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Analysis *in vitro* of the neuroprotective action of recombinant human TAT-parkin and parkin over-expression in the 6-hydroxydopamine model of Parkinson's disease

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ABSTRACT

Parkinson's disease is a chronic progressive neurodegenerative movement disorder characterized by a profound and selective loss of nigrostriatal dopaminergic neurons. A major hurdle in the development of neuroprotective therapies is due to limited understanding of disease processes leading to the death of neurons. The etiology of dopaminergic neuronal demise in Parkinson's disease is elusive, but a combination of genetic and environmental factors seems to play a critical role. Mutations in *parkin* are known to be the predominant cause of autosomal recessive, early-onset parkinsonism. Parkin is an E3 ubiquitin ligase, an essential component of the ubiquitin-proteasomal system, and its over-expression is neuroprotective in several cellular models of apoptosis. Cell-penetrating peptides, e.g. linked to the transactivator of transcription (TAT) provide an attractive delivery system across biological membranes.

In the first part of the study, we describe the construction, purification, and characterization of a TAT-parkin fusion protein, and its neuroprotective activity in an *in vitro* model of Parkinson's disease. Western blot and immunocytochemical analyses showed TAT-parkin to transduce PC12 pheocromocytoma cells and Chinese hamster ovary cells and to localize in the nucleus, cytoplasm, and mitochondria. Moreover, TAT-parkin exhibited ubiquitination activity *in vitro*. Importantly, TAT-parkin at nanomolar concentrations protected nerve growth factor-differentiated PC12 cells against the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA), but only if present in the culture medium before, during, and after treatment with 6-OHDA.

Based on these results, the second part of the study was designed to assess the pro-survival action of parkin using a system in which parkin is over-expressed. To accomplish this, stable PC12 cells transfectants were established with plasmids carrying either human wild-type (WT) parkin, or the human R42P mutation (parkin^{R42P}). Non-transfected PC12 cells were used as

control. Clones were screened for the expression of human parkin gene by Reverse Transcription-Polymerase Chain Reaction and Western blotting. The selected clones and un-transfected PC12 cells were treated with 50 and 75 μM 6-OHDA for different times. Cell-viability decreased in a time-dependent manner in both clonal and control cells, but the PC12 parkin^{R42P} expressing clone and un-trasfected cells showed a statistically significant reduction in cell viability already from 2 h. In contrast, in the PC12 parkin (WT) expressing clone cell viability was significantly reduced only from 12 h. As abnormalities in the ubiquitin-proteasome system and in the autophagy-lysosome pathway are thought to be involved in the etiopathogenesis of Parkinson's disease, we treated the above cells with either a specific proteasome inhibitor (MG132) or the macroautophagy inhibitor 3-methyladenine (3-MA) for further 16 h. Improved survival was again evident in PC12 parkin (WT) expressing cells. This latter also showed an increase in the amount of ubiquitinated proteins and in the basal level of autophagy activation. Nevertheless, co-administration of the proteasome and autophagy inhibitors, neutralized the protective effect of PC12 parkin (WT) expression. Over-expression of WT parkin thus appears able to protect cells from oxidative stress by 6-OHDA and from the neurotoxicity of proteasome or macroautophagy inhibitors, and confirmed that the R42P amino-acid substitution affects parkin physiological action.

Taken together, our data suggest that parkin, either as a recombinant TAT-fusion protein or when over-expressed is strongly neuroprotective, preventing dopaminergic PC12 cell death under various stress conditions. Parkin may thus represent a potential new therapeutic target in Parkinson's disease. Moreover, these findings support also the view that protein transduction strategies are well suited for intracellular translocation of proteins and, in particular, point to the application of designing fusion proteins with a protein transduction domain for potential therapeutic benefit, including Parkinson's disease.

RIASSUNTO

La Malattia di Parkinson (*Parkinson's disease,* PD), descritta per la prima volta nel 1817 da James Parkinson, è la più importante affezione del sistema extrapiramidale, le cui principali caratteristiche patologiche sono la progressiva e selettiva perdita di neuroni dopaminergici nella substantia nigra pars compacta, la conseguente diminuzione dei livelli striatali di dopamina e la presenza, nei neuroni dopaminergici nigrali sopravvissuti, di inclusioni citoplasmatiche, denominate Corpi di Lewy, che contengono soprattutto aggregati di α-sinucleina e parkina. La PD è sempre sporadica, eccetto alcuni rari casi associati a mutazioni geniche e che sono responsabili di forme di PD ad insorgenza precoce. A queste forme appartengono:

- forme della PD autosomiche dominanti, dovute a due mutazioni nel gene che codifica per l'α-sinucleina e una mutazione nel gene che codifica per la parte C-terminale dell' Ubiquitina idrolasi L1 (UCHL1);

- forme autosomiche recessive, dovute a mutazioni nel gene che codifica per la parkina, che portano alla maggior parte dei casi di PD familiare e giovanile sporadico e che si caratterizzano per la mancanza dei Corpi di Lewy. Forme autosomiche recessive della PD sono inoltre dovute a mutazioni nel gene che codifica per DJ-1 e PINK1.

Studi sulle forme ereditarie della PD si stanno concentrando verso un' ipotesi comune, cioè che l'accumulo normale e anormale di proteine intracellulari (mutate, disassemblate o danneggiate) ed il malfunzionamento del sistema ubiquitina-proteasoma possa condurre alla morte dei neuroni dopaminergici nigrali. In particolare, lo stress ossidativo generato dallo scompenso delle funzioni mitocondriali e dal metabolismo della dopamina potrebbe promuovere la formazione di proteine mal-ripiegate come risultato di modifiche post-traslazionali, specialmente a carico della α-sinucleina e della parkina. La parkina è una E3-ubiquitina ligasi

coinvolta nei processi di degradazione di proteine danneggiate o mal ripiegate mediante l'interazione con il complesso proteasomico. La perdita di questa funzione da parte della parkina, conseguente a mutazioni (per esempio la mutazione puntiforme Arg42Pro (R42P)) o a stress ossidativo, sembra costituire il meccanismo patogenetico del PD giovanile, portando ad un accumulo delle proteine e alla disregolazione del metabolismo della dopamina. In questo contesto è rilevante notare che recenti studi, sia *in vitro* che *in vivo*, hanno attribuito un probabile ruolo protettivo alla parkina nella sopravvivenza dei neuroni dopaminergici nigrali, aprendo così interessanti prospettive per lo studio e lo sviluppo di strategie terapeutiche innovative della PD, basate sulla neuroprotezione endogena. Alla luce di queste ultime evidenze, il progetto di ricerca ha avuto lo scopo di indagare il potenziale effetto neuroprotettivo della parkina nella risposta cellulare allo stress ossidativo indotto dalla tossina dopaminergica 6-idrossi-dopamina (6-OHDA). Tale obbiettivo è stato perseguito avvalendoci di due approcci sperimentali:

1. produzione, purificazione e caratterizzazione di una proteina TAT-parkina umana da *Escherichia coli* e valutazione della potenziale capacità protettiva mediante somministrazione esogena della stessa.

2. sovra-espressione della parkina wild-type (WT) umana e valutazione del possibile effetto protettivo mediante confronto con cellule di controllo e con cellule sovra-esprimenti la forma mutata R42P (parkina ^{R42P}).

Il modello *in vitro* utilizzato è costituito da cellule surrenali adrenergiche PC12, di feocromocitoma di ratto, sia indifferenziate, sia indotte a sviluppare tratti fenotipici che caratterizzano i neuroni dopaminergici in seguito all'esposizione al fattore di crescita nervoso.

Nella prima parte dello studio è stata clonata ed espressa, attraverso l' uso di sistemi d'espressione in procarioti, una proteina parkina umana. Tale proteina è stata fusa, nella

porzione ammino-terminale, con una coda di 6 istidine (His6) necessaria per la successiva purificazione, seguita dalla sequenza TAT di traslocazione cellulare, derivante dall' immunodeficienza umana (HIV). La sequenza TAT fa parte di una più ampia classe di domini di trasduzione *(protein transduction domains (PTD))* che hanno lo scopo di agevolare la diffusione di macromolecole attraverso le membrana cellulari. La proteina di fusione His(6)TAT-parkina è stata espressa in *Escherichia coli* e purificata secondo la tecnica standard del DNA ricombinante. Le varie fasi di purificazione sono state confermate mediante analisi elettroforetica SDS-PAGE. L'analisi in Western blotting e l'immunocitochimica hanno invece evidenziato come tale proteina sia in grado di entrare nelle cellule attraverso la sequenza TAT, di localizzare preferenzialmente nel nucleo e nel citoplasma, di co-localizzare nei mitocondri; mentre un saggio di ubiquitinazione *in vitro* ha rivelato la sua attività biologica di ubiquitina E3-ligasi. Questa proteina è stata pertanto testata su cellule PC12 differenziate sottoposte precedentemente a stress ossidativo indotto da 6-OHDA. I risultati ottenuti hanno dimostrato che TAT-parkina umana, a concentrazioni nanomolari, è in grado di proteggere i neuroni simil-dopaminergici purchè sia presente prima, durante e dopo il danno indotto da 6-OHDA. Sulla base di quest'ultimi risultati e su recenti studi che hanno dimostrato come la sovra-espressione della parkina protegge dalla tossicità indotta dall' α-sinucleina e in modelli cellulari di apoptosi, nella seconda parte dello studio si è indagata la potenziale proprietà neuroprotettiva della parkina nei confronti dello stress ossidativo, valutando gli effetti dell'aumento dell'espressione della proteina umana (WT) a confronto con la forma patologica mutata R42P. A tale scopo le cellule PC12 sono state transfettate stabilmente con un plasmide d'espressione codificante in parte per la proteina parkina umana (WT) e in parte per la forma umana mutata R42P. Le cellule non transfettate sono state usate come controllo. I cloni sono stati selezionati mediante Reverse Transcription-Polymerase Chain Reaction e Western blotting. I cloni selezionati e le cellule non

transfettate sono state trattate con 6-OHDA (50 e 75 μM) a tempi differenti. Per entrambe le dosi e per tutte le cellule, è stata osservata una riduzione della vitalità cellulare tempo-dipendente; tuttavia in maniera già significativa a partire dalle 2 ore per le cellule di controllo e per il clone sovra-esprimente la parkina mutata R42P, mentre il clone sovra-esprimente la parkina WT ha mostrato una significativa riduzione della vitalità solo a partire dalle 12 ore. Una successiva analisi in Western blotting ha inoltre evidenziato che questo ultimo clone è caratterizzato da un incremento della quantità di proteine ubiquitinate e da un aumento basale dell'autofagia. Poiché molti studi indicano che un mal funzionamento del sistema proteasoma-ubiquitina e del sistema autofagico sembra essere uno dei meccanismi di induzione e promozione della PD, per verificare se l'effetto protettivo della parkina nei confronti dello stress ossidativo potesse essere mediato da entrambi questi processi, è stato inibito il *pathway* proteosomico con l'inibitore specifico del proteasoma (MG132), o il *pathway* autofagico con l'inibitore selettivo dell' autofagia, 3-metil-adenina (3-MA). Anche in questo caso è apparsa evidente una maggior capacità di sopravvivenza cellulare nelle cellule sovra-esprimenti la parkina (WT). Tuttavia, la contemporanea somministrazione di entrambi gli inibitori ha determinato una riduzione della vitalità cellulare simile sia per i cloni, sia per le cellule di controllo, annullando pertanto l'effetto protettivo della parkina (WT). Questi ultimi risultati hanno dimostrato l'effetto protettivo della sovra-espressione della parkina sui neuroni simil-dopaminergici in condizioni di danno da stress ossidativo indotto da 6-OHDA. La sovra-espressione della parkina protegge inoltre dalla tossicità indotta da disfunzioni del sistema proteasoma-ubiquitina o del sistema autofagico, confermando infine che la mutazione R42P compromette la funzione fisiologica della proteina.

Complessivamente i dati ottenuti confermano che la parkina, sia come proteina TAT-parkina ricombinante, sia quando sovra-espressa, è dotata di una spiccata capacità neuroprotettiva nei

confronti di differenti stimoli tossici. La parkina potrebbe quindi rappresentare un possibile target di strategia terapeutica basato sulla neuroprotezione endogena, così come l'applicazione dei domini di trasduzione potrebbero rappresentare un valido strumento di somministrazione di farmaci o sostanze potenzialmente terapeutiche per la cura di molteplici patologie, compresa la PD.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ANOVA	analysis of variance
APS	ammonium persulfate
AR-JP	autosomal recessive juvenile parkinsonism
ATP	Adenosine Tri-Phosphate
BCA	bicinchoninic acid
BG	basal ganglia
BSA	bovine serum albumin
cDNA	complementary DNA
CHIP	Carboxy terminus of Hsp70-Interacting Protein
CHO	chinese hamster ovary
CMA	chaperone-mediated autophagy
CNS	central nervous system
CPP	cell penetrating peptide
Cys	cysteine
DA	dopamine
DAPI	4',6-diamidino-2-phenylindole
DCFH-DA	2',7'-dichlorofluorescein diacetate
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiolthreitol
DUBs	deubiquitinating enzymes
EIF4G1	elongation initiation factor 4G1
ER	endoplasmic reticulum
^{18}F-DOPA	^{18}Fluorodopa
GBA	Glucocerebrosidase
GFP	green fluorescent protein
GPe	globus pallidus pars externa
GPi	globus pallidus pars interna
GSH	glutathione
GWASs	genome-wide association studies
h	hours
His	histidine
HRP	horseradish peroxidase
IBR	in-between RING
LB	Lewy body
LBs	Lewy bodies
LC	locus coeruleus
L-dopa	L-3,4-dihydroxyphenylalanine
Levodopa	L-3,4-dihydroxyphenylalanine
LN	Lewy neurite
LNs	Lewy neurites
LPO	lipid peroxidation

LRRK2	Leucine-rich repeat kinase 2
Lys	lysine
3-MA	3-methyl-adenine
MG132	N-(benzylloxycarbonyl) leucinyl-leucinyl-leucinal[Z-Leu-Leu-Leu-al]
min	minutes
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic Resonance Imaging
MTT	3-(4-5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H- tetrazolium bromide
NF-kB	Kinase/Nuclear Factor-kB
NGF	nerve growth factor
NMR	Nuclear Magnetic Resonance
NO	nitric oxide
6-OHDA	6-hydroxydopamine
Pael-r1	parkin associated endothelin receptor like receptor
PBS	phosphate-buffer saline
PBS-T	phosphate-buffer saline wih Triton
PC12	rat adrenal pheocromocytoma cells
PCR	Polymerase Chain Reaction
PFA	paraformaldheyde
PET	Positron Emission Tomography
PIGD	postural imbalance gait disorder
PINK1	Phosphatase and Tensin (PTEN)-induced Kinase 1
pmito-GFP	plasmid-mito green fluorescent protein
PD	Parkinson's disease
PTD	protein transduction domains
RBR	RING between RING fingers
rhNGF	recombinant human nerve growth factor
RING	really interesting new gene
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
sec	seconds
SD	standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S.E.M	standard error medium
SN	substantia nigra
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
SPECT	Single Photon Emission Computed Tomography
STN	subthalamic nucleus
α-Syn	α-synuclein
TAE	Tris-acetate-EDTA
TAT	trans-activating transcriptional activator
TBS	Tris buffer saline
TBS-T	Tris buffer saline with tween-20

TEMED	tetramethylethylenediamine
Ub	ubiquitin
UBL	ubiquitin-like domain
UCHL1	Ubiquitin Carboxy-Terminal Esterase L1
UPS	ubiquitin proteasome system
WT	wilde-type

CHAPTER 1

INTRODUCTION

1.1 Parkinson's disease history

Parkinson's disease (PD) is widely believed to be a relatively modern condition. Actually, this disorder has been known for some time. In fact, there are references to PD symptoms throughout history. In India, for instance, an ancient population practiced a medical doctrine called Ayurveda, thought to be a divine revelation. They described the symptoms of PD, which they called Kampavata, already in 5000 B.C. To treat this disease, they used a tropical legume (Mucuna Pruriens) whose seeds are a natural source of L-3,4-dihydroxyphenylalanine (L-dopa). Mucuna Pruriens represents the oldest known method to treat the symptoms of PD, and is still in use for this purpose. In the sixteenth century, the Italian artist Leonardo da Vinci wrote, in one of his notebooks, about people with PD-like symptoms: "you will see…those who…move their trembling parts, such as their heads or hands without permission of the soul; the soul with all its forces cannot prevent these parts from trembling". There are references to this pathology also in the second part of Henry VI, written by William Shakespeare. However, PD was first formally described in "An Essay on the Shaking Palsy" published in 1817 by a London physician named James Parkinson (1755-1824). He described the medical history of six individuals by observing their daily walks. The purpose of his observations was to document the symptoms of the disorder, which he described as "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 intellect being uninjured" (Parkinson, 2002) (Figure 1.1). In 1861-1862 the neurologists Jean-Martin Charcot

and Alfred Vulpian added new symptoms to James Parkinson's description and subsequetly conferred the name Parkinson's disease to this syndrome. The two neurologists described the mask face, some forms of contraction of hands and feet, akathesia and rigidity as PD features. In the 1960s the main chemical differences between control and PD patient brains was found: low levels of the neurotransmitter dopamine (DA) that induces the selective degeneration of cells in a part of the brain called the *substantia nigra* (*SN*). This discovery led to the introduction of the drug Levo-dopa (the precursor of DA) as a therapeutic treatment for the disease (1968), and subsequently led the Swedish scientist Arvid Carlsson to win the Nobel Prize in Physiology or Medicine in 2000 along with co-recipients Eric Kandel and Paul Greengard. Since the 1960s research has continued to progress at a rapid rate. Although there is still no cure, the symptoms can now be effectively controlled and reduced in severity. *The Parkinson's Disease Foundation* was established in America in 1957 to assist sufferers and to fund and promote further research. Many other foundations assisting the cause have subsequently been established. A notable recent addition is the Michael J Fox Foundation, named after the much loved television and movie actor. The foundation has been very public about its goal of developing a cure for the disease within this decade. Since its inception in 2000 it has succeeded in raising over 90 milion US dollars.

Advocacy actions include April 11, the birthday of James Parkinson, as the world's Parkinson's disease day, and the use of a red tulip as the symbol of the disease.

Figure 1.1: Parkinson's Disease History. First pages of the treatise "An Essay on the Shaking Palsy", written by Dr. James Parkinson in 1817.

1.2 Neuropathological features of PD

1.2.1 Neuropathological lesions and their meaning

The pathological hallmark of PD is a region-specific selective loss of dopaminergic neuromelanin-containing neurons in the pars compacta of the substantia nigra (SNpc). However, cell loss in other brain regions such as the locus coeruleus (LC), dorsal nuclei of the vagus, raphe nuclei, nucleus basalis of Meynert, and ventrotegmental area are seen (Damier et al., 1999). This specific cell loss is accompanied by three different intraneuronal inclusions: the Lewy body (LB), the pale body, and the Lewy neurite (LN). LBs are subdivided into two families: classical (brainstem) and cortical types, on the basis of their morphology. Classical LBs are spherical structures of 8-30 μm with a hyaline core surrounded by a peripheral pale-staining halo. On the other hand, cortical LBs lack the inner core and halo and are especially common in small-to-medium sized pyramidal neurons of layers V and VI of the temporal, frontal, parietal, insular cortices, cingulum, and entorhinal cortex.

These latter bodies are present in small numbers in almost all cases of PD (Halliday et al., 2008). Pale bodies are large rounded eosinophilic structures that often displace neuromelanin and are the predecessor of the LBs. An abnormal, post-translationally modified and aggregated form of the presynaptic protein α-synuclein (α-syn) is the main component of LBs (Spillantini et al., 1997). Anti-α-syn antibodies stain LBs and LNs and have become the standard and most sensitive immunohistochemical method for diagnostic purposes (Figure1.2).
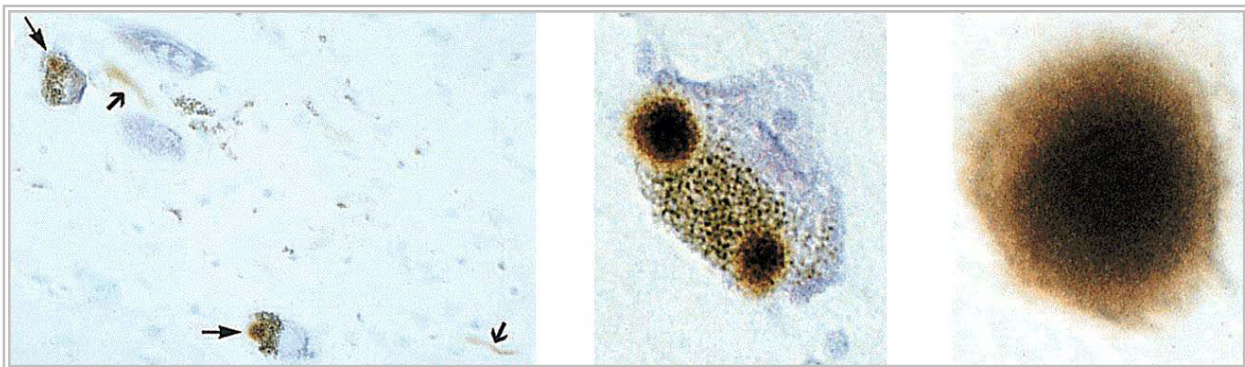
Figure 1.2: PD hallmarks. Lewy bodies (thin arrows) and Lewy neurites (thick arrows) in the substantia nigra of PD patients, immunostained for α-synuclein, at different magnifications (Spillantini et al., 1997).

To date, in addition to α-syn, more than 70 molecules have been identified in LBs, and can be divided into several groups:

• structural elements of the LB fibril (e.g. α-syn (Spillantini et al., 1997));

• α-syn-binding proteins (e.g. synphilin-1 (Wakabayashi et al., 2000) and tau (Ishizawa et al., 2003);

• synphilin-1-binding proteins (e.g. parkin (Schlossmacher et al., 2002);

• components of the ubiquitin proteasome system (e.g. ubiquitin, other ubiquitin ligases and ubiquitin hydrolases have also been found (Kuzuhara et al., 1988);

• proteins implicated in cellular responses (e.g. Hsp70 (Shin et al., 2005);

• proteins associated with phosphorylation and signal transduction (e.g. LRRK2 (Zhu et al., 2006);

• cytoskeletal proteins (e.g. tubulin (Galloway et al., 1989);

• cell cycle proteins (e.g. cyclin B (Lee et al., 2003);

• cytosolic proteins that passively diffuse into LBs (e.g.calbindin (Yamada et al., 1990);

• others (e.g. lipids (Gai et al., 2000).

What is the exact role of LBs in neurodegeneration?

Although LBs are a marker for neurodegeneration, to date their meaning remains elusive. Before the discovery of α-syn as the main component of LBs and LNs, these inclusions were considered to be a marker of neurodegeneration on the basis of:

I. significant loss of neurons in the brain areas ivolved in LBs localization, particularly in the SNpc;

II. the number of LBs in patients with mild-to-moderate loss of neurons in the SNpc was higher than in subjects with severe cell depletion, suggesting that inclusions-containing neurons could be dying cells.

Is the presence of these neuronal inclusion bodies the real cause of cell death? (Terry, 2000). Recent papers suggest that oligomers and protofibrils of α-syn could be the cytotoxic species and that aggregates may present a cytoprotective mechanism. Ding and co-workers, for example, demonstrated that α-syn protofibrils are able to bind lipid bilayers and form pore-like structures, increasing membrane permeability (Ding et al., 2002; Lashuel et al., 2002). On the other hand, Olanow proposed LB formation as a protective aggresome-related process (Olanow et al., 2004). Aggresomes are proteinaceous inclusions formed at the centrosome that segregate and facilitate the degradation of excess amounts of damaged, mutated, and cytotoxic proteins. In this case, LB formation could represent a protective mechanism.

1.2.2 Neuronal circuits involved in PD

The motor symptoms of PD result from the reduced activity of pigmented DA-secreting (dopaminergic) cells in the pars compacta region of the SN, projecting to the striatum. Striatum and SNpc are part of the basal ganglia (BG). This term refers to any gray matter structure located at the base of the cerebral hemispheres but is commonly applied to a group of interconnected subcortical nuclei including the striatum (caudate and putamen), the globus pallidus pars externa (GPe) and pars interna (GPi), the subthalamic nucleus (STN) and the SNpc and pars reticulata (SNpr). The dopaminergic system innervates all BG structures as well as its projection targets like the thalamus and brainstem motor centers. The BG are functionally sub-divided as motor, oculo-motor, associative, limbic, and orbitofrontal according to the main cortical projection areas (Alexander et al., 1986). The cortical motor areas (Area 4, Area 6 and supplementary motor area) project in a somatotopically organized way to the striatum (Takada et al., 1998) (Figure1.3).
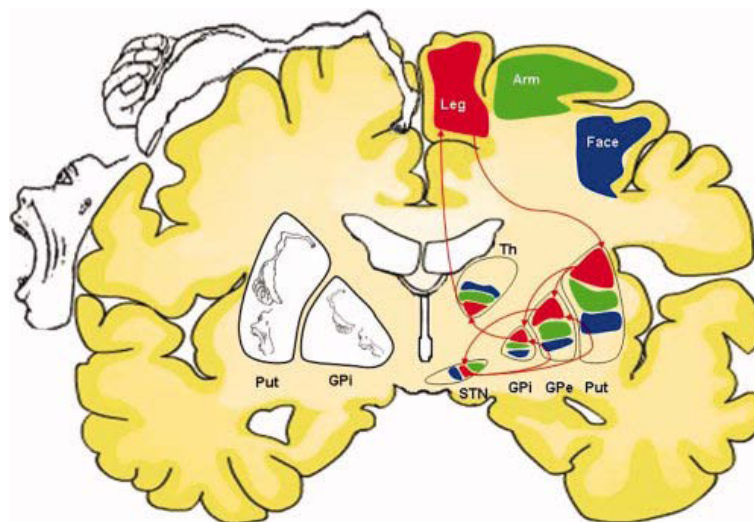
Figure 1.3: Somatotopically organization of the basal ganglia (homunculus).

Striatal efferent neurons are GABAergic and connect with the GPe and GPi by two different systems: the "direct" and "indirect" pathways. Neurons in the first pathway project directly from putamen to GPi/ SNpr, they bear DA-D1 receptors and they establish inhibitory connections. On the other hand, neurons in the indirect pathway have DA-D2 receptors and project to the GPe which influence the GPi/SNpr by inhibitory connections and indirectly through the GPe-STN-GPi projection. DA has a dual effect on striatal cells by exciting D1 neurons in the direct pathway and by inhibiting D2 neurons in the indirect system (Albin et al., 1989). In PD patients, DA depletion in the striatum leads to a series of changes that cause the motor features of the disease; in particular, there is an increased neuronal activity in the STN and GPi/SNpr leading to strong inhibition of thalamocortical and brainstem motor nuclei (Figure 1.4) (Hirsch et al., 2000).
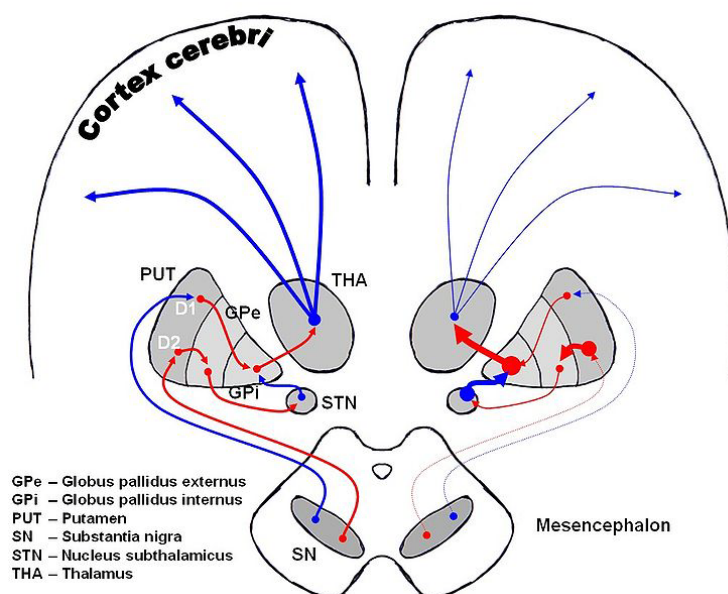
Figure 1.4: Dopaminergic pathways of the human brain.
Normal condition (left) and Parkinson's disease affected brain
(right).Red arrows indicate suppression of the target, blue
arrows indicate stimulation of target structures.

<u>Why are SNpc cells especially vulnerable?</u> DA metabolism (Sulzer et al.,2007) is considered to be critical for the preferential susceptibility of ventrolateral SNpc cells to damage in PD. DA metabolism produces highly reactive species that oxidize lipids and other compounds, increase oxidative stress, and impair mitochondrial function (Gluck et al., 2002; Sulzer et al.,2007; Naoi et al., 2009). At neutral pH, DA can auto-oxidize. Therefore, reduced sequestration of DA into synaptic vesicles, where the pH is lower and DA cannot auto-oxidize, may represent a vulnerability factor for neurons. Accordingly, dopaminergic neurons with low DA transporter activity are less sensitive to oxidative stress induced by DA or neurotoxins (Gonzalez-Hernandez et al., 2004) and are also less affected in PD (Damier et al., 1999). Interestingly, DA toxicity in the SNpc is reduced in α-syn knockout mice, thus suggesting a critical interaction between the cellular concentrations of α-syn and DA, and the inhibition (by DA) of chaperone-mediated autophagy in SNpc neurons (Cuervo et al., 2004). While DA toxicity hypothesis is appealing, it is supported only by indirect evidence, as differences in DA metabolism in the most vulnerable ventrolateral neurons are not readily apparent. The most obvious difference in relation to the regional pattern of cell loss in the SNpc occurs in the neuromelanin-containing neurons, which are more susceptible than neuromelanin-free DA neurons (Gibb et al., 1990). However, vulnerability within the ventrolateral SNpc is unrelated to the amount of neuromelanin per neuron (Gibb et al., 1990). Other factors that may selectively affect SNpc neurons compared to other catecholaminergic cells include differences in their handling of ionic fluxes, less capacity for calcium buffering (Esteves et al., 2009), and increased reliance on L-type calcium channels (Chan et al., 2007), and in their expression of specific transcription factors that regulate cell fate and survival (Alavian et al., 2008). Emphasis has recently been placed on calcium-mediated toxicity in SNpc neurons through Cav1.3 channels (Chan et al., 2007 and 2009), as compared to neurons of the ventral tegmental area, which use sodium

channels for pacemaking activity. Nevertheless, pacemaking is not a feature of the SNpc in awake primates, and the levels of DA required for toxicity are much higher than those seen under normal physiological conditions (Mosharov et al., 2009). Otherwise, SNpc degeneration would be extremely common. Important questions remain regarding how the levels of α -syn and DA are modified and maintained in SNpc neurons, how this might change with age to influence SNpc vulnerability, and whether there are cellular differences among SNpc neurons in features such as the number of synaptic contacts and the degree of neuronal activity and energy consumption (Moss et al., 2008) that explain the degeneration pattern observed in PD.

1.3 Clinical features

The traditional view of PD as a single clinical entity is under scrutiny (Langston, 2006; Selikhova et al., 2009). Clinically, the disease is heterogeneous, and subtypes may be recognized on the basis of age of onset, predominant clinical features, and progression rate. Two major clinical subtypes exist: a tremor-predominant form that is often observed in younger people, and a type known as "postural imbalance and gait disorder" (PIGD) that is often observed in older people (>70 years old) and is characterized by akinesia, rigidity, and gait and balance impairment. In very general terms, the first subtype leads to a slow decline of motor function, whereas the latter worsens more rapidly (Selikhova et al., 2009).

PD is characterized by several motor and non-motor symptoms. The core features of the disease are four motor symptoms that can be summarized with the abbreviation "TRAP": Tremor, Rigidity, Akinesia (or bradykinesia) and Postural instability. Several scales are used to evaluate the rate of impairment in PD patients but, to date, none of these have been fully validated; the most commonly used is the Unified Parkinson's disease Rating Scale (UPDRS) (Ebersbach et al., 2006). The current version (UPDRS 3.0) is comprised of four different parts covering the various aspects of the disease: behaviour and mood (part I), activities of daily living (part II),

motor symptoms (part III), and complications of therapy (part IV). All these aspects are evaluated by interview.

1.3.1 Motor symptoms

As mentioned before, the principal motor symptoms of PD are: tremor, rigidity, akinesia, and postural instability. Of these, the first is the most common and visible manifestation of the disease. Tremor is typically at rest and usually begins unilaterally; it is almost always prominent in the distal part of the extremity, in particular fingers and thumb. As disease worsens, the tremor becomes bilateral, occurs at a frequency between 4 and 6 Hz and is described as supination-pronation ("pill-rolling") type. PD patients can also show tremors involving legs, lips, chin and jaw, rarely neck, head and voice (Jankovic, 2009). This manifestation appears most pronounced when the patient is set and relaxed, while the tremor disappears with action and during sleep. In the late stages of PD, the face of patients is masked and expressionless, the speech is monotonous and rigidity, which occurs in neck, shoulders, wrist and ankles, leads to the typical stopped posture.

1.3.2 Non- motor symptoms

In addition to motor problems, James Parkinson noticed some non-motor abnormalities in PD patients. As a matter of fact, several studies have shown that the non-motor symptoms of PD (depression, psychosis, sensory, and sleep disorders) have a significant impact in the evaluation of the quality of life, institutionalization rates, and health economics (Chaudhuri et al., 2006). Depending on the criteria used, depression may be present in 10% - 45% of PD patients at some point during the pathology (Burn, 2002), although it is difficult to distinguish this condition from akinesia and facial masking. Subjects generally show dysphoria and pessimism, along with irritability and sadness. Risk of dementia exists (approximately 35% to 40% of patients), particularly in those patients who present with prominent gait and speech disorders, depression,

and a poor response to L-dopa. The greatest risk factor for dementia, however, is the age of the patient and not the duration of the disease (Levy, 2007).

1.4 Diagnosis

As PD is not considered a single disease entity, the term does not necessarily mean the same for all clinicians and researchers. Some use this term to indicate a strictly clinical diagnosis and might accept different pathological conditions underlying the syndrome; others use the term only for cases of idiopathic parkinsonism associated with the presence of inclusions bodies in the nigra cells and in some other brain regions (Gibb and Lees, 1988; Gelb et al., 1999). Typically diagnosis is based on medical history and neurological examination. The physician interviews and observes the patient in search of the cardinal motor symptoms, while also attending to other possible symptoms that would exclude a diagnosis of PD. Reduction of motor impairment in response to administration of L-dopa is considered a strong sign pointing to PD.

1.4.1 Clinical differential diagnosis

Possible PD requires at least two of the following four features: resting tremor, bradykinesia, rigidity, and asymmetry of onset. Differential diagnosis requires distinguishing PD from other types of tremors and also other causes of parkinsonism. Other tremors include postural and action tremors or intention tremor. Good clinical practice is to ask the patient to write a few lines of longhand script, although the correlation between abnormalities of finger tapping and micrographia is not always present. Moreover, especially early in the disease, signs and symptoms of different forms of parkinsonism have greater overlap, making very difficult an exact diagnosis. In fact, common presentations of the disease are usually easily diagnosed, and the most common misdiagnoses relate to other forms of degenerative parkinsonism, such as dementia with LBs, progressive supranuclear palsy, multiple system atrophy, and corticobasal degeneration (Hughes et al., 2002). Other common errors include essential tremor, drug-induced

parkinsonism, and vascular parkinsonism (Meara et al., 1999). On the other hand, diagnosis can be difficult when the symptomatology is not fully typical of PD, since parkinsonisms can occur due to a range of pathologies, with only slight clinical differences between them and PD. This difficulty is especially strong in the early stages when symptoms are not as marked.

1.4.2 Imaging

PD remains a clinical diagnosis; thus, brain imaging may be only supportive. Conventional imaging studies (e.g. Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) are not helpful in the diagnosis of PD except to exclude other causes of neurologic dysfunction (basal ganglia tumors, vascular pathology and hydrocephalus). However, it may be possible to longitudinally examine the involvement of nondopaminergic system in the nonmotor symptoms of the disease. Recent advances in understanding the relative roles of striatofrontal pathways and hippocampal circuits (Huang et al., 2007; Beauchamp et al., 2008;) provide new opportunities to evaluate progressive cognitive decline in PD through neuropsycological, anatomical, and neuroimaging methods. Moreover, functional imaging such as Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) may be useful for the early detection of PD. Dopaminergic function in the BG can be measured with the help of different PET and SPECT radiotracers. Examples are ioflupane (123I) (trade name DaTSCAN) and iometopane (Dopascan) for SPECT or ^{18}Fluorodopa (^{18}F-dopa) for PET (Figure 1.5).
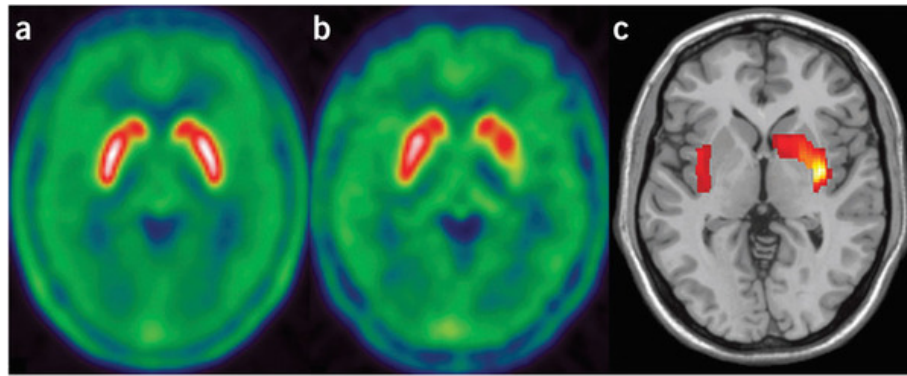
Figure 1.5: Striatal dopamine innervation assessed by ^{18}F-dopa positron emission tomography. (a)Mean control values for eight control subjects shows high uptake (highest value in white) in the striatum. (b) Subject with Parkinson's disease (right) featuring slowness and rigidity on the right limbs but minimal signs on the left limbs. Uptake is markedly reduced (70% below normal) in the left posterior putamen and reduced to a minor extent in the anterior putamen and caudate of the left hemisphere. (c) SPM2-based analysis (yellow represents the largest statistical difference and red the smallest one), showing the difference in uptake between **a** and **b** to highlight the caudorostral pattern of denervation. The statistical map is rendered over the MRI for anatomical localization.

Although PD is classically diagnosed by the insidious onset of motor manifestations, the concept of premotor PD has gained support (Langston, 2006; Hawkes, 2008). There is increasing evidence that olfactory dysfunction, sleep abnormalities, cardiac sympathetic denervation, constipation, depression, and pain may antedate the onset of motor signs of PD (O'Sullivan et al., 2008). It would be very informative to perform longitudinal studies of individuals who do not have motor signs of PD but show the full constellation of premotor signs, as they could be thought of as high-risk candidates to develop the disease. Such longitudinal studies not only will enhance our basic understanding of disease onset and progression but also may provide us with biomarkers that would enable us to start therapeutic intervation much earlier than is currently possible. Long-term longitudinal studies suggest that the rate of decline in motor function in PD is not linear; it is faster in subjects with very mild motor impairment than in those with marked impairment at fisrt evolution (Schrag et al., 2007; Hawkes, 2008). Age is the best predictor of PD progression rate and remains the most

prominent risk factor for developing the disease (Post et al., 2007). Cognitive impairment is also more frequent and begins earlier in individuals who are older at symptom presentation (> 70 years old) (Aarsland et al., 2008). Although the interplay between aging and PD is confounded by comorbidities that normally occur in the elderly, statistical methods might control for these issues and tease apart the role of normal aging in PD outcomes. More refined studies that analyze the effect of genetic and environmental factors on clinical presentation may lead to the identification of further subtypes that could allow us to stratify subjects during clinical studies and, eventually, to start thinking about personalized therapies for the disease.

