), we show that DJ-1 KD enhance ROS levels (Paper III). When balancing ROS by adding the precursor to glutathione, glutathione ethyl ester (GSH), both lipid peroxidation, mitochondrial motility and $\Delta\Psi_m$ were normalized to levels equivalent to the control. DJ-1 has previously been shown to up-regulate glutathione synthesis in response to oxidative stress (Zhou & Freed 2005). Our findings suggest that DJ-1-mediated regulation of glutathione is crucial for evading mitochondrial depolarization and fission. Since oxidative stress is known to induce mitochondrial fragmentation (Barsoum *et al.* 2006), we suggest that the mitochondrial phenotype described in Paper III is a downstream consequence from enhanced oxidative stress resulting from loss of DJ-1.

Neuritic motility was reduced in PINK1 and DJ-1 KD cells

In Paper III and IV we employed non-differentiated cells, with only short neurite extensions. To further validate if these finding may be of relevance for brain neurons exhibiting a different cellular morphology, we differentiated the same cells with retinoic acid and BDNF. This resulted in a neuronal-like phenotype, allowing for neuritic mitochondrial anterograde and retrograde motilities to be quantified (Paper V).

We found that mitochondrial motility was decreased in cells expressing either PINK1 or DJ-1 shRNA. Yet, neither axonal mitochondrial density nor length of mitochondria were altered. However, in undifferentiated cells, mitochondrial length was reduced after PINK1 KD (Paper III). This difference between studies may derive from the fact that mitochondrial length in neurites depends on both anchoring to microtubuli, motor transport along microtubuli and fusion and fission proteins. Another possibility is that only the mitochondria with a certain length are transported into the neurite. In Paper V we show that the mitochondria in the soma and neurites of PINK1 KD cells show a lower $\Delta\Psi_m$ compared to control cells. This may suggest that either normal $\Delta\Psi_m$ is not a prerequisite for neuritic transport, or $\Delta\Psi_m$ is decreased as a consequence from impaired motility. A photoactivatable dye in combination with a probe to measure $\Delta\Psi_m$ would be desirable to determine the effect from PINK1 KD on the process of mitochondrial transport from the soma into the neurite.

In order to see if we could associate the discrepant mitochondrial fusion, seen in Paper III and IV, to the decreased motility in Paper V, we down-regulated Drp1. Indeed, Drp1 siRNA abolished the difference between PINK1 and DJ-1 KD cells to control. Yet, the Drp1 KD had a negative effect on motility in control cells and positive

effect in PINK1 and DJ-1 KD. Thus, we propose that a balanced mitochondrial dynamic network is required for adequate mitochondrial transport.

It has been debated whether mitochondrial motility is regulated by intracellular calcium (Beltran-Parrazal *et al.* 2006, Rintoul *et al.* 2003). However, it remains to be confirmed whether the decrease in mitochondrial motility in PINK1 KD cells may be connected to deficiency in calcium handling, presented in a previous paragraph (p. 30).

PARKIN, PINK1 AND DJ-1 DO NOT FORM A COMPLEX

Several attempts have been made to connect parkin, PINK1 and DJ-1. Although these three proteins are seemingly different in localization and nature, parkin being an E3 ligase, PINK1 a mitochondrial kinase and DJ-1 a putative oxidative stress-sensitive chaperone, their mechanisms of action somehow converge seemingly toward the aim of protecting mitochondria (reviewed in (Henchcliffe & Beal 2008)). It is however possible that the underlying cause for such a mitochondrial phenotype arises from different sources, and that the similar traits are in fact shared downstream effects.

When it comes to connecting DJ-1 with parkin and PINK1, we found that DJ-1 influences PINK1 maturation, by increasing the level of mature protein (Paper III). In the same study, we could however not confirm previous reports that suggested that PINK1, parkin and DJ-1 forms a complex (Xiong et al. 2009) either with or without the induction of mitochondrial uncoupling by CCCP.

CONCLUSIONS

- Parkin interact with and ubiquitinates PLCγ1
- Lack of function of either parkin or PINK1 impair calcium homeostasis
    - Mutations in parkin or parkin KD disrupts PLC mediated calcium signaling
    - Calcium release from mitochondrial uncoupling is enhanced in PINK1 KD cells
- DJ-1 or PINK1 deficiencies alter mitochondrial dynamics and motility
    - Lack of DJ-1 results in increased ROS levels, depolarized mitochondria and mitochondrial fission
    - PINK1 KD depolarize mitochondria and induces mitochondrial fission
    - Mitochondrial fission in PINK1 and DJ-1-deficient cells could be rescued by inhibiting calcineurin and supplementing antioxidants respectively
    - Neuritic mitochondrial motility is reduced in both PINK1 and DJ-1 KD cells and could be enhanced by inhibiting fission