1.5 Epidemiology

PD is the most common neurodegenerative disorder after Alzheimer's disease. Two main measures are used in epidemiological studies: incidence and prevalence. Incidence is the number of new cases per unit of person–time at risk (usually number of new cases per thousand person–years); prevalence is the total number of cases of the disease in the population at a given time. The prevalence is estimated at 0.3% of the whole population in industrialized countries, rising to 1% in those over 60 years of age and to 4% of the population over 80. The mean age of onset is around 60 years, although 5-10% of cases, classified as "early" or "young" onset, begin between the ages of 20 (very rare) and 40. Onset of PD befor the age of 20 is termed " autosomal recessive juvenile PD" (AR-JP). PD affects all racial groups with a weak higher prevalence in Caucasian races than in African and Asian ones (Jendroska et al. 1994; Inamdar et al., 2007); this difference is thought to reflect the exposure to distinct environments rather than genetic factors (Jendroska et al., 1994). Some studies have proposed that it is more common in males than in females with a ratio of 5:4. Studies of the incidence of PD have reported that it is between 8 and 18 individuals per 100,000 person-years (Twelves et al., 2003) and rises with age. Many risk factors and protective factors have been proposed, sometimes in relation to

theories concerning possible mechanisms of the disease; however, none have been conclusively related to PD by empirical evidence. Combined with better stratification of PD subjects in clinical studies, epidemiological observations (Inzelberg and Jankovic , 2007; Gao et al., 2009) may provide insights into the causal mechanisms that trigger the disease.

PD may affect anyone at any time. Well known personalities with PD include Muhammad Ali, Michael J Fox, Pope John Paul II, and Adolf Hitler.

1.6 Pathogenesis of PD

1.6.1 Environmental factors

After decades of research, a single cause for PD has yet to be found and is unlikely to emerge. Most cases of PD are classified as sporadic and occur in people with no apparent family history of the disorder. Whereas some forms of PD are genetic, most cases are idiopathic, and the underlying environmental causes (if any) remain to be discovered, it is well established that PD is a multifactorial pathology, caused by both genetic and environmental factors that act on an ageing brain (Tanner, 2003). Intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983) and postencephalitic parkinsonism are the only examples of neuronal degeneration in the dopaminergic SNpc that are clearly induced by environmental factors, but neither one fully reproduces the clinical and pathological features of true PD. Research has concentrated on environmental susceptibility factors such as viruses (Encephalitis Lethargica), toxins (e.g. MPTP), other agents like herbicides and insecticides (e.g., paraquat, rotenone), exposure to pesticides and heavy metals, well-water ingestion, head injury, and lack of exercise (Bower et al., 2003; Elbaz and Tranchant, 2007; Thacker et al., 2008). Epidemiological studies that have identified adverse risk factors (e.g. pesticide exposure) as well as protective associations (e,g. consumption of coffee, tobacco and nonsteroidal anti-inflammatory drugs), that reduce the risk of PD are intriguing (Powers et al., 2008).

1.6.2 Genetic factors

In the past 10 years, significant progress has been made in the understanding of PD pathogenesis, based on the discovery of mutations in seven different genes. About 5 to 10% of PD patients have a family history of this disorder and carry a mutation in one of these genes (monogenic familial forms of PD), characterized by early-onset and an autosomal dominant or recessive pattern of inheritance. To date, there is a growing list of mutations linked to PD (Table 1.1). They account for 2–3% of the late-onset cases and ~50% of early-onset forms (Farrer, 2006; Schiesling et al., 2008).

TABLE 1.1 Monogenic forms of Parkinson's disease

Locus[a]	Gene	Protein function	Phenotype[b]
PARK1	*SNCA*[c]	Synaptic?	PD/DLBD
PARK4 (AD)		Lipid binding?	Onset from age 30 to 60, rapid course. Fulminant Lewy bodies
PARK2 (AR)	*Parkin*	E3 ligase	Parkinsonism onset from teenage to 40s, slow course. No Lewy bodies, except
PARK3 (AD)	Unknown Chr2p13	—	PD, dementia onset from age 50 to 60s Lewy bodies, tangles and plaques.
PARK5 (AD?)	*UCHL1*	Ubiquitin hydrolase/ligase	Typical PD onset at about age 50 Unknown pathology
PARK6 (AR)	*PINK1*	Protein kinase	Parkinsonism onset from age 30 to 50s Unknown pathology
PARK7 (AR)	*DJ-1*	Oxidative stress response?	Parkinsonism onset from age 20 to 40s, slow course. Unknown pathology
PARK8 (AD)	Unknown; Chr12p1-q13	—	Parkinsonism onset from age 40 to 60s Variable pathologye[d]

[a]PARKloci are shown for the monogenic forms. Inheritance is shown in parentheses beloweach loci: AD, autosomal dominant; AR, autosomal recessive.
[b]PD, Parkinson's disease; DLBD, diffuse Lewy body disease.
[c]*SNCA* is the gene name for *α*-synuclein.
[d]PARK8-linked families have been described by two groups as either Lewy body negative or variably Lewy body and tangle positive.

1.6.2.1 Autosomal dominant PD

Typical, late-onset PD with LB pathology is linked to mutations in three genes: *SNCA* (encoding α-syn) (Polymeropoulos et al., 1997), *LRRK2/dardarin* (encoding leucine-rich repeat kinase 2) (Zimprich et al., 2004) and *EIF4G1* (elongation initiation factor 4G1) (Farrer Matthew, 2009, unpublished data). Gain of function mutations in these genes lead to an autosomal dominant form of PD, resulting in the clinical manifestation of Parkinsonism.

α–Syn (PARK1)

α-Syn was the first gene identified to be associated with PD. This gene had been cloned previously as a precursor to a small peptide found in the Alzheimer's disease (Iwai et al., 1996). The protein was named for its localization to <u>s</u>ynapses and <u>n</u>uclei. In fact, α-syn is part of a gene family including β-and γ-synucleins and synoretin (George, 2002). All synucleins have a series of imperfect repeats including the sequences motif KTKEGV and a variable C-terminal tail, which is highly acidic in α-syn. Synucleins are also basally phosphorylated at serine and tyrosine residues. There is little or no detectable secondary structure in solution, and hence α-syn is referred to as natively unfolded. To date, the exact physiological functions of the synucleins are not well understood, although, given the location at synaptic membranes, they are suspected to play a role in regulating the reverse pool of synaptic vesicles in brain: they are involved in the regulation of neurotransmitter vesicle transport and release at the presynaptic membrane because of their ability to bind and stabilize lipid membrane bilayers, forming an amphipatic helix (Clayton and George, 1998), and their apparent enrichment in presynaptic terminals (Wood-Kaczmar et al., 2006). Missense mutations in *SNCA* (A53T, A30P and E46K) were first linked to familial parkinsonism with late onset (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004), and subsequent *SNCA* duplications were found in kindreds in which age of onset, progression and associated comorbidities relate to gene dosage (Singleton et

al., 2003; Ross et al., 2008). α-Syn is one of several proteins associated with neurodegeneration diseases that have a high propensity to aggregate. The end production of α-syn aggregation is the formation of heavily insoluble protein polymers known as fibrils. It is thought that fibrillar α-syn is the building block of LBs. Althougt the A53T mutation promotes the formation of such fibrillar species, A30P does not. In fact, A30P slows the rate of fibril accumulation but strongly promotes the formation of oligomeric species (Giasson et al., 1999; Conway et al., 2000). No studies on the E46K mutation have been performed to date, but the pathology in these cases suggests the mutation would have an effect on fibril formation. The fact that all mutations promote the formation of oligomeric rather than fibrillar species had led some to suggest that oligomers, not fibrils, are toxic. In fact, oligomers, also referred to as protofibrils (Lashuel et al., 2002), can form annular structures that may have pore-like properties that might damage membranes (Volles and Lansbury, 2002). In the past few years accumulating evidence suggests that phosphorylation of α-syn, especially at Ser^{129}, is important for fibril formation, both *in vitro* and *in vivo*, using a *Drosophila* model and in brains of patients with PD and other related synucleinopathies (Fujiwara et al., 2002). However, recent papers by Paleologou and Azeredo da Silveira demonstrated that $pSer^{129}$ is not relevant for aggregation (Paleologou et al., 2008), although α-syn aggregation is the key step that drives both pathology and cellular damage. In particular, the development of nonmotor features correlates with α-syn gene copy number as well as gene and protein expression (Farrer et al., 2004). These studies suggest that increased neuronal α-syn protein levels are a primary factor in the disease. The causes and consequences of α-syn aggregation in neurons are not yet fully understood, despite a large number of molecular studies (Cookson, 2009). Mutations in and overexpression of α-syn seem to be especially toxic to dopaminergic neurons, as DA-syn adducts may inhibit chaperone-mediated autophagy (Cuervo et al., 2004). Even with the limited mechanistic insight currently available,

reduction of α-syn expression may represent a potential therapeutic approach (Lewis et al., 2008).

Recently, genome-wide association studies (GWASs) have provided evidence for a contribution of common genetic variability in α-syn and microtubule-associated protein tau to PD (Satake et al., 2009; Simòn-Sànchez et al., 2009; Edwards et al., 2010). Array-based GWASs have limitations in that they only test the hypothesis that common variants (or single nucleotide polymorfisms in linkage disequilibrium) cause common disease and only consider those variants that are present in the arrays. In the coming decade, massively parallel sequencing methods, which can comprehensively survey the entire genome, may provide far more genetic insight than previous GWASs or linkage studies.

Leucine-rich repeat kinase 2 (LRRK2; PARK8)

Mutations in *LRRK2* represent the highest risk of familial and, seemingly, sporadic PD (Paisàn-Ruiz et al., 2004; Zimprich et al., 2004). Among mutation carriers, disease penetrance markedly increases as a function of age (Healy et al., 2008). LRRK2 encodes a rather large protein (2,527 amino acids) containing multiple, independent domains. Sequence analysis suggests that these domains include an armadillo and ankyrin repeat region, leucine-rich repeats (LRR) and contains both Rab GTPase and tyrosine kinase-like (TKL), as well as other domains (WD40 repeat), suggestive of a multimeric protein scaffold (Smith et al., 2005). More than 20 mutations have been reported to date and the majority of these lie within these functional domains. Pathogenicity has been confirmed for six of these: I1122V, R1441C, R1441G, Y1699C, G2019S, and I2020T (Mata et al., 2006). The most common mutation found (Gly2019Ser mutation) has a worldwide frequency of 1% in sporadic cases and 4% in patients with hereditary parkinsonism (Paisan-Ruiz et al., 2004). The pleomorphic pathology associated with LRRK2 mutations suggests that this protein plays multiple roles within the secretory pathway, and it

may contribute to adult neurogenesis, remodeling of cytoskeletal architecture and membrane dynamics, and dopaminergic signaling (Smith et al., 2005). The protein functions as both a kinase and a GTPase. To date, there is no clear evidence about a direct interaction between LRRK2 and α-syn or tau, but the identification of α-syn-positive LB pathology or tau-positive neurofibrillary tangle pathology in LRRK2 patients, suggests a possible role of LRRK2 in the phosphorylation of these two proteins (Zimprich et al., 2004). However, a recent paper by Qing et al.(2009) showed an interaction between LRRK2 and α-syn during oxidative stress. The LRRK2 also binds to parkin's RING domains *in vitro* (Smith et al., 2005), but the physiologic significance of this needs further investigation.

EIF4G1 (elongation initiation factor 4G1)

Recently identified point mutations affecting eIF4G1 act in a dominant-negative fashion to perturb complex assembly, eIF4E or eIF3e binding, and subsequent recruitment of the 40S ribosome for 5′ cap–dependent mRNA translation (Chartier-Harlin et al., 2009, unpublished data). Pathogenic eIF4G1 mutations are rare but can affect families with late-onset LB disease within many populations. eIF4G1 normally links mammalian target of rapamycin (mTOR)-dependent nutrient sensing to regulation of protein translation and cell proliferation; loss of function downregulates mitochondrial biogenesis and enhances autophagy (Ramirez-Valle et al., 2008).

Glucocerebrosidase (GBA)

Recently, heterozygous mutations in *GBA* (encoding glucocerebroside and famously linked to Gaucher disease) have been associated with a typical phenotype of PD and LB pathology (Neumann et al., 2009; Sidransky et al., 2009). It is now clear that this heterozygous loss-of-function mutation also leads to a > 5-fold–increased risk of PD in all populations as well as to earlier disease onset (typically in the early 50s) (DePaolo et al., 2009; Mitsui et al., 2009). The pathogenic mechanism is unclear (DePaolo J., et al. 2009), and possibilities such as lysosomal

dysfunction, interference with the helical binding of α-syn to lipid membranes or decreased ceramide metabolism are under scrutiny.

1.6.2.2 Autosomal recessive PD

Additional mutations linked to early-onset PD are found in affected individuals under the age of 45 and account for about 1% of cases of all types of PD. They are autosomal recessive loss-of-function mutations in both alleles of the genes encoding parkin (Kitada et al., 1998), DJ-1 (Bonifati et al., 2003), and PINK1 (Bonifati et al., 2005), resulting in the clinical manifestation of parkinsonism.

Parkin (PARK2)

Parkin mutations are the second most common genetic cause of parkinsonism. Parkin is a 465 amino acid E2-dependent E3 ubiquitin ligase and its main cellular function is to ubiquitinate proteins, targeting them for proteasomal or autophagosomal degradation (Tanaka et al., 2004; Olzmann and Chin, 2008). Further details will be described in a later paragraph about parkin.

DJ-1 (PARK7)

DJ-1 mutations, the most uncommon, affect a protein implicated in redox sensing (Ishikawa et al., 2009). PD causing DJ-1 mutations are rare and account for only 1-2% of early onset PD cases. Its cellular and subcellular localization is unclear, but is enriched in the brain and peripheral tissues and is primarily localized in the cytoplasm, with a small pool associated with the mitochondria (Kotaria et al., 2005; Zhang et al., 2005). Bonifati and colleagues (2003) suggested that DJ-1 maintains neuronal cell viability by modulating gene expression under conditions of cellular stress, either as a redox sensor protein than can prevent the aggregation of α-syn or an antioxidant (Mitsumoto and Nakagawa, 2001; Zhou et al., 2006). DJ-1 can also act as a direct scavenger of reactive oxygen species (ROS); it has been shown to eliminate hydrogen peroxide through auto-oxidation (Taira et al., 2004). The first description of DJ-1

mutations was a point mutation, L166P, resulting in a loss of protein function. The L166P mutation causes destabilization through unfolding of the C-terminus, inhibiting dimerization and enhancing degradation by the proteasome (Miller et al.2003; Moore et al. 2003; Olzmann et al., 2004). Consequential to this instability, L166P reduces the neuroprotective function of DJ-1 (Taira et al., 2004).

Phosphatase and Tensin (PTEN)-induced Kinase 1 (PINK1; PARK6)

PINK1 (or PTEN-induced kinase 1) is a 581-amino acid mitochondrial protein kinase, that was first identified in cancer cell expression profile experiments and was shown to be transcriptionally activated by PTEN (Phosphate and TENsin homolog), thus its name. PINK1 is ubiquitously expressed, containing a mitochondrial-targeting motif and a conserved serine/threonine kinase domain (Silvestri et al., 2005) A G309D missense and a W437X truncating mutations in PINK1 were discovered in three large consanguineous families, one Spanish and two Italian (Valente et al., 2004). This protein shares the same mitochondrial pathway as parkin (Clark et al., 2006; Park et al., 2006), suggesting that mitochondrial dysfunctions could be the key reason for at least some of the autosomal recessive forms of parkinsonism.

It now appears that both PINK1 and parkin are functionally linked, as their expression induces mitochondrial fission (Lutz et al., 2009) and the survival of nigrostriatal neurons. Wild-type PINK1 may play a neuroprotective role against mitochondrial dysfunction and proteasomally-induced apoptosis (Valente et al., 2004). Parkin is recruited to dysfunctional mitochondria to promote their autophagic degradation and rescues degeneration in PINK1-null flies (Narendra et al., 2009). A physical interaction between DJ-1 and PINK1, which protected cells against oxidative stress, was also demonstrated (Tang et al., 2006). However, the relevance of the findings from animal models to the human disease is uncertain, as aged parkin/DJ-1/PINK1

triple-knockout mice fail to develop nigral neurodegeneration (Kitada et al., 2009), and the impact of these proteins in sporadic PD seems to be low (Brooks et al., 2009).

Other gene mutations (such as those that encode the recessive loss-of-function of tyrosine hydroxylase, **ATP13A2**, which encodes for a protein member of the P-type ATPase superfamily that make use of ATP, and **PANK2** proteins have been linked to early-onset PD, but often with atypical symptoms and no LBs or loss of dopaminergic nigral neurons.

Lastly, mutations affecting **Ubiquitin Carboxy-Terminal Esterase L1 (UCHL1)**, **FGF20**, **Omi/HTRA2** (Strauss et al., 2005) and **GIGYF2** may be linked to PD, but the data remain equivocal (Hardy et al., 2009).

UCHL1 (PARK 5)

The *UCHL1* gene encodes a neuron-specific ubiquitin C-terminal hydrolase, whose enzymatic roles include the recycling of ubiquitin chains back to monomeric ubiquitin and adding ubiquitin to already monoubiquitynilated *α*-syn (Liu et al., 2002). UCHL1 expression is restricted to neurons, making it an interesting candidate for neurodegeneration. However, there is controversy regarding the UCHL1 mutation (Heavly et al., 2004). There have been no additional families with UCHL1 mutations, despite extensive searches, that would strengthen the case that this gene can be pathogenic for PD. Therefore, whether UCHL1 mutations are truly causal for PD is unclear. There is, however, a relatively common S18Y polymorphism in *UCHL1* that has been associated with risk for sporadic PD in several, but not all, studies (Heavly et al., 2004). It is possible that UCHL1 plays some role in PD, but this requires further clarification.

In summary, mutations may help define the molecular pathways underlying neurodegeneration in PD (Hardy et al., 2009). Ideally, genetic studies should identify critical pathways (such as mTOR (Ramirez-Valle et al., 2008) or ceramide metabolism (DePaolo et al., 2009)), which may

be affected by mutations in several of their components. Genetic studies could also help clarify some clinical findings, such as a possible association between the tau locus and dementia in PD. However, despite extensive experimental scrutiny, the process by which mutations in the genes, which here mentionated, lead to SNpc cell death and LB formation is not understood (Lees et al., 2009; Naoi et al., 2009; Yang et al., 2009). Moreover, causative factors may differ among individuals with different clinical subtypes of the disease. An emerging concept is that SNpc homeostasis is vulnerable to different genetic, cellular, and environmental factors that independently or concomitantly cause cell death over time (Perier et al., 2007; Sulzer, 2007). These factors may lead to mitochondrial dysfunction and oxidative stress, to abnormal protein degradation due to alterations in the ubiquitin system or in chaperone-mediated autophagy (CMA), and to other forms of subcellular dysfunction (Figure 1.6). Combined, these alterations can precipitate cell death. Which (if any) of these mechanisms is more important to disease pathogenesis is not known.
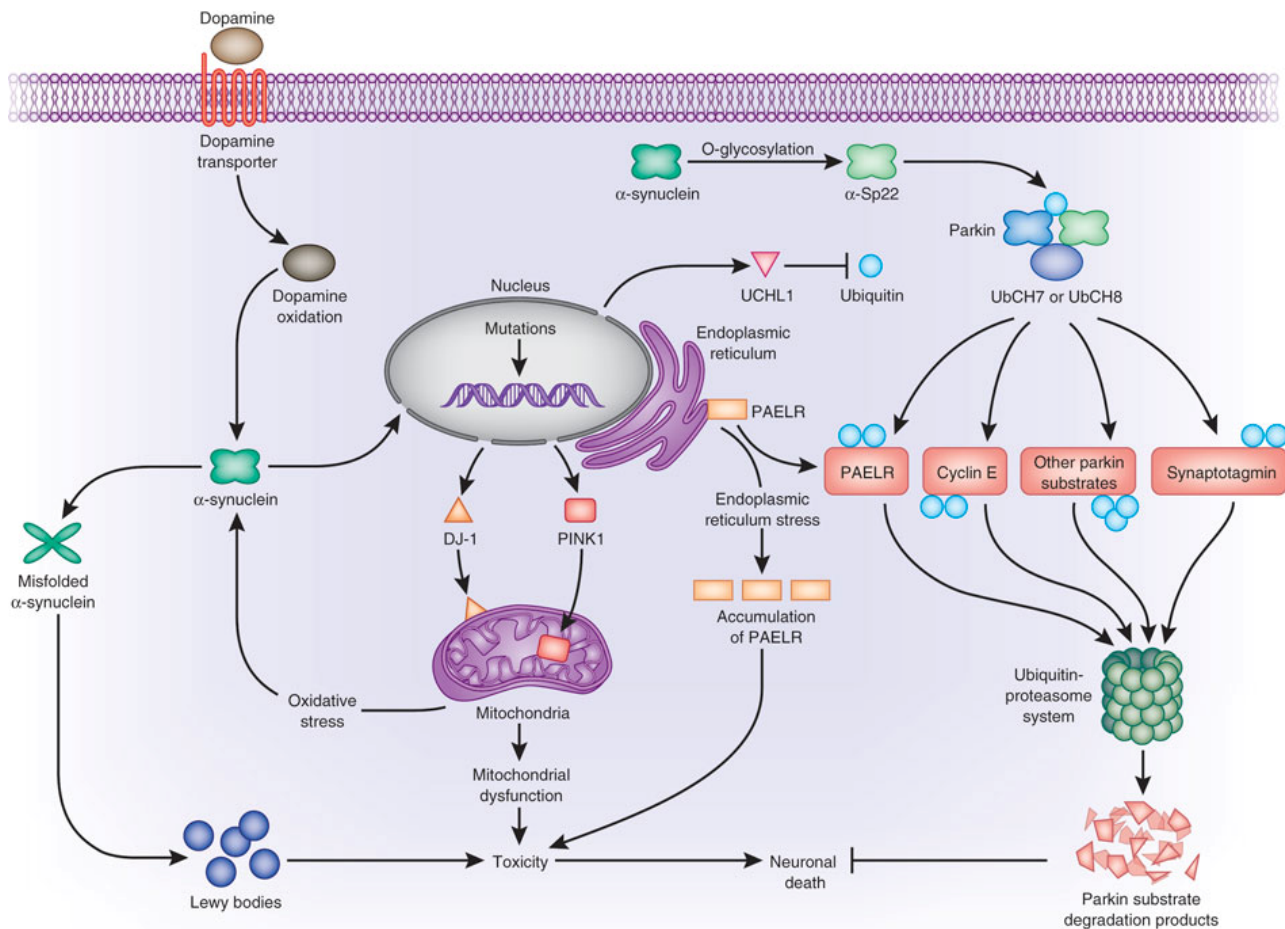
Figure 1.6 : Schematic summary of established etiopathogenic mechanisms and interactions in the dopaminergic cells of the substantia nigra in Parkinson's disease. Cell death may be caused by α -syn aggregation, proteosomal and lysosomal (not shown) system dysfunction, and reduced mitochondrial activity. Gene mutations are associated with impairment of one or several of these mechanisms. In addition, secondary changes (not shown) such as excitotoxicity and inflammation are likely to play a relevant role in progressive neuronal degeneration.

α -Sp22, a 22-kilodalton glycosylated form of α -synuclein; PAELR, parkin-associated endothelin receptor-like receptor; UbCH7, ubiquitin-conjugating enzyme 7; UbCH8, ubiquitin-conjugating enzyme 8; UCHL1, ubiquitin carboxy-terminal hydrolase L1.

1.7 Pathways to PD: is there a multiple connection?

PD involves several causations that ultimately lead to neuron death. It is now becoming clear that the genes and constituent proteins are partners in a complex network, each being connected by at least one branch. The remaining questions are: what are the upstream pathways that lead to dopaminergic-specific neuronal cell death? Do all of these known genes converge to form a common pathogenetic pathway? As mentioned above, there is evidence for the neuroprotective effect of some PD-linked genes (parkin, DJ-1, PINK1) against loss of mitochondrial function. It is unlikely that PINK1 and DJ-1 physically interact, because they are probably on opposite sides of the mitochondrial membrane. The mitochondrial leader peptide of PINK1 should direct the kinase through the mitochondrial import machinery into the mitochondrion. In contrast, when DJ-1 overlaps with mitochondria, it localizes to the outer mitochondrial surface (Canet-Aviles et al., 2004). Therefore, if there is a common mitochondrial pathway for DJ-1 and PINK1, it is at the level of the whole organelle. This is reasonable, given the prominent roles that mitochondria play in determining cellular life or death. However, if all these genes suppress cell death under many circumstances, it would be surprising that cell death is restricted to a subset of neurons only. It is interesting that DJ-1 might be excluded from mitochondria unless cells are stressed (Canet-Aviles et al., 2004). If the cell survival pathway is more important under specific conditions, then one might expect the pattern of cellular damage to be restricted to cells that undergo these stresses or are especially vulnerable to them. If mitochondrial pathways are implicated in this scheme, the neurons affected in recessive parkinsonism should be susceptible to mitochondrial damage. Although exposure to MPTP produces a parkinsonian syndrome, toxicity is dependent on uptake by the DA transporter (Langstone, 1996), so restriction to dopaminergic neurons is not surprising. However, rotenone also inhibits mitochondrial complex I without requiring uptake via the DA transporter, so this is a test of whether some neurons are

more sensitive than others to mitochondrial damage. Chronic administration of rotenone selectively damages dopaminergic neurons in the nigra (Betarbet et al., 2000), although some studies also report more a generalized toxicity. The mechanism appears to involve free radicals (Sherer et al., 2003); hence, DJ-1 should protect neurons from rotenone toxicity. Therefore, mitochondrial complex I with attendant oxidative damage might account for some of the neuronal cell damage in parkinsonian conditions, and we can relate this to loss-of-function mutations in DJ-1 and PINK1. Moreover, DA is an oxidant neurotrasmitter that is normally packaged in vesicles. Exposure to high cytosolic amounts of DA could increase oxidative stress as well as the promotion and stabilization of a-syn protofibrils (Conway et al., 2001).

Parkin does not easily fit into the above scheme. Given the ubiquitination activity of this enzyme, cell death is most obviously related to proteasome function. We can ask whether proteasome inhibition would be sufficient to induce cell death, and whether such cell death would be restricted to the mosaic of cells susceptible in PD. This experiment was performed recently, and the patterns of cell death closely resemble those in sporadic PD (McNaught et al., 2004). Proteasome inhibitors also preferentially affect catecholaminergic neurons in some (Petruccelli et al., 2002), but not all (Hoglinger et al., 2003), *in vitro* models. This implies that susceptible neurons in PD are linked by sensitivity to proteasome dysfunction. Therefore, there are at least two pathways that can lead to parkinsonian syndromes: mitochondrial and proteasomal. Is there a link between the ubiquitin proteasome system (UPS) and mitochondria? Logically, there are three ways to consider these two pathways. Firstly, perhaps each is sufficient to induce cell death and is independent of the other. In this scheme, the fact that some groups of neurons are affected by these stresses is coincidental, and the human syndromes are phenocopies of each other. A second possibility is that both mitochondrial and proteasomal damage are required for neuronal cell injury, having initially distinct events but converging on a

later, common pathway. The third possibility: the three genes mark a single pathway that we can connect in an ordered way. However, it is hard to separate proteasomal from mitochondrial damage because they interact with each other. Increased oxidative damage has also been found following proteasomal impairment (McNaught et al., 2004). In fact addition of proteasome inhibitors increases the sensitivity of catecholaminergic neurons to rotenone or MPTP *in vitro* (Hoglinger et al., 2003): complex I inhibitors caused a decrease in proteasome activity. This may be the result of ATP depletion, as the UPS is very heavily ATP-dependent, or a consequence of oxidation, or both. Increased oxidative damage has also been found following proteasomal impairment (McNaught et al., 2004). Reciprocally, proteasome inhibitors have been reported to cause mitochondrial damage (Sullivan et al., 2004). Therefore, proteasomal and mitochondrial damage interact in both directions to converge on cell death as an outcome. The evidence that parkin has an effect on mitochondria (Shen and Cookson, 2004) is surprising for an E3 ligase with no mitochondrial substrates. The effects of parkin can be specific, as parkin is effective against apoptosis pathways that proceed through mitochondrial signaling but not other triggers (Darious et al., 2003). A more powerful example is when proteomics was used to examine the brains of parkin knockout mice. Although many proteins were present on two-dimensional gels, mitochondrial proteins were specifically represented (Palacino et al., 2004). These observations led to the idea that mitochondria may be important in parkin disease, as well as DJ-1 and PINK1, but specificity is unclear. Such considerations become much more complex when we add α-syn to the equation. The Dawson laboratory recently articulated these difficulties by proposing that there are two logical models (Von Coelln; Dawson VL. and Dawson TM., 2004). In the "unifying model," parkin and $\alpha-$syn have differential effects on a common pathway, whereas in the "distinguishing model," PD and recessive parkinsonism have different pathways (Von Coelln; Dawson VL. and Dawson TM., 2004). This centers on whether

parkin plays an essential role in α-syn disease and vice versa. Alternatively stated, the problem is: what causes disease in these different conditions? We can be sure that α-syn is causal in the PD/DLBD families, and it is likely that protein aggregation underscores the disease process. What happens downstream of protein aggregation to cause cell death is less clear. α-Syn has detrimental effects on both proteasomal (Petrucelli et al., 2002; Willingham et al., 2003) and mitochondrial (Hsu et al., 2000; Tanaka et al., 2001) function. Interactions between mitochondria and proteasomal function were discussed above, but aggregated α-syn can inhibit the proteasome *in vitro*, suggesting that it might directly affect the UPS (Snyder et al., 2003; Lindersson et al., 2004). Adding to the confusion, mitochondrial damage may exacerbate α-syn aggregation, promoting the accumulation of the protein post-translationally (Lee et al., 2004). This leads to many schemes of the pathogenesis of PD that evoke amplifying circles of mitochondrial damage, proteasomal dysfunction, and protein accumulation.

If α-syn affects both mitochondria and the proteasome and if mitochondrial/proteasomal genes cause parkinsonism, does this mean that α-syn mediates neuronal cell damage in the recessive diseases? α-Syn is a good candidate for being an endogenous stressor, as we know the wild-type protein can be toxic when present at high levels. One might imagine that lack of protective gene products (such as parkin, DJ-1, or PINK1) might render some neurons susceptible to the same causal agent at normal expression levels. An argument against α- syn involvement in cell death is the lack of obvious LB pathology in most parkin cases, although there are reported exceptions (Farrer et al., 2001). However, if LB formation is not required for toxicity, as implied by A30P, then inclusion body pathology might not be needed for α-syn to be toxic. Another argument is that parkinsonism is a component of diseases caused by other aggregating proteins, including tau mutations (Hutton, 2001). There are several parallels between tau and α-syn. Both are intracellular proteins with a tendency to aggregate, perhaps co-aggregating (Lee et al., 2004),

and both proteins can cause cell death (Hardy, 2003). Therefore, α-syn is not the only brain protein that can aggregate and kill nigral cells, although it is one of the few that aggregates so readily in its wild-type form; tau is the other major one in brain. In the absence of α-syn pathology in parkin, DJ-1, and PINK1 cases, we cannot be certain about the causative agent in the same way as α-syn mutations. However, parkin can protect cells against α-syn toxicity. This implies that the pathways triggered by the aggregating protein must converge at some point on the positive effects of parkin and other recessive gene products. A critical set of experiments will be to compare whether all three recessive parkinsonism genes are important in protecting against α-syn toxicity specifically, or against toxic proteins in general. More importantly, we need to better define the relationships between the different recessive gene products and understand where their effects are specific and where they only coincidentally affect the same cellular processes.

Why should we care about the distinction between the concept of a single pathway and multiple roads to the same output, even if it is a tractable problem? The most practical benefit from more fully understanding the nature of cell death in PD and related disorders is the possibility of providing new therapeutic avenues. In this sense it is not critical whether events are early or late in the pathogenic process; each is an avenue for intervention. Identifying the earliest and most specific events that cause neuronal cell loss in these disorders might also indicate where to aim strategies with the highest level of specificity. Independent of the underlying cause of cell loss in parkin-DJ-1-or PINK1-associated diseases, all three of genes impact neuronal ability to survive in the face of stress, and it can be no coincidence that all three produce such a similar phenotype. Perhaps recessive genes tell us why neurons are damaged in parkinsonian conditions, but is quite crude at this point. Certainly, identifying why different neuronal groups rely on these proteins more than others may be key to the problem of parkinsonism in many

diseases. The challenges for the PD field are to describe in detail the routes that lead to toxicity and answer whether, or not, these different pathogenic cascades overlap.

1.8 PARKIN

1.8.1 AR-JP

The first gene that causes recessive parkinsonism was identified in 1998 in Japanese families, and was named *parkin* by Mizuno and colleagues (Kitada et al.,1998). These patients had early-onset parkinsonism (teens to twenties) with slow progression and additional features such as dystonia. They have a good response to L-dopa replacement and developed L-dopa induced-dyskinesias, whereas dementia seems to be rare (Lohmann et al., 2003). Subsequent studies suggest that *parkin* mutations are the most numerous cause of recessive, early-onset parkinsonism (average onset before 40 years of age). Identification of additional mutations shows that the phenotype can be expanded to include cases with features more typical of sporadic PD, such as hyperactive tendon reflexes and less frequent resting tremor (Klein et al., 2000). Pathologically, patients with *parkin* mutations display neuronal cell loss in the SNpc and LC. One discrepancy between parkin disease (typical recessive) and PD is the absence of LBs (typical of idiopathic disease), although there is one exception: LBs have been demonstrated in a patient carrying an R275W substitution and an exon 3 deletion (Farrer et al., 2001). This suggests that LB formation is not required to evoke nigral cell death, i.e., that there are other ways to kill neurons. So parkin disease is a phenocopy of PD, parkinsonism without LB. There are other forms of parkinsonism with these features, notably exposure to the toxin MPTP (Langston, 1996). On the other hand, parkin and several parkin substrates, including α−syn, p38, parkin associated endothelin receptor like receptor (Paelr1) and synphilin-2, were found to localize to LBs in sporadic disease, leading to the attractive hypothesis that functional parkin may be required for the formation of LBs (Schlossmacher et al., 2002).

1.8.2 The parkin gene

The gene responsible for AR-JP maps to chromosome 6q25.2-q27, based on linkage identification to markers D6S305 and D6S253. The D6S305 marker was deleted in one AR-JP patient (Matsumine et al., 1997). By positional cloning within this microdeletion, Kitada et al. (1998) used positional cloning to isolate a cDNA clone of 2960 bp with a 1395 bp open reading frame, and termed this PARK2. The PARK2 gene is the second largest human gene reported to date (over 1.3 Mb), with 12 exons. Interestingly, the parkin gene is highly conserved across species, not only in vertebrates, such as human, rat, and mouse, but also in invertebrates (*Caenorhabditis elegans* and *Drosophila melanogaster*) (Kahle et al., 2000), suggesting that it plays a common role in various organisms. Mutations such as exon deletions, exon multiplications, or point mutations resulting in missense and nonsense changes of parkins have been reported in AR-JP patients. Parkin mutations account for about 50% of familial cases and about 70% of sporadic cases with age of onset < 20 years (Lucking et al., 2000; Periquet et al., 2003; Mata et al., 2004). Since parkin-linked PD is recessively inherited, a deleterious alteration can be presumed on both alleles, and heterozygous carriers may be unaffected. However, some studies suggest that a large proportion of the total number of cases identified with parkin mutation had only a single heterozygous mutation (Lucking et al., 2000; Kann et al., 2002; Periquet et al. 2003; Mata et al., 2004). Moreover, the frequency of same variants in cases and controls suggests these could be polymorphisms rather than disease-causing mutations.

1.8.3 The parkin protein: structure

The parkin protein consists of 465 amino acids with a molecular weight of about 52 kDa. Its unique modular structure can be divided into three parts: the ubiquitin-like domain (UBL) (aa 1-76) at the N terminus, the RING-box domain close to the C terminus, and the linker region containing caspase cleavage sites, which connects the two termini. The C-terminal RING-box

region, a special zinc finger configuration, consists of two RING ("really interesting new gene")

finger motifs, termed RING1, RING2 (aa 238-293 and 418-449, respectively), separated by a

cysteine-rich IBR (in-between RING) domain (aa 314-377). The whole RING-box is also called

RBR (RING between RING fingers) domain. (Figure 1.7)
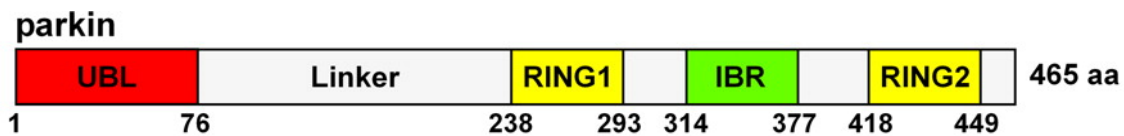
Figure 1.7 Schematic representation of the parkin protein.

The RING-IBR-RING domain is also called the TRIAD or DRILL domain, which functions by

interacting with ubiquitin-conjugating enzymes (E2s), suggesting it is the catalytic site of this

class of E3-enzyme family. The RING-IBR-RING motif is important in recruiting substrates

and the E2 enzyme (UbcH7 or UbcH8) that carries activated ubiquitin (see Box 1). RING

fingers are found in a number of E3 ligases and have varying numbers of cysteine (Cys) and

histidine (His) residues that coordinate a structurally important zinc atom (Marin and Ferrus,

2002) Intriguingly, this RING-IBR-RING type E3 ligase is strongly expressed in brain.

The UBL region of parkin exhibits moderate similarity to ubiquitin, displaying approximately

20% sequence identity. Nuclear Magnetic Resonance (NMR) studies have indicated that the

three-dimensional structure of the UBL domain of parkin resembles that of ubiquitin (Sakata et

al., 2003). As shown in Figure 1.8, ubiquitin (Ub) is a small protein with two α-helical and five

β-sheet structures, which arrange in the order of ββαββαβ in the secondary structure. Overall,

these structures are conserved in the UBL of parkin, indicating that both molecules appear to be

structurally very similar. However, its role is largely unknown. Intriguingly, inspection of

chemical shift perturbation data revealed that UBL binds the Rpn10 subunit of the 26S

proteasome (see a model in Figure 1.11). On the other hand, accumulating evidence suggests

that various proteins harboring the UBL domain, e.g., Rad23, Dsk2, and their human homologues (hHR23a/h and hPLIC1/2, respectively), provide links between the 26S proteasome and the ubiquitinylation machinery (Kleijnen et al., 2000). In this context, it has been reported that a 50-kDa subunit Rpn10 of the human 26S proteasome, originally called S5a, could bind to polyubiquitin conjugates *in vitro* and, hence, could possibly function as a polyubiquitin chain-binding subunit (Kawahara et al., 2000). Rpn10 also binds the UBL domain of hHR23a/h and hPLIC-2 (Walters et al., 2002). Thus, it is likely that the UBL domain tethers parkin close to the proteasome, directing poly-ubiquitinated proteins to the latter, contributing to the recognition of target proteins (Box 1).
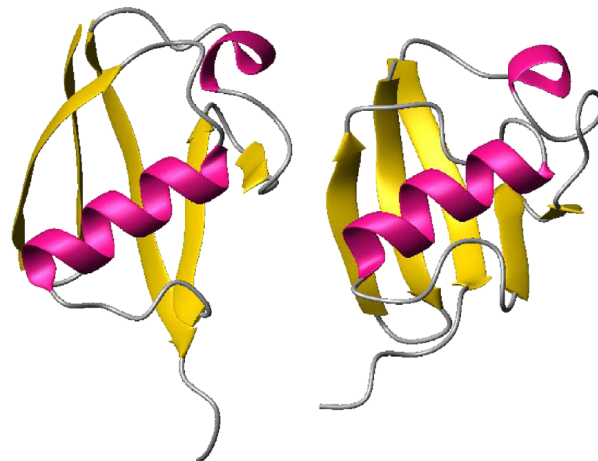
Figure 1.8. The tertiary structures of ubiquitin (left) and the ubiquitin-like domain of parkin (right). α-helices and β-sheets are shown in red and yellow, respectively.

All of these domains appears to be functionally important because PD mutations cluster in them (Kahle and Haass, 2004). Endogenous parkin's normal cellular location appears to be largely cytosolic, and it may colocalize to synaptic vesicles, the Golgi complex, endoplasmic reticulum (ER), and mitochondria outer-membrane (Shimura et al., 1999; Kubo et al., 2001; Darios et al., 2003; Mouatt-Pringet et al., 2004). As many as one-half of the single amino acid substitutions reported to date appear to alter wild-type parkin cellular localization, solubility, and propensity to aggregate (Cookson et al., 2003; Gu et al., 2003; Wang et al., 2005).

1.8.4 The parkin protein: function

In 2000 Shimura et colleagues demonstrated that the parkin protein is an E3 ubiquitin-protein ligase, which mediates the covalent attachment of ubiquitin to target proteins with a polymerization step to form a degradation signal (Imai et al., 2000; Shimura et al., 2000). These marked polyubiquitinated proteins ultimately are degraded by the 26S proteasome complex. E3 ligases control the key step in the cycle of Ub-mediated hydrolysis of damaged or misfolded proteins that are degraded via the proteasome. The reaction promoted by E3 ligases is the addition of a lysine (Lys)-linked chain of four or more Ub molecules to the target protein, which is recognized by subunits in the proteasome lid (Box 1).

Box 1: The Ubiquitin-proteasome system (UPS)

The UPS, is an element of major intracellular machinery whose purpose is to dispose of abnormal proteins (Ding and Keller, 2001). UPS is capable of catalyzing rapidly, timely, and unidirectionally a multitude of biological reactions including cell-cycle progression, DNA repair, cell death (e.g., apoptosis), signal transduction, transcription, metabolism, and immunity (Hershko et al., 2000; Pickart et al., 2001; Weissman et al., 2001). In addition to regulating the functions of divergent proteins, UPS plays a major role in the stress response and in protein homeostasis, i.e., protein quality control, not only in the ER but also in the cytosol of eukaryotic cells: misfolded ER proteins are translocated into the cytosol and then degraded by the proteasome (Sherman et al., 2001; Yoshida et al., 2001).

1. Ubiquitin (Ub)

Ub, consisting of 76 amino acid residues, is a highly conserved small protein that acts as a degradation marker for a wide spectrum of cellular proteins and a unique molecule of intracellular proteolysis (Hershko et al., 2000) . It is first activated by an ATP-dependent E1 (activating enzyme), forming a high-energy thioester bond between Ub and an E1, and the activated Ub is then transferred to an E2 (conjugating enzyme), forming a similar thioester linkage between Ub and an E2. In some cases, E2 directly transfers Ub to the target proteins, but the reaction often requires the participation of an <u>E3</u> (ligating enzyme, and thus referred as <u>ubiquitin-protein ligase</u>). Through a cascade of enzymatic reactions, Ub is covalently attached through its C-terminal Gly residue to the ε-NH2 group of the Lys residue on the target proteins. Finally, a polyubiquitin chain is formed by repeated reactions through which another Ub links a Lys residue at position 48 within one Ub associated with the target protein (proteasomal degradation requires a chain of at least four Ub moieties in length) (Figure 1.9). Ub has seven Lys residues, which are all used for polymerization catalyzed by this Ub-modifying system

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(Pickart et al., 2001; Peng et al., 2003), but a polyubiquitin chain formed via Lys48 functions mainly as a marker for proteolytic attack by the 26S proteasome (Coux et al., 1996; Baumeister et al., 1998). In addition, the Lys63-linked polyubiquitination and monoubiquitination without the formation of an Ub tree has many biological roles other than proteolysis (Pickart et al. 2001; Weissman et al., 2001) e.g. transcriptional regulation, signal transduction, DNA repair, and finally, marking substrate proteins for degradation in lysosomes (Welchman et al., 2005). Monoubiquitination is involved in endocytosis, membrane trafficking, histone regulation, and DNA repair. Multiple monoubiquitination also plays a role in the regulation of endocytosis.

Ubiquitination is a reversible process. In fact, eukaryotic cells contain an unexpectedly large number of deubiquitinating enzymes (DUBs), which are also called Ub-specific proteases (USPs) (Figure 1.9). They belong to a family of cysteine proteases subclassified into at least two gene families that are structurally unrelated; the UCH (ubiquitin C-terminal hydrolase) family and the UBP (ubiquitin-specific processing protease) family (Kim et al., 2003). DUBs may contribute to the production of a functional Ub moiety from its precursors as well as disassembly of degradation intermediates generated by the 26S proteasome. Indeed, Ub is reutilized, but not degraded in the breakdown of ubiquitinated proteins. DUBs are also thought to catalyze the reversal of the ubiquitination reaction for proofreading of incorrectly ubiquitinated proteins or trimming of abnormal polyubiquitin structures, which play an essential role in facilitated proteolysis mediated by the 26S proteasome (Wilkinson, 1997).
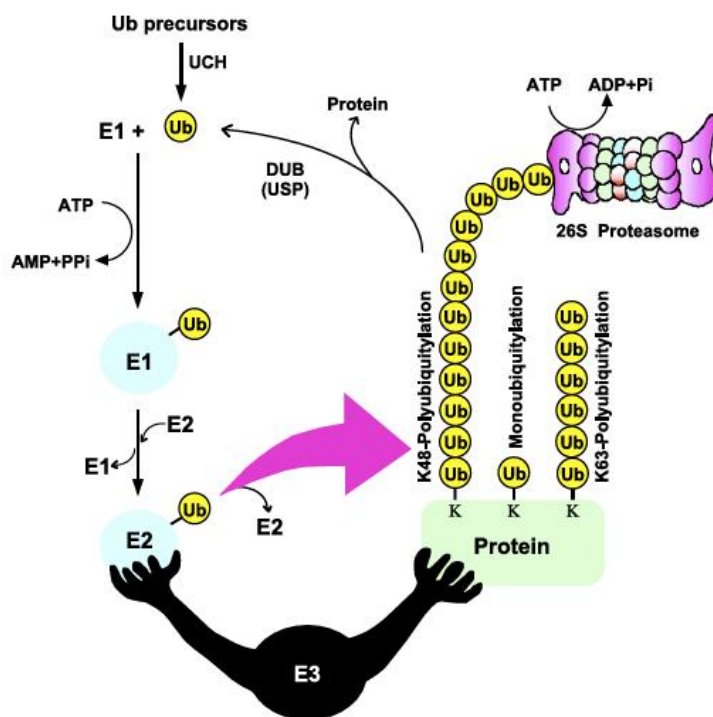
Fig. 1.9 The ubiquitin–proteasome system. Ub, ubiquitin; E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes; DUB, deubiquinating enzyme; USP, ubiquitin-specific protease; HCH, ubiquitin C-terminal hydrolase. The 26S proteasome is a eukaryotic ATP-dependent, multi-subunit proteolytic complex

To date, it is known that there is a single E1 for ubiquitination, whereas E2 enzymes consist of a family of proteins, with over a dozen isoform in mammals. Moreover, E3s are considered to exist as molecules with a large diversity, presumably in more than hundreds or thousands species, because E3 can trap not only target protein(s) but also Ub activated by the E1–E2 coupled reaction, and thus is capable of catalyzing the successive transfer of Ub to the protein (Figure 1.9). Thus, in the UPS pathway, E3 plays a critical role in the selection of target proteins for degradation, because each distinct E3 usually binds a protein substrate with a degree of selectivity for ubiquitination in a temporally and spatially regulated fashion. So far, E3s are classified into several groups, categorized into four types as shown in Table 1.2.

2. E3s enzymes

One is the HECT-type E3 encompassing E3 with a domain capable of binding ub as a thioester bond, termed bHECTQ (Schneffner et al., 1994). The major group of E3s is named RING-type E3, a general term for ubiquitin-ligases with a RING-finger motif(s) consisting of the Cys-rich consensus sequence flanked by one or two His residue(s) (Borden, 2000; Jackson et al., 2000). The RING-finger motif is capable of binding Zn^{2+}, and is subcategorized into typical and atypical forms. The typical RING-type E3s contain three classes with subtle differences in their structure: RING–HC (C_3HC_4), RING-H2 (C_3H2C_3), and RING-IBR-RING. These RING-IBR-RING type E3s, which belong to parkin, are strongly expressed in brain. The atypical RING-type E3s are structurally somewhat divergent compared with the typical types. The third type of E3s have the U-box domain whose tertiary structure resembles that of the RING-finger domain (Aravind et al., 2000; Ohi et al., 2003), but does not show a binding potency to Zn^{2+}, which is probably required for keeping the domain structure in RING-type E3s. The fourth group of E3 consists of very unique E3s [ICPO, TAFII250, (UCHL1)2, and p300] that have no sequence homology to known E3 enzymes. ICPO has two catalytic sites: one RING-HC and another novel HUL-1 motif (Hagglund et at., 2002). TAFII250 has intrinsic E1 and E2 activities within a single molecule, and exhibits no homology to other E3s (Pham et al., 2000). (UCHL1)2 is the dimeric form of UCHL1 (functioning as a de-ubiquitination enzyme in monomeric form) and exhibits E3 activity (Liu et al., 2002). p300 exhibits E4-like activity in the presence of Mdm2 E3 ligase (Grossman et al., 2003). However, whether the above E3s are truly ubiquitin-ligases remains elusive at present. It is of note that all E3s except HECT-type E3s are probably not covalently bound to Ub. It is plausible that certain domains, such as RING-finger or U-box, recruit E2s to the vicinity of proteins to be ubiquitinated and thus mediate ubiquitination by facilitating the direct transfer of ub from E2-ubiquitin to the target Lys residue.

Table 1.2 Classification of E3 ubiquitin-protein ligase

HECT-type:	AIP4, Ceb1, E6-AP, Herc2, Hul4, Hul5, hHYD/EDD, Itch, Nedd4, Pub1/2, Rsp5, Smurf1/2, SU/DX,Tom1, Ufd4, WWO1, WWP2, etc.
RING-type:	
(1) RING-HC (C_3HC_4)-finger:	BBAP, BRCA1, (Bre1), Cbls, Chfr, DTX3 (Deltex3), Efp, Hakai, HEI10, IAPs, ICP0, IE2, LNX, Mahogunin (mahoganoid), Mdm2, Mdmx, MID1, Mind Bomb (Mib), Momo, Neuralized (Neu), Nrdp1/FLRF, RAG1, Rma1, RNF2/HIPI-3, Sakura, Siah-1,SINAT5, Staring, TRAF6, etc.
(2) RING-H2 (C_3H2C_3)-finger:	AO7, Apc11, ARNIP, CIP8, DTX1 (Deltex1), DTX2/Deltex2, EL5, gp78, GRAIL/GREUL1, Hrd1, kf-1, NFX-1, Pirh2, Praja1/PJA1, Rbx1, RLIM, TRC8, Tul1, Ubr1
(3) <u>RING-IBR-RING-finger</u>:	Dorfin, HOIL-1, Parc, <u>Parkin</u>, etc.
(4) Atypical RING-finger:	K3/MIR1, K5/MIR2, MEKK1, Doa10, Pib1, CNOT4, etc.
U-box type:	ARC1, CHIP, CYC4, PRP19, Ufd2, Ufd2b UIP5, etc.
Others:	ICP0 HUL-1 domain, TAFII250, (E1+E2 activity), (UCHL1)2, p300, etc.

HECT, homologous to E6-AP carboxyl terminus; RING, really interesting new gene; HUL-1, herpes virus ubiquitin ligase-1.

3. Proteasome

Most cellular proteins in eukaryotic cells are targeted for degradation by the 26S proteasome, usually after they have been covalently attached to Ub in the form of a polyubiquitin chain functioning as a degradation signal. The 26S proteasome is a eukaryotic ATP-dependent protease responsible for selective degradation of polyubiquitin-tagged proteins. It is an unusually large multisubunit proteolytic complex, consisting of a central catalytic/core particle (equivalent to a 20S proteasome) and two terminal regulatory particles (also termed PA700 or 19S complex), which are attached to both ends of the central portion in opposite orientations to form the enzymatically active proteasome (Coux et al., 1996; Baumeister et al., 1998) (Figure 1.10). It appears to act as a highly organized apparatus designed for efficient and exhaustive hydrolysis of proteins, and can in fact be regarded as a protein-destroying machine. The 20S proteasome is a barrel-like particle formed by the axial stacking of four rings made up of two outer α-rings and two inner β-rings, which are each made up of seven structurally similar α- and β-subunits, respectively, being associated in the order of αββα. Three β-type subunits of each inner ring have catalytically active threonine residues at their N-terminus (in which of β1, β2, and β3 correspond to caspase-like, trypsin-like, and chymotrypsin-like activities, respectively), and these active sites face the interior of the cylinder and reside in a chamber formed by the centers of the abutting β rings (Bochtler

et al., 1999). Thus, substrates gain access to the active sites only after passing through a narrow opening corresponding to the center of the α rings, while the amino-termini of the α subunits form an additional physical barrier for substrates to reach the active sites. Interestingly, the center of the α-ring of the 20S proteasome is almost closed, preventing penetration of proteins into the inner surface of the β−ring where the proteolytically active sites are located. Deubiquitination is an important step in the proteasomal degradation of polyubiquitinated proteins. Ub moieties are necessarily removed from the substrate protein prior to its insertion into the narrow opening of the proteasome. On the other hand, the lid-complex is thought to be involved in the recognition of target proteins, deubiquitination for reutilization of Ub, and interactions with various proteins including proteins with an ubiquitin-like domain or certain E3(s).
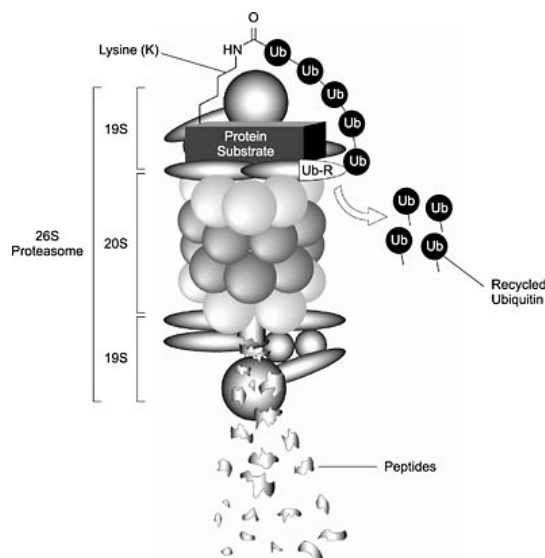
Figure 1.10 A schematic representation of 26S proteasome

Figure 1.11 A parkin-centric view of the ubiquitin proteasome system. Ubiquitin is activated by the enzyme E1 (*yellow*), represented here by a red circle around the black dot of ubiquitin. After activation, ubiquitin is transferred to an E2 enzyme (*blue*), which docks with E3s including parkin (*green*). Parkin contains two RING (really interesting new gene) domains separated by an IBR (in-between ring) motif, and the E2 is probably recruited to this region. Substrates (*red diamond*; see text for description of the different candidates) then bind to the same region of parkin. For simplicity, substrates are shown binding to RING1 and E2 to RING2, but as there are no data on how parkin is folded, we cannot be sure of the exact spatial arrangement of these components. Activated ubiquitin is transferred from the E2 to the target and, by analogy to other E3 ligases there is no transfer to parkin itself. This process is repeated until a string of four or more ubiquitin molecules, linked by lysines to each other and to the substrate, is formed. This is recognized by the proteasome, which degrades the protein into small peptides and amino acids. During all or some of this process, parkin may be tethered to the proteasome by interactions of its N-terminal ubiquitin-like (Ubl) domain. Prior to substrate degradation, the polyubiquitin chain is removed and recycled to monomeric ubiquitin by a series of enzymes including the ubiquitin C-terminal hydrolases. Parkin is also freed to participate in further reactions.

An outline of the reaction scheme catalyzed by parkin is shown in Figure 1.11, where parkin's domain structure reflects this role, as described above.
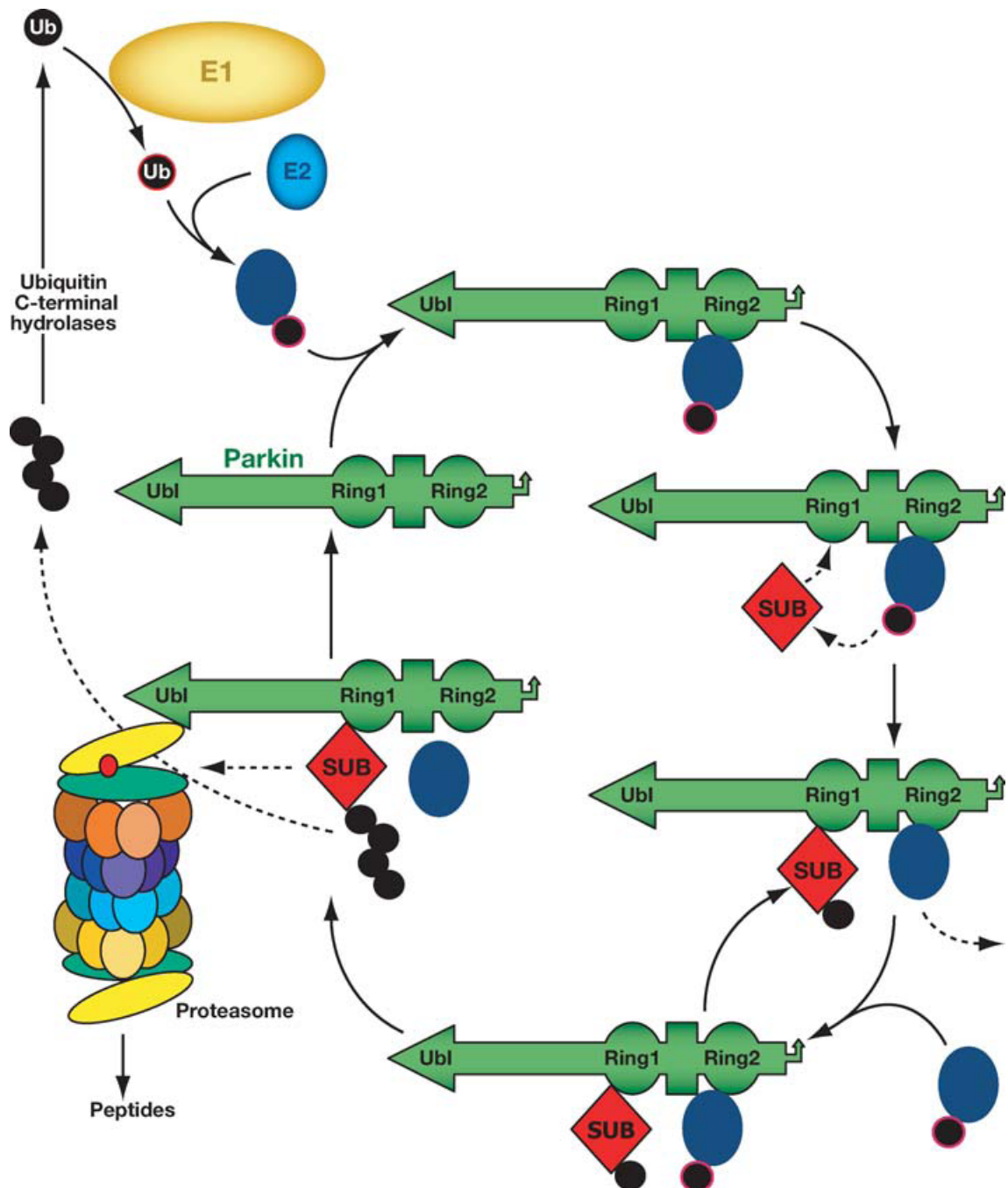
Nevertheless, the precise mechanism by which parkin promotes ubiquitination of its substrates is not fully defined, in part because the structure of parkin has not been solved. However, other E3 ligases that have similar RING domains act as scaffolds to bring the E2-bound ub close to target lysines on the substrate protein. These E3 ligases are not catalysts as the reaction does not proceed via a thiol intermediate as on the E3, but is based on a proximity effect. A by-product of this reaction scheme is that these E3 enzymes undergo autoubiquitination (probably throughout the activation of the transcription factor Kinase/Nuclear Factor-kB (NF-kB)), a phenomenon that is easily seen with parkin. Under experimental conditions, parkin can apparently catalyze all modes of ubiquitination, suggesting that ubiquitination catalyzed by parkin is not only Lys48-linked (Doss-pepe at al., 2005), thus destined for proteasomal degradation. Matsuda et al. (2006) developed a sensitive E3 assay system using recombinant maltose-binding protein (MBP)-parkin and revealed that the mode of ubiquitination catalyzed by parkin *in vitro* is multiple monoubiquitination rather than polyubiquitination. They demonstrated that if there is an additional factor(s) like E4 that cooperates with parkin *in vivo*, it is possible that monoubiquitination catalyzed by parkin is used as scaffold for further polyubiquitination and finally for proteasomal degradation. Recent research revealed that parkin does not necessarily have to deal with proteasomal degradation because it can mediate degradation-independent ubiquitination, implicated in the regulation of signal transduction pathways. On the other hand, it was recently reported that parkin also catalyzes the formation of the Lys63-linked polyubiquitination chain, targeting protein substrates in autophagic and/or lysosomal degradation (see Box 2). Thus, it is plausible that parkin shares two roles as an E3 ligase: one linked to, and the other independent of, the proteasome. However, much still remains to be understood concerning parkin-catalyzed ubiquitination.

The abnormal enrichment of Ub in inclusion bodies was first reported more than 20 years ago (Mori et al., 1987; Lowe et al., 1988). This has been used as a diagnostic feature of many neurodegenerative disorders, including Alzheimer's disease and PD. At that time, ubiquitination was equivalent to the proteasomal degradation signal, and thus it was natural for many scientists to think that dysfunction of the UPS contributes to the pathogenesis of these neurodegenerative disorders. In 2006, Matsuda and co-workers revealed that impairment of the autophagy system in mouse neurons causes neurodegeneration and Ub-positive inclusion formation (Hara et al., 2006; Komatsu et al., 2006). The later discovery that Ub-binding receptors (p62 and NBR1) function in autophagic clearance of protein aggregates (Komatsu et al., 2007; Pavkin et al., 2007; Kirkin et al., 2009) made it clear that it is premature to conclude that "dysfunction of parkin impairs UPS and consequently predispones to PD".

Box 2: Autophagy/Lysosome pathway: a role for ubiquitin

The generic term "autophagy" comprises several processes by which the lysosome acquires cytosolic cargo, with three types of autophagy being discerned in the literature: (1) **macroautophagy**, characterized by the formation of a crescent-shaped structure (the phagophore) that expands to form the double-membrane autophagosome, capable of fusion with the lysosome; (2) **microautophagy**, in which lysosomes invaginate and directly sequester cytosolic components; and (3) **chaperone-mediated autophagy (CMA)**, which involves translocation of unfolded proteins across the lysosomal membrane (Mizushima et al., 2008). While autophagosomes can sequester cytosolic material nonspecifically, for example, as a response to starvation, there is ample evidence for selective autophagic degradation of various cellular structures, including protein aggregates, mitochondria, and microbes (Xie and Klionsky, 2007) for CMA. The mechanism of selective autophagy is not well understood; however, the involvement of Ub in this process is evident: analogous to the proteasome, where ubiquitinated cargo is delivered by Ub receptors (Elsasser and Finley, 2005; Husnjak et al., 2008), autophagic clearance of protein aggregates requires Ub-binding receptors p62 and NBR1 (Kirkin et al., 2009; Komatsu et al., 2007; Pankiv et al., 2007). It is envisaged that by simultaneous binding to both Ub and the autophagosome-associated Ub-like (UBL) proteins (i.e., LC3/GABARAP, parkin proteins), these molecules can mediate docking of ubiquitinated protein aggregates to the autophagosome, thereby ensuring their selective degradation. The attachment of Ub moieties to various cellular cargos

constitutes a universal degradation signal recognized by two major intracellular proteolytic systems: the proteasome and the lysosome.

What determines whether a given Ub-labeled protein substrate will enter one or the other pathway? Classically, conjugation with Lys48-linked polyUb chains allows recognition of the proteolytic substrate by UBD-containing proteasomal receptors, whereas the Lys63-linked chains have been associated with nonproteolytic functions of Ub (Welchman et al., 2005). However, more recently, Lys63-linkage has been implicated in proteolytic degradation of misfolded and aggregated proteins (Olzmann et al., 2007; Tan et al., 2008; Wooten et al., 2008). Given the reported preference of the known Ub-binding autophagy receptors for Lys63-linked Ub chains, Lys63 Ub chain-marked cargo may be preferentially targeted to the autophagy/lysosomal degradation pathway *in vivo*. On the other hand, p62 competes for ubiquitinated cargo with the classical proteasome. Although polyUb chains are most frequently associated with proteolytic degradation, monoubiquitination may be sufficient as a signal for selective autophagy. The autophagosome is able to take up bulky substrates, ranging from protein aggregates to membrane-bound organelles, and deliver them for degradation in the lysosome, Yet, deubiquitination may be involved in autophagy to reduce bulkiness of a complex substrate and/or to allow Ub recycling. The autophagosomal membrane is envisaged to enwrap structures of varying size and geometry. This property of the autophagosome ensures that highly complex cytosolic cargo, including ribosomes and mitochondria, is efficiently degraded by autophagy, for instance, during a starvation response (Klionsky et al., 2008).

Ubiquitin in Selective Degradation of Mitochondria

Mitochondria provide an important example of selective autophagy of organelles. Damage and loss of mitochondrial potential are proposed to lead to targeted autophagic degradation of this organelle, aptly named mitophagy (Elmore et al., 2001). Opening of the mitochondrial permeability transition pore and subsequent rupture of the outer mitochondrial membrane is likely to cause release of as yet unknown autophagy-promoting factors.

In the absence of published evidence for a clearly defined signal for autophagic targeting of superfluous or damaged mitochondria, Vladimir et al. (2009) considered the possibility that induced conjugation of monoUb/Ub chains to an exposed mitochondrial protein may provide the missing link between the mitochondrion and the autophagosome. E3 ligases, residing in or at mitochondrial membranes (Li et al., 2008; Chu et al., 2009), may regulate this process. Alternatively, induced recruitment of cytosolic E3 ligases can also be envisaged. Recently, parkin has been shown to be selectively recruited to depolarized mitochondria and to mediate their autophagic degradation (Narendra et al., 2008). Moreover, this phenomenon is significant in the pathogenesis of PD, suggesting that the genuine physiological substrate(s) of parkin resides on the outer membrane of mitochondria (Matsuda et al., 2010, unpublished data). Selective autophagy is coming to be recognized as a new pathogenetic factor

in neurodegenerative disease. It remains to be determined what the ubiquitinated substrates on the mitochondrial membranes are and whether the known autophagic receptors, p62 and NBR1, could provide the mechanistic link between mitochondrial depolarization, ubiquitination, and mitophagy. Interestingly, early studies on mitochondrial degradation in reticulocytes suggested that the process was Ub-dependent (Rapoport et al., 1985). More work is necessary to unequivocally demonstrate the role of mitochondrial ubiquitination in mitophagy.

1.8.5 Putative parkin substrates

There are several targets for parkin's E3 ligase activity. These include the septins CDC-rel1 (a synaptic vesicle associated GTPase) and CDC-rel2 (Zhang et al., 2000; Choi et al., 2003), cyclin E (Staropoli et al., 2003), p38 transfer RNA synthase (p38, a key structural component of the mammalian aminoacyl-tRNA synthetase complex) (Corti et al., 2003), Paelr1 (parkin associated endothelin receptor like receptor) (Imai et al., 2001), synaptotagmin XI (Huynh DP., et al. 2003), $\alpha-$ and β-tubulin, L166P mutant DJ-1, *O*-glycosylated form of α-syn (named αSp22), and synphilin-1 (an α-syn interacting protein) (Chung et al., 2001). Since parkin is autoubiquitinated, parkin itself can be considered as a substrate (Shimura et al., 2000; Zhang et al., 2000). It is notable that some of these proteins are synaptic, as parkin is tethered to synaptic densities by an interaction with the PDZ protein Cask (Fallon et al., 2002). This implies that parkin could have a role in synaptic function. In most cases, parkin can ubiquitinate substrates without additional binding proteins, but parkin does require an additional protein (hSel10) for activity against cyclin E (Staropoli et al., 2003). Abnormal accumulation of one or more of these substrates due to loss of parkin function, which then leads to cell death of nigral neurons, may be the cause of neurodegeneration in parkin-related parkinsonism. Support for this idea comes from experiments where over-expression of the parkin substrate Paelr1 (Yang et al., 2003) produces dopaminergic cell death that can be rescued by parkin but not its E3 inactive mutants. When over-expressed in cells, this receptor tends to become unfolded, insoluble, and ubiquitinated *in vivo*. The insoluble Pael-R leads to unfolded protein-induced cell death.

Moreover, the insoluble form of Pael-R accumulates in the brains of AR-JP patients. Accumulation of the unfolded Pael-R in the ER of dopaminergic neurons induces ER stress leading death of dopaminergic neurons in AR-JP. Like α-syn, Pael-R has a propensity to misfold and aggregate. Another substrate, CDC-rel1, also causes cell death restricted to the nigra *in vivo* (Dong et al., 2003). Parkin ubiquitinates and promotes the degradation of CDCrel-1, whereas its familial-linked mutations impair CDCrel-1 degradation. On the other hand, parkin is tightly bound to microtubules that are polymers of tubulin α/β heterodimers, ubiquitinates highly toxic misfolded tubulin monomers, and promotes their degradation (Ren et al., 2003). It is interesting that the *O*-glycosylated form of α−syn (αSp22) becomes a target for parkin (Shimura et al., 2001). In contrast to normal parkin, mutant parkin associated with ARJP failed to bind and ubiquitinate αSp22. Thus, αSp22 is a substrate for parkin's E3 ligase activity in normal human brain and loss of parkin function causes pathological αSp22 accumulation. These findings demonstrate a critical biochemical reaction between the two PD-linked gene products and suggest that this reaction underlies the accumulation of α−syn ubiquitinated in conventional PD. Note that non-glycosylated α−syn, the major species in the brain, is not a parkin substrate *in vivo* or in the brain.

Although direct links between these factors and dopaminergic cell death have not yet been established, accumulation of substrate(s) for ubiquitination mediated by parkin is likely to be critical to our understanding of the pathogenesis of AR-JP.

1.8.6 Parkin-interacting molecules

Parkin may be present as part of a modular complex in the brain with additional proteins that act to control substrate specificity. As mentioned, parkin interacts with E2s and Rpn10 (and hence the 26S proteasome) through the RING-IBR-RING and UBL domains, respectively. At least one additional protein can also bind to parkin, the Carboxy terminus of Hsp70-Interacting

Protein (CHIP) (Imai et al., 2002). CHIP interacts with Hsp70 to ubiquitinate misfolded proteins. Hsp70 and CHIP are important components of the decision-making machinery of the cell to direct either protein refolding in the ER or retrotranslocation to the cytosol and degradation. Recently, it was reported that parkin forms a complex with CHIP, Hsp70, and Pael-R both *in vitro* and *in vivo* (Imai et al., 2002). The amount of CHIP in the complex is increased during stress of the ER. CHIP promotes the dissociation of Hsp70 from parkin and Pael-R, thus facilitating parkin-mediated Pael-R ubiquitination. Moreover, CHIP enhances parkin-mediated *in vitro* ubiquitination of Pael-R in the absence of Hsp70. Thus, CHIP acts as a mammalian E4-like enzyme that positively regulates parkin E3 activity. On the other hand, it is also reported that parkin forms a complex with expanded poly-Gln protein, Hsp70 and the proteasome, which may be important for the elimination of the poly-Gln protein (Tsai et al., 2003). In addition, Hsp70 enhances parkin binding and ubiquitination of poly-Gln protein, suggesting that Hsp70 may help to recruit misfolded proteins as substrates for parkin E3 ligase activity. A recent study reported that parkin is a component of an SCF (Skp1, Cullin-1, Roc1, and F-box protein)-like ubiquitin ligase (Staropoli et al., 2003). Indeed, parkin functions in a multiprotein ubiquitin ligase complex that includes the F-box/ WD repeat protein hSel-10 and Cullin-1.

1.8.7 Pathogenic mutations

The number of identified mutations of the parkin gene has recently increased in patients with early-onset parkinsonism, as described above, to include a single point mutation that causes Arg-Pro substitution at position 42 of the UBL domain, and naturally occurring **parkin**$^{\text{R42P}}$**,** identified in one family of AR-JP patients (Terreni et al., 2001). Intriguingly, the NMR data also indicate that Arg42 is located in the Rpn10- binding site. It is likely that the UBL domain tethers parkin close to the proteasome, directing poly-ubiquitinated proteins toward their

proteolysis. The recessive parkin mutation R42P disrupts this interaction (Sakata et al., 2003). It is quite conceivable that this mutation induces a significant conformational change in the Rpn10- binding site of UBL, resulting in impaired proteasomal binding of parkin, which could be the structural basis of AR-JP. This mutated parkin retains the ability to bind E2 (UbcH7), but fails to co-immunoprecipitate ubiquitinated proteins, such as *O*-glycosylated α-syn (Shimura et al., 2001), suggesting that the UBL domain functions as a module necessary for binding with ubiquitinated proteins. This finding provides direct evidence that parkin is linked to cellular proteolysis, and its dysfunction presumably causes AR-JP. This is of particular importance, because even if parkin is an E3 ligase, the possibility that it has actions other than proteolysis cannot be excluded. Conversely, RING-box mutants lacking either RING1 or RING2 domains, e.g. parkin harboring the **K161N** mutation, or the C terminal **T415N** and **T240R** substitutions in RING1, are unable to bind UbcH7 or UbcH8 enzymes, but mantain normal E3 activity, suggesting that the phenotype is not entirely attributable to loss of catalytic function. Matsuda et al. (2006) demonstrated that most PD-relevant missense mutations, such as **K161N, K121N, K211N,** RING1 **T240R, R256C, R275W, D280N and C289G** variants do not abrogate E3 activity of parkin. *In vitro* analysis of the E3 ubiquitin- protein ligase activity of these parkin variants revealed that most promoted their own ubiquitination as efficiently as wild-type parkin. Only mutations replacing essential amino acids in the RING2 domain or truncating this domain e.g. **T415N, G430D, C431F, M434K, C418R and C441R** abolish E3 enzymatic activity, revealing that not the first but the second RING finger motif is the catalytic core of parkin (Figure 1.12). Collectively, the bulk of published studies conclude that parkin dysfunction is not simply attributable to catalytic impairment of its E3 activity, and that loss of ligase activity is a minor pathogenic mechanism. However, it is also possible that some of the missense point mutations reported are, in fact, polymorphisms without pathogenic consequences. This may

well be the case of the **A82E** substitution, which affects an amino acid position that is poorly conserved throughout evolution. Disease-relevant mutations cause not only attenuation of E3 activity but also a variety of primary defects such as sequestration into aggresome and dissociation from its partner protein. Possibly a complex of such defects may eventually lead to parkin disfunction and AR-JP (Gu et al., 2003; Sriram et al., 2005). Corti and colleagues (Hampe et al., 2006) demonstrated that mutations in the RING fingers or in the UBL domains decreased protein solubility in detergent and increased its tendency to form visible aggregates. In general, the solubility of the truncated isoform is similar to that of the full-length protein: it is rather soluble in the case of normal parkin and its A82E, K161N, K211N, R256C, and G328E variants, whereas it tended to be at least as insoluble as the full-length protein in the case of the C289G, C418R, and C441R variants. However, in the case of the R42P and R275W variants, the truncated isoform was consistently more soluble than the 52-kDa protein. This configuration is expected for R42P, as its truncated isoform does not carry the corresponding amino acid substitution, but is surprising for the RING1 R275W variant. R256C was significantly less soluble in Triton X-100 than normal parkin, whereas A82E tended to be more soluble. In addition, the relative protein levels of R42P, C418R, and C441R in total cell extracts tended to be lower than those of the other variants, suggesting that the former group had shorter half-lives. Cells containing aggregates were frequently observed following overproduction of the R275W, C289G, C418R, and C441R variants. In contrast, R42P and R256C behaved similar to normal parkin and A82E, K161N, K211N, R256C, and G328E, which only rarely formed aggregates in transfected cells. This observation is consistent with a previous attempt to categorize parkin variants into 'soluble', 'insoluble with low propensity to form inclusions', and 'insoluble with high propensity to form inclusions' (Wang et al., 2005).