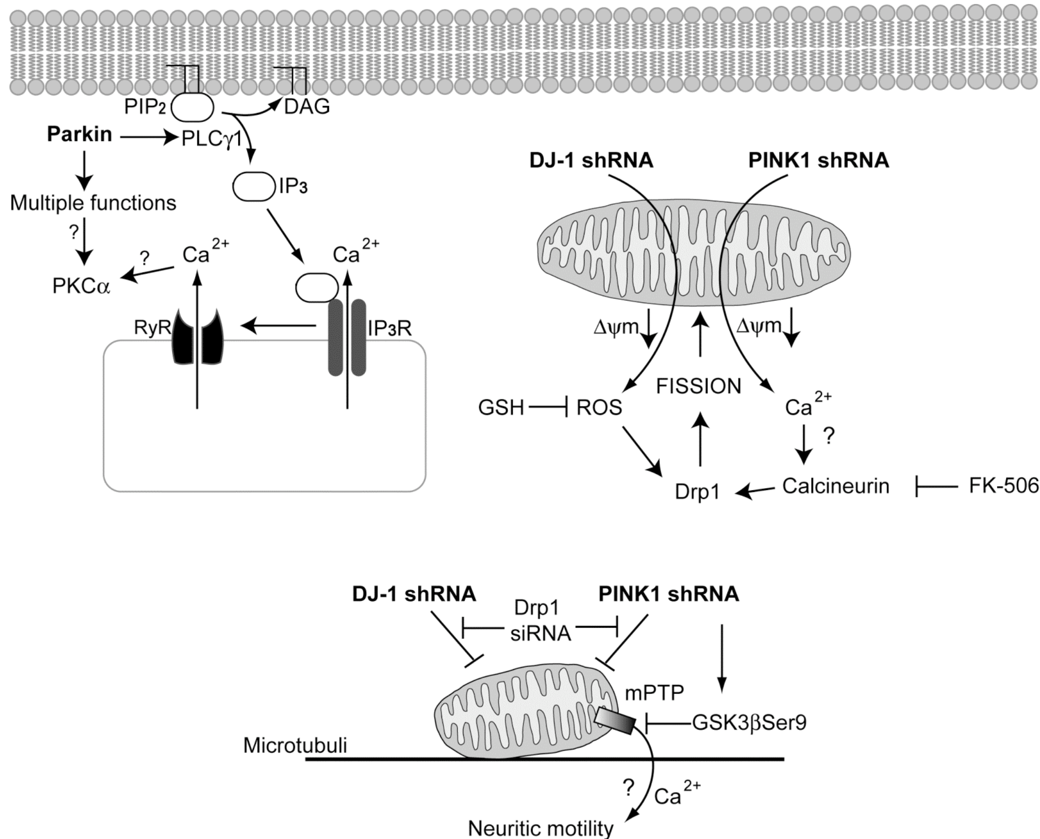

Figure 5. Summary of the results presented in this thesis.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

PD is a complex disorder with apparently many involved factors. It is not clear how the neuropathology develops and if the features arise from a common origin or from several confounding pathways. In the papers included in this thesis we have studied putative pathways involved in the onset of AR-JP. Although this is a rare form of parkinsonism the similarities in pathology between sporadic and familiar disease may be a reflection of common underlying cellular pathways.

In Paper I and II we found that parkin ubiquitinates PLCγ1 and regulates PLC mediated calcium signaling. Parkin mutations or KD resulted in increased levels of intracellular calcium. Calcium toxicity is indeed believed to be a contributing factor in PD pathogenesis, but the underlying causes are not clear (reviewed by (Chan et al. 2009, Surmeier 2007)). The unique autonomous activity and reliance on L-type calcium channels to generate action potentials may be problematic for SNpc neurons if intracellular calcium homeostasis is impaired. The conclusion from Paper II is that parkin is a crucial component for maintaining calcium homeostasis. Some further questions that arose are:

- Can multiple mono-ubiquitination be associated to increased PLCγ1 degradation?
- Do the sites of PLCγ1 ubiquitination converge with the sites of phosphorylation?
- Is PLCγ1 more active in PD affected regions in humans?

Neurons require tremendous amounts of energy. In fact, 20 % of the oxygen is destined for supplying the brain, yet it only constitutes 2 % of the body weight. Mitochondria are the key organelles for supplying neurons with energy and thus their function are inevitably very important for the brain. Indeed, mitochondrial impairment has been implicated for many neurological diseases including PD. The discovery of familial parkinsonism-associated genes has supported the importance for mitochondria in PD pathogenesis. Of the AR-JP associated genes, a convincing body of evidence suggest a link between their functions and mitochondria (reviewed in (Beal 2007, Henchcliffe & Beal 2008)).

In Paper III-V we studied the dynamics and motility of mitochondria in DJ-1 and PINK1 KD cells. We found that both DJ-1 and PINK1 are important for mitochondrial

network dynamics and motility in neurites of differentiated cells. Some of the new questions from these studies were:

- Is mitochondrial GSK3βSer9 related to mitochondrial calcium storage and motility?
- How does DJ-1 associate to the mitochondria and exert its protective functions?

An important challenge for the future is to identify new diagnostic markers for PD. Today, the majority of SNpc are already degenerated at the time of diagnosis. This means that even if medications to target degenerating cellular mechanisms are developed, it may not be sufficient for recovery. An early diagnosis would thus improve the benefit of such drugs.

I think that the amount and quality of research papers elucidating the roles of genes associated with parkinsonism has advanced the field rapidly. It is not yet clear however, what are the primary mechanisms involved in nigral degeneration. We have today many ideas and links between the different gene products. Ultimately the goal would be to find a way to converge all the parkinsonism-associated genes into one cellular pathway, but this is a difficult task. Parkin especially has a wide range of different substrates and exhibits effects in the cell making it very hard to dissect any particular important function.

A less tempting, but perhaps more realistic idea discussed in the scientific community today is that PD is in fact a syndrome rather than a unified disease, with many confounding factors together generating a similar pathology. Even so, common downstream features such as calcium toxicity and altered mitochondrial dynamics could serve as interesting candidate targets in drug development.

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