Some parkin mutations compromise the protein's binding to a series of known partners (Hsp70, γ-tubulin, proteasomal α4 subunit) / substrates (p38, CDCrel-1, α-tubulin). Staropoli et al. (2003) reported abrogation of the interaction of parkin with the F-box protein hSel-10 by the RING1 T240R mutation, whereas parkin binding to the chaperone-like protein 14-3-3η, was abolished by the R42P, K161N, and T240R mutations in another study (Sato et al., 2006). However, in other reports, mutations had little or no effect on the interactions between parkin and selected proteins (Chung et al., 2001; Imai et al., 2002; Huynh, 2003; Chung et al., 2001; Shiram et al., 2005). Conflicting results have generally been obtained in attempts to explore the conseguences of parkin gene mutations. Similarly, Shimura et al. (2000 and 2001) reported the R42P variant to be inactive, whereas Ko et al. (2005) and Sriram et al. (2005) concluded that it promoted the ubiquitination of synphilin or p38 even more efficiently than normal parkin. These descrepancies are based on different approaches to study pathogenetic parkin variants. The mechanisms underlying the pathogenic effects of a series of missense parkin gene mutations that seem to preserve the stability, subcellular distribution, protein interactions, and enzymatic activity of parkin remain to be elucidated. Increasing understanding of the dynamic regulation of parkin distribution within the cell, of the network of parkin intermolecular interactions, and of the cooperation between these parameters in modulating the ubiquitination capacity of this protein, should help to better understand the functional consequence of parkin gene mutations. This will also be an essential step towards a fuller comprehension of the multiple physiological functions of parkin and their relationship with disease.
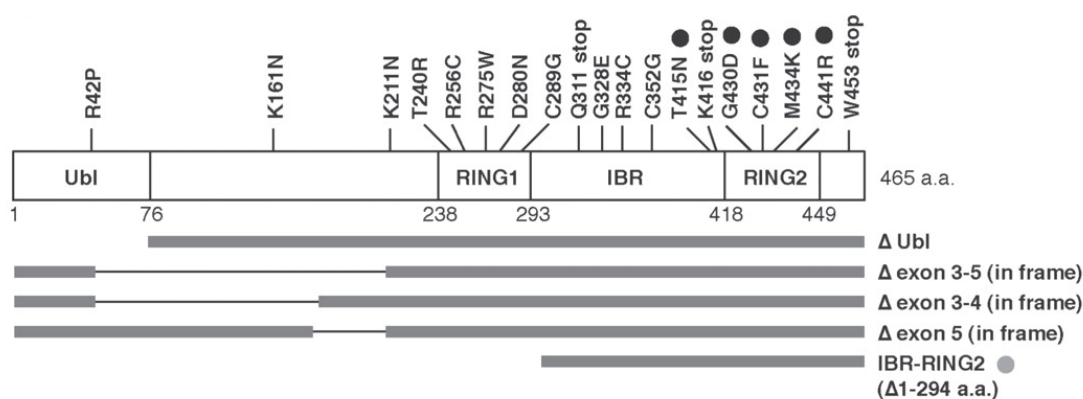
Figure 1.12 Schematic diagram of disease relevant mutations and exon mutations of parkin Note that mutation neighboring the second ring finger motif (solid circles) abolished E3 activity of parkin. Conversely, pathogenic mutants other than RING 2 mutants retain E3 activities equivalent to that of the wild-type (Matsuda et al., 2006).

1.8.8 Mechanisms of parkin inactivation

There are suggestions that parkin inactivation may play a role in typical PD. A growing body of evidence indicates that **misfolding** and **aggregation of parkin** is a major mechanism of parkin inactivation, accounting for the loss-of-function phenotype of various pathogenic parkin mutants. Remarkably, wild-type parkin is also prone to misfolding under certain cellular conditions, suggesting a more general role of parkin in the pathogenesis of PD. (Box 3).

Box 3. Protein Misfolding and Aggregation

Protein misfolding, which results in the exposure of hydrophobic residues normally confined within the folded proteins, accompanies normal protein synthesis but can be dramatically enhanced by numerous stimuli, including ER and oxidative stress, starvation, mutation, and heat shock (Goldberg, 2003). Misfolded polypeptides are recognized by molecular chaperones of the heat shock protein (HSP) family, which bind to and shield exposed hydrophobic surfaces from the cytosol while promoting protein refolding (Goldberg, 2003). In addition, HSPs interact with Ub E3 ligases, such as CHIP and parkin (Imai et al., 2002), which promote substrate polyubiquitination and, thus, prime terminally misfolded polypeptides for degradation by the proteasome.

Protein aggregation occurs when protein misfolding is left unresolved by the chaperone-assisted refolding or proteasomal degradation. Polymerization of misfolded proteins is mediated by nonspecific hydrophobic interactions of partially unfolded polypeptide chains that can lead to the formation of microscopically visible structures known as inclusion bodies and aggresomes (Kopito, 2000) (Figure 1.13). For simplicity, inclusion bodies can be viewed as multiple intracellular foci into which misfolded

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protein oligomers are sequestered, whereas the aggresome is a structure formed via the retrograde transport of aggregated proteins on microtubules and is usually found at the microtubule organizing center (MTOC) (Kopito, 2000) (Figure 1.13). The biological role of protein inclusions is not entirely clear. They may play a protective role by sequestering toxic misfolded protein species and providing the cell with an opportunity of delayed protein degradation (Kopito, 2000; Bjorkoy et al., 2005). Alternatively, they may inactivate the proteasome and mediate cytotoxicity (Bence et al., 2001). The dual role of inclusions highlights the vital importance of alternative degradation pathways that would be amenable to degradation of bulky substrates. Thus, inhibition of the proteasome potently induces autophagy, which serves as a compensatory mechanism for degradation of accumulating polyubiquitinated misfolded proteins.
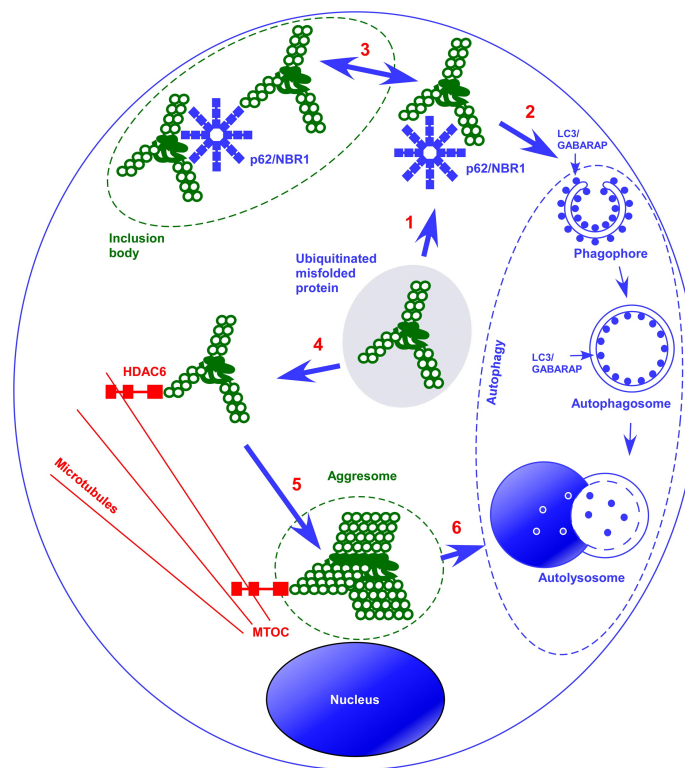
Figure 1.13 A Model for the function of p62, NBR1, and HDAC6 proteins in selective autophagy of ubiquitinated misfolded proteins. Oligomerized misfolded proteins are ubiquitinated and recognized by the Ub-binding domain of oligomeric p62 and NBR1 proteins, drawn as spokes of a wheel (although polyUb chains are depicted, it is possible that monoubiquitination is sufficient for target recognition) (1), which target them for selective degradation by autophagy (2). Oligomeric p62 and NBR1 also mediate formation of proteinacious inclusion bodies (3). Binding of HDAC6 to ubiquitinated proteins ensures their transport along the microtubules toward the MTOC (4), where excess misfolded proteins can be organized into an aggresome (5). Inclusion bodies (3) and aggresomes (6) may allow autophagic degradation of stored misfolded proteins. Closed boxes, Ub-binding domains; empty circles, Ub; filled circles, conjugated LC3/GABARAP proteins

1.8.8.1 Proteotoxic stress induces misfolding of wild-type parkin

Oxidative stress is believed to be a major pathogenic mechanism in PD that contributes to the selective vulnerability of dopaminergic neurons (Mattson et al., 1999; Beal MF., 2002; Lotharius and Brundin, 2002) (Box 4). DA metabolism leads to the formation of various reactive species, while DA autoxidation generates semiquinone and quinone radicals, superoxide radicals, and hydrogen peroxide. Enzymatic deamination of DA catalyzed by monoamine oxidase is also a source of hydrogen peroxide. Cytotoxic species result from further reactions: superoxide can be converted into peroxynitrite in the presence of nitric oxide (NO), and hydrogen peroxide reacts with ferrous ions to form hydroxyl radicals (Fenton reaction). Reactive oxygen and nitrogen species interfere with the cellular integrity by damaging DNA, proteins, and lipids. Two groups have recently shown that exposure to NO alters parkin's E3 ligase activity (Chung et al., 2004; Yao et al., 2004). The mechanism involves NO-derived radical species that attack cys residues in RING1. As these cysteines are important in coordinating the structural zinc molecule, this will affect protein folding and, hence, enzyme activity. In support of this concept, the E3 ligase activity of parkin is impaired by nitrosative stress, and there is indeed evidence for the presence of *S*-nitrosylated parkin in the brains of PD patients (Chung et al., 2004; Yao et al., 2004). In one study, decreased activity of parkin toward synphilin-1 was noted (Chung et al., 2004), whereas increased autoubiquitination was seen in the other study (Yao et al., 2004). Whether small differences in experimental conditions are responsible for these apparent discrepancies remains to be resolved. One notable difference is that shorter exposures to nitrosylating agents produce increased activity, whereas longer exposures inhibit activity. Parkin is nitrosylated both in human tissue from PD patients and in the MPTP and rotenone animal models (Chung et al., 2004; Yao et al., 2004). NO-derived radicals are important mediators of MPTP toxicity (Dauer and Przedborski, 2003). Even wild-

type parkin is prone to misfolding under severe oxidative stress (Winklhofer et al., 2003). Remarkably, insoluble, catechol-modified parkin could be detected in the SNpc of patients suffering from sporadic PD, suggesting a more general role of parkin in the pathogenesis of PD (LaVoie et al., 2005). Based on these finding that the deletion of C-terminal amino acids results in misfolding and aggregation of parkin, it is obvious that the high cys content found in the RBR domain predisposes parkin to oxidative stress-induced inactivation and misfolding. Interestingly, in comparison to other RBR proteins, parkin seems to be uniquely sensitive to DA-induced inactivation (LaVoie et al., 2007; Wong et al., 2007). If proteotoxic stress, typical for dopaminergic neurons, induces misfolding of wild-type parkin and as a consequence, a significant fraction of parkin is inactivated by aggregation, parkin aggregates do not accumulate but are cleared by the proteasome. The sensitivity of parkin to oxidative stress might indicate a more general role of this protein in the pathobiology of PD. An increase in the oxidative burden combined with a decrease in the capacity to scavenge reactive species is a characteristic feature of the aging SNpc; consequently, it is conceivable that parkin dysfunction contributes to the pathology of sporadic PD.

Box 4.

Oxidative stress in the brain

Why is the central nervous system particularly vulnerable to oxidative insult? There are several reasons: its high rate of O_2 utilization, the relatively poor concentrations of classical antioxidants and related enzymes present and the high content of polyunsaturated lipids, the biomacromolecules most susceptible to oxidation. In addition, there are high concentrations of redox-active transition metals capable of the catalytic generation of reactive oxygen species (ROS). Thus, oxidative stress may be a common feature of neurodegenerative diseases, where damage to neurons can reflect both an increase in oxidative processes and a decrease in antioxidant defenses (Figure 1.14).
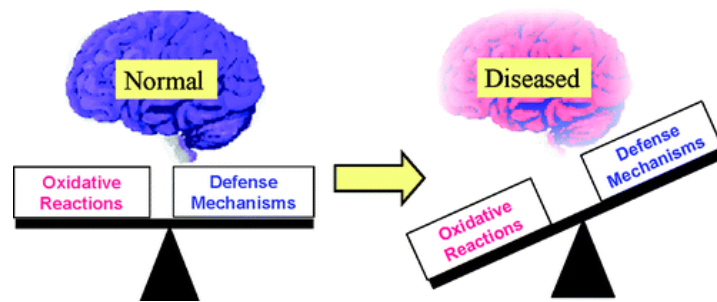
Figure 1.14 Imbalance between ROS production and the defense mechanisms in diseased brain.

For three different age-related neurodegenerative diseases, Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS), in addition to the more common sporadic forms, there are rare familial forms, the hereditary bases of which continue to be defined. The sporadic forms are predominant of unknown origin but are thought to reflect a combination of hereditary, environmental, and lifestyle factors. The same is true for multiple sclerosis (MS), a progressive autoimmune demyelinating disease. In contrast, Huntington's disease (HD) is a strictly autosomal, dominantly inherited, progressive neurodegenerative disorder. Concerning the etiology, for all these pathologies there is evidence for some component of oxidative stress. The central question is whether oxidative stress is a consequence of degenerative processes initiated by some other factors or whether oxidative stress is an early event that contributes to the etiology of the disease. Often both primary and secondary oxidative stress components occur simultaneously. Indeed, it has been relatively straightforward to evaluate an association between oxidative stress and neurodegeneration by finding increased levels of oxidative stress markers or immunocytochemical evidence for oxidative damage to biomacromolecules in affected brain regions. What is still unclear is the nature of the relationship and mechanism between oxidative stress and cell death.

Oxidative stress and neurodegenerative disorders

There are four key mechanisms that present in the spectrum of neurodegenerative diseases, although not every disease has all features. First, there is increasing evidence of an interaction between neuroinflammation and chronic oxidative stress. In recent years, NO was discovered as a common second messenger in inflammation; in fact, it is released from macrophages (or activated microglia in the CNS), along with superoxide. High levels of diffusible NO and superoxide give rise to peroxynitrite. Peroxynitrite and related reactive nitrogen species (RNS) induce both oxidation and nitration (Alvarez and Radi, 2003), resulting in a condition known as "nitrosative stress". Growing evidence suggests that ROS and RNS act together to mediate damage in neurodegenerative disease (Floyd, 1999; Chung et al., 2005). A second common feature is the accumulation of unfolded or misfolded proteins in brain cells, leading some researchers to refer to AD, PD, HD, and ALS as "conformational protein diseases". The third common feature, most prominent in AD, PD, and MS, is dyshomeostasis of both redox-active

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(e.g., copper and iron) and redox-inactive (e.g. zinc) metal ions (Sayre et al., 2005). The last feature is a dysfunction of mitochondria (Lin and Beal, 2006), which plays a crucial role in metabolism and regulates the life cycle of cells (e.g. in mediating apoptosis). Of course, these four features are not independent. For example, small-molecule products of oxidative stress can mediate protein misfolding, leading to neurotoxicity (Bieschke et al., 2006). The major source of intracellular ROS is localized in mitochondria (oxidative phosphorylation), suggesting the presence of a link between mitochondrial abnormalities in neurodegenerative diseases and the involvement of oxidative stress. To protect itself under physiological conditions, the inner membrane of mitochondria presents several free radical scavengers and enzymatic ROS removal systems. However, in certain pathological conditions, mitochondrial defenses can become compromised, due to either genetic mutations or an increase in radical production. Although it is difficult to distinguish whether mitochondrial defects are the primary cause of toxicity, or instead, represent a secondary collateral damage, there is evidence to indicate that mitochondrial-derived oxidative stress is a primary event associated with neurodegeneration (Mancuso et al., 2006). Moreover, the absence of protective histones in mitochondrial DNA (mtDNA) and the limited repair capacity render mtDNA an easy target for ROS. AD and PD, in particular, are diseases in which there are clear elements in support of an involvement of oxidative stress (Jenner 2003); in fact, in the brain of AD and PD patients hight levels of redox metals, particularly iron, have been found.

Oxidative stress and PD

Neurons in the SNpc are particularly sensitive to oxidative stress-induced damage because of their distinct physiological and biochemical features, such as the existence of neuronal melanin, an abundance of iron content, and a deficiency in oxidative stress and free radical scavenging mechanisms in dopaminergic neurons. In the early 1990s, Jenner and Olanow (1996) proposed the oxidative stress hypothesis to account for the pathogenesis of PD. A variety of discoveries in PD patients and animal models indicate involvement of oxygen free radicals and oxidative stress in the pathogenesis of PD. Many researchers discovered that the concentration of iron and lipid peroxidation (LPO) in the SNpc of PD patients were considerably elevated, whereas the activity of mitochondrial complex I and glutathione (GSH) content were reduced. When GSH content was reduced to such an extent that it could no longer effectively scavenge hydrogen peroxide, iron ions at high concentration would convert hydrogen peroxide to an even more toxic species, OH$^-$, thus inducing LPO to cause oxidative damage to cells. In this context,, DA may be the major contributor as it can be generated endogenously by SNpc neurons (Barzilai et al., 2001; Betarbet et al., 2002). An increase in DA may lead to mitochondrial dysfunction and impaired proteolysis via its reactive metabolites, dihydroxyphenylacetic acid and the protein-modifying DA-quinones, or directly due to its oxidative nature (Berman et al., 1996; Berman and Hastings 1997; Kuhn and Arthur 1998; Stokes et al., 1999; Khan et al., 2001). Neuronal catechols are subject to spontaneous oxidation and polymeralization within the cytoplasm to form the primary

component of the insoluble, intracellular deposits of neuromelanin that give the human SNpc and LC their distinctive colors (Sulzer et al., 2000; Zecca et al., 2003). The presence of neuromelanin in the adult human brains shows that the oxidation of DA, and the generation of its reactive metabolites occurs *in vivo*. The neurotoxin 1-methyl-4-phenylpyridinium, a reactive metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, is a mitocondrial complex I inhibitor and is selectively taken up into dopaminergic neurons via the DA transporter leading to severe oxidative damage and neuronal degeneration resulting in parkinsonism in rodents, primates, and humans (Javitch et al., 1985; Ramsay et al., 1986; Mizuno et al., 1987). Metabolism of DA leads to 6-hydroxydopamine (6-OHDA) production (Napolitano et al., 1995; Linert et al., 1996). The toxicity of 6-OHDA is thought to be mediated by selective uptake through the transporter for DA. 6-OHDA and the autooxidation of DA generate free radicals (Cohen and Heikkila 1974; Graham 1978) and the induction of apoptosis in catecholaminergic neurons (Walkinshaw and Walters 1994; Mayo et al., 1998). 6-OHDA-induced cell death is widely used as an experimental model of PD both *in vivo* and *in vitro* (Blum et al., 2001; Hanrott et al., 2006) since this neurotoxin induces cell death of such neurons, as well as symptoms of the disease (Marti et al., 2002).

Oxidative stress in AR-JP

Takanashi et al. (2001) found that iron staining in the brains of AR-JP patients was stronger than that in nonaffected individuals and that the distribution patterns are different between AR-JP and primary PD patients. Furthermore, the neuronal axons in the SNpc of AR-JP patients showed very intensity iron staining. They inferred that oxidative stress might play a pivotal role in the neurodegenerative process in AR-JP. Hung et al. (2003) reported that three parkin mutation (Del 3-5, T240R, and Q311X) lead to an elevation of protein and LPO levels in *in vitro* cultured NT-2 and SK-N-MC cells and to an increase in expression of neuronal NO synthases. Palacino et al. (2004) recently reported decreases in serum antioxidant capacity and increased protein and LPO, rendering cells more vulnerable to oxidative stress, in parkin knockout mice. Thus, increasing evidence supports the concept that functional defects of the parkin gene is closely related to oxidative stress.

1.8.8.2 Pathogenic missense mutations induces misfolding of wild-type parkin

Another observation suggesting parkin may play a role in sporadic PD is the reported association with promoter polymorphisms (West et al., 2002), although conflicting results have been reported (Oliveira et al., 2003). Winklhofer et al. (2008) shown that misfolding of parkin can lead to two phenotypes: the formation of detergent-insoluble-aggregated parkin, or

destabilization of parkin resulting in an accelerated proteasomal degradation. Different lines of evidence indicate that pathogenic parkin mutations result in a loss of parkin function. This study revealed that misfolding and aggregation is characteristic for C-terminal deletion mutants of parkin. Alterations in the detergent solubility of parkin and formation of parkin aggregates/inclusion bodies have also been reported for various parkin missense mutants (Cookson et al., 2003; Gu et al., 2003; Wang et al., 2005; Hampe et al., 2006). However, the propensity to misfold upon C-terminal truncations was specific for parkin. This strategy was based on the commonly held view that RING2 of the RBR domain extends into the adjacent C-terminal region to stabilize its fold. A biochemical analysis of different C-terminal deletion mutants revealed that deletion of more than three amino acids interfered with the native folding of parkin. Destabilization of parkin is also induced by some pathogenic missense mutations within the UBL domain (Henn et al., 2005), which may explain the instability of the R42P mutant. Safadi and Shaw (2007) showed by NMR spectroscopy that the R42P mutation causes the complete unfolding of the UBL. Notably, a smaller parkin species, which occurs in human brain attributable to the presence of an internal initiation site and which lacks the N-terminal UBL (ΔN parkin), is significantly impaired in activating the nuclear factor-kB (NF-kB) pathway and thus in protecting cells from toxic insults. This observation is consistent with the finding that a mutation in the authentic initiation codon of parkin is pathogenic (Rawal et al., 2003; Mata et al., 2004). In this case, the second initiation codon is presumably used, giving rise to N-terminally truncated, functionally less active parkin. The internal initiation site at codon 80 is only present in human parkin; thus, two parkin species with different functional activities coexist in neurons, which might explain why humans are particularly vulnerable to inactivation of full-length parkin. These data highlight the relevance of the RING and ubiquitin-like domains for the correct folding of parkin. The integrity of these domains may also be important

for protein stability (Muqit et al., 2004). Interestingly, differences in the conformational state were not be observed for two parkin mutants with an amino acid substitution within or close to the second RING finger motif (G430D and T415N). However, these mutants did not assemble into a high molecular weight complex, as does wild-type parkin when subjected to cellular stress. That misfolding of parkin can occur in two facets, aggregation or destabilization is an interesting feature which merits further analysis. Conceptually, pathogenic mutations might induce the formation of different parkin conformers or might affect parkin folding at distinct stages of the folding pathway. Mutations of several genes leading to various abnormalities of cellular signaling pathways, infections, and toxins (Marras et al., 2010) are associated with neuronal cell loss in the ventrolateral SNpc. Currently, there is no definitive explanation for why these abnormalities affect dopaminergic neurons earlier and more profoundly than other cell types. One major common theme for most mutations and toxins is the impairment of mechanisms related to cellular energy production leading to oxidative stress. It may be that the selective vulnerability of nigrostriatal cells is determined by their profuse arborization, which may result in high levels of energy consumption.

1.8.8.3 Post-translational modifications regulate parkin enzymatic activity

Whereas the effects of mutations on the structure and function of parkin have been intensely studied, post-translational modifications of parkin and the regulation of its enzymatic activity are poorly understood. Nitrosylation of parkin was recently found to occur in PD, leading to an inhibition of its ubiquitin ligase activity (Chung et al., 2004; Yao et al., 2004). The phosphorylation status of parkin also has an impact on its activity (Meffert and Baltimore, 2005; Yamamoto et al., 2005). Yamamoto et al. (2005) demonstrated that parkin is phosphorylated both in non-neuronal and neuronal cell lines. The turnover of parkin phosphorylation is rapid, because inhibition of phosphatases with okadaic acid was necessary to stabilize phosphoparkin.

Phosphoamino acid analysis revealed that phosphorylation occurred mainly on serine residues, rather than threonine residues. At least five phosphorylation sites were identified, including Ser[101], Ser[131], and Ser[136] (located in the linker region) as well as Ser[296] and Ser[378] (located in the RING-IBR-RING motif). Casein kinase-1, protein kinase A, and protein kinase C phosphorylated parkin *in vitro*, and inhibition of casein kinase-1 caused a dramatic reduction of parkin phosphorylation in cell lysates. Unfolded protein stress mediated by proteasomal inhibition or ER stress, but not oxidative stress, reduced the overall phosphorylation of parkin. Proteasomal inhibition with MG-132 also reduced parkin phosphorylation, but less so than ER stress. Unphosphorylated parkin isolated from eukaryotic cells or purified as recombinant fusion protein from bacteria showed a small but significant increase of autoubiquitin ligase activity, compared with parkin phosphorylated *in vivo* and *in vitro*. Thus, this suggests that modulation of the phosphorylation state of parkin has a regulatory role on its E3 ubiquitin ligase activity. In cells exposed to PD-relevant protein folding stress (Giasson et al., 2001), overall parkin phosphorylation decreased. Unphosphorylated parkin tends to be more active. These findings suggest that phosphorylation of parkin contributes to the regulation of its ubiquitin ligase activity upon unfolded protein stress. Although the RING-IBR-RING motif is crucial for parkin ubiquitin ligase function, single site phosphorylation in this domain appears to have no effect on autoubiquitination. Nevertheless, hydrogen peroxide-induced oxidative stress-mediated overall phosphorylation of parkin slightly reduced its E3 enzymatic activity. The regulation of parkin E3 activity must be due to multiple phosphorylation sites. Induction of protein folding stress in cells reduced parkin phosphorylation, and unphosphorylated parkin had slightly but significantly elevated autoubiquitination activity. Thus, complex regulation of the phosphorylation state of parkin may contribute to the unfolded protein response in stressed cells.

How can the inactivation of parkin by misfolding be prevented?

Molecular chaperones interfere with parkin misfolding. The former are ubiquitous, highly conserved proteins destined to assist in the folding of nascent proteins and in the refolding of damaged proteins (Morimoto et al., 1994; Rutherford et al., 1994; Bukau and Horwich, 1998). In response to environmental stress, the expression of chaperones is increased to counteract proteotoxic effects. In the aging brain, the levels of several chaperones, like small heat shock proteins and Hsp73 (Hsc70), are constitutively elevated; however, the induction of Hsp72 (Hsp70) is impaired (Fargnoli et al., 1990; Schults et al., 2001). In combination with a decreased activity of the UPS, the reduced inducibility of stress proteins might significantly impair cellular homeostasis, increase neuronal cell vulnerability, and promote neurodegeneration. Molecular chaperones are associated with protein aggregates occurring in a variety of neurodegenerative diseases, including sporadic PD, AD, polyglutamine diseases, and prion diseases, which might reflect a general cellular mechanism to deal with misfolded proteins (Welch and Gambetti, 1998; Sherman and Goldberg, 2001). Several cell culture studies revealed that chaperones have an impact on the conversion of the prion protein and the aggregation of polyglutamine proteins (Jana et al., 2000; Wyttenbach et al., 2002). The potential of chaperones in preventing or reducing the toxicity of misfolded proteins has been established in animal models where increased expression of Hsp70, together with its co-chaperone Hsp40, suppressed the toxicity of α-syn and polyglutamine proteins (Warrick et al., 1999; Chan et al., 2000). In a study by Winklhofer et al. (2003), the authors observed that misfolded parkin co-localized with Hsp70 which, together with Hsp40, significantly increased the amount of natively folded parkin. Furthermore, increased expression of molecular chaperones promoted folding of the W453Stop mutant and interfered with stress-induced misfolding of wilde-type parkin. These observations revealed the propensity of parkin to misfold, and suggest that also

chemically denatured recombinant parkin is strictly dependent on molecular chaperones to adopt a native conformation. Elucidating the *in vivo* folding pathway of parkin will help to identify molecular chaperones and cofactors involved in order to design strategies to prevent the misfolding and inactivation of parkin.

Although prevention of parkin misfolding induced by pathogenic mutations is difficult to achieve, the inactivation of wild-type parkin induced by massive oxidative stress might be amenable to anti-oxidative strategies. Different free radical scavengers, such as α-tocopherol, and iron chelators (VVK-28 and M30), exhibited neuroprotective activity in cell culture (Behl, 2000; Zheng et al., 2005). Unfortunately, a beneficial effect of antioxidants has not be demonstrated conclusively in PD patients so far. The failure of anti-oxidative approaches in clinical trials might be explained by the fact that neuroprotective strategies applied only when patients already show parkinsonian symptoms. At this stage of PD, 50-60% of dopaminergic neurons in the SNpc have degenerated. Thus, neuroprotective drugs should ideally be given in a preclinical stage, underling the necessity of defining biomarkers.

1.8.9 What is the functional role of parkin?

A consistent observation in cell culture and animal models is the neuroprotective capacity of parkin. Parkin protects cultured cells against death induced by excitotoxicity (kainate or glutamate), mitochondrial disfunction (in particular inhibition of complex I of the electron transport chain, induced by rotenone, and inhibition of complex IV of the electron transport chain, induced by 6-OHDA), ER stress, unfolded protein stress mediated by protesome inhibitors, α-syn, ceramide, manganese, DA, tau or expanded polyglutamine fragments (Imai et al., 2000; Petrucelli et al., 2002; Darios et al., 2003; Staropoli et al., 2003; Higashi et al., 2004; Jiang et al., 2004; Muqit et al., 2004). In *Drosophila*, over-expression of parkin can suppress loss of dopaminergic neurons induced by α-syn or Pael-R (Yang et al., 2003; Haywood and

Staveley, 2004). Furthermore, lentiviral delivery of parkin prevents dopaminergic degeneration caused by mutant α-syn in a rat model and protects mouse skeletal muscle cells against mitochondrial toxins (Lo Bianco et al., 2004; Rosen et al., 2006). Parkin is neuroprotective against stresses in which the direct relationship to its substrates is unclear.

Because of its unambiguous contribution to dominantly inherited PD, several laboratories have examined whether there is a relationship between α-syn and parkin. For example, parkin, but not its E3 inactive mutants, protects cells against mutant α-syn (Petrucelli et al., 2002; Kim et al., 2003; Chung et al., 2004). Parkin also suppresses mutant α-syn toxicity in *Drosophila* models (Yang et al., 2003; Haywood and Staveley, 2004). Demonstrating again that the difference between wild-type and mutant α-syn is qualitative rather than quantitative, parkin can suppress the toxicity associated with expression of high levels of α-syn *in vitro* (Oluwatosin-Chigbu et al., 2003). The simplest explanation for this observation is that α-syn might be a parkin substrate. The steady-state level of α-syn is not affected by the expression of parkin in cell lines (Chung et al., 2001) or in *Drosophila* (Yang et al., 2003). Although there have been suggestions that α-syn levels might respond to proteasome inhibition *in vitro*, most studies have not noted any effect. One study found that α-syn can be degraded by the proteasome in an ubiquitin-independent fashion (Tofaris et al., 2001), which would not require an E3. There is also evidence for α-syn degradation by lysosomal proteases (Paxinou et al., 2001; Lee et al., 2004). There is evidence for a glycosylated form of α-syn, sp22, that can be purified from human brain and is a substrate for parkin (Shimura et al., 2001). To date, there are no replications of this important result, so it is not quite clear whether sp22 is present in some of the model systems where α-syn is demonstrably toxic and, hence, whether formation of sp22 is required for α-syn toxicity.

Creating knockout models for parkin is one way to model the disease and understand the pathogenic process. Two groups have produced mice with targeted deletion of exon 3 of parkin. Neither shows loss of nigral neurons, although there are subtle changes in dopaminergic neurotransmission (Goldberg et al., 2003; Itier et al., 2003). Recently, mice with an exon 7 deletion have been reported to have loss of neurons in the LC with attendant behavioral changes (Von Coelln et al., 2004). Although there is no loss of catecholaminergic neurons, *Drosophila* parkin knockouts show mitochondrial damage and apoptosis of flight muscles (Green et al., 2003). Interestingly, one of the knockout mouse models shows deficits in mitochondrial respiration (Palacino et al., 2004). Non unsurprisingly, the major phenotype reported in parkin models is mitochondrial. Parkin rather specifically prevents mitochondrial cytochrome *c* release and apoptosis *in vitro* (Darios et al., 2003). This observation has gained greater weight with the realization that other recessive genes for parkinsonism also impact on mitochondrial function.

What is the possible mechanism underlying the broad neuroprotective capacity of parkin?

Parkin can protect neuronal cells against a remarkably wide array of intrinsic and extrinsic stressors. Most studies reporting on the protective potential of parkin are consistent concerning the nature of the stressors tested, and discrepancies can be explained by the fact that high-level stress conditions inactivate parkin because of its tendency to misfold (Winklhofer et al., 2003; LaVoie et al., 2005; Wang et al., 2005; Winklhofer and Tatzelt, 2006) (Figure 1.15). In support of a role of parkin in coping with cellular stress, stressors significantly increased the amount of parkin-specific mRNA in cultured neuroblastoma cells as well as in primary neurons, resulting in an increased expression of parkin protein (Henn et al., 2007). These observations are in line with a recent publication showing activation of the parkin promoter after hydrogen peroxide treatment (Tan et al., 2005).

Because of the broad cytoprotective activity of parkin, was reasoned that it may have an impact on a key survival pathway (Figure 1.15). Reporter assays specific for stress-inducible pathways revealed that parkin stimulates the NF-kB-dependent transcription. In the course of several studies, three lines of evidence have converged to suggest that activation of the NF-kB signaling cascade is essential for the neuroprotective activity of parkin. First, there is a correlation between the neuroprotective activity of parkin and its ability to activate NF-kB. Pathogenic parkin mutants impaired in their neuroprotective capacity are also compromised in their ability to efficiently stimulate NF-kB-dependent transcription. In this context, it should be pointed out that pathogenic parkin mutants do not show a complete loss of function, at least during over-expression in cultured cells. However, subtle differences in the activity of parkin may be pathophysiologically relevant over decades, especially under conditions of increased cellular stress. Second, when the NF-kB pathway is blocked, parkin loses its protective activity. In the presence of the NF-kB super-repressor IkBΔN or the kinase-inactive IKKβ mutant IKKβ K/A, parkin no longer protects cells from kainate-induced toxicity. Finally, downregulation of endogenous parkin by an RNA interference approach results in an increase in cell death along with a decrease in NF-kB signaling in response to stress. Notably, a smaller parkin species, which occurs in human brain attributable to the presence of an internal initiation site and lacks the N-terminal UBL (ΔN parkin), is significantly impaired in activating the NF-kB pathway and thus in protecting cells from toxic insults.

The NF-kB pathway is a key pro-survival pathway, which is paradigm for the role of ubiquitination in mediating degradation-dependent and degradation-independent functions. Conventional roles of ubiquitin in the NF-kB pathway include targeting of IkBα for degradation as well as inducing the proteasomal processing of the NF-kB precursors p105 and p100. Unconventional degradation-independent polyubiquitin chain attachment is essential for the

activation of the IKK signalosome and involves ubiquitination of tumor necrosis factor receptor associated factors 2, and 6 (TRAF2, TRAF6) , receptor-interacting protein, and IKKγ/NEMO (NF-kB essential modulator), which is an essential component of the IkBα kinase complex (Chen, 2005; Krappmann and Scheidereit, 2005). In another study, it was reported that parkin can delay epidermal growth factor receptor (EGFR) internalization and degradation via degradation-independent ubiquitination of Eps15, thereby promoting phoshoinositide 3'-kinase (PI3K) /Akt signaling (Fallon et al., 2006).

<u>What might be the role of parkin, particularly in dopaminergic neurons that are exposed to oxidative and excitotoxic stress even under physiological conditions</u>?

By modulating the NF-kB pathway, parkin may initiate a neuroprotective program under low-level and moderate stress. In line with this scenario, parkin induces a supra-additive stress response under conditions that only weakly stimulate the NF-kB pathway, indicating a sensitizing effect. Moreover, parkin is upregulated in response to cellular stress, raising the question of whether parkin itself is regulated by NF-kB. Several stress-responsive binding elements are located in the promoter region of parkin, although a NF-kB-responsive element was not identified. In some experimental models, over-expression of parkin is sufficient to activate the NF-kB signaling cascade, but the E3 ligase activity of parkin is regulated rather than constitutive under physiological conditions (Henn et al., 2007). In this context, it is interesting to note that the phosphorylation status of parkin has an impact on its activity (Meffert and Baltimore, 2005; Yamamoto et al. 2005). Yamamoto et al. (2005) demonstrated that the reduced phosphorylation of parkin in ER stressed cells contributes to the up-regulation of parkin E3 ubiquitin ligase activity, which is believed to suppress cytotoxicity due to unfolded protein stress.
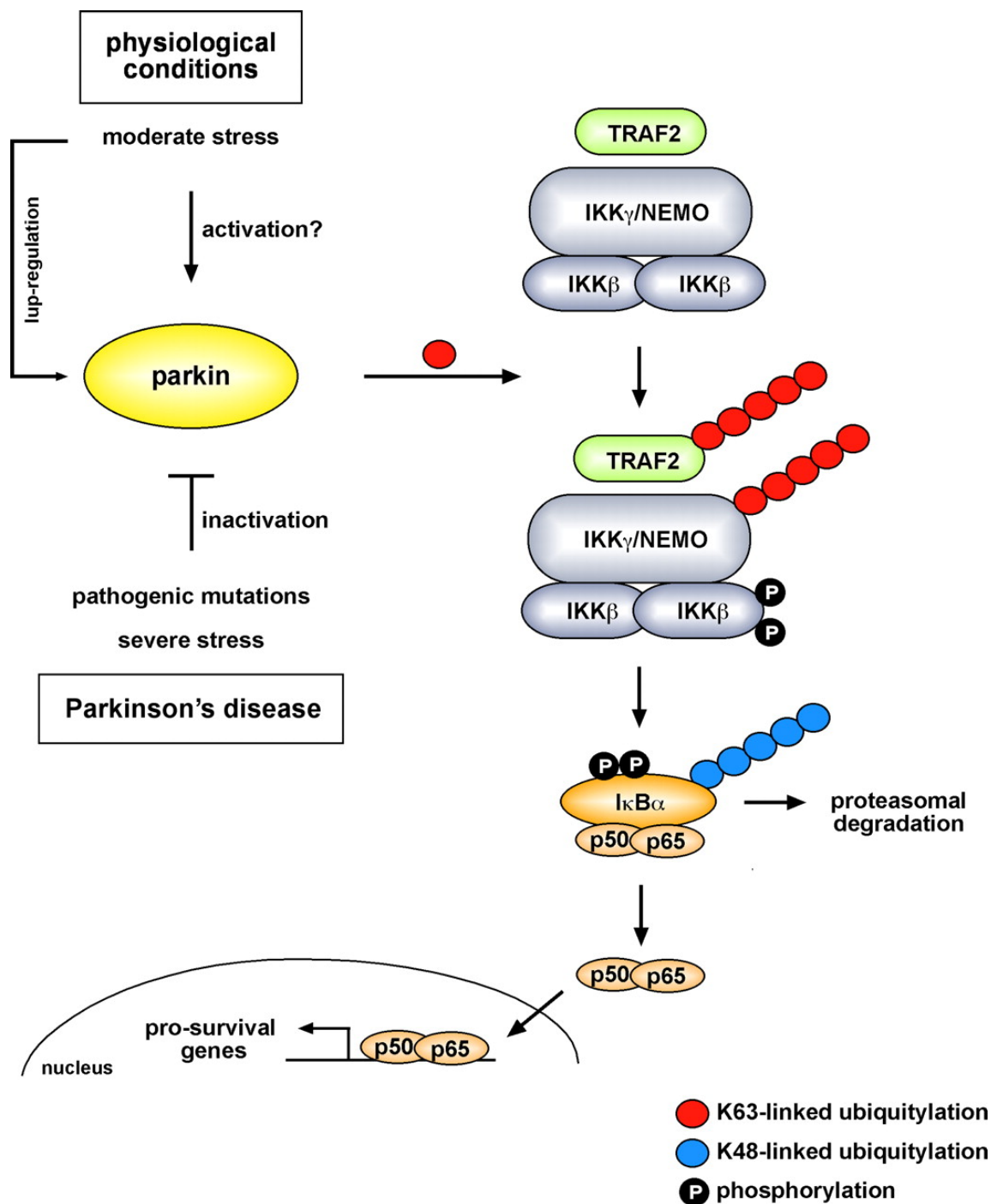
Figure 1.15 Model of the functional role of parkin. Under physiological conditions, parkin promotes regulatory ubiquitination of TRAF2 and IKKγ during stress, leading to the activation of NF-kB. As a consequence, transcription of prosurvival genes is upregulated. The activity of parkin can be modulated by stress. Under moderate stress, parkin is upregulated. For an immediate response, it is conceivable that parkin activity can be regulated by post-translational modifications. In contrast, severe proteotoxic stress, for example induced by oxidized dopamine, causes misfolding and thus inactivation of parkin. Mutations in the parkin gene linked to familial PD also interfere with the capacity of parkin to stimulate the IKK/NF-kB signaling pathway.TRAF2, tumor necrosis factor receptor associated factors 2. IKKγ/NEMO,NF-kB essential modulator.

How can these novel insignts be translated into therapeutic srategies?

The association of parkin with PD and its wide neuroprotective activity marks parkin as an attractive candidate for the development of prophylactic or therapeutic strategies. The neuroprotective action of parkin point to a link between parkin function and IKK/NF-kB signaling, suggesting that dysregulation of this neuroprotective pathway plays a pathophysiological role in PD. Three approaches are conceivable: first, to increase the expression of parkin via gene therapy; second, to prevent the inactivation of parkin; third, to modulate the signaling pathway affected by lack of functional parkin. These approaches are speculative at the present, but might be considered as a component of future strategies to target specific disease-associated events, such as mitochondrial dysfunction and the oxidative stress response, thereby shifting therapy from symptomatic to disease modifying. However, there are still severe limitations to the development of new therapies at the preclinical and clinical stages: the lack of animal models, the need for biomarkers to identify PD patients in a presymptomatic stage, and difficulties in designing appropriate clinical studies to accurately monitor the effect of an intervention on PD progress.

1.9 Concluding perspectives

Recognizing that PD does not result from a single cause but from many interacting factors goes a long way in explaining the many clinical observations, as well as the plethora of biochemical abnormalities that have been identified in individuals and experimental systems. Genetic studies have revealed proteins involved in the initiation of some forms of PD, and establishing their relative roles and interactions should be a priority. Molecular and cellular abnormalities occur to different degrees in the SNpc of individuals with PD (Pan et al., 2008; Vila et al., 2008; Brar et al., 2009). DA metabolism seems to interact with and enhance these abnormalities, but a specific sequence of events has not been defined. Understanding the vulnerability of the SNpc

and the mechanism whereby pathology becomes widespread are primary objectives of basic and clinical research in PD. In this context, *in vivo* monitoring of nondopaminergic pathways and their correlation with impairment of DA pathways and symptom progression in the different PD subtypes should be a priority, in order to better assess degree and pattern of cell loss throughout disease progression.

New therapeutic avenues

Box 5: <u>TREATMENTS FOR PD</u>

CURRENT SYMPTOMATIC THERAPIES

Oral medications

Levodopa + a dopadecarboylase inhibitor ± a catechol-O-methyltransferase inhibitor

DA agonists, including slow-release formulations such as ropinirole, pramipexole

Monoamine oxidase B inhibitors (MAOB): for example, selegiline, rasagiline

Anticholinergics: for example, trihexyphenidyl

Antiglutamatergics: for example, amantadine

Continuous delivery therapies

DA agonists: subcutaneous or intravenous, such as apomorphine and lisuride

Transdermal patch: for example, rotigotine

Intraduodenal levodopa: for example, Duodopa

Surgical therapies

Deep brain stimulation of the subthalamic nucleus, globus pallidum pars interna

Lesions: for example, subthalamotomy, pallidotomy

FUTURE SYMPTOMATIC THERAPIES, INCLUDING ANTI-DYSKINETICS

Partial DA agonists: for example, pardoprunox

Adenosine A2a antagonists

Safinamide—MAOB inhibitor, anti-glutamatergic and sodium-channel blocker

Zonisamide—MAOB inhibitor, glutamate release blocker

mGluR5 antagonists

Alpha-adrenoreceptor antagonists: for example, fipamexole

AMPA antagonists: for example, perampanel, talampanel

5HT2A partial agonists: for example, pimavanserin

PUTATIVE NEUROPROTECTIVE DRUGS IN CLINICAL TRIALS

Pramipexole—DA agonist

Coenzyme Q10—respiratory-chain enhancer and antioxidant

Creatine—ATP synthesis enhancer

Green tea polyphenol—antioxidant

Inosine—urate elevator

Isradipine—calcium channel blocker

Cogane—GDNF, BDNF synthesis stimulator

Before L-dopa (Box 5), PD was essentially a motor disorder. After the arrival of L-dopa, the development of motor complications and psychiatric manifestations, such as hallucinations and delirium, came to the fore and became the prevailing clinical problems in PD for the next two decades. More judicious use of L-dopa, the introduction of DA agonists and of atypical neuroleptics (clozapine, quetiapine) and the possibility of treating severely affected individuals with surgery have reduced the urgency of these problems. Indeed, there is general agreement that new PD treatments should tackle two unresolved problems: moving from symptom-alleviating to disease-modifying therapies, and reducing the growing prevalence of non-motor disease symptoms such as loss of balance, autonomic dysfunction and cognitive impairment, which are the real causes of disability in long-term PD.

Transplant: starting in the 1980s, many people with PD received striatal grafts from various sources (fetal tissues, porcine fetal SNpc neurons, carotid body cells, and immature retinal cells). Despite evidence for a beneficial effect of mesencephalic fetal grafts in some open-label studies, two double-blind, placebo-controlled trials failed to show clinical improvement (Freed et al., 2001; Olanow et al., 2003). The best result of any transplant study in PD does not surpass the clinical benefits of either deep-brain stimulation (Box 5) of the subthalamic nucleus or parenteral delivery of L-dopa and apomorphine (Olanow et al., 2009). To date, all cell-replacement studies have used fetal ventral mesencephalic tissue or paraneural DA cells. Recently, there has been great interest in human stem cells, which survive, innervate to some

extent, and reverse motor dysfunction in rodent and monkey models of PD (Bjorklund et al., 2002; Redmond et al., 2007; Cai et al., 2009). It is clear that human embryonic stem cells are the easiest to manipulate, but they can form teratomas and have raised ethical concerns (Bjorklund et al., 2002). The possible application of induced pluripotent stem cells to treat PD and other disorders (Soldner et al., 2009; Kiskinis et al., 2010) may address the ethical concerns and provide a means to deliver cells of autologous origin, eliminating immunological reactions after transplantation. However, there is no evidence yet that pluripotent stem cells cells will be more efficacious than deep-brain stimulation. We would therefore argue that the high hopes for cell-replacement therapy need to be tempered until more experimental data are available.

Gene therapy: currently, there are four clinical trials testing different gene therapy approaches for PD. One finding common to all of these studies is that no serious adverse events have yet been reported for any of them. One approach uses adeno-associated viral vector serotype 2 (AAV2) to deliver aromatic amino acid decarboxylase (AADC), the enzyme that converts L-dopa to DA. The idea is to make this conversion more efficient, allowing for optimal therapeutic benefit with lower L-dopa doses and avoiding treatment-related side effects. This procedure has been through a successful phase 1 trial (Eberling et al., 2008) and is currently in phase 2. However, it is difficult to see how this technique will avoid the tendency of L-dopa to induce motor complications. A second approach uses AAV2 to deliver glutamic acid decarboxylase (GAD) to the STN (Kaplitt et al., 2007). As glutamic acid decarboxylase synthesizes γ-aminobutyrate, the main inhibitory neurotransmitter in the nervous system, the underlying idea is that delivering this enzyme will increase inhibitory tone. In a sense, this approach is a gene therapy version of deep-brian stimulation, and the advantages of this gene therapy–based approach over are deep-brian stimulation unclear.

The third approach (Palfi et al., 2008), which is in phase 1 trials, involves a tricistronic vector encoding tyrosine hydroxylase, AADC and GTP cyclohydrolase hydroxylase—the last one of which is an enzyme necessary for tetrahydrobiopterin synthesis, an essential cofactor for AADC. Last, AAV2-mediated delivery of neurturin, a functional analog of glial cell–derived neurotrophic factor, aims to provide neuroprotective benefits in addition to symptomatic improvement. Neurturin provides robust neuroprotection and upregulation of DA function in a variety of rodent (Gasmi et al., 2007) and nonhuman primate models (Kordower et al., 2006), and has completed a successful phase 1 clinical trial (Marks et al., 2009). However, it is now known (Siffert et al. 2010) that neurturin failed in phase 2 clinical testing. New trials are in the planning stages.

In this study we propose the use of protein transduction domains (PTD), such as Trans-activating transcriptional activator (TAT) domain, to deliver parkin protein. The TAT from human immunodeficiency virus 1 (HIV-1) is the first cell penetrating peptide (CPP) discovered, in 1988. CCPs are short peptides that facilitate cellular uptake of various molecular cargo (from small chemical molecules to nanosize particles and large fragments of DNA) (Crombez et al., 2008; Esposito et al., 2009). Their function is to deliver the cargo into the cells, thanks to their ability to translocate across the plasma membranes. CPPs have typically an amino acid composition containing either several positively charged, cationic amino acids (such as lysine or arginine) or have sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids. To date, the exact mechanism of CPP's membrane translocation is not fully understood and CPP transduction is an area of ongoing research. Theories of CPP translocation can be classified into three main entry mechanisms: direct penetration in the membrane, endocytosis-mediated entry, and translocation through the formation of a transitory structure (Gump and Dowdy, 2007) (Figure 1.16).
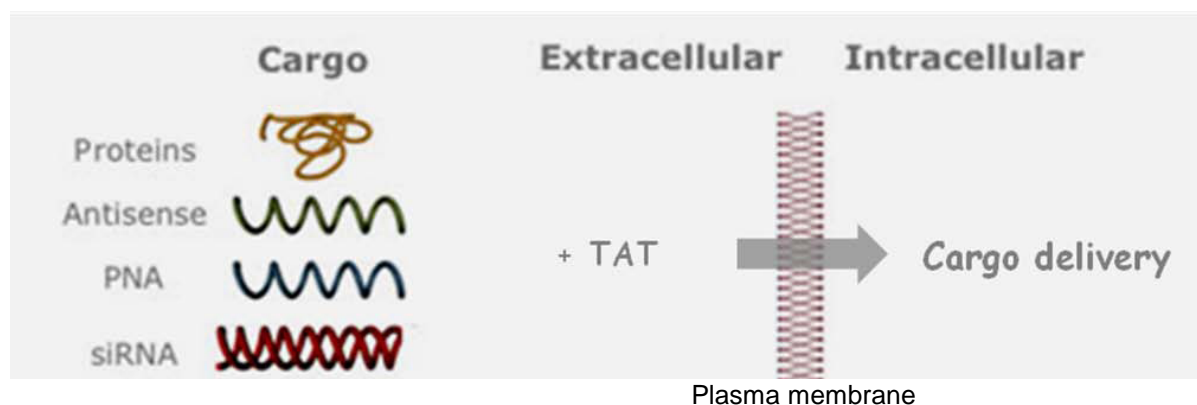
Figure 1.16: CPPs. The cell penetrating peptides (CPPs) are natural peptides identified as cellular membrane-crossing molecules, in particular through their potency to carry various kinds of compounds to the cytoplasm and nucleus of living cells.

Gene therapy remains a viable and apparently safe procedure, particularly if aiming to deliver neuroprotective molecules. However, its actual clinical value is unknown, and further research is required to draw firmer conclusions.

What are the prospects for neuroprotective treatments? Several molecules have been proposed (TCH346, CEP-1347), glutamate antagonists, promitochondrial drugs (coenzyme Q10, creatine), calcium channel blockers (isradipine), and growth factors (such as glial cell-derived neurotrophic factor) (Schapira et al., 2009). However, none of these molecules has definitively shown neuroprotective effects in clinical trials (LeWitt et al., 2008). This may indicate the ineffectiveness of these compounds, but may also be a consequence of the limitations of clinical-trial design (Hung et al., 2008), use of the wrong dose, recruitment of too broad a patient population or selection of inappropriate endpoints (Hung et al., 2008; Olanow et al., 2008).

Despite these limitations, some PD neuroprotection trials—pramipexole and ropinirole versus L-dopa (Whone et al., 2003; Holloway et al., 2004), coenzyme Q10 (Storch et al., 2007) and selegiline (Shoulson et al., 2002) have had positive outcomes in terms of reducing the progression of motor deficits in early PD. However, the interpretation of these trials is

confounded by potential drug modulation of therapeutic endpoints, symptomatic (as opposed to true disease-modifying) effects or trial size (Olanow et al., 2008; Hart et al., 2009). The results of the ADAGIO trial using rasagiline in individuals with PD are relevant to some of these problems. This large (>1,000 subjects), randomized, placebo-controlled, delayed-start trial showed that those receiving 1 mg (but not 2 mg) of rasagiline, as compared to placebo, had slower motor progression over 9 months and improved motor outcome after 18 months compared to those who started the drug later (Olanow et al., 2009). The ADAGIO design was intended to avoid confounding symptomatic effects on the primary clinical endpoints, but conclusions about rasagiline's real long-term impact and putative mechanism of action remain premature.

The management of PD has improved considerably in the past two decades thanks to new therapies and better use of old ones. Most affected individuals now have a relatively good quality of life for most of the natural history of their disease. Nevertheless, a better understanding of the biochemical pathogenesis of PD is the best route to lead us to new disease-modifying therapies. A breakthrough has remained elusive, but there is increasing information about the mechanisms underlying neuronal cell death and regional vulnerability.

AIM of the study

Parkinson's disease (PD) is chiefly a sporadic neurodegenerative disorder affecting about 3% of the population over 65 years (Di Monte, 2003; Fahn, 2003). From the neuropathological point of view, PD is characterized by progressive loss of nigrostriatal dopaminergic neurons and a concomitant reduction in the striatal concentration of dopamine, and by the appearance of intracytoplasmic, ubiquitin-positive inclusion bodies named Lewy bodies and Lewy neurites in surviving neurons (Baba et al., 1998). Genetic studies have contributed greatly to our knowledge on the etiopathogenesis of PD. To date, ten genes (α-synuclein, LRRK2, EIF4G1, GBA, parkin, DJ-1, PINK1,UCHL1, others) have been identified, as well as several genetic loci linked to this disease (Hardy et al., 2003; Gosal et al., 2006; Lesage and Brice, 2009). The gene coding for parkin protein (PARK2) has been linked to a familiar autosomal recessive, early-onset form of PD (West and Maidment, 2004) and is characterised by the absence of Lewy bodies. As such, the latter is a phenocopy of PD, i.e. parkinsonism without Lewy bodies. Shimura and colleagues (Shimura et al., 2000; Zhang et al., 2000) have shown that parkin encodes an E3 protein-ubiquitin ligase. E3 ligase controls the key step in the cycle of ubiquitin-mediated hydrolysis of damaged or misfolded proteins that are degraded via the proteasome. Numerous *in vitro* (Oluwatosin-Chigbu et al., 2003; Henn et al., 2007) and *in vivo* (Lo Bianco et al., 2004) studies have demonstrated a protective effect of parkin against various toxic injuries linked to PD etiopathogenesis (oxidative stress, mitochondrial dysfunction, excitotoxicity, endoplasmic reticulum stress, proteasome inhibition and over-expression of α-synuclein, tau, or expanded polyglutamine fragments) (Moore, 2006). This opens attractive prospects to explore parkin as a new target for PD treatment, based on endogenous neuroprotection.

The present study was designed to investigate the biochemical and biological properties of parkin and its neuroprotective effect against 6-hydroxydopamine (6-OHDA)-induced neurotoxicity. Rat adrenal pheochromocytoma PC12 cells, naïve or induced to develop phenotypic traits of dopaminergic neurons by exposure to nerve growth factor (NGF) will be utilized as a model in which parkin is given either exogenously or over-expressed.

In the first part of this study we will generate from *Escherichia coli*, a recombinant parkin protein fused to a protein transduction domain derived from the human immunodeficiency virus-1 transactivator of transcription (TAT) protein (TAT-parkin) to facilitate diffusion across cell membranes. The fusion protein will be expressed and purified following standard techniques (Dietz et al., 2004), and subsequently tested for its ability to prevent cytotoxicity against 6-OHDA.

The second part of the project will involve developing an inducible *in vitro* model of parkin over-expression in PC12 cells by generating stable clones expressing wild-type and pathogenic mutated R42P protein, thereby allowing a comparison of the effect of over-expression of human wild-type and the R42P mutation on parkin antioxidant function. Lastly we will evaluate the protective effect of parkin over-expression against proteasome inhibitor or/and macroautophagy inhibitor-induced toxicity.

If successful, these date may serve to encourage further, *in vivo* studies in animal PD models to support the therapeutic potential of parkin in this neurodegenerative disease.

CHAPTER 2

MATERIALS AND METHODS

2.1 PARKIN: CLONING AND TAT-FUSION PROTEIN GENERATION

2.1.1 Construction of pTAT-parkin bacterial expression vector

The pTAT-parkin plasmid was constructed essentially as described previously for TAT-α-syn (Albani *et al.,* 2004), and was provided by Prof. Alessandro Negro (CRIBI, University of Padova, Italy). A human placenta cDNA library (Clontech, Palo Alto, CA) was used to amplify the human *parkin* gene by PCR, using a specific primer complementary to the double-strained cDNA and based on the published sequence of the human *parkin* gene. The following primers were used:

 Forward: 5'-CTGCTAGCATGATAGTGTTTGTCAGGTTC-3'

 Reverse: 5'-CTGGAATTCCCTGGAGACACGTGGAACCAGTG-3'

The purified PCR product was cut at the *NheI* and *EcoRI* restriction sites and cloned directly into the bacterial expression vector pRSETB in the same restriction sites (plasmid pParkin). A pTAT vector was constructed as reported for the pTAT-hemagglutinin expression vector (Becker-Hapak *et al.,* 2001) and the sequence of *parkin* was cloned after the TAT sequences between the *NheI* and *EcoRI* restriction sites (plasmid pTAT-parkin). To generate the fusion protein TAT-parkin, the sequence containing six histidine residues and the minimal translocation domain of the HIV-1 protein TAT (YGRKKRRQRRR) was inserted in-frame before the N-terminus of the corresponding parkin cDNA. All clonings were verified by sequence analysis. The fusion protein was then expressed and purified adapting standard recombinant techniques (Dietz et al., 2004).

2.1.2 Expression and purification of TAT-parkin

BL21(DE3) pLysS *Escherichia coli* were transformed with the pTAT-parkin plasmid. Two liters of cells were grown in LB/ampicillin broth with shaking at 37°C until reaching an absorbance of OD_{600} = 0.6. Protein expression was induced by adding 500 μM isopropy-l-β-thiogalactoside. After 3 h the cells were harvested by centrifugation, washed with phosphate-buffer saline (PBS) pH 7.4, and lysed by sonication in denaturing conditions with 6 M guanidinium-HCl, pH 8.0. The lysate was cleared by centrifugation (20 min at 11,000*xg*) and then loaded onto a pre-equilibrated Ni-nitrilotriacetic-acid (NTA) agarose column (Quiagen, Hilden, Germany). The column was exhaustively washed with 6 M guanidium-HCl/8 M urea, pH 8.0 and the TAT-parkin fusion protein was eluted with 8 M urea and 250 mM imidazole, pH 4.5. The recovered protein was further purified, from imidazole and urea, using a gel filtration G25 column (Sephadex^{TM} G-25 M, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) conditioned by a buffer containing 10 mM Tris/HCl, 20% (v/v) glycerol, 274 mM NaCl, 0.1 % pluronic acid, and 0.02% Tween 20, pH 10.0. The concentration of the eluted protein was determined by measuring absorbance at 280 nm. The purity of TAT-parkin was evaluated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue.

2.2 CELL CULTURES

2.2.1 Coating of flasks, multiwall plates and chamber slides

Poly-D-lysine (100X, MW 30-70 kDa, Sigma Aldrich Co., St. Louis, Missouri, USA) was dissolved in H_2O at 2 mg/ml final concentration and filter sterilised through a Millipore filter, 0.22 μm pore size, and stored at 4°C.

Cell cultures were plated at the appropriate density in 25 or 75 cm^2 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) or 24-multiwell ^{TM} (24 well; Falcon, Oxnard, CA, USA) coated with 500 μL of 20 μg/mL poly-D-lysine, at 37°C for 30 min, washed with H$_2$O and let dry. Plates were then maintained at 4°C until used.

Chamber slides were coated with 250 μL of 20 μg/mL poly-D-lysine solution at 37°C for 30 min. The coating solution was then removed, washed with H$_2$O and let dry. Chamber slides were then maintained at 4°C until used

2.2.2 PC12 cell line

Rat adrenal pheocromocytoma cells (PC12) were a generous gift from Professor Maria Speranza Desole, University of Sassari, Italy. PC12 cells were cultured at 37°C in a 5% CO$_2$/95% air incubator in Dulbecco's modified Eagle's medium/F12 medium (InVitrogen, Carlsbad, CA, USA) supplemented with 10% horse serum (Euroclone, Milan, Italy), 5% fetal calf serum (Euroclone), 100 IU/mL penicillin and 100 μg/mL streptomycin solution (InVitrogen) (*growth medium*). Cells were seeded in 25 cm^2 flasks or dishes (Becton Dickinson) coated with poly-D-lysine at 2 x 10^4 cells/cm^2 and were routinely split by removing medium from confluent flasks. After washing with PBS to remove any trace of serum (that inhibits trypsin), cells were incubated with a minimal volume of trypsin/EDTA solution (500 μL/25 cm^2 flask) (InVitrogen) for 2 min at 37°C. Trypsin was inhibited by adding 5 mL of fresh medium and cells were seeded in the appropriate flasks or dishes. When necessary, cells were counted by a Burker chamber. For this purpose, 10 μL of cell suspension were pipetted in the chamber and counted; the average number of cells was calculated counting three independent squares. For experimental treatments, cells were seeded into 24-well dishes coated with poly-D-lysine, at a density of 2 x 10^4 cells/cm^2.

PC12 cells differentiation: Neuronal-like cell differentiation was induced by culturing cells in 24-well plates at 1 x 10^4 cells/cm^2 for 5 days in the presence of 50 ng/mL recombinant human nerve growth factor (rhNGF) (Sigma Aldrich), in Dulbecco's modified Eagle's medium /F12 medium supplemented with 1% horse serum and 100 IU/mL penicillin and 100 μg/mL streptomycin solution (*differentiation medium*). All experiments were carried after 5 days of incubation with rhNGF, at which time ≈ 90% of the cells had developed at least one neurite ≥ two cell body diameters in length (Figure 2.1).
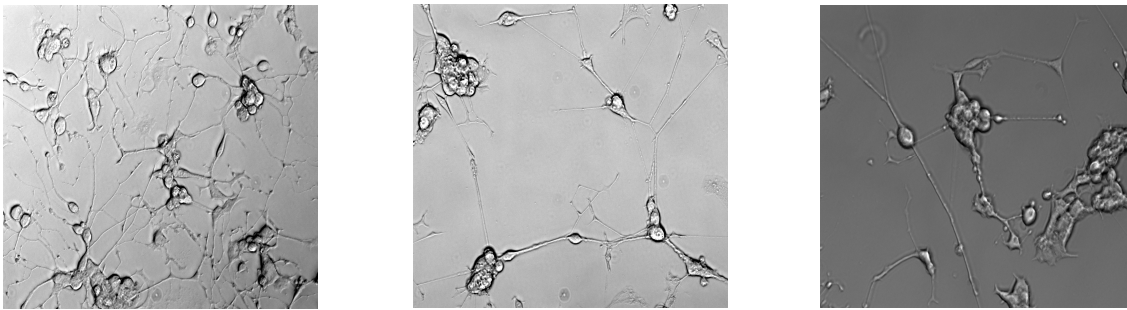
Figure 2.1. Images of PC12 cells differentiated with50 ng/ml rhNGF.

2.2.3 CHO cell line

Chinese hamster ovary (CHO) cells were cultured at 37°C, 5% CO_2 in Ham's F12 nutrient mix supplemented with 10% fetal calf serum, 2 mM L-glutamine (Invitrogen), 100 IU/mL penicillin, and 100 μg/mL streptomycin solution (*culture medium*). Cells were seeded in 75 cm^2 flasks at 2 x 10^4 cells/cm^2 and routinely split by trypsinization and extensively dissociated by pipetting to reduce the formation of cell clumps. Cells were seeded on 12 mm diameter glass coverslips (flamed with absolute ethanol and then transferred to sterile 24-well plates), placed in 24-well dishes at a density of 1 x 10^4 cells/cm^2 and transfected with pmito-green fluorescent protein (pmito-GFP) (Nunnari et *al*.,1997) using Lipofectamine Plus reagent (InVitrogen). Specific methods are described in detail on page 109.

2.2.4 Cell freezing and thawing

➢ For freezing, cells were seeded in 25 cm^2 flask, coated with poly-D-lysine as described for PC12 cells, and grown until confluence was reached. Cells were subsequently washed with PBS, trypsinized and re-suspended in 10 mL of growth/culture medium. The cell suspension was transferred in a 15-mL sterile tube and centrifuged at 900 rpm for 5 min in a 5415R centrifuge (Eppendorf AG, Hamburg, Germany). The pellet was re-suspended in 5 mL of cold medium containing 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich) and transferred into 5 criovials (Nalgene Company, Rochester, New York). Vials were subsequently placed in a criobox (Nalgene Company, Rochester, New York) and pre-frozen at - 80°C for one night before storing in liquid nitrogen (- 196°C).

➢ To thaw cells, the frozen vial was incubated at 37°C for 1 min. The cell suspension was immediately transferred to a 15-mL tube containing 10 mL of growth/culture medium and centrifuged at 900 rpm for 5 min in a 5415R centrifuge. The cell pellet was re-suspended in 5 or 10mL of growth/culture medium and transferred a 25 or 75 cm^2 flask (coated with poly-D-lysine as for PC12 cells).

2.3 GENERATION OF PC12 STABLE CLONES EXPRESSING WILD- TYPE HUMAN PARKIN AND THE PATHOGENIC PARKIN MUTANT R42P (PARKINR42P)

2.3.1

Human parkin full-length cDNA was cloned into the pcDNA3.1 vector (InVitrogen) and fully sequenced. To generate the mutated form parkin[R42P], a GeneTailor Site-Directed Mutagenesis System kit was used (InVitrogen) was used, using the following primers:

PARK2 Forward: 5'-ATG TTG CTA TCA CCA TTT AAG GG -3'

PARK2 Reverce: 5'-AGA ATT GGC AGC GCA GGC GGC ATG -3'

The presence of the R42P mutation in exon 2 of the *parkin* was analysed by genomic DNA extraction and denaturing high performance liquid chromatography (dHPLC).

2.3.2 Stable transfection

Transfection is the process of introducing nucleic acids into cells by non-viral methods. There are various methods of introducing foreign DNA into eukaryotic cells; a very efficient technique is the incapsulation of the nucleic acid to be transfected into liposomes that are able to fuse with biological membranes, releasing their cargo into the cell. If it is desired that the transfected gene remains in the genome of the cell and its daughter cells, a stable transfection should be performed. To accomplish this, we generated PC12 cells stably expressing wild type parkin protein and parkin[R42P] using Lipofectamine™ 2000 (InVitrogen) as transfection agent, using the following protocol:

the day before transfection, PC12 cells were plated, in 10 cm Ø dishes (Becton Dickinson), to obtain 70-80% confluency next day. The pcDNA3.1-wild-type parkin vector (4μg), and pcDNA3.1-R42P mutant parkin vector (4μg) (InVitrogen) were mixed with liposomes (4μL) in serum-free Optimem (InVitrogen) and incubated for 15-30 min at room temperature to allow complex formation. Culture medium was exchanged with 5 mL Optimem and the mixture was

added to the cells and incubated for 4 h at 37°C. At the end of the incubation, medium was changed. At about 16 h post transfection, the selection agent Geneticin G418 (500 ng/mL) (Sigma Aldrich) was added. Medium with selection agent was changed twice a week and after about 4 weeks, at which time isolated colonies began to appear. Individual clones were picked up by pipetting and seeded in a 24-well plate coated with poly-D-lysine. At confluence, each clone was split and progressively expanded in 6 cm Ø dishes (Becton Dickinson). Some clones were subjected to Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for detection of mRNA expression, while Western blotting was used to assess target protein expression. Positives clones were expanded in 25 cm^2 flasks at 2 x 10^4 cells/cm^2; non-expressing clones were discarded.

2.4 RNA ANALYSIS: RT-PCR

RT-PCR is a highly sensitive and specific method for the detection of mRNA expression levels. The general steps of a RT-PCR experiment are the followings:

2.4.1 Total RNA extraction

TRIZOL$^®$ Reagent (InVitrogen) was used for total RNA isolation from cells grown in monolayer or in suspension, according to the manufacturer's instructions. The reagent is a monophasic solution of phenol and guanidine isothiocyanate, which maintains RNA integrity during sample homogenization while disrupting cells and dissolving cell components. Cells were pelleted by centrifugation (200xg for 5 min), and lysed in TRIZOL$^®$ Reagent by repetitive pipetting. After homogenization, samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Two hundred microlitres of chloroform per 1 mL of TRIZOL$^®$ were added, the samples shaken manually for 15 sec, and re-incubated at room temperature for a few minutes. Samples were then centrifuged at 11,000xg

for 15 min at 4°C to separate the mixture into 3 phases: a lower red, phenol-chloroform organic phase containing proteins, an interphase containing DNA, and a colorless upper aqueous phase containing RNA. The aqueous phase was transferred to a fresh vial and RNA precipitated by mixing with an equal volume of isopropyl alcohol (0.5 mL of isopropyl alcohol per mL of TRIZOL® Reagent, approximately). Samples were incubated at 4°C for 15 min and centrifuged at 12,000xg for 15 min at 4°C. RNA appeared as a gel-like pellet, and the supernatant was removed. RNA was washed with 75% ethanol in RNAse-free water (1 mL ethanol per mL TRIZOL®) and centrifuged at 11,000xg for 15 min at 4°C. The RNA was then briefly air-dried, then dissolved in RNAse-free water (0.01% (v/v) diethylpyrocarbonate (DEPC), autoclaved distilled water in RNAse-free glass bottles) and stored at -80°C.

2.4.2 Preparation of RNA samples prior to RT-PCR

Duplicate tubes were prepared, as positive and negative reverse transcriptase (RNA) samples were to be used in the amplification reaction. To avoid DNA contamination all samples were treated for 15 min at room temperature with deoxyribonuclease I, amplification grade (DNase I, Amp Grade) (InVitrogen). To a RNase-free, 0.5-ml microcentrifuge tube were added the following:

17 μL	RNA sample
2 μL	10X DNase I reaction buffer (200 mM Tris-HCl pH 8.4, 20 mM MgCl$_2$, 500 mM KCl)
1 μL	DNase I, Amp Grade, 1U/μL
10 μL	0.01% (v/v) diethylpyrocarbonate-treated water

DNase I was inactivated by the addition of 2 μL of 25 mM EDTA solution (pH 8.0) (InVitrogen) to the reaction mixture. DNase was completely denatured by heating at 70°C for 15 min.

2.4.3 Agarose gel electrophoresis of RNA for quality determination

Agarose (ultrapure, electrophoresis grade, InVitrogen) was dissolved in boiling 1X Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) (TAE) . The gel was then cast on a gel bed with a suitable comb using a horizontal gel apparatus. The gel was placed in an electrophoresis tank containing 1X Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). buffer to a level just above the gel surface. For gel electrophoresis, to each RNA sample was added 0.62 mM Ficoll 400 (Sigma Aldrich), a highly branched sucrose and epichlorohydrin copolymer that acts as a electrolyte stabilizer, and 4µg/mL Orange G sodium salt (Sigma Aldrich), as tracking dye for gel electrophoresis of nucleic acids. Electrophoresis was carried out applying an electrical potential difference of 6 V/cm to the gel. The gel was placed on a 3UV transilluminator for viewing. A single fluorescent band near the loading lane represents high molecular weight RNA, while a fluorescent smear directed to the anode suggests partial or total RNA degradation.

2.4.4 RNA spectrophotometric quantification

The spectrophotometric A_{260}/A_{280} ratio was determined and used to asses purity and yield of the RNA sample. A_{260} should be higher than 0.15, with an absorbance of 1 unit at 260 nm corresponding to 40 µg RNA per ml. This relationship is valid only for measurements at neutral pH. The concentration of purified RNA was calculated using the following equation:

[RNA] in µg/µl = (A_{260} x D)/1000

Where 1 optical density unit is equivalent to 40µg/µl single-stranded RNA and D is the dilution factor.

2.4.5 Reverse transcription reaction

First-Strand cDNA Synthesis

Retrotranscription reaction mixture was prepared in a final volume of 20 µL. The following

components were added to a nuclease-free microcentrifuge tube:

2 µL	75 ng/µl random hexamers
1 µL	10 mM deoxyribonucleotide triphodphates (dNTPs) Mix (10 mM each dATP, dGTP, dCTP and dTTT at neutral pH)
10 µL	total RNA

The mixture was heated to 65°C for 6 min for primer annealing and incubated on ice for at least

1 min. To the tubes was then added:

4 µL	5X first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)
1 µL	0.1 M dithiothreitol (DTT)
1 µL	Recombinant Ribonuclease Inhibitor (RNase OUT ,40 units/µl); storage buffer: 20mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 8 mM DTT, 50% (v/v) glycerol.
1 µL	SuperScript^{TM} III reverse transcriptase (200 units/µl); storage buffer: 20mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0,01% (v/v) Nonidet$^{®}$ P-40, 50% (v/v) glycerol.

Retrotranscription reaction was performed at 50°C for 70 min and inactivated by heating at

75°C for 15 min. cDNA was used as a template for amplification in PCR.

2.4.6 PCR Reaction

cDNA samples were amplified using HotStarTaq® DNA polymerase (5 units/µl) (Quiagen;

storage and dilution buffer: 20mM Tris-HCl, pH 9.0, 100 mM KCl, 0.1 mM EDTA, 1 mM

DTT, 0,5% (v/v) Nonidet$^{®}$ P-40, 0,5% (v/v) Tween$^{®}$20, 50% (v/v) glycerol) in a MX 3000P

thermal cycler (Stratagene, La jolla, USA). All reaction mixtures were prepared in an area

separated from that used for DNA preparation, and disposable tips containing hydrophobic

filters were used to minimize cross-contamination. PCR mix composition:

0.25	μL	25 mM MgCl$_2$
0.25	μL	dNTPs mix (10 mM of each)
500	nM	forward primer*
500	nM	reverse primer*
2.5	units/reaction	HotStarTaq® DNA polymerase
1	μL	cDNA
11.5	μL	0.01% (v/v) DEPC

A negative control containing RNA instead of cDNA was usually performed to rule out genomic DNA contamination and to evaluate accidental reagent contamination.

Each PCR program started with an initial activation step at 95°C for 15 min and a final extension step at 72°C for 7 min. The cycling program was obtained for each template target and primer pair.

* RT-PCR primer pairs

Forward primer : 5'-AGCCACATCAGCATTCACAG-3'
Reverse primer : 5'-AGAAGGGAGCCACCTGATTT-3'

PCR products were identified by subjecting samples to 1.5 % agarose gel for electrophoresis (See earlier section 2.4.3 for details).

2.5 BIOCHEMICAL ANALYSIS

2.5.1 Protein extraction

Cell extracts were prepared by lysing cells in an appropriate volume of lysis buffer (50 mM Tris-HCl pH 7.4, SDS 0,5%, 75 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X100), in the presence of a broad-range protease inhibitor cocktail (Sigma Aldrich) (1:100) to obtain a total protein extract. Lysates were then boiled at 100°C for 5 min and centrifuged at 12,000xg for 5 min at room temperature. Protein concentration was determined as describe below.

2.5.2 Protein quantification

Protein extracted from bacterial and cellular lysates were quantified using the BCA Protein Assay Reagent Kit (Pierce Inc., Rockford, Illinois, USA). This method combines the reduction of Cu^{++} to Cu^+ by a protein in an alkaline medium with the colorimetric detection of the cuprous ion (Cu^+) by bicinchoninic acid (BCA). The first step consists in the chelation of copper by a protein in an alkaline environment to produce a light blue-to-violet compound. In the second step, two molecules of BCA react with one Cu^+ ion to form a purple-colored compound. The intensity of the colour developed is proportional to the protein concentration and may be measured at any wavelength between 550 nm and 570 nm. To perform the assay, 25 μL of cellular lysate (diluted 1:5 or 1:10 in H_2O) is added to a microplate well. A fresh set of protein standards is prepared by serially diluting bovine serum albumin (BSA) stock solution (2 mg/mL). A 200 μL reaction mix, prepared by mixing 40 parts of BCA reagent A with 1 part of reagent B, was then added to each well and incubated for 30 min at 37°C. The absorbance of the final solution was read at 570 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Protein lysates were re-suspended in either 1X sample loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5% (v/v) 2-mercaptoethanol) or 1X Laemmli sample buffer (125 mM Tris-HCl pH 6.8, 10%, SDS, 0,1 mM DTT, 10% glycerol, 0.006% bromophenol blue) and denatured at 100°C for 5 min. Equal amonts of protein (25-30 μg/lane) were separated by 10% SDS-PAGE according to Laemmli (Laemmli, 1970).

10% Resolving gel (5 ml) compositum :

1.9	mL	distilled water
1.7	mL	30 % (w/v) acrilamide-N,N'-methylenebisacrilamide
1.3	mL	1,5 M This-HCl PH 8.8

0.05	mL	10% (w/v) SDS
0.05	mL	10% (w/v) ammonium persulfate $((NH_4)_2S_2O_8)$
0.002	mL	N,N,N',N'-Tetramethylethylenediamine (TEMED)

5% Stacking gel (3 ml) compositum:

2,1	mL	distilled water
0,5	mL	30% (w/v) acrilamide-N,N'-methylenebisacrilamide
0,38	mL	1 M This-HCl PH 6.8
0,03	mL	10% (w/v) SDS
0,03	mL	10% (w/v) ammonium persulfate
0,003	mL	N,N,N',N'-Tetramethylethylenediamine (TEMED)

Molecular weight markers (Full Range Rainbow Molecular Weight Markers, GE Healthcare Life Sciences, Milan, Italy) were also loaded, and the gel run in 1X running buffer (10% Tris-glycine, 1% SDS) at a constant current of 25 mA (Mini-PROTEAN® electrophoresis cell unit (Biorad Laboratories Inc.,,Hercules-CA, USA). Proteins were stained with Coomassie brilliant blue (see Figure 3.2 in Chapter 3) or transferred by an electric field to a sheet of a nitrocellulose blotting paper for Western blotting analysis, as described below.

2.5.4 Western blotting

2.5.4.1 Evaluation of TAT-parkin cellular half-life

TAT-parkin-treated PC12 cells (naïve or differentiated) and CHO cells after different times were washed twice with PBS, scraped off the plate into PBS, and pelleted by centrifugation at 12,000xg for 5 min at room temperature. Proteins were denatured by boiling for 5 min at 100 °C in 1X sample loading buffer and then analyzed on 10% SDS-PAGE gels. The gel was run in 1X running buffer 1X for about 2 h at 25 mA in a Mini-PROTEAN® electrophoresis cell unit. Proteins were then transferred in transfer buffer (Tris-Glycine 10%, SDS 1%, Methanol 20%) to a nitrocellulose membrane (Bio-Rad, Hercules-CA, USA) using a Mini Trans-blot® apparatus (Biorad Laboratories Inc.) applying a constant current of 350 mA for 75 min. Blots were pre-

incubated with 3% BSA (Sigma Aldrich) overnight, then probed with a mouse monoclonal anti his(6)tag antibody (1:3000) for 1 h at room temperature, followed by 3 x 10 min rinses with Tris-buffered saline/1% BSA (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 7.5). Immunoreactivity was visualized by incubation with an alkaline phosphatase-conjugated anti-mouse secondary antibody (1:300, Sigma Aldrich) for 45 min and developed using 5-bromo-4-chloro-3-indolyl phosphate dipotassium/ nitrotetrazolium blue chloride, pH 9.8 (Roche Molecular Biochemical, Mannheim, Germany).

2.5.4.2 Evaluation of expression of human parkin

PC12 cells (un-transfected, transfected with pcDNA3.1-witd-type parkin and pcDNA3.1-R42P mutant parkin), protein extracts were denatured by heating for 5 min at 95°C in 1X Laemmli sample buffer and resolved by 10% SDS-PAGE as describe in the preceding section. Proteins were transferred to a nitrocellulose membrane and this latter then blocked with 5% non-fat-dry milk (Sigma Aldrich) in Tris buffer saline with 1% tween-20 (T-TBS) for 1 h at room temperature, as describe in the section on Western blotting. The membrane was then incubated with the primary anti-parkin rabbit polyclonal antibody (Cell Signaling Technology, MA, USA), diluted 1:1000 in 5% non-fat-dry milk in T-TBS at 4°C overnight. Membrane was washed 2 x 15 min in T-TBS to remove unbound antibody, followed by incubation for 1 h at room temperature with a peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was next washed 3 x 10 min in T-TBS before detection with Immobilion Western Chemiluminescent horseradish peroxidise substrate (luminal) (Millipore Corporation, Billerica-MA, USA). The oxidation of luminal by horseradish peroxidise was detected using the ChemiDoc^{TM} XRS instrument (Biorad Laboratories Inc.), designed to capture chemiluminescence images by a CCD camera without using a photographic darkroom and analyzed with Biorad Quantity One® software.

2.5.4.3 Evaluation of protein ubiquitination

PC12 cells (un-transfected, transfected with pcDNA3.1-witd-type parkin and pcDNA3.1-R42P mutant parkin), were processed as describe above for Western blotting, and blots probed with a primary anti-ubiqitin rabbit polyclonal antibody (DakoCytomation, Denmark), diluted 1:200 in 5% non-fat-dry milk in T-TBS at 4°C overnight, followed by a rabbit peroxidase-conjugated secondary antibody, diluted 1:200.

2.5.4.4 Evaluation of autophagy pathway activation

PC12 cells (un-transfected, transfected with pcDNA3.1-witd-type parkin and pcDNA3.1-R42P mutant parkin), were processed as describe above for Western blotting, and blots probed with a primary anti-LC3B rabbit polyclonal antibody (Abcam, Cambridge, UK), diluted 1:2000 in 5% non-fat-dry milk in T-TBS at 4°C overnight, followed by a rabbit peroxidase-conjugated secondary antibody, diluted 1:5000.

For all Western blotting signal quantification was carried out by densitometry, using National Institutes of Health software (ImageJ, version 1.38X).

Primary antibodies

The following primary antibodies were used for Western blotting:

- anti-polyHistidine mouse monoclonal antibody (1:3000) (Sigma Aldrich);

- anti-parkin rabbit polyclonal antibody (1:1000) (Cell Signaling Technology, MA, USA);

- anti-α-tubulin mouse monoclonal antibody (1:5000) (Abcam, Cambridge, UK);

- anti-ubiqitin rabbit polyclonal antibody (1:200) (DakoCytomation, Denmark);

- anti-LC3B rabbit polyclonal antibody (1:2000) (Abcam).

2.6 IN VITRO UBIQUITINATION ASSAY

CHO cells were treated or not (control cells) with 100 nM of TAT-parkin, washed twice with PBS, scraped off the plate into PBS, and pelleted by centrifugation. Cell pellets were sonicated in 100 µl PBS for 20 s, and then centrifuged for 5 min at 10,000*xg*. The *in vitro* ubiquitination assay was performed according to Imai et al. (2003) and Matsuda et al. (2001, 2005). Briefly, extracts from CHO cells were incubated at 32°C for 2 h in a reaction buffer (50 mM Tris-HCl, 2 mM DTT, 5 mM $MgCl_2$ and 4 mM ATP, pH 8.8) containing 1.6 µg/ml of recombinant mouse ubiquitin-activating enzyme E1, 20 µg of the ubiquitin-conjugating E2 enzyme His-UbcH7, and 50 µg His(6)ubiquitin/ml (Sigma Aldrich) (total volume 30 µl). The reaction was terminated by adding 25 µL of SDS-PAGE sample buffer and boiling for 5 min, and then 20µL was subjected to 10% SDS-PAGE and immunoblotting, as described above. Ubiquitinated proteins were visualized with mouse monoclonal anti(his)6tag antibody(1:3000).

2.7 CELL IMAGING: IMMUNOFLUORESCENCE

2.7.1 Evaluation of TAT-parkin cellular translocation in PC12 cells.

Cells were incubated with 100 nM TAT-parkin for 24 h, followed by fixation with 4% (w/v) paraformaldehyde (Sigma Aldrich). After rinsing with PBS, the cells were permeabilized (0.1% Triton X-100 in PBS) and blocked with 3% (w/v) BSA/PBS. Cells were then incubated for 1 h with a primary antibody (anti-parkin polyclonal antibody, 1:1000, Santa Cruz Biotechnology) at room temperature. After extensive washing with PBS, a secondary antibody (AlexaFluor 488-conjugated goat anti-rabbit; 1:1000, InVitrogen) was added for 1 h at room temperature. The cells were then washed extensively with PBS and mounted using Mowiol mounting medium (Sigma Aldrich). Cells were analyzed using a fluorescence microscope coupled to a digital camera (Leica Imaging Systems, Leica DMI4000, photocamera Leica DCF480) with "Leica Application Suit" Software, version 2.8.1 (Leica Microsystems GMS Gmblt, Germany.).

2.7.2 Evaluation of TAT-parkin cellular translocation in CHO cells.

Cells were seeded at a density of 1 x 104 cells/cm2 on 12 mm diameter glass coverslips (flamed with absolute ethanol) in 24-well plates, and transfected with pmito-GFP, using Lipofectamine Plus[TM] Reagent (InVitrogen). The CHO cells were plated the day before transfection so as to obtain 70-80% confluency the following day. pMito-GFP (0,4 μg) was mixed with the liposomes (4 μL) in serum-free Optimem and incubated for 15-30 min at room temperature to allow complex formation. The mixture was added to the cells and incubated for 4 h at 37°C. At the end of the incubation, medium was exchanged with fresh culture medium and incubated for 48 h. Cells were then treated with or without (control cells) 100 nM TAT-parkin for 4 h. After this time cells were washed with PBS and fixed in 2% paraformaldehyde. Cells treated with TAT-parkin only were permeabilized using 0.1% Triton X-100 in PBS. All cells were then incubated for 1 h at 37 °C with a primary antibody (mouse monoclonal anti his(6)tag antibody) diluted 1:300 in 1% BSA. Cells were washed twice with PBS, followed by incubation for 1 h at 37°C with a monoclonal anti-mouse rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:100 in 1% BSA. After extensive washing with PBS, cells were mounted with Mowiol (EMD Bioscience, La Jolla, San Diego, CA, USA). Immunostained cells were visualized with an inverted fluorescence microscope (Axiovert 100, Zeiss), equipped with a computer-assisted CCD camera (AxioCam, Zeiss).

2.7.3 Evaluation of expression of human parkin

Differentiated PC12 cells (un-transfected, transfected with pcDNA3.1-wild type parkin, and pcDNA3.1-R42P mutant parkin) were seeded at a density of 1 X 10^{4} cells/cm2 on poly-D-lysine -coated plastic chamber slides, fixed with 4% paraformaldehyde for 15 min and permeabilized using 0.5% Triton X-100, 0.2% fetal calf serum in PBS for 10 min. After washing with PBS (2-3 times for 5 min each) non-specific binding sites were blocked with 5% horse serum in PBS. Cells were then incubated overnight at 4°C with a primary anti-parkin rabbit polyclonal

antibody (Cell Signaling Technology) diluted 1:100 in 1% horse serum/PBS, followed by a FITC-conjugated secondary antibody (AlexaFluor 488-conjugated goat anti-rabbit; Jackson Immuno Laboratories, West Grove-PA, USA) diluted 1:500 in 1% horse serum/PBS for 1 h. Cells were washed with PBS for 5 min and labelled with 3 μg/ml 4',6-diamidino-2-phenylindole (Boehringer, Mannheim, Germany), to identify nuclei, diluted 1:500 in PBS for 5 min. Cells were washed twice with PBS and analyzed with a fluorescence microscope coupled to a digital camera (Olympus Corporation, Tokyo, Japan).

2.7.4 Evaluation of protein ubiquitination

PC12 cells (un-transfected, transfected with pcDNA3.1-wild type parkin, and pcDNA3.1-R42P mutant parkin) were labeled using the primary anti-ubiquitin rabbit polyclonal antibody described earlier, diluted 1:200 in 1% horse serum/PBS, followed by a FITC-conjugated secondary antibody (AlexaFluor 488-conjugated goat anti-rabbit) diluted 1:500 in 1% horse serum/PBS. Cells were analyzed with a fluorescence microscope coupled to a digital camera.

2.7.5 Evaluation of autophagy pathway activation

PC12 cells (un-transfected, transfected with pcDNA3.1-wild type parkin, and pcDNA3.1-R42P mutant parkin) were labeled using a primary anti-LC3B rabbit polyclonal antibody (Abcam) diluted 1:200 in 1% horse serum/PBS, followed by a FITC-conjugated secondary antibody (AlexaFluor 488-conjugated goat anti-rabbit) diluted 1:500 in 1% horse serum/PBS. Cells were then analyzed with a fluorescence microscope coupled to a digital camera.

Primary antibodies

The following primary antibodies were used for immunocytochemistry:

- anti-polyHistidine mouse monoclonal antibody (1:300) (Sigma Aldrich);

- anti-parkin rabbit polyclonal antibody (1:100) (Cell Signaling Technology);

- anti-ubiqitin rabbit polyclonal antibody (1:200) (DakoCytomation);

- anti-LC3B rabbit polyclonal antibody (1:200) (Abcam).

2.8 CELL VIABILITY ASSAYS

2.8.1 3-(4-5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H- tetrazolium bromide (MTT) assay

Cell viability was measured 2 h after initiating treatment of differentiated PC12 cells using 3-(4-5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H- tetrazolium bromide (MTT) assay, as described by Denizot and Lang (1986). MTT is a tetrazolium salt upon reaction with mitochondrial dehydrogenases in living cells is reduced to a blue formazan. The formazan product is dissolved and the resulting color spectrophotometrically evaluated. Once treatments were completed, culture medium was replaced with fresh medium containing MTT (0.176 mg/ml in PBS). After 2 h of incubation at 37°C, the MTT-containing medium was replaced with 300 μL of DMSO. The absorbance was measured with an automatic microplate reader (Titertek ELISA multiscan) at A_{570} and A_{630}. Results are expressed as percentage of control.

2.8.2 CellTiter 96® Aqueous One Solution Cell Proliferation Assay

Cell viability was measured 2, 4, 6, 12 and 24 h after initiating treatment of differentiated PC12 cells using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay. The CellTiter 96® Aqueous One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl-)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] inner salt (MTS) and an electron coupling reagent (phenazine ethosulfate-PES). Cell viability is assessed by measuring the level of cellular conversion of the reagent into a water-soluble formazan product. The assay is performed by adding the reagent (10% v/v) directly to the culture medium, incubating for 1-4 h and then recording formazan absorbance at 490 nm that is directly proportional to the number of living cells.

2.9 CELL TREATMENTS

2.9.1 6-OHDA treatment and evaluation of toxicity

A) 6-OHDA solutions were prepared in 0.9% saline/0.1% ascorbic acid, fresh before use and

kept on ice in the dark. PC12 cells were plated (1×10^4 cells/cm^2) in a poly-D-lysine-

coated 24-well plate, and differentiated with 50 ng/mL rhNGF for 5 days. The cells were

then incubated for 2 h with 6-OHDA diluted to give final concentrations of 5, 10, 25, 50,

100, 200, and 300 μM. The medium was changed immediately after 6-OHDA treatment

and the number of viable cells was determined by the MTT assay. Values are given as

means ± SEM of at least three experiments.

B) 6-OHDA solutions were prepared as described above. Un-transfected PC12 cells, PC12 cells

transfected with either pcDNA3.1-wild type parkin or pcDNA3.1-R42P mutant parkin were

plated in a poly-D-lysine-coated 24-well plate (1×10^4 cells/cm^2), and differentiated with

50 ng/mL rhNGF for 5 days. The cells were then incubated with 50 or 75 μM 6-OHDA for

2, 4, 6, 12 and 24 h. Culture medium was changed immediately after 6-OHDA treatment

and the number of viable cells was determined by CellTiter 96® Aqueous One Solution

Cell Proliferation Assay. Values are given as means ± SEM of at least three experiments.

2.9.2 Evaluation of intracellular reactive oxygen species

Intracellular ROS were evaluated using fluorescent probe 2',7'-dichlorofluorescein diacetate
(DCFH-DA). This molecule is cleaved intracellularly by non-specific esterases to 2',7'-
dichlorofluorescin which leads to the fluorescence compound 2',7'-dichlorofluorescein upon
oxidation by reactive oxygen species. The method follow that described by Wang and Joseph
(1999). Un-transfected PC12 cells, PC12 cells transfected with either pcDNA3.1- wild-type
parkin or pcDNA3.1-R42P mutant parkin were plated in a poly-D-lysine-coated 24-well plate at
1×10^4 cells/cm^2, and differentiated with 50 ng/mL rhNGF for 5 days. Then cells were pre-

incubated with 100 μM DCFH-DA (Sigma Aldrich) for 30 min at 37°C in the dark. The cells were then twice PBS and treated with 50 or 75 μM 6-OHDA for 2, 4, 6, 12 and 24 h. The fluorescence was evaluated in a microplate fluorimeter FLX-800 at an excitation wavelength of 488 nm and an emission wavelength of 543 nm. The increase in fluorescence for each treatment was calculated as the relative fluorescence of each treatment compared with un-treated cells and normalized to the number of cells as determined by the MTT assay (Goldshmit et al., 2001).

2.9.3 TAT-parkin treatment and evaluation of TAT-parkin toxicity

rhNGF-differentiated PC12 cells were plated in a poly-D-lysine-coated 24-well plate at 1×10^4 cells/ cm^2, and incubated with TAT-parkin solution diluted to give final concentrations of 100-500 nM. After incubation for a further 24, 48, and 72 h, viable cell number was determined by MTT assay. Values are given as means ± SEM of at least three experiments.

2.9.4 Oxidative stress and TAT-fused protein treatments: evaluation of TAT-parkin protective effect against 6-OHDA induced cytotoxicity

rhNGF-differentiated PC12 cells were plated in a poly-D-lysine-coated 24-well plate at 1×10^4 cells/ cm^2, pre-incubated with 100 nM TAT-parkin for 24 h, and then treated with 50 μM 6-OHDA for 2 h. Upon removel of the 6-OHDA solution, cells were incubated in fresh differentiation medium ± 100 nM TAT-parkin for different periods of time. Cell viability was determined by MTT assay. Values are given as means ± SEM of at least three experiments.

2.9.5 Evaluation of parkin over-expression against treatment with proteasome/ autophagy drugs

N-(benzylloxycarbonyl)-leucinyl-leucinyl-leucinal[Z-Leu-Leu-Leu-al] (MG132) (Sigma Aldrich), a specific proteasomal inhibitor, was dissolved in DMSO and used at a final concentration of 2.5 μM. 3-methyl-adenine (3-MA) (Sigma Alderich), a specific inhibitor of autophagy, was made up in water and used at a final concentration of 10 mM.

Un-transfected PC12 cells, and PC12 cells transfected with either pcDNA3.1- wild-type parkin or pcDNA3.1-R42P mutant parkin were plated in a poly-D-lysine-coated 24-well plate at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/mL rhNGF for 5 days. Then cells were then treated with 2.5 μM MG132 or/and 10 mM 3-MA for a further 16 h. Alternatively, other group of cells were treated with 50 μM 6-OHDA + 2.5 μM MG132 or 50 μM 6-OHDA + 10m M 3-MA. The number of viable cells was determined by CellTiter 96® Aqueous One Solution Cell Proliferation Assay. Values are given as means ± SEM of at least three experiments.

2.10 STATISTICAL ANALYSIS

At least three replicates per group were used in each experiment. Results shown are representative of three independent experiments. Significance was tested using one-way ANOVA or two-way ANOVA followed by Bonferroni's Multiple Comparison *post-hoc* test, or Dunnett's or Tukey's *post-hoc* test. Differences were considered significant at $p < 0.05$.

CHAPTER 3

REULTS

3.1 Expression of TAT-parkin

The TAT fusion protein strategy is well suited for the intracellular translocation of proteins (Dietz and Bähr, 2007; Gump and Dowdy, 2007). To this end a cell-permeable parkin fusion protein was produced by constructing an expression vector bearing the TAT-parkin gene in place of the TAT-cyano fluorescent protein (CFP) sequence (Figure 3.1). This plasmid encoded the parkin protein whose N-terminal portion was fused to a sequence of six histidine residues, which permitted protein purification by immobilized-metal ion affinity chromatography. A parkin plasmid lacking the TAT gene was constructed as control. Figure 3.2 shows the expression of His6-parkin and TAT-parkin on Coomassie blue-stained SDS-PAGE gels before (left panel) and after (right panel) purification. The His6-parkin protein was evident as a band at 52 kDa, while the TAT-parkin fusion protein presented as a slightly larger, 54 kDa band accounting for the 11 amino acids of the TAT sequence.
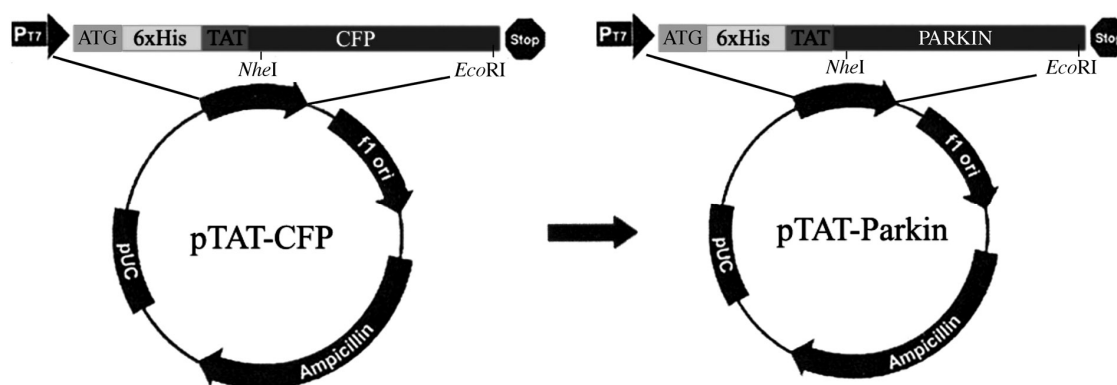
MRGSHHHHHHGMARGYGRKKRRQRRRGARLMIVFVRFNSSHGFPVEVDSDTSIFQLKEVVAKRQGVP
ADQLRVIFAGKELRNDWTVQNCDLDQQSIVHIVQRPWRKGQEMNATGGDDPRNAAGGCEREPQSLTR
VDLSSSVLPGDSVGLAVILHTDSRKDSPPAGSPAGRSIYNSFYVYCKGPCQRVQPGKLRVQCSTCRQATL
TLTQGPSCWDDVLIPNRMSGECQSPHCPGTSAEFFFKCGAHPTSDKETPVALHLIATNSRNITCITCTDVR
SPVLVFQCNSRHVICLDCFHLYCVTRLNDRQFVHDPQLGYSLPCVAGCPNSLIKELHHFRILGEEQYNRY
QQYGAEECVLQMGGVLCPRPGCGAGLLPEPDQRKVTCEGGNGLGCGFAFCRECKEAYHEGECSAVFE
ASGTTTQAYRVDERAAEQARWEAASKETIKKTTKPCPRCHVPVEKNGGCMHMKCPQPQCRLEWCWN
CGCEWNRVCMGDHWFDV

Figure 3.1: Schematic organization of the TAT-parkin expression vector. The amino acid sequence
of the fusion protein is shown in the lower portion of the panel. HHHHHH., six histidine residues.
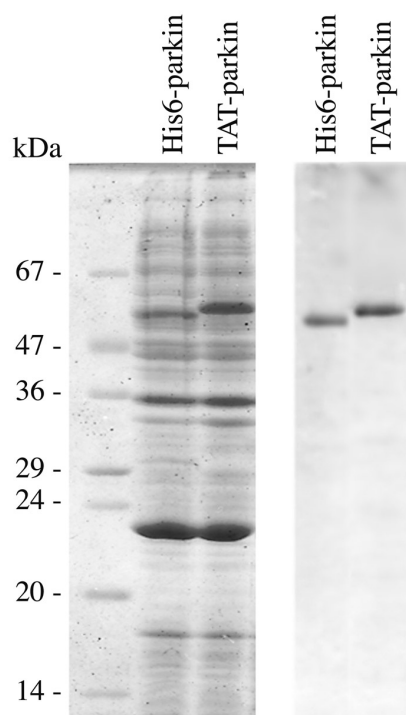YGRKKRRQRRR, 11 amino acids of the TAT sequence. See text for further details.

**Figure 3.2: SDS-PAGE of His6-parkin and TAT-parkin expressed in BL21(DE3)pLysS before
(left panel) and after (right panel) purification.** The gel was stained with Coomassie brillant blue.
Molecular weight markers are indicated on the left.

3.2 Transduction of PC12 and CHO cells with TAT-parkin

To examine the stability of transduced TAT-parkin, PC12 and CHO cell lysates were prepared after 2, 4, 8, 24, and 48 h incubation with 100 nM TAT-parkin. Western blot analysis using an anti-His tag antibody showed that TAT-parkin quickly transduced both cell types and remained inside for: at least 48 h (the longest time examined), in naïve PC12 cells (Figure 3.3(A)); 24 h, in rhNGF-differentiated PC12 cells (Figure 3.3(B)) and CHO cells (Figure 3.3(C)). The efficient transduction of TAT-parkin in rhNGF-differentiated PC12 cells was confirmed by immunocytochemistry (Figure 3.4). The confocal microscopic image shown demonstrates that TAT-parkin readily entered rhNGF-differentiated PC12 cells. However, because of the relative difficulty in observing a detailed intracellular localization of the fusion protein in PC12 cells due to their size, the assay was repeated utilizing CHO cells. The latter cells were transfected with pmito-GFP (to selectively label mitochondria) and then treated without or with 100 nM TAT-parkin (Figure 3.5). The images show that TAT-parkin diffuses inside CHO cells at the nuclear, cytoplasmic (Figure 3.5, panel B) and mitochondrial (Figure 3.5, panel C) levels.
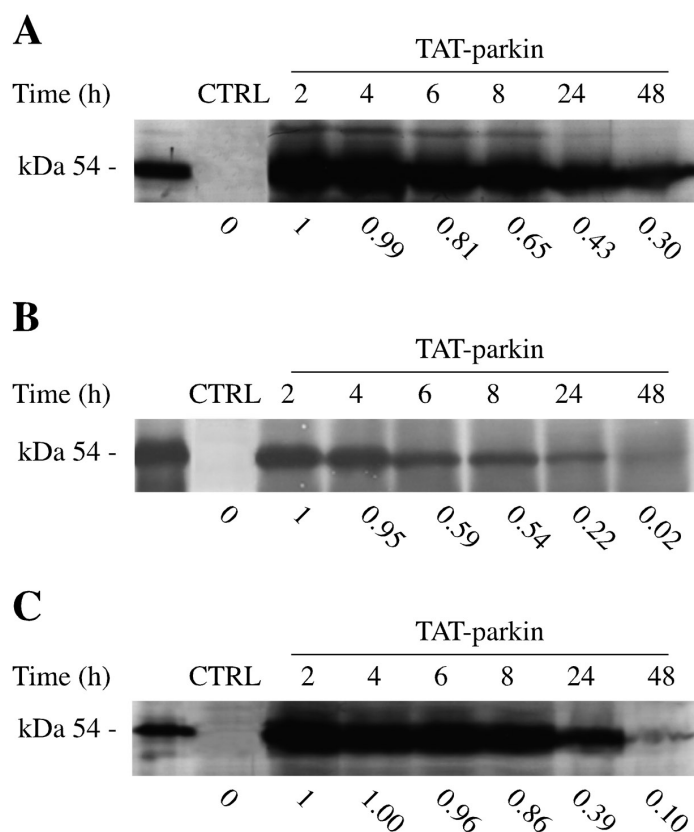
A

B

C

Figure 3.3 (A) TAT-parkin translocation in naïve PC12 cells. Undifferentiated PC12 cells were seeded into poly-D-lysine-coated 24-well dishes, at 2 x 10^4 cells/cm^2, then incubated without (CTRL, control cells) or with 100 nM TAT-parkin for the times indicated. Cell lysates were then prepared and subjected to Western blot analysis with a mouse monoclonal anti-his(6)tag antibody. Recombinant TAT-parkin was loaded in the far left lane (molecular weight 54 kDa). Note that parkin immunoreactivity is detectable up to at least 48 h (the longest time examined). **(B) TAT-parkin translocation in differentiated PC12 cells.** PC12 cells were seeded into poly-D-lysine-coated 24-well dishes, at 1 x 10^4 cells/cm^2, with 50 ng/ml rhNGF for 5 days, and then incubated without (CTRL, control cells) or with 100 nM of TAT-parkin for the times indicated. Cell lysates were then prepared and subjected to Western blot analysis with a mouse monoclonal anti-his(6)tag antibody. Recombinant TAT-parkin was loaded in the far left lane. Note that parkin immunoreactivity is detectable up to 24 h. **(C) TAT-parkin translocation in CHO cells**. CHO cells were seeded into 24-well dishes, at 1 x 10^4 cells/cm^2, and incubated without (CTRL, control cells) or with 100 nM of TAT-parkin for the times indicated. Cell lysates were then prepared and subjected to Western blot analysis with a mouse monoclonal anti-his(6)tag antibody. Recombinant TAT-parkin was loaded in the far left lane. Note that parkin immunoreactivity is detectable up to 24 h. Numbers under each lane indicate relative band intensity with respect to alpha-tubulin (not shown for purposes of simplification).
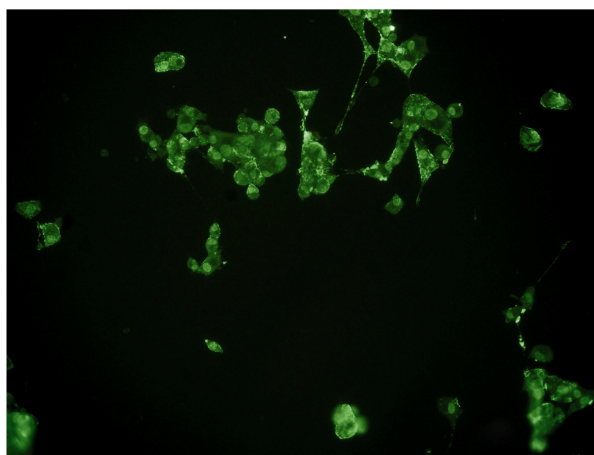
Figure 3.4. Fluorescence microscopic image of rhNGF-differentiated PC12 cells. PC12 cells were cultured for 5 days in 24-well plates in *differentiation medium* and then incubated with 100 nM TAT-parkin for 24 h. Cells were fixed with 4% PFA and processed for parkin immunocytochemistry as described in Materials and Methods. The image clearly shows entry of TAT-parkin into the cells. Cells not treated with TAT-parkin, or TAT-parkin-treated cells in which primary antibody was omitted, displayed no immunoreactivity above background (not shown).
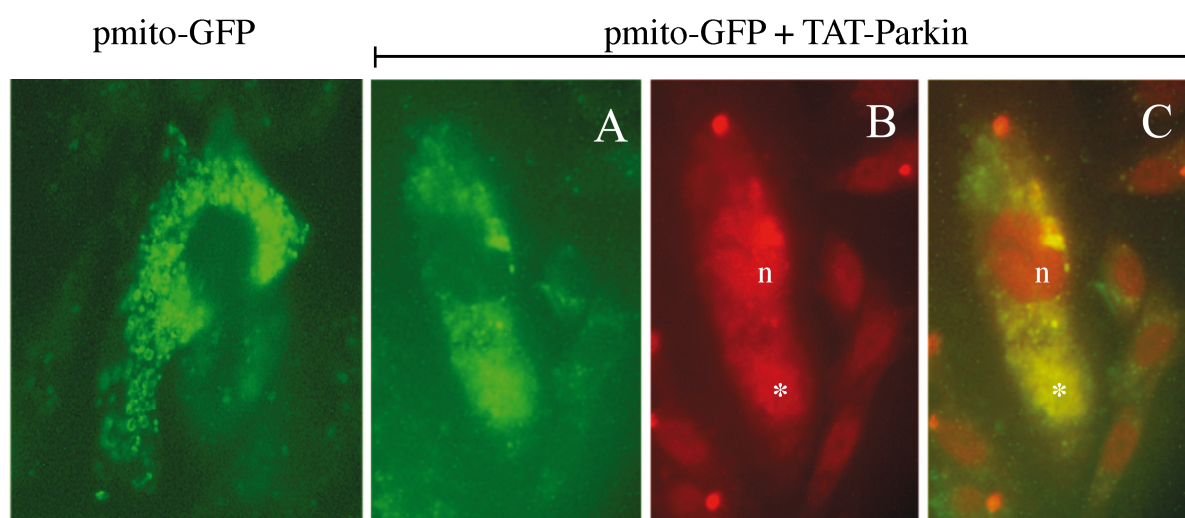

Figure 3.5. Intracellular localization of TAT-parkin in transduced CHO cells. CHO cells were cultured in 24-well plates and transfected with pmito-green fluorescent protein (GFP), then incubated with 100 nM TAT-parkin for 4 h. Cells were fixed with 4% PFA and processed for parkin immunocytochemistry as described in Materials and Methods. Panel A) shows transduced pmito-GFP within CHO cells. Panel B) shows transduced fusion protein in the nucleus and cytoplasm. Panel C) merge of panels A and B, where is clearly visible the trasduced of fusion protein in mitochondria. n, nucleus; *, cytoplasm.

3.3 *In vitro* ubiquitination assay

Parkin consists of two functionally distinct domains: the C-terminal RING-finger box, which recruits a specific ubiquitin-conjugating enzyme (E2, UbcH7), and the N-terminal UbL domain required for recognition of a target substrate protein, designated "X", for ubiquitination before proteasomal degradation (Shimura et al., 2000) (Figure 3.6, left panel). Rankin et al. (2001) reported that glutathione S-transferase-tagged parkin purified from *E. coli* possesses E3 activity. In order to demonstrate directly that TAT-parkin also has E3 activity, we devised an *in vitro* assay for ubiquitination utilizing lysates from CHO cells treated or not (control cells) with TAT-parkin (100 nM) purified from *E. coli*. Cell lysates were incubated with E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme UbcH7, his6-ubiquitin and ATP, in the presence of recombinant DJ^{L166P}, a mutant DJ-1 purified from *E. coli* that is incorrectly folded and highly susceptible to ubiquitination, thus a presumptive substrate "X". Cell lysates were then subjected to immunoblotting with a monoclonal anti-polyhistidine antibody. As Figure 3.6 (right panel, lane *b*) shows, transduction of TAT-parkin into CHO cells results in activation of the fusion protein (presumably being folded by intracellular chaperones), leading to the appearance of ubiquitinated higher molecular weight species: DJ^{L166P} undergoes ubiquitination as evidenced by an increase in its molecular weight from 25 to 33 kDa for the first ubiquitination (accounted for by the molecular weight of about 8 kDa for ubiquitin), from 33 to 41 kDa for the second ubiquitination and so on, until the nth ubiquitination. Yet higher molecular laddering was also observed, indicating the presence of polyubiquitinated species. In contrast, untreated (control) CHO cells, incubated under the same conditions, did not show evidence of polyubiquitination (Figure 3.6 right panel, lane *a*). Taken together, these results confirm that TAT-parkin acts as an E3 ubiquitin ligase.
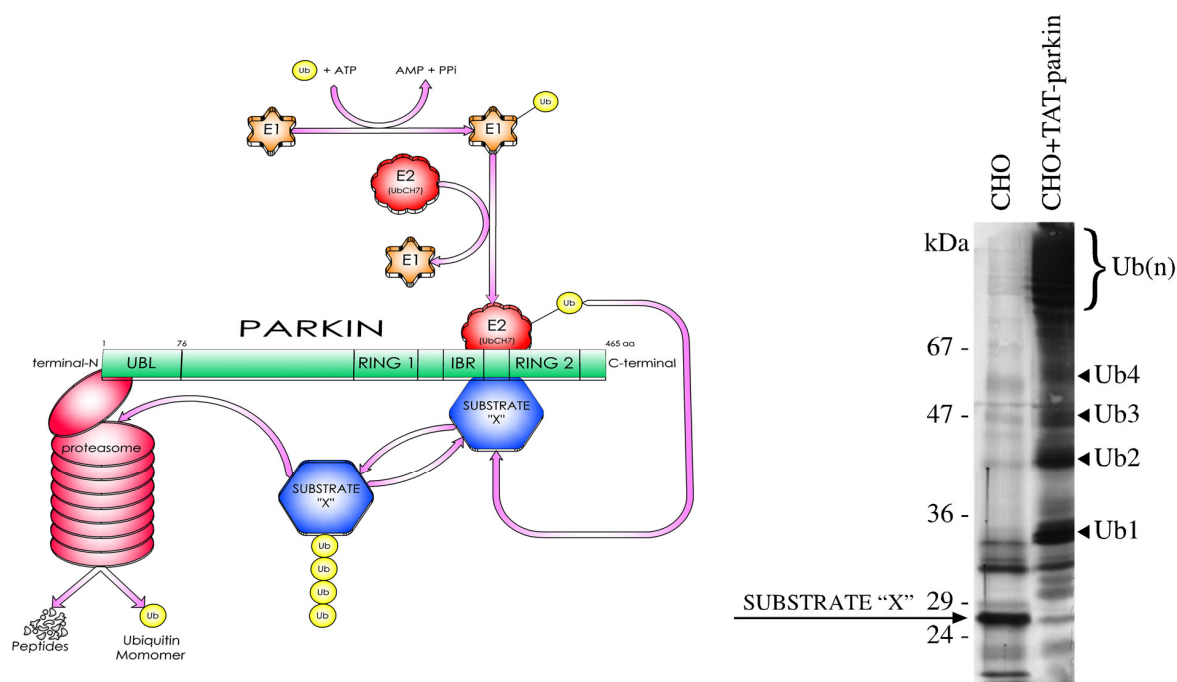
Figure 3.6. *In vitro* **TAT-parkin ubiquitination assay.** *Left panel*: schematic of the parkin-directed ubiquitination pathway. Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme (Ubch7); UBL, ubiquitin-like domain; RING1-IBR-RING2, RING-finger box; substrate 'X', presumptive substrate protein. *Right panel:* CHO cells were treated or not (lane *a*) with 100 nM TAT-parkin, lysated, then added to the ubiquitination assay buffer (50 mM Tris-HCl, 2 mM dithiothreitol, 5 mM $MgCl_2$ and 4 mM ATP, pH 8.8, 1.6 μg/ml E1, 20 μg ubcH7 enzyme, 50 μg/ml his6-ubiquitin) in the presence of the DJ-1 mutant DJ^{L166P} used as a target substrate (substrate "X") (arrow). High molecular weight ubiquitinated signals were detected by immunoblotting with anti-poly-histidine-tag antibody and are designated Ub1, Ub2, Ub3, Ub4 (arrowheads) and (Ub)n (parenthesis) on the right. Molecular weight markers are indicated at left.

3.4 6-OHDA toxicity in PC12 cells

6-OHDA is believed to cross cell membranes via DA uptake transporters and to inhibit mitochondrial respiration with subsequent generation of intracellular reactive oxygen species (Blum et al., 2000). In order to establish the optimal concentration for 6-OHDA cytotoxicity to rhNGF-differentiated PC12 cells, a concentration-response study was first carried out. High concentrations ($\geq$100 μM) of 6-OHDA induced approximately 80% cell death after 2 h, while 50 μM 6-OHDA induced about 45% cell death (Figure 3.7). The latter concentration of 6-OHDA was used in all subsequent neuroprotection experiments. rhNGF-differentiated PC12 cells were used, as these cells take on characteristics of catecholaminergic sympathetic neurons and represent a useful *in vitro* system for neurobiological studies (Greene and Tischler, 1976). Interestingly, naïve PC12 cells were resistant to 6-OHDA toxicity (data not shown).
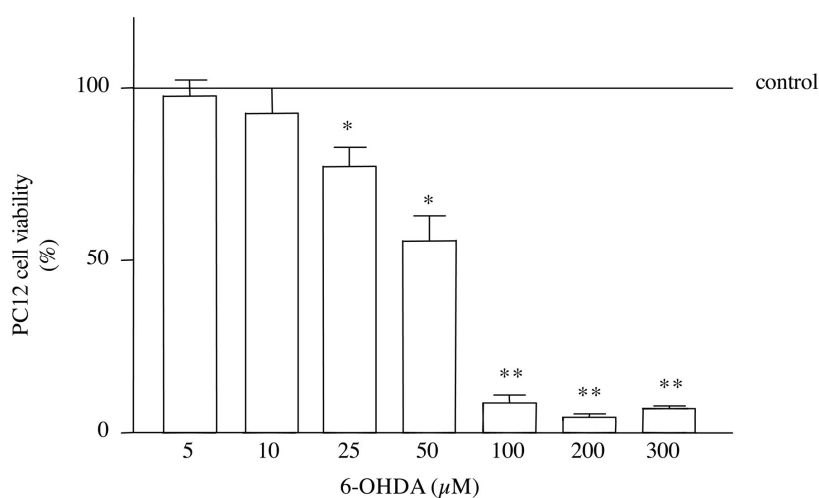

Figure 3.7: Dose-response curve of 6-hydroxydopamine (6-OHDA) toxicity in differentiated PC12 cells. PC12 cells were seeded into poly-D-lysine-coated 24-well dishes, at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. Then cells were incubated for 2 h with increasing concentrations of 6-OHDA (5-300μM), after which time cell viability was determined by MTT assay. Data are expressed as a percentage of vehicle-treated control cells, and are the mean $\pm$ S.D. of 32 determinations from 4 independent experiments run in duplicate. Statistical significance was evaluated by one-way ANOVA followed by Bonferroni *post-hoc* Multiple Comparison Test. *p <0.01 and **p <0.001 *vs* control.

3.5 Effect of TAT-parkin on viability of differentiated PC12 cells

Treatment of rhNGF-differentiated PC12 cells with increasing concentrations of TAT-parkin for 24, 48, and 72 h caused significant reductions in cell viability only at higher concentrations (300 to 500 nM) at all incubation times (Figure 3.8), reaching maximum effect at the longest incubation time (72 h). While 150 nM TAT-parkin was cytotoxic only after 72 h, the lowest concentration of TAT-parkin (100 nM) was not cytotoxic up to 72h. The latter concentration of TAT-parkin was used in all subsequent neuroprotection experiments.
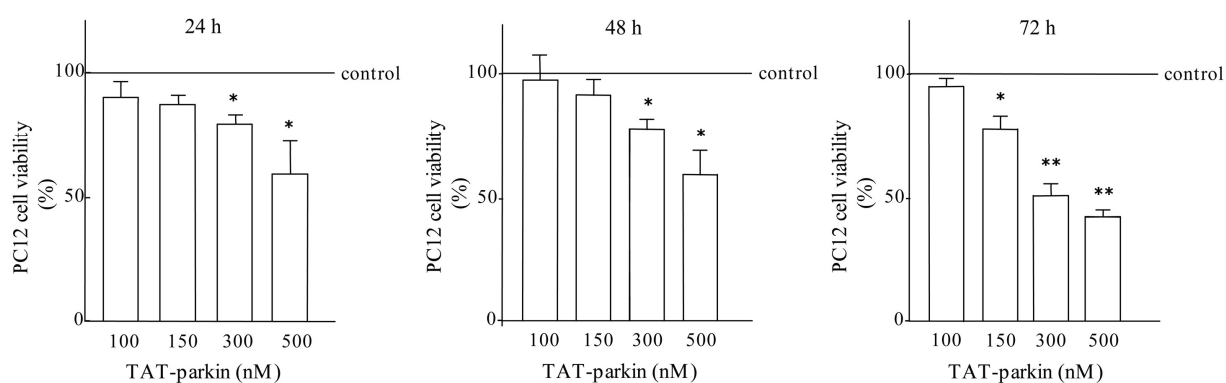

Figure 3.8: Dose-response curve of TAT-parkin toxicity in differentiated PC12 cells. PC12 cells were seeded into poly-D-lysine-coated 24-well dishes, at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. Cells were then incubated with increasing concentrations of TAT-parkin (100-500 nM) for 24, 48, and 72 h, after which times cell viability was evaluated by MTT assay. Data are expressed as a percentage of vehicle-treated control cells, and are the mean ± S.D. of 80 determinations from 10 independent experiments run in duplicate. Statistical significance was evaluated by one-way ANOVA followed by Bonferroni *post-hoc* Multiple Comparison Test. *p < 0.05 and **p < 0.01 *vs* control.

3.6 TAT-parkin protective effect against 6-OHDA-induced cytotoxicity in differentiated PC12 cells

The potential protective effect of TAT-parkin against cytotoxicity induced by 6-OHDA was evaluated in rhNGF-differentiated PC12 cells (Figure 3.9). Cells were pre-incubated for 24 h with 100 nM TAT-parkin followed by a 2 h exposure to 50 μM 6-OHDA in the absence (Fig.9, open bars) or continued presence (Figure 3.9, shaded and solid bars) of 100 nM TAT-parkin. At the end of the treatment, PC12 cells were placed in fresh medium without (Figure 3.9, open and shaded bars) or with (Figure 3.9, solid bars) 100 nM TAT-parkin, and cell viability was determined by MTT immediately (time 0), and after a further incubation for 2, 4, or 6 h. In PC12 cells treated with only 6-OHDA viability was reduced by approximately 40% already at time 0, and increased with post-incubation time reaching a maximum of 70% at 6 h (Figure 3.9, open bars). This time-dependent increase in 6-OHDA cytotoxicity could, conceivably, be due to accumulation of intracellular ROS resulting from neurotoxin-induced mitochondrial complex I dysfunction, which was not prevented by pre-incubation with TAT-parkin. The ability of TAT-parkin to protect PC12 cells against 6-OHDA cytotoxicity required that the fusion protein be present both during and continually following toxin exposure; delaying readdition by even 2 h renderd TAT-parkin ineffective (Figure 3.9, shaded bars). However, when post-incubation was performed in the presence of TAT-parkin, the protective action was maintained at all post-incubation times (Figure 3.9, solid bars).
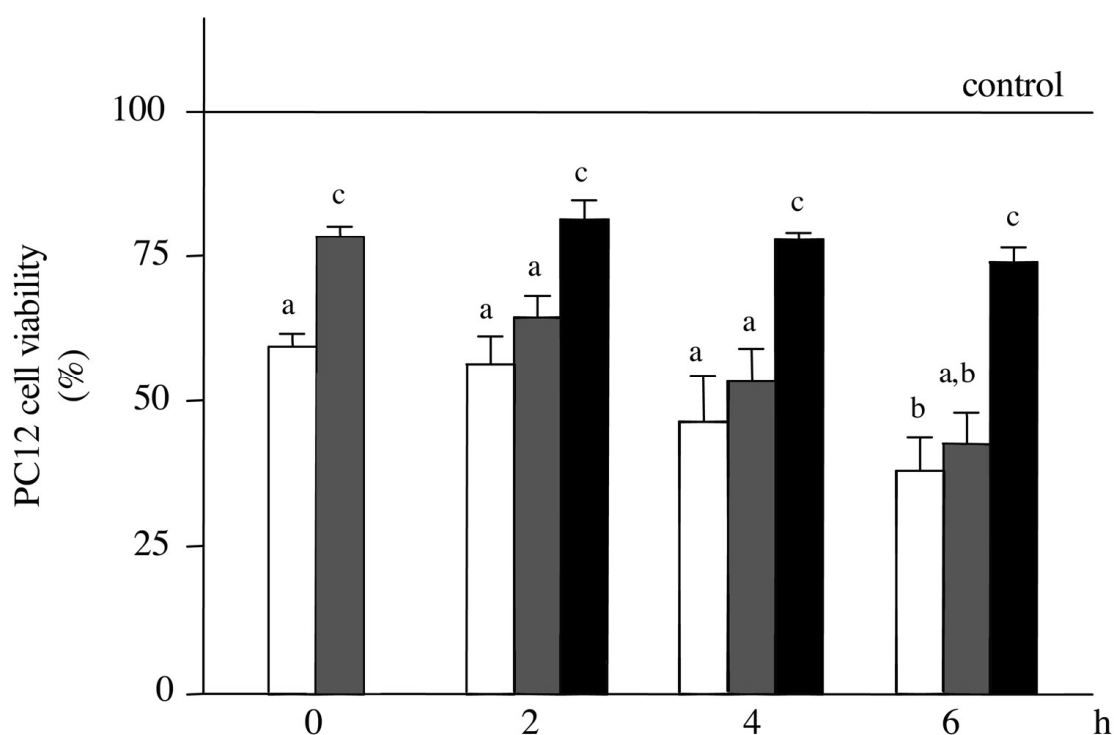
Figure 3.9: TAT-parkin protection of differentiated PC12 cells against 6-OHDA cytotoxicity.
PC12 cells were seeded into poly-D-lysine-coaed 24-well dishes, at 1 x 10^4 cells/cm2, and differentiated
with 50 ng/ml rhNGF for 5 days. rhNGF-differentiated cells were pre-incubated with 100 nM TAT-
parkin for 24 h before treatment with 50 μM 6-OHDA for 2 h in the absence (open bars) or presence
(shaded and solid bars) of 100 nM TAT-parkin. Cells were then reincubated in fresh differentiation
medium without (open and shaded bars) or within (solid bars) 100 nM TAT-parkin for 2, 4, and 6 h.
Cell viability was determined by MTT assay. Data are expressed as a percentage of vehicle-treated
control cells, and are the mean ± S.D. of 32 determinations from 4 independent experiments performed
in duplicate. Different superscript letters indicate statistically significant differences (p < 0.01, one-way
ANOVA followed by Bonferroni's *post-hoc* Multiple Comparison Test).

3.7 Generation of PC12 stable clones expressing human parkin wild-type and the pathogenic parkin mutant R42P (parkin^{R42P})

In order to verify the extent to which parkin protects PC12 cells from oxidative damage, we over-expressed human parkin protein by stable transfection of cells. We generated PC12 stable clones expressing wild-type (WT) human parkin and the pathogenic parkin mutant R42P (parkin^{R42P}). The parkin PD-linked mutation R42P is localized in exon 2 and resultes in a conservative amino acid substitution of arginine for proline at position 42 (Terreni et al., 2001). The mutation occurs in the UBL domain at the N-terminus of the protein. It is likely that the UBL domain tethers parkin close to the proteasome, directing poly-ubiquitinated proteins toward their proteolytic end. The recessive parkin mutation R42P disrupts this interaction (Sakata et al., 2003), resulting in impaired proteasomal binding of parkin, which may be a contributing factor in AR-JP pathogenesis.

Following transfection, expansion and screening 4 clones for WT parkin were isolated, together with 5 clones for parkin mutant R42P. These clones were tested for expression of human parkin gene by RT-PCR and Western blotting.

3.7.1 RT-PCR analysis

RT-PCR analysis showed the presence of mRNA-positive clones among the stably transfected PC12 cells, compared with control (un-transfected) PC12 cells. We selected those clones displaying a more intense band: clone 1 for cells transfected with pcDNA3.1-R42P mutant parkin and clone 9 for pcDNA3.1- WT parkin (Figure 3.10).

3.7.2 Western blotting analysis

The increased expression of the respective parkin mRNA was confirmed at the protein level by Western blotting. Cells lysates from clone 1 and clone 9 were subjected to Western blot analysis using an anti-parkin antibody. Both clones exhibited a significant, 5 fold higher level of parkin

protein expression than un-transfected cells, as confirmed by densitometric analysis (Figure 3.11(A,B)). Interesting, PC12 cells stably expressing human WT parkin and parkin[R42P], but not un-transfected cells, displayed 52- and 38-kDa anti-parkin-immunoreactive bands (Figure 3.11(A)). In fact, and in accord with Kahns et al., 2002, parkin is cleaved during apoptosis by caspase-mediated proteolysis, generating a 38-kDa C-terminal fragment and a 12-kDa N-terminal fragment, with cleavage occurring at Asp-126/Ser-127. While this 38-kDa fragment may be a product cellular stress, one cannot rule out that the process of cellular transfection per se contributed to generation of this fragment.

3.7.3 Parkin expression was also assessed by immunocytochemical means. As shown in Figure 3.11(C), fluorescence staining was more intense signal in the two PC12 cell clones, while in un-transfected PC12 cells only a faint signal was detectable. Moreover, parkin was predominantly located in the cytosol, in accord with Shimura et al., 1999; Darios et al., 2003; Kuroda et al., 2006.
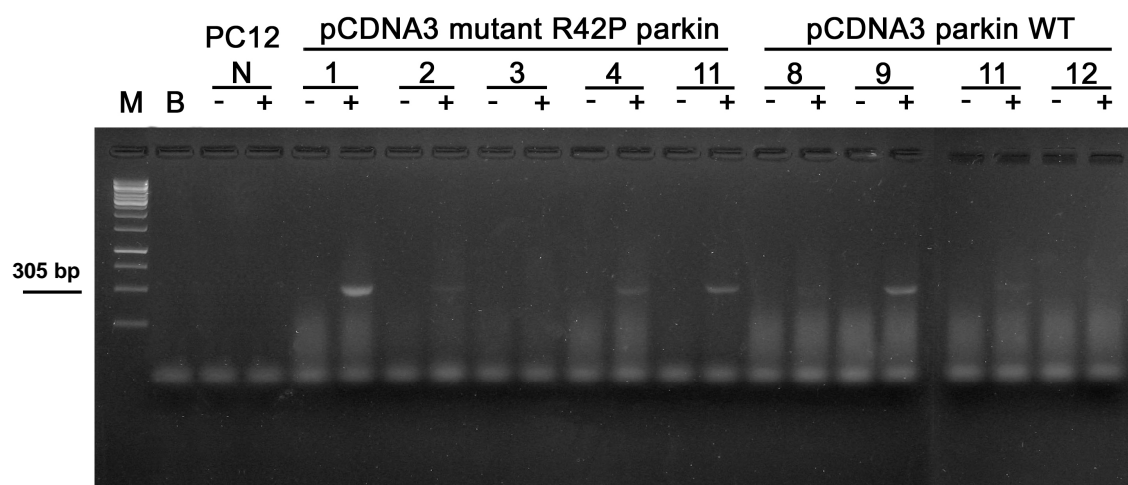
Figure 3.10: Parkin (WT) and Parkin[R42P] clone screening by RT-PCR analysis. Digital image of an agarose gel assessing human parkin mRNA positive clones. PC12 cells were transfected with plasmid pcDNA3.1- carrying either human-WT or -R42P mutant parkin full-length cDNAs as described in Materials and Methods. Following selection total mRNA was extracted, reverse-transcribed and amplified to detect human-parkin (305 bp band). M: marker. B: No template control (blank). +: RT positive. -: RT negative. PC12 N: PC12 un-transfected cells.

(A)
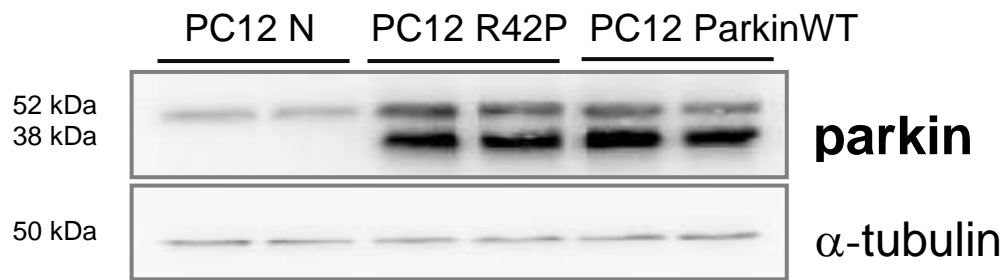
(B)
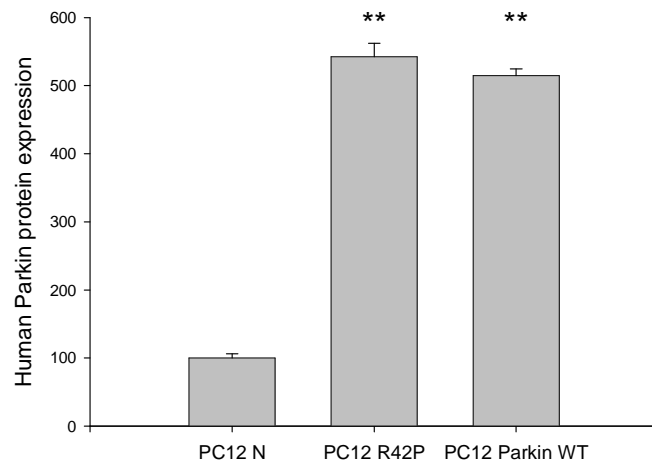
(C)

Figure 3.11: Parkin WT and Parkin^{R42P} clone screening by Western Blot analysis. PC12 clones were subjected to Western blot analysis to quantify parkin protein expression. PC12 cells were transfected with plasmid pcDNA3.1- carrying either human-WT or -R42P mutant parkin full-length cDNAs as described in Materials and Methods. Following clone selection un-transfected PC12 cells and the PC12 stable cell clones were seeded in 24-well plates at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days, after which time cells w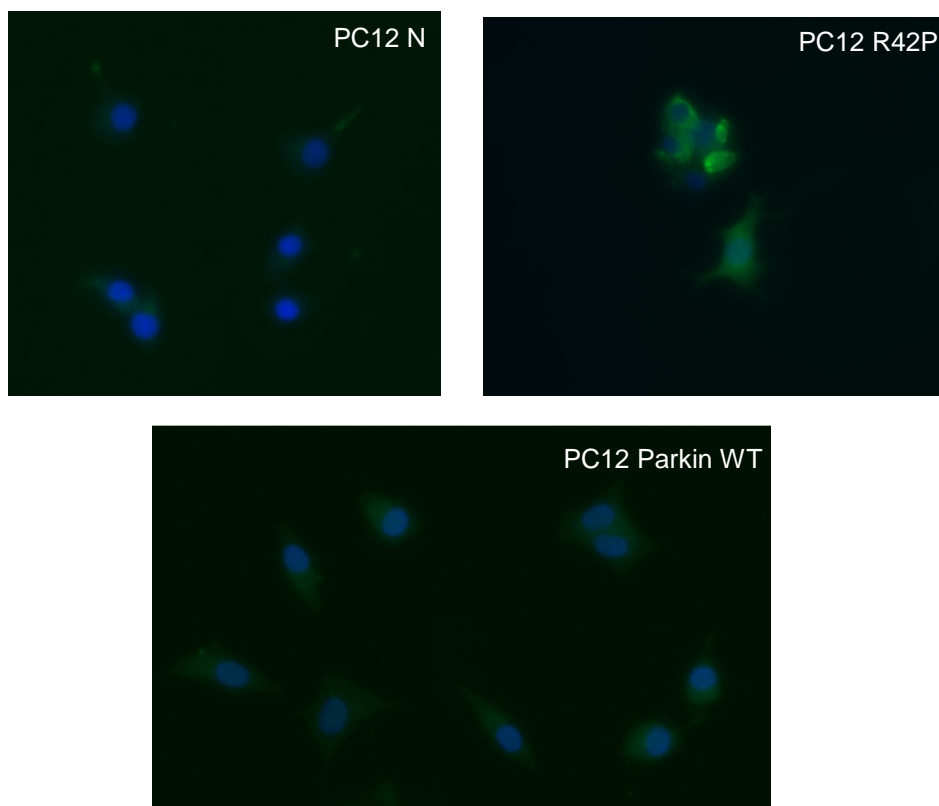ere harvested and lysates prepared. **(A)** Western blot showing parkin protein level in un-transfeced PC12 cells (PC12 N, control cells) and in PC12 expressing WT human parkin (clone 9) and human parkin^{R42P} (clone 1). All parkin stable clones show an higher level of human parkin, compared to control. α-Tubulin immunoreactivity was used to control for equal protein loading. **(B)** Bar graph showing the values normalized to α-tubulin and is representative of one of 3 independent experiments (n=3 for each group). Statistical significance was evaluated by one-way ANOVA followed by Tukey's *post-hoc* test. **$p<0.01$.

PC12 N: un-transfected PC12 cells. PC12 R42P: PC12 cells stably transfected with pcDNA3.1-R42P mutant parkin (clone 1). PC12 Parkin WT: PC12 cells stably transfected with pcDNA3.1-WT parkin (clone 9).

(C) Immunocytochemistry of human parkin expression (WT and mutant R42P) in stably transfected clones. Un-transfected PC12 cells and PC12 stable cell clones were seeded on poly-D-lysine-coated plastic chamber slides at 1 X 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. Then cells were fixed with 4% paraformaldehyde (PFA) and stained with a primary anti-parkin rabbit polyclonal antibody (1:100) as described in Materials and Methods. The photomicrographs in PC12 N (un-transfected PC12 cells) is indicative of basal (endogenous) parkin. The photomicrographs in panels PC12 R42P and PC12 Parkin WT show parkin immunoreactivity in PC12 cells stably transfected with pcDNA3.1-R42P mutant parkin (clone 1) and PC12 cells stably transfected with pcDNA3.1-WT parkin (clone 9) respectively. Note the greater intensity of immunostaining in these cells compared to control PC12 cells.

3.8 Effect of WT parkin and mutant R42P expression on cell viability after treatment with the dopaminergic neurotoxin 6-OHDA

In order to investigate the effect of parkin over-expression on PC12 cell survival under conditions of oxidative stress-induced damage by the dopaminergic neurotoxin 6-OHDA, rhNGF-differentiated PC12 cells, transfected with pcDNA3.1-R42P mutant parkin (clone 1) and with pcDNA3.1-WT parkin (clone 9) and rhNGF-differentiated PC12 un-transfected cells were treated with 50 and 75 μM 6-OHDA for different times. As Figure 3.12 shows, cell viability was reduced in all cells under all treatment condition. However, the PC12 parkin[R42P] expressing clone (cyano bar) and un-transfected PC12 cells (orange bar) showed heightened sensitivity compared with PC12 WT parkin expressing cells (blue bar). There was, in fact, a statistically significant reduction in cell viability already from 2 h for both un-transfected PC12 cells (orange bar) and for PC12 transfected with mutant R42P parkin (cyano bar). In contrast, PC12 transfected with WT parkin (blue bar) showed a reduction in cell viability which became statistically significant only from 12 h. In this situation, PC12 WT parkin expressing cells (blue bar) showed a cell viability about 30% higher compared with PC12 parkin[R42P] expressing cells (cyano bar) and control group (orange bar), for all time points, while no significant difference was detectable between PC12 parkin[R42P] expressing (cyano bar) and control cells (orange bar). Thus, WT parkin appears to protective, while the R42P substitution may adversely affect the parkin physiological function, including its protective activity.
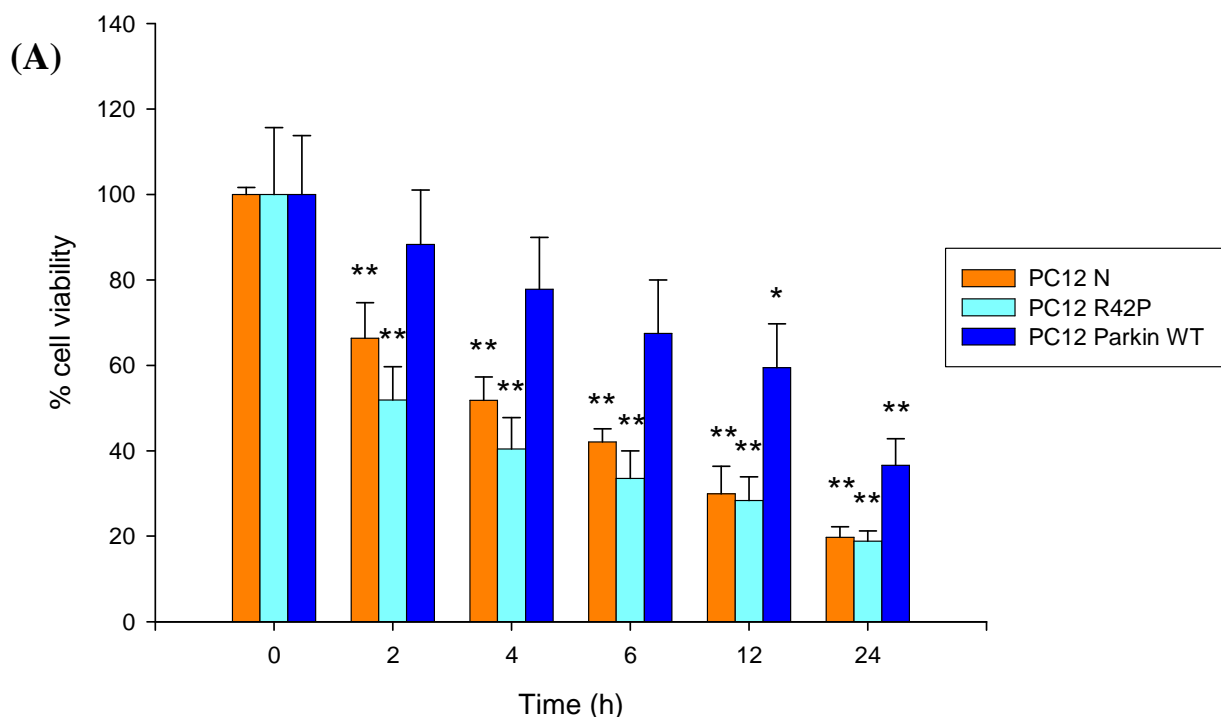
(A)

PC12 Parkin WT VS PC12 R42P: p value < 0,01
PC12 Parkin WT VS PC12 N: p value < 0,05
Two-ways ANOVA, Method: Student-Newman-Keuls al 95.0%

(B)
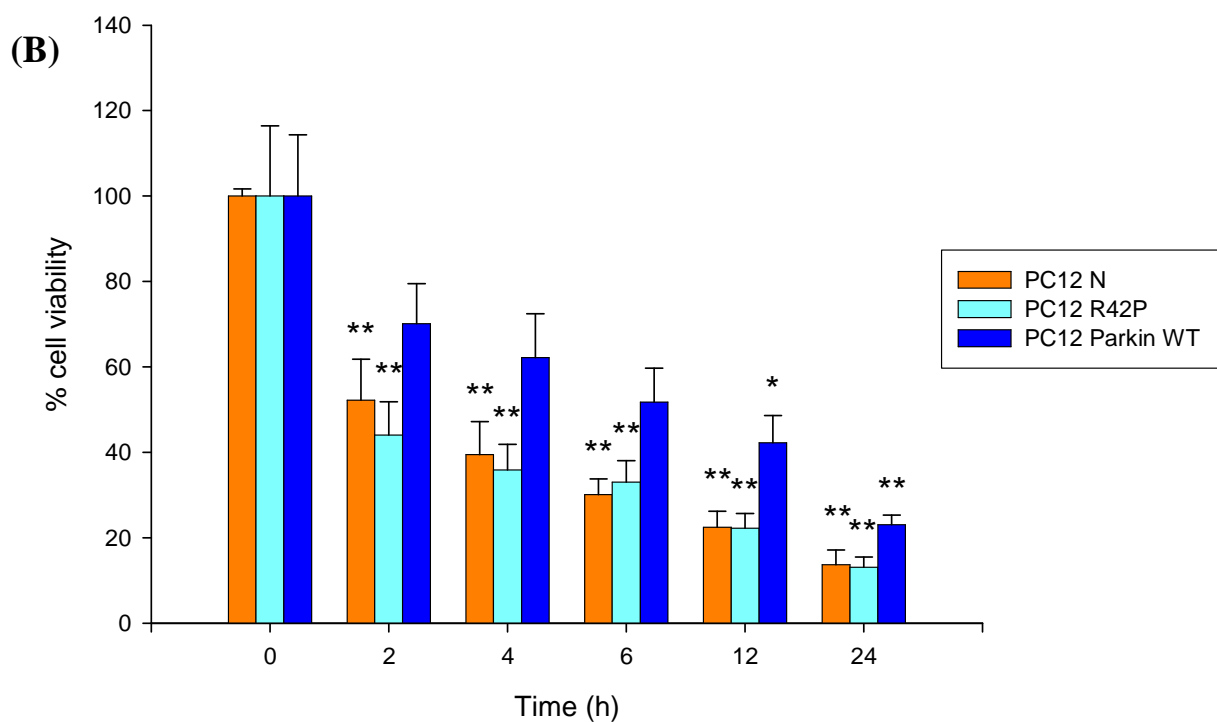
PC12 Parkin WT VS PC12 R42P: p value < 0,01
PC12 Parkin WT VS PC12 N: p value < 0,05
Two-ways ANOVA, Method: Student-Newman-Keuls al 95.0%

Figure 3.12: Protective effect of WT parkin over-expression in PC12 cells against 6-OHDA-induced cytotoxicity. Un-transfected PC12 cells and PC12 stable cell clones were seeded in poly-D-lysine-coated 24-well plates at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. Differentiated control PC12 cells (orange bar), WT parkin cells (blue bar) and parkin^{R42P} cells (cyano bar) were treated with 50 **(A)** and 75**(B)** µM 6-OHDA for 2, 4, 6, 12 and 24 h. Cell viability was assessed by MTT assay. All results are expressed as the percentage *vs* the group without 6-OHDA (corresponding vehicle control values), and are the mean ± S.D. of 4 independent experiments performed in duplicate. Statistical significance was evaluated by two-way ANOVA followed by Student-Newman *post-hoc* test and one-way ANOVA followed by Dunnett's *post-hoc* test. * $p < 0,05$; ** $p < 0.01$ *vs* control group.

PC12 N: Un-transfected PC12 cells (control cells, orange bar). PC12 R42P: PC12 cells stably transfected with pcDNA3.1-R42P mutant parkin (clone 1, cyano bar). PC12 Parkin WT: PC12 cells stably transfected with pcDNA3.1-WT parkin (clone 9, blue bar).

3.9 Evaluation of intracellular levels of reactive oxygen species

The cytotoxicity of 6-OHDA is thought to involve the production of reactive oxygen species (ROS) (Blum et al., 2001). In order to assess whether the observed protective effect of parkin, was mediated by decreased levels of intracellular ROS, we examined 6-OHDA-induced production of free radicals at the same time points used to measure 6-OHDA toxicity. Oxidative stress, induced by treatment with 50 µM 6-OHDA increased in a time-dependent manner and was statistically significant already after 2 h. Moreover, this behaviour was seen in both the WT parkin clone (Figure 3.13, blue bar), and the Parkin^{R42P} clone (Figure 3.13, cyano bar), as well as in control cells (Figure 3.13, orange bar). The protective effect of parkin is thus not due to a lower production of ROS.
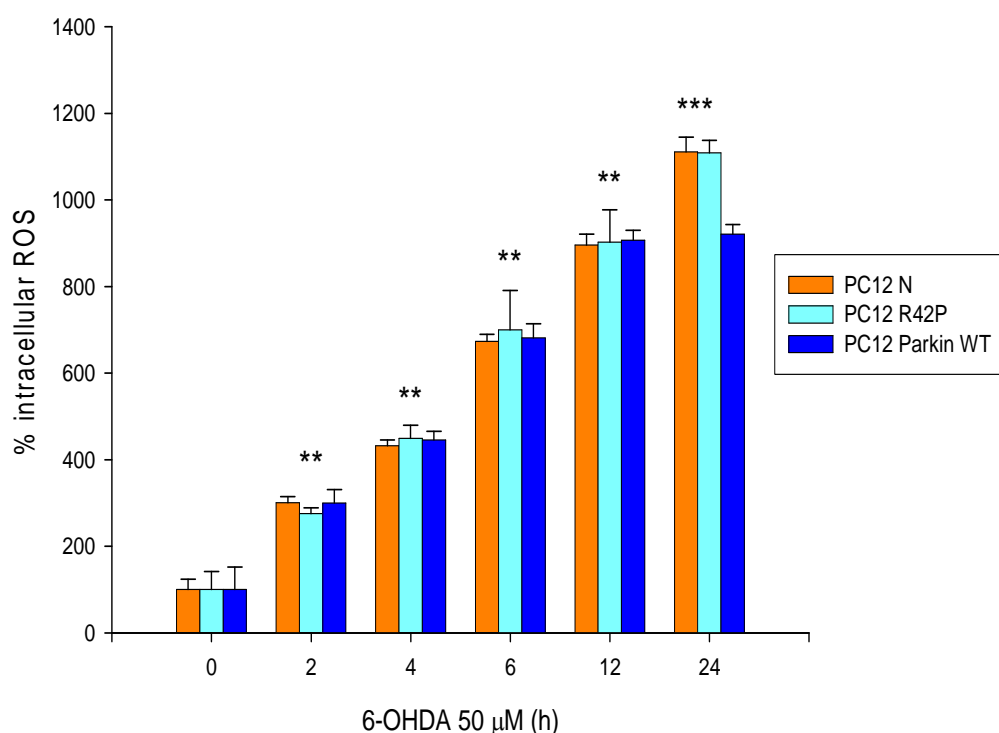
Figure 3.13: Intracellular levels of reactive oxygen species (ROS)

Un-transfected PC12 cells and PC12 stable cell clones were sedeed in poly-D-lysine-coated 24-well plates, at 1×10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. Differentiated un-transfected PC12 cells (orange bar), WT parkin cells (blue bar) and parkin^{R42P} clone (cyano bar) were treated with 50 and 75 μM 6-OHDA for 2, 4, 6, 12 and 24 h. Intracellular ROS were determined using the fluorescent probe DCFH-DA. Values of ROS generation were normalized to the number of viable cells at the same time-point measured as MTT reduction. All results are expressed as the percentage *vs* the group without 6-OHDA (corresponding vehicle control values), and are the mean ± S.D. of 3 independent experiments performed in duplicate. Statistical significance was evaluated by one-way ANOVA followed by Dunnett's *post-hoc* test. ** $p < 0.01$; *** $p < 0,001$ *vs* control group.

PC12 N: Un-transfected PC12 cells (orange bar). PC12 R42P: PC12 cells stably transfected with pcDNA3.1-R42P mutant parkin (clone 1, cyano bar). PC12 Parkin WT: PC12 cells stably transfected with pcDNA3.1-WT parkin (clone 9, blue bar).

3.10 Evaluation of protein ubiquitination

3.10.1 Western blotting analysis

Shimura and colleagues (Shimura et al., 2000) demonstrated that parkin is an E3 ubiquitin-protein ligase that polyubiquitinates abnormal proteins. These polyubiquitinated proteins ultimately are degraded by the 26S proteasome complex. As assessed by Western blotting (Figure 3.14(A)), the PC12 WT parkin expressing clone 9 was more immunoreactive to the antibody against ubiquitin, showing a greater level of high molecular weight ubiquitinated proteins compare to un-transfected PC12 cells (PC12 N) and even more so compared to the PC12 parkin^{R42P} expressing clone 1. Quantitatively, PC12 cells expressing WT parkin contained about 20% more of the protein than control, un-transfected cells (PC12N) (Figure 3.14(B)). Conceivably, this increase could explain the increased survival and protection against 6-OHDA in the former cells. Furthermore, ubiquitinated proteins levels were reduced about 30-fold and 50-fold, respectively, in control (PC12 N) and in parkin^{R42P} expressing. This result suggests that the presence of the R42P substitution may affect the protein's physiological behaviour(s), including biological activity.

3.10.2 Immunocitochemical analysis

Immunocitochemical analysis confirmed a more intense signal in PC12 clones expressing WT parkin, and showed that ubiquitinated inclusions were dispersed throughout the cytoplasm and distributed in the perinuclear region (Figure 3.14(C)).

(A)
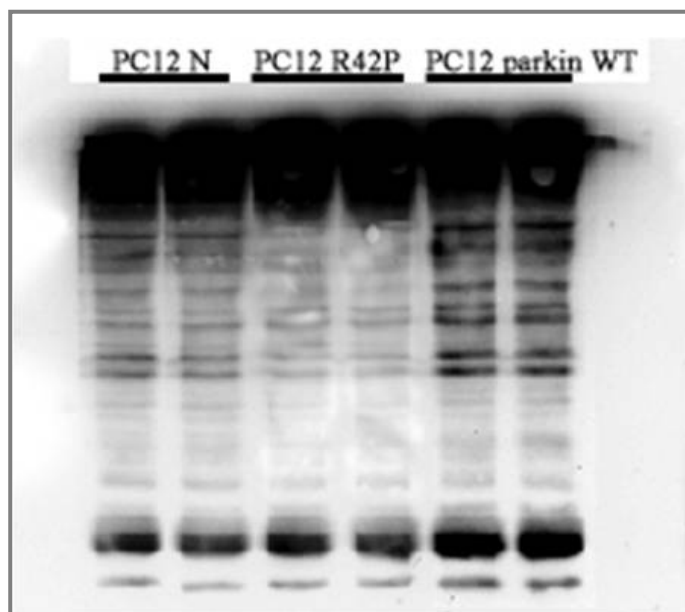
(B)
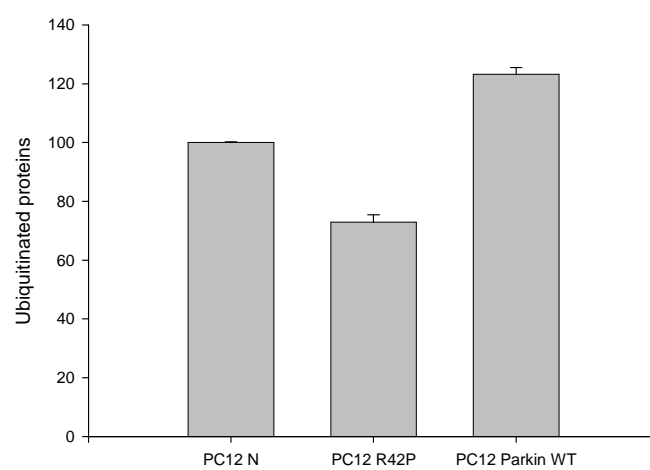
(C)
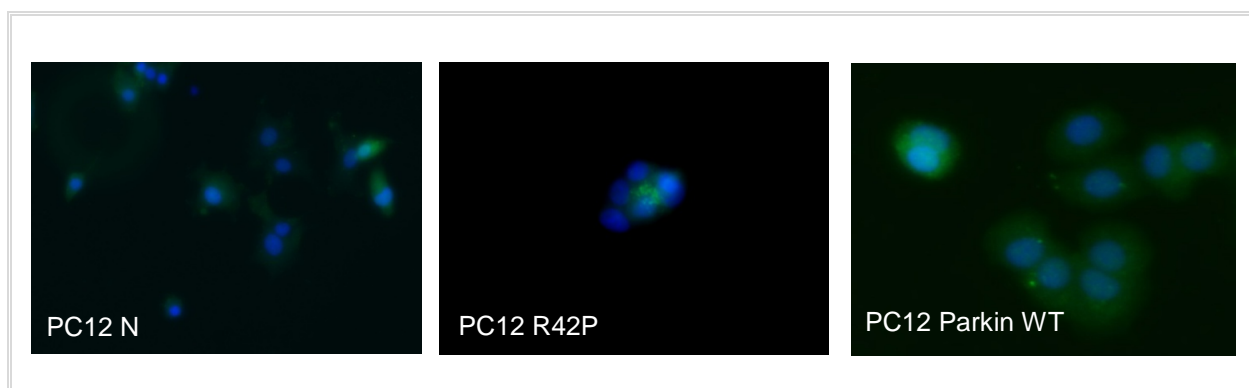
Figure 3.14.: Over-expression of WT parkin increases protein ubiquitination. (A) Western blot assessing the amount of high molecular weight ubiquitinated proteins in un-transfected PC12 cells, and in the WT parkin and parkin^{R42P} clones. Un-transfected PC12 cells and PC12 stable cell clones were seeded in poly-D-lysine-coated 24-well plates at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days, then harvested and lysates prepared for Western blot analysis. **(B)** Western blot quantification. Values are normalized to ponceau staining and are rapresentative of one of 3 independent experiments (n=3 for each group). **(C)** High molecular weight ubiquitinated proteins were also evaluated by immunocytochemistry. Un-transfected PC12 cells (PC12 N) and PC12 stable cell clones (PC12 R42P and PC12 Parkin WT) were seeded on poly-D-lysine- coated plastic chamber slides at 1 X 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. The cells were then fixed with 4% PFA and stained with a primary anti-ubiquitin rabbit polyclonal antibody (1:200) as described in Materials and Methods.

PC12 N: Un-transfected PC12 cells. PC12 R42P: PC12 cells stably transfected with pcDNA3.1-R42P mutant parkin (clone 1). PC12 Parkin WT: PC12 cells stably transfected with pcDNA3.1-WT parkin (clone 9).

3.11 Evaluation of autophagy pathway activation

To understand whether the observed protective effect of parkin, correlated with an increase in autophagy, we evaluated the activation of this pathway by Western blotting and immunocitochemical detection of LC3, a protein that localizes to autophagosomal membranes (Figure 3.15). During autophagy, the cytoplasmic form (LC3 I) is processed and recruited to the autophagosomes, where LC3 II is generated by site-specific proteolysis and lipidation near the C-terminus. The hallmark of autophagic activation is thus the formation of cellular autophagosome punctae containing LC3 II, while autophagic activity is measured biochemically as the amount of LC3 II that accumulates in the absence or presence of lysosomal activity. Tracking the conversion of soluble LC3-I to lipid-bound LC3-II is indicative of autophagic activity. Immunoblotting of LC3 usually reveals two bands: LC3-I (18 kDa) and LC3-II (16 kDa). The antibody can detect both forms of LC3. The amount of LC3-II correlates well with the number of autophagosomes. We took advantage of this characteristic conversion of LC3-I to LC3-II to monitor autophagic activity. Western blot analysis showed a clear increase in LC3 II protein level due to over-expression of WT parkin (Fig 3.16(A)). There was a statistically significant, approximate 40% increase of LC3 II, normalized to LC3 I, when compared to both control (un-transfected) and parkin^{R42P} PC12 cells (Figure 3.16(B)). This result could also contribute, at least in part, to the protective effect of WT parkin.
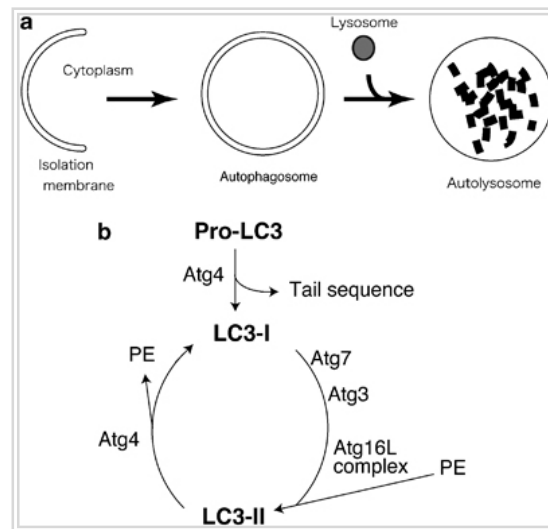
Figure 3.15 a) Representative stages of autophagy. The isolated membrane/phagophore traps a portion of the cytosol to become an autophagosome. The autophagosome fuses with the lysosome to become an autolysosome and its contents are degraded. **b) The LC3 reaction pathway**. LC3 translated from mRNA is called pro-LC3 and possesses an unnecessary C-terminal tail. Subsequently, Atg4 protease cleaves the tail sequence to generate form-I LC3, which has a C-terminal glycine. Form-I LC3 is activated by Atg7 (E1) to become a high-energy thio-ester intermediate conjugated to Atg3 (E2) enzyme. Atg3-LC3 conjugate is recruited to the site where lipidation occurs by directly binding to Atg16L complex, and the C-terminus of LC3 forms an amino bond to the head group of phosphatidylethanolamine (PE), generating form-II. Form-II LC3 becomes membrane-bound. Form-II LC3 is then cleaved at the PE by Atg4 to regenerate form-I.

(A)
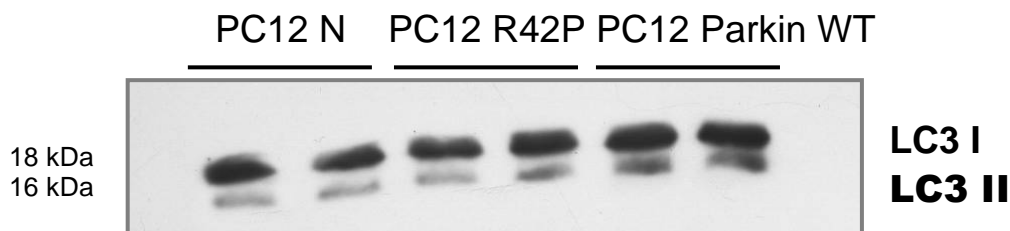
(B)
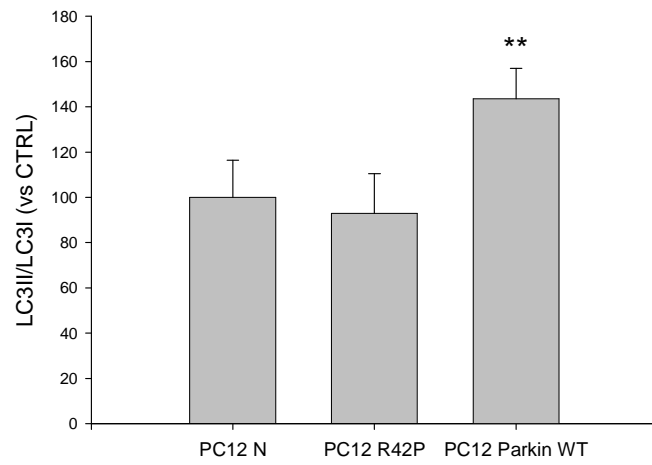
(C)
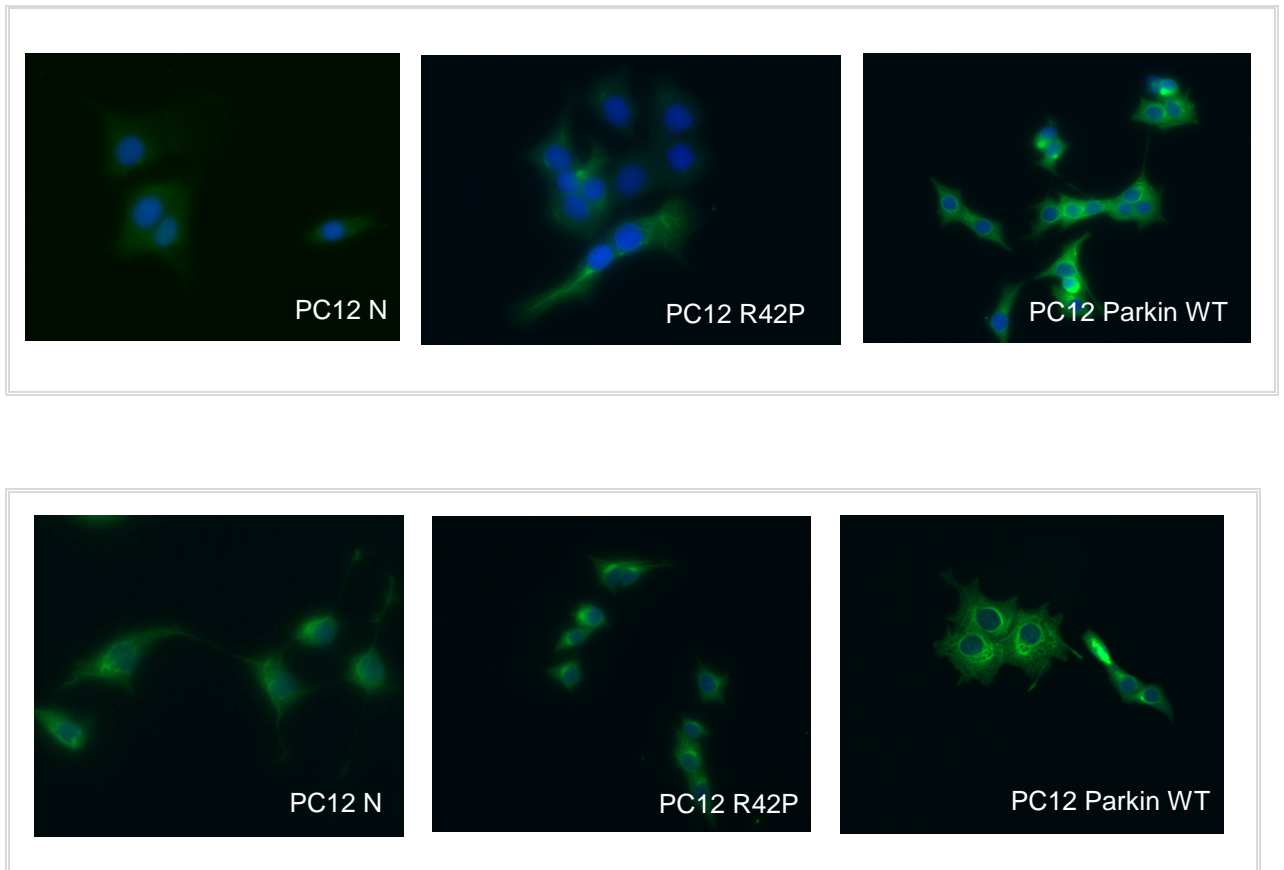
Figure 3.16: Over-expression of WT parkin increases basal activation levels of autophagy. (A) Western blot assessing the basal autophagic expression level in PC12 un-transfected cells (PC12 N), and in WT parkin clone 9 and parkin^{R42P} clone 1. Un-transfected PC12 cells and PC12 stable cell clones were seeded in poly-D-lysine-coated 24-well plates at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days, then harvested and lysates prepared for Western blot analysis. **(B)** Western blot quantification: bar graph showing the values normalized to LC3-II/LC-3-I ratio and is representative of one of 3 independent experiments (n=3 for each group). Statistical significance was evaluated by one-way ANOVA followed by Tukey's *post-hoc* test. ** $p < 0.01$ *vs* control group (PC12 N). **(C)** LC3 immunoreactivity. Un-transfected PC12 cells (PC12 N) and PC12 stable cell clones (PC12 R42P and PC12 Parkin WT) were seeded on poly-D-lysine-coated plastic chamber slides at 1 X 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. The cells were then fixed with 4% PFA and stained with a primary anti-LC3B rabbit polyclonal antibody (1:200) as described in Materials and Methods. For un-transfected PC12 cells (PC12 N) and for each clone (PC12 R42P and PC12 Parkin WT), the first frame illustrates basal expression of LC3, while the second image represents the level of LC3 after 16 h of treatment with 10 mM 3-MA.

PC12 N: Un-transfected PC12 cells. PC12 R42P: PC12 cells stably transfected with pcDNA3.1-R42P mutant parkin (clone 1). PC12 Parkin WT: PC12 cells stably transfected with pcDNA3.1-WT parkin (clone 9).

3.12 Effect of WT parkin over-expression on cell viability after proteasome or/and lysosomal pathway inibition

Having established that stable expression of WT parkin is characterized by an increased level of ubiquitinated proteins and basal autophagy, we decided to evaluate which pathway was involved in the parkin neuroprotection effect in our cellular model. We selectively blocked macroautophagy with 3-MA (10 mM) or the ubiquitin proteasome system (UPS) with MG132 (2.5 μM), for a further 16 h, and then evaluated cellular viability by MTT assay. The results are shown in Figure 3.17: this experiment confirmed the increase in cell viability in our PC12 WT parkin expressing clone (clone 9, blue bar), compared to un-transfected cells (PC12 N, orange bar) and to the PC12 parkin^{R42P} expressing clone (clone 1, cyano bar) after treatment with 50 μM 6-OHDA. It was also apparent that clone 9 exhibits enhanced survival when

macroautophagy is inhibited, rather than the proteasome, with a statistically modest reduction in cell viability only when 6-OHDA treatment was combined with either MG132 or 3-MA. Moreover, PC12 un-transfected (orange bar) and PC12 parkin^{R42P} cells (cyano bar) treated with 6-OHDA, MG132 or 3-MA all showed statistically significant increases of cell toxicity. On the other hand, PC12 un-transfected and PC12 parkin R42P cells did not differ significantly from each other, except for PC12 parkin R42P incubated with 6-OHDA + 3-MA, where cell viability decreased (Figure 3.17(A)). Whereas the UPS and the autophagic pathway are the two main routes of protein and organelle clearance in eukaryotic cells, the protective effect seen in cells expressing WT parkin might simply reflect pathway redundancy, whereby the autophagic pathway remains active when the UPS pathway is inhibited, and vice versa. This interpretation is supported by the observation that inhibiting both pathways by combined treatment with MG132 plus 3-MA decreased cell viability about 70% in un-transfected PC12 cells (orange bar), as well as in both clones (cyano and blue bars), neutralizing the protective effect of WT parkin over-expression (Figure 3.17(B)).

(A)
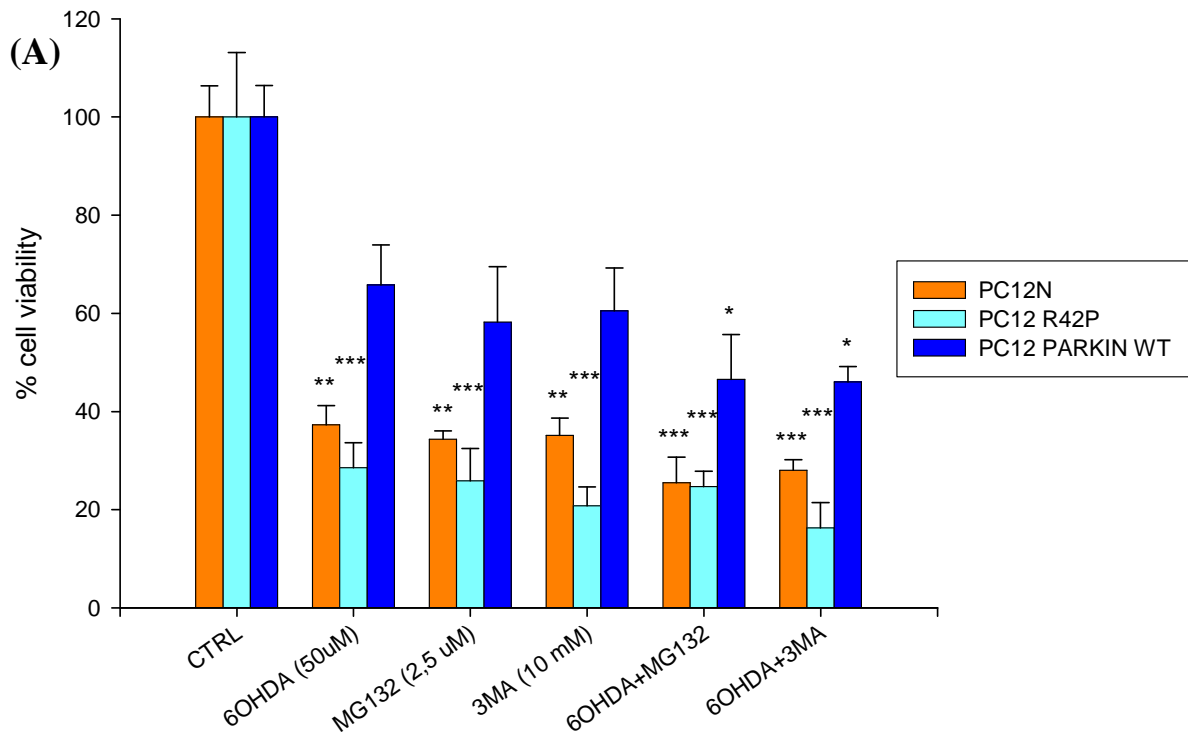
PC12 Parkin WT VS PC12 R42P: p value < 0,01
PC12 Parkin WT VS PC12 N: p value < 0,05
One-way ANOVA, Method: Bonferroni's Multiple ComparisonTest

(B)
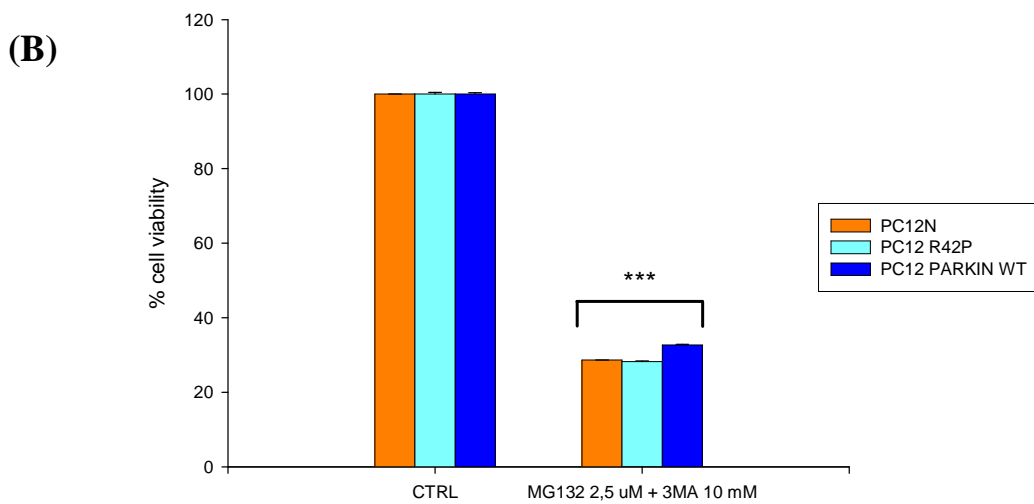
Figure 3.17: (A) Protective effect of WT parkin over-expression against toxicity of MG132 or 3-MA treatment. Un-transfected PC12 cells and PC12 stable cell clones were seeded in poly-D-lysine-coated 24-well plates, at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. Un-transfected PC12 cells (orange bar), parkin WT (blue bar) and parkin R42P (cyano bar) were treated with 50 μM 6-OHDA, 2.5 μM MG132, 10 mM 3-MA, 50 μM 6-OHDA + 2.5 μM MG132, 50 μM 6-OHDA + 10 mM 3-MA for a further 16 h. Cell viability was assessed by MTT assay. All results are expressed as the percentage *vs* the group without treatments (corresponding vehicle control values), and are the mean ± S.D. of 4 independent experiments performed in duplicate. Statistical significance was evaluated by one-way ANOVA followed by Bonferroni *post-hoc* Multiple Comparison Test. * $p < 0,05$; ** $p < 0.01$; *** $p < 0,001$ *vs* control group.

(B) The protective effect of WT parkin over-expression is cancelled by the combined treatment with of proteasome and autophagy inhibitors. Un-transfected PC12 cells and PC12 stable cell clones were seeded in poly-D-lysine-coated 24-well plates, at 1 x 10^4 cells/cm^2, and differentiated with 50 ng/ml rhNGF for 5 days. Un-transfected cells (orange bar), parkin WT (blue bar) and parkin R42P (cyano bar) were treated with 2.5 μM MG132 + 10 mM 3-MA for a further 16 h. Cell viability was assessed by MTT assay. All results are expressed as the percentage *vs* the group without treatments (corresponding vehicle control values), and are the mean ± S.D. of 4 independent experiments performed in duplicate. Statistical significance was evaluated by one-way ANOVA followed by Tukey's *post-hoc* test. *** $p < 0,001$ *vs* control group.

PC12 N: Un-transfected PC12 cells (orange bar). PC12 R42P: PC12 cells stably transfected with pcDNA3.1-R42P mutant parkin (clone 1,cyano bar). PC12 Parkin WT: PC12 cells stably transfected with pcDNA3.1-parkin WT (clone 9, blue bar).

CHAPTER 4

DISCUSSION

A milestone in PD research was the discovery of genes that are responsible for familial variants of this disease. Sporadic and familial variants of PD share important pathological features, most notably the demise of dopaminergic neurons in the SN. Consequently, insights into the function of PD-associated genes may facilitate a better understanding of the pathomechanism not only of hereditary PD, but also of sporadic PD. In this context, the parkin gene (PARK2) seems to play a prominent role accounting for the majority of autosomal recessive PD cases (Mata et al., 2004; West and Maidment, 2004). A consistent observation in cell culture and animal models is the neuroprotective capacity of parkin. Parkin have been shown to protect cultured cells against death induced by kainate, proteasomal inhibition, α-syn, ceramide, manganese, DA, and unfolded protein stress (Imai et al., 2000; Petrucelli et al., 2002; Darios et al., 2003; Staropoli et al., 2003; Higashi et al., 2004; Jiang et al., 2004; Muqit et al., 2004). In *Drosophila*, over-expression of parkin can suppress loss of dopaminergic neurons induced by α-syn or Pael-R (Yang et al., 2003; Haywood and Staveley, 2004). Furthermore, lentiviral delivery of parkin prevents dopaminergic degeneration caused by mutant α-syn in a rat model and protects mouse skeletal muscle cells against mitochondrial toxins (Lo Bianco et al., 2004; Rosen et al., 2006). The mechanism(s) underlying parkin's protective action remains to be elucidated. As it turns out, parkin is an E3 ligase (Shimura et al., 2000), and parkin ability to protect cells from various cellular toxic stimuli, including proteasome inhibitors (Muqit et al., 2004), appears to depend on its E3 ubiquitin-protein ligase activity.

To gain insight into the mechanism underlying the broad neuroprotective capacity of parkin, we investigated a possible role of parkin in the stress response pathway. We analyzed, *in vitro,* the protective activity of parkin in a cellular model of oxidative stress using the dopaminergic neurotoxin 6-OHDA. Oxidative stress-induced cytotoxicity is believed to play a major role in neurodegenerative disorders, including PD. 6-OHDA is ofen use to produce an in *vivo* animal model of PD (Beal, 2009). Parkin involvement in the antioxidant response has been demonstrated by its over-expression, which correlates positively with a pro-survival outcome. In this project we focused on the protective potential of parkin by two routes: first, by exogenous addition of a TAT-fusion protein; second, by up-regulating its expression via generation of stable transfected clones.

In the first part of this study, a TAT-parkin fusion protein was designed, synthesized, and purified. TAT-parkin efficiently transduced naïve or NGF-differentiated rat adrenal pheocromocytoma PC12 cells and CHO cells, with localization to the nucleus, cytoplasm, and mitochondria. TAT-parkin exhibited ubiquitination activity *in vitro* and, importantly, at nanomolar concentrations protected NGF-differentiated PC12 cells against 6-OHDA. As mentioned in the Introduction, fusion of parkin protein to the TAT peptide allowed diffusion through neuronal cell membranes. This vehicle is very important because the delivery of therapeutic proteins into tissues and across the cell membrane and the blood-brain barrier is severely limited by the size and biochemical properties of the respective proteins. The blood-brain barrier is a major handicap in CNS drug development and is probably the single most important factor limiting the future growth of neurotherapeutics (Pardridge, 2005). The protein transduction domains derived from the TAT-protein of HIV-1, the antennapedia homeodomain of drosophila, and the simplex herpes virus VP22 protein (Leifert et al., 2003) are all capable of

crossing the blood-brain barrier and delivering fusion peptides and proteins into the brain parenchyma. In particular, the TAT protein can mediate heterologous protein passage across the plasma membrane in nearly all eukaryotic cells (Schwarze et al., 2000). Indeed, in recent years, protein transduction technology has found utilization in numerous paradigms of neurodegeneration (Dietz et al., 2002; Wheeler et al., 2003; Dietz and Bahr 2004, 2005; Nagel et al., 2008).

After synthesis and purification of the recombinant protein, and in order to verify protein intracellular availability through this route of delivery, we first performed Western blotting to determine the kinetics of TAT-parkin availability inside cells. Immunoblot analysis showed that TAT-parkin had a half-life of 24-48 h, depending upon the cell type transduced. The shorter half-life in CHO cells (approximately 24 h) compared to PC12 cells may reflect differences in metabolism between the two cell types, with faster degradation of TAT-parkin in CHO cells. The mechanism by which TAT-linked proteins enter cells remains unclear. The TAT motif can be dissected functionally into two parts: GRKKR acts as a potential nuclear localization signal, whereas the RRR sequence appears to be critical for protein translocation (Vives et al., 1997). Hauber et al. (1989) and Ruben et al. (1989) reported that TAT-GFP localized predominantly in the nucleus, especially within the nucleolus. Under physiological conditions endogenous parkin is largely cytosolic in location or associated with the ER or mitochondria (Shimura et al., 1999; Darios et al., 2003; Kuroda et al., 2006).

While immunocytochemical analysis showed that TAT-parkin entered PC12 cells, its subcellular location was not clear. Repeating the assay with CHO cells demonstrated that the fusion protein displayed a nuclear and cytoplasmic distribution, in agreement with earlier reports (Hauber et al., 1989; Ruben et al., 1989). In order to investigate further the subcellular localization of TAT-parkin, a plasmid encoding mitoGFP was transfected into CHO cells. At 48

h post-transfection cells were treated with TAT-parkin for 4 h, fixed, and processed for parkin immunocytochemistry. pMito-GFP in control cells (CHO cells not treated with TAT-parkin) was evident within mitochondria, while TAT-parkin was predominantly located in the nucleus and cytoplasm, resulting in a very strong fluorescent signal (Figure 3.5, panel B), consistent with earlier reports for TAT fusion proteins (Hauber et al., 1989; Ruben et al., 1989). In contrast, CHO cells treated with TAT-parkin but not processed for parkin immunocytochemistry (Figure 3.5, panel A) showed only the plasmid pmito-GFP, similar to pmito-GFP control cells. These results demonstrate that TAT-parkin successfully transduced cells *in vitro*, indicating that the state of TAT-parkin folding is not critical for this process and suggesting that TAT-mediated delivery of proteins such as parkin may be a viable therapeutic approach.

Recent *in vitro* (Oluwatosin-Chigbu et al., 2003; Henn et al., 2007) and *in vivo* (Lo Bianco et al., 2004) studies have attributed a protective role for parkin in DA neuron survival. Parkin can protect cells against a wide array of stressors (Moore, 2006). Parkin acts as an ubiquitin-protein isopeptide ligase, and loss-of-function mutations in the gene encoding parkin (Kitada et al., 1998; Imai et al., 2000; Zhang et al., 2000) compromise its neuroprotective activity. Substrates destined for proteasomal degradation via parkin might thus conceivably accumulate in parkin-deficient cells, and constitute a factor mediating AR-JP. The E3 ubiquitin-ligase activity of parkin appears to be an important aspect of its neuroprotective action in PD models, although the mode of parkin-catalyzed ubiquitination remains poorly understood. We tested the enzymatic activity of TAT-parkin using an *in vitro* ubiquitination assay system. Ubiquitin is a small, covalent modifier that forms a poly-ubiquitin chain on proteins, which constitutes a degradation signal for the 26S proteasome (Coux et al., 1996; Hershko et al., 1998). The poly-ubiquitin chain is synthesized by a reaction cascade involving E1 (ubiquitin-activating enzyme),

E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-ligating enzyme), the last acting as a substrate-recognition molecule (Hochstrasser, 1996; Hershko et al., 1998) (see Box 1, Introduction). With regard to parkin in AR-JP, the presence of carboxy-terminal two RING-finger motifs, which are suggested to interact with E2 (Lorick, 1999; Moynihan et al., 1999; Joazeiro et al., 1999; Xie and Varshavsky, 1999) prompted us to investigate TAT-parkin function as an E3 ligase. Recombinant TAT-parkin provides the advantage of a protein free from other contaminating E3 enzyme(s) (Matsuda et al., 2006). Although many putative substrates for parkin have been reported, the lack of a good *in vitro* substrate makes it difficult to check the intrinsic E3 activity of the fusion protein. We thus chose the recombinant DJ^{L166P}, a mutant DJ-1 purified from *E. coli*, as a pseudo-substrate to monitor the E3 activity of TAT-parkin. DJ^{L166P} is incorrectly folded and should be highly susceptible to ubiquitination. Our results suggest that when TAT-parkin is transduced in CHO cells it becomes active presumably being folded by intracellular chaperones, and is able to recognize DJ^{L166P} as a substrate. This was evident by an increase in molecular weight, which remained unchanged in CHO cells not incubated with TAT-parkin (Figure 3.6). TAT-parkin, which is purified under denaturing conditions and is incorrectly folded, was unable to ubiquitinate DJ^{L166P} *in vitro* (data not shown). These observations demonstrate that TAT-parkin, once transduced in cells, possesses E3 activity.

In this context, we evaluated whether TAT-parkin exerted a protective effect against 6-OHDA-induced cytotoxicity *in vitro*. The mechanism of 6-OHDA toxicity involves inhibition of mitochondrial complexes I and IV, generation of intracellular reactive oxygen species, and apoptosis of catecholaminergic neurons (Blum et al., 2000). As already mentioned, oxidative stress is believed to be a key feature in mediating neuronal cell damage in PD. NGF-differentiated PC12 cells become post-mitotic and take on characteristics of catecholaminergic

neurons (Greene and Tischler, 1976), and display sensitivity to 6-OHDA toxicity in a concentration- and time-dependent manner after 2 h. Given concurrently, TAT-parkin limited 6-OHDA-induced cell death only at time 0 of post-incubation. Efficacy in the post-incubation period required the fusion protein to be present throughout that period. Most studies on the protective potential of parkin report consistent findings concerning the nature of stressors tested although discrepancies, when observed may be explained by the fact that robust stress conditions inactivate parkin because of its tendency to misfold (Winklhofer et al., 2003; La Voie et al., 2005; Wang et al., 2005; Winklhofer and Tatzelt, 2006). Thus, one explanation for our findings is that the proteotoxic stress induced by 6-OHDA (to which dopaminergic neurons are highly sensitive) (Winklhofer et al., 2003) induced misfolding of parkin, leading to a loss of protective activity with time. This is consistent with the possibility that TAT-parkin undergoes auto-degradation (data not shown). Moreover, molecular chaperons, such as Hsp70 and Hsp40, interfere with the misfolding and aggregation of parkin (Winklhofer et al., 2003) and may account for their neuroprotective action (Nagel et al., 2008). In fact, chemically denatured recombinant parkin is strictly dependent on molecular chaperones to adopt a native conformation.

These experiments suggested the antioxidant effect of TAT-parkin is not long-lasting, as a nearly continuous administration was required. The second part of this study was thus designed to explore the protective effect of parkin in cells over-expressing human parkin. Two different stable PC12 cell clones were generated: PC12 human WT parkin and the pathogenic parkin mutant R42P. These cell lines were then used to evaluate the cytoprotective activity of parkin when subjected to three stressors relevant to PD, namely oxidative stress (induced by 6-OHDA, at the same conditions of the previous experiments with Tat-parkin), proteasome inhibition induced by a specific inhibitor (MG132), and autophagy inhibition with the selective inhibitor

3-MA. Multiple studies indicate that abnormalities in the autophagy-lysosome pathway and the UPS are involved in the development of PD (see e,g. McNaught et al., 2001, 2006). These authors reported decreased proteasomal activity in the SN of patients with sporadic cases of PD. It is therefore possible that dysfunction of the UPS is a common pathogenetic trigger in both familial and sporadic PD. When challenged by 6-OHDA-induced oxidative stress, we found that the parkin R42P transfected cells were more susceptible to the toxin in comparison to both un-transfected PC12 cells and also the WT parkin cell clone. These data thus confirmed the involvement of WT parkin in the antioxidant cellular response and suggested that one effect of parkin inactivation by genetic alterations (point mutation or deletion) is to impair its protective effect against oxidative stress, thus exposing dopaminergic neurons to an increased level of ROS, whether of exogenous or endogenous origin, as well as to exogenous or endogenous harmful stimuli. In fact Hyun et al. (2002) found that abnormal proteins such as mutated parkins impact on the UPS, leading to oxidative stress and excessive NO production. This in itself is not sufficient to kill the cells, at least in short-term culture, although it may render them more sensitive to other insults. Under this condition the pathogenic parkin mutant R42P showed a reduced neuroprotective capacity, confirming that the N-terminal UBL domain of parkin plays an important role in neuroprotection. Interestingly, Henn and colleagues (2007) showed that a smaller parkin species lacking the N-terminal UBL-domain (ΔN parkin) was impaired in protecting cells from stress-induced death. Conceivably, the survival-enhancing nature of the PC12 WT expressing clone is due to its E3 ubiquitin ligase activity, allowing parkin to bind cellular protein substrates that undergo ubiquitination before degradation by the 26S proteasome. Indeed, our Western blot analysis showed an increase in the amount of ubiquitinated proteins in PC12 WT expressing cells compared to control cells, while PC12 parkin^{R42P} cells displayed a reduced level of ubiquitinated proteins. This underlies the

importance of the N-terminal UBL-domain. Shimura et al. (2000) demonstrated that mutant parkin lacking the UBL domain and naturally occurring parkin[R42P] failed to co-immunoprecipitate ubiquitinated proteins, suggesting that the UBL domain functions as a module necessary for binding with ubiquitinated proteins. Moreover, biochemical and cellular data demonstrate that deletion of the UBL domain abrogates *in vitro* and *in vivo* ubiquitin ligase activity and binding of some parkin substrates (Imai et al., 2000, 2001; Shimura et al., 2001). Collectively, these results indicate that parkin is required for this ubiquitination, possibly through its function as an E3. The loss of parkin E3 function may underlie AR-JP. At the same time, our results appear to contrast with a previous study of Kahns and co-workers (2002) who described proteolysis of human parkin after Asp126, generating a 38-kDa C-terminal fragment and a 12- kDa N-terminal fragment. The 38- and 12- kDa bands are seen in also our immunoblots (Figure 3.11(A)). Proteolysis of human parkin after Asp126 will liberate the UBL domain from the remaining large polypeptide containing the RING-box domain, which is incompatible with a functional parkin enzyme. Clinical data demonstrate that a single missense mutation in the UBL domain, R42P, or in-frame deletion of exons 2 and 3 results in the loss of amino acid residues 3-137, causing early onset AR-JP as a consequence of parkin dysfunction (Lucking et al., 2000; Shimura et al., 2000). Our results agree with Petrucelli and co-workers (2002) who demonstrated that over-expression of parkin decreases sensitivity to proteasome inhibitors in a manner dependent on parkin's ubiquitin-protein E3 ligase activity, and showed that R42P was ineffective against insult by the proteasome inhibitor lactacystin. In fact, our results suggest that treatment with the proteasome inhibitor MG132 did not change significantly the already low survival of PC12 parkin mutant cells, as we blocked the proteasome whose binding is already compromised by the loss-of-function mutation. Thus, neither 6-OHDA nor

MG132 treatment significantly affected PC12 parkin ^{R42P} cell viability, except for 3-MA treatment. Rather, we showed that PC12 WT expressing cells were also characterised by an increase in the basal activation level of autophagy, which might explain its positive outcome on cell survival. As described in the Introduction, the protein degradation machinery in eukaryotic cells consists of two main systems, one mediated by the ubiquitin-proteasome and the other by the autophagy-lysosome. These two degradation machineries are interrelated, with ubiquitin functioning in both systems. However, recent work suggests that parkin can catalyze degradation-independent ubiquitination (Lim, 2007). There is a controversy concerning whether ubiquitination catalyzed by parkin is destined for proteasomal degradation. In fact, parkin acts on mitochondria and is involved in mitochondrial degradation. In 2008, Youle and colleagues (Narendra et al., 2008, 2009) reported that parkin is selectively recruited to depolarized/damaged mitochondria and leads it to degradation by selective autophagy. Because mitochondria are too large for proteasome degradation, these results again suggest that ubiquitination catalyzed by parkin functions as a signal for proteolysis not only by the proteasome, but also by the autophagic and/or the lysosomal systems. Parkin may simultaneously ubiquitinate proteins involved in mitochondrial dynamics and induce a pro-fission state by Lys48 ubiquitination and degradation, and tag unidentified mitochondrial proteins for autophagosome recruitment by Lys63 ubiquitination (Tanaka, 2010). These considerations posit that there are various modes of ubiquitination such as mono-ubiquitination and Lys63-linked poly-ubiquitination that are not essentially associated with proteasomal targeting, and that ubiquitination is relevant to the autophagic system. Our observed protective effect of WT parkin over-expression after inhibition of proteasome or macroautophagy was greater than would have expected. In the case of WT parkin expressing cells, inhibiting the

proteasome remains leaves unaffected the autophagic pathway for degradation, while inhibiting the autophagy system parkin remains able to degrade its substrates via the UPS pathway (Figure 3.17(A)). This observation is supported by the concurrent treatment with proteasome and autophagy inhibitors neutralizes the protective effect of WT parkin over-expression (Figure 3.17(B)). A comparable result was obtained inhibiting only macroautophagy in the R42P mutant parkin clone. This result is in contrast with Yang et al. (2008) who showed that inhibiting the proteasome together with macroautophagy in human (WT) α-syn and mutant (A30P) PC12 cells did not have an additive effect in promoting apoptosis. Many studies showed that proteasome inhibitors, such as epoxomicin, lactacystin, and MG132 promote apoptosis (Rego and Oliveira, 2003), and that 3-MA, a macroautophagy inhibitor, also increased the apoptosis ratio (Yang at al., 2009). Proteasome and autophagy pathway activity may thus antagonize apoptosis (Cheng et al., 2008). Accelerating apoptosis is one mechanism of inducing and promoting PD provoked by proteasome or macroautophagy inhibitors. Inhibition of proteasome/autophagy accelerates cell death that manifests the hallmarks of apoptosis, including chromatin condensation margination, karyopyknosis, nuclear fragmentation in cells, cytoplasmic vacuolation, and activation of casapse (Gonzalez-Polo, 2005; Yang et al., 2009). The same phenomena were observed by Chen and colleagues (2005), who found that PC12 cells treated with lactacystin underwent apoptosis, but that stable over-expression of parkin distinctly reduced lactacystin-induced apoptosis. A report from Darios et al. (2003) also supports this finding. Parkin over-expression inhibited cell death, delayed mitochondrial swelling and cytocrome *c* release, and reduced caspase-3 activation induced by ceramide, indicating that parkin may play an important role in the mitochondrial-dependent apoptosis pathway. Other studies have demonstrated that ubiquitin-protein ligases with Ring finger structures, such as the inhibitor of apoptosis protein

family, inhibit apoptosis, and may bind and degrade caspases (Suzuki et al., 2001). Whether the anti-apoptotic effect of parkin is due to its role in degradation of one or more proteins in the apoptosis pathway requires further research. Based on these observations, we can say that over-expression of WT parkin protects PC12 cells from MG132- or 3-MA-induced toxicity through an anti-apoptotic mechanism. Using Hoechst fluorescence staining, Yang et al., (2009) demonstrated that following treatment with lactacystin, the number of apoptotic cells in their parkin over-expressing cell lines was significantly lower than in cells transfected with empty vector. Unfortunately, in our study we have been unable to detect any morphological changes in the PC12 cell clones, perhaps because 4',6-diamidino-2-phenylindole nuclear staining was not sufficient, and would have required other approaches to assess the execution phase of apoptosis, such as measuring caspase-3 activation and/or release of cellular lactate dehydrogenase into the culture medium.

The specific function of the UPS pathway in apoptosis is still controversial (Naujokat, 2002), and the mechanisms by which inhibition of autophagy may favour cell death are not entirely clear. Other investigations also demonstrated that depending on the cellular context and stimulus, apoptosis is preceded by and even depends on the occurrence of autophagy (Xue et al., 1999; Cui et al., 2007). Some studies have reported that autophagy prevents or halts apoptosis (Bauvy et al., 2001). Thus, the differences between published findings and our study may be related with different cell lines, as well as different stimuli and their intensity. Faced with the same situation, multiple connections between apoptotic and proteasome/autophagic processes may converge to seal the fate of cells. Clearly, the relationships between autophagy, the proteasome system and apoptosis are very complicated, with many factors participating.

As PD is the second most prevalent neurodegenerative disorder, analysis of parkin is clearly important from a global health perspective. Indeed, a large number of articles on parkin have

been, and continue to be published. In this study, a TAT-parkin fusion protein was synthesized which effectively transduced cells. TAT-parkin retained ubiquitin ligase E3 activity, and protected against cytotoxicity mediated by the dopaminergic neurotoxin 6-OHDA. We further demonstrated that parkin over-expression protects dopaminergic cells against the toxicity of 6-OHDA. These results are in agreement with those riported in the first part of the study and concerning a role of this TAT- recombinant fusion protein in the antioxidant response. Over-expression of parkin increases the accumulation of ubiquitin-protein conjugates. Finally, over-expression of parkin protects cells from inhibitors of the proteosome and autophagy, addressing the question of whether impairment of the UPS or the autophagy-lysosome pathway predisposes individuals to neurodegenerative disorders such as PD. Incidentally, mutation of parkin, in which parkin protein has lost its ubiquitination capacity, results in loss of neuroprotective activity: the pathogenetic mutant form R42P of parkin was no longer able to protect against the same toxic stimulus.

What might be the implications of these findings for PD? First, these observations strengthen the concept of applying protein transduction domain technology as a therapeutic approach to deliver neuroprotective agents, with emphasis on parkin for PD. Second, the *in vitro* data highlight the importance of parkin function in counteracting oxidative stress and proteasomal or autophagic system dysfunctions, paving the way for new pharmacological interventions. Parkin may be a new target for a specific neuroprotective or therapeutic approach to prevent or halt the loss of dopaminergic neurons. For this purpose it will be important in future studies to examine the neuroprotective capacity of parkin utilizing agents potentially involved in the early phases of PD, for example α−syn, as well as parkin neuroprotection in animal models of PD.

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